Effect of high hydrostatic pressure (HHP) on proteins, lipids, and water state of three different thawed seafood products

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Abstract

The aim of this research is to investigate the changes in the state of different components of seafood muscle subjected to High Hydrostatic Pressure (HHP), namely water, proteins, and lipids. Three seafood products were subjected to three pressure levels (400, 500, and 600 MPa) for 10 min and analyzed through a multi-analytical approach, including differential scanning calorimetry and protein solubility assay, time-domain nuclear magnetic resonance (TD-NMR), and various lipid oxidation and hydrolysis assays. Results showed a similar level of variation for some parameters, such as whitening and water mobility, across all species and pressure levels investigated, while protein denaturation, protein solubility, and texture were more effective in discriminating the samples—particularly for grey mullet, which appeared to be the most affected and, therefore, the least suitable for HHP treatment. Only a slight increase was observed in lipid oxidation parameters. In conclusion, the results highlight the importance of selecting raw material based on its response to pressure application.

Keywords: deep-water rose shrimp; grey mullet; lipid oxidation; protein functionality; tiger prawn

Introduction

High Hydrostatic Pressure (HHP) is a non-thermal technology that has found wide application in the food sector as a cold pasteurization strategy, capable of reducing microbial load with less detrimental effect on the product's nutritional quality compared to thermal treatments (Huang *et al.*, 2014). Its effect is based on the inactivation of vegetative cells of pathogenic and spoilage microorganisms, thus increasing microbiological shelf life. However, significant changes in some food components, such as proteins and lipids, as well as in food structure, are unavoidable and could lead to alterations in quality characteristics, ultimately reducing consumer acceptability of HHP-treated products.

Fish products are generally very perishable due to microbial spoilage, lipid oxidation, and proteolytic reactions (Tavares *et al.*, 2021); therefore, the processing industry is continually searching for sanitation strategies that increase the safety and shelf life of the product. In addition, the increasing consumption of raw fish has highlighted this need. HHP has been found effective in increasing seafood shelf life by reducing microbial load

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and maintaining freshness indexes (Lan *et al.*, 2022). Moreover, HHP also appears to be capable of devitalizing *Anisakis* larvae (Brutti *et al.*, 2010), and although European legislation still requires a freezing-thawing cycle for fish products intended for raw consumption, it might be considered a valid alternative in the future.

Nevertheless, the qualitative changes induced by the application of high pressure might be even more relevant when related to a product intended for raw consumption. Indeed, protein denaturation occurring as a consequence of pressure may promote significant changes in color, leading to an appearance typical of a cooked product (Kaur *et al.*, 2016). Also, the texture is generally strongly affected, although the effect appears to be strictly dependent on the specific fish/seafood species considered.

Changes in protein structure also affect their interaction with water, resulting in modifications to hydration level, solubility, and functional properties. According to Zhang *et al.* (2015), the level of applied pressure affects protein–water interactions differently; at pressures below 300 MPa, the hydrogen bonds between water and proteins are favored, resulting in higher water-holding capacity, while at higher values, the opposite effect is observed, as protein–protein bonds become predominant.

Seafood products are also rich in polyunsaturated lipids, which are known to have beneficial effects on human health but are also highly susceptible to oxidation. Various authors have reported an increase in oxidation after HHP application on seafood products (Medina-Meza *et al.*, 2014); however, reports are often contradictory, likely due to the many factors involved, such as processing parameters (level of pressure and process time) and intrinsic characteristics of the matrix (lipid content, fatty acid profile, age and fiber type, presence of oxygen, heme-proteins, and enzymes) (Truong *et al.*, 2015).

Although the literature is rich with examples of HHP application on seafood products, contrasting results have been presented, mainly due to different pressure profiles applied to different species.

Therefore, three seafood products—grey mullet, tiger prawn, and deep-water rose shrimp—were subjected to three pressure levels (400, 500, and 600 MPa) for 10 minutes, and the effect on microbial inactivation and shelf life was analyzed previously (de Aguiar Saldanha Pinheiro *et al.*, 2023). The aim of this research is to investigate the changes in the state of different components of seafood muscle subjected to HHP, namely water, proteins, and lipids. To this end, the same three seafood species examined in the previous study were analyzed through a multi-analytical approach, including differential scanning calorimetry and protein solubility assay, time-domain nuclear magnetic resonance (TD-NMR), and various lipid oxidation and hydrolysis assays.

Materials and Methods

Fish samples preparation

Grey mullet (*Mugil cephalus*), tiger prawn (*Melicertus kerathurus*), and deep-water rose shrimp (*Parapenaeus longirostris*) were fished in the Adriatic Sea. Because samples were meant for raw consumption, they were fast frozen at a temperature of -18°C by the company Economia del Mare (Cesenatico, Italy). After 24 h, the seafood was thawed for 16 h at refrigeration temperature. The samples were then mechanically deboned (grey mullet) and peeled (rose shrimp and tiger prawn). Flesh was cut into pieces and packed in polypropylene (PP) trays containing 6 mono-portions of about 15-20 g, vacuum packed with a PP film. The packaged samples were refrigerated and stored at 2°C for 24 h until HHP treatment. For each specie, 3 trays were considered for each of the selected pressure conditions.

HHP treatment

Vacuum-packed samples were subjected to HHP treatment with a Hiperbaric_420 Model (Hyperbaric, Spain) at the company HPP Italia s.r.l (Parma, Italy). Packed samples were placed inside cylindrical baskets that entered a 420 L chamber and were subjected to 400, 500, and 600 MPa for 10 min. Untreated samples (0.01 MPa) were used as controls. Considering that the duration of the pressure rise ramp was 2.7, 3.1, and 3.5 min for the increasing pressure levels, while the intended pressure was maintained for 10 min, the total cycle durations were 13.9, 14.4, and 14.7 min.

The pressure levels and holding times were selected according to a previous study (de Aguiar Saldanha Pinheiro *et al.*, 2023) showing their effectiveness in significantly increasing the product's shelf life. All analytical determinations were carried out 24 h after the treatment, at 2°C.

Analytical determinations

Differential scanning calorimetry

A differential scanning calorimeter DSC Q20 (TA Instruments, Germany), equipped with a low-temperature cooling unit Intercooler II (Perkin-Elmer Corporation, USA), was used to determine the protein denaturation status of control and treated samples.

Calibrations of temperature and melting enthalpy were carried out with distilled water (mp 0.0°C) and indium (mp 156.6°C), and the heat flow was calibrated using the fusion heat of indium (ΔH = 28.71 J/g), applying the same heating rate and dry nitrogen gas flow of 50 ml/min used for the analysis.

According to Baldi *et al.* (2019), each sample was weighed (about 15 mg) into a 50-µL aluminum pan, sealed hermetically, and then loaded into the DSC instrument at room temperature. The heating rate of DSC scans was 5°C/min over a range of 20 to 90°C. Empty aluminum pans were used as references and for baseline corrections. Three replications for each sample were performed.

