Effect of Thermal Processing on Astaxanthin and Astaxanthin Esters in Pacific White Shrimp *L. vannamei*

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Abstract: The red color of processed shrimp, one of the most attractive attributes and an important criterion for consumers, is often limited by thermal processing (microwaving, boiling and frying), due to astaxanthin degradation. The effect of thermal processing on astaxanthin in Pacific white shrimp (*L. vannamei*) were investigated. A High-performance liquid chromatographic - atmospheric pressure chemical ionization mass spectrometry (LC-(APCI)-MS/MS) method was used to identify and quantify all-trans- and cis-isomers of astaxanthin, and molecular species of astaxanthin esters in fresh and thermal processed shrimps. Total astaxanthin loss ranged from 7.99% to 52.01% in first 3 min under three thermal processing. All-trans-astaxanthin was most affected, with a reduction from 32.81 to 8.72 μg kg⁻¹, while 13-cis-astaxanthin had a rise (from 2.38 to 4.58 μg kg⁻¹). Esterified astaxanthin was shown to hydrolyze and degrade, furthermore astaxanthin diesters had a better thermostability compare to astaxanthin monoesters. Astaxanthin monoesters with eicosapentamcioc acid (EPA, C₂₀:₅) and docosahexaenoic acid (DHA, C₂₂:₆), had a lower thermal stability than those with saturated fatty acids, however, it was the opposite of astaxanthin diesters. The findings suggested that the method of thermal processing should be carefully used in the manufacturing and domestic cooking of shrimps. The results also could be useful in calculating the dietary intake of astaxanthin and in assessing astaxanthin profiles and contents of shrimp containing products.

Key words: astaxanthin, astaxanthin esters, *L. vannamei*, processing

1 INTRODUCTION

Astaxanthin (3,3'-dihydroxy-α,α-carotene-4,4'-dione) is the main carotenoid pigment found in a variety of aquatic animals, and provides bright red or pink color of Crustacea (shrimp, krill) and Salmonidae (salmon, rainbow trout)¹. Due to its special molecular structure, astaxanthin has strong antioxidative activity, which is several times higher than vitamin E², 10 times higher than other carotenoids, including zeaxanthin, lutein, β-carotene, and canthaxanthin³. Recent studies have demonstrated that astaxanthin showed 80% anti-lipid peroxidation activity in ethanol induced gastric ulcer rats and skin cancer rats⁴⁻⁵. Other effects of astaxanthin on human health nutrition, such as anti-aging⁶, improving lipid metabolism⁷, anti-inflammatory activity⁸, etc., have been published previously.

The molecule of astaxanthin bears two ionone rings held together by a long chain of conjugated double bonds, which indicates that many possible geometrical isomeric forms (Fig. 1). Free astaxanthin is considerably unstable and particularly susceptible to oxidation⁹. Hence, it is found in nature either conjugated with proteins (e.g., salmon muscle or lobster exoskeleton) or esterified with one or two fatty acids(monoester and diester forms) (Fig. 1), which stabilize the molecule⁹. In microalgae (Haematococcus pluvialis), astaxanthin occurs in three different forms that can be classified according to free (5%), monoesters (70%), and diesters (25%)¹⁰. For salmons and red yeast Phaffia rhodozyma, only free astaxanthin exists¹¹. A study has demonstrated that there were 5 astaxanthin monoesters and 8 astaxanthin diesters in arctic shrimp (Pandalus borealis), in which astaxanthin diesters clearly formed the main components and lauric acid was found to be one of the prevalent fatty acids¹². In krill (Euphausia superba Dana)¹³, diestered astaxanthin also accounts for nearly 70% in the carotenoid extract, and C₁₄:₀, C₁₆:₀, C₁₆:₁, C₁₈:₁, C₂₀:₀, C₂₀:₅, and C₂₂:₆ were found in astaxanthin.

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esters, while PUFAs (C20:5 and C22:6) were only identified in astaxanthin diesters. So far, little is known about the composition of astaxanthin and its esters, and fatty acids acylating astaxanthin in Pacific white shrimp.

