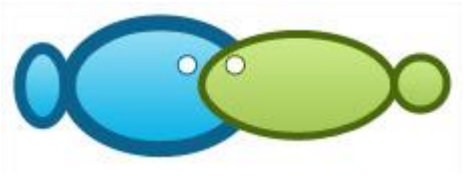

The natural antioxidant potential of astaxanthin extracted from the whiteleg shrimp (*Litopenaeus vannamei*) carapace

Rahman Karnila, Edison, Nintha Pratiwi, Santhy W. Sidauruk

Department of Fishery Products Processing, Faculty of Fisheries and Marine Science, Riau University, Riau, Indonesia. Corresponding author: R. Karnila, karnilarahman@gmail.com

Abstract. The use of whiteleg shrimp, *Litopenaeus vannamei*, produces carapace waste which is not properly used. Carapace of *L. vannamei* is assumed to have astaxanthin compounds as carotenoid pigments and has potential as natural antioxidant. This study aimed to determine: the best extraction period of astaxanthin compounds by using organic chloroform solvents, and the antioxidant capacity of astaxanthin in *L. vannamei*. A Completely Randomized Design (CRD) experimental method was used with three extraction periods, of 24, 48, and 72 hours, with a yield of astaxanthin raw extract of 2.49, 2.61 and 3.02%, respectively. Based on the results of HPLC chromatograms, astaxanthin in *L. vannamei* carapace has the same pattern as the standard astaxanthin, which is about 1 minute retention period. Moreover, astaxanthin level in *L. vannamei* carapace, show that an extraction of 72 hours is optimal, with the highest IC50 value: 27.18 mg mL^{-1}. However, the antioxidant activity is weak compared to standard astaxathin.
Key Words: pigment, extraction period, chloroform, HPLC, maceration.

Introduction. *Litopenaeus vannamei* (whiteleg shrimp) is one of the widely consumed shrimps species in Indonesia. The shrimp production generates wastes, in particular shell. In the shrimp freezing process, 60-70% of the weight of shrimp is removed, especially its skin and head. It is estimated that aggregated shell waste for all shrimp processing units in Indonesia is 325,000 tons $year^{-1}$ (Ministry of Maritime Affairs and Fisheries 2016). The waste of shell causes environmental problems, but it simultaneously contains several potential bioactive compounds, such as astaxanthin (Holanda & Netto 2006), carotenoids, protein, chitin, minerals (Pacheco et al 2009). According to Ngginak et al (2013), the shrimp shells or shrimp exoskleton parts contain 8.1 mg kg^{-1} astaxanthin, in higher concentration than its meat with 3.2 mg kg^{-1}.

Astaxanthin is a type of carotenoid, which are powerful antioxidants, 40 times more effective than beta carotene and 550 times stronger than vitamin E (Santocono et al 2006). The advantage of astaxanthin is not to switch ito pro-oxidant (McNulty et al 2006). Astaxanthin is a natural antioxidant with some advantageous properties: prevention of cardiovascular and immune systems diseases (Kurihara et al 2002), anti-cancer (Tanaka et al 1995), anti-aging, functional food, pharmaceutical, cosmetics and fodder (Holanda & Netto 2006; Goto et al 2001).

Among the for methods used for theastaxanthin compounds extraction can be mentioned: the oil-soluble method (Li et al 2015; Handayani et al 2008; Sachindra & Mahendrakar 2005; Dong et al 2014), the super crystalline carbon dioxide extraction method (Radzali et al 2014; Sanchez-Camargo et al 2012), the enzymatic hydrolysis methods (Holanda & Netto 2006) and the solvent extraction methods, including: ethanol solvents (Zhang et al 2014), acetone, methanol, hexane (Dong et al 2014), isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether (Sachindra et al 2006). However, further developments are needed to find the best method to extract astaxanthin compounds. One of the best method is extraction with chloroform organic solvents by maceration. The maceration period will determine the yield and antioxidant capacity. The current study aimed to: 1) determine the best extraction period of

astaxanthin compounds by using organic chloroform solvents and 2) determine the antioxidant capacity of astaxanthin in *L. vannamei.*

Material and Method

Materials. *L. vannamei* carapace waste, especially its skin and head, were collected from local markets (Pekanbaru, Indonesia). The solvents used for astaxanthin extraction were chloroform (Merck, Germany), KOH (Merck, Germany), and methanol (Merck, Germany). The analytes were asthin force (SOHO global health, Indonesia) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Merck, Germany).

Instruments. The following tools wereused: rotary evaporator (RV 10 digital V), vacuum, freezer, ultrasonic cleaner (Elma Elmasonic S40H, Germany), HPLC CDGU-20A5 (Shimadzu), UV spectrophotometer (Shimadzu), microplate reader TriStar LB 941 (Berthold Technologies GmbH).

