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Obtaining of Astaxanthin from Crab Exosqueletons and Shrimp Head Shells

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Abstract: Fresh wastes of Mexican headshell shrimp (*Litopenaeus vannamei*), blue crab (*Callinectes bellicosus*), and Mexican brown crab (*Callinectes sapidus*) exoskeletons were autoclaved, dried, and grounded at a particle size of 150 µm. Macerated samples were diluted to ethyl acetate, acetone, cyclohexane, isopropyl alcohol, hexane, heptane, and a combination of hexane-acetone-ethanol-toluene solvents. High-Performance Liquid Chromatography analysis determined the astaxanthin amount in every organic solvent extract. Acetone was the most efficient solvent: 114 µg/g (*L. vannamei*), 39 µg/g (*C. bellicosus*), and 44 µg/g (*C. sapidus*); the mixture of hexane-acetone-ethanol-toluene resulted a idoneus solvent when was used on crab exoskeletons: 39 µg/g (*C. bellicosus*) and 51 µg/g (*C. sapidus*). The astaxanthin characterization was performed without saponification, in *L. vannamei* chromatograms, the amount of trans astaxanthin was 6.23 µg/g (5.47 % of total area), in *C. bellicosus* was 26.13 µg/g (67 % of total area) and in *C. sapidus* was 28.42 µg/g (64.6 % of total area).

Keywords: acetone extract; HPLC analysis; saponification.

1. Introduction

Mexico is an important source of crustaceans. It is attained through fishing and aquaculture; Mexico's shrimp production was around 224 thousand tons, more recently, FAO (2020) reported for Mexico a total annual crab production of 51 thousand tons [1]. In Mexico, there are not installed processing plants that could take advantage of such waste products and, therefore, represent a high business opportunity. It is important to mention that crabs exoskeletons and shrimp shells can be used as a raw material to recover products of high added value such as chitin, chitosan, glucosamine, and astaxanthin [2-4]. Astaxanthin belongs to the xanthophyll chemical family; these are oxygenated compounds derived from carotenoids [5]. Carotenoids presented an orange-red appearance and are commonly used as a pigment of

salmon meat, trout, crustaceans, and poultry meat [6]. Some of the works reported that astaxanthin showed the highest biological activity with respect to other carotenoids [7]. The Food and Drug Administration of the United States (USFDA) has been approved the use of astaxanthin as a colorant in animal feed and fish food [8]; also, the European Commission considers the astaxanthin as a food colorant (Roche, 1987) [8]. Today, astaxanthin has gained special strength cause of its different applications in several industries, mainly as a food supplement agent in human and animal feed, as well as the pigment collection sources such as crab and shrimp [9]. Several methods to extract astaxanthin include fermentative process extraction and not fermentative extraction method from crab wastes combined with organic solvents, enzymatic methods, extraction with plant or marine oil sources, and supercritical fluids [10,11]. However, the most used method for astaxanthin extraction is organic solvents, among them alcohol and ethyl acetate, isopropanol and/or acetone, ethanol, and methanol n-hexane, and isopropyl alcohol [12]. Besides, the yield astaxanthin determination is a key factor in choosing the astaxanthin extraction method; one of the most used is the spectrophotometry determination and HPLC method [13]. The last technique is a rapid and accurate method that allows establishing a quality control, specifically by the presence of esters, where the most common chemical structure is: free form, monoester, and diester. To avoid its chemical behavior is necessary to remove the astaxanthin esters' fatty acid chains by alkaline saponification or enzymolysis, then only after this, HPLC can easily separate astaxanthin isomers. Identification esters can be determined by High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) [14]. Therefore, in this work, we used HPLC as a standard method to quantify and identify astaxanthin main esters and isomers obtained from shrimp headshells, blue crab, and brown crab, a common waste on the northwest coasts of Mexico. The main purpose of the current research is to propose a sustainable method to extract astaxanthin from shrimp (*Litopenaeus vannamei*), headshells, blue crabs (*Callinectes sapidus*), and brown crabs (*Callinectes bellicosus*) exoskeletons. It means the most common Mexican crustaceans produced by aquaculture or fishing. For this, we proposed a solvent extraction method of fresh exoskeletons by using an autoclave oven and grounded by mean a manual mill to obtain a size particle of 150 mm. Later, a powder sample of *L. vannamei*, *C. sapidus*, and *C. bellicosus* was exposed to a common organic solvent (ethyl acetate, acetone, cyclohexane, isopropyl alcohol, hexane, heptane, ethanol, and the mix hexane-acetone-ethanol-toluene: HAET). Finally, the HPLC method determined the amount of astaxanthin to quantify and determine the type of astaxanthin molecules extracted.

