

# Effects of Shrimp Waste Types and Their Cooking on Properties of Extracted Astaxanthin and Its Characteristics in Liposomes

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**Abstract** This study was aimed to evaluate the effects of shrimp waste types (fresh head, cooked head, fresh shell and cooked shell) and of cooking these wastes on the properties of astaxanthin. The astaxanthin was subject to liposomal encapsulation and its characteristics were determined. Astaxanthin was extracted from fresh and cooked (90°C for 15 min) materials, and then yield, astaxanthin content, antioxidant activities (DPPH and ABTS scavenging assays) and fatty acid profiles were determined. Phospholipid was used at various concentrations (2, 3 and 4 % w/v) to prepare astaxanthin-loaded liposomes, which were further evaluated for particle size, zeta potential, antioxidant activity (DPPH scavenging assays) and outer structure by confocal laser scanning microscope. The results showed that fresh shrimp heads gave the highest percent yield (30.49 mg extract/g raw material), but the highest astaxanthin content was found in fresh shrimp shells (14.65 mg/g). Shrimp heads and shrimp shells, both fresh and cooked, were significantly different ( $p < 0.05$ ) in terms of their antioxidant activity (based on DPPH and ABTS scavenging assays). Three fatty acids, palmitic acid, oleic acid and linoleic acid were most abundant and found in all samples. However, extract from fresh shrimp shells showed the highest EPA and DHA content of 10.48 % and 11.62 %, respectively. Astaxanthin-loaded liposomes derived were different in size in that the higher concentration, the larger liposomes produced. Liposomes prepared from 4% (w/v) phospholipid at 50 amplitude gave the highest %DPPH scavenging activity of 79.23.

**Keywords:** Astaxanthin, shrimp waste, cooked shrimp head, cooked shrimp shell, fatty acid, liposome

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## 1. Introduction

Approximately 6-8 million tons of crustacean waste is generated annually worldwide, based on a recent FAO report [1]. In Thailand, Pacific white shrimp (*Litopenaeus vannamei*) is the most common species produced, with an estimated production of 263,000 tons each year [2]. Depending on the processing method, 45–60 % of the shrimp becomes by-products (primarily heads and shells) [3]. These materials may not be useful as food products, but they do contain valuable nutrients and other compounds, including chitin, protein, lipid, and carotenoid pigments [4,5,6,7].

Astaxanthin (3,3-dihydroxy- $\beta$ , $\beta$ -carotene-4,4-dione) is a member of the xanthophyll family of carotenoid pigments, and is present in aquatic animals, including shrimps, crabs, salmon, as well as many other species [8].

Astaxanthin is of increasing interest as an antioxidant, as it exhibits antioxidant activity 100-times higher than  $\alpha$ -tocopherol, and 10-times higher than other carotenoids such as lutein, canthaxanthin, zeaxanthin and  $\beta$ -carotene [9]. Astaxanthin has been linked to a reduced risk of cancers, diabetes, metabolic syndrome, chronic inflammatory diseases and cardiovascular diseases in humans [10].

Besides astaxanthin, lipids are also found in shrimp by-products. [11] indicated that the hepatopancreas of shrimp are rich in PUFAs (polyunsaturated fatty acids). [12] reported that Northern shrimp (*Pandalus borealis* Kreyer) by-products are a source of omega-3 polyunsaturated fatty acids. Because of their antioxidant properties, both astaxanthin and polyunsaturated fatty acids (in particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) may be beneficial for human health such as reducing cardiovascular risk, cancer and anti-inflammation [13]. To our knowledge, few studies have been reported on the difference in properties

of astaxanthin and in fatty acid profiles of astaxanthin extracted from fresh and cooked shrimp heads and shrimp shells. [14] reported that heat can lead to degradation and isomerization of astaxanthin. In addition, [15] examined the effects of cooking on astaxanthin with fatty acids extracted from Pacific white shrimp, and reported that astaxanthin monoesters with EPA and DHA had a lower thermal stability than those with saturated fatty acids. Currently, the application of astaxanthin in different food formulations is limited due to their easy degradation in the presence of heat, oxygen and light. Encapsulation technology is considered to be a way of solving these problems.

