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OPTIMIZATION OF EMERGING TREATMENTS FOR SEAFOOD PRODUCTS AND BY-PRODUCTS VALORIZATION

Presentata da: Ana Cristina De Aguiar Saldanha Pinheiro

Coordinatore Dottorato

Massimiliano Petracci

Supervisore

Pietro Rocculi

Co-supervisore

Silvia Tappi

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Abstract

The increasing demand for fresh fish and new fish-based preparations has led the food industry to develop innovative preservation technologies to extend the shelf-life of these foods, whose fresh raw material is highly perishable. The development of emerging technologies in seafood processing can contribute significantly to meet consumer needs toward safe, healthy, and minimally processed foods. Moreover, these innovative technologies can lead to more sustainable and environmentally friendly seafood processing.

This PhD thesis is focused on the qualitative and quantitative evaluation of the effects of emerging treatments applied to extend shelf-life of seafood processing and to preserve the qualitative characteristics of raw materials. Moreover, the application of innovative non-thermal technologies to recover bioactive substances from crustacean by-products were also studied.

Several experimental procedures were evaluated individually by the following studies: i) study of the effect of modified atmosphere packaging (MAP) with unconventional gas mixtures on the modification of the main qualitative parameters of sardine fillets during refrigerated storage; ii) valorization of an underutilized crustaceans by the application of mechanical flesh separation and freezing iii) application and evaluation of the main effects of high pressure processing (HPP) treatments on the quality and stability of different seafood products; iv) study and application of innovative non-thermal technologies, such as accelerated solvent extraction (ASE) and pulsed electric fields (PEF) for the recovery and valorization of value-added products from crustacean processing by-products.

Based on the obtained results, the use of argon for MAP of sardine fillets showed an inhibitory effect on bacterial spoilage and on the development of hypoxanthine during storage, increasing their shelf-life. The effect of HPP treatment on the different types of considered seafood products, intended for raw consumption, highlighted a significant microbiological shelf-life increase at the highest applied pressure levels for all the considered species. Fat oxidation was only minimally affected and remained at low levels during refrigerated storage. Mechanical separation and freezing of mantis shrimp flesh allowed to develop a high added value product, overcoming the issue related to seasonality of this crustacean.

The application of ASE and PEF showed high yield of astaxanthin and seemed to be an effective tool to recover high antioxidant compounds from crustacean by-products.

The overall results of this PhD thesis highlighted the great advantages in the application of emerging technologies for both seafood products and crustacean by-product valorization, therefore contributing to the potential increase of the sustainability of the seafood sector.

List of papers

This PhD thesis is based on the work contained in the following Papers, which are referred to in the text by their Roman numerals. The Papers are attached at the end of the thesis.

I. **Pinheiro, A. C. D. A. S**., Urbinati, E., Tappi, S., Picone, G., Patrignani, F., Lanciotti, R., Romani, S., & Rocculi, P. (2019). The impact of gas mixtures of Argon and Nitrous oxide (N_2O) on quality parameters of sardine (*Sardina pilchardus*) fillets during refrigerated storage. Food Research International, 115, 268-275.

II. **De Aguiar Saldanha Pinheiro, A. C.**, Tappi, S., Patrignani, F., Lanciotti, R., Romani, S., & Rocculi, P. (2020). Effects of novel modified atmosphere packaging on lipid quality and stability of sardine (*Sardina pilchardus*) fillets. International Journal of Food Science & Technology, 56(2), 945-953.

III. Tappi, S., **De Aguiar Saldanha Pinheiro, A. C.**, Mercatante, D., Picone, G., Soglia, F., Rodriguez-Estrada, M. T., ... & Rocculi, P. (2020). Quality Changes during Frozen Storage of Mechanical-Separated Flesh Obtained from an Underutilized Crustacean. Foods, 9(10), 1485.

IV. **De Aguiar Saldanha Pinheiro, A. C**., Tappi, S., Braschi, G., Genovese, J., Patrignani, F., & Rocculi, P. (2021). Quality and stability of different seafood products treated with high hydrostatic pressure (HPP) intended for raw consumption, (submitted)

V. **Pinheiro, A. C. D. A. S.,** Martí-Quijal, F. J., Barba, F. J., Tappi, S., & Rocculi, P. (2021). Innovative Non-Thermal Technologies for Recovery and Valorization of Value-Added Products from Crustacean Processing By-Products—An Opportunity for a Circular Economy Approach. Foods, 10(9), 2030.

VI. **Pinheiro, A. C. D. A. S.**, Barba, F. J., Tappi, S., & Rocculi, P. Pulsed Electric Fields (PEF) and Accelerated Solvent Extraction (ASE) for valorization of Red shrimp (*Aristeus antennatus*) and Camarote shrimp (*Melicertus kerathurus*) by-products: Recovery of astaxanthin and antioxidant extracts (Manuscript).

Contents

I. Introduction and Objective……..…….....2

II. Emerging strategies for seafood processing and valorization……...4

1. Introduction…….....4

2. Modified atmosphere packaging (MAP).....4

2.1 Principle of modified atmospheric packaging (MAP)…….....5

2.2 Modified atmosphere packaging of seafood products…….....5

2.2.1 MAP with unconventional gases……..…….....6

2.2.2 Effect of MAP on microorganisms and biochemical parameters…….....7

2.2.3 Effect of MAP on color and lipid quality……..….....13

3. High Pressure Processing (HPP).....19

3.1 Principles of HPP……..……19

3.2 HPP on seafood products…….....20

3.2.1 Microbial inactivation……..……21

3.2.2 Changes in color and texture parameters……..……...25

4. Value addition of fishery products ……30

III. Innovative non-thermal technologies for crustacean by-products valorization…..34

1.Introduction……...34

2.Crustacean by-products as a source of valuable compounds……35

3.Innovative non-thermal technologies for the recovery of valuable compounds…….37

3.1 Supercritical Fluid Extraction (SFE)……...38

3.2 High-Pressure Extraction (HPE).....40

3.3 Pulsed electric field (PEF) 41

3.4 Ultrasound-Assisted Extraction (UAE).....42

3.5 Accelerated solvent extraction (ASE)……..……43

IV. Conclusions……..49

References……..51

Acknowlegments……..64

I. Introduction and Objective

The consumption and popularity of seafood has been steadily increasing in recent years because it is more and more recognized as an important source of nutrients for human health (Alasalvar, 2002). Fresh fish and shellfish are one of the most highly perishable food products due to endogenous enzymes that promote proteolysis of muscle protein and connective tissue, hydrolysis of fat followed by deterioration of muscle, caused by the metabolic activities of microorganisms (Wu et al., 2014). Nowadays, consumers have a greater awareness of food and the consequences associated with food consumption in both positive and negative ways, and they are very concerned about the quality, safety, and nutritional benefits of food products (Pinnaduwa et al., 2020). In this context, there is a trend toward consumers who are willing to purchase high-quality minimally processed seafood, in order to obtain better nutritional value and other functional benefits associated with seafood consumption.

Considering the short shelf-life of seafood products with conventional refrigeration, the main problems faced by the current commercial seafood industry are the shorter shelf-life and the possible presence of pathogenic microorganisms, presenting problems in commercialization, thus, highlighting the need for improved preservation methods that allow their shelf-life extension.

Traditional heat treatments, such as pasteurization and sterilization applied by the seafood industry, can be highly efficient in inactivating or inhibiting bacterial pathogens, but they can result in undesirable nutritional, chemical/biochemical, and sensory changes in foods. These changes reduce consumer acceptance, as they seek minimally processed products with greater safety, added value, and longer shelf-life (Ekonomou & Boziaris, 2021)

In this sense, the application of emerging food processing and preservation technologies, (i.e., innovative modified atmosphere packaging, high-pressure processing, pulsed electric fields, ultrasound, cold gas plasma etc.) could contribute significantly to meet consumer needs for safe, healthy, and minimally processed foods. Furthermore, innovative technologies can lead to more sustainable and environmentally friendly food production techniques (Toepfl et al., 2006). The development of non-thermal processes has several advantages, such as longer shelf-life, preservation of organoleptic characteristics and no regulatory limitations regarding consumer safety (Raso & Barbosa-Cánovas, 2003).

Also, it is important to consider that the industrial processing of fish products generates relatively large flows of by-products. These by-products contain very interesting compounds

from a nutritional and technological point of view, such as peptides, proteins, natural pigments, collagen, fatty acids, biopolymers, etc. (Kim & Mendis, 2006; Suresh et al., 2018). The optimization of new technologies for their further processing, such as separation, extraction, conversion, and stabilization, is very promising in order to raise the quality standards of ingredients and intermediate products, and for the economically and environmentally sustainability of the applied processes. Furthermore, concepts such as circular economy, have been regarded as leading principles for eco-innovation, that aims a "zero waste" society and economy, in which waste and by-products are exploited as raw material for the development of new products and applications (Mirabella et al., 2014).
Innovative extraction technologies, based on non-thermal methods such as ultrasound, high-pressure processing, pulsed electric fields, supercritical fluid extraction and accelerate solvent extraction, have been proposed for use within the food industry including the extraction of valuable components from wastes and by-products (Pinheiro et al., 2021).

This PhD thesis is focused on the qualitative and quantitative evaluation of the effects of emerging processes and packaging strategies to increase the quality and extend the shelf-life of different seafood products, as well as the valorization of Mediterranean wild seafoods with a high seasonality. The effect of innovative modified atmosphere packaging (MAP) with unconventional gas mixtures on sardine fillets during storage was investigated (***Paper I and II***). Moreover, the effect of HPP on different seafood products, grey mullet, tiger prawn and rose shrimp, intended for the raw consumption was evaluated (***Paper IV***). Regarding the valorization of products with a high seasonality, changes in some qualitative indices of mechanically separated *S. mantis* flesh during deep-freezing storage were investigated (***Paper III***). Concerning the seafood by-products valorization, the application of innovative non-thermal technologies to recover valuable compounds from crustacean by-products was studied (***Paper V***). Finally, the application of accelerate solvent extraction (ACE) and pulsed electric filed (PEF) to recover astaxanthin and antioxidant extracts from crustacean by-products was also investigated *(**Paper VI***).

The results of this dissertation contribute to the knowledge and benefit of exploring some important gaps in science and industry to meet the trend of consumer interest in minimally processed, fresh, and natural seafood and to increase the value of seafood industry byproducts.

II. Emerging strategies for seafood processing and valorization

1. Introduction

Global seafood production has quadrupled over the past 50 years. In 2018, global production reached an estimated 179 million tons. Of the combined total, 156 million tons were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita and it is expected to increase further over the next decade (FAO, 2020) highlighting its critical role in global food and food security.

To provide high-quality and safe seafood products with longer shelf-life, the development of new technologies has become the top priority for many researchers and food industries. Non-thermal techniques have been adopted and are under continuous development in the last years. High pressure processing (HPP), ultrasound (US), cold plasma (CP) and pulsed electric fields (PEF) are some methods that show potential to be applied by the fish industry (Kulawik & Kumar Tiwari, 2019; Zhang et al., 2019).

Packaging technologies like modified atmosphere, active and Intelligent packaging also plays an important role in fish preservation (Nagarajarao, 2016). In this last context, in this PhD research work, the effect of modified atmosphere packaging (MAP) with unconventional gas mixtures (Argon and N_2O) on the main qualitative parameters of sardine fillets during refrigerated storage was investigated.

2. Modified atmosphere packaging (MAP)

In the last decades, Modified Atmosphere Packaging (MAP) has received increasing attention as a food preservation method. This technology has brought great changes in the preservation, distribution, and marketing of raw and processed products to meet consumer demands. Advances in the application of MAP to preserve quality and extend shelf-life are occurring at a fast pace. This is evident by the large amount of scientific literature available not only addressing applications in fishery products but also in muscle foods in general (Dewitt & Oliveira, 2016). However, an excessive amount of the product in the package may result in limited preservative effect of MAP on the food product (e.g., due to insufficient amount of CO_2, which guarantees microbiological stability of stored raw material). Due to the interactions between preservative gases and the food product, it is essential to experimentally define the appropriate ratio, so that the protective atmosphere could be maintained (Tsironi & Taoukis, 2018).

2.1 Principle of Modified Atmospheric Packaging (MAP)

The principle of MAP is the replacement of air in the package with a fixed gas mixture. The product is usually packed in a barrier material along with a mixture of gases; once the gas mixture is introduced in the package, there is no further control of the gas composition (Sivertsvik et al., 2002). The headspace gas composition depends on the product properties, the expected shelf-life, the packaging material permeability properties, and the storage conditions (Floros & Matsos, 2005). Temperature is a very important parameter in MAP. This technology usually works well at reduced temperatures. During storage, the mixture of gasses within the package can change due to the respiration rate of the packed food in the case of fresh-cut fruit and vegetables, the solubility of the packaging gases in the product, the specific permeability of the packaging material to fixed gases, storage time, temperature and relative humidity (Kontominas et al., 2021).

The main gases used in food MAP are oxygen (O_2), nitrogen (N_2) and carbon dioxide (CO_2). For most food products different combinations of two or three of these gases are used, chosen to meet the needs of the specific product. However, the role and the importance of each gas in MAP are related to its properties (Mullan & McDowell, 2003). Nitrogen is an inert and tasteless gas, without any antimicrobial activity. It is not very soluble in water, and it is primarily used to displace oxygen and prevent package collapse. Oxygen inhibits the growth of anaerobic microorganisms but promotes the growth of aerobic microbes. Also, oxygen is responsible for several undesirable reactions in foods, including oxidation and rancidity of fats and oils, color changes, and spoilage due to microbial growth (Floros & Matsos, 2005). Carbon dioxide inhibits the growth of a wide range of microorganisms, and it slows down the respiration of many products. Unlike nitrogen, then, which is inert, carbon dioxide is highly reactive to biological systems. Carbon dioxide is soluble in both water and lipids, and its solubility increases with decreasing temperatures (Gill, 1988). The dissolution of CO_2 in the product can result in package collapse. The optimum level of each gas for each food product must be determined and used in order to maximize their positive and minimize their negative effects (Floros & Matsos, 2005).

2.2 Modified atmosphere packaging of seafood products

MAP extends the shelf-life of several fishery products by inhibiting bacterial growth and oxidative reactions. For raw fish, an increase of 50-100% in shelf-life is usually observed, and for cooked crustaceans, an extension of 100-200% in shelf-life can be obtained under ideal storage conditions (Sivertsvik et al., 2002; Stammen et al., 1990).

The MAP effectiveness in extending fish product quality/shelf-life depends on species, fat content, initial microbial load, gas mixture, gas/product ratio and storage temperature (Sivertsvik et al., 2002).

A variety of atmospheres have been investigated in the packaging of seafood products. However, the reported increases in shelf-life for fish and fish products vary markedly and are small in comparison with those reported for several other products (Alasalvar, 2002). Generally, MAP for fish products is obtained by partial or total removal of oxygen (O_2) and increased carbon dioxide (CO_2) concentration (Erkan et al., 2006; Özogul et al., 2004; Stamatis & Arkoudelos, 2007) that allowed to inhibit lipid oxidation and microbial growth. The composition of the atmosphere used for MAP of fish depends on the fat content of the product. In general, an atmosphere of 30% O_2, 40% CO_2, and 30% N_2 is used for lean fish. In the case of fat and smoked fish, however, the exclusion of O_2 is recommended to avoid oxidative reactions and rancidity development. Then in these cases, a gas mixtures consisting of 60% CO_2 and 40% N_2 are often used (Floros & Matsos, 2005).

2.2.1 MAP with unconventional gases

Beside the traditional gases used for MAP (O_2, CO_2 and N_2), alternative gases such as Argon (Ar) and nitrous oxide (N_2O) have been approved for food use in the European Union (EU). Hence, MAP system is a physical approach to increase fresh fish shelf-life and has already proved to work, however, its potential is not fully known, in particular in relation to the aforementioned gases as alternative to nitrogen. Indeed, while N_2 is completely inert and is used for the sole filling purpose, argon and N_2O have shown some biological effects.

The effects of Ar, that is a chemically inert gas are attributed to its physical properties, in particular its enhanced solubility in water compared to nitrogen, and its ability to interfere with enzymatic oxygen receptor sites (Rocculi et al., 2005; Spencer & Humphreys, 2003). According to Spencer and Humphreys (2003), the use of argon allowed to improve the overall acceptability of packed meat products through the reduction of CO_2 levels, in addition to the benefits of reduced O_2 level. While both nitrogen and argon are inert gases, their physical properties differ in significant ways. Since argon displaces and excludes oxygen more efficiently than nitrogen, it provides better control against oxidation of flavor and color components of foods.

To our knowledge, argon has been tested for seafood MAP in just few studies. Choubert et al. (2008) investigated the use of Ar for rainbow trout fillets MAP, while Randell et al. (1997) on rainbow trout and herring fillets. However, these authors observed contrasting results

regarding the increase of shelf-life related to this gas. Olatunde et al. (2019) studied the impact of an argon-based atmosphere on the shelf-life of Asian sea bass. These authors demonstrated the potential preservative effect of this gas that extended the shelf-life of sea bass by 9 days, when stored at 4 °C.

In recent years, there has been great attention to the potential benefits of nitrous oxide (N_2O) in MAP applications. N_2O has been found to have effect on inhibition of respiration and senescence in higher plants showing high potentiality for the packaging of fresh-cut fruits (Rocculi et al., 2004, 2005). Argon and N_2O are known to sensitize microorganisms to other antimicrobial agents (Qadir & Hashinaga, 2001; Thom & Marquis, 1984). Despite the potentialities shown by these two gases, their main disadvantage is related to the cost that is significantly higher than that of nitrogen (about 4 –5 times), that makes very relevant to evaluate if the qualitative benefits promoted by the use of argon are economically sustainable.

2.2.2 Effect of MAP on microorganisms and biochemical parameters

Most of the quality changes that have been noted in seafood packed in MAP are directly related to microbial spoilage, and can result in undesirable odors and flavors, muscle softening, discoloration, and increased muscle exudate during storage. Other important changes are due to the incorporation of CO_2 into the aqueous phase of the food, which causes a reduction in muscle pH and protein water retention capacity, resulting in increased fluid loss during storage (Dewitt & Oliveira, 2016). It is known that the effect of MAP on the shelf-life of food is primarily affected by the amount of carbon dioxide (CO_2) dissolved in the product and the storage temperature. During storage, the microflora changes owing to different abilities of the microorganisms to tolerate the preservation conditions (Gram & Dalgaard, 2002). Growth and survival of spoilage and pathogenic microorganisms are affected by MAP. Variation in the dominant microbiota in MAP fish depends mainly on geographic origin, water temperature, and storage conditions. Seafood products packaged in modified atmosphere can be increased considerably in their shelf-life, when the atmosphere is rich in CO_2 and the temperature is maintained below 2°C. This is mainly due to the inhibiting effect of carbon dioxide on the growth of *Pseudomonas* spp. and other psychrotrophic spoilage microorganisms (Floros & Matsos, 2005). Farber et al. (1991) highlight that one of the key MAP safety issues is not only the psychotropic and nonproteolytic clostridia, but the emergence of other psychotropic pathogens such as *Listeria monocytogenes*, *Aeromonas hydrophila*, and *Yersinia enterocolitica*. The same

authors emphasize that inhibition of spoilage microorganisms and extension of shelf-life by MAP may permit psychotropic pathogens sufficient opportunity for growth. Some authors have reported that Gram-positive microorganisms, such as lactic acid bacteria (LAB), which exhibit significant resistance to CO_2, play a significant role in the spoilage process of MAP finfish species from warm waters, such as the east Mediterranean basin (Dewitt & Oliveira, 2016; Sivertsvik et al., 2002; Stenstrom, 1985). Some studies have reported LAB as dominant bacteria in the final population in MAP sea bream fillets (Tsironi et al., 2011; Tsironi & Taoukis, 2010). During storage, LAB inhibit growth of other bacteria due to the formation of lactic acid and bacteriocins or by competition for nutrients which may contribute to their selective growth in fish products under anaerobic conditions (Gram & Dalgaard, 2002; Stamatis & Arkoudelos, 2007). MAP usually maintains an atmosphere poor in oxygen. Thus, this kind of atmosphere considerably inhibits the growth of strict aerobes, i.e., pseudomonads, which include the main spoilers of muscle foods (e.g., meat, fish, poultry). However, this poses a potential danger for the development of anaerobic pathogens *Clostridium botulinum* is a strict anaerobe, while, in addition, the non-proteolytic *C. botulinum* type E is psychotropic, capable of producing its deadly toxin under refrigerated conditions. Therefore, the O_2 concentration maintained within the package, in combination with the oxygen permeability of the packaging material, results in a trade-off between inhibition of oxidation and the potential risk of extreme anaerobiosis (Kontominas et al., 2021).