2. Materials and Methods

2.1. Chemicals and reagents.

Analitical solvents (Sigma Aldrich, USA) used were acetone (100% purity), absolute ethanol (99.5% purity), ethyl acetate (99.5% purity), hexane (99.5% purity), toluene (99.3% purity), cyclohexane (99.3% purity), heptane (99% purity), isopropyl alcohol (70% in water). The HAET mixture (hexane-acetone-ethanol-toluene) was prepared in a relationship of 10:7:6:7 mL.

2.2. Demineralization and sample powder obtention from Mexican shrimp head shells and exoskeleton of blue crab and brown crab.

14 kg of fresh shrimp head, 5 kg of a fresh shell of blue, and 5 kg of brown crab were washed with distilled water and autoclaved (Market Forge Model STM-E) at 121 ºC during 15 min at 15 lb in^2 of pressure. When the system achieved 100 ºC of temperature, the pressure was lowered at 0.5 lb in^2 during 10 min. The sample was withdrawn, dried in the shade for 12 h, and then dehydrated in an oven at 50 ºC for two hours (Yamato DX 302 oven). The dried sample was grounded in a manual meat mill No. 32 (SKU: MC-32) and sieved (No. 40 mesh) to obtain a particle size of 150 µm.

2.3. Astaxanthin extraction from Mexican shrimp and crab exoskeleton.

30.0 mg of powder sample was placed in a volumetric flask of 100 mL. Then it was added 100 mL of every reagent organic solvent proved in this investigation (ethyl acetate, acetone, cyclohexane, isopropyl alcohol, hexane, heptane, ethanol, and the mix hexane-acetone-ethanol-toluene HAET (Sigma Aldrich, USA), and the mixture was treated in an ultrasound device (Cole Parmer Model 0889021) during one minute. Then, the volumetric flasks were filled with hexane (Sigma Aldrich, USA) up to the mark. Every solution of the solvents prepared was taken 10 mL and were added 5 mL of acetone at 100 % of purity and gradually filled since up 50 mL and the final solution were well manually mixed.

2.4. Sample saponification.

We used a protocol reported previously by Hu *et al*. (2019); the method consisted of adding 4% KOH-ethanol solution to every 5 mL of sample to adjusted pH to 10.0. The sample was saponified for 5 minutes. Finally, the sample was filtered before to inject into the HPLC.

2.5. Molecular mass astaxanthin determination.

The seven solvents' astaxanthin hydrolysis capacity was estimated with a precolumn packed with silica (maxil silica column of 5 nanometers 4.0 x 5.0 mm), using a conventional Varian 9050 HPLC system with a UV detector at a wavelength of 472 nm. The mobile phase consisted of 75-25 hexane-ethyl acetate at 23 ºC; flow rate of 2.5 mL min-1 and isocratic pressure of 85 psi (Isoocratic pump Varian 9002). The columns were calibrated by a standard calibration method using astaxanthin and its isomers as standards of molecular mass (Sigma Aldrich, 596.84 g/mol). Hydrosilate samples of every solvent were filtered by using 0.22 µm Millipore membrane to remove excessive debris. A volume of 20 µL was injected into HPLC by a syringe. The astaxanthin concentration was determined by extrapolating the absorbance values using a standard curve.

2.6. Statistical analysis.

Statistical analysis was performed through the program SAS (2018), chromatographic dates were analyzed using the multiple means comparison test (ANOVA, $P < 0.05$).

3. Results and Discussion

3.1. Astaxanthin quantification.

HPLC method was used to quantify astaxanthin extracted with organic solvents proved in this work. Table 1 shows the results. It is evident that with acetone, the highest astaxanthin yields; in non saponified shrimp samples (L. vannamei), it was yielded 114 µg/g and with saponified samples was quantified 92 µg/g (Table 1). In shrimp, the second solvent was isopropyl alcohol with 99 µg/g in unsaponified samples and 92 µg/g on saponified samples; complete results are shown in Table 1. In the case of astaxanthin extracted from blue (*C. sapidus*) and brown crab (*C. bellicosus*) exoskeletons, the best solvents were acetone and the mix HAET.