One encapsulation technique uses liposomes, which are spherical colloidal systems of a phospholipid bilayer with an aqueous internal core, and are commonly used to encase the active agents in pharmaceuticals, cosmetics, foods and nutrition supplements, as well as coatings. [16] showed that the liposome can improve stability of natural astaxanthin derived from white shrimp shells. Liposomes have been studied as delivery systems for improvement of the physical and chemical stabilization of protein hydrolysates from croaker byproduct [17]. [18] reported that liposome decreased the thermal oxidation of astaxanthin extracted from shrimp waste. In addition, [19] indicated that liposome made from phospholipid (phosphatidyl choline 70%) at 2% (w/v) maintained the antioxidant activities of astaxanthin obtained from shrimp shell and the liposome showed a high bioavailability by cellular uptake using cell culture.

The objectives of this study were to determine the properties of astaxanthin and fatty acid profiles found in astaxanthin of fresh and cooked shrimp heads and shrimp shells. In addition, creation of astaxanthin-loaded liposomes using ultrasonic atomizer was conducted and their properties were evaluated.

## 2. Materials and Methods

Fresh heads and shells of white shrimp (*Litopenaeus vannamei*) were obtained from a frozen shrimp processor in Samut-Sakorn Province, Thailand. Astaxanthin standard (97% (w/w) purity) was obtained from Sigma-Aldrich Co. (Mo, USA). Phospholipid containing 70% phosphatidylcholine (70% PC, Lipoid®P75) was obtained from Lipoid GmbH (Ludwigshafen, Germany).

### 2.1. Extraction of Astaxanthin from Fresh Shrimp Heads and Shrimp Shells

Fresh shrimp heads and shrimp shells were extracted for astaxanthin using ethanol. In brief, the raw materials were mixed with ethanol (raw material:ethanol, 1:2 w/v) and filtrated through filter paper (Whatman No. 1). The extraction was repeated in triplicate. Solvent was removed with a rotary evaporator (under vacuum at 40°C, 175 MPa) and the resulting concentrate was analyzed for astaxanthin quantity using reverse phase High Performance Liquid Chromatography (HPLC) followed the method of [20]. The extracts were kept in the dark bottle at -18°C for further investigation.

### 2.2. Extraction of Astaxanthin from Cooked Shrimp Heads and Shrimp Shells

Fresh shrimp heads and shrimp shells were cooked at 90°C for 15 min. The cooked heads and shells were then further extracted for astaxanthin using the same procedure as for fresh materials as described above in 2.1.

### 2.3. The Yield of the Extract

The yield of the extract was calculated using the equation (1)

$$\%yield = (w_0 / w_1) \times 100 \quad (1)$$

Where  $w_0$  and  $w_1$  are weight of extract and raw material, respectively.

### 2.4. Analysis of Astaxanthin Content

The extract obtained from both fresh and cooked materials was determined for astaxanthin content by HPLC (Agilent Technologies, Santa Clara, CA, USA), UV/vis detector and C18 column (ZORBAX-Eclipse Plus, 150 mm x 4.6 mm). Samples (20 microliters) were eluted with a mobile phase consisting of 85% methanol, 5% acetonitrile, 5% dichloromethane, and 5% water at a flow rate of 1.0 ml/min and the eluate absorbance was measured at 480 nm. Astaxanthin was identified by its retention time against pure astaxanthin standard. The content was indicated as mg of astaxanthin per g of waste material [20].

### 2.5. Extraction of Lipid from the Extract

The extraction of lipids from astaxanthin extract was conducted using solvent according to [21]. The astaxanthin extract was mixed with chloroform: methanol: distilled water (2:1:2, v/v), was added. After extraction, the obtained lipid was kept for further analysis.