Seafood specific spoilage organisms can grow and produce spoilage metabolites, especially volatile bases, hypoxanthine, organic acids and biogenic amines. Some spoilage metabolites are indicators of fish and shellfish spoilage. Various individual or groups of volatile organic compounds have the potential to be used as spoilage/freshness indicators but the most common chemical methods for monitoring microbial activity in fish and crustaceans are TVB-N and TMA determination (Parlapani et al., 2015).

Trimethylamine-N-oxide (TMAO-N) is a substance which is present in almost all marine fish, and in some freshwater fish, but in different amount depending on species, age, fish size, time of year, and environmental factors. Seawater fish contains 1–100 mg TMAO-N in every 100 g of muscular tissue (Hebard et al., 1982). Trimethylamine nitrogen (TMA-N) results from the reduction of TMAO-N by bacterial activity and partly by intrinsic enzymes and is often used as an index of spoilage of marine fish. TMA-N is considered a valuable tool in the evaluation of the quality of refrigerated fish, as it has been often related to the pungent odor and to the load of spoilage microorganism in many species of spoiled fish. The determination of TMA-N content are used widely in shelf-life studies of seafood products

packed in MAP (Erkan et al., 2006; Esteves et al., 2021; Fletcher et al., 2005; P. Li et al., 2020; T. Wang et al., 2008). However, some studies have observed significantly less TMA production for seafood products packed in MAP than for air packed ones (Li et al., 2020; Özogul et al., 2004). High O_2 concentrations in combination with CO_2 have been used to package lean marine fish species. O_2 directly inhibited the reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) (Boskou & Debevere, 1998).

Nucleotide degradation is the first biochemical change observed in post-mortem muscle, which is tightly connected to rigor mortis process. The amount of ATP-degradation products is considered as a good indicator of fish freshness (Shumilina et al., 2015). In the early post-mortem stages, the nucleotides produced by the ATP decomposition, such as ADP and AMP, Ino and IMP, as well as H_x, are considered useful indicators of fish freshness (Mendes et al., 2001), and their amount is combined to define the K-value parameter (Ocaño-Higuera et al., 2011). Despite its importance for assessing the quality of fresh seafood, quantifying ATP decomposition, or measuring K value, some studies have shown that CO_2 atmosphere does not alter K values compared to those determined for product held aerobically (Boyle et al., 1991; Sivertsvik et al., 2002). Moreover, López-Gálvez et al. (1998) found no effect of the atmosphere on the K value of sole fillets stored aerobically in ice or CO_2-enriched atmospheres, despite differences observed in the microbial load. Thus, the changes in the K-index may not always be representative of microbial activity. Hypoxanthine (H_x) accumulation in fish tissue reflects the initial phase of autolytic deterioration, as well as subsequent contribution through bacterial spoilage and has been related to loss of freshness and taste (Ocaño-Higuera et al., 2011). Özogul et al. (2000) found that Hx concentration increased with storage time om herring held under both MAP and ice. However, the Hx content of herring held in ice increased more rapidly than herring held in carbon dioxide (60%), suggesting that the presence of carbon dioxide influenced Hx accumulation.

New findings

In ***paper I*** the effect of modified atmosphere packaging (MAP) with unconventional gas mixtures on the main qualitative parameters of sardine fillets during refrigerated storage was investigated. Fresh fillets of Sardines (*Sardina pilchardus*) were packed in four different atmosphere conditions, Air (20.8% O_2+79.2% N_2), N_2 (30% CO_2+70% N_2), N_2O (30% CO_2+70% N_2O) and Ar (30% CO_2+70% Ar) and following stored at 4 °C for 12 days. The gas composition in MAP and air packed samples was monitored through the storage period. The results obtained from oxygen (O_2) and carbon dioxide (CO_2) analysis can be seen in

Figure 1A and B. In sample packed in air, O_2 concentration decreased, and CO_2 increased rapidly until the 8th day when they reached values respectively around 1% and 6.5% and then remained fairly similar until the end of the storage. The change in headspace composition is likely due to the development of aerobic microorganisms.

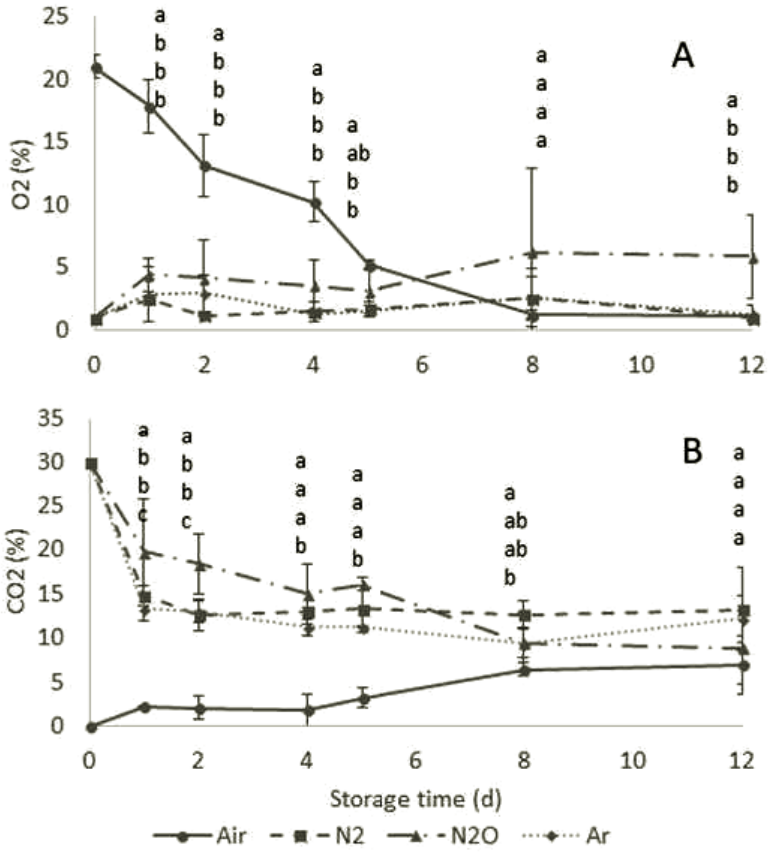

Figure 1. Concentration (%) of O_2 (A) and CO_2 (B) in the headspace of packages of sardine fillets in MAP during storage at 2-4 °C. (corresponding to Figure 1 A and B in ***paper I***).

Complete oxygen elimination was not achieved in the samples packed with modified atmosphere, and a residual content of 1% was measured in all samples after packaging. The CO_2 concentration of MAP samples decreased rapidly on the first day from an initial value of 30% in all samples and reached a relatively stable value between 10 and 15% at the end of storage. The reason for the decrease in CO_2 concentration over time is probably due to the solubilization of this compound in the liquid muscle fraction until equilibrium is reached (Sivertsvik et al., 2004). The O_2 concentration in the MAP samples increased slightly during storage, probably due to CO_2 dissolution, but remained below 3% in the N_2 and Ar samples. In the N_2O sample, however, it reached a concentration of 6.2%, probably

due to the simultaneous partial dissolution of N_2O, which has solubility values similar to CO_2 (Spilimbergo et al., 2011).

The evolution of the different microbial groups was affected by the adopted MAP conditions, as reported in Table 1. Mesophilic and psychrotrophic bacteria were constantly higher in samples packed in air, compared to the MAP samples until the end of storage, and reached the threshold value (6 log CFU/g for total mesophilic bacteria), between the 5th and the 8th day.

Table 1. Cell loads (log CFU/g) of different microbial groups detected in sardine products in relation to the packaging atmosphere (Corresponding to Table 2 ***paper I***).

	Time (d)	TMB*	TPB**	*Pseudomonas* spp	*Enterobacteriaceae*	Total coliforms	Fecal coliforms
Air	0	4.0±0.1^{a}	4.1±0.2^{a}	2.2±0.1^{a}	-***	-	-
	2	4.6±0.3^{a}	4.2±0.1^{a}	3.1±0.3^{a}	-	-	-
	5	5.0±0.2^{a}	5.5±0.3^{a}	4.5±0.2^{a}	1.7±0.2^{a}	2.2±0.2^{a}	-
	8	6.8±0.2^{a}	6.1±0.1^{a}	7.1±0.1^{a}	2.2±0.2^{a}	2.4±0.3^{a}	.
	12	7.2±0.1^{a}	7.3±0.2^{a}	5.1±0.3^{a}	2.3±0.2^{a}	2.9±0.1^{a}	1.1±0.1^{a}
N_2	0	4.1±0.2^{a}	4.1±0.2^{a}	2.2±0.1^{a}	-	-	-
	2	2.9±0.2^{b}	2.7±0.1^{b}	2.7±0.2^{a,b}	-	-	-
	5	2.9±0.2^{b}	2.9±0.1^{b}	2.8±0.2^{b}	1.0±0.1^{b}	1.0±0.1^{b}	-
	8	6.1±0.2^{b}	5.7±0.2^{b}	5.7±0.3^{b}	2.2±0.3^{a}	2.3±0.1^{a}	-
	12	6.5±0.3^{b}	7.0±0.2^{a}	4.7±0.3^{a,b}	2.3±0.1^{a}	2.5±0.1^{b}	1.1±0.1^{a}
N_2O	0	4.0±0.1^{a}	4.1±0.2^{a}	2.2±0.1^{a}	-	-	-
	2	2.9±0.2^{b}	3.1±0.1^{c}	2.5±0.2^{b}	-	-	-
	5	2.8±0.2^{b}	3.3±0.4^{b}	2.6±0.3^{b}	-	1.2±0.1^{b}	-
	8	6.2±0.2^{b}	5.3±0.4^{b}	5.6±0.2^{b}	2.6±0.4^{a}	2.3±0.1^{a}	1.1±0.2
	12	5.7±0.1^{c}	6.4±0.2^{b}	4.3±0.2^{b}	2.0±0.3^{a}	2.4±0.1^{b}	1.1±0.1^{a}
Ar	0	4.0±0.1^{a}	4.1±0.2^{a}	2.2±0.1^{a}	-	-	-
	2	3.1±0.3^{b}	3.1±0.1^{c}	2.5±0.2^{b}	-	-	-
	5	3.1±0.1^{b}	3.2±0.1^{b}	2.6±0.2^{b}	1.0±0.0^{b}	1.1±0.1^{b}	-
	8	4.6±0.3^{c}	5.2±0.4^{b}	5.6±0.1^{b}	2.1±0.0^{a}	2.2±0.1^{a}	-
	12	5.9±0.2^{c}	6.3±0.2^{b}	4.3±0.3^{b}	2.3±0.1^{a}	2.3±0.3^{b}	-

* Total mesophilic bacteria

** Total psychrotrophic bacteria

***under the detection limit

Although N2 and N2O samples showed significantly lower ($p < 0.05$) values for mesophilic bacteria, the threshold was reached contemporaneously to the air packed samples, while for psychotropic microorganism, they reached spoilage only at the last day (Table 1).The samples packaged in argon reached the fixed threshold for total mesophilic and psychrotrophic bacteria after 12 days of storage, resulting the best MAP condition adopted able to increase the sardine shelf-life of 3 days with respect to the other tested conditions. At the end of the shelf-life, the product stored in argon was also characterized by the lowest level of total coliforms (2 log CFU/g), while fecal ones were lower than 1 log CFU/g.

The initial TMAO-N values were 374.8 mg/kg; as expected, the content decreased in all samples during the storage period reaching 43.7 mg/kg at the end of the storage (12 days). At this storage condition, no significant differences were observed between the samples. At the beginning of the storage period, no significant changes in trimethylamine-N (TMA-N) content were observed for all samples. The initial mean TMA-N content of sardine muscle was 32.4 ± 2.0 mg N/kg, slightly higher compared to the value observed by Erkan et al. (2006). For all samples, TMA-N content remained fairly stable until the 6th day, then a slight increase was observed for the Air samples. At the end of the storage period of 12 days, the values were similar in all the samples, between 41 and 47 mg N/kg with no significant differences. In general, the upper limit for TMA-N before consumer rejection of fish is usually 50 to 100 mg/kg. Changes in the Hx content of sardines stored under different modified atmosphere packaging conditions at 4 °C are shown in Figure 2.

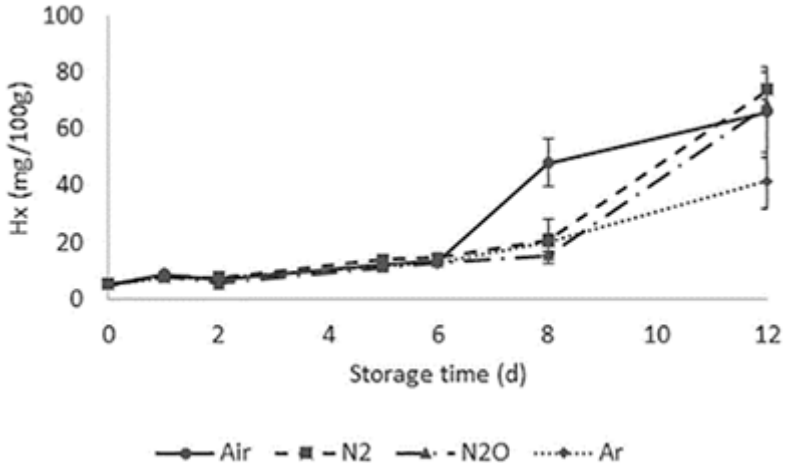

Figure 2. Changes in Hx of sardine fillets in MAP during storage at 4 °C (corresponding to Figure 6 B in ***paper I)***.

In this study, Hx concentration increased with the storage period, as reported for sardines (Özoğul et al., 2007). At day height, air packed samples showed significantly higher ($p < 0.05$) Hx content (50 mg/100g) compared to the rest of the MAP samples (20 mg/100g). At the end of the storage period at 12 days, while Air, N_2 and N_2O samples were characterized by similar values (around 60–70 mg/100g), the sample packed in Ar gas mixture showed a significant lower value ($p < 0.05$) (around 40 mg/100g). This result is in agreement with the lower microbial load found in Ar samples at the end of the storage. The initial level of H-index was about 22% and increased fairly slowly and in a similar way up to 29% in all samples until the 6^{th} day. In the second part of the storage period, a sharp increase was observed in all samples, in particular for N_2 at the 8^{th} day and at the 12^{th} day for the other samples. However, Ar sample showed a lower value compared to the others, confirming the trend observed for H_x formation. Results were fairly similar to the ones observed by Özogul et al. (2004) in sardine fillets stored in different packaging conditions. The initial level of H-index was about 22% and increased fairly slowly and in a similar way up to 29% in all samples until the 6^{th} day (Figure 2). In the second part of the storage, a sharp increase was observed in all samples, in particular for N_2 at the 8^{th} day and at the 12^{th} day for the other samples. However, Ar sample showed a lower value compared to the others, confirming the trend observed for H_x formation.

2.2.3 Effect of MAP on color and lipid quality

The composition of MAP can affect the color of the seafood products. Meat color can vary dramatically depending on the chemical state of myoglobin, and its relative proportions present from the purple-red color of deoxymyoglobin (with the reduced form of iron), and the bright red color of the oxygenated oxymyoglobin form, to the oxidized brown color of the metmyoglobin form (with the oxidized form of iron) (Figure 3) (Feiner, 2006).

Atmospheres with high concentrations of carbon dioxide can bleach fish species that contain carotenoid pigments (e.g., salmon) during prolonged storage in MAP (Randell et al., 1999). Oxygen is preferentially excluded or set as low as possible to reduce oxidative deterioration of foods, particularly in high fat seafood product. However, O_2 also plays a role in affecting the color of packed red fish meat (e.g., tuna, yellowtails). Fresh tuna fillets are highly perishable due to hemoglobin/myoglobin oxidation, which causes detrimental color changes. However, tuna fillets are often packaged under super atmospheric O_2 concentrations as this can reduce these color changes (Torrieri et al., 2011).

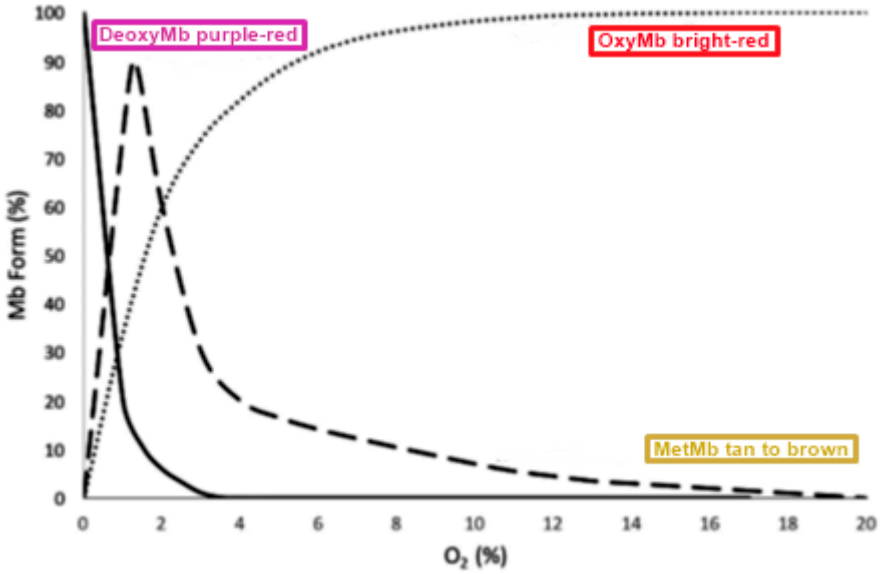

Figure 3. Relationship of oxygen concentration on myoglobin chemical state (modified from Thippareddi & Phebus, 2002).

One of the major deteriorative processes, that occurs during the storage of fish and which has a major influence on quality, is lipid oxidation. Lipid oxidation may affect both odour and flavour of fish and, in severe cases, its nutritional value (Pacetti et al., 2015). Fish and shellfish are excellent sources of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5v3) and docosahexaenoic acid (DHA, 22:6v3). PUFAs are known to be markedly susceptible to peroxidation, and to be readily incorporated into the mechanism of lipid peroxidation to yield free radicals and lipid peroxy radicals. Therefore, their protection against oxidation by use of novel packaging technologies, as well as storage conditions, is essential. During the advanced stages of lipid oxidation, the breakdown of hydroperoxides generates low molecular-weight carbonyl and alcohol compounds that could lead to the changes in food quality (Pacetti et al., 2015). Moreover, the resulting products of reaction between protein and oxidized lipid are yellow pigment formation (Masniyom, 2011). Rancidity due to the oxidation of PUFA in some fish may be a problem in modified atmosphere with O_2 (Stammen et al., 1990). Thus, under MAP, oxygen is preferentially excluded or set as low as possible to reduce oxidative deterioration of foods, particularly in high fat seafood product. The increase in TBARS indicates the formation of secondary lipid oxidation products (Chaijan et al., 2006). Arashisar et al. (2004) reported that no significant differences were observed between vacuum packaged and 100% CO_2 group with respect to TBARS value. The highest average TBARS value was obtained from fillets packed with 30% O_2 than other atmospheres (100% CO_2, 2.5%O_2 + 7.5%N_2 + 90%CO_2 and 30%O_2 +

30% N_2 + 40%CO2) in rainbow trout (*Oncorynchus mykiss*) fillets. Gimenez et al. (2002) reported that lipid oxidation was significantly higher in gas packages with 20% and 30% O_2 than in those with 10% O_2 in rainbow trout fillets.

New findings

In ***paper II,*** the effect of MAP, with unconventional gas mixtures (Ar and N_2O) on the color and different lipid oxidation indicators of sardine fillets, was evaluated during refrigerated storage.

Figure 4. Images of fresh sample (a), and of samples air (b), N_2 (c), N_2O (d) and Ar (e) after 12 days of storage at 3 °C (corresponding to Figure 2 in ***paper II***).

Changes in the L*, a* and H° colour parameters of sardine fillets packed in air and MAP during storage are shown in Table 2. During storage, L* (lightness) showed a slight decrease in all samples, but at the end, sardine fillets packed in N_2O showed a significantly lower L* value compared to the N_2 and air samples ($p < 0.05$). On the contrary, a* (redness) values increased in all samples, but after the fifth day until the end of storage, in fillets packed in N_2 and Ar, it was significantly higher compared to samples in air.

Table 2. Changes in the colour parameters of sardine fillets under different MAP conditions at 4°C (corresponding to Table 1 in ***paper II***).

