

Application of matrix solid-phase dispersion on astaxanthin extraction from white leg shrimp (*Litopenaeus vannamei*) by-product

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Abstract. The extraction of astaxanthin (ATX) from white leg shrimp (*Litopenaeus vannamei*) shells by matrix solid-phase dispersion was conducted to evaluate the conditions and efficiency of the extraction. The first experiment was conducted to determine the effect of the ratio of raw material to dispersion agent on the extraction efficiency of ATX from white leg shrimp shells. The second experiment investigated the effect of moisture of raw materials on extraction efficiency. The results showed that white leg shrimp shells mixed with silica gel at the ratio of silica gel to raw material of 5% and moisture of raw materials of 30% produce the highest ATX extraction efficiency. In these conditions, ATX yield was $17.2 \mu\text{g g}^{-1}$ raw material at the given moisture. After evaporation, the ATX content was 3.4 mg g^{-1} final extracted product.

Key Words: astaxanthin, *Litopenaeus vannamei*, matrix solid-phase dispersion extraction, shrimp shell.

Introduction. Astaxanthin (ATX) is a xanthophyll carotenoid found in microalgae, yeast, salmon, and the shells of crustaceans such as shrimp and crabs (Ushakumari & Ravi 2013). The content of ATX in carotenoids accounts for about 86 to 98% (Armenta-López et al 2002). Its antioxidant activity is much stronger than that of β -carotene, α -tocopherol, lycopene, lutein or vitamin E (Shimizu et al 1996; Nishida et al 2007). Therefore, ATX is considered to have potential activities against cardiovascular problems, various cancers, and certain diseases of the immune system (Pickova et al 1998; Kavitha et al 2013). This leads to the consideration of ATX as an agent in human and animal health improvement (Higuera-Ciapara et al 2006).

Shrimp farming in the Mekong Delta (Vietnam) accounts for a high proportion of farmed shrimp production in the country. Farmed shrimp mainly includes white leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*). In shrimp processing, the by-products account for about 40-50%, with mainly shrimp heads and shells (Xu et al 2008). Regarding white leg shrimp, head accounts for 28% and shells for 9% of the waste (Trung et al 2015). Therefore, reducing the amount of waste from processing or finding solutions to reuse them could increase profits in the seafood industry. In Vietnam, most of shrimp product related studies are directed to the recovery of chitin-chitosan. ATX extraction and optimization of ATX recovery has received little attention. The method of matrix solid-phase dispersion (MSPD) has an outstanding ability to recover carotenoids with high extraction efficiency and it is also ecologically friendly. The method has many advantages, such as using less solvent, being easy to automate, less time-consuming, and low cost. Most studies use solvents such as acetone and petroleum ether. A feature of these solvents is that it is possible to extract ATX in high concentrations. However, these solvents are toxic, and can cause adverse effects on the environment and human health, especially for workers who contact the solvents directly (Dick 2006). Therefore, the application of an effective extraction method with less solvent use on shrimp processing by product is needed to reduce the environmental pollution and produce a natural antioxidant compound.

Material and Method

Shrimp by product. The shells and heads of white-leg shrimp were selected as research subjects. Raw materials were collected at Co Chien Seafood Joint Stock Company, Tra Noc 2 Industrial Park, Phuoc Thoi Ward, O Mon District, Can Tho City and Sao Ta Food Joint Stock Company on National Highway 1A, Ward 2, City Soc Trang, Soc Trang province, Vietnam. Raw materials were transported to the laboratory of the Department of Seafood Science and Technology, College of Aquaculture and Fisheries, Can Tho University, in thermally insulated foam boxes and kept cold with ice (temperature <5°C). The raw material was washed to remove impurities, drained, weighed, packed in PE bags, and stored at -20°C.

Chemicals. The chemicals used include methanol, ethanol (Merck, Germany), glass wool, silica-gel (60–200 mesh) (J.T. Barker, USA), and astaxanthin standard (DR Ehrenstorfer, Germany).