Protein solubility

Protein solubility was measured according to Warner *et al.* (1997) with slight modifications. Specifically, sarcoplasmic protein solubility was determined by homogenizing 1 g of muscle tissue in 10 mL of ice-cold 25 mM potassium phosphate buffer (pH 7.2) for 30 s at 13,500 rpm using the UltraTurrax T25 Basic (IKA-Werke, Germany). The homogenates were stored at 4°C for 20 hours and then centrifuged at 2,600 × g for 30 min. Protein concentration of the supernatant was measured by Bradford assay, using bovine serum albumin as the standard. Total protein solubility (i.e., myofibrillar and sarcoplasmic protein fractions) was similarly determined by homogenizing the same muscle tissue aliquot in 1.1 M KI and 0.1 M potassium phosphate buffer (pH 7.2). Myofibrillar protein solubility was then calculated as the difference between total and sarcoplasmic protein solubility. Three replications for each sample were performed.

TD-NMR

The samples for TD-NMR consisted of cylinders weighing about 400 mg, with a height and diameter of 10 mm. They were analyzed with a Bruker spectrometer mod. Minispec PC/20 operating at 20 MHz, using the CPMG pulse sequence (Meiboom and Gill, 1958), employed to obtain signals weighted by transverse relaxation time (T2) (Meiboom and Gill, 1958). Each signal's decay was digitized into 30K points, spaced 0.080 ms. This spacing prevented sample overheating and allowed the observation of protons with a T2 higher than a few milliseconds. A recycle delay of 3.5 s was set up to separate subsequent scans. UPEN software (Borgia *et al.*, 1998) was used to obtain an overview of the protons' T2 distributions, known as relaxograms. This was done through the inversion of the T2-weighted signals into a semi-continuous distribution of exponential decays, according to Eq. (1):

$$I(2\tau n) = \sum_{i=1}^{M} I_0(T_{2,i}) \exp\left(\frac{-2\tau n}{T_{2,i}}\right) \quad (1)$$

With 2τ being the time between subsequent pulses of the CPMG sequence, n is the index of each CPMG point, while I_0 represents the intensities of each T2 component extrapolated at t = 0, sampled logarithmically. To separately observe some components of the relaxograms, even when partially overlapped, a fitting of the T2-weighted signals to the sum of an increasing number of exponential curves was performed. The optimum ratio between fitting ability and model complexity was reached for most samples with three exponentials, as assessed by an F-test. Six measurements were performed for each of the experimental sets.

Color and texture

The color was evaluated by measuring the lightness (L*), redness (a*), and yellowness (b*) with a spectrophotocolorimeter mod. ColorFlex™ (Hunterlab, Reston, Virginia). The tristimulus L*, a*, b* measurement mode (CIE, 1976) was used.

The texture was evaluated with a Texture Analyser mod. TA.HDi 500 (Stable Micro Systems, Godalming, UK) equipped with a 25 kg load cell. Briefly, 15 g of the sample, removed from refrigeration 10 min prior, were inserted into a cylindrical cup (diameter 3 cm), and a piston was used to compress the sample at 1 mm/s up to 50% of its height. The pressure was held for 60 s. The force at the end of compression was considered as an index of resistance to compression (F2, N).

Color and texture were evaluated using at least 6 replicates for each sample.

Lipid oxidation and hydrolysis indexes

Total lipid extraction

Lipids were extracted using a method previously described by Bligh and Dyer (1959), according to the modification of Vernocchi *et al.* (2007). Peroxide value (PV) was used to determine primary lipid oxidation, thiobarbituric acid reactive substances (TBARS) were used to determine secondary oxidative products, while lipid hydrolysis was measured by free fatty acids (FFA) content. Extraction was performed in triplicate for each sample, and PV, TBARS, and FFA were determined in duplicate for each extract.

Peroxide value (PV)

The value of PV was determined by the ferrothiocyanate method (Chapman and Mackay, 1949). Results were expressed as millimoles of O_2 per kg of lipid.

Thiobarbituric acid reactive substances (TBARS)

The 2-thiobarbituric acid-reactive substances (TBARS) were measured according to the method described by

Bao and Ertbjerg (2015). Results were expressed as mg of malondialdehyde (MA) per kg of fillet, calculated using a standard curve of 1,1,3,3-tetraethoxypropane.

Free fatty acid (FFA)

FFA content was determined using the method of Lowry and Tinsley (1976), modified according to Bernárdez *et al.* (2005). The absorbance was read at 710 nm, and the amount of FFA was determined using a standard curve prepared from oleic acid. Results were expressed as g oleic acid/100 g lipids.

Statistical analysis

All data were subjected to one-way ANOVA for mean comparisons, with standard deviation (SD) calculated, and significant differences were determined according to the Tukey HSD post-hoc test. Pearson correlation was used as a measure of linear association between treatments.

Principal Components Analysis (PCA), based on the Spearman correlation matrix, was performed using all the variables to reduce dimensionality and better represent the variation present in the dataset.

All data were processed using XLSTAT, a Microsoft Office Excel add-in software (XLSTAT 2021, Addinsoft, Paris, France).

Results and Discussion

Differential scanning calorimetry (DSC)

Figure 1 reports examples of thermograms obtained from grey mullet (A), tiger prawn (B), and deep-water rose shrimp (C) samples subjected to different pressure levels. As muscle tissue is a complex system comprising different classes of proteins, the DSC curves are also generally quite complex. In each thermogram, several peaks can be recognized in the temperature range of 40–85 °C, which represent the different protein fractions. According to various reports on chicken flesh (Baldi *et al.*, 2019) and various fish species (Jantakoson *et al.*, 2012; Schubring, 2005), the peaks with increasing temperature can be assigned to myosin (region 1: range 45–55°C),