Samples	Storage time (days)						
	0	1	2	5	6	8	12
L*							
Air	46.56±0.0^{a}	45.52±0.5^{a}	43.71±2.8^{a}	43.68±2.6^{a}	45.07±2.7^{a}	43.94±2.6^{a}	45.55±2.4^{a}
N2	46.56±0.0^{a}	45.47±1.8^{a}	43.98±3.1^{a}	43.15±1.74^{a}	43.43±2.2^{a}	42.03±2.6^{a}	44.33±1.6^{a}
N2O	46.56±0.0^{a}	42.85±1.3^{b}	43.63±2.9^{a}	41.91±1.95^{a}	41.25±2.5^{a}	41.95±2.8^{a}	42.66±2.7^{b}
Ar	46.56±0.0^{a}	43.78±1.2^{ab}	45.30±2.0^{a}	42.61±1.92^{a}	43.50±2.1^{a}	42.66±2.4^{a}	43.70±1.6^{ab}

a*							
Air	1.22±0.0[a]	1.96±2.1[a]	3.07±1.8[a]	2.35±1.1[b]	4.41±1.8[a]	4.36±2.7[b]	3.89±2.5[b]
N2	1.22±0.0[a]	0.99±1.5[a]	3.52±1.6[a]	5.05±2.4[a]	5.71±1.3[a]	6.53±2.3[a]	5.94±2.6[a]
N2O	1.22±0.0[a]	1.45±1.0[a]	4.36±2.2[a]	4.40±2.8[a]	5.89±1.6[a]	6.64±1.4[a]	4.96±2.9[ab]
Ar	1.22±0.0[a]	2.05±1.4[a]	2.94±2.3[a]	4.55±2.6[a]	5.89±1.3[a]	6.24±1.5[a]	6.26±2.6[a]
H⁰							
Air	80.21±0.0[a]	79.02±19.7[a]	70.29±13.4[a]	75.68±12.3[a]	62.91±11.2[a]	65.88±13.8[a]	54.45±13.3[a]
N2	80.21±0.0[a]	83.89±11.6[a]	66.59±13.6[a]	59.84±11.6[b]	54.12±5.0[b]	51.48±9.1[b]	56.20±11.1[a]
N2O	80.21±0.0[a]	77.95±8.4[a]	63.91±10.7[a]	62.99±14.6[b]	52.68±6.1[b]	50.54±5.6[b]	54.19±14.2[a]
Ar	80.21±0.0[a]	74.25±10.4[b]	71.22±14.4[a]	62.37±13.2[b]	54.18±5.2[b]	52.56±6.4[b]	56.73±10.9[a]

Different letters in the same column indicate significant differences ($p < 0.05$).

The hue angle (H°) decreased during storage showing differences among the tested atmospheres. However, at the end of the storage, no significant differences were observed among the different MAP samples ($p > 0.05$). The concentration of oxygen in the package determines the oxidative state of the myoglobin in fresh meats (Figure 3). From a macroscopic point of view, this phenomenon was evident considering the images of sardine fillets acquired in conditions of standardized illumination, as shown in Figure 4. After 12 days of storage, while sample packed in air (b) evidenced a yellow/brown colour, the N_2, N_2O and Ar samples (c, d, e) evidenced an evident purple-red colour component, even more evident also respect to the fresh sample (a). The yellow-brown tint and the inhomogeneous distribution of colour in the surface of the sample packed in air was indicating an advanced state of oxidation. On the contrary, the absence of oxygen or an oxygen concentration very close to 0% promoted deoxy-myoglobin.

Regarding the lipid oxidation indexes (in ***paper II***), primary lipid oxidation was evaluated by peroxide value (PV). Evolution in the PV during storage is shown in Figure 5. At the beginning, the value was rather low (1.56 meq O_2 kg^{-1} fat), indicating a good oxidative quality of the raw material. In the sample packed in air, the increase in PV began just on day one, following the typical trend of peroxide formation. At the end of the 12 days, PV was significantly greater (8.34 meq O_2 kg^{-1} fat) in Air samples compared to the MAP samples, in which PV remained almost constant during storage with values that never exceeded 4 meq O_2 kg^{-1} fat. The low peroxide values in the MAP samples were probably due to the low concentration of oxygen in the headspace of the packages.

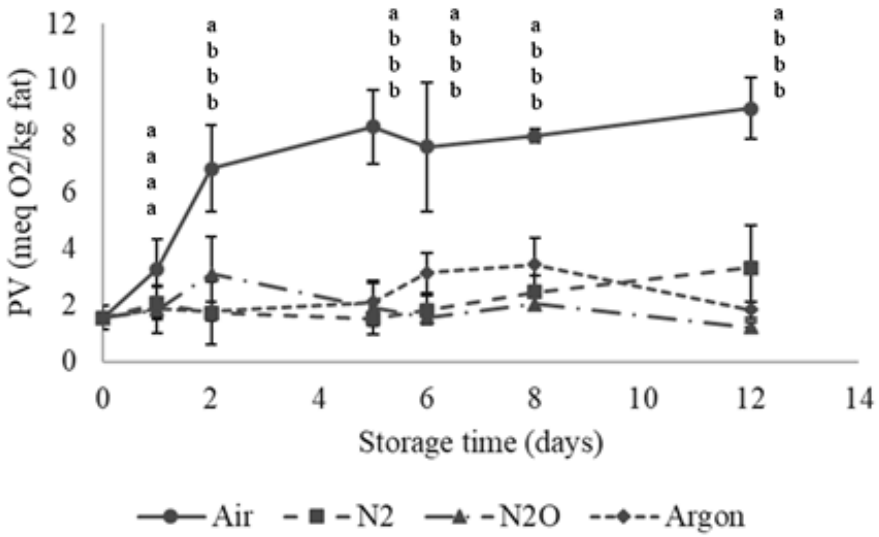

Figure 5. Peroxide values measured in sardine fillets in MAP during storage at 3 °C. Different letters indicate significant differences among samples at the same storage time (P < 0.05) (corresponding to Figure 3 (a) in ***paper I***).

According to the classification reported by Pinheiro et al. (2019), samples in air could be considered 'spoiled' (PV = 8–10) at the end of the storage, while the other samples were still 'good' (PV = 2–5).

Thiobarbituric acid reactive substances (TBARS) values in the sardine fillets stored under different MAP conditions are shown in Figure 6. The measured lipid oxidation increased with the storage time. However, after the 5th day, for MAP samples the TBARS values were significantly lower ($p < 0.05$) compared to Air sample and remained quite low throughout the entire period of storage.

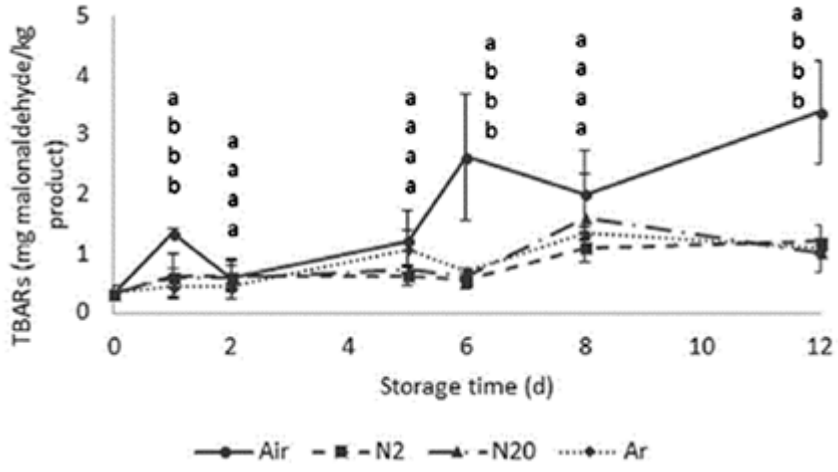

Figure 6. TBARs values of the sardine fillets in MAP during storage at 4 °C (corresponding to Figure 2 in ***paper I***).

After 12 days, a value of 3.39 mg MA kg−1 was recorded for the Air sample, while in MAP samples, the higher value was 1.09 mg MA kg−1.

The evolution of lipid hydrolysis is shown in Figure 7**.** The initial fresh fillets value (0.09 ± 0.01 g oleic acid/100 g lipids) was similar to that obtained by Chaijan et al. (2006) for another sardine species (*S. gibbosa*). Free fatty acids (FFAs) values for all samples increased slightly with storage time. In Ar samples, the higher value was observed at the day 8 (0.73 ± 0.22 g/100 g lipids) followed by a decrease at the 12th day. At the end of the storage period, the samples packed in Ar gas mixture were lower than all the other ones.

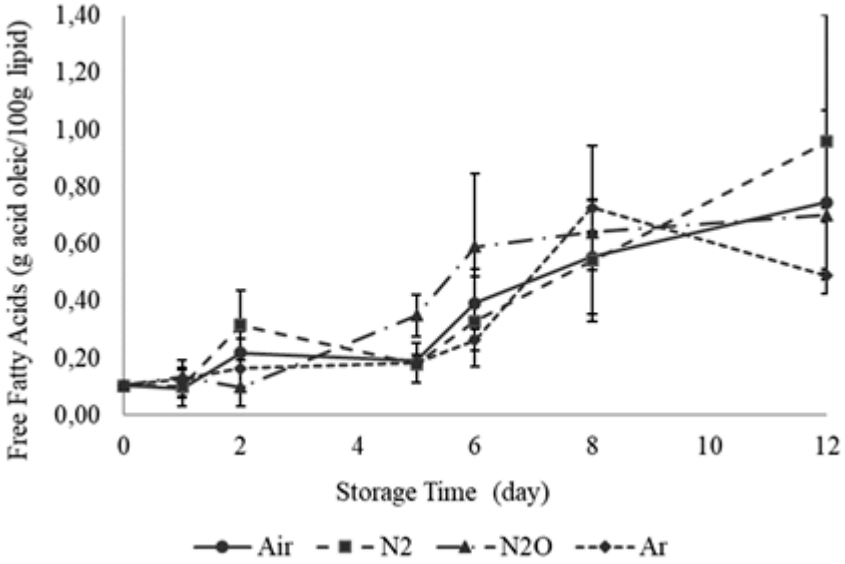

Figure 7. Free fatty acids content measured in sardine fillets in MAP during storage at 3 °C (corresponding to Figure 3 (d) in ***paper II***).

Chaijan et al. (2006), reported a high lipid hydrolysis (>6 g FFA/100 g lipid) in iced sardines at the end of the storage (15 days), probably because the authors used whole fish in which hydrolytic enzymes can derive also from internal organs. In this study, the highest FFAs value (0.96 ± 0.44 g/100 g lipids) were found on N_2 samples on day 12th and was similar to the values reported by Fagan et al. (2004) for mackerel and salmon fillets packed in MAP (60% N_2/40% CO_2), combined with freeze-chilling. However, no significant differences were found among Air, N_2 and N_2O samples throughout the storage period. Probably, the removal of oxygen from the packages confirmed to reduce the lipid oxidation in a similar way in all the samples. While the use of N_2O instead of N_2 did not show any advantages in terms of the investigated parameters, the use of Ar allowed to increase sardine shelf-life up to 12 days, showing an inhibitory effect on bacterial spoilage and on the development of

hypoxanthine during storage. The promising results obtained with Ar should be confirmed by extending the investigation to other fish species.

3. High-pressure processing (HPP)

High pressure processing (HPP) is a non-thermal technology that emerged as a new food preservation method, that has begun to be widely implemented in the meat industry, mainly due to its ability to inactivate tissue enzymes, pathogenic and spoilage microorganisms, maintaining freshness characteristics and nutritional value (Barba et al., 2015). In recent years, research on HPP of fish muscles has mainly focused on extension of chilled/frozen shelf-life, pressure-induced texturization (gel formation), and HP freezing/thawing. Recent studies have shown that HPP can have beneficial effects on microbial inactivation, shelf-life extension, microstructure, drip loss, and texture of several fresh and frozen fish muscles (Truong et al., 2015). However, significant changes to some quality characteristics are unavoidable and might reduce consumer acceptability of HPP treated products

Despite a significant number of machines operating in the industry, HPP is still considered an emerging technology. Innovations and technological changes are often associated with economic risks and successes, but they can also represent a long-term prospect for sustainable development and competitive production. In this perspective, continuous research and development efforts are still needed to make the technology commercially viable for more seafood products. In this context, in this PhD research work, the effect of HPP on different seafood products, grey mullet (*Mugil cephalus*), striped prawn (*Melicertus kerathurus*) and deep-water rose shrimp (*Parapenaeus longirostris*), intended for the raw consumption was evaluated (***paper IV***). Three pressure levels (400, 500 and 600 MPa) were applied and microbiological quality, safety, chemical parameters, colour and texture were analyzed during refrigerated storage.

3.1 Principles of HPP

The governing principles of HPP are based on the assumption that, foods which experience HP in a vessel, follow the isostatic rule regardless of the size or shape of the food. The isostatic rule states that pressure is instantaneously and uniformly transmitted throughout a sample whether the sample is in direct contact with the pressure medium or hermetically sealed in a flexible package. Therefore, in contrast to thermal processing, the time necessary for HPP should be independent of the sample size (Rastogi et al., 2007).

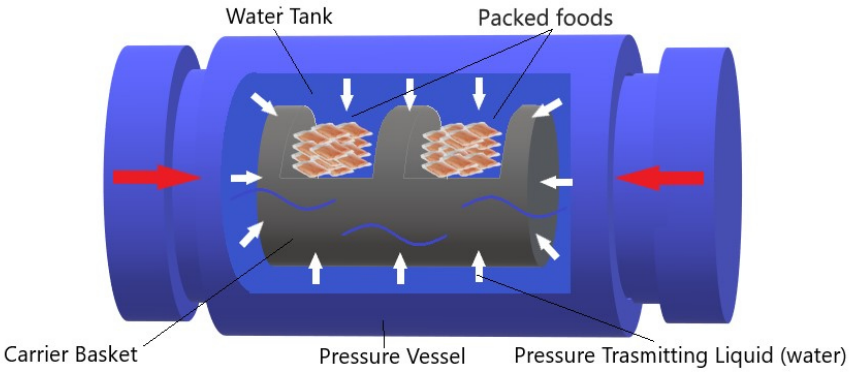

Figure 8. Schematic representation of HPP-vessel

The HP system consists of a pressure chamber, a pressure generation and intensification system, and a compression fluid (usually water) (Figure 8). In operation, the pressure intensifying compression fluid is forced into the chamber containing the sample. Industrial food processing equipment has a capacity of about 500 L, able to operate at maximum pressures of 900 and 1200 MPa, though the most common is about 400 and 600 MPa (Balasubramaniam et al., 2016).

3.2 HPP on seafood products

Applications of HPP in the seafood industry have received great attention in the last decade. Currently, HPP is applied on seafood products for several purposes, such as extension of shelf-life and preservation of freshness attributes, increase the safety of raw seafood, create value-added seafood products and shucked meat from shellfish (Truong et al., 2015). However, effects such as discoloration, increased hardness, changes in water holding capacity, pH variations, lipid oxidation, protein oxidation among others, can occur in more intensive treatments (Oliveira et al., 2017). The extent of microbial inactivation depends on treatment parameters such as pressure level, holding time, temperature, but also on the characteristics of the microflora in the product. Besides, in complex matrices like food products, the desired effect on microbial inactivation may also produce physical and biochemical changes which may affect the product properties in a negative manner. The denaturation of protein in fish muscle is often cause of significant changes in important parameters for consumer acceptability. In particular, the application of high pressures is known to lead to a cooked appearance (Matser et al., 2000), that can be specifically detrimental in products intended for the raw consumption. Moreover, the effect on protein

structure and on enzymatic activity can lead to variation in the texture of seafood product, not only after the treatment but also a modification during refrigerated storage. The effect has been studied in a variety of fish and seafood matrixes, but results are very variable depending on process parameters but also on specific product characteristics (Truong et al., 2015). Although the literature is rich of examples of application of HPP on seafood products, contrasting results have been presented, mainly because different pressure profiles have been applied to different species (Oliveira et al., 2016). Hence, it is essential to understand the mechanisms of these changes, in order to propose alternatives that can reduce the negative effects.

3.2.1 Microbial inactivation

The inactivation of microorganisms by HPP is the result of a combination of factors including changes in the cell membranes, cell wall, proteins and enzyme-mediated cellular functions (Campus, 2010). The inactivation by HPP depends on the type of microorganism and its growth phase, the pressure applied, the processing time, the composition of the food, its pH and water activity and the temperature, (Tewari et al., 1999). In general, it is assumed that Gram-negatives and cells in the growth phase are more sensitive than Gram positives and cells in the stationary phase (Farkas & Hoover, 2000), respectively, but there are some exceptions to this generalization; for instance, certain strains of *E. coli* O157 are exceptionally pressure resistant (Patterson et al., 1995). Nevertheless, investigations have shown that cell disruption is highly specific to the geometry of the bacteria rather than to the Gram type.

Many vegetative bacteria including spoilage and pathogenic microorganisms, yeasts, molds and viruses, are sensitive to HP. However, it should be noticed that spores are very resistant to HP; for example, spores of *Clostridium botulinum* can still survive under extreme conditions of 827 MPa for 30 min at 75 °C (Farkas & Hoover, 2000).

HPP can effectively reduce the initial microbial load in many fish muscles (Truong et al., 2015). In general, the application of pressure higher than 300 MPa for few minutes at room temperature has shown to significantly reduce the initial microbial load, slowing microbial growth during chilled storage in various fish muscles such as hake, sea bass, rainbow trout, mahi mahi, salmon, red mullet, gilthead sea bream and tuna (Truong et al., 2015). Typically, the inactivation of micro-organisms in fish muscles is increased with the increase in pressure and holding time. For example, the inactivation rate of bacteria in albacore muscle is

enhanced when pressure level increased from 275 to 310 MPa at 10 °C, and holding time increased from 2 to 6 minutes (Ramirez-Suarez & Morrissey, 2006). Besides the treatment conditions, the efficacy of HPP on microbial inactivation is also influenced by the characteristics of the microbiota in fish muscles. Psychrotrophic bacteria, the dominant microorganisms in temperate sea water fish species during chilled storage are more sensitive to HPP at elevated temperature (Chéret et al., 2005; Ojagh et al., 2011).

HPP is also an effective processing method to inactivate *Anisakis simplex* in some fish products. Treatment at a pressure of 200 MPa and a temperature between 0 and 15 °C for 5 and 10 min kills all *Anisakis* larvae in hake and finfish muscle, without producing visual changes in fish muscle appearance (Molina-García & Sanz, 2002).

New findings

Paper IV reports the effects of HPP treatments (400, 500 and 600 MPa), compared to the untreated controls, on the microbiological quality of packed grey mullet, striped prawn and rose shrimp. In all the tested conditions, *Salmonella* spp. and *Listeria monocytogenes* were never detected during the shelf-life of the considered products. Also, coagulase positive staphylococci were never found in untreated and HPP treated samples of grey mullets and striped prawn. In general, the application of the HPP treatments increased the microbiological shelf-life of the considered products and the inactivation effect became more severe as the pressure increased. The microbiological threshold to define product shelf-life was fixed at the attaining of 6 log CFU/g for total mesophilic bacteria (TMB) even if, according to the Regulation 2073/2005, other important microbiological criteria such as the cell load of *Escherichia coli* and positive coagulase staphylococci were considered in the data discussion. More specifically, the detected microbiological data for grey mullets, untreated and in relation to the pressure applied and time of storage, are reported in Table 2. As clearly showed, the control sample spoiled within 6 days of storage at 2°C, reaching a cell load of total mesophilic bacteria of 6.22 log CFU/g, with a corresponding level of total coliforms of 1.8 log CFU/g. In contrast, the application of treatments of 400, 500 and 600 MPa prolonged the product shelf-life to 12, 32 and >32 days, respectively. The application of HPP treatments ranging between 400 and 600 MPa decreased the cell loads of *E. coli*, compared to the untreated sample, under the detection limit (1 log CFU/g).

Table 2. Evolution of microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB), *Lactobacillus spp.*, *Pseudomonas spp.*, total Coliforms, sulfite reducing anaerobic bacteria (AB), E. coli during the refrigerated storage of packaged Mullet (*Mugil cephalus*) flesh in relation to the High Pressure Processing (HPP) treatments applied (400, 500, 600 MPa).