Naturally, astaxanthin is predominantly present in its all-trans-isomeric forms, which are easily converted into their cis-isomeric forms (9-cis-astaxanthin and 13-cis-astaxanthin) under processing conditions such as heat, light, and oxygen. This isomerization may result in changes in the nutritional function and bioavailability of astaxanthin (14). Zhao et al. (15) studied the stability of standard astaxanthin (dissolved in ethanol) by microwaving and ultrasound at different intensity. The study demonstrated that microwave only induced the conversion of all-trans-astaxanthin to its cis-forms, preferentially to 13-cis-astaxanthin, while ultrasound probably degraded this pigment into colorless compounds. Zeb and Murkovic (16) added astaxanthin into refined olive oils and heated at 110 °C for 1–14 h. Astaxanthin was found to degrade as well as isomerize with the increment of thermal treatment time. However, their experiments were performed using astaxanthin organic solution, did not consider the isomerization and degradation of astaxanthin in food system during thermal processing or domestic processing. Other investigations studied the effect of boiling (17), smoking (18), and microwaving (19) on salmon or rainbow trout containing astaxanthin. In addition, numerous epidemiological studies suggested that the effects of thermal processing on carotenoids in many fruits and vegetables, such as broccoli (20), oranges (21), spinach (22), etc. in the past years. However, there is also little data dealing with the change of astaxanthin mono- and diesters, which account for a big ratio of the total astaxanthin content in aquatic foods during thermal processing.

With increasing consumer demand of aquatic foods, which are convenient, instant and with various flavors, thermal food processing techniques such as microwaving, frying, boiling, drying, and baking are gaining interest. Boiling, frying, microwaving were the main thermal processing methods during shrimp manufacturing or domestic cooking. The red color of processed shrimp product is one of the most attractive attributes and an important quality criterion for consumers in the marketplace (23). Due to the high tolerance to environmental stress, the year-round availability of healthy post-larvae, and high profit for investment, Pacific white shrimp (Litopenaeus vannamei), is the most commonly farmed shrimp species in the world. We used high-performance liquid chromatography coupled triple quadrupole mass spectrometric (LC-MS/MS) to detect and obtain a deeper insight into the changes of composition, molecular species and pattern of astaxanthin and astaxanthin esters in Pacific white shrimp Litopenaeus vannamei. The objective of this study was to investigate how different thermal processing affected the stability of astaxanthin and astaxanthin esters in shrimp.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

The all-trans astaxanthin standard (≥ 97.3%) was purchased from Dr. Ehrenstorfer (Augsbury, Germany). Methanol and tert-butyl methyl ether (TBME) were purchased from Merck Chemical Company (Hohenbrunn, Germany). Light petroleum ether, ethyl acetate, and other chemicals were analytical grade. Fresh Pacific white shrimps (Litopenaeus vannamei) were purchased from local supermarkets (Qingdao, Shandong, China).

2.2 Iodine-catalyzed photoisomerization to obtain cis-isomers

The all-trans astaxanthin standard was photoisomerized into an equilibrium mixture of several geometrical isomers.
Effect of thermal processing on astaxanthin from Pacific white shrimp

according to a published procedure. The detailed procedures were as follows. A stock solution (5 mL) of all-trans-astaxanthin standard was prepared at a concentration of 2 mg mL\(^{-1}\) in nitrogen-degassed dichloromethane, and mixed with 100 μL of 0.1 g mL\(^{-1}\) iodine-dichloromethane solution. The resulting solution was exposed to natural sunlight at room temperature for 15 min. The solution was then washed twice with Na\(_2\)S\(_2\)O\(_3\) solution (1 mol L\(^{-1}\)) to remove excess iodine and evaporated under nitrogen gas. Residues were dissolved in a mixture of methanol/TBME (1:1, v/v) containing 0.1% BHT and made up to 10 mL (final concentration 200 μg mL\(^{-1}\)). These geometric isomers were characterized by their ultraviolet-visible (UV-vis) spectra, retention times compared to the elution order of the astaxanthin isomers.