Table 1. Asthaxanthin extracted from shrimp powder (*L. vannamei*) quantified by HPLC.

Solvent	**µg/g**	**SD**	**µg/g**	**SD**
	Astaxanthin without saponification	**µg g^{-1}**	**Astaxanthin saponification**	**µg g^{-1}**
Ethyl acetate	68 d	±0.3	65 b	±0.1
Acetone	114 a[1]	±0.4	92 a*	±0.2
Cyclohexane	81 c	±0.5	60 b	±0.5
HAET	83 c	±0.1	53 c	±0.4
Isopropyl alcohol	99 b	±0.15	92 a*	±0.3
Hexane	83 c	±0.2	38 e	±0.1
Heptane	80 c	±0.2	47 d	±0.5
Ethanol	86 c	±0.3	66 b	±0.4

[1]$p < 0.05$ Significant differences; SD standard deviation of three trials.

With acetone were quantified 39 µg/g (*C. sapidus*) and 44 µg/g (*C. bellicosus*), while with the mix HAET was obtained 39 µg/g (*C. sapidus*) and 51 µg/g (*C. bellicosus*), respectively; complete results are showed in Table 2.

Table 2. Astaxanthin extracted from crab powder exoskeletons quantified by HPLC.

Solvent	**µg/g**	**SD**	**µg/g**	**SD**
	Astaxanthin *C. sapidus*	**µg g^{-1}**	**Astaxanthin *C. bellicosus***	**µg g^{-1}**
Ethyl acetate	16 d	±0.3	22 c	±0.1
Acetone	39 a[1]	±0.4	44 a*	±0.2
Cyclohexane	13 d	±0.5	26 c	±0.5
HAET	39 a*	±0.1	51 a*	±0.4
Isopropyl alcohol	34 b	±0.1	31 c	±0.3
Hexane	20 c	±0.2	30 b	±0.1
Heptane	21 c	±0.2	25 c	±0.5
Ethanol	31 b	±0.3	37 b	±0.4

[1]$p < 0.05$ Significant differences; SD standard deviation of three trials.

3.2. Identification of astaxanthin type extracted.

Acetone was one of the most effective solvents to extract astaxanthin in this work; then, the next step was to identify the pigment types extracted by the use of chromatograms obtained by HPLC. Figure 1A shows that unsaponified extracts from shrimp headshells (L. vannamei) were mainly ester astaxanthin (more than 90 %). On the other hand, in brown crab (*C. bellicosus*) Figure 1B, 64.67 % of total astaxanthin was found in trans astaxanthin (peak 18) with 26.2 µg/g, and finally, in blue crabs (*C. sapidus*) 67.17 of total astaxanthin was found as a trans astaxanthin (28.43 µg/g) (Figure 1C). Table 3 shows the astaxanthin types identified in chromatograms, main astaxanthin esters and several geometrical isomers such as all-trans

astaxanthin, 9-cis astaxanthin, and 13-cis astaxanthin with their specific concentrations. It is important to mention that in carotenoids, the trans configuration has major useful applications than cis isomers. As shown in Figure 1a), the astaxanthin molecule in *L. vannamei* was found in an esterified form, normally with fatty acids.

