**The effect of high pressure treatment on functional components of shrimp
(*Litopenaeus vannamei*) cephalothorax**

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ABSTRACT

High pressure (HP) technology has been applied to extend the shelf life of shrimps by inhibiting enzymes with PPO activity or microorganisms. However, there is very little information on its effect on relevant compounds from a nutritional or functional point of view, such as fatty acids, α -tocopherol, astaxanthin, and hemocyanin, which constitutes the main objective of the present work. Shrimp cephalothoraxes were HP-processed at 200, 400, or 600 MPa/18 °C/15 min or three consecutive 5 min cycles. It was found that hemocyanin was partially denatured at pressures up to 400 MPa, resulting in lower PPO activity, and it was totally denatured at 600 MPa, although 20% residual PPO activity remained. Astaxanthin, α -tocopherol, and total antioxidant activity were stable whichever HP treatment was applied, whereas 600 MPa caused a slight reduction of eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA). Despite this reduction, the -6/ -3 fatty acids ratio was very low (1).

24 **Keywords**

25 High pressure, Hemocyanin, polyunsaturated fatty acids, astaxanthin, α -tocopherol,
26 Shrimp

27

28 **1. Introduction**

29 Shrimps are much-appreciated, high-value fishery products worldwide. In particular,
30 Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important aquaculture
31 species, mostly imported to Europe in a frozen state from farms located in Ecuador and
32 Thailand. Shrimps are appreciated not only from a sensory point of view but also
33 because they are a source of high biological value proteins (high digestibility, essential
34 amino acids), polyunsaturated fatty acids, carotenoids, and α -tocopherol (Bono, Gai,
35 Peiretti, Badalucco, Brugiapaglia, Siragusa, et al., 2012; Dararat, Lomthaisong, &
36 Sanoamuang, 2012; Özogul, Özogul, & Kuley, 2011). Indeed, most of the health
37 benefits provided by frequent seafood consumption come from adequate uptake of ω -3
38 (specially eicosapentaenoic (EPA) and docosahexaenoic acids (DHA)) and ω -6
39 polyunsaturated fatty acids and antioxidants (Barros, Poppe, & Bondan, 2014).
40 Astaxanthin is the most abundant carotenoid found in aquatic animals such as salmon,
41 trout, shrimp, and lobster, and its potential beneficial effects are associated with its
42 antioxidant activity (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006), which is
43 even higher than that of α -tocopherol (Sowmya & Sachindra, 2012).

44 Shrimps are highly perishable fishery products, mainly as a result of microbial growth
45 coupled with the onset of melanosis or blackspot formation. Rapid melanosis
46 development in the cephalothorax of Pacific white shrimp (*L. vannamei*) has been
47 related to the presence of highly stable, active polyphenoloxidase (PPO) (Nirmal &

Benjakul, 2012). PPO in crustaceans, normally involved in cuticle sclerotization and immune response *in vivo*, is mainly responsible for catalyzing post-mortem oxidation of phenolic substrates to quinones. After non-enzymatic polymerization of quinones, an accumulation of black, high molecular weight pigments (melanins) in cephalothorax and cuticle takes place. Although these pigments are apparently harmless to consumers, they may cause an important loss of market value (Montero, Ávalos, & Pérez-Mateos, 2001). Moreover, shrimps (*L. vannamei*) subjected to repeated freeze-thawing cycles, which often occur in retail shops, have been shown to suffer pronounced melanosis promoted by PPO activation (Manheem, Benjakul, Kijroongrojana, & Visessanguan, 2013).

Hemocyanin (Hc), which is a copper-containing respiratory protein, is the main constituent protein in crustacean hemolymph, comprising 90–95% of the total plasma proteins (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005). Hc has been found to acquire diphenoloxidase activity in deepwater pink shrimp (*P. longirostris*) upon post-mortem protease-induced activation (Martínez-Alvarez, Gómez-Guillén, & Montero, 2008). The conversion of Hc to an active PPO form by the action of trypsin or a denaturing treatment with SDS has also been reported in whiteleg shrimp (*P. vannamei*) (García-Carreño, Cota, & Del Toro, 2008). Both PPO and Hc, which have been described as complex, high molecular weight proteins with a strong resemblance to each other, are composed of several subunits capable of aggregating into dimers, trimers, hexamers, or dodecamers, playing a crucial role in melanosis development (García-Carreño, Cota, & Del Toro, 2008; Martínez-Alvarez, Gómez-Guillén, & Montero, 2008). The two main strategies currently employed to reduce melanosis and/or microbial growth in order to extend the shelf life of shrimps are the addition of preservatives (antioxidants, antimicrobials, reducing agents, specific inhibitors, etc.) and

the application of preservation technologies, either thermal or non-thermal (Huang, Ji, Liu, Zhang, Chen, Guo, et al., 2014). However, on the one hand, consumers increasingly demand the marketing of foods with lower amounts of added preservatives, and, on the other, the use of thermal treatments affects the nutritional value of shrimps as well as their sensory properties (Delfieh, Rezaei, Hosseini, Hosseini, Zohrehbakhsh, & Regenstein, 2013; Jantakoson, Kijroongrojana, & Benjakul, 2012). These are the reasons why the application of novel non-thermal food preservation technologies such as modified atmosphere packaging, dense high carbon dioxide processing, pulsed electric field, or high pressure processing have been gaining interest in recent years (Huang, et al., 2014).