### 2.6. Analysis of Fatty Acids Profile

Fatty acids were analyzed by methylation method of [22]. The extracted lipid (25 mg) was derivatized into FAMES (fatty acid methyl esters) using 0.5 M sodium methoxide in anhydrous methanol and acetyl chloride in anhydrous methanol. The resulting FAMES were analyzed by gas chromatograph and Supelco 37 component FAME MIX was used as a standard. Fatty acid profiles were determined using an Agilent 7820A gas chromatograph equipped with FID detector. Separation was performed with an Agilent HP-88 column with split injection (40:1) and helium at a constant flow of 1.2 mL/min. Detector temperature was 260°C and injector temperature was 250°C. The temperature profile of the oven was 170°C which then increased by 5°C /min to 240°C and then held at 240°C for 45 min. Identification was based on comparison of the retention times with standards. Relative percentages of the identified fatty acids were calculated.

## 2.7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH radical scavenging activity of the extracts was measured following the method of [23]. A 0.2 mM solution of DPPH in ethanol was mixed with 2 mL of sample solution at different concentrations (10, 25, 50 µg/ml). The solution was incubated for 30 min in the dark. The absorbance of samples was measured at 517 nm using a 96-well micro-plate (PowerWave XS2, BioTek, Winooski, VT, USA). The %DPPH scavenging effect was found using the following equation (2):

$$\text{DPPH scavenging effect (\%)} = \left[ A_0 - \left( (A - A_b) / A_0 \right) \right] \times 100 \quad (2)$$

Where  $A_0$ ,  $A_b$  and  $A$  are the absorbance of DPPH solution without the sample, the sample without DPPH solution and the test sample mixed with DPPH solution, respectively.

## 2.8. ABTS 2,2'-azino-bis(3 ethylbenzothiazoline-6-sulphonic acid) (Radical Scavenging Activity)

ABTS radical scavenging activity was measured using the method of [24]. The working solution was prepared by combining 20 mL of 7 mmol/L ABTS<sup>•+</sup> with 352 µl of a 140 mmol/L potassium persulphate solution. This mixture was allowed to react for 12–16 h at room temperature in darkness. Various concentrations of samples (100 µL) were allowed to react with 100 µL ABTS<sup>•+</sup> working solution. Absorbance at 734 nm of samples and control was measured in a 96-well microplate in the dark using a Power Wave XS2 (BioTek, Winooski, VT, USA). The %ABTS scavenging effect was determined by the following equation (3):

$$(1 - A_t / A_0) \times 100\% \quad (3)$$

Where  $A_0$  and  $A_t$  are absorbance of control (the ethanol and ABTS with a working solution) and test sample, respectively.

## 2.9. Preparation of Astaxanthin-loaded Liposome

Liposomes were prepared following the method of [25]. Briefly, phospholipid was dissolved in methanol (2, 3 and 4 % w/v), then astaxanthin (2% w/v) was added in solution and evaporated using evaporator to obtain a film. The dried film was dissolved in methanol and evaporated, then dissolved with 50 ml distilled water, giving a homogeneous suspension of phospholipid. Liposomes were produced using ultrasonic atomizer at an amplitude of 50 and 65 for 15 min. The liposomes were determined for DPPH and ABTS activity as described above.

## 2.10. Sizes and Zeta Potentials of Liposomes

The particle size and zeta potential of the liposomes were acquired by the dynamic light scattering technique using a Zetasizer Nano ZS model (Malvern Instruments,

Worcestershire, UK). The liposomes were diluted with distilled water 10 times prior to determination. Measurements were made at least three times, from which a mean diameters ( $D_{43}$ ) value $\pm$ S.D. was calculated.

## 2.11. Confocal Microscopy

The liposomes were added with Nile red (0.1% v/v) and stirred for 1 hr at room temperature. The samples were transferred onto a concave microscopy slide and covered with a coverslip. Images were taken using a confocal laser-scanning microscope (OLYMPUS FV10i-DOC (Tokyo, Japan)) with a 639 magnification oil immersion objective at 543 nm.

## 2.12. Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare treatments. Differences between means were evaluated based on confidence intervals using the Duncan test, with a significance level of  $p < 0.05$ .