2.3 Preparation of shrimp samples

Fresh Pacific white shrimps were divided into four equal sets, and three sets were subjected to different thermal treatments, boiling, microwaving, and frying, and the fourth set was used as the control. One set of whole shrimps was placed in a pan, with water up to 3–4 cm above the shrimps, and boiled for different time. For microwaving, shrimps were cooked in a microwave oven (Model R-2V15, SHARP, Japan) for different time at different cooking levels (200/400/600 W, 2450 MHz). The other set of shrimps was fried using a pan preheated to 160°C. All the samples were stored in the dark at -20°C until the extraction.

2.4 Extraction of pigment from shrimp samples

The following extraction procedures were according to Breithaupt with minor modifications. Shrimps (50 g) were minced with an Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany)/5,000 rpm for 1 min. An aliquot (1 g) of the homogenized sample was extracted three times (3 mL each) using a ternary solvent mixture (methanol/ethyl acetate/light petroleum, 1:1:1, v/v/v) containing 0.1% BHT. After centrifuged at 4,000 rpm, 4°C for 5 min, the supernatants were collected and added with 1 mL of a saturated sodium chloride solution. The water phase was discarded, and the organic layer was evaporated under nitrogen gas. The residues were resolved with methanol/TBME (1:1, v/v) containing 0.1% BHT. These samples were filtered through a 0.22 μm nylon membrane filter and stored at -20°C until analyzed by LC-MS/MS. All the above procedures were carried out under dim light and 4°C conditions to protect the pigment from isomerization and photodegradation.

2.5 Analysis of astaxanthin content in shrimp samples

The above pigment extract was analyzed with an Agilent 1260 series system HPLC (Agilent, Waldbronn, Germany) consisting of a G1314A pump. An automatic injector and a G1315B diode array detector (DAD) equipped with work-station computer. Reversed-phase separation was performed on a YMC carotenoid C30 column (4.6 mm × 250 mm, 5 μm), which was kept in a column oven at 35°C. The mobile phase consisted of mixtures of methanol/TBME/water [90:8:2, v/v/v (A) and 8:90:2, v/v/v (B)]. The following gradient was used (min%/A): 0/99; 39/44; 45/50; 99/55. The flow rate was 1.0 mL min\(^{-1}\) and the injection volume was 20 μL. Monitoring was performed at 476 nm, and analyses were performed in duplicate.

Eight-point standard calibration graphs of all-trans-astaxanthin standard were prepared for quantification of free astaxanthins. Calibration graphs were recorded with sample concentrations ranging from 0.05 to 35.00 μg mL\(^{-1}\) by plotting the respective peak area against concentration. Both trans-isomer and cis-isomer concentrations were calculated using the corresponding all-trans standard calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of diluted solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio (S/N) equal to 3 and 10, respectively.

2.6 Analysis of astaxanthin molecular species in shrimp samples

For LC-MS/MS analysis of astaxanthin molecular species, the C30-HPLC module with DAD was coupled to an Agilent 6410B Spectrometer (Agilent, Waldbronn, Germany). The detection was performed using atmospheric pressure chemical ionization (APCI) in the positive ionization mode. The voltage of the corona needle was set to 4 kV. Nitrogen was used as the drying gas, and the carrier gas at a flow rate of 5 L min\(^{-1}\), with a nebulizer was 45 psi and the dry gas temperature was held at 350°C. Mass spectra was monitored in the mass range m/z 200–2000. MS\(^{2}\) was detected in the modes of product ion scan and precursor ion scan. The fragmentor voltage and collision energy were set to 60 V and 15 eV respectively.

2.7 Statistical analysis

The obtained data were subjected to one-way ANOVA and comparisons among samples were performed with Tukey multiple comparison test using SPSS (version 18.0, SPSS Inc., Chicago, IL) statistical software. Significant differences were accepted at \(p<0.05\).

3 RESULTS AND DISCUSSION

3.1 Identification of geometric isomers of astaxanthin and molecular species of astaxanthin esters in Litopenaeus vannamei

In this experiment, a C30 reversed-phase column was used to obtain the separation of astaxanthin geometric isomers as well as astaxanthin esters in the shrimps (Fig.
According to reported analysis, spectra were obtained by a DAD and shown in Fig. 2. As reported previously, astaxanthin monoesters typically showed retention times 22-32 min, and astaxanthin diesters were eluted after 35 min. To further confirm the structures of astaxanthins and its isomers, we used LC-(APCI)-MS/MS with positive ion mode. The identification of 26 peaks (Fig. 2) was shown in Table 1.