	Mullet	Log CFU/g		
		M-0	M-400	M-500	M-600
Days of storage					
0d	TMB	4.67 ± 0.43	<1	<1	<1
	Lactobacillus spp.	3.18 ± 0.33	<1	<1	<1
	Sulfite reducing AB	4.20 ± 0.54	<1	<1	<1
	Pseudomonas spp.	4.45 ± 0.22	<1	<1	<1
	Total Coliforms	2.56 ± 0.38	<1	<1	<1
	E. coli	1.12 ± 0.10	<1	<1	<1
2d	TMB	5.14 ± 0.44	<1	<1	<1
	Lactobacillus spp.	3.30 ± 0.29	<1	<1	<1
	Sulfite reducing AB	5.15 ± 0.51	<1	<1	<1
	Pseudomonas spp.	5.20 ± 0.45	<1	<1	<1
	Total coliforms	1.80 ± 0.12	<1	<1	<1
	E. coli	1.22 ± 0.10	<1	<1	<1
6d	TMB	**6.22 ± 0.52**	<1	<1	<1
	Lactobacillus spp.	4.00 ± 0.28	<1	<1	<1
	Sulfite reducing AB	5.54 ± 0.46	<1	<1	<1
	Pseudomonas spp.	5.27 ± 0.35	<1	<1	<1
	Total coliforms	1.85 ± 0.16	<1	<1	<1
	E. coli	1.50 ± 0.20	<1	<1	<1
12d	TMB	-*	**5.67 ± 0.31**	<1	<1
	Lactobacillus spp.	-	<1	<1	<1
	Sulfite reducing AB	-	<1	<1	<1
	Pseudomonas spp.	-	2.46 ± 0.29	<1	<1
	Total coliforms	-	<1	<1	<1
	E. coli		<1	<1	<1
19d	TMB	-	7.52^{a} ± 0.43	4.22^{b} ± 0.41	2.20^{c} ± 0.23
	Lactobacillus spp.	-	1.06 ± 0.32	<1	<1
	Sulfite reducing AB	-	<1	<1	<1
	Pseudomonas spp.	-	5.11 ± 0.29	<1	<1
	Total coliforms	-	<1	<1	<1
	E. coli		<1	<1	<1
32d	TMB	-	-	**5.55^{a} ± 0.42**	**3.10^{b} ± 0.33**
	Lactobacillus spp.	-	-	<1	<1
	Sulfite reducing AB	-	-	<1	<1
	Pseudomonas spp.	-	-	<1	<1
	Total coliforms	-	-	<1	<1
	E. coli	-	-	<1	<1

-*: not more analyzed since the microbiological threshold fixed at 6 log CFU/g was reached in the previous time of analysis
In the same raw, values with different letter are significantly different ($p < 0.05$).

Pseudomonas spp were strongly affected by the level of HPP applied since cell loads of 2.4 log CFU/g were detected at 400 MPa after 12 days from the treatment to further increase on the 19th day of storage. In all the other treated samples (500 and 600 MPa), and for each time of sampling considered, Pseudomonas spp was always under the detection limit (< 1 Log CFU/g). Regarding untreated striped prawn, the total mesophilic bacteria reached the threshold level of 6.0 log CFU/g after 6 days of refrigerated storage, while the application of HPP pressure levels of 500 and 600 MPa determined a significant increase of the product shelf-life to 19 and 32 days, respectively, and a cell load of E. coli always under the detection limit. However, for the sample treated at 500 MPa, after 19 days, Lactobacillus spp. and Pseudomonas spp. were able to recover and reach cell load of 4.59 and 5.72 log CFU/g, respectively, differently from the samples treated at 600 MPa, where these microbial groups were found to always be under the detection limit. The application of pressure level of 400 MPa determined a shelf-life of about 12 days.Similar trends were observed for rose shrimp products for which the application of pressure level of 500 and 600 MPa allowed to reach the threshold shelf-life after 21 and 28 days respectively. In general, according to the literature data, the HPP treatments on fish samples are commonly applied between 150 and 450 MPa (Perez-Won et al., 2020), since higher pressures, aimed to increase the microbial inactivation, are generally associated with significant changes in physico-chemical, texture and sensory properties such as increase in discoloration, cooked appearance or lipid oxidation (Truong et al., 2015). Among the treated samples, the results showed that those treated at 400 MPa had the lowest shelf-life for all the species investigated. However, this pressure level seemed adequate to inactivate *E. coli* during the shelf-life of the considered fish samples. On the other hand, other microbial groups that play a role in the spoilage and safety problem, such as *Pseudomonas* spp. or coagulase-positive staphylococci, were able to recover during storage, highlighting the critical problem of viable but non-culturable cells (VBNC). In fact, although the efficiency of microbial inactivation is influenced by various factors, including the food matrix characteristics and food processing parameters, also the physiological diversity within a microbial population has to be taken into consideration, especially in the validation of the effectiveness of a treatment on a specific food product (Patrignani et al., 2019). Particularly, *Pseudomonas* spp., strict aerobic bacteria, whose growth decreased in vacuum conditions, were able to recover in all considered samples treated at 400 MPa during their shelf-life period. *Pseudomonas*, having also a psychrotrophic behavior and being able to produce specific H_2S off-flavors, could have a

negative impact also for the production of specific proteases potentially able to affect the textural properties of the food matrix.

3.2.2 Changes in color and texture parameters

High pressure treatment can affect seafood color depending on the pressure level. However, the mechanisms and reactions that lead to color changes after HP have not been fully elucidated. Several studies indicate that the L* parameter increases in pressurized fish, which is lighter, and takes on a typical cooked meat appearance when subjected to pressure above 150 MPa (Chéret et al., 2005; Jantakoson et al., 2012; Kaur et al., 2013; Truong et al., 2015; Yagiz et al., 2007). Although there are some differences between the results, most studies have shown the decrease in a* (loss of red) and increase in b* (up yellow), which varies with the species and the pressurization parameters. However, the increase in L* parameters depends on the intensity of the HP process (pressure and time) and may be associated with globin and myofibrillar denaturation. Otherwise, the change in the a* color coordinate may be related to oxidation reactions (Oliveira et al., 2017). In shrimp and salmonid, the red colour is mainly determined by astaxanthin, canthaxanthin and beta carotene. The astaxanthin occurs naturally in tissues in its free form, lipid esterified and complexed with proteins (carotenoprotein), and changes in these structures can cause meat whitening (Gudbjornsdottir et al., 2010; Jantakoson et al., 2012). This phenomenon can be attributed to proteins unfolding, that are complexed to the pigment (Truong et al., 2015) or to the pigment oxidation. Tuna meat is rich in myoglobin pigment, which is a hemo-protein; myoglobin denaturation with possible heme release can lead to color changes (Oliveira et al., 2017).

Texture is a very important parameter for seafood products appreciation. An increase in fish muscle hardness after HP treatment compared to fresh muscle has been observed in several species such as trout and mahi-mahi (Yagiz et al., 2007), cod and shrimp (Angsupanich & Ledward, 1998; Jantakoson et al., 2012; Yagiz et al., 2007). However, carp showed an increase in hardness at 100 MPa, with lower values at 200 and 300 MPa (Yoshioka, Yamada, & Maki, 1996). Sea bass subjected to 100 and 300 MPa for 5 min at 10 °C showed lower hardness than the control; similar values were also observed at 400 and 500 MPa for 5 min at 10 °C (Chéret et al., 2005). The changes in texture can be directly related to the effects of HPP on proteins, such as protein denaturation and aggregation, changes in actin-myosin interaction, α-actinin release, and denaturation of myofibrillar

proteins (Chevalier et al., 2001). High pressure promotes tissue compression by forming additional protein interactions (Jantakoson et al., 2012), with consequent compression of fibers and connective tissue rearrangement; The compaction of tissues and fibers caused by HP treatments may be due to the reduction of the sarcomere length, and a possible softening effect related to fragmentation of myofibrils structures can also be observed after HPP (Ashie & Simpson, 1996).

New findings

In ***paper IV,*** the effect of HPP treatment (400, 500 and 600 MPa) on color and texture parameters were evaluated. Regarding color results, L* and h° values of the three seafood species are reported in Figure 9.

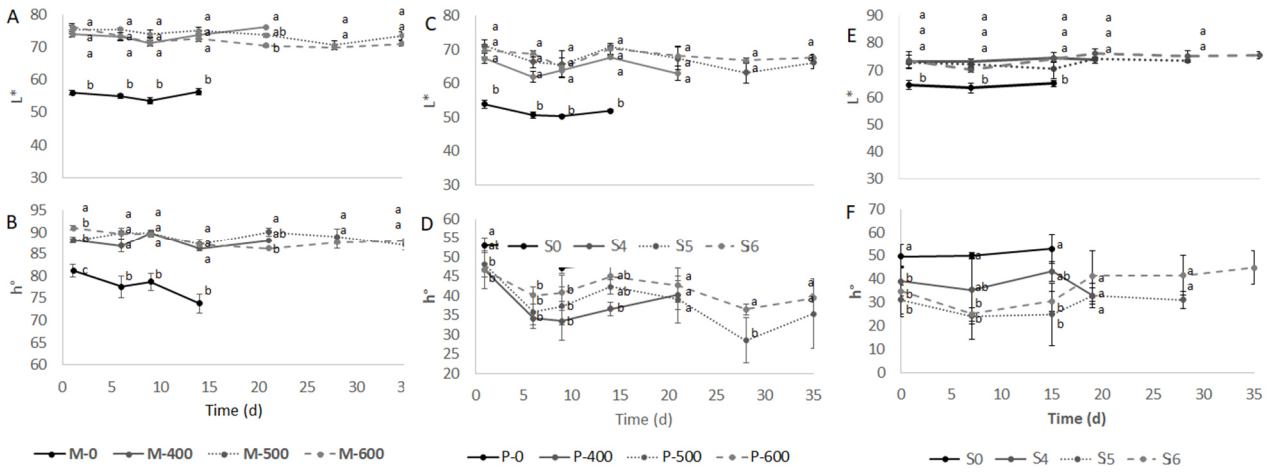

Figure 9. Colour coordinates of luminosity (L*) and hue angle (h°) of mullet (A and B), prawn (C and D) and rose shrimp (E and F). Different letters indicate significant differences among samples ($p<0.05$) (corresponding to Figure 3 in ***paper IV***).

The luminosity (L*) of the flesh was significantly increased in all three species considered, by about 20 units immediately after treatment. This effect has been largely observed in many fish species, and it has been attributed to protein denaturation. Considering that 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 level adopted, it can be assumed that protein denaturation occurred in all samples to a similar extent for 400 to 600 MPa. During storage, all L* values were very close to the initial ones, for the control and the pressure treated samples. Similarly, just after treatment, significant differences between

control and treated samples were observed for the hue angle (h°). However, while for grey mullet h° was increased by HPP, for shrimp and prawn, the values of this chromatic parameter were decreased. h° is calculated using both red and yellow indexes; in all samples, a* was remarkably decreased, while the changes in b* were higher in shrimp and prawn as compared to grey mullet. According to the literature, the variation of colour during storage can depend on the degradation of myofibrillar proteins and the disorganization of myofibrils caused by enzymatic and non-enzymatic reactions, but also on the possible oxidation of pigments (Yagiz et al., 2007).

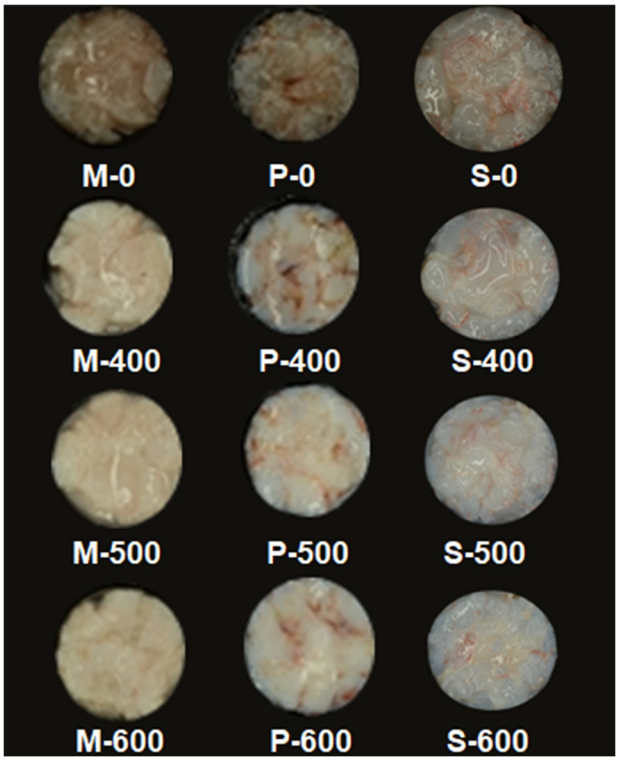

Figure 10. Digital images of grey mullet (M), striped prawn (P) and rose shrimp (S) samples treated at 400, 500 and 600 MPa, compared to the untreated ones.

Hence, colour change occurring during storage in seafood products subjected to high pressures can differ significantly according to the species and the adopted treatment conditions. In the present study, the final effect for all samples was a general whitening and the occurrence of a cooked appearance (Figure 10), that is typical for muscle food subjected to pressurization. Considering that the visual quality is a very important parameter for consumer acceptability, and that these products are intended for raw consumption, the cooked appearance might represent a problem that could be probably addressed with a marketing and/or informative strategy.

In ***paper IV***, texture profile analysis (TPA) was performed on the fish fillets and, considering the specific characteristics of the products studied, compression with subsequent application of constant pressure was also applied. In the present paper, the observed effect was different for the three considered species.

For grey mullet (Figure 11 A-B), a reduction in the initial hardness was observed for all applied pressures (400, 500 and 600 MPa), just after the treatments. Moreover, the increasing pressure promoted a decrease in the resistance to compression of the tissues proportionally to the applied pressure level. During the storage, for both parameters a decrease was observed in control and all treated samples. For striped prawn (Figure 10 C-D), a slight but significant reduction of hardness was observed for the 500 and 600 MPa treatments, while the resistance parameter was reduced only for the 600 MPa.

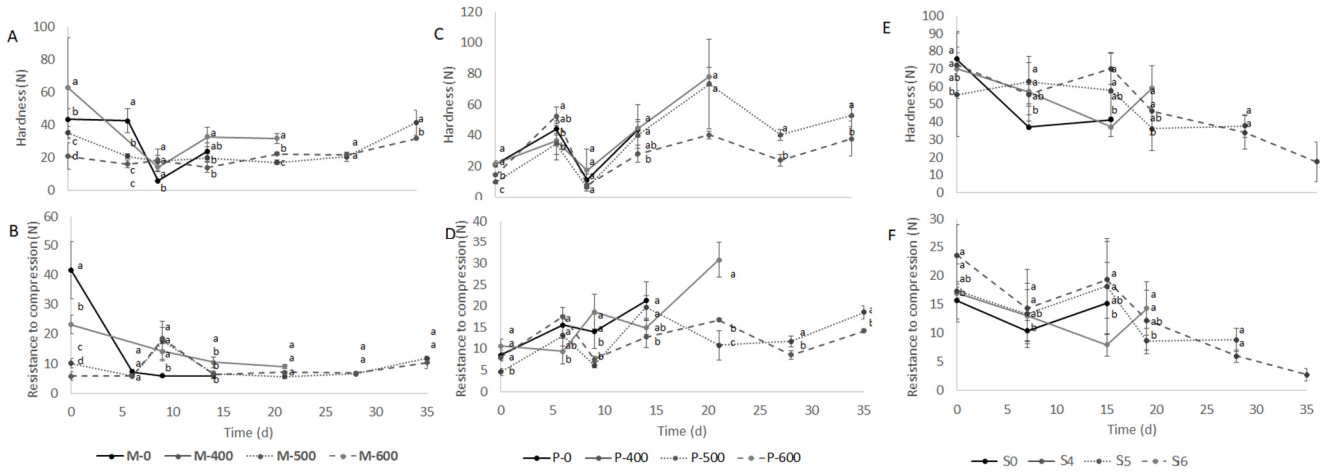

Figure 11. Texture parameters of Hardness (N) and resistance to compression (N) of mullet (A and B), shrimp (C and D) and rose shrimp (E and F). Different letters indicate significant differences among samples ($p<0.05$) (corresponding to figure 2 in ***paper IV***).

Resistance was shown to increase during storage only in the control and 400MPa samples. On the contrary, in rose shrimp (Figure 11 E-F), hardness was not influenced by any pressure applied while the resistance to compression was found significantly higher after the 600 MPa treatment. During storage, both parameters showed a decreasing trend for all samples. However, values were highly variables and very few significant changes were observed. These results are in contrast with the increase in hardness measured after high pressure application by Bindu et al. (2013) in Indian white prawn and by Jantakoson et al.

(2012) ad Kaur et al. (2013) in black tiger shrimp. Moreover, the behaviour of the two considered parameters during storage was different between the two crustaceans. A tendency to increase was found in prawn, while in shrimp values progressively decreased for the considered period. Decrease of hardness during storage was also observed by Kaur et al. (2013), that attributed this phenomenon to the effect of proteolytic enzymes. However, values were mostly constant for hardness in the stripe prawn at 600 MPa and for resistance to compression at 500 and 600MPa. This effect might be explained by a partial inactivation of such enzymes. Generally, after HPP an increase in hardness has been observed by many authors on different fish species such as rainbow trout and mahi mahi (Yagiz et al., 2007), cod (Angsupanich and Ledward 1998) and tuna (Zare, 2004). On the other hand, Briones-Labarca et al. (2012) found no differences in red abalone treated with HPP up to about 500 MPa compared to the control. Beside the modification to the myofibrillar proteins, HPP also promotes pH changes and modification of hydrogen and hydrophobic bonds that result in changes in the structural characteristics of proteins. Moreover, an effect on collagen and connective tissue of red abalone was observed by Briones-Labarca et al. (2012) through scanning electric microscope, that confirmed a significant change in the microstructure of the flesh upon high pressure application. Hence, the effect of HPP on fish texture is the resultant of all the modifications to water bonding and holding capacity, activity of enzymes such as proteases that can be inhibited or enhanced, and structural modification of myofibrillar and sarcoplasmic protein.

The results obtained in the present study (***paper IV***) confirmed that the effect of HPP on texture is strictly dependent on the considered species of the raw material and the specific tissue structure. Moreover, the effect observed during storage probably depends on the possible inactivation of proteolytic enzymes, that again, is probably matrix dependent. The effect of HPP on proteolytic enzymes in fish has been studied on many species by different authors, but results appear to be quite variable depending on seafood species, pressure level and holding time, as well as on type and structure of the enzyme.

4. Value addition of fishery products

Fish is a very perishable food product that needs proper handling and storage to increase its shelf-life and maintain its quality and nutritional attributes. Value addition is a topic of great importance in the seafood processing industry, especially in the export-oriented seafood processing industry (Datta, 2015), but also for small companies that process mainly local species and highly seasonal ones. Value can be added to fishery products according

to the needs of different markets. These products can vary from live fish and shellfish to ready-to-eat products. Overall, value addition means any additional activity that in one way or another changes the nature of the product, thus adding to its value at the time of sale. Many seafood products are characterized by significant changes in quality and abundance throughout the year. Therefore, it would be convenient, from both economic and sustainability standpoints, to promote strategies for their valorization, which lead to the development of innovative, high-added value products with prolonged shelf-life and that are available throughout the year, thereby reducing waste.

Mechanical separation of fish flesh could represent an opportunity for the development of fish-based innovative products from seafood that would otherwise be discarded. This technology that is successfully applied in the fish sector, even though the loss or modification of the normal structure of the muscle fiber often occurs during this operation (Secci et al., 2016).

Storage under deep-freezing conditions greatly increases the shelf-life of seafood products by arresting microbial growth and, in general, slowing down all other chemical and enzymatic degradation reactions. Freezing could help providing high-quality product constantly throughout the year and prevent product waste. However, freezing temperatures are only able to slow down enzymatic activity and oxidation. The main limiting factor of the shelf-life of frozen fish products is, in fact, represented by lipid degradation, due to both oxidative and hydrolytic reactions which affect their nutritional and sensory profile.

New findings

In ***paper III***, mechanical separation and freezing of mantis shrimp flesh (*Squilla mantis*) was carried out with the aim of valorizing this underutilized fish species from the Mediterranean characterized by high seasonality. For this purpose, changes in some qualitative indices of mechanically separated mantis flesh during deep-freezing storage were evaluated. The mantis shrimp was separated, deep-frozen, vacuum-packed and stored at three different temperatures of storage, -18, -26 °C and thermal abuse condition (-10 °C).

The evolution of the colorimetric parameter of luminosity L* (Figure 12 A), measured in the mechanically separated *Squilla mantis* flesh during frozen storage at the three selected temperatures, showed that at the temperatures of -18 and -26 °C values were roughly constant (37–42) throughout the 12 months storage. On the contrary, the sample stored at -10 °C showed a significant increase during the entire storage period, reaching values of 56.