High pressure has been applied to shrimps, although the amount of work found is much lower than for meat, vegetables, or fish species. For example, Jantakoson, Kijroongrojana, and Benjakul (2012) applied pressures up to 800 MPa/20 min/28 °C on black tiger shrimp (*Penaeus monodon* Fabricius) that caused lower modifications (weight loss, texture, color) than heat treatment at 100 °C for 2 min. With regard to enzymatic activity, results from the literature reveal that pressurizing a PPO enzyme extract from *L. vannamei* up to 300 MPa/2 min/25 °C increased enzyme activity, whereas pressures of 400–600 MPa/2 min/25 °C inactivated it (Huang, Ji, Liu, Zhang, Chen, Guo, et al., 2014). In spite of this, when pressurizing whole shrimps (400–600 MPa/2 min/25 °C or 400 MPa/10 min/7 °C), no melanosis inhibition was observed, or it was even higher than in unpressurized samples (Huang, et al., 2014; Montero, Lopez-Caballero, & Perez-Mateos, 2001). It is evident, and also previously documented, that the effect of high pressure is very dependent on the pressure/processing time/temperature trinomial as well as on the application on food extracts, whole or minimally processed foods, etc. (Van der Plancken, Grauwet, Oey, Van Loey, &

Hendrickx, 2008). With regard to the antimicrobial action of high pressure, it has been reported to extend the shelf life of shrimps effectively (200 or 400 MPa/10 min/7 °C) (Lopez-Caballero, Perez-Mateos, Borderias, & Montero, 2000). High pressure technology has also been proposed as a means for improving astaxanthin extraction from shrimp waste consisting of cephalothoraxes and cuticles (Du, He, Yu, Zhu, & Li, 2013). Although no differences were observed in comparison with a sample processed at atmospheric pressure, results suggest that astaxanthin was stable to high pressure treatment. Therefore, the few works found in the literature on the application of high pressure treatment on shrimps deal with the effect on muscle proteins, sensory properties, microorganisms, or PPO activity/melanosis development, but there is very little information on its effect on relevant compounds from a nutritional or functional point of view, such as fatty acids, α -tocopherols, astaxanthin, and hemocyanin, which constitutes the main objective of the present work.

2. Materials and methods

2.1. Sample preparation and high pressure (HP) treatment

Among the different shrimp body parts, cephalothorax contains the greatest amount of carotenoids, lipids, and hemolymph proteins (Sachindra, Bhaskar, & Mahendrakar, 2005); for this reason, previously separated cephalothorax was employed for the study of the effects of HP treatment, considering also, from a methodological point of view, that it may represent an intermediate material type between unprotected extracts and fully protected whole shrimps. Ten kilograms of frozen shrimps (*L. vannamei*), kindly provided by Angulas Aguinaga Burgos (Burgos, Spain), was thawed at room temperature and peeled manually. Cephalothoraxes were homogenized to a particle size

of ≈ 5 mm. Aliquots of the homogenate (20 g) were mixed with 10 mL of distilled water and vacuum packed in flexible bags (type BB4L, Cryovac, Barcelona, Spain). HP treatment was performed using a Stansted Fluid Power Iso-lab 900 High Pressure Food Processor (Model: FPG7100:9/2C, Stansted Fluid Power Ltd., Harlow, Essex, UK) at 200, 400, or 600 MPa for 15 min in one cycle (samples named as 200, 400, or 600, respectively) or three consecutive cycles of 5 min each without waiting time between cycles (samples named as 200C, 400C, or 600C, respectively) at 18 °C. A control sample was held at atmospheric pressure for comparative purposes (sample AP). The samples were freeze dried in a BenchTop 6KB lyophilizer (VirTis Co., Gardiner, NY, USA) and subsequently pulverized in a grinder (Moulinex, Madrid, Spain).

2.2. Molecular weight profile

The molecular weight profile of the cephalothorax protein material was determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The freeze-dried sample powders were mixed with loading buffer (2% SDS, 5% mercaptoethanol, and 0.002% bromophenol blue) to achieve a final protein concentration of ≈ 2 mg/mL. Sample was heat-denatured at 95 °C for 5 min and then centrifuged at $13000 \times g$ for 10 min and analyzed according to Laemmli (1970) in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA, USA) using 7.5% resolving gels (Bio-Rad Laboratories) at 25 mA/gel. Protein bands were stained with Coomassie Brilliant Blue R-250. A molecular weight protein standard acquired from Bio-Rad Laboratories (Precision Plus Protein™ Dual Xtra Standards, ref. 161-0377-MSDS) with molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15, 10, 5, and 2 kDa was employed.