For astaxanthin geometric isomers, due to their similar mass spectra, identification of cis-isomers of astaxanthin was based on the UV-vis spectra, retention times and elution order. Peaks 1, 2 and 5 all had dominant ion of [M + H]⁺ at m/z 597, and produced major MS² ions at m/z 579 ([M-H₂O + H]⁺), and 561 ([M-2H₂O + H]⁺), which were thought as isomers of astaxanthin. As reported previously, due to the introduction of cis double bond, the maximum absorption in UV-vis spectra of all-trans-astaxanthin shifts to a shorter wavelength in shape to those of cis-isomers. Compared to all-trans-astaxanthin, 9-cis, and 13-cis isomers showed hypsochromic shifts between 4 nm and 8 nm. To identify three species isomers, their UV-vis spectra were obtained by a DAD and shown in Fig. 3. According to reported analysis, cis-isomer of astaxanthin could be identified by its Q ratio, which was defined as the absorbance ratio of the cis- peak to the middle maximum absorption peak. The Q ratio of peak 1, 2 and 5 were 0.541, 0.120 and 0.210, respectively. This has been compared with the values reported in the literature and found to be very consistent with the cis-isomers of astaxanthin. Combining all these evidences, we concluded that peaks 1, 2 and 5 were 13-cis-astaxanthin, all-trans-astaxanthin and 9-cis-astaxanthin, respectively. Figure 2A illustrated the HPLC separation of compounds for the standard mixture of all-trans astaxanthin and their cis-isomers (obtained after iodine-catalyzed photoisomerization of all-trans astaxanthin standard).

The positive ion mode mass spectrum of peak 3 and 4 in the HPLC chromatogram (Fig. 2) had strong quasi-molecular ions at m/z 595.4 and 593.6. In MS², the molecules showed a basic peak of m/z 577.4 and 575.4 produced by parent molecules losing water. As detected by HPLC-DAD, these compounds had maximum absorbance at 400 nm, whereas astaxanthin had maximum absorbance at 476 nm. Therefore, they were suggested to be semi-astacene and astacene.

As previous study, astaxanthin monoesters usually have protonated quasi-molecular ions [M + H]⁺ and fragment ions m/z 579 [M + H-FA]⁺ in MS¹, and the characteristic astaxanthin skeleton ion m/z 561 [M-FA-H₂O + H]⁺ produced from m/z 579 can be observed in MS². Astaxanthin diesters also showed a protonated quasi-molecular ions [M + H]⁺, and fragment ions of losing one fatty acid in MS¹. In MS², the diesters had a typical peak of [M + H-FA]⁺, and the fragment ions [M + H-FA₂]⁺ in a weaker relative intensity and the skeleton fragment of astaxanthin m/z 561. Based on the mass fragmentation of astaxanthin esters, we referred that peaks 6-14 were astaxanthin monoesters and peaks 15-26 were astaxanthin diesters. The detailed data for the identification of free astaxanthin and astaxanthin esters were summarized in Table 1.

In Pacific white shrimp, the monoesterified astaxanthin was the major component with a relative percentage of 58.65% among the pigment extract (Table 1), whereas free astaxanthin also accounted for a large percentage (32.95%), which is different from the pattern of *Pandalus borealis*. Both astaxanthin monoesters and diesters in Pacific white shrimp were rich in polyunsaturated fatty acids (PUFAs; 61.74% of total fatty acids bound to astaxanthin), in particular C20:5 and C22:6. Note that nearly every molecule of diestered astaxanthin contained a fatty acid chain of PUFAs. Interestingly, C12:0, and C14:0, which is abundant in total lipids of Pacific white shrimp and other shrimp species reported previously, was not identified in astaxanthin esters. In contrast to krill, red crab langostilla and arctic shrimp, the fatty acid profile of astaxanthin esters in Pacific white shrimp is typical with higher content of PUFAs and both presence in the fraction of astaxanthin monoester and diesters.
3.2 Effects of thermal processing on total astaxanthin content in Pacific white shrimps