This parameter was significantly influenced by storage temperature, time, and their interaction (Table 2 in ***paper III***).

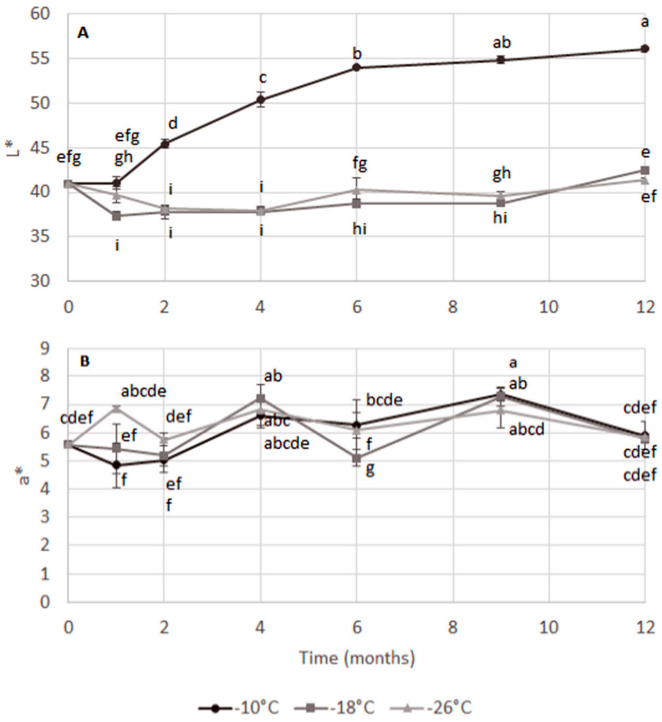

Figure 12. Colorimetric parameters of luminosity (L*) (**A**) and red index (a*) (**B**) of mechanically separated mantis shrimp flesh during frozen storage at -10, -18 and -26 °C. Different letters indicate significant differences ($p < 0.05$) among samples (corresponding figure 1 ***paper III***).

By contrast, the red index (Figure 12B) was significantly affected only by storage time and by the interaction between time and temperature. Although some significant variations were observed among samples during storage, there was not a clear trend and values remained between 5 and 7. Sundararajan et al. (2011) observed an increase in a* value for peeled frozen shrimp stored at –21 °C for 180 days, while no significant changes in L* values were observed. These authors suggested that the decrease in a* values could be mainly attributed to the degradation of astaxanthin and lipid oxidation. Shrimp flesh is highly perishable and normally high product quality can be obtained when immediately frozen after capture (Tsironi et al., 2009). Generally, results showed that the main color differences occurred during processing rather than during storage and that white flesh led to lower changes, proving to be more suited for the development of fish processed product (Secci et al., 2016). However, color fading, lipid oxidation, protein denaturation, and dehydration can occur during the frozen storage of shrimp and other crustaceans (Tsironi et al., 2009). Color variations observed in the sample stored at −10 °C may be related to enzymatic and

non-enzymatic reactions that result in degradation of myofibrillar proteins and disorganization of myofibrils.

Concerning the distribution of the main lipid classes, FFA was found to be influenced by both storage temperature and time, increasing from 9% up to around 40% in samples stored at −10 °C after 6 months. Similarly, monoacylglycerols (MAG) rose by increasing storage time and storage temperature, while triacylglycerols (TAG) and diacylglycerols (DAG) content showed the opposite trend. These results evidence the occurrence of lipid hydrolysis during frozen storage, being more intense at storage temperatures above −26 °C. The accumulation of FFA in frozen marine species is related to some extent with lack of acceptability. FFA, in fact, are known to cause deterioration of seafood products through their interaction with proteins and have been reported to exert a great effect on lipid oxidation development (Torres et al., 2014). FFA have also been shown to oxidize faster than higher molecular-weight lipids, i.e., TAG and phospholipids, due to their higher accessibility caused by their lower steric hindrance to oxygen and other prooxidant molecules (García-Soto et al., 2015).

In the present study, TMA-O (Figure 13A) decreased rapidly in the first month and then remained fairly constant in samples stored at −18 and −26 °C. In the sample stored at −10 °C, instead, lower values were observed during the rest of the storage; in fact, at the end of the 12-month storage period, TMAO was half as much the initial value. In parallel to the decrease of TMAO, both TMA (Figure 13B) and DMA (Figure 13C) increased in samples stored at −18 and −10 °C, while roughly the same values were observed in the sample at −26 °C.

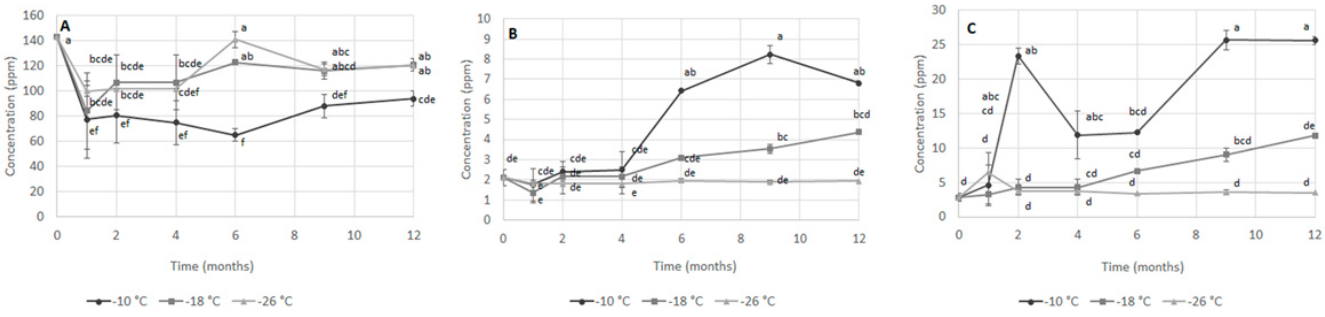

Figure 13. Concentration (ppm) of trimethylamine-O (TMA-O) (A), trimethylamine (TMA) (B) and dimethylamine (DMA) (C) measured by 1HR-NMR in extracts of mantis shrimp mechanically separated flesh during frozen storage at −10, −18 and −26 °C. Different letters indicate significant differences (at $p < 0.05$) among samples (modified by figure 3 ***paper III***).

In particular, TMA increased from the 4th month in both samples stored at −10 °C and −18 °C proportionally to the storage temperature. DMA started to increase after the first month for samples stored at –10 °C, whereas in samples kept at −18° C it rose just after 4 months and to a lower extent. Sotelo et al. (1995) found an increase of TMA during storage at −5 °C, but not at −12 °C. These authors suggested that some residual bacterial activity could still be found at temperatures slightly below zero. However, the TMA increase observed at −10 and −18 °C in the present study is probably related to enzymatic degradation. According to García-Soto et al. (2015), the formation of TMA during frozen storage of crustaceans can also be due to biochemical breakdown of proteins and non-protein nitrogen (NPN) compounds. Free amino acids in fish are the main components of non-protein nitrogen and, since some of them are precursors of aromatic components, they are directly responsible for the development of flavor and taste during cooking (Özden, 2005). Amino acids have also been used as quality indices for various fish and crustacean species. Some of them are precursors of biogenic amines obtained by decarboxylation, which are very important from the toxicity standpoint, and as quality control indices for fish spoilage. During storage, changes in amino acids are caused by muscle autolysis and the concentration of the single components depends on a dynamic balance between their production and destruction, this balance being associated with muscle enzymes (Ruiz-Capillas & Moral, 2001).

III. Innovative non-thermal technologies for seafood processing by-product valorization

1. Introduction

Seafood processing creates a huge volume of non-edible portion (50-80%) as by-products, which are thrown away or underutilized in many parts of the world. An important category of by-products from seafood processing includes crustacean ones. Approximately 6-8 million tons of crustacean waste is produced worldwide every year (FAO, 2014). A great number of bioactive compounds can be obtained from seafood by-products, such as collagen, chitin, enzymes, gelatin, glycosaminoglycans, polyunsaturated fatty acids (PUFA), minerals, protein and peptides and vitamins (Khawli et al., 2019). Several approaches (physical, mechanical, chemical, and biological) have been attempted by seafood industries to treat or minimize the disposal of seafood by-products. For the responsible and proper use of marine resources, it is essential to establish efficient and safe methods for the extraction of target nutrients and bioactive compounds. Conventional extraction techniques are already widely used for the separation, selective concentration and extraction of target compounds, such as fish meal and fish oil or in the production of EPA and DHA rich oil. These methods are efficient, but their main drawback is related to high energy consumption and potential thermal degradation of target compounds due to high processing temperatures (Khawli et al., 2019). In addition, many conventional extraction methods involve the use of organic solvents, present risks to human health and to the environment. Considering these aspects, innovative food processing technologies, based on non-thermal methods (i.e., ultrasounds, high-pressure processing, pulsed electric fields, cold plasma, supercritical fluid extraction) have been proposed for the use within the food industry including the extraction of valuable components from wastes and by-products (Suresh et al., 2018).

This chapter focuses on innovative non-thermal processes for recovering high value products from crustacean processing by-products. *In* ***paper V*** a literature review has been presented to provide a summary of the main compounds that can be extracted from crustacean by-products and of the results obtained by applying the main innovative non-thermal processes for recovering such high value products. In ***paper VI*****,** the application of accelerate solvent extraction (ACE) and pulsed electric filed (PEF), independently and in combination, was investigated to recover astaxanthin from crustacean by-products. This work was developed at University of Valencia, Spain, in cooperation with Prof. Francisco

Barba (Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine, Faculty of Pharmacy, University of Valencia, Spain).

2. Crustacean by-products as a source of valuable compounds

Crustacean by-products contain several valuable components such as proteins, lipids, and carotenoids, especially astaxanthin and chitin. When isolated, these valuable compounds are characterized by bioactivities such as anti-microbial, antioxidant, and anti-cancer ones, and that could be used as nutraceutical ingredients or additives in the food, pharmaceutical, and cosmetic industries. Different innovative non-thermal technologies have appeared as promising, safe, and efficient tools to recover these valuable compounds.

From the literature review (***paper V***), it is possible to conclude that crustacean by-products are mainly used for the recovery of chitin and chitosan, which is its deacetylated form. These compounds have been correlated to important biological activities, such as antioxidants, antimicrobial, and various other properties that could be exploited for food formulation to improve safety, quality, and shelf-life. Moreover, other valuable components could be applied in the food and pharmaceutical industries, in particular, crustacean by-products can be exploited for the extraction of protein (hydrolysates), lipids rich in polyunsaturated fatty acids (PUFA), and carotenoids could be also recovered from crustacean by-products.

Chitin, Chitosan and Derived Compounds

Crustacean exoskeletons are the main source of α-chitin aimed at commercial use on account of their high content and easy accessibility. These compounds (chitin and its derivatives) gained increasing attention in various fields, from the pharmaceutical, biotechnology, biomedical to the food sector (Hamed et al., 2016; Younes & Rinaudo, 2015) on account of their various beneficial properties, as they are biocompatible, biodegradable, and safe. However, chitin has a limited application due to its insolubility in water and many solvents. Therefore, water-soluble derivatives are produced, chitosan being the most important. It shows interesting biological activities, such as antimicrobial and antioxidant characteristics that make it attractive for preservation as a possible alternative to chemical preservatives and for food packaging for producing edible antimicrobial films based on its good film-forming properties (Alishahi & Aïder, 2012; No et al., 2007).

Proteins

Carotenoprotein isolated from shrimp by-products has shown high antioxidant activity, as well as being a rich source of essential amino acids and carotenoids (Pattanaik et al., 2021; Sowmya et al., 2011) and has the potential to be used as an additive to enrich foods and promote human health benefits (Santos et al., 2012). With respect to other seafood species, proteins obtained from crustaceans are characterized by a higher content of some amino acids such as glycine, glutamic acid, arginine, and alanine, resulting in increased palatability compared to finfish proteins (Nguyen et al., 2017). Moreover, on account of its optimal essential amino acid profile, the nutritional value of crustacean protein is similar or even higher compared to red meat (Venugopal, 2008) or soya bean (Yan & Chen, 2015)]. For this reason, protein hydrolysates from shrimp by-products have been used for the fortification of different types of food products, such as biscuits (Sinthusamran et al., 2019) and bread (Karimi et al., 2020). Moreover, the functional properties of protein extracts from crustaceans have also been investigated for the production of an edible film (Joaquín Gómez-Estaca et al., 2015).

Lipids

Crustaceans have appreciable proportions of ω-3 (omega-3) long-chain PUFA, particularly eicosapentaenoic and docosahexaenoic acid (EPA and DHA) (Nguyen et al., 2015). The PUFAs are probably the most successful bioactive components isolated from marine sources, because they have been widely recognized to be related to excellent health benefits (Sahena et al., 2009). However, lipid content in crustacean by-products may be variable depending on the species, the fishery's geographical location, and the kind of by-products. Among crustacean waste products, cephalothorax and hepatopancreas have also been used as an excellent source of lipids with high PUFAs content (Takeungwongtrakul et al., 2012, 2015), with a yield of approximately 2.7 and 11.6%, respectively.

The lipid extract from crustacean cephalothorax processing by-products, containing high levels of PUFAs (including DHA and EPA), α-tocopherol, and astaxanthin, has recently been suggested to be added as a natural ingredient to food formulation where it could exert different effects, as a food coloring and functional ingredient (Albalat et al., 2016; Gómez-Guillén et al., 2018). Biological activities that have been attributed to lipids derived from shrimp by-products include antioxidant, anti-proliferative, anti-mutagenic, and anti-inflammatory effects (Gómez-Guillén et al., 2018; López-Saiz et al., 2013, 2016). Lipids from crustacean by-products are oxidatively unstable, and the processes involved in their

extraction may significantly affect their yield, quality, and stability (Takeungwongtrakul et al., 2012). The presence of astaxanthin and α-tocopherol seems to increase lipidic extract stability on account of their antioxidant properties (J. Gómez-Estaca et al., 2017). However, their content was found to decrease during storage. Therefore, to expand their industrial application and utilization, recovery strategies that can improve yields without causing detriment to the quality of the extracted oil are necessary (Aryee & Simpson, 2009).

Carotenoids Pigments

Crustacean by-products represent important natural sources of carotenoid, among which astaxanthin (AX) is the major one. The content of AX in crustaceans can vary substantially, the variations observed in different shrimp species were in the range between 24 and 199 μg/g (Li et al., 2017). The observed differences could be due to variations in the amounts of carotenoids available in the feed, environmental conditions, and species, as well as due to the methods used for extraction. The main application of AX is as a coloring agent added in the formulation of diets for various aquaculture species, in particular salmon, and it has been used for functional foods development (Dmytrów et al., 2021), but it also finds various uses in the cosmetic and pharmaceutical industries (Higuera-Ciapara et al., 2006). The use of microencapsulation has shown great potential for the use of AX as a food ingredient for maintaining its coloring ability and overcoming some of its drawbacks such as odor (Gomez-Estaca et al., 2018), and improving its bio-accessibility and antioxidant capacity (Montero et al., 2016).

3. Innovative non-thermal technologies for recovery valuable compounds

Recently, the development of novel technological processes characterized by reduced energy consumption and impact on the environment increased the quality and safety of the final products, that can be applied for by-product valorization, have gained growing interest (Knez et al., 2014). For these reasons, various modern non-thermal processes, such as supercritical fluid extraction (SFE), high-pressure processing (HPP), pulsed electric field (PEF), and ultrasound (US), have recently been suggested with the aim of shortening the processing time, increasing recovery yield, improving the product quality, and enhancing the functionality of extracts from crustacean by-products (Figure 14) (Khawli et al., 2019).

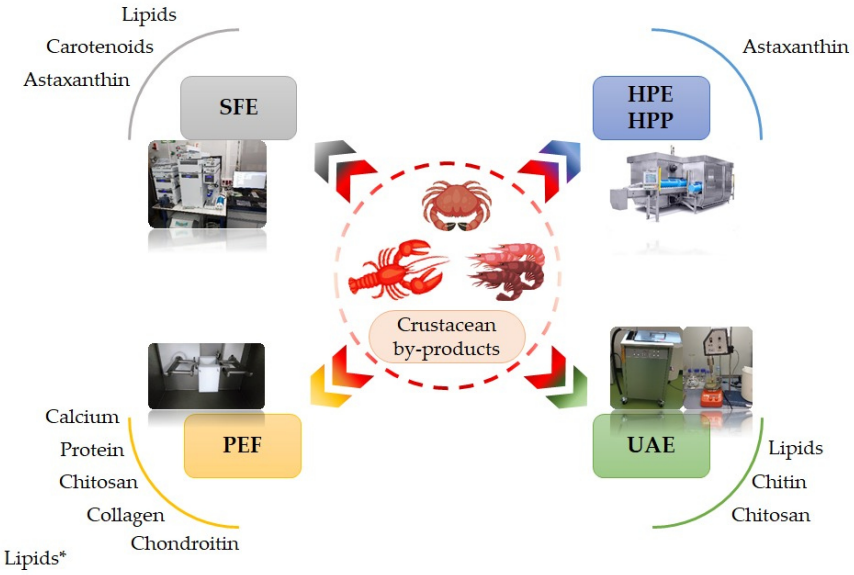

Figure 14. Main compounds extracted using non-thermal technologies. SFE: Supercritical Fluid Extraction; HPE: High Pressure Extraction; HPP: High Pressure Processing; PEF: Pulsed Electric Fields; UAE: Ultrasounds Assisted Extraction. * = used as a pre-treatment (published by pinheiro et al., 2021).

In ***paper V***, an overview of the most relevant research of the last 20 years for the optimization of innovative non-thermal extraction technologies reported for biomolecules from crustacean by-products obtained from their industrial processing was provided.

3.1 Supercritical fluid extraction (SFE)

SFE technology is based on the separation of one component from a matrix, solid or liquid, using a supercritical fluid (Figure 15). Supercritical fluids are particularly suited for the extraction process because they are characterized by physico-chemical properties that fall between those of a liquid and those of a gas, for instance, low viscosity, high diffusivity, and low surface tension (Prameela et al., 2017).

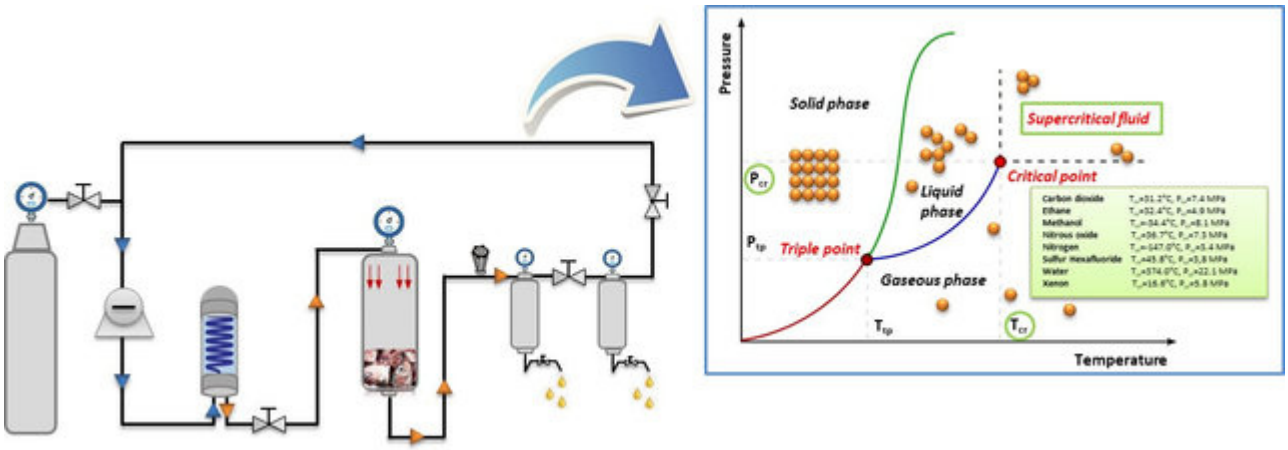

Figure 15. Schematic representation of supercritical fluid extraction (SFE) and the mechanism involved in this extraction technique (modified from Khawli et al., 2019).

SFE is a modern technology for extracting bio-compounds from various matrices that can be applied in the pharmaceutical and food industries. SFE has shown various advantages compared with traditional extraction processes, such as high yields, reduced processing times, and the use of solvents generally recognized as safe (GRAS), which make it a very popular green extraction method (da Silva et al., 2016). For seafood by-products, much research is focused on the recovery of components producing high added value products, principally lipids and lipophilic components, such as carotenoids (Herrero et al., 2010). For the SFE of carotenoids, the five most critical parameters are processing temperature, pressure, time, CO_2 density (solvent power) and flow rate, and entrainers concentration (Saini & Keum, 2018).