Astaxanthin extraction. The extraction of astaxanthin from the carapace of *L. vannamei* starts with the preparation by reducing the size using blender and by sifting its flour with a mesh (Karnila 2012). Moreover, according to Saleha & Murniana (2009), with some modifications, the extraction process by maceration uses a flour to chloroform ratio 1:4 (w/v), with different treatment periods: 24, 48 and 72 hours. After 12 hours of maceration, the mixture is centrifuged at 4,000 rpm. The resulting supernatant is saponified with additional 100 mL of saturated KOH in methanol, then packaged in dark colored bottles and coated with aluminum foils to be stored for 24 hours. Separation of the soap is done by vacuum filtering. The filtrate was placed in separating funnel, washed by methanol and concentrated with vacuum rotary evaporator at 30-40°C temperature to obtain raw extract.

Identification of astaxanthin content. Identification of astaxanthin content using HPLC, i.e. 0.1 g of *L. vannamei* shell extract was reacted in 5 mL of methanol then exposed to ultrasonic waves, so that the sample and solution were homogeneous. Afterwards, 20 µL of sample (with particles size of 5 µm) were taken by syringe and injected into the HPLC through the ODS column (25 cm X 4.6 mm). The sample was eluted by mixture of water methanol (elution gradient of 50-90 methanol). The elution process was monitored with a visible detector with a wavelength of 479 nm. The results are obtained in the form of two chromatograms (Lee et al 1999 with some modifications).

Measuring antioxidant capacity. The measurement of antioxidant capacity of astaxanthin extract in *L. vannamei* carapace uses the DPPH method (Zhang et al 2006) at wavelength of 520 nm. Free radical capture activity was measured as the degradation of DPPH absorbance with microplate reader, against an astaxanthin positive control of 10,000 µg mL^{-1} concentration. The antioxidant activity of the sample is determined by the magnitude of DPPH radical absorption inhibition, calculated using the formula (Zhang et al 2006):

$$\text{Inhibition value (\%)} = 100 \times (1 - \text{absorbance of sample})/\text{absorbance of control}$$

Based on the IC50 value, the concentration of the sample solution needed to reduce DPPH free radicals is 50%.

Statistical analysis. The experimental method of this study was a completely randomized design (CRD) for three treatment levels (24, 48 and 72 hours). The collected data from each treatment were analyzed with a significance level of $P<0.05$ using the analysis of variance (ANOVA). All essays were repeated 3 times. When the results were significantly different, data was further analyzed using the Tukey advanced test.

Results and Discussion

The yield of astaxanthin raw extract. The yield of astaxanthin raw extract in *L. vannamei* carapace has different results in each treatment, that can be seen in Figure 1.

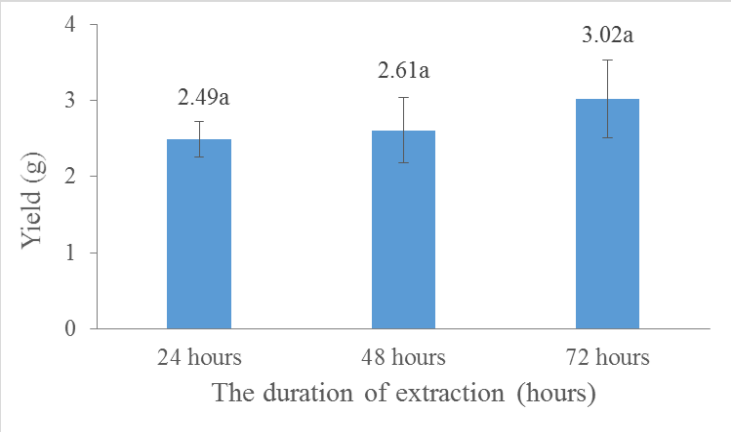

Figure 1. The raw extract of *Litopenaeus vannamei* carapace using chloroform solvent.

The increase of the processing period will effect on the yield value, due to the a higher penetration of the solvent into the raw material. The solubility of the material's components in increases slowly with the extraction period increase, but after reaching the optimal period the extracted quantity of components will reduce, due to the limited concentrations of components in the material and to the solvent's efficiency limitation, so that even though the extraction period is extended, the solute in the material will not be accessible anymore (Yulianti et al 2014).