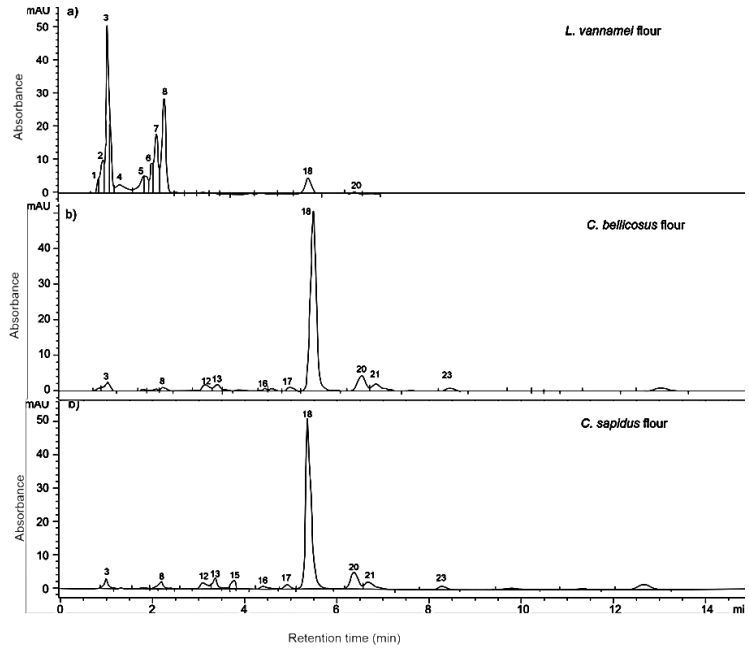

Figure 1. Chromatograms obtained with acetone hydrolysis of flour from: a) *L. vannamei*, b) *C. Sapidus*, and c) *C. bellicosus*.

3.3. Saponification of acetone hidrosilates from L. vannamei.

In order to de-esterify astaxanthin esters obtained from shrimp samples, it was necessary to apply a saponification process which under alkaline conditions results in its degradation, the use of 4% KOH achieved it, Table 3 show the results of ester separation in *L. vannamei*.

Table 3. Astaxanthin type molecules identified with acetone hydrolysis on HPLC.

Peak	Shrimp	µg/g	Blue crab	µg/g	Brown crab	µg/g
	L. vannamei		***C. sapidus***		***C. bellicosus***	
1	Ast ester	1.73 g	[1]ND	ND	ND	ND
2	Ast ester	6.61 e	ND	ND	ND	ND
3	Ast ester	26.22 a	Ast ester	1.0 c	Ast ester	0.97 d
4	Ast ester	9.72 d	ND	ND	ND	ND
5	Ast ester	5.60 f	ND	ND	ND	ND
6	Ast ester	5.65 f	Ast ester	0.1 d	Ast ester	0.26 d
7	Ast ester	12.54 c	Ast ester	0.24 d	Ast ester	0.28 d
8	Ast ester	20.40 b	Ast ester	0.37 d	Ast ester	0.67 d
10	ND	ND	ND	ND	ND	ND
11	Ast isomer	1.35 g	ND	ND	Ast isomer	1.32 c
12	Ast isomer	0.94 h	Ast isomer	1.23 c	Ast isomer	1.38 c
13	ND	ND	Ast isomer	0.85 d	ND	ND
14	ND	ND	ND	ND	ND	ND
15	Ast isomer	1.63 g	Ast isomer	0.22 d	Ast isomer	0.62 d
16	ND	ND	Dicis1-ast	0.29 d	ND	ND
17	Dici2-ast	0.78 h	Dicis2-ast	0.29 d	Dicis2-ast	0.75 d
18	Trans ast	6.25 e	Trans ast	26.2 a	Trans ast	28.43 a
19	ND	ND	ND	ND	ND	ND
20	9cis-ast	0.81 h	9cis-ast	2.9 b	9cis-ast	3.32 b
21	13cis-ast	0.38 h	13cis-ast	1.98 c	13cis-ast	1.8 c
22	ND	ND	Epoxy ast	0.70 d	Epoxy ast	0.85 d

[1]ND: not detected.

The main fraction obtained was trans astaxanthin (29.17 %) (with a specific concentration of 26.7 µg/g (Table 4), and as it happens in *C. sapidus* and *C. bellicosus*, also was obtained 9-cis astaxanthin and 13-cis astaxanthin.

Table 4. Astaxanthin type molecules identified with acetone hydrolysis and a posteriori saponification process.

Signal	*L. vannamei* Shrimp	µg g^{-1}
1	Ast ester	1.70 gh
2	Ast ester	2.88 fg
3	Ast ester	1.21 gh
4	Ast ester	1.33 gh
5	Ast ester	3.50 ef
6	Ast ester	6.26 bc
7	Ast ester	6.43 bc
8	Ast ester	4.49 de
10	[1]ND	ND
11	ND	ND
12	Ast isomer	4.65 de
13	ND	ND
14	ND	ND
15	ND	ND
16	Ast isomer	2.62 fg
17	Dici1-ast	2.64 fg
18	Dici2-ast	3.77 ef
19	Trans-ast	26.7 ab
20	ND	ND
21	9cis-ast	5.75 cd
22	13cis-ast	3.38 ef

[1]ND: not detected.