The study investigated the stability as well as degradation of free astaxanthin and astaxanthin esters in Pacific white shrimps during common thermal processing. As expected, the total astaxanthin content of thermal processed shrimps showed a significant loss (Fig. 4). The total astaxanthin in boiled shrimps for 3, 10, 15, and 20 min lost 40.76%, 56.31%, 77.48% and 79.38%, while in the fried shrimps lost 52.01%, 93.54%, 94.53%, and 96.59%, respectively. A larger decrease was shown for the concentration of total astaxanthin in frying processing extract, and nearly no astaxanthin were detected after 10 min. Simultaneously, the disappearance, visible macroscopically by a

Table 1  Positive ion LC-(APCI)-MS/MS Data Used for Identification of Molecular Species of Astaxanthin and Astaxanthin Esters in Raw Pacific White Shrimps.

<table>
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<th>peak</th>
<th>m/z</th>
<th>[M+H]⁺</th>
<th>[M+H-FA₁]⁺</th>
<th>[M+H-FA₂]⁺</th>
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<th>relative content (%)</th>
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Note. Asta, astaxanthin; FA, fatty acid.
loss of color, was more rapid in fried shrimps. This is probably due to the fact that astaxanthin are fat-soluble compounds and solubilize readily in oil during frying, in addition, the high temperature of frying is also a significant attribute to the deduction of astaxanthin. However, for the microwave processing, the change of total astaxanthin in shrimps had shown a similar but slower trend compared to boiling and frying, which might be due to the mild heating mechanics of microwave reported in various food processing reports.

To investigate the correlation between the decrease of total astaxanthin and processing intensity and treatment time, we monitored the change of total astaxanthin in Pacific white shrimp under different microwaving conditions (Fig. 4). With increasing the microwave intensities from 200 W to 600 W, the levels of total astaxanthin decreased at all the three examined microwave intensities. Likewise, the content of total astaxanthin also decreased as the treatment time was increased from 0 min to 20 min. For example, the content of total astaxanthin decreased by 10.69% (200 W), 14.88% (400 W), and 47.40% (600 W) after 3 min treatment with microwave, whereas total astaxanthin decreased by 19.24% (200 W), 50.06% (400 W), and 63.59% (600 W) at 10 min, which is a usual treatment time for industrial thermal processing as well as domestic cooking. Thus the decreasing level of total astaxanthin is a function of both processing intensity and time, which is consistent with the previous study presented by Zhao.

Degradation of all-trans-astaxanthin in ethanol during the microwaving treatment increased with higher microwaving power and prolonged treatment time. Combining with the experimental results of boiling and frying processing, it is suggested that thermal processing has a marked influence on the stability of total astaxanthin in Pacific white shrimp, especially at higher processing intensity and longer treatment time.

Among three thermal processing methods, microwaving caused relatively high retention of astaxanthin content in Pacific white shrimps, as compared to boiling and frying. Microwaving may cause a lower increase in solution temperature through interaction with the polar molecules. When boiling, each part of shrimps was submerged in water, in which, the penetration of heat throughout the boiling medium was easier because the heat transfer coefficient was higher in aqueous solutions; hence oxidation and destruction of the heat-sensitive free astaxanthin and astaxanthin esters was expected to be greater. Blessington reported that boiling significantly reduced carotenoids in potatoes, comparing with microwaving. Other studies were also in agreement with these results. The most destructive thermal processing is frying. Frying is usually under high temperature (100-220°C), which will significantly increase the rate of degradation of astaxanthin. In addition, greater amounts of free astaxanthin and astaxanthin esters may be extracted out of the shrimp matrix and into the oil used for frying, as compared to microwaving and boiling since the pigment are oleophilic.