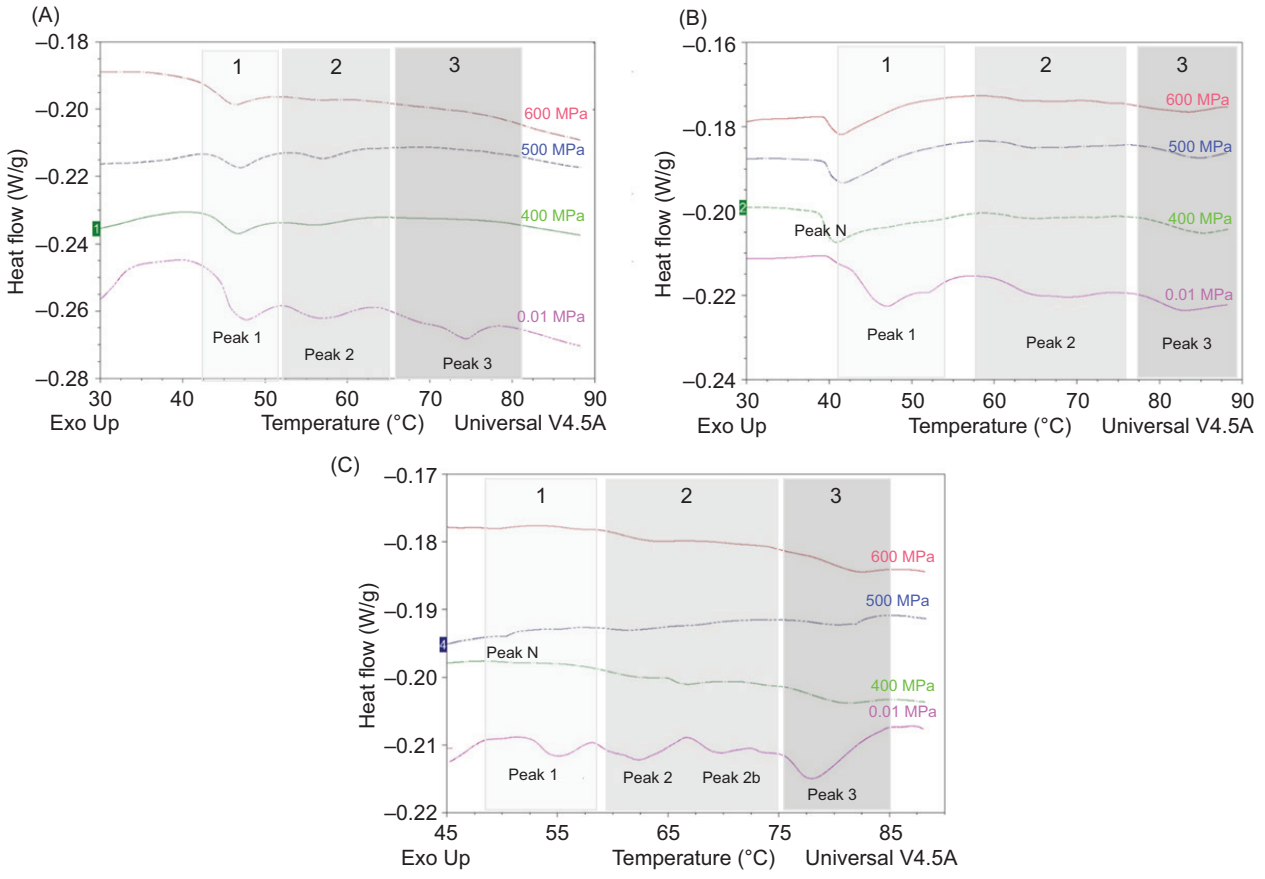

Figure 1. Thermograms obtained for the three seafood species, grey mullet (A), tiger prawn (B), and rose shrimp (C) treated with different pressure levels compared to the untreated samples.

sarcoplasmic proteins and/or collagen (broader peak in region 2: range 55–70°C), and actin (region 3: range 70–80°C). In the DSC pattern of untreated rose shrimp, two different transition peaks can be observed in the second region, which can be assumed to be related to sarcoplasmic proteins and collagen/connective tissue.

Pressure above 300 MPa is known to affect quaternary, tertiary, and secondary protein structures, while the primary structure remains unchanged. According to various authors, myosin starts denaturing between 100 and 200 MPa, while sarcoplasmic proteins, which are more resistant, start denaturing at 400 MPa (Hedges and Goodband, 2003; Jantakoson *et al.*, 2012). According to Hsu and Ko (2001), after the application of 100 MPa, the denaturation enthalpy was reduced by 85%, but increasing exposure time did not influence the denaturation levels, indicating gelling and aggregation phenomena.

In the present study, for all seafood species, all individual peaks were greatly reduced and eventually disappeared completely after high-pressure treatment, due to the denaturation process, with a reduction in total enthalpy that increased with the applied pressure. Table 1 reports the total denaturation enthalpy for all samples and the reduction (%) due to pressure application. In particular, for grey mullet, the reduction in total denaturation enthalpy ranged from 65% to 80%; for tiger prawn, it was only 21% for the 400 MPa treatment and around 37-38% for the higher pressures; and for rose shrimp, it was 53% for the 400 MPa treatment and around 80% for the higher pressures.

Considering the singular peaks, Table 1 also reports the specific enthalpy (ΔH) and denaturation temperature (Td). For all three species, sarcoplasmic proteins and/or collagen and actin appear to be more sensitive to pressure compared to myosin, in agreement with the observations of Larrea-Wachtendorff *et al.* (2015). At pressures above 150 MPa, actin has been reported to undergo irreversible denaturation. In this study, the actin peak showed a lower temperature (Td) after the 400 MPa treatment and was no longer detectable at pressures above 500 MPa for grey mullet. In tiger prawn and rose shrimp, the denaturation temperature did not change, but a significant decrease in peak enthalpy was observed.

With regard to sarcoplasmic proteins and/or collagen, a remarkable reduction was observed in all three species. For rose shrimp, the two separate peaks identified in the control sample merged into a broader one for all pressure-treated samples, with a shift in the temperature.

Moreover, while for grey mullet, the myosin denaturation temperature did not decrease significantly (around 47 °C), in tiger prawn and rose shrimp treated samples, a shift of the peak to a lower temperature (slightly above 40 °C), identified as Peak N, was observed. A similar effect was observed by Jantakoson *et al.* (2012) on black tiger shrimp. This behavior appears to be species-specific (Schubring, 2005) and was not observed in grey mullet. Peak N can probably be attributed to a structure formed following myosin denaturation. It is known that protein structural modifications by HHP are related to the rupture of noncovalent interactions and the subsequent reformation of intramolecular and intermolecular bonds within and between protein molecules. According to the authors, the denaturation of myosin alone gave rise to the new transition, dominantly stabilized by hydrogen bonds, which was not modified by further denaturation of sarcoplasmic proteins, connective tissue, and actin (Larrea-Wachtendorff *et al.,* 2015).

Protein solubility

The solubility of proteins plays an important role in defining various functional properties of meat, including gelling and emulsifying properties, as well as water retention capacity. In particular, the solubility of proteins depends on the interaction between myofibrillar proteins and other components present in the muscle (Xiong, 2018).

As can be clearly seen from Figure 2 (A and B), regardless of the treatment, both sarcoplasmic (Figure 2A) and total proteins (Figure 2B) of all seafood species underwent a remarkable ($p < 0.05$) decrease in their solubility after HHP, with grey mullet showing the greatest reduction, reaching values 4 to 6 times lower for the higher pressures applied.

On the other hand, the measurements of protein solubility and texture showed different behavior depending on the species investigated. Protein solubility refers to the quantity of total muscle proteins that are solubilized under specific conditions and depends on various factors such as protein structure, pH, salt content, temperature, and other intrinsic factors (Mirmoghtadaie *et al.*, 2016). Indeed, it is generally believed that HHP can modify electrostatic and hydrophobic interactions, which are the major forces involved in maintaining proteins' tertiary structure (Campus, 2010).

Results obtained in the present research indicate a decrease in protein solubility, both for sarcoplasmic and total proteins, corroborating the outcomes of a previous study carried out on cod (Angsupanich and Ledward, 1998). Such a reduction can be mainly ascribed to the modification of proteins' structure induced by the treatment.

Solubility is a relevant property of muscle proteins, so much so that, being strictly related to their functionality,

Table 1. Denaturation temperature (T_d) and enthalpy (ΔH) of individual peaks and the total enthalpy, corresponding to the DSC thermograms of the three seafood species, according to the applied pressure levels.