# 3. Results and Discussion

## 3.1. Yield of Extract and Astaxanthin

Table 1 shows yields of extract and astaxanthin content from shrimp heads and shells. The yields of extract from fresh shrimp heads, cooked shrimp heads, fresh shrimp shells and cooked shrimp shells were 30.49, 29.50, 15.33, 13.61 mg/g raw material, respectively, while astaxanthin content was 3.64, 2.38, 14.65 and 11.76 mg/g of crude extract, respectively. It was found that shrimp heads gave a higher yield but lower astaxanthin content than shrimp shells. This might be explained by the fact that shrimp heads contain more lipid, which normally come from the hepatopancreas, than the shells [26]. The results are in agreement with [4] who found that the extract from shrimp heads had higher yield but less astaxanthin than shrimp shells. [27] also mentioned that more astaxanthin was found in shrimp shells than other carotenoids. When comparing fresh and cooked material, fresh shrimp shells had the highest astaxanthin content among treatments ( $p < 0.05$ ). Heat affects the degradation of astaxanthin; this can be shown by decrease in astaxanthin from fresh shrimp shells (14.65 mg/g of crude extract) to cooked shrimp shell (11.76 mg/g of crude extract), a reduction of 19.73%. According to a study by [15] showed that thermal processing (microwave, boiling and frying) decreased the amount of astaxanthin compared to fresh material (Pacific white shrimp).

**Table 1. Yield of extract and astaxanthin content obtained from different parts of shrimp waste**

Samples	Yield (mg/g of material)	Astaxanthin content (mg/g of crude extract)
Fresh head	30.49 $\pm$ 0.39 <sup>a</sup>	3.64 $\pm$ 0.33 <sup>c</sup>
Cooked head	29.50 $\pm$ 0.54 <sup>a</sup>	2.38 $\pm$ 0.20 <sup>d</sup>
Fresh shell	15.33 $\pm$ 0.19 <sup>b</sup>	14.65 $\pm$ 0.31 <sup>a</sup>
Cooked shell	13.61 $\pm$ 0.33 <sup>c</sup>	11.76 $\pm$ 0.46 <sup>b</sup>

Values are mean  $\pm$  standard deviation of three different experimental results. Means within columns that share letter, are significantly different ( $p < 0.05$ ).