3.3 Effects of thermal processing on free astaxanthin isomerization and degradation

The quantitation of astaxanthin isomers was based on the standard calibration of all-trans-astaxanthin standard obtained from DAD detection. The LOD and LOQ of all-trans-astaxanthin were 0.016 μg mL⁻¹ and 0.032 μg mL⁻¹, respectively. The calibration range was 0.08-20.00 μg mL⁻¹, and the correlation coefficients (R²) of calibration graphs were 0.9995. The amounts of free astaxanthin (both trans- and cis-isomers) and their percentage change in processed shrimps extracts relative to the raw shrimps extracts were
isomerization reaction of free astaxanthin was favored at the beginning of the treatment time. All experimental statistics indicated that the loss of all-trans-astaxanthin and the increase of 13-cis- and 9-cis-isomers implied that all-trans-astaxanthin in Pacific white shrimp was readily converted to cis-isomers, preferentially to 13-cis- and 9-cis-astaxanthin isomers.

Several studies showed that heating causes the degradation and isomerization of all-trans-astaxanthin in model systems, i.e. organic solvents or various oils. As was shown previously by Zhao et al., microwave only induced the conversion of all-E-astaxanthin to its cis-forms, preferentially to 13-cis-astaxanthin first, then the 13-cis-isomer decrease and subsequent increase in the amount of 9-cis-isomer, which suggested that 9-cis-isomer may be a more stable form. Zeb and Murkovic dissolved all-trans-astaxanthin into refined olive oil and heated the sample at 110°C. It was observed that extending thermal treatment time increased the degradation and isomerization of all-trans- and cis-isomer of astaxanthin. Compared these investigations with our study, it was demonstrated the degradation and isomerization of free astaxanthin in complex food systems, was much more severe than that in organic solutions or oils. The presence of fat and lipid might be a factor that accelerate the isomerization reaction and protected by the trans- and cis-astaxanthin isomers against oxidation. Additionally, our results also reflected a possible isomerization and degradation of free astaxanthin owing to the time–temperature regime.

Liu and Osawa showed that cis-astaxanthin, especially 9-cis-astaxanthin, exhibited a higher antioxidant effect.

### Table 2 Quantities of Free Astaxanthin Isomers in Raw and Thermal Processed Shrimps.

<table>
<thead>
<tr>
<th></th>
<th>all-trans-astaxanthin</th>
<th>13-cis-astaxanthin</th>
<th>9-cis-astaxanthin</th>
<th>total free astaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>content (μg/100 g)</td>
<td>change (%)</td>
<td>content (μg/100 g)</td>
<td>change (%)</td>
</tr>
<tr>
<td>raw</td>
<td>3.28 ± 0.03</td>
<td>n. d.</td>
<td>0.24 ± 0.04</td>
<td>n. d.</td>
</tr>
<tr>
<td>microwaving (600 W)</td>
<td>2.65 ± 0.07</td>
<td>19.27</td>
<td>0.46 ± 0.03</td>
<td>92.68</td>
</tr>
<tr>
<td>3 min</td>
<td>2.04 ± 0.05</td>
<td>38.04</td>
<td>0.32 ± 0.05</td>
<td>32.69</td>
</tr>
<tr>
<td>10 min</td>
<td>1.58 ± 0.04</td>
<td>51.84</td>
<td>0.28 ± 0.03</td>
<td>17.59</td>
</tr>
<tr>
<td>15 min</td>
<td>0.60 ± 0.09</td>
<td>81.59</td>
<td>0.11 ± 0.02</td>
<td>54.46</td>
</tr>
<tr>
<td>20 min</td>
<td>1.72 ± 0.01</td>
<td>47.54</td>
<td>0.28 ± 0.02</td>
<td>17.1</td>
</tr>
<tr>
<td>boiling</td>
<td>1.61 ± 0.02</td>
<td>50.89</td>
<td>0.25 ± 0.03</td>
<td>3.83</td>
</tr>
<tr>
<td>10 min</td>
<td>0.75 ± 0.02</td>
<td>77.25</td>
<td>0.15 ± 0.02</td>
<td>35.74</td>
</tr>
<tr>
<td>15 min</td>
<td>0.71 ± 0.02</td>
<td>78.35</td>
<td>0.13 ± 0.02</td>
<td>44.31</td>
</tr>
<tr>
<td>frying</td>
<td>0.87 ± 0.07</td>
<td>73.42</td>
<td>0.24 ± 0.04</td>
<td>2.69</td>
</tr>
<tr>
<td>10 min</td>
<td>0.79 ± 0.09</td>
<td>75.85</td>
<td>n. d.</td>
<td>100</td>
</tr>
<tr>
<td>15 min</td>
<td>0.08 ± 0.03</td>
<td>97.4</td>
<td>n. d.</td>
<td>100</td>
</tr>
<tr>
<td>20 min</td>
<td>n. d.</td>
<td>100</td>
<td>n. d.</td>
<td>100</td>
</tr>
</tbody>
</table>