Seafood specie	Pressure level (Mpa)	Region 1				Region 2				Region 3		Total ΔH (J/g)	Reduction (%)
		Peak N		Peak 1		Peak 2		Peak 2b		Peak 3			
		T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)		
Grey Mullet	0.01	-	ND	47.2	1.06	56.3	0.800	-	ND	73.6	0.302	2.162	–
	400	-	ND	46.6	0.453	56.9	0.302	-	ND	67.3	0.005	0.760	64.85
	500	-	ND	46.7	0.314	56.9	0.126	-	ND	-	ND	0.440	79.65
	600	-	ND	46.2	0.398	56.5	0.127	-	ND	-	ND	0.525	75.70
Tiger Prawn	0.01	-	ND	46.9	0.721	64.5	0.241	-	ND	81.3	0.150	1.112	–
	400	41.1	0.616	-	ND	63.8	0.045	-	ND	84.5	0.218	0.879	20.93
	500	41.1	0.598	-	ND	64.0	0.032	-	ND	83.7	0.052	0.682	38.61
	600	40.9	0.456	-	ND	65.0	0.123	-	ND	82.4	0.121	0.700	37.02
Rose Shrimp	0.01	-	ND	50.9	0.122	59.6	0.163	67.49	0.079	77.9	0.444	0.807	–
	400	47.1	0.283	-	ND	63.1	0.032	-	ND	79.0	0.062	0.376	53.40
	500	40.7	0.054	-	ND	63.4	0.028	-	ND	81.9	0.064	0.147	81.84
	600	44.9	0.025	-	ND	61.6	0.045	-	ND	79.7	0.093	0.163	79.76

ND: Not detected

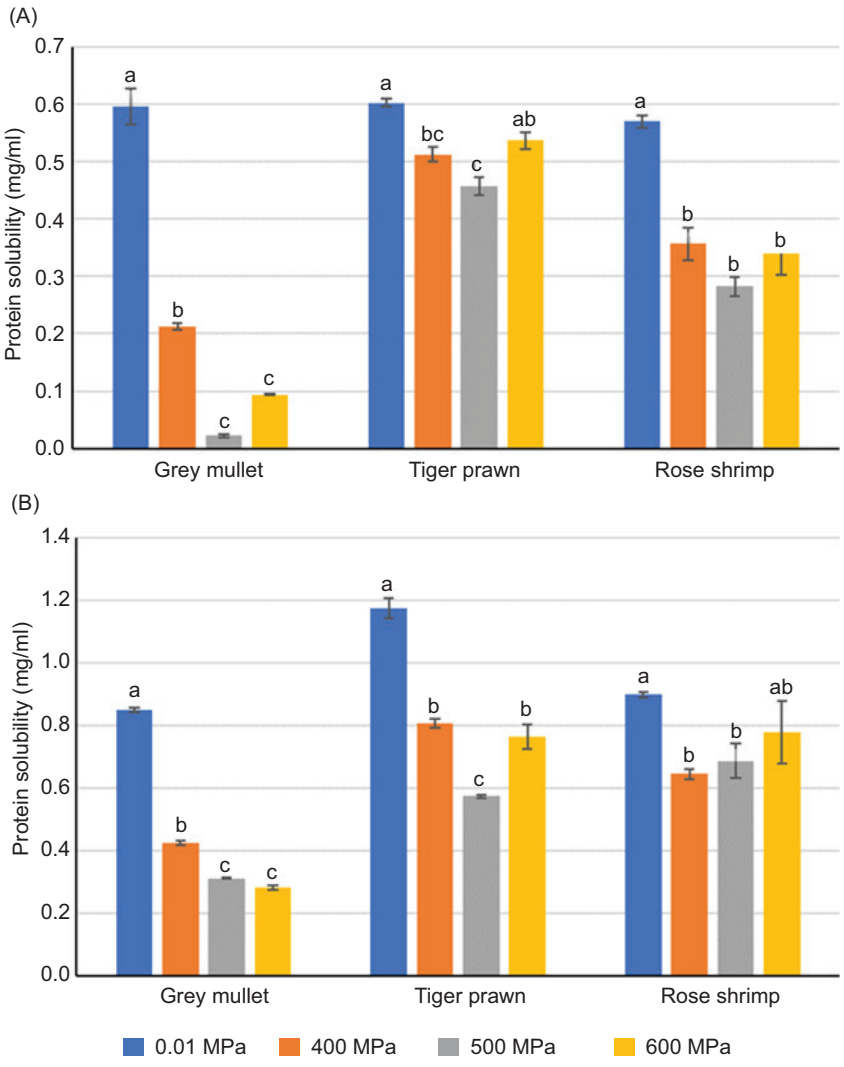

Figure 2. Solubility of sarcoplasmic (A) and total (B) proteins in the three seafood species samples subjected to different pressure levels, compared to the untreated samples. Different letters indicate significant differences ($p<0.05$) among samples within the same seafood species.

it is generally considered an indicator of protein denaturation level (Ghelichpour and Shabanpour, 2011). Indeed, the conformation of proteins, which is closely related to the environment to which they are exposed, also plays an important role in defining the functional properties of the protein itself.

From the comparison between the three matrices considered (grey mullet, tiger prawn and deep-water rose shrimp), it emerges that the solubility of the sarcoplasmic and myofibrillar proteins of grey mullet is more influenced by the treatment with HHP and is therefore the least suited to the use of this technology presenting a significant reduction in protein functionality; both from a technological and nutritional point of view (reduction in protein digestibility digestibility) (Zhang *et al.*, 2019).

Time domain nuclear magnetic resonance (TD-NMR)

TD-NMR has proved to be a useful tool to assess water mobility and distribution in fish and meat samples subjected to storage and different technological treatment (Aursand *et al.*, 2008; Gudjónsdóttir *et al.*, 2011; da Silva Carneiro *et al.*, 2016; Cropotova *et al.*, 2021). According to previous literature reports (Cropotova *et al.*, 2021), 3 water populations, characterized by different relaxation times, were identified. The first (WB), with short proton relaxation times (T2 = 1–10 ms), refers to water that is bound to the proteins by secondary bonds and is therefore closely associated with macromolecules. The second (W1), characterized by medium proton relaxation times (T2 = 40–80 ms), describes capillary water found in the myofibrillar network, while the third (W2), with long

proton relaxation times (T2 = 100–190 ms), is mechanically immobilized water or extra-myofibrillar, which can be further released as drip loss.

Figure 3 shows the relative intensities, expressed as arbitrary units (AU), of water populations WB (a), W1 (b), and W2 (c) for the three seafood species according to the HPP treatment applied. The higher percentage (65–85% in the untreated sample) is represented by W1, while the sum of W1 and W2 accounts for more than 90% of the total, in agreement with literature (Aursand *et al.,* 2008; Gudjónsdóttir *et al.,* 2011).

In all seafood species, a similar trend was observed. While no differences were detected for population WB, increasing pressure levels led to a decrease in the intensity of W1 and a corresponding increase in population W2 (an increase of the extra-myofibrillar water at the expense of the myofibrillar one). However, due to the high variability of the data, these values were not statistically different for tiger prawn.