In ***paper V***, examples of SC-CO_2 for the recovery of lipids and astaxanthin from by-products derived from crustacean processing have been reported. Most of the published results showed that, when SC-CO_2 was used alone, pressure and temperature did not impact the yield of oil extraction leading to low quantity of recovered lipids and astaxanthin (Charest et al., 2001; Sánchez-Camargo et al., 2011). However, some authors have reported that adding co-solvents, generally ethanol or methanol, improved the extraction yields of both lipids and astaxanthin from by-products of crustacean processing. Despite the various advantaged of SFE, several concerns have been raised about the environmental and safety impact but also about the high energy consumption of the process. Other disadvantages of SFE include the limited sample size, extraction efficiency affected by matrix type, analyte type and moisture content of the matrix, and high cost of SFE equipment (Esquivel-Hernández et al., 2017). Possible solutions investigated to increase its efficiency are the

combination with other pre-treatments such as enzymatic treatment or the addition of co-solvents (Herrero et al., 2010).

3.2 High-Pressure Extraction (HPE)

Recently, the use of high pressure has been suggested also for extraction purposes (High Pressure Extraction - HPE) with the aim of reducing extraction time, solvent consumption, increasing extraction yields, and improving quality of the obtained extracts (Shouqin et al., 2004). HPE is based on the same principles of HPP (isostatic and Le Chatelier's principles), the applied pressure levels usually range from 100 to 600 MPa, not affecting the covalent bonds, and the use of room or refrigerated temperatures allows to avoid thermal degradation (Du et al., 2013). HPP produces physical damages to the plant tissue, cellular wall, membrane, and organelles, making cells more permeable to solvents, increasing the mass transfer rate and facilitating the release of extracts. For this reason, HPE can be a useful strategy to valorize by-products facilitating the recovery of bioactive compounds. Compared to the conventional used methods such as thermal or solvent extraction, HPE is faster, allowing to increase extraction yields, to reduce impurities and to preserve the bioactivity of the extracted compounds, in particular thermo-sensitive ones (Shouqin et al., 2004). Another important advantage of HPE relies is the ability to use different solvents (and solvent ratios), with distinct polarities, in combination enabling to extract different components, and to minimize the presence of impurities present (Li et al., 2017). HPE has been actively used to recover some biologically active substances from natural biomaterials; however, only few researches have evaluated the extraction from crustacean by-products.

Paper VI shows some examples of the application of this technology for the extraction of astaxanthin from crustacean by-products. Despite the small number of scientific publications, a wide range of pressure (0.1-600MPa), holding time, solvents (acetone, dichloromethane, and ethanol) and shrimp species (seven species) has been studied. High extraction yield (89.12 μg/g) was obtained by applying a pressure of 210 MPa for 10 min and a liquid-to-solid ratio of 32 mL/g *of Litopenaeus vannamei* shrimp by-product, using ethanol as the extraction solvent (Du et al). Compared with acetone and dichloromethane, ethanol showed the higher extraction yield (71.1 μg/g) obtained in only 5 min, using a solvent/solid rate of 20 mL/g and pressure range from 200 to 400 MPa (Li et al., 2017). Moreover, Irna et al., (2017) observed that the astaxanthin from shrimp carapace (*Penaeus monodon*) extracted by HPE was characterized by a higher antioxidant activity and a greater

zone of inhibition against four bacterial strains (*E. coli, E. aerogenes, S. aureus* and *B. subtilis*) compared to the chemically extracted one.

3.3 Pulsed electric fields (PEF)-assisted extraction

PEF processing represents a novel, non-thermal method that has been shown as a potential tool to recover bioactive compounds from agri-food by-products (Puértolas & Barba, 2016). Compared to conventional techniques, PEF offers several advantages such as non-thermal behavior, high selectivity, less time, and energy consumption, and does not require any additional chemicals. PEF technology involves the application of series of short high voltage pulses to a biological material (plant, animal or microbial cells) placed between two electrodes. Pulses generally have duration in the range of microsecond to millisecond, and a pulse amplitude that ranges from 100 to 300 V/cm to 20–80 kV/cm depending on the characteristics of the material. PEF treatment causes a phenomenon known as “electroporation”, related to the formation of pores in the cell membrane that facilitates cell's intracellular content release (Figure 16).

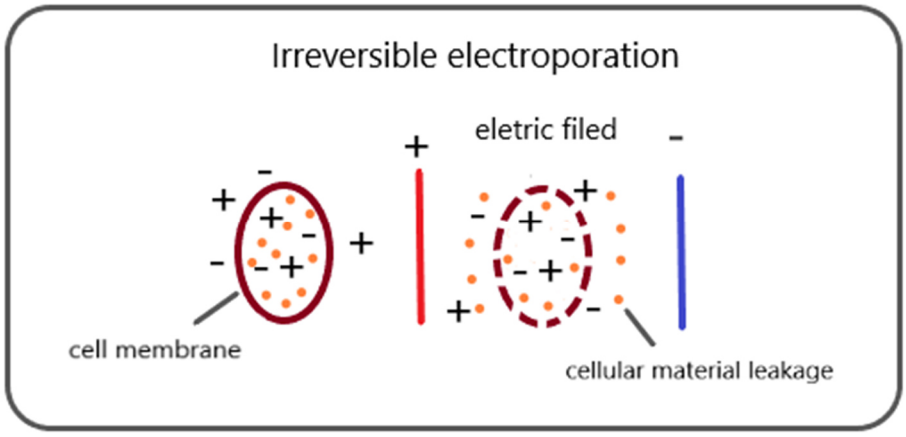

Figure 16. Mechanism of action of used pulsed electric field assisted extraction.

PEF treatment may be a promising method for the isolation and extraction of different components from seafood by-products such as calcium, chondroitin, collagen, chitosan, and protein (He et al., 2017; M. Li et al., 2016; Luo et al., 2010). However, the study of this technology for the extraction of compounds from crustacean by-products has been limited. PEF pretreatment (to extract lipids from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) (electric field strengths in the range 4-16 kV/cm and pulse number in the range 120-240)) in combination with an ultrasound-assisted process (UAE) allowed to maximize

lipid yield (30.34 g/100 g) and to reduce lipid oxidation. Indeed, lipids from PEF-pretreated samples extracted using UAE process had showed an increased content of PUFAs and carotenoids, but peroxide value (PV) and thiobarbituric acid reactive sub-stances (TBARS) were decreased. The authors suggested that the negative effects on lipid quality due to UAE might have been, to some degree, mitigated by PEF pretreatment, however they did not put forward a possible mechanism for this observed phenomenon (Gulzar & Benjakul, 2020).

3.4. Ultrasound-Assisted Extraction (UAE)

Application of ultrasound (US) has proven to be a powerful method in food technology for processing, preservation, and extraction. US offer a significant advantage in productivity, yield and selectivity, reduced processing time, improved quality, reduced presence of chemical and physical hazards, being overall considered environmentally friendly (Chemat et al., 2011). The major effects obtained by the application of US in a liquid medium are related to the cavitation phenomena, compression and decompression of molecules leading to the creation, enlargement and implosion of micro bubbles of gases dissolved in the liquid (Figure 17). The mechanical effects of US promote an increased penetration of solvent into the cellular material, an improved mass transfer thanks to micro-streaming, and the release of cell con-tent due to with the disruption of biological cell walls (Picó, 2013).

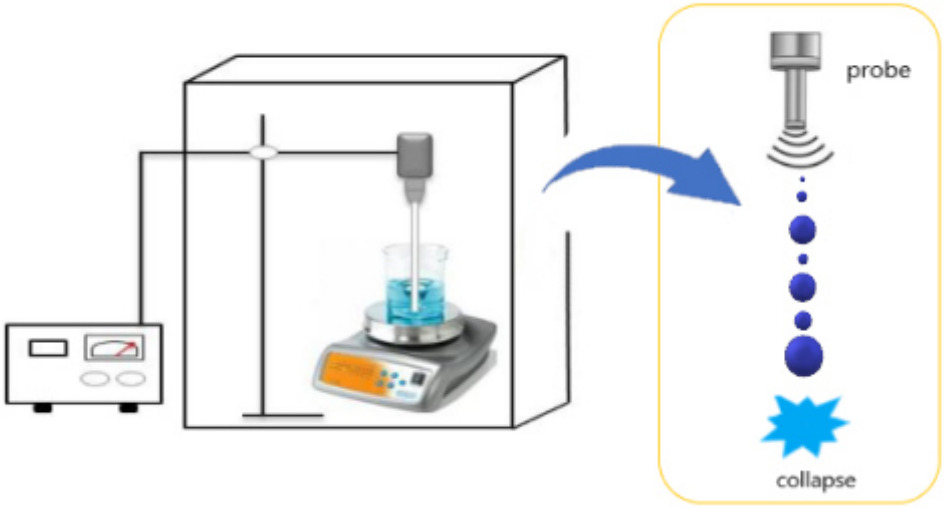

Figure 17. Schematic representation of the ultrasound-assisted extraction (UAE) process and the bubble cavitation phenomenon (modified by Khawli et al. 2019).

In ***paper VI***, several recent studies, that have demonstrated UAE is a powerful method for extracting lipids from crustacean processing by-products, are summarized. UAE increases the extraction yield of lipids and carotenoids; however, in some cases it can lead to degradative processes such as lipid oxidation and hydrolysis that can be explained with the incorporation of oxygen and mechanical effects, and with increased exposure of substrates to enzymes (Gulzar & Benjakul, 2018; Sinthusamran et al., 2018). The addition of an antioxidant combined with UAE is a potential approach to reduce the disadvantages brought along by cavitation, in particular the accelerated oxidation. As observed by Gulzar & Benjakul, (2019), pre-heating along with 0.1% tannic acid addition allowed to reduce lipid oxidation during UAE of Pacific white shrimp. Currently, UAE is widely used for the recovery of chitin and chitosan from crustacean by-products. The ultrasound-assisted deacetylation (USAD) has been reported as an efficient process to produce chitosan. Moreover, some authors confirmed that the sonication of chitosan significantly reduces the molecular weight (MW) of this polymer and has become an alternative method for degrading chitosan into low-molecular-weight chitosan (LMWC), chitosan oligomers and glucosamine (Baxter et al., 2005; Liu et al., 2006; Savitri et al., 2014). Intrinsic viscosity and average MW decreased exponentially with increasing sonication time, which is often desirable for its increase in antimicrobial activity and its use in pharmaceutical and biological applications.

3.5 Accelerate solvent extraction (ASE)

Accelerated solvent extraction (ASE) is an emerging extraction procedure that uses organic solvents at elevated pressures and temperatures above the normal boiling point of solvents, therefore increases the efficiency of extraction of organic compounds from solid and semisolid matrices (Richter et al., 1996). Using ASE, a solid sample is enclosed in a sample cartridge that is filled with an extraction fluid and used to statically extract the sample under elevated temperature (40-200 °C) and pressure (500-3000 psi) conditions for short time periods (5-25 min). Compressed gas is used to purge the sample extract from the cell into a collection vessel. Increased temperature accelerates extraction kinetics and elevated pressure keeps the solvent below its boiling point, allowing for fast, safe, and efficient extraction of target analytes from various matrices. Usually, extraction is completed in 15-25 min, consuming only 15-45 mL of solvent (Mottaleb & Sarker, 2012). Recently, some studies have investigated the application of ASE to obtain aqueous protein extracts with in vitro antioxidant capacity from fish by-products of rainbow trout, sole, sea bass, sea bream and salmon (de la Fuente et al., 2021a; de la Fuente et al., 2021b; Wang et al., 2021). Fang

et al. (2018) found an optimal conditions for extracting oil from tuna liver, with an oil yield of 17.46%. For crustacean by-product, only one scientific publication was found in the literature, where a response surface design was applied to study the effects of temperature (46-114 °C), pressure (43-77 bar) and extraction time (7-24 min) on astaxanthin recovered from shrimp by-products. The result showed that the maximum yield of astaxanthin of 24 mg kg^{-1} from shrimp by-products was achieved with an extraction temperature, pressure, and extraction time of 87 °C, 49 bar, and 14 min, respectively (Quan & Turner, 2009).

New findings

Paper VI reports the effects of the application of pulsed electric field (PEF) and accelerated assisted extraction (ASE) independently and combined to recovery astaxanthin from *M. kerathurus* and *A. antennatus* shrimp by-products, using two organic solvents (dimethyl sulfoxide, DMSO and ethanol, EtOH). The following ASE (50 °C, 15 min,103.4 bars) and PEF (3 kV/cm, 100 kJ/kg, 74 pulses) conditions were applied as extraction processes. The antioxidant capacity of the extracts was evaluated by and Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.

The astaxanthin (ASX) content in extracts derived from shrimp by-products is shown in Figure 18(a) for *M. Kerathurus* and Figure 18(b) for *A. antennatus*. In this study, shrimp by-products showed from 47.32 ± 1.5 µg/g on a dry weight basis (dw) (EtOH) to 85.8 ± 1.9 µg/g dw (DMSO) and from 132.8 ± 1.2 µg/g dw (EtOH) to 169.6 ± 2.6 µg/g dw (DMSO) the content of astaxanthin for *M. Kerathurus* for *A. antennatus* respectively. The content of ASX in crustaceans can vary substantially, due to variations in the amounts of carotenoids available in the feed, environmental conditions, species, and body parts as well as due to the methods and solvent used for extraction (Nakkarike M. Sachindra et al., 2005; Su et al., 2018). Ogawa et al. (2007) reported that the total carotenoid content in the heads of Brazilian shrimp (*Litopenaeus vannamei*) was 47.1µg/g waste dry wt. In Atlantic shrimp (*Pandalus borealis*) by-products, the astaxanthin content was 284.48 µg/g, when extracted with hexane/isopropanol (3:2 (v:v)) (Dave et al., 2020). Takeungwongtrakul et al., (2015) reported that the highest carotenoid content of 378.95 mg/kg was extracted from hepatopancreas of *Litopenaeus vannamei* by using isopropanol:hexane (50:50 (v/v)). ASX content among the two shrimp species was significantly different ($p<0.05$), independent of the extraction process and solvent. *A. antennatus* showed higher astaxanthin content regardless of the method and solvent extraction, ranging from 132.8 ± 1.2 µg/g dw to 585.9 ± 6.9 µg/g dw, while the maximum value for *M. kerathurus* was 213.1 ± 3.4 µg/g dw.

However, considering values from the literature, the content found in both species can be considered high and it indicates the potential of valorizing shrimp processing by-products into high value astaxanthin product. Moreover, the obtained results showed that both the extraction processes and the solvents used had significant effects on the recovery of astaxanthin content.

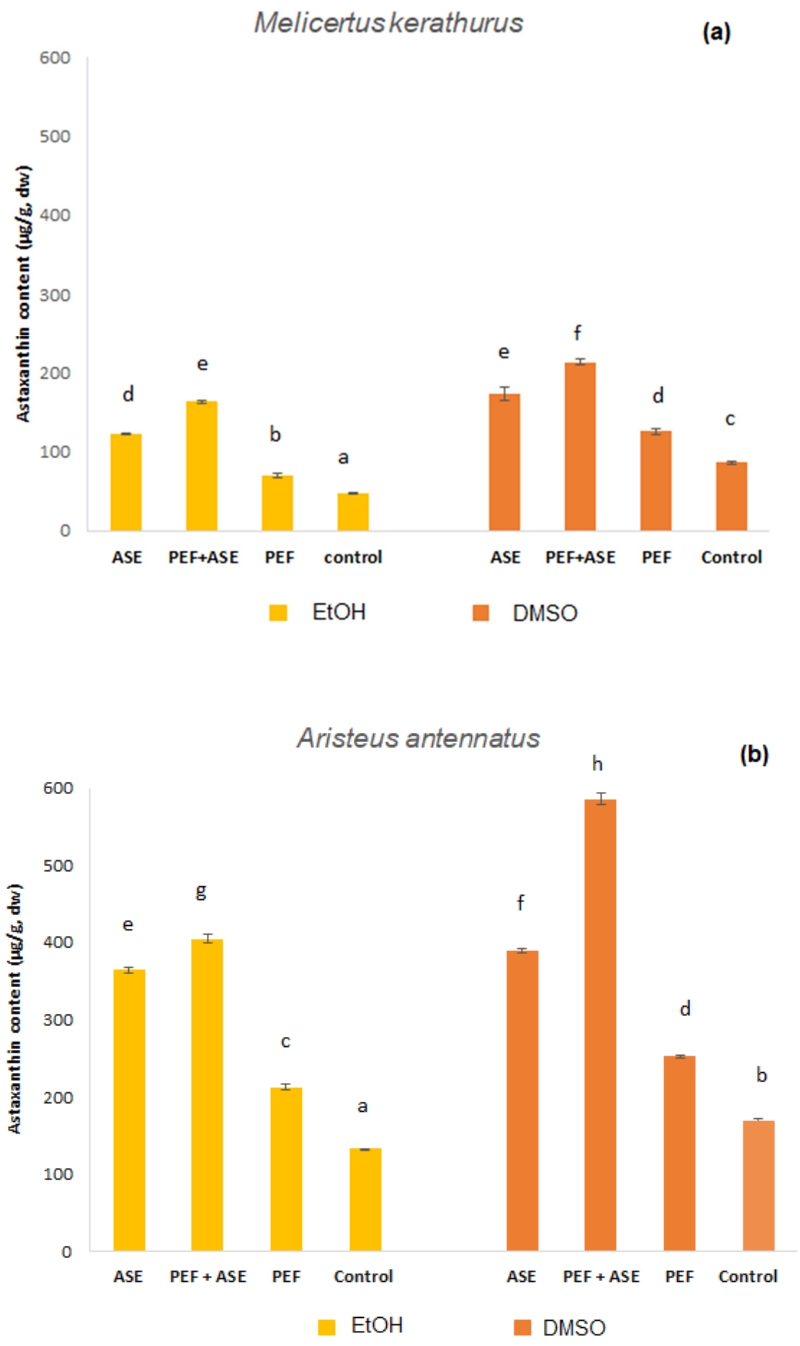

Figure 18. Astaxanthin content in *M. kerathurus* (a) and *A. antennatus* (b) by-products. Different letters above the bars indicate statistically significant differences between treatment averages ($P < 0.05$) (corresponding to figure 2 in ***paper VI***).

PEF treatment followed by solvent extraction resulted in an increase in extracted astaxanthin in the range of 46-47% and 48%-59% (depending on solvent) in *M. kerathurus and A. antennatus*, respectively, compared to the respective control.

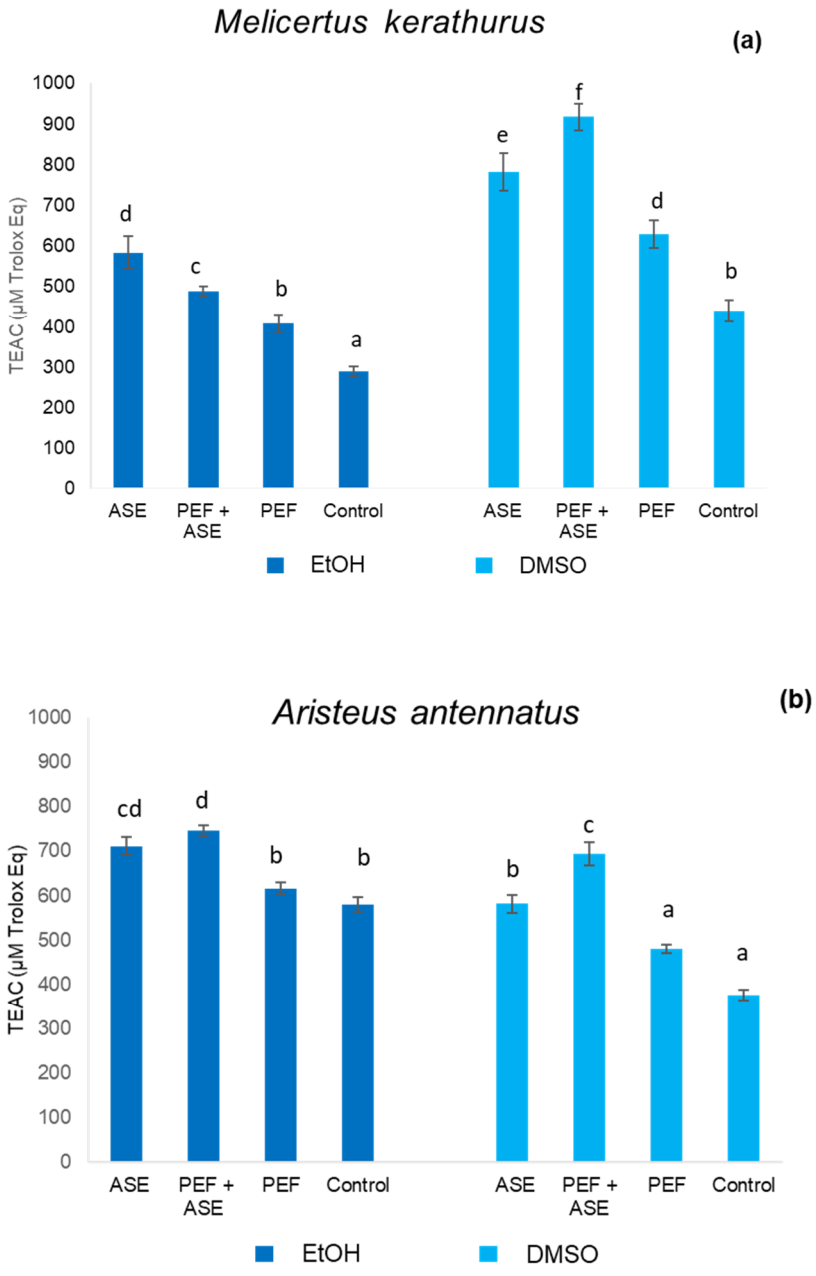

Figure 19. Trolox equivalent antioxidant capacity (TEAC) of the extracts from *M. kerathurus* (a) and *A. antennatus* (b) by-products. Different letters above the bars indicate statistically significant differences between treatment averages ($P < 0.05$) (corresponding to figure 3 in ***paper VI***).