**Table 2. Fatty acid profiles of lipid extract of astaxanthin from different types of shrimp waste**

Fatty Acid	Name	%fatty acid			
		fresh head	cooked head	fresh shell	cooked shell
C6:0	Caproic acid	-	-	0.10±0.00 <sup>a</sup>	0.05±0.00 <sup>b</sup>
C8:0	Caprylic acid	-	-	0.22±0.01 <sup>a</sup>	0.15±0.00 <sup>b</sup>
C10:0	Tridecanoic acid	0.22±0.02 <sup>a</sup>	0.22±0.02 <sup>a</sup>	0.16±0.01 <sup>b</sup>	0.11±0.00 <sup>c</sup>
C14:0	Myristic acid	0.73±0.01 <sup>a</sup>	0.78±0.01 <sup>a</sup>	0.43±0.06 <sup>b</sup>	0.46±0.01 <sup>b</sup>
C15:0	Pentadecanoic acid	0.51±0.01 <sup>a</sup>	0.55±0.00 <sup>a</sup>	0.35±0.01 <sup>b</sup>	0.37±0.00 <sup>b</sup>
C16:0	Palmitic acid	19.43±0.23 <sup>b</sup>	19.71±0.06 <sup>a</sup>	16.11±0.28 <sup>d</sup>	17.34±0.21 <sup>c</sup>
C17:0	Heptadecanoic acid	1.64±0.02 <sup>c</sup>	1.22±0.02 <sup>d</sup>	3.11±0.09 <sup>a</sup>	2.24±0.03 <sup>b</sup>
C18:0	Stearic acid	8.09±0.09 <sup>b</sup>	7.42±0.05 <sup>d</sup>	10.23±0.23 <sup>a</sup>	7.89±0.04 <sup>c</sup>
C20:0	Arachidic acid	-	-	0.13±0.02 <sup>a</sup>	0.06±0.01 <sup>b</sup>
C21:0	Heneicosanoic acid	0.98±0.01 <sup>b</sup>	0.91±0.03 <sup>c</sup>	1.21±0.04 <sup>a</sup>	0.97±0.05 <sup>b</sup>
C22:0	Behenic acid	0.14±0.00 <sup>c</sup>	0.18±0.10 <sup>b</sup>	0.20±0.01 <sup>a</sup>	0.15±0.02 <sup>d</sup>
C24:0	Lignoceric acid	-	-	0.11±0.01	-
∑SFA		31.78±0.30 <sup>b</sup>	31.11±0.07 <sup>c</sup>	32.35±0.71 <sup>a</sup>	29.78±0.21 <sup>d</sup>
C14:1	Methyl myristoleate	0.14±0.00 <sup>b</sup>	0.16±0.00 <sup>a</sup>	0.07±0.00 <sup>c</sup>	-
C15:1	cis-10-Pentadecenoic acid	-	-	0.08±0.04	-
C16:1	Palmitoleic acid	1.60±0.02 <sup>b</sup>	1.72±0.01 <sup>a</sup>	0.96±0.03 <sup>d</sup>	1.10±0.02 <sup>c</sup>
C17:1	cis-10-Heptadecenoic acid	0.26±0.00 <sup>b</sup>	0.27±0.01 <sup>a</sup>	0.18±0.00 <sup>c</sup>	0.18±0.03 <sup>c</sup>
C18:1n9t	trans-9-octadecenoic acid	0.42±0.01 <sup>a</sup>	0.34±0.06 <sup>b</sup>	0.17±0.08 <sup>d</sup>	0.28±0.17 <sup>c</sup>
C18:1n9c	Oleic acid	14.62±0.08 <sup>b</sup>	14.54±0.06 <sup>a</sup>	12.77±0.08 <sup>d</sup>	12.98±0.04 <sup>c</sup>
C20:1	cis-11-Eicosenoic acid	0.18±0.01 <sup>a</sup>	0.15±0.04 <sup>b</sup>	0.18±0.02 <sup>a</sup>	0.11±0.02 <sup>c</sup>
C24:1n9	Nervonic acid	-	-	0.18±0.01 <sup>a</sup>	0.17±0.00 <sup>b</sup>
∑MUFA		17.07±0.30 <sup>a</sup>	16.96±0.38 <sup>a</sup>	14.56±0.12 <sup>c</sup>	14.83±0.22 <sup>b</sup>
C18:2n6t	Linolelaidic acid	0.24±0.01 <sup>b</sup>	0.23±0.01 <sup>c</sup>	0.27±0.01 <sup>a</sup>	0.16±0.01 <sup>d</sup>
C18:2n6c	Linoleic acid	18.14±0.16 <sup>b</sup>	18.87±0.07 <sup>a</sup>	16.04±0.05 <sup>c</sup>	16.06±0.20 <sup>c</sup>
C18:3n3	Linolenic acid	1.22±0.02 <sup>b</sup>	1.29±0.05 <sup>a</sup>	0.91±0.01 <sup>c</sup>	0.90±0.01 <sup>c</sup>
C18:3n6	γ-Linolenic acid	0.20±0.00 <sup>b</sup>	0.18±0.01 <sup>c</sup>	0.27±0.01 <sup>a</sup>	0.16±0.01 <sup>d</sup>
C20:2	Eicosadienoic acid	1.72±0.01 <sup>c</sup>	1.68±0.01 <sup>d</sup>	2.33±0.00 <sup>a</sup>	2.12±0.01 <sup>b</sup>
C20:3n6	Eicosatrienoic acid	-	-	0.12±0.01 <sup>a</sup>	0.11±0.00 <sup>a</sup>
C20:3n3	Eicosatrienoic acid	0.34±0.00 <sup>b</sup>	0.32±0.01 <sup>c</sup>	0.35±0.01 <sup>a</sup>	0.28±0.00 <sup>d</sup>
C20:4n6	Arachinodic acid	3.73±0.03 <sup>c</sup>	3.64±0.04 <sup>d</sup>	4.56±0.03 <sup>a</sup>	4.46±0.05 <sup>b</sup>
C22:2n6	Docosadienoic acid	0.15±0.00 <sup>b</sup>	0.17±0.00 <sup>a</sup>	0.11±0.00 <sup>c</sup>	0.11±0.00 <sup>c</sup>
C20:5n3	Eicosapentaenoic acid	8.47±0.07 <sup>c</sup>	8.43±0.11 <sup>c</sup>	10.48±0.16 <sup>a</sup>	9.38±0.18 <sup>b</sup>
C22:6n3	Docosahexaenoic acid	8.87±0.05 <sup>d</sup>	9.19±0.13 <sup>c</sup>	11.62±0.16 <sup>a</sup>	11.38±0.04 <sup>b</sup>
∑PUFA		43.04±0.35 <sup>c</sup>	43.28±1.40 <sup>b</sup>	43.95±0.33 <sup>b</sup>	46.14±0.47 <sup>a</sup>
other		8.11±0.83 <sup>c</sup>	8.08±0.95 <sup>c</sup>	9.14±0.50 <sup>b</sup>	9.24±0.55 <sup>a</sup>