A similar effect was observed by Thorarinsdottir *et al.* (2011) and Gudjónsdóttir *et al.* (2011) after salting of fish muscle and was related to protein denaturation, with a consequent shrinking of fibers and an increase in intercellular space. Similarly, Gudmundsson and Hafsteinsson (2001) showed microscopic images of high-pressure-treated salmon cells, in which proteins and collagen were stained with orange and blue colors, respectively. After the application of 300 MPa, a clear reduction in cell size and a gaping effect were visible.

Table 2 reports the relaxation times (T2) of the three water populations and their changes according to the pressure level applied. Regarding water molecules located within organized protein structures (W1), a decrease in T2 was observed for grey mullet, while an increase was observed for tiger prawn and rose shrimp. No significant differences were found among the different applied pressure levels for all seafood species. Therefore, in all investigated species, the water movements were similar, despite the differences observed by DSC.

Color and texture

Table 3 reports the whitening index values measured for the three seafood species according to the pressure applied. For all three species considered, a significant increase of between 10 and 20 units was observed after treatment. The final effect for all samples was a general

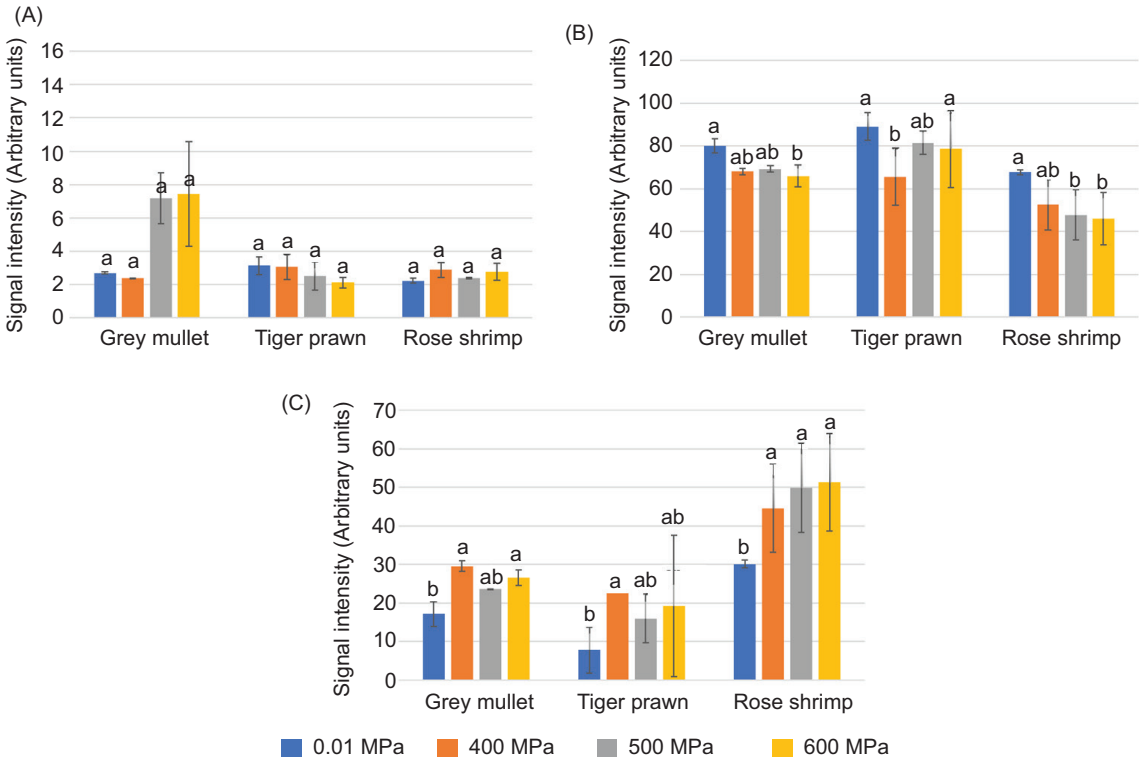

Figure 3. Intensity (Arbitrary Units) of the different water populations—WB (A), W1 (B) and W2 (C)—measured in the three seafood species samples subjected to different pressure levels compared to the untreated sample. Different letters indicate significant differences (p<0.05) among samples within the same seafood species.

Table 2. Relaxation times (T_2) (ms) of the three water populations and their changes according to the applied pressure level.

Applied pressure MPa	Grey mullet	Tiger prawn	Rose shrimp
W_{B}–T_2			
0.01	2.16^{a} ± 0.24	3.59^{a} ± 0.77	3.42^{a} ± 0.36
400	3.745^{a} ± 0.84	3.87^{a} ± 1.77	5.49^{a} ± 1.40
500	12.21^{a} ± 1.54	3.92^{a} ± 1.65	4.55^{a} ± 0.14
600	10.05^{a} ± 1.18	2.85^{a} ± 0.60	6.00^{a} ± 0.20
W_{1}–T_2			
0.01	45.515^{a} ± 1.00	38.40^{b} ± 1.43	46.14^{b} ± 2.98
400	39.75^{b} ± 1.02	45.39^{a} ± 1.02	53.27^{a} ± 0.54
500	38.84^{b} ± 0.88	46.31^{a} ± 0.13	54.72^{a} ± 0.45
600	35.23^{b} ± 0.08	44.77^{a} ± 0.94	55.87^{a} ± 4.38
W_{2}–T_2			
0.01	98.15^{b} ± 17.83	86.28^{b} ± 5.37	86.69^{b} ± 1.67
400	112.36^{a} ± 0.96	83.10^{b} ± 0.80	131.77^{a} ± 10.66
500	154.96^{a} ± 23.07	94.78^{ab} ± 16.40	121.62^{a} ± 0.43
600	144.2^{a} ± 33.55	105.98^{a} ± 22.95	138.65^{a} ± 16.70

Values are reported as average (± Standard deviation). Different letters indicate significant differences among sample within the same column and the same species.

Table 3. Whitening index and compression force of the three seafood species and their changes according to the applied pressure level.

Applied pressure MPa	Grey Mullet	Tiger Prawn	Rose Shrimp
Whitening Index			
0.01	53.31 ± 0.66^{b}	52.25 ± 1.59^{b}	62.66 ± 1.96^{b}
400	70.14 ± 0.54^{a}	66.46 ± 1.35^{a}	72.50 ± 2.25^{a}
500	71.57 ± 1.01^{a}	69.96 ± 1.35^{a}	72.26 ± 0.66^{a}
600	71.81 ± 0.93^{a}	68.22 ± 2.11^{a}	73.00 ± 3.21^{a}
Force (N)			
0.01	41.6 ± 9.71^{a}	8.01 ± 3.36^{b}	15.64 ± 3.26^{b}
400	23.2 ± 2.33^{b}	10.67 ± 2.94^{a}	16.94 ± 5.08^{b}
500	10.01 ± 4.21^{c}	4.54 ± 1.71^{c}	17.33 ± 1.23^{b}
600	5.72 ± 1.25^{d}	7.93 ± 1.20^{b}	23.56 ± 5.52^{a}

Values are reported as average (± Standard deviation). Different letters indicate significant differences among

whitening and the occurrence of a cooked appearance, which is typical for muscle food subjected to pressurization. The whitening effect has been largely observed in many fish species, and it has been attributed to protein denaturation, and in particular to pressure-induced coagulation of sarcoplasmic and myofibrillar proteins. However, although DSC results showed some differences according to the applied pressure, in particular between 400 MPa and the higher ones, for each investigated species, there were no significant differences in the values of the treated products just after the treatment as a function of the different pressure levels adopted.