For the shrimp species with the lowest carotenoid content (*M. kerathurus*), the application of ASE showed an increase in the amount of astaxanthin extracted from 2.0 (DMSO) to 2.6 (EtOH) fold compared to control. The extracts from shrimp by-products exhibited notable ABTS+ radical scavenging activity. Anyway, the radical scavenging in shrimp by-products extracts showed significant differences ($p < 0.05$), among the extraction process adopted for each species (Figure 19). For *M. kerathurus*, the TEAC values were in the range of 398.2 ± 11.5 to 915.4 ± 52.4 μM TE/g dw (Figure 19a) and the highest value was found in samples pre-treated with PEF and submitted to ASE using DMSO (PEF + ASE-DMSO). For *A. antennatus*, TEAC values ranged from 475.8 ± 12.4 and 746.0 ± 12.5 μM TE/g dw, reaching the maximum value for samples pre-treated with PEF and extracted by ASE using EtOH (PEF + ASE-EtOH). ASE and PEF processes have significantly increased the TEAC values ($p < 0.05$) for *M. kerathurus* independently of the solvent used, while for *A. antenntus* PEF processes had no significant effect ($p > 0.05$) compared with control.

The ORAC values varied from 5567 ± 424 μM TE/g dw to 22600 ± 306 μM TE/g dw. The highest ORAC value was observed in the extracts of *M. keranthurus* extracted by ASE and ASE + PEF-EtOH. On the same line, for *A. antennatus* extracts, the highest ORAC value (19130 ± 459 μM TE/g) was for the extraction processes ASE + PEF-EtOH. The higher ORAC value of the *A. antennatus* extracts found in samples extracted by ASE + PEF-EtOH was in accordance with the higher ABTS+ scavenging activity. In particular, it was observed that *M. kerathurus* extracts showed a higher ORAC value than *A. antennatus* extracts although this species showed significantly lower ($p<0.05$) astaxanthin values than *A. antennatus*. Shrimp by-products contains astaxanthin and its esters as the major pigments (Sachindra et al., 2006); their antioxidant activity is well documented. However, shrimp by-products extract contains other antioxidants, such as phenolics, in addition to carotenoids (Seymour et al., 1996). Moreover, crustaceans are rich of several other lipophilic antioxidants, such as tocopherol and ubiquinol (Passi et al., 2002). The presence of other antioxidants at the same time also affects the antioxidant potential of the extracts, as antioxidants are known to have synergistic action (Milde et al., 2007; Shixian et al., 2005). In this study, it is possible that components other than carotenoids have influenced the radical-scavenging activity of the extracts. Moreover, TEAC and ORAC are based on different antioxidant activity mechanism. Despite both assays using Trolox as a reference antioxidant and expressing results based on Trolox equivalents, results obtained for TEAC and ORAC can lead to different conclusions, in agreement with the data reported in the

literature (Zuluaga et al., 2017). Therefore, to better understand the obtained results, a more comprehensive characterization of the extract should be carried out.

From results obtained in this **study**, it is possible to conclude that the application of accelerated solvent extraction (ASE) and pulsed electric fields (PEF), used separately or in combination, increased the ASX content in the extracts for both shrimp species despite the solvent used. Moreover, these technologies seem to be an effective tool to recover extracts with strong antioxidant activity from shrimp by-products. Both techniques are eco-friendly and safe and can increase the extraction astaxanthin content reducing the processing time. However, these techniques are poorly developed and tailored for shrimp by-products application, lacking in standardization at industrial scale. These promising results should be confirmed by extending the investigation to other valuable compounds derived from seafood by-products.

IV. Conclusion

Based on the overall results obtained in the present PhD research work, it can be concluded:

- Modified atmosphere packaging (MAP) using a novel gas argon increased sardine shelf-life, showing an inhibitory effect on bacterial spoilage and on the development of hypoxanthine, and a slight decrease of fat oxidation in lipid hydrolysis during storage. The promising results obtained with argon should be confirmed by extending the investigation to other fish species. Considering that argon is at present more expensive compared to nitrogen, before a possible utilization at industrial level, a careful consideration of benefits *vs.* costs should be carried out.
- The application of HPP treatment on different types of seafood products, intended for raw consumption, highlighted a significant microbiological shelf-life increase at the highest applied pressure levels for different seafood species, while the degree of fat oxidation during storage remained rather low. However, in terms of visual quality, the final effect for all samples was a general whitening and the occurrence of a cooked appearance, that is typical for muscle food subjected to pressurization.
- The combined approach of the use of mechanical separation and freezing on mantis shrimp pulp helped to develop a frozen product that can be characterized by a high added value that aims to enhance the value of this fish product, keeping in mind the problems related to its preservation bound to its seasonality.
- Innovative food processing technologies based on non-thermal concepts have the potential to be applied for the extraction of several bio compounds from crustacean by-products. These techniques are eco-friendly and safe and can increase the extraction yield reducing the processing time. However, many of these techniques are poorly developed and tailored for crustacean by-products application, lacking in standardization at industrial scale. Moreover, crustacean by-products are very different and complex; considering these aspects, it is essential to define the appropriate extraction technology that allow minimizing processing and maximizing the quality for the target compounds.
- The application of accelerated solvent extraction (ASE) and pulsed electric fields (PEF), used separately or in combination, have shown good results in the extraction of ASX from shrimp by-products with EtOH and DMSO. Both techniques are eco-friendly and safe and seem to be an effective tool to recover rich astaxanthin extracts

with strong antioxidant activity from shrimp by-products. However, these promising results should be confirmed by extending the investigation to other valuable compounds derived from seafood by-products.

The overall results of this doctoral study highlighted the great advantages in the application of emerging technologies for both seafood products and crustacean by-product valorization. New strategies have been investigated to obtain safe seafood products with good shelf-life and high quality. Among these, non-thermal technologies have the potential to increase sustainability, overall value, and expand the variety of processed seafood products in the market with a positive impact on the fishing and aquaculture industry. However, considering the variety of seafood species, further research, scientific and industrial efforts are needed to define the appropriate process for seafood products and the extraction technology to valorized other important seafood species.

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Paper I

The impact of gas mixtures of Argon and Nitrous oxide (N2O) on quality parameters of sardine (Sardina pilchardus) fillets during refrigerated storage.

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The impact of gas mixtures of Argon and Nitrous oxide (N_2O) on quality parameters of sardine (*Sardina pilchardus*) fillets during refrigerated storage

Ana Cristina De Aguiar Saldanha Pinheiro[a], Eleonora Urbinati[a], Silvia Tappi[b,*], Gianfranco Picone[a], Francesca Patrignani[a,b], Rosalba Lanciotti[a,b], Santina Romani[a,b], Pietro Rocculi[a,b]

[a] Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Campus of Food Science, Via Ravennate 933, Cesena, (FC), Italy

[b] Department of Agricultural and Food Science, Alma Mater Studiorum, University of Bologna, Campus of Food Science, Piazza Goidanich 60, Cesena, (FC), Italy

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ABSTRACT

The effect of modified atmosphere packaging (MAP) with unconventional gas mixtures on the main qualitative parameters of sardine fillets during refrigerated storage was investigated. Four different atmospheres conditions were tested: air; 30% CO_2 + 70% N_2; 30% CO_2 + 70% N_2O and 30% CO_2 + 70% Ar. All samples were packaged in polypropylene trays sealed with a high barrier film and stored at 2–4 °C for 12 days. The quality and the freshness of sardine fillets packed in MAP were evaluated by microbiological, physical and chemical analyses after 0, 1, 2, 5, 6, 8 and 12 days of the storage period. The 2-thiobarbituric acid-reactive substances (TBARS) values for MAP samples were lower compared to air samples, reaching a final value of 1.09 mg malonaldehyde (MA)/kg and 3.39 mg MA/kg, respectively. The samples packed in Ar reached the fixed threshold for total mesophilic and psychrotrophic bacteria after 12 days of storage, resulting the best MAP condition adopted, able to increase the sardine shelf-life of 3 days with respect to the other tested conditions. Air packed samples showed significantly higher ($p < 0.05$) Hx content (50 mg/kg) compared to the rest of the MAP samples (20 mg/kg). At the end of the storage period, the sample packed in Ar showed a significantly lower value ($p < 0.05$) (around 40 mg/kg), than the other MAP conditions.

1. Introduction

The consumption and popularity of seafood has consistently increased during recent years since they are increasingly recognized as important sources of nutrients for human health (Alasalvar, 2002). Among them, fresh fish is one of the most highly perishable food products due to endogenous enzymes promoting proteolysis of muscle proteins and connective tissue, fat hydrolysis followed by muscle spoilage caused by metabolic activities of microorganisms (Wu et al., 2014). These phenomena lead to a short shelf life in fish and other seafood products, evidencing the need for improved preservation methods that allow extension of shelf life.

The definition of quality related to seafood is quite complex. Beside the safety aspects, the overall quality perceived by consumers is strictly related to its flavor and other sensory parameters based on its chemical composition. Hence, the determination of shelf-life of fish products has to take into account all of these aspects.

Proton nuclear magnetic resonance (^{1}H NMR) has emerged as a powerful technique to evaluate quality changes of fish products in relation to different harvesting and post-harvesting procedures (Ciampa, Picone, Laghi, Nikzad, & Capozzi, 2012; Picone et al., 2011; Savorani et al., 2010). By evaluating the whole metabolic profile of a fish product, it is possible to identify various components related to the quality, such as free aminoacids, nucleotide degradation components and biogenic amines proving high potential for assessing evolution of fish quality during storage (Ciampa et al., 2012).

Modified atmosphere packaging (MAP), along with refrigeration, has become increasingly popular preservation techniques, which have brought major changes in storage, distribution, and marketing of raw and processed products to meet consumer demands. MAP extends shelf-life of most fishery products by inhibiting bacterial growth and oxidative reactions. The MAP effectiveness in extending fish product quality/shelf-life depends on species, fat content, initial microbial load, gas mixture, the gas/product ratio and the storage temperature (Sivertsvik, Jeksrud, & Rosnes, 2002). A wide range of atmospheres has been examined for use with fish. However, the reported increases in shelf-life

* Corresponding author.
E-mail address: silvia.tappi2@unibo.it (S. Tappi).

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for fish and fish products vary markedly and are small in comparison with those reported for several other products (Alasalvar, 2002).

Generally, MAP for fish products is obtained by partial or total removal of oxygen (O_2) and increased carbon dioxide (CO_2) concentration (Erkan, Özden, Alakavuk, Yildirim, & İnuğur, 2006; Özogul, Polat, & Özogul, 2004; Stamatis & Arkoudelos, 2007), that allowed to inhibit lipid oxidation and microbial growth. Beside the traditional gases used for MAP (O_2, CO_2 and N_2), alternative gases such as Argon (Ar) and nitrous oxide (N_2O) have been approved for food use in the European Union (EU).

Hence, MAP system is a physical approach to increase fresh fish shelf-life and has already proved to work, however, its potential is not fully known, in particular in relation to the aforementioned gases as alternative to nitrogen. Indeed, while N is completely inert and is used for the sole purpose of filling, Ar and N_2O have shown some biological effects.

The effects of Ar, that is a chemically inert gas are attributed to its physical properties, in particular its enhanced solubility in water compared to nitrogen and the ability to interfere with enzymatic oxygen receptor sites (Rocculi, Romani, & Dalla Rosa, 2005; Spencer & Humphreys, 2003). According to Spencer and Humphreys (2003), the use of Ar allowed to improve the overall acceptability of packaged meat products through the reduction of CO_2 levels in addition to the benefits of reduced O_2 level.

To our knowledge, Ar has been used for MAP of fish products only by Choubert, Brisbarre, Parfouru, and Baccaunaud (2008) on rainbow trout fillets and by Randell, Hattula, and Ahvenainen (1997) on rainbow trout and herring fillets. However, these authors observed contrasting results regarding the increase of shelf-life related to this gas.

Nitrous oxide (N_2O) has been found to have effect on inhibition of respiration and senescence in higher plants showing high potentiality for the packaging of fresh-cut fruits (Rocculi et al., 2005; Rocculi, Romani, & Dalla Rosa, 2004).

Argon and N_2O are known to sensitize microorganisms to other antimicrobial agents (Qadir & Hashinaga, 2001; Thom & Marquis, 1984). However, to our knowledge, N_2O has never been tested for the packaging of fish products.

However, despite the potentialities shown by these two gases, their main disadvantage is related to the cost that is significantly higher than that of nitrogen (about 4–5 times), that makes very relevant to evaluate the whether the qualitative benefits promoted by the use of argon are economically sustainable.

Italian fishery sector is one of the most important and competitive industry of the Mediterranean area (Crescimanno, Galati, & Bal, 2014). Sardines constitute 14.6% (25,729 tons) of the total Italian seafood production, respectively (INEA, 2015). For these reasons, the implementation of innovative processes for the transformation and preservation of sardines might represents an important economic opportunity for several manufacturing companies located along the Mediterranean basin (Alfonzo et al., 2017).

The aim of the present research was to evaluate the effect of MAP with unconventional gas mixtures (Ar and N_2O) on the main qualitative parameters of sardine fillets. During refrigerated storage. Packed sardines were stored at 4 °C for 12 days and were evaluated for pH, moisture and colour, lipid oxidation indices, microbiological quality and presence of pathogens and for the evolution of freshness indicators.

2. Material and methods

2.1. Chemicals

All reagents used for microbiological analysis were purchased from Oxoid (Cambridge, UK), all the other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

2.2. Fish samples

Fresh Sardines (*Sardina pilchardus*) were caught in the Adriatic Sea (Cesenatico, Italy) in November 2017. The length of the fillets was in the rage of 8.5 ± 0.5 cm and the average weight was 10 ± 1 g. After fishing, the fish were placed in polystyrene boxes and distributed uniformly between the layers of ice and stored in for 12 h before mechanical filleting. The sardine fillets (about 35 kg) were placed in boxes of polystyrene covered with ice and transported immediately to the laboratory where they were packed.

2.3. Modified atmosphere packaging (MAP)

The samples were packed in polypropylene trays sealed with a high barrier film. The realization of the protective atmospheres was performed using a gas quaternary mixer (KM100-4, Witt-Gasetechnik, Witten, Germany) connected to compressed gas cylinders and a gas-flushing welding machine (Multiple 315, Orved Srl, Venezia, Italy). Four different atmospheres conditions were tested: Air (20.8% O_2 + 79.2% N_2), N_2 (30% CO_2 + 70% N_2), N_2O (30% CO_2 + 70% N_2O) and Ar (30% CO_2 + 70% Ar). For each tray, the average weight of fillets placed was 250 g. For each MA tested a total of 24 packages/sample were prepared and stored in a cold room at 2–4 °C for 12 days. The samples were subjected to microbiological, physical and chemical analyses on the 0, 1, 2, 5, 6, 8, 12 days of the storage period. At each sampling time 2 trays from each gas condition were randomly taken and subjected to analysis.

2.4. Headspace gas analysis, pH and drip loss

The headspace gas composition (% v/v CO_2/O_2) was analyzed with a gas analyzer (check point O_2/CO_2 mod. MFA III S/L – Witt-Gasetechnik, Witten, Germany), the percentages in the headspace of 4 packages for each sample were measured. The pH values were measured using a pH meter (Crison, Barcellona) after homogenization of each 5 g fish muscle sample in 10 mL distilled water. Drip loss was measured as the weight loss during the storage compared with the initial weight of the sample. The results were expressed as per cent of fillet initial weight.

2.5. Thiobarbituric acid reactive substances (TBARS)

The 2-thiobarbituric acid-reactive substances (TBARS) analysis were performed in the fish fillets according to Bao and Ertbjerg (2015) to evaluate the oxidation changes during storage at 2–4 °C. An aliquot of 5.0 g of each sample was homogenized with 15 mL trichloroacetic acid (5% w/v) and 0.5 mL butylated hydroxytoluene (4.2% in ethanol, w/v) in ice bath. The homogenization was done by IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) at 13.000 rpm for 30 s. The slurry was filtered with filter paper (Whatman 42, GE Healthcare), and an aliquot of 2 mL filtrate was mixed with 2 mL thiobarbituric acid (0.02 M) in a test tube and boiled in a water bath (100 °C) for 40 min. After cooling, absorbance was read at 532 nm (UV-1800 spectrophotometer; Shimadzu, Kyoto, Japan). A standard curve of 1,1,3,3-tetraethoxypropane was used to calculate the amount of malondialdehyde produced. TBARS content was expressed as mg of malonaldehyde (MA)/kg of fillet. The determination was carried out in triplicate for each sample and sampling time.

2.6. Microbiological analyses

For each sampling time, microbiological analyses were performed in triplicate, on sardine samples packed in four different atmospheres sampling 10 g of product from three packages for sample. Total mesophilic and psychrotrophic bacteria were determined on Plate Count Agar, incubating the plates at 28 °C for 48 h and 10 °C for 7 days,

respectively. *Enterobacteriaceae* were determined on Violet Red Bile Glucose agar and plates were incubated at 37 °C for 24 h. For *Pseudomonas spp* the Pseudomonas Agar Base medium has been used, with the addition of selective supplements. The plates were incubated at 25 °C for 24 h. Furthermore, also Total and fecal coliforms were counted on VRBA medium, incubating the plates at 37 and 44 °C, respectively, for 24 h. The medium was also added of MUG supplement to highlight the presence of E. coli.

For the evaluation of the presence of L. *monocytognes* and *Salmonella* spp. in 25 g of sardine homogenates, the method of the Intl. Organisation for Standardisation (ISO 11290 and 6579, 1993) was followed.

Qualitative *Vibrio* spp. and *Aeromonas hydrophila* analyses were carried out on 25 g of sample according to the method described by Vernocchi, Maffei, Lanciotti, Suzzi, and Gardini (2007). *Clostridia* were enumerated on Reinforced Clostridial Agar Base, with addition of selective supplements and the plates were incubated at 37 °C for 24 h under anaerobic conditions.

2.7. Freshness indicators

2.7.1. Sample preparation

For each sample, 4 g of samples were homogenized whit 8 mL of 7% perchloric acid. The acid mixtures, transferred into 2 mL centrifuge tubes, were neutralized to pH 7.8 using 9 M KOH and then centrifuged at 14 k rpm for 10 min at 4 °C in order to remove potassium perchlorate precipitate. 720 µL of supernatant was aliquoted and placed in Eppendorf microfuge tube adding 80 µL of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM and then centrifuged at 14 k rpm for 10 min at 4 °C. 800 µL of the centrifuged sample were placed in a standard 5 mm NMR tube and measurement were performed. The determination was carried out in triplicate for each sample and sampling time.