Values are mean ± standard deviation of three different experimental results. Means within columns that share letter, are significantly different ( $p < 0.05$ ).

### 3.2. Fatty Acid Profile of Lipid Extract of Astaxanthin

Table 2 shows a summary of polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) from shrimp waste extracts. The results showed that SFAs were found in a range of 29.78 - 32.35% of all fatty acids, whereas MUFAs comprised 14.56 % to 17.07 % and PUFAs comprised 43.04 % to 46.14 %. Linoleic acid, oleic acid

and palmitic acid were the three main fatty acids found in the extract, and shrimp heads contained higher amounts of these than shrimp shells. These are major fatty acids found in triglycerides of organisms that can reduce cholesterol and LDL, which cause fatty deposits in the blood vessels [28]. However, the highest EPA and DHA was found in fresh shrimp shells (10.48 and 11.62 respectively). Both have powerful anti-inflammatory functions within the body [29] and also have antioxidant properties [30]. [31] reported that the EPA and DHA content in shrimp waste was 6.71 % and 9.85 %, respectively.

### 3.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) Radical Scavenging Activity

DPPH and ABTS scavenging assays are frequently used to determine primary antioxidant. The DPPH antioxidant assay relies on the characteristics of DPPH, a stable free radical that contains an odd electron, while the ABTS radical cation is stable for several minutes at room temperature and reacts immediately and quantitatively with several antioxidants over a wide pH range [32].

The antioxidant activity of the four different samples is shown in Table 3. Percent scavenging of DPPH and ABTS were similar; there was very small significant difference ( $p < 0.05$ ) of % scavenging between fresh shrimp shell and fresh shrimp head but cooked shrimp heads showed markedly lower activity ( $p < 0.05$ ). More astaxanthin was found in shrimp shells, (Table 1) while shrimp heads had higher total fat content than shrimp shells by approximately three times (Table 1). Therefore both astaxanthin and some fatty acids, especially EPA and DHA, might contribute to overall antioxidant activity. This is supported by a previous report of [30], which indicated that both EPA and DHA had antioxidant properties. Moreover, astaxanthin contains long, conjugated double bonds which act as antioxidants by scavenging radicals and by quenching singlet oxygen. According to the report of [26], they also found high total lipid in both the cephalothorax (10.5% d.w.) and exoskeleton (3.78% d.w.).

**Table 3. % DPPH and %ABTS scavenging of the extract from shrimp heads and shrimp shells**

Sample	%DPPH scavenging	%ABTS scavenging
Fresh head	78.36±0.30 <sup>c</sup>	90.07±0.12 <sup>b</sup>
Cooked head	67.18±0.08 <sup>d</sup>	79.19±0.06 <sup>e</sup>
Fresh shell	79.23±0.01 <sup>a</sup>	91.63±0.14 <sup>a</sup>
Cooked shell	78.51±0.10 <sup>b</sup>	91.32±0.21 <sup>a</sup>

Values are mean ± standard deviation of three different experimental results. Means within columns that share letter, are significantly different ( $p < 0.05$ ).