For grey mullet, the increasing pressure promoted a remarkable decrease in the resistance to compression (Table 3) of the tissues, proportionally to the applied pressure level. For the higher pressure applied (600 MPa), values were 10 times lower compared to the untreated sample.

In tiger prawn, values were slightly higher after the application of 400 MPa, while they decreased after 500 MPa, and no changes were observed for the 600 MPa. On the contrary, in deep-water rose shrimp, the resistance to compression was found to be significantly higher after the 600 MPa treatment.

Results from texture measurements clearly indicate that grey mullet was the most affected species, with a remarkable decrease in the compression force, while only slight differences were observed for tiger prawn, and a slight increase was obtained in deep-water rose shrimp only after the higher pressure. Contradictory reports are found in the literature with regards to the effect of pressure application on the texture of seafood products. Many authors observed an increase in hardness after HPP application to cod (Angsupanich and Ledward, 1998), rainbow trout and mahi mahi (Yagiz *et al.*, 2007), tuna (Zare, 2004), and black tiger shrimp (Jantakoson *et al.*, 2012). This effect was attributed to the unfolding of actin and sarcoplasmic proteins and the formation of new hydrogen-bonded networks, as well as an increase in protein–protein interactions and bond formation. On the other hand, Chéret *et al.* (2005) found a softening of seabass fillets after a 300 MPa treatment, but no changes after the application of 400 and 500 MPa. Briones-Labarca *et al.* (2012) observed no differences in red abalone treated with HPP up to about 500 MPa compared to the control; however, the authors observed an effect on collagen and connective tissue using scanning electron microscope, suggesting a significant modification of the microstructure due to the applied pressure.

On one hand, it needs to be considered that the discrepancies might be due to the different species investigated and the method used for texture evaluation. Most of the cited studies apply pressure on whole fillets (Chéret *et al.*, 2005; Yagiz *et al.*, 2007) or portions (Angsupanich and Ledward, 1998), while red abalone was hand-shucked and packed (Briones-Labarca *et al.*, 2012). Texture Profile Analysis (TPA) was the most commonly used method

applied on samples excised from the fillets, but with different compression strains. In our case, the three species were manually cut into small pieces to represent a fish tartare, and since the TPA test was not applicable, we selected a compression test that allowed us to measure the resistance to compression as an indicator of the structural modifications.

The results obtained in the present study confirmed that the effect on texture is strictly dependent on the species of the raw material considered and the specific tissue structure.

Moreover, besides protein denaturation, other parameters affect the structural characteristics of proteins, such as possible pH changes resulting in modification of hydrogen and hydrophobic bonds, modifications to water bonding and holding capacity, and the activity of enzymes such as proteases, which can be inhibited or enhanced.

Fat oxidation and hydrolysis

Very important for seafood products' quality are also lipids, whose composition is considered to have a variety of benefits for human health, particularly in the prevention of cardiovascular diseases (Innes and Calder, 2020). Nevertheless, seafood species are highly susceptible to oxidation because they contain high amounts of polyunsaturated fatty acids, as well as heme-containing proteins (hemoglobin), and some pro-oxidant compounds such as transition metals and enzymes.

The application of HPP has often been found to increase susceptibility to lipid oxidation, although the effects are believed to vary according to different factors such as pressure level, process time, type of muscle, age, fatty acid profile, presence of other pro- or anti-oxidant compounds, handling, and other processing processes (Truong *et al.,* 2015).

The three seafood species considered in this study are characterized by a generally low fat content—about 2.5 g/100 g for grey mullet, 0.8 g/100 g for tiger prawn, and 1.1 g/100 g for rose shrimp—and for this reason, they are not considered particularly susceptible to lipid oxidation.

However, Angsupanich and Ledward (1998) found a significant increase in the TBARS of cod when pressure levels rose above 400 MPa. Moreover, it should be considered that, because the samples are cut into small pieces compared to fillets, there is a higher exposure to oxygen, increased interaction between pro-oxidants and their targets, and disruption of cell membranes, which results in the leakage and spreading of pro-oxidants (Beltran *et al.,* 2004).

Lipid oxidation was measured by PV value (Figure 4A), which represents a primary oxidation level, and by TBARs (Figure 4B), which quantifies secondary oxidation products, in particular MDA. These indexes followed

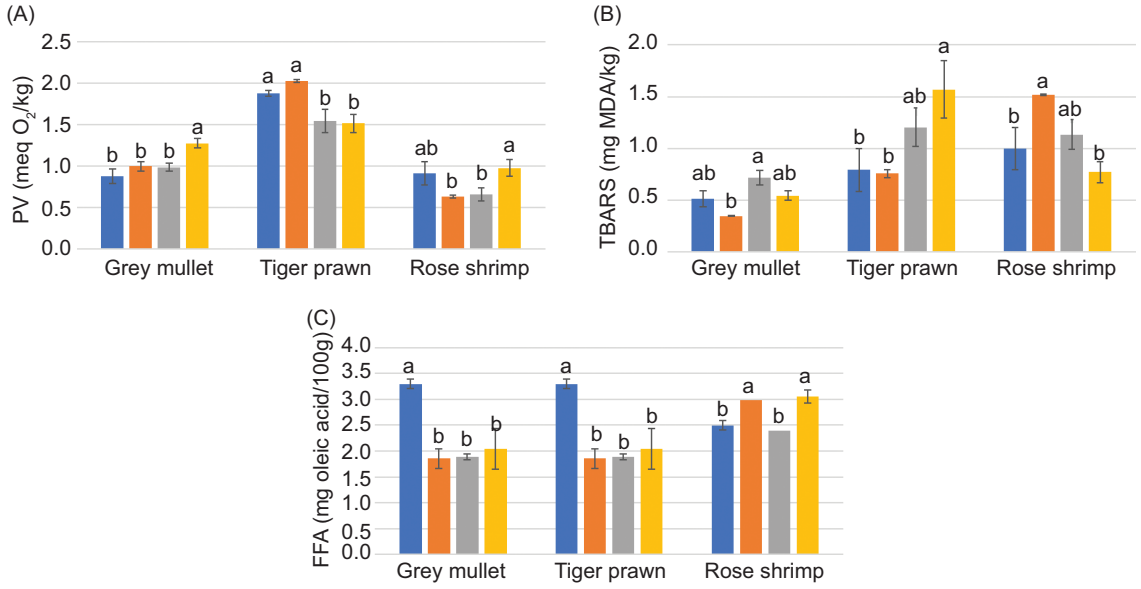

Figure 4. Peroxide value (PV) (A), TBARs (B), and Free Fatty Acids (FFAs) (C) content in the three seafood species subjected to HPP compared to the control. Different letters indicate significant differences ($p<0.05$) among samples within the same seafood species.

different behaviors in the three species. For grey mullet, PV increased significantly only at the higher pressure (600 MPa), while no significant changes were observed in TBARs values. In tiger prawns, instead, at 500 and 600 MPa, PV values underwent a significant decrease, paralleled by an increase in TBARs values. In rose shrimp, PV did not change compared to the control sample, but a higher TBARs value was observed after the 400 MPa treatment. It is important to remember that the lipid oxidation process is a balance between the formation and degradation of hydroperoxides. Indeed, during the propagation and termination steps typical of the oxidative process, the simultaneous formation and decomposition of primary compounds occurs (Frankel, 1991).