Matrix solid-phase dispersion (MSPD) method optimization

Proportion determination of dispersion agent. Shrimp by-product material was dried at 55–65°C (Muoi et al 2014). The raw material was dried until the moisture content reached 50%, and finely ground. The experiment was set with one variable, i.e. the proportion of silica gel to raw material and was repeated 3 times, as described by Castillo et al (2020). 6 g of finely ground material was mixed with 200–300 µm silica gel at different proportions of silica gel to materials of 5%, 10%, 15%, and 20% (w/w). The mixture was embedded into a glass column containing a layer of glass fiber and 0.2 g of silica gel below the column to create a filter layer. The column was covered with a layer of glass fiber to dissolve the solvent evenly. 60 mL 99% ethanol was pumped through the sample column at a flow rate of 10 mL min⁻¹ and the extract from the column was collected. The ATX content in the elute was determined by high-pressure liquid chromatography (HPLC) with an ultraviolet (UV) detector (Shimadzu, Japan).

The moisture content of shrimp shell determination. The result of the above experiment was applied in the next experiment. The raw materials were dried until they reached different moisture levels: 10%, 30%, 50%, and 70%. Then they were finely ground to increase the contact area between the material and the solvent. Samples at different moisture levels will be extracted according to the extraction conditions obtained in the first experiment. The extracts were collected and the ATX content was determined by high-pressure liquid chromatography (HPLC) with a UV detector (Shimadzu, Japan). The elute was then processed in a vacuum rotary system (Buchi, Switzerland) at 40°C to evaporate all ethanol solvent to collect ATX.

Sample and data analysis

Calibration curve preparation. Standard solutions were prepared from astaxanthin standards provided by Dr. Ehrenstorfer (Germany). Astaxanthin standard substance was weighed and dissolved in methanol in a brown volumetric flask to obtain 1 mg mL⁻¹ of stock solution, which was stored in a -18°C refrigerator without light. The working range of the calibration curve was prepared at concentrations of 0.05, 0.10, 1.00, 2.00 and 4.00 mg L⁻¹ in methanol/water (97:3 v/v). The linear regression was then applied to calculate the standard curve.

HPLC identification and quantification. A LC-10A HPLC (Shimadzu, Japan) equipped with LC-10ATvp binary pump was used for ATX determination and quantification. Chromatograms were recorded at 480 nm with a UV detector (Shimadzu, Japan). The system was computer controlled and installed with Lab Solutions chromatography workstation for the analysis of data. The chromatographic Hypersil Gold C18 column (4.6×250 mm, 5 µm) was used for the separation of ATX. The flow rate was 1 mL min⁻¹,

the column temperature was 25°C and the injection volume was 20 µL. The mobile phase was a mixture of methanol and water with a ratio of 97:3 (v/v) (Tzanova et al 2016).

Determination of DPPH free radical-scavenging activity of extracted ATX. Due to the hydrogen-donating ability of antioxidants, they may reduce the free radical DPPH• to a stable form. Based on this principle, various concentrations of ATX were measured for their DPPH radical scavenging activities. The radical scavenging activity of extracted ATX was evaluated using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay according to Navarro-Hoyos et al (2018), with modifications. 100 µL of 0.25 mM DPPH solution in methanol were added to 100 µL of various concentrations of the ATX in methanol (5-40 µg mL⁻¹), then mixed vigorously and allowed to stand in the dark at room temperature for 30 min. The absorbance of the sample solution was measured at 492 nm using a microplate reader (Multiskan EX, Labsystems, Finland). Curves of absorbance vs. concentration were plotted to obtain IC₅₀ (inhibitory concentration when 50% oxidative radical was scavenged), which is the concentration of sample needed to obtain 50% of radical-scavenging activity. This value is known as IC₅₀ and was expressed in units of µg mL⁻¹. This assay was carried out twice and the mean values were used to calculate the EC₅₀. Alpha-tocopherol was used as a positive control. The scavenging effect on the DPPH inhibition was calculated as a percentage (%), according to the equation:

$$\% \text{DPPH radical scavenging} = [A_{\text{control}} - (A_{\text{sample}} - A_{\text{sample blank}})/A_{\text{control}}] \times 100$$

Where: A_{control} , A_{sample} , and $A_{\text{sample blank}}$ are the absorbance of the DPPH solution in methanol, ATX solution with DPPH, and ATX solution without DPPH, respectively.