Analysis using Duncan’s multiple range test, a, b, c and d indicate significant difference (n = 3, p < 0.05); n. d., not detected.
than the all-trans-isomer in a DPPH-scavenging activity test and also in rat microsome and membrane lipid peroxidation systems. Thus, thermal processing or cooking of shrimps might enhance the function and anti-oxidation ability of astaxanthin, which helped to inhibit the oxidation of other functional lipids, such as cholesterol, phospholipid, triglycerides, in the processed products of shrimps.

3.4 Effects of thermal processing on astaxanthin esters

The chromatogram of astaxanthin and astaxanthin esters in raw, microwaved, boiled, fried and shrimps were shown orderly in Fig. 2B-D. Thermal processing of shrimps did not generate new astaxanthin esters. All the 28 astaxanthin esters detected in the extracts of thermal processed shrimps were present in raw shrimps, but their amounts varied. In raw shrimp extract, there was no peak 6 (Fig. 2B), while in thermal processed shrimps, we identified the peak 6 was astaxanthin monoester (Asta-C20:5), which was produced in relatively small amounts. This probably was the geometric isomer of Asta-C20:5, which indicating that thermal processing may also generate the geometric isomers of astaxanthin esters. The stability and degradation of total astaxanthin esters during thermal processing was shown in Fig. 5. As can be seen, in the first 3 min, microwaving, boiling, and frying processing of shrimps diminished the astaxanthin esters content with a loss of 40.06%, 53.26%, 70.50%, respectively. Expectedly, the content of astaxanthin esters in fried shrimps can not be detected at 20 min. Surprisingly, in contrast to the loss of free astaxanthin during thermal processing (Table 2), astaxanthin esters in processed shrimps underwent more serious thermal degradation during microwaving and boiling.

Astaxanthin esters are the main part in the pigment extract in both raw and processed shrimps. It is shown in previous study that astaxanthin esters, which is the main form of astaxanthin existed in marine animals, may possess better singlet oxygen quenching ability and oral-absorbability than free astaxanthin. These experiments on the effect of thermal processing astaxanthin esters may be correlated with daily cooking and industrial processing, which will provide a guiding and profound meaning. However, it has been shown in previous studies that esterified carotenoids, including astaxanthin, are more thermoresistant than free forms. Another investigation by Miao et al. also demonstrated that astaxanthin esters are more stable than free astaxanthin in Haematococcus pluvialis alga powder under different storage conditions. The contrary results of the slower degradation rate of free astaxanthin obtained in our study probably suggested that astaxanthin esters undergo hydroxylation caused by thermal processing, which convert astaxanthin esters into free astaxanthin and fatty acids, particular during microwaving.