Although an increase in the oxidation indexes considered was observed in some samples, the values remained quite low, probably due to the low fat content and the very low oxygen levels in the packages. However, it is important to emphasize that the analytical determinations were carried out only after 24 hours, which is a very short time for oxidative and hydrolytic alterations of lipids to occur. Therefore, the effect on lipid oxidation should be further investigated during storage.

Moreover, the reduced protein solubility found in this study might also be due to the possible onset of oxidation processes affecting the protein fraction, induced by HHP. This phenomenon may have led to the formation of intermolecular cross-links that, by promoting protein aggregation, ultimately reduce solubility, since it has been suggested that lipid-derived radicals and hydroperoxides can promote protein oxidation (Lund *et al.*, 2011). However, in the present study, the species most affected in terms of protein solubility (grey mullet) did not show a higher lipid oxidation status compared to the others.

Lipid hydrolysis was measured through the FFA content (Figure 4C). In grey mullet and tiger prawn, a significant decrease in FFA was observed at all applied pressure levels, with no differences among them. On the other hand, in rose shrimp, a slight increase compared to the untreated sample was found after 400 and 600 MPa. There are contrasting reports in the literature regarding the effect of HPP on lipid hydrolysis.

Some authors reported an increase in FFA content proportional to the applied pressure in horse mackerel (Torres *et al.*, 2013) and carp (Sequeira-Muñoz *et al.*, 2006), while Kaur *et al.* (2013) found no effect on the FFA content of rose shrimp. An increase in FFA might also indirectly influence oxidation, since unesterified fatty acids are more susceptible to oxidative degradation. On the other hand, a decrease in FFA after pressurization has also been observed and was attributed to the inactivation of lipolytic enzymes (Vázquez *et al.*, 2013).

However, studies on the effect of pressure on the activity of enzymes responsible for both lipid oxidation and hydrolysis are still scarce; therefore, this aspect should be further clarified in order to better understand the differences among the species.

Data correlation

Tables 4, 5, and 6 show the correlation matrix of the investigated parameters for grey mullet, tiger prawn, and deep-water rose shrimp. A significant correlation was found between protein solubility (sarcoplasmic and/or total) and denaturation enthalpy (ΔH), whitening index, and some water mobility parameters for grey mullet and deep-water rose shrimp. For tiger prawn, fewer significant correlations were observed among the parameters; in particular, the whitening index was negatively correlated with denaturation enthalpy, total sarcoplasmic solubility, free fatty acids, and the relaxation time of water population W1.

The textural index was found to have a significant positive correlation with protein solubility and with the W1-I parameter of water mobility only for grey mullet. Moreover, the indices of lipid oxidation and hydrolysis did not show significant correlations with the other investigated parameters, except for a few sporadic cases.

To provide an overall overview of the correlation among parameters and treatments, a principal component analysis (PCA) based on the Spearman correlation matrix was carried out. Graphical results are shown in Figure 5. For grey mullet (Figure 5A), PC1 and PC2 explain 67.04% and 27.05% of the total variance, respectively. It is quite clear that the samples are discriminated along PC1 according to the level of applied pressure. The untreated sample forms a cluster characterized by protein solubility parameters, denaturation enthalpy, texture, and high mobility of the W1 water population, while samples treated with increased pressure are more associated with an increased whitening index, mobility of the W2 population, and lipid oxidation parameters.

A similar trend was observed for rose shrimp (Figure 5C). In this case, PC1 and PC2 accounted for 59.50% and 25.00% of the variance, respectively. On the other hand, for tiger prawn (Figure 5B), where PC1 and PC2 explain 52.31% and 37.77% of the total variance, the untreated sample is clearly separated from the treated ones. However, there is no such clear effect of pressure level.

To clarify the differences in response to the treatment among the three investigated species, PCA was also performed on all data (Figure 5D). For all three species, the untreated sample is clearly separated from the treated

Table 4. Correlation matrix of the measured parameters for grey mullet.

Variables	FFA	P.V.	W_1-t_2	F (N)	P.S.	W_1-int	WI	ΔH	S.S.	W_2-t_2	W_2-int	TBARS
FFA	**1**											
P.V.	0.712	**1**										
W_1-t_2	–0.589	–0.902	**1**									
F (N)	–0.345	–0.792	**0.961**	**1**								
P.S.	–0.414	–0.721	0.950	**0.971**	**1**							
W_1 int	–0.618	–0.762	**0.951**	0.905	**0.968**	**1**						
WI	0.453	0.656	–0.914	–0.922	**–0.988**	**–0.980**	**1**					
ΔH	–0.375	–0.642	0.910	0.943	**0.993**	**0.961**	**–0.995**	**1**				
S. S.	–0.257	–0.617	0.892	**0.959**	**0.985**	0.917	**–0.971**	**0.989**	**1**			
W_2-t_2	–0.024	0.586	–0.793	–0.928	–0.858	–0.706	0.783	–0.837	–0.905	**1**		
W_2-int	0.750	0.562	–0.751	–0.637	–0.783	–0.903	0.857	–0.803	–0.708	0.354	**1**	
TBARS	–0.666	0.044	–0.164	–0.405	–0.250	–0.002	0.137	–0.233	–0.371	0.709	–0.392	**1**

*Peroxide value (P.V.); Total Enthalpy ΔH; Protein solubility (P.S.); Sarcoplasmic Solubility (S.S.). Free Fatty Acids (FFA). Water population moblitiy (W_1-t_2) (W_2-t_2); Whitening index (WI). Values in bold are different from 0 with a significance level p<0,05.

Table 5. Correlation matrix of the measured parameters for tiger prawn.

Variables	F (N)	P.V.	TBARS	ΔH	P.S.	WI	FFA	W_1-t_2	S.S.	W_2-t_2	W_2-int	W_1 int
F (N)	**1**											
P.V.	0.774	**1**										
TBARS	–0.458	–0.917	**1**									
ΔH	0.442	0.734	–0.777	**1**								
P.S.	0.442	0.560	–0.539	0.946	**1**							
WI	–0.234	–0.494	0.577	**–0.953**	**–0.967**	**1**						
FFA	0.045	0.285	–0.405	0.860	0.916	**–0.974**	**1**					
W_1 t_2	–0.170	–0.366	0.439	–0.897	**–0.959**	**0.987**	**–0.992**	**1**				
S.S.	0.448	0.393	–0.303	0.821	**0.961**	–0.887	0.867	–0.918	**1**			
W_2 t_2	–0.209	–0.173	0.076	0.531	0.711	–0.761	0.881	–0.851	0.776	**1**		
W_2-$t_2$int	0.366	0.005	0.253	–0.643	–0.669	0.818	–0.910	0.849	–0.599	–0.871	**1**	
W_1-$t_2$int	–0.606	–0.393	0.142	0.317	0.425	–0.576	0.742	–0.660	0.439	0.894	–0.920	**1**

*Peroxide value (P.V.); Total Enthalpy ΔH; Protein solubility (P.S.); Sarcoplasmic Solubility (S.S.). Free Fatty Acids (FFA). Water population mobility (W_1-t_2) (W_2-t_2); Whitening index (WI). Values in bold are different from 0 with a significance level p<0,05.

ones, along PC1 for tiger prawn and rose shrimp, and along both PC1 and PC2 for grey mullet. This clustering suggests that the parameters most associated with the level of treatment differ according to the species considered.