Data analysis. Collected data were analyzed using descriptive statistics (mean, standard deviation). The difference between treatments was analyzed by one-way ANOVA and Duncan's test (when differences were observed for $p < 0.05$), using SPSS 16.0 software.

Results and Discussion

Linear range and standard curve determination. The retention time of ATX was 4.7 min with methanol/water (97:3, v/v) used as mobile phase at 1 mL min⁻¹ flow rate. The coefficient of determination (R^2) value (0.9999) revealed a good linearity over the selected range for ATX (0.1–4 mg L⁻¹), as presented in Figure 1.

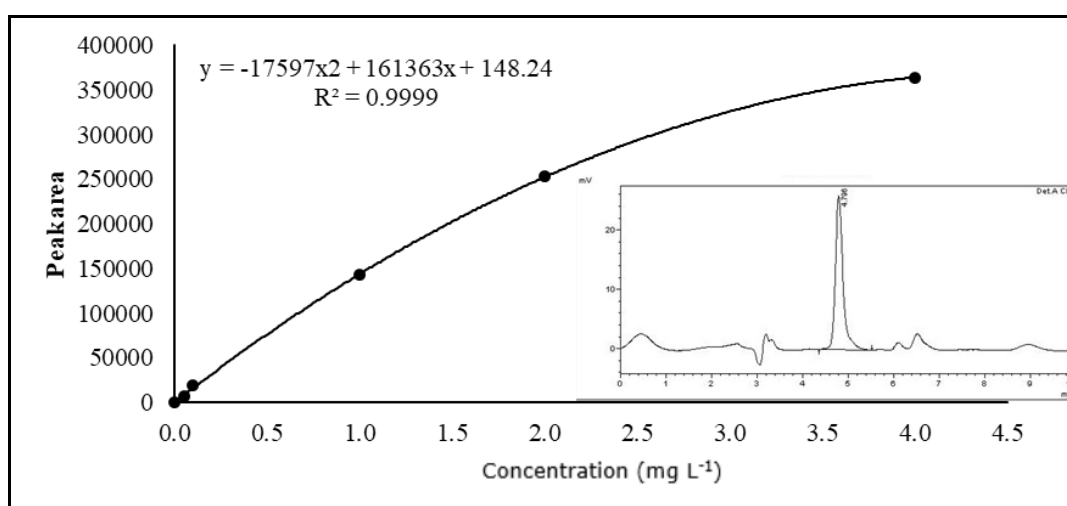


Figure 1. Astaxanthin calibration curve and chromatography graph.

Effects of dispersion agent proportion on ATX extraction efficiency. According to the statistical results, the silica gel ratio influences the extraction efficiency of ATX ($p < 0.05$). Experimental results showed that the ATX contents were significantly higher in treatments with 5% and 10% of dispersion ratio and gradually decreased when

increasing the silica gel ratio (Table 1). The dispersion ratio was much lower than in other previous MSPD applications in crustaceans and other raw materials (Grynbaum et al 2005; Castillo et al 2020). However, the recovery rate in the current study was lower than the results of Ushakumari & Ravi (2012), for the extraction of ATX from shrimp waste by a mixture of acetone and hexane solvents with a ratio of 3:7, where the ATX content obtained was 43.44 $\mu\text{g g}^{-1}$. According to Hu et al (2009), silica gel has excellent separation and purification abilities, but the irreversible adsorption is unavoidable; the irreversible adsorption rate was 14.05%. Fortunately, in the actual purification process, a large amount of fatty acids and other polar substances in the crude extract of ATX compete with ATX for the adsorption center of silica gel. Therefore, the irreversible absorption of ATX on silica gel during the purifying experiment was probably lower. The yield of ATX recovered in this study was lower than that obtained by Dalei & Sahoo (2015), where the ATX content extracted with acetone with a sample/acetone ratio of 1:10 in 1 hour was 48.64 $\mu\text{g g}^{-1}$ ATX. The reason for this difference is that the ATX content varies with species, habitat, extraction solvent, and material moisture. However, both acetone and hexane solvents are toxic and expensive. In addition, the conditions of storage can obviously reduce the content of ATX and the main reason may be that ATX has strong antioxidant activity. The ATX in shrimp shells will be destroyed by the oxygen in the air and sunlight during drying (Hu et al 2009).