2.3. Thermal behavior

It was determined with a model TA-Q1000 Differential Scanning Calorimeter (DSC) (TA Instruments, New Castle, DE, USA). The freeze-dried powders were reduced to ~1 mm particle size in a mortar for proper sampling. Samples of approximately 5–8 mg ($\pm$ 0.002 mg) were weighed out using a model ME235S electronic balance (Sartorius, Goettingen, Germany), tightly encapsulated in hermetic aluminum pans and scanned under dry nitrogen (50 mL/min) purge. An empty capsule was used as reference. Samples were heated from 5 to 100 °C at 10 °C/min and a second scan was occasionally run to check whether protein denaturation was already complete. Peak temperatures (T_{peak} , °C) and transition enthalpies (ΔH , kJ/g_{dm}) by linear baseline integration were calculated after normalization to dry matter content by encapsulated-sample desiccation. Samples were analyzed in triplicate.

2.4. Lipid extraction

Aliquots of 2 g of the freeze-dried powders were mixed with ethyl acetate (50 mL) and stirred at room temperature in darkness for 30 minutes; after extraction, the sample was filtered through Whatman No. 1 filter paper, and the filtrate was evaporated under vacuum to dryness; afterwards it was stored at –30 °C in darkness. The extraction yield was calculated and expressed as g/100 g dry sample.

2.5. Quantification of carotenoids

The lipid extracts were diluted with hexane and the absorbance was measured at 470 nm using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan). The carotenoid concentration was calculated using the equation described by Britton (1995),

where A is absorbance, V is the dilution volume (mL), P is the molecular weight of astaxanthin (597), and ϵ is the molar absorption coefficient reported by Britton (1995) for astaxanthin (125100). Results are expressed as mg astaxanthin/100 mg lipid extract.

2.6. Fatty acid profile

The fatty acid profile was determined in duplicate in an Agilent 7820A gas chromatograph with FID detector equipped with an Agilent HP-88 (ref. 112-8867) column, after preparing fatty acid methyl esters from the lipid extracts obtained. Identification was accomplished by comparison of the retention times with standards, and results were expressed as mg fatty acid/g lipid extract.

2.7. α -Tocopherol content

The lipid extract was subjected to saponification and subsequent extraction with hexane. An aliquot was dried and dissolved in methanol and then injected in an Agilent 1100 high resolution liquid chromatograph equipped with a Supelcosil LC-F column with pre-column (ref. 59158) and coupled to an Agilent G1946D simple quadrupole mass spectrometer. The mobile phases used were isocratic 1:10 water:methanol at a flow rate of 1 mL/min. Identification was accomplished by comparison with a DL- α -tocopherol standard (Supelco, ref. 4-7783) which was subjected to the same preparation steps as the samples. Results are expressed as mg α -tocopherol/100 mg lipid extract.

2.8. Antioxidant activity of lipid extracts

It was accomplished by a photochemiluminescence assay which involves the photochemical generation of superoxide free radical combined with chemiluminescence detection of luminol, which acts as a photosensitizer and also as an oxygen radical

detection reagent. This reaction takes place in the PHOTOCHEM® equipment (Analytik Jena AG). The ACL kit provided by the manufacturer was used to measure lipophilic antioxidant capacity, using Trolox as the calibration reagent. All samples were measured in duplicate and the results were expressed as $\mu\text{g eq. Trolox/mg lipid extract}$.

2.9. Polyphenol oxidase (PPO) activity

The differences in PPO activity among the samples were estimated from the changes in the absorbance of a L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma Chemical, St. Louis, USA) solution incubated in the presence of the obtained freeze-dried powders. L-DOPA (20 mM) was dissolved in 0.05 M pH 6.5 phosphate buffer, mixed with the freeze-dried powder (1 g/100 mL solution), and then incubated in a water bath with vigorous agitation at 30 °C for 20 min. Afterwards, the mixtures were centrifuged at $5000 \times g/4 \text{ }^{\circ}\text{C}/15 \text{ min}$, and the absorbance of the supernatant was measured at 475 nm. The higher the absorbance, the higher the PPO activity (Zamorano, Martínez-Álvarez, Montero, & Gómez-Guillén, 2009). Relative PPO activity was expressed as a percentage with respect to the activity found in the unpressurized sample.