Conclusions

This study evaluated the effect of HPP application on different components of seafood species using various analytical methods. The response to the applied pressure was found to differ according to the species investigated. Some parameters, such as TD-NMR and colorimetric analysis, showed significant modifications after treatment, but no differences were observed among the levels of pressure applied. On the other hand, denaturation enthalpy, protein solubility, and texture were more effective at discriminating the samples. Lipid oxidation was only slightly affected by HPP, probably due to the low susceptibility to oxidation resulting from the low lipid content and the absence of oxygen in the packages. However, the effect should be further investigated during the storage of the samples.

Table 6. Correlation matrix of the measured parameters for rose shrimp.

Variables	FFA	F (N)	W_2–t_2	W_2–int	ΔH	W_1–int	W_1–t_2	WI	S.S.	P.S.	P.V.	TBARS
FFA	**1**											
F (N)	0.649	**1**										
W_2–t_2	0.707	0.691	**1**									
W_2–int	0.395	0.670	0.918	**1**								
ΔH	–0.306	–0.626	–0.878	**–0.995**	**1**							
W_1–int	–0.416	–0.673	–0.928	**–1.000**	**0.993**	**1**						
W_1–t_2	0.467	0.678	0.949	**0.996**	**–0.984**	**–0.998**	**1**					
WI	0.519	0.562	**0.969**	**0.960**	–0.941	**–0.966**	**0.977**	**1**				
S.S.	–0.254	–0.445	–0.863	**–0.963**	**0.972**	**0.962**	**–0.957**	**–0.958**	**1**			
P.S.	–0.206	0.106	–0.641	–0.597	0.598	0.604	–0.622	–0.757	0.766	**1**		
P.V.	0.201	0.540	–0.216	–0.235	0.265	0.236	–0.243	–0.393	0.483	0.886	**1**	
TBARS	0.041	–0.623	0.099	–0.063	0.069	0.051	–0.020	0.184	–0.161	–0.758	–0.876	**1**

*Peroxide value (P.V.); Total Enthalpy ΔH; Protein solubility (P.S.); Sarcoplasmic Solubility (S.S.). Free Fatty Acids (FFA). Water population mobility (W_1-t_2) (W_2-t_2); Whitening index (WI). Values in bold are different from 0 with a significance level p<0,05.

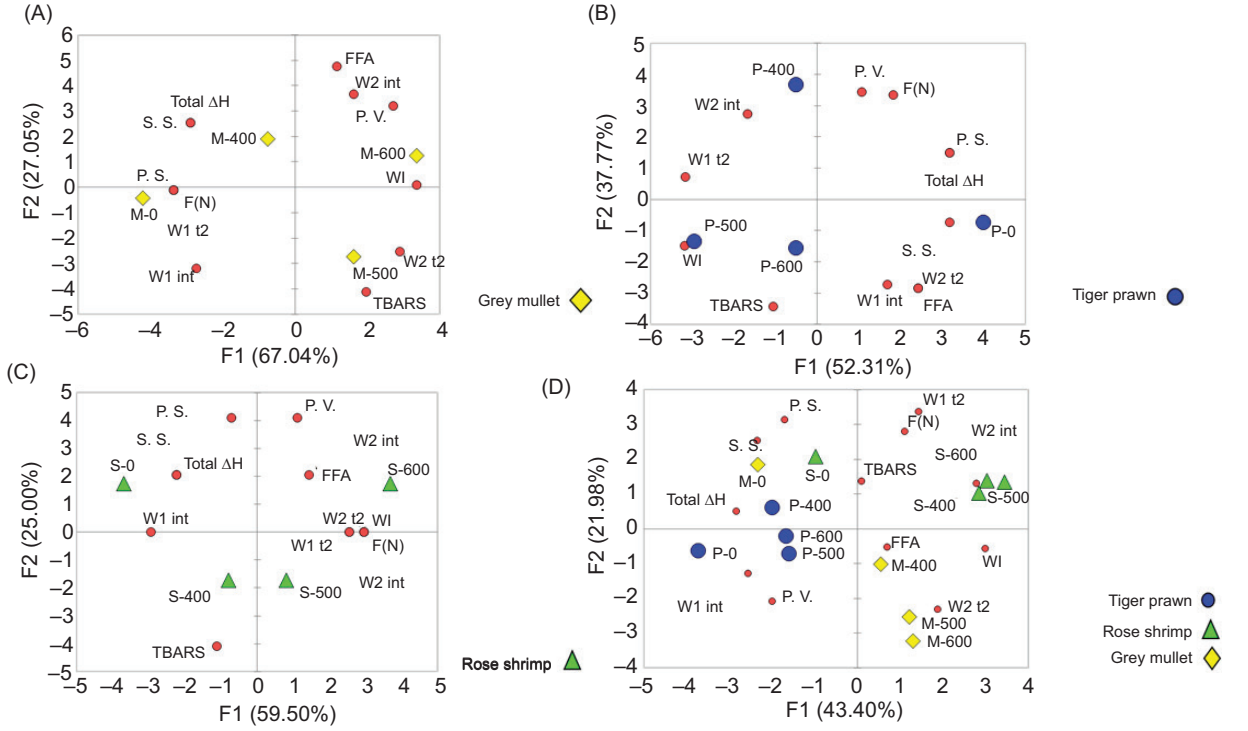

Figure 5. PCA biplot (loading and scores plot) of the first two principal components, PC1 and PC2, showing the variance obtained by analyzing the observations from untreated (control) and treated sample. PV, Peroxide value; ΔH, Total Enthalpy; PS, Protein solubility; SS, sarcoplasmic solubility. FFA, free fatty acids. W_1-t_2)-(W_2-t_2), Water population mobility; WI, Whitening index.

Therefore, although previous literature reports indicate that microbiological shelf life was significantly increased for all the species, the technological properties show that, among the species considered, grey mullet appeared to be the most affected and, therefore, possibly the least suitable for HPP treatment.

Data correlation confirmed that the complexity of the investigated matrix makes it difficult to identify parameters for monitoring the effect of pressure on seafood species, as the effect is strictly species-dependent. Finally, the results highlight the importance of selecting the raw material based on its response to pressure application.

Author Contributions

All authors contributed equally to this article.

Conflicts of Interest

The authors declare no conflict of interest.

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