Table 1

Astaxanthin content recovery from shrimp shells with different silica gel ratio

<i>Silica gel ratio (%)</i>	<i>Astaxanthin recovery ($\mu\text{g g}^{-1}$)</i>
5	20 \pm 1.4 ^a
10	18.5 \pm 0.7 ^a
15	15.3 \pm 0.6 ^b
20	14.2 \pm 0.5 ^b

Note: data are expressed as mean \pm SD; different superscripts show significant differences ($p < 0.05$).

Effect of shrimp shell moisture content on ATX extraction efficiency. The results showed that the moisture content of the raw materials influences the extraction efficiency of ATX ($p < 0.05$). The highest ATX content was obtained at 30% moisture content, 20.5 $\mu\text{g g}^{-1}$. When reducing the moisture content of the raw materials from 30% to 10% or increasing the moisture content to 50% and 70%, the obtained ATX content tends to decrease, the lowest, 9.93 $\mu\text{g g}^{-1}$, occurring at 70% moisture content (Table 2). The reason is that the high water content in the material will react with the protein and other hydrophilic substances to prevent the migration of the lipid into the material, hindering the diffusion process (Nga 2012).

Table 2

Astaxanthin content recovery from shrimp shells with different moisture contents

<i>Moisture content (%)</i>	<i>Astaxanthin recovery ($\mu\text{g g}^{-1}$)</i>
10	16.3 \pm 0.36 ^b
30	20.5 \pm 2.8 ^c
50	17.2 \pm 2.54 ^{bc}
70	9.93 \pm 0.95 ^a

Note: data are expressed as mean \pm SD; different superscripts show significant differences ($p < 0.05$).

When reducing the moisture content of the raw materials to 10%, the ATX content obtained was 16.3 $\mu\text{g g}^{-1}$ of shrimp shell, lower than the ATX content obtained at 50% moisture content, 17.2 $\mu\text{g g}^{-1}$, causing the ATX content to decrease. Due to the long drying time, the raw materials are exposed to oxygen, causing the oxidation of carotenoids in shrimp shells. Russia (2012) extracted ATX from black tiger shrimp shells using soybean oil at 92.2°C for 169 min, obtaining an ATX content of 41 $\mu\text{g g}^{-1}$. The reason for this difference is that the ATX content varies by breed, species, habitat,

extraction solvent, raw material moisture, and shell size. The results showed that the optimum moisture content of the material selected in this experiment was at 30% humidity for the highest ATX extract content.

After evaporation, the ATX concentration was 3.4 mg g⁻¹. This concentration is much lower than that of some commercial products in the market, which have about 10% (100 mg g⁻¹) of ATX, but also lower than the results of other studies. Hu et al (2009), in a study on ATX extraction from *Procambarus clarkia* shrimp, obtained a purity of ATX extract of 85% after applying complicated steps of extraction and purification. Therefore, the crude ATX recovered in this study requires further purification. However, the ATX content in commercial medicine products mostly varies from 1 to 12 mg in a pellet. Thus, the extract obtained in the current study meets the ATX concentration available in commercial supplements.

Antioxidant activity of shrimp shell ATX. All samples at tested concentrations showed obvious scavenging activities on DPPH radicals in a dose-dependent manner. The lower EC50 value means a more powerful antioxidant capacity. Ethanol extract of ATX presented antioxidant potential with the EC50 value of 22.3 µg mL⁻¹. This EC50 value was similar to the result obtained by Chintong et al (2019) (17.5 µg mL⁻¹).

Conclusions. ATX could be extracted from white shrimp shells by dispersed solid phase extraction (MSPD) with a 5% silica gel rate and a moisture content of the raw materials at 30%. The yield of ATX was 17.2 µg g⁻¹ of shrimp shell material. In the final extract, the content of ATX was 3.4 mg g⁻¹.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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