3. Results and discussion

3.1. The effect of high pressure treatment on bioactives

A lipid extract with an intense red color was obtained from all samples, irrespective of the HP treatment applied. The extraction yield of lipids was slightly increased ($p \leq 0.05$) when pressurizing, and the highest extraction yield was obtained in the sample pressurized at 200 MPa in three consecutive cycles (Table 1). In spite of the higher lipid

extraction, the amount of astaxanthin and α -tocopherol per gram of lipid extract was unchanged ($p > 0.05$), irrespective of the treatment applied (Table 1), indicating that both molecules were stable to high pressure treatment in the conditions used. Similar results were found by Du, He, Yu, Zhu, and Li (2013), who did not find changes in astaxanthin extraction yield of shrimp by-products by high pressure treatment (210 MPa/9.6 min). However, astaxanthin standard dissolved in methanol has been found to be pressure sensitive (13% and 25% reduction for treatments at 250 or 500 MPa/18–20 °C/10 min, respectively, as well as isomerization) (Lerfall & Birkeland, 2014). There are two possible causes for this difference. On the one hand, it is feasible that the food matrix protected astaxanthin from degradation. On the other hand, the chemical form in which astaxanthin is commonly found in shrimp, that is, not only as free astaxanthin but also mono- or diesterified with fatty acids or in the form of carotene-proteins (Breithaupt, 2004; Lorenz & Cysewski, 2000; Yang, Zhou, Yang, Xue, Xu, & Xue, 2015), can also influence its stability to HP treatment, as esterified forms are generally recognized as more stable forms (Lorenz & Cysewski, 2000; Miao, Geng, Lu, Zuo, & Li, 2013; Schweiggert, Kurz, Schieber, & Carle, 2007). Similar results were found for lycopene, as the pressurized standard was degraded at pressures ≥ 500 MPa/20 °C/12 min, whereas it was stable when tomato puree was pressurized (Qiu, Jiang, Wang, & Gao, 2006). There are numerous works studying the effect of HP treatment on carotenoid and tocopherol stability of fruits and vegetables, generally indicating the higher stability of such compounds in comparison with thermal treatment (Sanchez-Moreno, De Ancos, Plaza, Elez-Martinez, & Cano, 2009). Furthermore, some reports on the application of HP treatment to fruits, vegetables, or their products point to the higher extraction of certain functional compounds, e.g., the extraction of lycopene from tomato (Krebbers, Matser, Hoogerwerf, Moezelaar, Tomassen, & van den Berg, 2003) or β -

244 carotene, α -carotene, zeaxanthin, lutein, and β -cryptoxanthin from orange juice (De
245 Ancos, Sgroppo, Plaza, & Pilar Cano, 2002), but little work is found in crustaceans. The
246 explanation for this lies in the denaturation of intracellular membranes that facilitates
247 extraction (Barba, Terefé, Buckow, Knorr, & Orlén, 2015). From results of the present
248 work it seems clear that astaxanthin and α -tocopherol in shrimp are more easily
249 extractable than in vegetables, as HP treatment did not cause an increase in their
250 extraction. In contrast to vegetable cells, lipids in animal cells are in the cytoplasm as
251 large free drops. In shrimps, astaxanthin is mainly in the cephalothorax (hepatopancreas
252 and gonads), included in fat globules or as carotene-protein complexes within the
253 cuticle (Nègre-Sadargues, Castillo, & Segonzac, 2000).

254 No significant differences ($p \leq 0.05$) in antioxidant activity were found among non-
255 pressurized and pressurized samples, in good agreement with the high stability to
256 pressure exhibited by astaxanthin and α -tocopherol, as they are the compounds mainly
257 responsible for the antioxidant activity of lipid extracts of a similar nature to those
258 obtained in the present work (Gómez-Estaca, Calvo, Sánchez-Faure, Montero, &
259 Gómez-Guillén, 2015; Sanches-Silva, Costa, Bueno-Solano, Sendón, Sánchez-
260 Machado, Soto Valdez, et al., 2011; Sowmya & Sachindra, 2012). Reports on the
261 antioxidant activity of such compounds can be found elsewhere (Bauerfeind, Hintze,
262 Kschonsek, Killenberg, & Böhm, 2014; Higuera-Ciajara, Félix-Valenzuela, &
263 Goycoolea, 2006).