### 3.4. The Physical and Chemical Properties of Astaxanthin-loaded Liposome

Zeta potential reflects the difference in electric potential between a substance containing insoluble, suspended

particles (colloid) and the layer of fluid attached to those particles. The measurement of zeta potential is commonly used to predict colloidal system stability [33]. Colloids with high zeta potential (either negative or positive) are more stable, while colloids with zeta potential near zero are more likely to coagulate or flocculate [34]. The results showed that zeta potential of the astaxanthin-loaded liposomes was in the range of -17.05 to -35.75 mV (Table 4). Particles with zeta potential value more negative than -30 mV or more positive than 30 mV have relatively high repulsive interaction and are considered to be stable [35].

Phospholipids contain up to 70% of phosphatidylcholine; the electrostatic charge of phosphatidylcholine is neutral. Therefore, when the amount of phospholipid is increased, the charge will be more neutral. The results are in agreement with [36], who studied the effect of phosphatidylcholine from sunflower oil (50%, 65%, 75% and 90%), and found that the zeta potential reached nearly zero with an increase of the phosphatidylcholine.

Results of the particle size study showed that astaxanthin-loaded liposomes had a diameter ranging from 98.32 to 114.33 nm, as shown in Table 4. As the phospholipid content increased, the size of liposomes significantly increased ( $p > 0.05$ ) as was also reported by [37]. The phospholipids are an important part of the formation of liposomes; as the amount of phospholipids increases, the particle size increased.

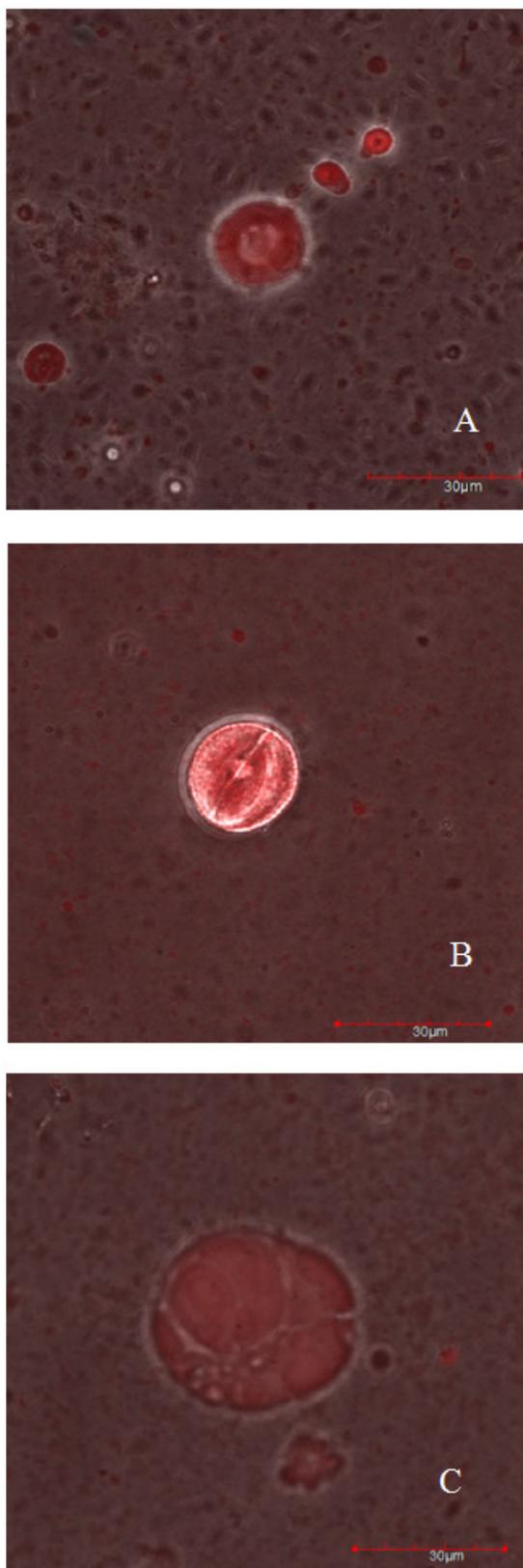
The antioxidant activity of astaxanthin extract and astaxanthin-loaded liposomes is reflected by the percentage of scavenging of DPPH (Table 4). It was found that the percentage of scavenging of DPPH of the astaxanthin extract was 79.36, while the percentage of scavenging of DPPH of the astaxanthin-loaded liposomes was in the range of 68.31 to 79.23. When observing the antioxidant efficacy of astaxanthin after storage in liposomes, it was found that astaxanthin still retains antioxidant efficiency close to that of astaxanthin before storage. Moreover, the amplitude used in generating liposomes did not significantly affect antioxidant activity.