The extraction of raw astaxanthin using chloroform for 72 hours has the highest yield with 3.02 g of fresh weight. Some reported studies state that yield of pure astaxanthin from waste of *Aristeus alcocki* using 41.46 μg g^{-1} of acetone solvent (Sindhu & Sherief 2011), waste of *Penaeus indicus* using 40.6 μg g^{-1} of acetone (Sanchez-Camargo et al 2011), *Saccharina japonica* using 29.3 μg g^{-1} of dichloromethane solvent (Zhou et al 2011), shrimp waste using 17.8 μg g^{-1} of ethanol solvent (Yoon et al 2012), *Pandalus borealis* shrimp using 147.7 μg g^{-1} of chloroform solvent and snow crab *Chionoecetes opilio* using 119.6 μg g^{-1} of chloroform solvent (Shadidi & Synowiecki 1991). Compared to the yield of other solvents, the chloroform solvent has the highest astaxanthin yield value.

Identification of astaxanthin. Identification of standard astaxanthin („Asthin force") using HPLC with a wavelength of 479 nm is presented in Figure 2.

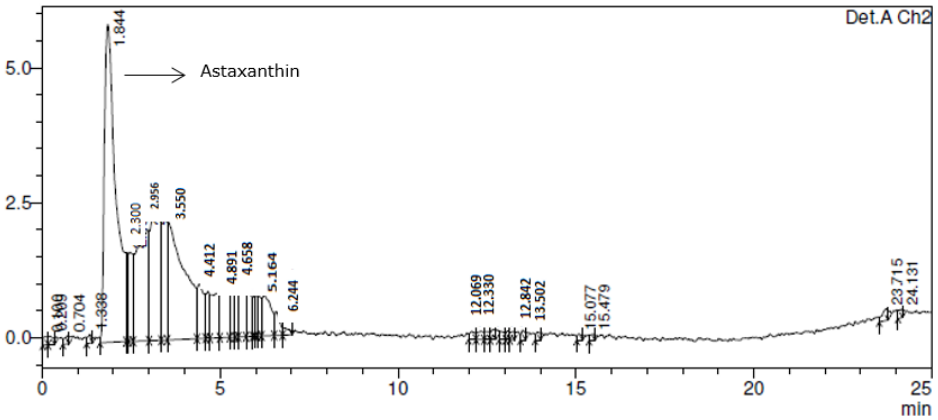

Figure 2. Standard astaxanthin chromatogram pattern.

Figure 2 shows that the standard astaxanthin pattern formed a peak of retention period with 1,844 minutes in the area of 132,461. Chromatograms from the astaxanthin standard were used as reference to find the astaxanthin chromatogram patterns in the extraction results. Chromatogram results from each sample showed that peaks appear for almost the same retention periods (simultaneously) as for standard astaxanthin, as shown in Figure 3.