264 The fatty acid composition of the lipid extracts from shrimp cephalothorax is shown in
265 Table 2. In general, HP treatment did not cause drastic changes in the fatty acid
266 composition; however, depending on the fatty acid, HP caused a significant ($p \leq 0.05$)
267 increase or decrease in their contents in the extracts. The extraction of saturated fatty

acids (SFA) was higher with all HP treatments except for 400C, in comparison with the unpressurized sample (AP), and the increase in C16:0 was particularly large. Similar results were obtained for monounsaturated fatty acids (MUFAs), although in this case the total amount was significantly ($p \leq 0.05$) higher than that of the control (AP sample) only for 200, 200C, and 400 samples. The total amount of polyunsaturated fatty acids (PUFAs) extracted rose with the least intense HP treatments (200 and 200C), mainly as a result of the higher content of C18:2n6c, whereas in the most intense HP treatments (600 and 600C) the total PUFAs decreased; specifically, C20:4n6, C20:5n3, and C22:6n3 were the fatty acids that were significantly ($p \leq 0.05$) reduced, in comparison with unpressurized sample. A slight ($p \leq 0.05$) decrease of C20:4n6 with the 200C treatment was also observed. These changes in the PUFA content gave rise to an increase in the ω -6/ ω -3 fatty acids ratio in pressurized samples; however, it was ≈ 1 for all of them, which is a very low value. The WHO has recommended that the daily ratio of ω -6/ ω -3 in the total human diet should be no higher than 5, because it is the key factor for balanced synthesis of eicosanoids in organisms (Deng, Padilla-Zakour, Zhao, & Tao, 2015). As the ω -6/ ω -3 fatty acids ratio is nowadays estimated to range between 15 and 16.7 (Simopoulos, 2002), the inclusion of any of the lipid extracts studied in the present work would contribute to reduce the overall ratio in the human diet. Results from the literature reveal contradictory results on the effect of HP treatment to fatty acid profile, depending on the type of food and processing conditions. For example, pressurization of various fish or shellfish species such as salmon, mackerel, turbot, or oyster at pressures up to 800 MPa and at temperatures ranging from 4 to 20 °C did not cause significant modifications of the fatty acid profiles, whereas pressures up to 800 MPa at temperatures up to 60 °C on beef or pressures up to 400 MPa/15 °C in orange juice-milk beverage induced modifications of the fatty acid composition (Barba, Terefe,

Buckow, Knorr, & Orlie, 2015). It is well known that the effect of high pressure on food components and microorganisms depends on the treatment applied (pressure/time/temperature) and the type of foodstuff.

3.2. The effect of high pressure treatment on proteins or enzymes with PPO activity

Cephalothorax contains not only most of the bioactives of crustaceans but also enzymes and other proteins that contribute to deterioration. Among them, PPO and hemolymph proteins with potential PPO activity stand out because of their implication in the development of melanosis (García-Carreño, Cota, & Del Toro, 2008; Martínez-Alvarez, Gómez-Guillén, & Montero, 2008).

Figure 1 represents the molecular weight distribution, determined by SDS-PAGE, of proteins present in the cephalothorax subjected to different high pressure treatments. Two main protein bands at around 75 kDa were clearly predominant in the electrophoretic profile of the unpressurized sample (AP), as well as in the samples pressurized at 200 and 400 MPa, with or without three consecutive cycles (200, 200C, 400, and 400C). These bands disappeared completely in the case of samples subjected to 600 MPa (600 and 600C). According to Zhang, Wang, Xu, Chen, Lin, and Peng (2006), these proteins would correspond to different hemocyanin (Hc) subunits. Those authors identified as Hc three proteins of 77, 75, and 64 kDa from the hemolymph of shrimp (*L. vannamei*), and specifically the 75 kDa protein was shown to react with anti-human IgA, and also had the ability to agglutinate bacteria and erythrocytes. Similarly, Hc subunits with molecular masses of 75 and 82 kDa have also been reported in whiteleg shrimp (*P. vannamei*) hemolymph (García-Carreño, Cota, & Del Toro, 2008). Crustacean hemocyanins have been reported to adopt a typical hexameric structure, in

which each subunit, in the range of around 75 kDa, can bind an oxygen molecule (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005). Hc hexamers can cluster to form supramolecular aggregates up to 48 (8×6) subunits (García-Carreño et al., 2008). As the electrophoretic study in the present work was performed under denaturing conditions (SDS-PAGE), the aggregation status of Hc in the different samples could not be elucidated. Furthermore, it is interesting to note that the electrophoretic protein bands associated with the presence of PPO subunits or oligomers in cephalothorax crude enzyme extracts are normally very hard to visualize using conventional staining with Coomassie Brilliant Blue, as the amount of PPO in crustaceans is about 1000 times lower than Hc (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005; Martínez-Alvarez, Gómez-Guillén, & Montero, 2008). However, on the basis of activity staining with L-DOPA, Manheem, Benjakul, Kijroongrojana, and Visessanguan (2012) reported two partially purified isoforms of PPO from Pacific white shrimp (*L. vannamei*) cephalothorax with molecular weights of 210 and 220 kDa.

The electrophoretic protein pattern of 600 and 600C samples clearly indicates a strong pressure-induced denaturation of Hc subunits. Trace protein bands at around 50 kDa appeared in samples pressurized at 200 and 400 MPa, probably corresponding to products of possible hydrolysis of Hc subunits (García-Carreño, Cota, & Del Toro, 2008). These small bands were absent in the samples pressurized at 600 MPa (600 and 600C), in which diffuse lower molecular weight protein bands arose. These findings strongly suggest noticeable protein breakage induced by the high pressure treatment in Hc subunits, being maximum at the highest pressure intensity level tested.