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The measurement of zeta potential is commonly used to predict colloidal system stability [33]. While, the colloids with high zeta potential (negative or positive) are electrically stabilized, on the other hand, colloids with low zeta potentials tend to coagulate or flocculate [34]. The results showed that zeta potential of the astaxanthin-loaded liposome was in range -17.05 to -35.75 mV (Table 4).

**Table 4. The physical-chemical properties of astaxanthin -loaded liposome**

concentration of phospholipid (%w/v)	an amplitude of ultrasonic atomizer	size of particles	zeta potential	%scavenging (DPPH)
crude extract		-	-	79.36±0.30 <sup>a</sup>
2		104.70±0.66 <sup>b</sup>	-35.75±0.78 <sup>e</sup>	77.42±0.06 <sup>c</sup>
3	50	101.90±0.56 <sup>c</sup>	-20.35±0.35 <sup>b</sup>	77.69±0.13 <sup>c</sup>
4		113.43±1.10 <sup>a</sup>	-30.95±0.64 <sup>d</sup>	79.23±0.01 <sup>a</sup>
2		98.32±0.76 <sup>d</sup>	-30.95±0.64 <sup>d</sup>	68.31±0.29 <sup>e</sup>
3	65	104.97±1.00 <sup>b</sup>	-24.50±0.00 <sup>c</sup>	76.43±0.06 <sup>d</sup>
4		114.33±0.52 <sup>a</sup>	-17.05±0.92 <sup>a</sup>	78.07±0.14 <sup>b</sup>

Values are mean ± standard deviation of three different experimental results. Means within columns that share letter, are significantly different ( $p < 0.05$ ).



**Figure 1.** Images of liposomes produced using ultrasonic atomizer at 50 amplitude at various concentrations of phospholipid: (A) 2% phospholipid (w/v), (B) 3% phospholipid, (C) 4% phospholipid

### 3.5. Microstructure

The microstructure of the astaxanthin-loaded liposomes was examined by confocal microscopy. The astaxanthin extract can be dyed with Nile red, which appears red as

shown in Figure 1. It was found that the liposome particles were spherical shape by forming of phospholipid in liposome. Phospholipids are known as amphipathic, which consists of two parts: polar or water soluble and non-polar or fat soluble [38]. Therefore, when added to water, the water-soluble part of the phospholipid interacts with the water and the oil-like part avoids the water. Afterward, it arrange to form bilayer, which then curls into liposome [39]. The bilayer of liposomes was filled with molecules of astaxanthin that are oil-like and can be encapsulated in the bilayer of the liposome thus the results showed the red part in particle of liposome.

## 4. Conclusion

Extract obtained from fresh shrimp shells yielded the highest astaxanthin, EPA and DHA content compared to other treatments, and can contribute to enhanced antioxidant properties. Our results indicated that cooking slightly affected the astaxanthin content and antioxidant activity in fresh shrimp shells but in shrimp heads. In terms of application, astaxanthin-loaded liposomes prepared with a higher concentration of phospholipid generally had a larger particle size and zeta potential. However, all liposomes had spherical shape and were effective in terms of their antioxidant properties. The results of this study could be applied in various food products.

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