To get a deeper insight of effect of thermal processing on astaxanthin esters, each astaxanthin ester molecular species was determined by LC-MS/MS. Due to lack of astaxanthin esters standards, the determination of each astaxanthin ester molecular species were assessed by integrating the corresponding ester molecule peaks in an extracted ion chromatogram in MS. The degradation of eight main astaxanthin ester molecular species in different processed shrimps was illustrated in Fig. 5. As can be seen from Fig. 5, the losses of astaxanthin diesters tended to be lower than those of monoesterified astaxanthin. For example, the microwaved, boiled and fried shrimps lost 73.51%, 76.88% and 85.34% of Asta-C22:6, at the treatment time of 3 min, whereas the content of Asta-C22:6/C22:6 reduced 43.81%, 48.88% and 65.26%, respectively. The other mono- and di- ester molecular species also had
similar declined trends during all the three thermal processing. The same phenomenon was described by another investigation\(^{40}\) explaining the higher stability of astaxanthin diesters in *Haematococcus pluvialis* during the storage of alga power under different conditions. The possible reason is that astaxanthin diesters have longer chain than the monoester form, which may need more energy to break up. Another reason may be that diester molecules have lower polar and greater lipophilic property than astaxanthin monooesters, which facilitate the diester molecule to conjunct with biological tissues. The higher stability of the esterified astaxanthin seemed to be related to their more lipophilic property and hence, to their better integration into membrane structures, which might protect the esters from thermal degradation.\(^{42}\)

Moreover, susceptibility of astaxanthin monooesters was found to differ, with Asta-C20:5 and Asta-C22:6 losing 73.50\% and 76.76\% in microwaved shrimps, 78.31\% and 76.88\% in boiled shrimps, 87.78\% and 85.34\% in fried shrimps, after 3 min of processing. In contrast, Asta-C18:0 and Asta-C18:2 had a higher retention and a slower descending trend of the content in thermal processed shrimps (Fig. 5). A similar degradation behavior was observed in other monoester species during boiling and frying in this study, confirming that the astaxanthin monooesters with saturated fatty acids have a better stability than those with EPA and DHA. Esterified carotenoids, including astaxanthin, composed of saturated fatty acids are more stable, being less prone to photo- and thermo-oxidative degradation and enzyme assisted decomposition.\(^{39}\) Another investigator\(^{41}\) observed that zeaxanthin esters with unsaturated fatty acids have a lower stability than those with saturated fatty acids. However, with regard to astaxanthin diesters, degradation rates of Asta-C22:6/C22:6 and Asta-C22:5/C20:5 in thermally treated shrimps were lower in comparison to other diester molecules (Fig. 5). The lower degradation of Asta-C22:6/C22:6 and Asta-C22:5/C20:5 may suggest a higher thermostability, which is probably due to the high relative content in shrimps and the lower molecular polarity, which is beneficial for the tight connection to biological tissues, thus protects the diestered molecules.

4 CONCLUSIONS

In this study, the stability of free astaxanthin and astaxanthin esters in *Litopenaeus vannamei* during microwaving, boiling and frying was discussed. The amount of total astaxanthin decreased in three thermal processing, and microwaving was the mildest among the employed processing method in this study. However, their effects on free astaxanthin and astaxanthin esters are slightly different. For free astaxanthin, thermal processing induces the conversion of all-trans-astaxanthin to its cis forms, preferentially to 13-cis-astaxanthin and 9-cis-astaxanthin, simultaneously, degradation also happened.

In contrast to free astaxanthin, esterified astaxanthin was found to be hydrolyzed and degraded during thermal processing. But during the process, astaxanthin diesters had a better stability than the monoester form. In addition, astaxanthin esters with EPA or DHA may have a lower thermal stability than those with saturated fatty acids.

This work was the first time to use LC-(APCI)-MS/MS method to identify and quantify all-trans- and cis-isomers of astaxanthin, and molecular species of astaxanthin esters in raw and thermal processed shrimps. The results will facilitate to improve the current knowledge about the effects of different thermal processing methods on astaxanthin and astaxanthin esters, and thus may provide a guiding significance in application of thermal processing in the manufacturing of shrimp and domestic cooking.

Furthermore, these results also demonstrate high relative content of DHA and EPA esterified astaxanthin existing in Pacific white shrimps, which have great value and meaningful references in effective utilization of shrimp processing waste and the development of functional food products containing EPA and DHA.

ACKNOWLEDGMENT

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References

4) Rao, A. R.; Sindhuja, H. N.; Dharmesh, S. M.; Sankar, K. U.; Sarada, R.; Ravishankar, G. A. Effective Inhibition of Skin Cancer, Tyrosinase, and Antioxidative Properties by Astaxanthin and Astaxanthin Esters from the


Effect of thermal processing on astaxanthin from Pacific white shrimp