Normalized DSC traces of the AP and pressurized samples are shown in Figure 2, where, despite structural differences concerning processing parameters (pressure, temperature, time), hemocyanin denaturing effects followed the same pattern as

reported previously for myofibrillar proteins (Fernández-Martín, Fernández, Carballo, & Colmenero, 1997; Fernández-Martín, Pérez-Mateos, & Montero, 1998). Samples AP, 200, 200C, 400, and 400C exhibited an endothermic asymmetrical event with T_{peak} at 77–78 °C, overlapping a smaller one with T_{peak} at ~85 °C. It was assigned to thermal denaturation of the shrimp hemocyanin oligomer with hexameric molecular structure, which was consistent with the electrophoretic 75 kDa band (the minimum functional unit of arthropod hemocyanins) in Figure 1. The buried second peak corresponded to an isomeric form of the main one.

Total enthalpy data (ΔH , J/g_{dm}) progressively diminished with increasing pressure level and processing time until total endotherm disappearance in samples 600 and 600C (flat DSC trace): 1.18, 1.12, 1.10, 1.02, and 0.82 for samples AP, 200, 200C, 400, and 400C, respectively. The protein thermal behavior was consistent with the evolution of the 75 kDa band (Figure 1) in pressurized samples and its final disappearance in samples 600 and 600C, with the simultaneous appearance of bands at well below 25 kDa.

We have no previous DSC literature on shrimp hemocyanin for comparison, but corresponding data for lobster hemocyanin (Guzman-Casado, Parody-Morreale, Mateo, & Sanchez-Ruiz, 1990) indicated occurrence of the same type of phenomenology (as could be expected, as both animals are crustaceans), although shifted down around 20 °C, probably due to species differences. It was also shown by gastropod hemocyanin (Idakieva, Parvanova, & Todinova, 2005), which was not so foreseeable. However, ΔH data were extremely low for a protein denaturing at this high transition temperature range (Lyubarev & Kurganoy, 2001). AP, the non-pressurized sample, showed a very small ΔH , around 4.5% of the value reported for lobster (27 J/g for the hexamer) by Guzman-Casado, Parody-Morreale, Mateo, and Sanchez-Ruiz (1990); the different species, sample manipulation, and experimental conditions in the cited work and the

present one (particularly, not being able to express ΔH in relation to protein content, by far the smallest component of dry matter) may largely explain the difference.

Relative diphenoloxidase activity, determined using L-DOPA as substrate, of cephalothorax protein constituents after high pressure treatment is presented in Figure 3. Pressure intensity levels of 200 and 400 MPa led to around 25% reduction of PPO activity with respect to the unpressurized sample. In samples pressurized at 600 MPa, the residual PPO activity was strongly diminished by up to around 20% of the initial activity. The treatment with three consecutive high pressure cycles did not induce any significant difference ($p \leq 0.05$) in the residual activity. These findings are in agreement with SDS-PAGE and DSC results, where pressure-induced denaturation and/or breakage of Hc subunits were clearly evidenced. Since Hc has previously been reported to exhibit PPO activity through reaction with L-DOPA (García-Carreño, Cota, & Del Toro, 2008; Martínez-Alvarez, Gómez-Guillén, & Montero, 2008), the contribution of hemocyanin to PPO-like activity in Pacific white shrimp is apparently of great importance, being considerably reduced only at a high pressure such as 600 MPa. The ability of Hc to oxidize phenols could be of considerable economic significance, given the large amount of this protein in crustaceans (Adachi, Endo, Watanabe, Nishioka, & Hirata, 2005). However, PPO activity was not completely inhibited even at the highest pressure intensity tested, which may be largely due to the contribution of very active PPO subunits or oligomers present in shrimp hemolymph (Manheem, Benjakul, Kijroongrojana, & Visessanguan, 2012), which could not be visualized in the electrophoretic profile.

It's worth noting that, regardless the pressure intensity, HP treatments induced slight whitening (barely visible to the naked eye) in the corresponding cephalothorax homogenates, as compared to the non-pressured sample, largely attributed to the above

reported pressure-induced denaturation of hemocyanin. To this respect, the muscle of *Penaeus japonicus* has been also found to exhibit slight whiter appearance after high pressure treatment of 400 MPa for 10 min (López-Caballero, Pérez-Mateos, Borderías & Montero, 2000). In contrast, the intense red color was apparently highly preserved in the corresponding lipid extracts, in good accordance to the relatively high stability of astaxanthin, α -tocopherol and most fatty acids. Moreover, although PPO-like activity was not completely inhibited by any of the HP treatments tested, no signs of melanosis were evidenced in both pressurized and non-pressurized homogenates under the studied conditions.