It is well known that astaxanthin can be esterified in one or both hydroxyl groups with some fatty acids such as palmitic, oleic, or linoleic acids [15]. In shrimp wastes, astaxanthin is found mainly in esterified form and normally associated with fatty acids (monoester or diester), and usually, less than 10 % is found in free form. With respect to the astaxanthin quantified in this work, Hu *et al*. (2019) reported 239 µg/g of astaxanthin extraction from shrimp shells of *Procamburus clarkia* when was used alcohol at 95% and ethyl acetate [16]. In this context, Khanafari *et al*. (2007) reported 23.28 µg/g of astaxanthin and its ethers extracted from shrimp wastes (*Penaeus semisulcatus*) [17]. Radzali *et al*. (2014) reported that with ethanol was obtained the highest amount of astaxanthin from *Peanus monodon* shrimp (70.26 µg/mg of astaxanthin) but when was used 50% methanol, the amount of astaxanthin was only 14.57 µg/g of astaxanthin [18]. Dalei and Sahoo (2015) reported the use of acetone as a better solvent to extract astaxanthin from shell shrimp wastes (48.64 mg/g) [19]. Other authors mentioned the use of solvent extraction, for example, some researchers reported the use of n-hexane: isopropyl alcohol (1:1 v/v) for astaxanthin extraction from shrimp waste (*Fanaeus indicus*) with 43.9 µg/g; acetone for pigment extraction from *Penaeus indicus* as a better choice with 40.6 µg/g and n-hexane isopropyl alcohol (6:4 v/v) from *Farfantepenaeus paulensis* with 53 µg/g. On the other hand, Yoon *et al*. (2012) reported a yield of 17.8 µg/g of astaxanthin extracted when ethanol was used [20]. As we can see, the amount of astaxanthin extracted on shrimp, blue crab, and brown crab with different solvents used in this work fall in the range reported by several authors. Khanafari *et al*. (2007) reported the extraction of astaxanthin esters from shrimp wastes *Penaeus semisulcatus* when was used diethylamine, hexane, acetone, and ethyl acetate as an organic solvent [17]. In *C. sapidus* (blue crab), Suganya and Asheeba (2015) reported the extraction of monoesters and diesters when astaxanthin was extracted with isopropanol; monoesters when was extracted with DMSO acetone, but its molecules were absent when was used acetone as a solvent [21]. Coral-Hinostroza and Bjerkeng (2002),

reported in *Pleuroncodes planipes* diesters; C14:0 (5-9%), C16:0 (17-30%), C20:0 (0-23%), C16 (12-15%) and C18:1 (15-24%) while C20:5 was the most predominant fraction (47%) [22]. Breithaupt (2004) identified astaxanthin esters in extracts of *Pandulus borealis*: astaxanthin-C16:0, astaxanthin-C16:0/C16:0, and free astaxanthin [23]. The method to remove it is by a saponification process; however, the de-esterification of natural astaxanthin esters is rarely published. In this context, the same author reported the highest efficiency of cholesterol esterase to remove astaxanthin's fatty acids than the saponification process with NaOH. The effectiveness depends basically on time. The effectiveness depends basically on time between 60 to 90 minutes. In this work, all-trans astaxanthin was the predominant isomer, followed by astaxanthin isomers, dici1, dici2, 9-cis, and 13-cis astaxanthin.

4. Conclusions

Fresh wastes of Mexican headshell shrimp (*Litopenaeus vannamei*), blue crab (*Callinectes bellicosus*), and Mexican brown crab (*Callinectes sapidus*) exoskeletons were autoclaved, dried, and grounded at a particle size of 150 µm. Macerated samples were diluted to ethyl acetate, acetone, cyclohexane, isopropyl alcohol, hexane, heptane, and a combination of hexane-acetone-ethanol-toluene solvents. High-Performance Liquid Chromatography analysis determined the astaxanthin amount in every organic solvent extract. Acetone was the most efficient solvent; the mixture of hexane-acetone-ethanol-toluene resulted in an idoneus solvent used on crab exoskeletons.

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Conflicts of Interest

The authors declare no conflict of interest.

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