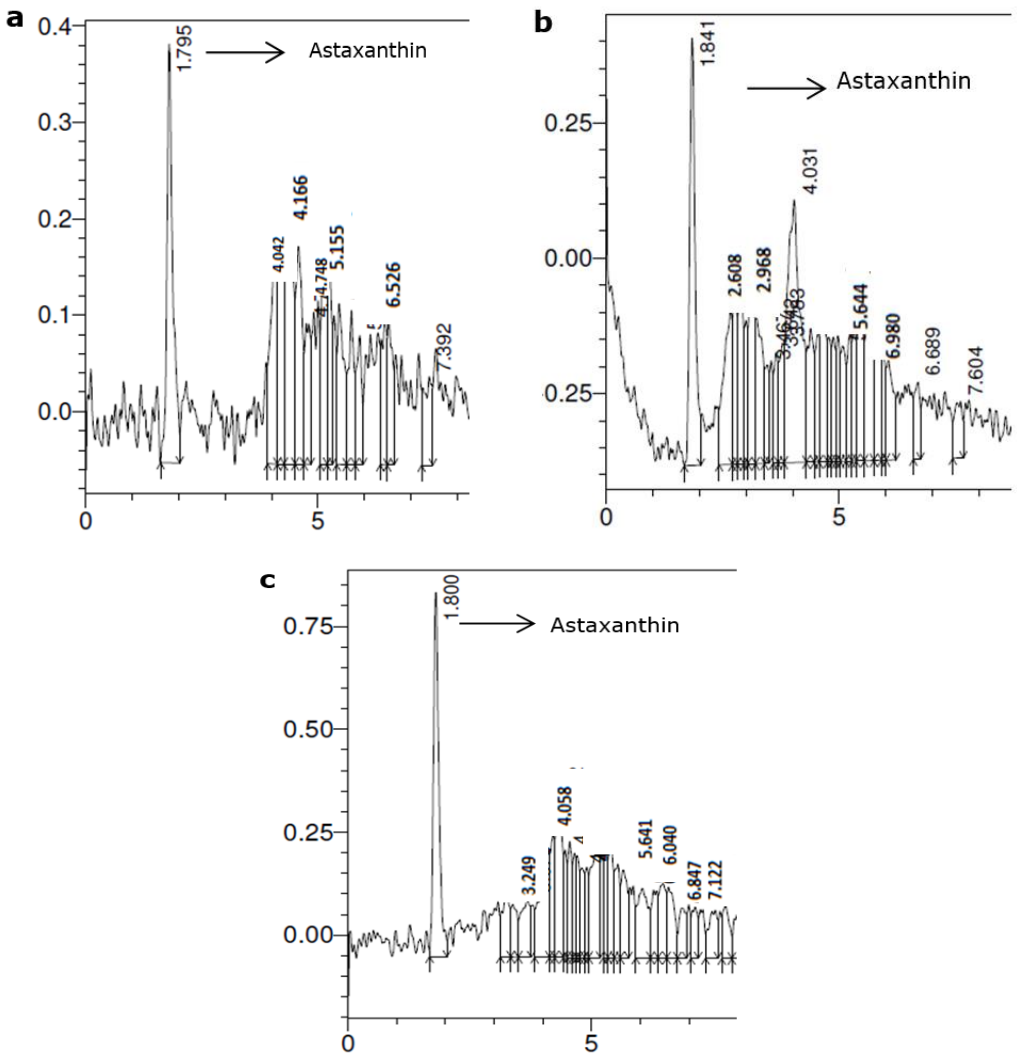

Figure 3. Chromatogram pattern of astaxanthin from the *Litopenaeus vannamei* carapace (a) extraction period 24 hours; (b) extraction period of 48 hours; (c) extraction period of 72 hours.

Figure 3 shows that the retention period of chromatogram on astaxanthin from the *L. vannamei* carapace for 24 hours, 48 hours, and 72 hours was 1,796 with area of 3,836, 1,841 with area of 6,571, and 1,800 with area of 6,937, respectively. The results of the chromatogram showed that peak appearance at retention period is 1 minute based on astaxanthin standards. Moreover, the results of chromatogram also showed that there were some other impurities peaks which indicated there were other rubberonoid pigments in the *L. vannamei* carapace. Babu et al (2008) state that shrimp heads have various gumonoid pigments, such as astaxanthin, α-carotene, β-carotene, cantaxanthin, lutein, zeaxanthin and crustacyanin.

Astaxanthin antioxidant activity. Astaxanthin from *L. vannamei* carapace was measured by using the DPPH method in order to determine the IC50 values characterizing its antioxidant activity (Zhang et al 2006), presented in Table 1. „Asthin

force" is the standard astaxanthin used as positive control. Molyneux (2004) states that the smaller the IC50 value, the higher its antioxidant activity.

Table 1

The antioxidant activity of astaxanthin from *Litopenaeus vannamei* carapace

Samples		*IC_{50} (mg mL^{-1})*
Standard astaxanthin (positive control)	*Asthin force*	*3.22*
Astaxanthin from carapace	24 hours	69.22 ± 0.02^{c}
	48 hours	48.77 ± 0.13^{b}
	72 hours	27.18 ± 0.35^{a}

Values followed by different letters indicate significant effect on 99% confidence level.

Table 1 shows that the longer the extraction period with chloroform solvents, the higher the antioxidant activity of astaxanthin in *L. vannamei* carapace. Salamah (2008) stated that when the extraction period is longer, chloroform can dissolve other components that can provide a synergistic effect with the antioxidant compounds that have been extracted previously, improving their effectiveness.

Astaxanthin in *L. vannamei* carapace has a lower antioxidant activity value compared to the standard astaxanthin (with an IC_{50} of 3.22 mg mL^{-1}), due to the fact that the solvent extraction process result represents a raw extract still containing impurities. The solvent type influences the extract yield, but it cannot increase the antioxidant activity of the extract.

Conclusions. The carapace of *L. vannamei* contains astaxanthin, whose optimal extraction time is 72 hours, with the highest yield of 3.02 g and an IC50 value of 27.18 mg mL^{-1}. However, the level of the antioxidant activity is weak, compared to the standard astaxathin.

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Authors:
Rahman Karnila, Riau University, Faculty of Fisheries and Marine Science, Department of Fishery Products Technology, 28293 Riau, Indonesia, e-mail: karnilarahman@gmail.com
Edison, Riau University, Faculty of Fisheries and Marine Science, Department of Fishery Products Technology, 28293 Riau, Indonesia, e-mail: edisonsona@yahoo.co.id
Nintha Pratiwi, Riau University, Faculty of Fisheries and Marine Science, Department of Fishery Products Technology, 28293 Riau, Indonesia, e-mail: ninthapratiwi@gmail.com
Santhy Wisuda Sidauruk, Riau University, Faculty of Fisheries and Marine Science, Department of Fishery Products Technology, 28293 Riau, Indonesia, e-mail: santhy.sidauruk@lecturer.unri.ac.id

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