4. Conclusion

HP treatment at pressures ranging from 200 to 400 MPa/18 °C applied in one cycle of 15 min or three consecutive cycles of 5 min each caused a significant reduction of PPO activity due to partial denaturation of hemocyanin, which was total at pressures of 600 MPa, although some residual PPO activity ($\approx 20\%$ of the initial) remained. Under these processing conditions, neither astaxanthin nor α -tocopherol were degraded, and the lipid extracts obtained showed no changes in antioxidant activity, in comparison with unpressurized sample. Only small changes in the fatty acid profile in relation to HP treatment were observed, the most notable one being a significant reduction of C20:4n6, C20:5n3, and C22:6n3 in the most intense treatments (600 MPa), resulting in a slight increase in the -6/ -3 fatty acids ratio from 0.95 (control sample) to 1. Therefore, high pressure treatment is an effective means to inactivate enzymes with PPO activity from shrimp with a minor modification of their nutritional value.

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590 **Legend to Figures**

591 Figure 1. SDS-PAGE patterns of shrimp cephalothorax subjected to different HP
592 treatments.

593

594 Figure 2. Normalized typical DSC patterns of samples: a) AP, 200, 200C, 400, and
595 400C. b) 600 and 600C. Vertical bar, Ordinate scale (0.05 W/gdm). Endothermic
596 effects, Traces Downshifting.

597

598 Figure 3. Relative PPO activity (%) determined using L-DOPA as substrate.

Figure 1

Figure 2.

Figure 3.- . Relative PPO activity (%) determined using L-DOPA as substrate.

Table 1. Lipid extraction yield (g/100 g dry sample), astaxanthin (mg/100 mg lipid extract), and α -tocopherol (mg/100 mg lipid extract) contents of the lipid extract, and antioxidant activity (μ g eq. Trolox/mg lipid extract) of the lipid extract, after different HP treatments of shrimp cephalothorax.

	Total lipids	Astaxanthin	α -Tocopherol	Antioxidant activity
AP	1.7 $\pm$ 0.0a	0.52 $\pm$ 0.11a	4.7 $\pm$ 0.3a	3.4 $\pm$ 0.4ab
200	2.3 $\pm$ 0.0bc	0.46 $\pm$ 0.04a	4.1 $\pm$ 0.0a	3.0 $\pm$ 0.3ab
200C	2.5 $\pm$ 0.1c	0.46 $\pm$ 0.04a	3.7 $\pm$ 0.8a	2.8 $\pm$ 0.3a
400	2.0 $\pm$ 0.2ab	0.49 $\pm$ 0.05a	4.8 $\pm$ 0.2a	3.2 $\pm$ 0.2ab
400C	2.0 $\pm$ 0.1b	0.50 $\pm$ 0.02a	4.3 $\pm$ 0.5a	3.3 $\pm$ 0.1ab
600	2.1 $\pm$ 0.1b	0.52 $\pm$ 0.05a	5.0 $\pm$ 0.6a	3.2 $\pm$ 0.2ab
600C	2.1 $\pm$ 0.0b	0.54 $\pm$ 0.04a	4.5 $\pm$ 0.3a	3.4 $\pm$ 0.1b

Table 2. Fatty acid composition (mg fatty acid/g lipid extract) of the lipid extract from shrimp cephalothorax subjected to different high pressure treatments.

Fatty Acid	AP	200	200C	400	400C	600	600C
C12:0	0.6±0.1a	0.5±0.1a	0.5±0.1a	0.5±0.0a	0.5±0.1a	0.6±0.0a	0.6±0.0a
C14:0	5.4±0.0a	6.0±0.1b	6.2±0.1b	6.0±0.0b	5.3±0.2a	6.2±0.2b	6.0±0.2b
C15:0	4.7±0.0ab	4.6±0.1ab	4.5±0.0a	5.0±0.1c	5.1±0.0c	5.0±0.1bc	5.0±0.1c
C16:0	94.0±0.2a	108.9±0.6c	119.2±0.3d	104.3±1.8b	90.9±1.0a	112.2±1.7c	102.9±1.3b
C17:0	6.9±0.0a	7.0±0.2a	7.1±0.3a	7.0±0.2a	6.9±0.0a	6.9±0.1a	6.7±0.2a
C18:0	29.2±0.2cd	28.4±0.1bc	30.0±0.1d	27.7±0.6ab	26.8±0.4a	27.4±0.7ab	27.2±0.2ab
C20:0	2.1±0.1a	2.4±0.2a	2.5±0.1a	2.5±0.2a	2.3±0.1a	2.4±0.0a	2.4±0.0a
C21:0	1.3±0.1a	1.6±0.2a	1.5±0.1a	1.4±0.0a	1.3±0.1a	1.4±0.1a	1.4±0.0a
C22:0	2.1±0.2a	2.4±0.4a	2.4±0.1a	2.2±0.1a	2.0±0.0a	2.2±0.2a	2.1±0.0a
C23:0	1.2±0.1a	1.3±0.2a	1.2±0.0a	1.2±0.1a	1.3±0.0a	1.1±0.0a	1.2±0.0a
C24:0	2.3±0.4a	2.9±0.7a	2.8±0.3a	2.6±0.2a	2.5±0.2a	2.6±0.1a	2.4±0.4a
Σ SFA¹	150±1	166±3	178±2	160±3	145±2	168±3	158±3
C14:1	0.6±0.0a	0.7±0.0a	0.7±0.0a	0.7±0.0a	0.6±0.0a	0.6±0.0a	0.7±0.1 ^a
C16:1	14.0±0.2a	15.4±0.2c	15.3±0.0c	14.7±0.3b	13.7±0.1a	13.6±0.2a	13.8±0.1a
C18:1 ^{n7c}	14.8±0.1b	15.6±0.1c	15.1±0.0bc	15.4±0.3c	14.7±0.1b	14.2±0.2a	14.6±0.1ab
C18:1 ^{n9t}	1.8±0.1a	1.7±0.1a	1.6±0.1a	2.1±0.5a	1.7±0.3a	1.6±0.5a	1.9±0.3a
C18:1 ^{n9c}	97.6±0.2ab	112.6±1.4d	114.1±0.0d	104.7±2.1c	94.4±0.9a	97.1±1.6ab	98.9±0.7b
C20:1	5.7±0.1a	6.4±0.0c	6.4±0.0c	6.0±0.1b	5.5±0.0a	5.7±0.1a	5.8±0.1a
C22:1 ⁿ⁹	1.4±0.1a	1.6±0.3a	1.2±0.1a	1.3±0.1a	1.7±0.5a	1.3±0.1a	1.4±0.0a
C24:1	2.4±0.1a	2.7±0.3a	2.6±0.0a	2.4±0.0a	2.5±0.0a	2.4±0.0a	2.4±0.1a
Σ MUFA²	138±2	157±2	157±1	147±5	135±3	136±5	140±3
C18:2 ^{n6c}	80.0±0.3a	91.5±0.9c	92.7±0.3c	84.7±1.4b	76.8±0.6a	77.8±1.5a	78.6±0.7a
C18:3 ⁿ³	6.2±0.1b	7.0±0.1c	7.1±0.0c	6.4±0.1b	5.7±0.1a	5.8±0.1a	5.9±0.0a
C18:3 ⁿ⁶	0.7±0.1a	0.5±0.1a	0.6±0.0a	0.7±0.1a	0.6±0.1a	0.6±0.0a	0.6±0.0a
C20:2	12.1±0.8a	12.5±0.3a	12.5±0.5a	12.4±0.5a	11.8±0.2a	11.6±0.1a	11.9±0.3a
C20:3 ⁿ⁶	1.2±0.0ab	1.4±0.1c	1.2±0.0abc	1.3±0.0bc	1.2±0.0abc	1.1±0.0a	1.2±0.0abc
C20:4 ⁿ⁶	25.1±0.1e	24.0±0.0cd	22.8±0.0ab	24.7±0.5de	25.1±0.3e	22.1±0.4a	23.3±0.3bc
C20:5 ⁿ³	39.7±0.5b	39.5±0.3b	39.0±0.0b	39.3±1.0b	39.3±0.4b	35.1±0.7a	36.6±0.4a
C22:5 ⁿ³	7.8±0.2a	8.5±0.8a	8.0±0.0a	7.8±0.0a	7.5±0.1a	7.2±0.1a	7.2±0.2a
C22:6 ⁿ³	58.5±0.9b	60.5±0.0b	60.4±0.4b	59.0±1.4b	57.9±0.6b	53.3±1.2a	55.0±0.4a
Σ PUFA³	231±3	245±5	244±1	236±5	226±3	215±4	220±3
Σ PUFAⁿ⁻³⁴	112±2	115±4	114±1	113±7	110±3	101±6	105±3
Σ PUFAⁿ⁻⁶⁵	107±7	117±9	117±3	111±10	104±6	102±10	104±6
Σ PUFAⁿ⁻⁶/Σ PUFAⁿ⁻³⁶	0.95	1.02	1.03	0.99	0.94	1.00	0.99

¹Σ SFA: total amount of saturated fatty acids. ²Σ MUFA: total amount of monounsaturated fatty acids. ³Σ PUFA: total amount of polyunsaturated fatty acids. ⁴Σ PUFA -3: total amount of -3 polyunsaturated fatty acids. ⁵Σ PUFA -6: total amount of -6 polyunsaturated fatty acids. ⁶Σ PUFA -6/PUFA -3: -6/ -3 polyunsaturated fatty acids ratio.