2.7.2. Proton nuclear magnetic resonance (^{1}HR-NMR) measurements

All ^{1}H NMR spectra were recorded at 300 K on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). For each sample 256 scans were collected into 32 K data points covering a 12 ppm spectral width and requiring 32 min of measurement time. A recycle delay of 5 s and a 90° pulse of 11.4 µs were set up. Acquisition time (2.27 s) and recycle delay was adjusted to be 5 times longer than the T1 of the protons under investigation, which has been considered to be no longer than 1.4 s. Saturation of the residual water signal was achieved by irradiating it during the recycle delay at δ equal to 4.703 ppm. The phase correction and baseline adjusted with TOPSPIN software version 3.0 (Bruker Biospin) and successively the data in ASCII format were exported in R (R Project, 3.4.1). The spectra were calibrated taking the chemical shift of trimethylsilyl propionate (TSP) signal and integrals of the areas of the different diagnostic areas were calculated.

Concentration of trimethylamine-N (TMA-N), trimethylamine-O (TMA-O) and Hypoxanthine (Hx) were determined as indicators of fish freshness. Also, other ATP related components (adenosine-5′-diphosphate-ADP, adenosine-5′-monophosphate-AMP, inosine-Ino, inosine-monophosphate-IMP, and hypoxanthine-Hx) were quantified according by Picone et al. (2011) and their contents used for the determination of K-index (Eq. 1) and H-index (Eq. 2) as reported by Karube, Matsuoka, Suzuki, Watanabe, and Toyama (1984) and Luong, Male, Masson, and Nguyen (1992), as follow:

$$K\ index\ (\%) = (Ino + Hx)/(ATP + ADP + IMP + AMP + Ino + Hx) * 100 \quad (1)$$

$$H\ index\ (\%) = (Hx)/(IMP + Ino + Hx) * 100 \quad (2)$$

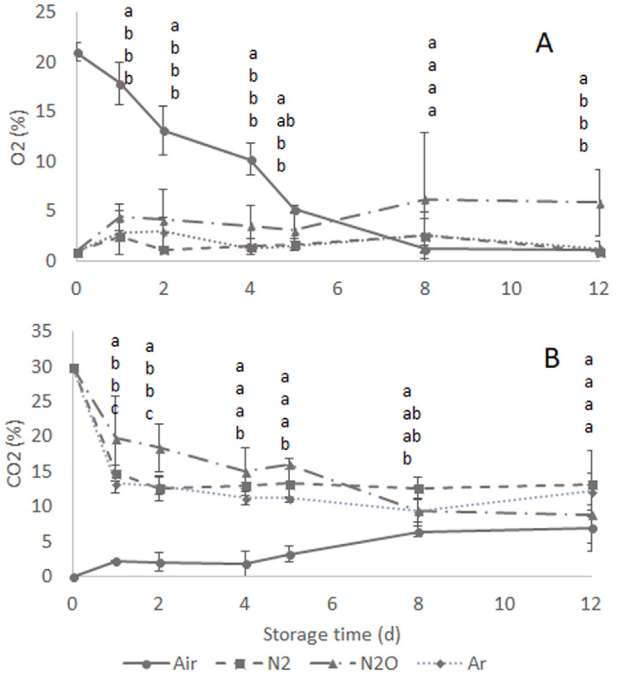

Fig. 1. Concentration (%) of O_2 (A) and CO_2 (B) in the packages headspace of sardine fillets in MAP during storage at 2–4 °C.

2.8. Statistical analysis

For all evaluated parameters, Kolmogorov-Smirnov test were compiled to determine whether the data were distributed normally and Levene's test was used to test for homogeneity of variance. Significant differences (p-level < 0.05) between the means at different storage times were explored by means of the analysis of variance (ANOVA with post-hoc Tukey HSD); Kruskal-Wallis test was used if significant differences emerged by the Levene test.

3. Results and discussion

3.1. Headspace gas analysis, pH and drip loss

The gas composition in MAP and air packed samples was monitored through the storage period. The results obtained from oxygen (O_2) and carbon dioxide (CO_2) analysis can be seen in Fig. 1A and B. In sample packed in air, O_2 concentration decreased and CO_2 increased rapidly until the 8th day when they reached values respectively around 1% and 6.5% and then remained fairly similar until the end of the storage. The modification of the head space composition can probably be attributed to the development of aerobic microorganism.

In sample packaged with modified atmosphere, the total elimination of oxygen was not obtained, and a residual 1% content was measured in all samples after packaging. The CO_2 concentration on MAP samples decreased rapidly in the first day from the initial value of 30% or all samples to reach a fairly steady state between 10 and 15% until the end of the storage. Probably, the reason of the diminution of CO_2 concentrations throughout time is because of the solubilization of this compound in the liquid muscle fraction until equilibrium is attained (Sivertsvik, Jeksrud, Vågane, & Rosnes, 2004). The O_2 concentration on MAP samples showed a slight increased during storage, probably because of the dissolution of CO_2, but remained below 3% in N_2 and Ar samples. In the N_2O sample, instead, it reached concentration of 6.2%, probably due to the partial contemporary dissolution of N_2O, which exhibits solubility values similar to CO_2 (Spilimbergo, Matthews, & Cinquemani, 2011).

A slight tendency to increase in pH values were observed for all

Table 1
Evolution of drip loss (%) during storage of sardine fillets in MAP.

Samples	Storage time (days)						
	0	1	2	5	6	8	12
Air	–	2.96 ± 0.50[a]	2.75 ± 0.66 [a]	3.20 ± 1.72[b]	1.83 ± 0.33[c]	3.00 ± 1.22[b]	5.64 ± 0.88[b]
N_2	–	2.32 ± 0.52[a]	1.79 ± 0.80 [a]	3.00 ± 0.39[b]	3.01 ± 0.42[b]	1.97 ± 1.46[b]	3.42 ± 1.31[c]
N_2O	–	3.02 ± 0.71[a]	3.89 ± 1.22 [a]	8.19 ± 3.46 [a]	10.13 ± 2.86 [a]	8.21 ± 1.39 [a]	10.03 ± 2.38 [a]
Argon	–	2.13 ± 1.12[a]	2.3 ± 0.94 [a]	2.10 ± 0.04[b]	2.44 ± 0.94[b]	2.45 ± 1.37[b]	3.34 ± 0.65[c]

Different letters indicate significant differences among samples at the same storage time ($p < 0.05$).

samples during the period of storage. Actually the initial pH values of sardine samples were 6.24 and a slight pH increment (pH = 6.52) was observed after 12 days of the storage, in agreement with Erkan et al. (2006) for the same species and for Provincial et al. (2010) for sea bass (*Dicentrarchus labrax*) fillets stored at 4 °C. However, the changes were not significantly different (data not reported) nor during storage, neither among samples. Similarly, Arashisar, Hisar, Kaya, and Yanik (2004) did not find significant differences between packing atmosphere in air (control), vacuum and modified atmosphere packaging (MAP) with various gas mixtures conditions at 4 °C.

The changes in drip loss of sardine fillets during storage are shown in Table 1. Drip loss from the samples increased with storage time for all samples. However, starting from day 5 the N_2O samples presented a significantly higher drip loss compared with N_2, Ar and Air samples, with the maximum drip loss value 10.0% at 6th day of storage. A possible reason for a relatively high drip loss in N_2O samples was due to the creation of a partial vacuum inside the package (vacuum effect) following the solubilization of both CO_2 and N_2O in the sample, as detected from a visual examination. After 12 days of storage, drip loss was less pronounced in N_2 and Ar samples than other samples with a value around 4%, which is in accordance with the values reported at the end of shelf life of fresh MAP cod.

3.2. Thiobarbituric acid reactive substances (TBARS)

The highly unsaturated lipids of fish are easily susceptible to oxidation, resulting in a rancid smell and taste as well as alterations in texture, colour and nutritional value (Ocaño-Higuera et al., 2011). TBARS is a good indicator of the quality of the fish and is widely used to determine the secondary oxidative products for interpreting the lipid oxidation degree (Özogul et al., 2011). It has been suggested that a maximum TBARs value indicating the good quality of the fish stored with ice is 5 mg malonaldehyde/kg, while the fish may be consumed up to a level of 8 mg MA/kg in TBARS value (Ocaño-Higuera et al., 2011). The TBARS values in the sardine fillets stored under different MAP conditions are shown in Fig. 2.

In the present study, TBARS values of fresh fillets were about 0.33 mg MA/kg in agreement with those mentioned by some authors for the same species (Méndez et al., 2017; Nunes, Batista, & De Campos, 1992). The lipid oxidation, measured as TBARS values for all samples increased with time of storage. However, after the 5th day, for MAP samples the TBARS values were significantly lower ($p < 0.05$) compared to Air sample and remained quite low throughout the entire period of storage.

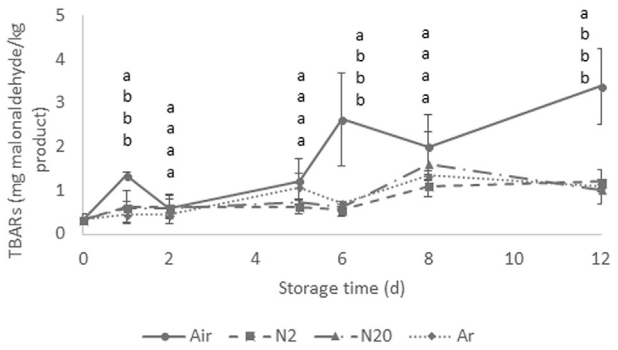

Fig. 2. TBARs values of the sardine fillets in MAP during storage at 2–4 °C.

Similar results have been observed by several authors (Caglak, Cakli, & Kilinc, 2014; Hassoun & Karoui, 2016; Messina, Bono, Renda, La Barbera, & Santulli, 2015). At the end of the storage period, the samples reached the maximum values of 3.39 mg MA/kg and 1.09 mg MA/kg for Air and MAP samples respectively, which is far below the reported critical values. Similar results were reported for fillets of dolphinfish and salmon preserved in MAP with addition of natural antioxidants. The low TBARS values were attributed to the antioxidants' addition and the absence of oxygen in the gas mixture (Giménez, Roncalés, & Beltrán, 2005; Messina et al., 2015).

In the present research, after 12 days of storage at 2–4 °C, the TBARS values observed in MAP samples were far lower to those found by Erkan et al. (2006) for the same species packed in two different modified atmospheres ($5\%O_2/35\%CO_2/60\%N_2$ and $5\%0_2/70\%CO_2/25\%N_2$) at 5 days of storage at 4 °C. Significant differences ($P < 0.05$) were not found between the MAP samples during storage. However, concerning TBARS values, the three different gas mixtures (containing N_2, N_20 and Ar) have shown to effectively minimize the lipid oxidation in the sardine fillets.

3.3. Microbiological analysis

In Table 2, the cell loads of the principal spoilage microbial groups detected in the samples in relation to the adopted packaging atmosphere and sampling time are reported. Since the Commission Regulation 2073/2005 and subsequent modifications fixed the shelf-life threshold for fish-based products and their preparations at 6 log CFU/g for total mesophilic bacteria, this cell load value was considered in this study to determine the microbial shelf-life of the considered samples. The initial cell loads of total viable mesophilic psychrotrophic bacteria recorded in the sardine samples was around 4 log CFU/g confirming the data found by Stamatis and Arkoudelos (2007) on the same type of products. As evidenced by the Table 2, the sardines were characterized by a good microbiological quality since total and fecal coliforms and *Enterobacteriaceae* were under the detection limit while the *Pseudomonas* spp. cell loads were of about 2 log CFU/g. This microbial group with *Shewanella* spp. can be considered as the dominating microbiota of fresh marine fish stored under refrigerated aerobic conditions (Kuuliala et al., 2018).

The evolution of the different microbial groups was affected by the adopted MAP conditions. Mesophilic and psycrotrophic bacteria were constantly higher in samples packed in air, compared to the MAP samples until the end of storage and reached the threshold value between the 5th and the 8th day. Although N2 and N2O samples showed significantly lower ($p < 0.05$) values for mesophilic bacteria, the threshold was reached contemporaneously to the air packed samples, while for psycotrophic microorganism, they reached spoilage only at the last day. The samples packaged in argon reached the fixed threshold for total mesophilic and psychrotrophic bacteria after 12 days of

Table 2
Cell loads (log CFU/g) of different microbial groups detected in sardine products in relation to the packaging atmosphere.

Sample	Storage time				
	0	2	5	8	12
	TMB*				
Air	4.0 ± 0.1^{a}	4.6 ± 0.3^{a}	5.0 ± 0.2^{a}	6.8 ± 0.2^{a}	7.2 ± 0.1^{a}
N2	4.1 ± 0.2^{a}	2.9 ± 0.2^{b}	2.9 ± 0.2^{b}	6.1 ± 0.2^{b}	6.5 ± 0.3^{b}
N2O	4.0 ± 0.1^{a}	2.9 ± 0.2^{b}	2.8 ± 0.2^{b}	6.2 ± 0.2^{b}	5.7 ± 0.1^{c}
Ar	4.0 ± 0.1^{a}	3.1 ± 0.3^{b}	3.1 ± 0.1^{b}	4.6 ± 0.3^{c}	5.9 ± 0.2^{c}
	TPB**				
Air	4.1 ± 0.2 ^{a}	4.2 ± 0.1 ^{a}	5.5 ± 0.3^{a}	6.1 ± 0.1^{a}	7.3 ± 0.2^{a}
N2	4.1 ± 0.2 ^{a}	2.7 ± 0.1^{b}	2.9 ± 0.1^{b}	5.7 ± 0.2^{b}	7.0 ± 0.2^{a}
N2O	4.1 ± 0.2 ^{a}	3.1 ± 0.1^{c}	3.3 ± 0.4^{b}	5.3 ± 0.4^{b}	6.4 ± 0.2^{b}
Ar	4.1 ± 0.2 ^{a}	3.1 ± 0.1^{c}	3.2 ± 0.1^{b}	5.2 ± 0.4^{b}	6.3 ± 0.2^{b}
	Pseudomonas spp				
Air	2.2 ± 0.1^{a}	3.1 ± 0.3^{a}	4.5 ± 0.2^{a}	7.1 ± 0.1^{a}	5.1 ± 0.3^{a}
N2	2.2 ± 0.1^{a}	2.7 ± 0.2^{a,b}	2.8 ± 0.2^{b}	5.7 ± 0.3^{b}	4.7 ± 0.3^{a,b}
N2O	2.2 ± 0.1^{a}	2.5 ± 0.2^{b}	2.6 ± 0.3^{b}	5.6 ± 0.2^{b}	4.3 ± 0.2^{b}
Ar	2.2 ± 0.1^{a}	2.5 ± 0.2^{b}	2.6 ± 0.2^{b}	5.6 ± 0.1^{b}	4.3 ± 0.3^{b}
	Enterobacteriaceae				
Air	–***	–	1.7 ± 0.2^{a}	2.2 ± 0.2^{a}	2.3 ± 0.2^{a}
N2	–	–	1.0 ± 0.1^{b}	2.2 ± 0.3^{a}	2.3 ± 0.1^{a}
N2O	–	–	–	2.6 ± 0.4^{a}	2.0 ± 0.3^{a}
Ar	–	–	1.0 ± 0.0^{b}	2.1 ± 0.0^{a}	2.3 ± 0.1^{a}
	Total coliforms				
Air	–	–	2.2 ± 0.2^{a}	2.4 ± 0.3^{a}	2.9 ± 0.1^{a}
N2	–	–	1.0 ± 0.1^{b}	2.3 ± 0.1^{a}	2.5 ± 0.1^{b}
N2O	–	–	1.2 ± 0.1^{b}	2.3 ± 0.1^{a}	2.4 ± 0.1^{b}
Ar	–	–	1.1 ± 0.1^{b}	2.2 ± 0.1^{a}	2.3 ± 0.3^{b}
	Fecal coliforms				
Air	–	–	–	.	1.1 ± 0.1^{a}
N2	–	–	–	–	1.1 ± 0.1^{a}
N2O	–	–	–	1.1 ± 0.2	1.1 ± 0.1^{a}
Ar	–	–	–	–	–

Different letters indicate significant differences among samples at the same storage time for each microbial group ($p < 0.05$).
* Total mesophilic bacteria.
** Total psychrotrophic bacteria.
*** Under the detection limit.

storage, resulting the best MAP condition adopted able to increase the sardine shelf-life of 3 days with respect to the other tested conditions.

Pseudomonas spp. cell loads increased particularly in the samples stored in air conditions that showed always statistically higher ($p < 0.05$) values compare to the other MAP conditions, reaching the level of 7.1 log CFU/g after 8 days of storage. The growth potential of this spoilage microbial group was decreased when the samples were stored in presence of the other MAP conditions, and particularly in argon when after 8 days of storage Pseudomonas spp. cell load was of 4.7 log CFU/g. However, although a decrease was observed in the first days of storage, no one of the adopted MAP conditions were able to completely inhibit the Pseudomonas spp. growth. On the other hand, according to Parlapani, Haroutounian, Nychas, and Boziaris (2015), *Pseudomonas spp.*, was dominating also in fish packed in MAP characterized by CO_2 (60%) and O_2 (10%). In the present research, after 8 days of storage at 2–4 °C, although the level of *Pseudomonas* spp. and psychrotrophic bacteria was under 6 log CFU/g in samples packed in N_2 and N_2O, their total mesophilic bacteria cell loads exceeded the fixed threshold suggesting a shift in the microbiota spoilage population for these preparations. These results are in agreement with those of Kuuliala et al. (2018) who found for raw Atlantic cod stored under MAP, that Lactic Acid Bacteria (LAB) and B. thermosphacta were co-dominating.

At the end of the shelf-life, the product stored in argon was also characterized by the lowest level of total coliforms (2 log CFU/g) while fecal ones were lower than 1 log CFU/g. All the pathogenic species investigated were never detected in any of the considered samples (data not showed).

3.4. Freshness indicators

Fig. 3 shows an example of the NMR spectrum acquired from a sardine extract at pH = 7.8 and the identified compounds.

3.4.1. Reduction of TMAO and formation of TMA

Trimethylamine-N-oxide (TMAO-N) is a substance which is present in almost all marine fish, and in some freshwater fish, but in different amount depending on species, age, fish size, time of year, and environmental factors. Seawater fish have 1–100 mg TMAO-N in every 100 g muscular tissue (Hebard, Flick, & Martin, 1982). Trimethylamine nitrogen (TMA-N) results from the reduction of TMAO-N by bacterial activity and partly by intrinsic enzymes and is often used as an index of spoilage of marine fish. TMA-N is considered a valuable tool in the evaluation of the quality of refrigerated fish, as it has been often related to the pungent odour and to the load of spoilage microorganism in many species of spoiled fish. Hence, it is possible to use this parameter not only to assess overall quality of sardine, but also differentiate between fish of good and moderate freshness (Erkan et al., 2006). Fig. 4A and B show the TMAO-N and TMA-N concentrations in the sardine fillets during the storage period at 4 °C. The initial TMAO-N values were 374.8 mg/kg; as expected, the content decreased in all samples during the storage period 43.7 mg/kg at the end of the storage (12 days). The

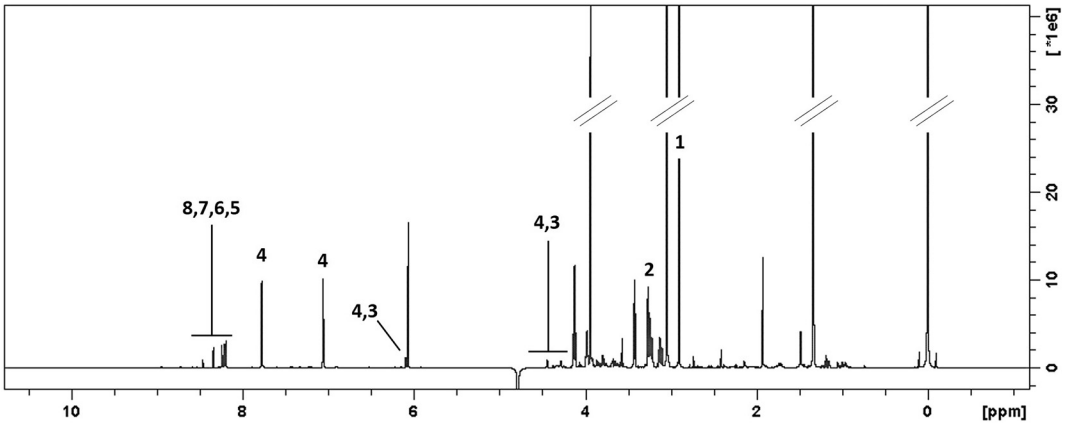

Fig. 3. 600.13 MHz ^{1}H NMR spectrum of sardine T12 extract at pH 7.80. Signals involved in the freshness evaluation have been indicated. 1) Trimethylamine-TMA (s: 2.901 ppm); 2) Trimethylamine N-oxide-TMAO (s: 3.231 ppm); 4) Inosine (dd: 4.283 ppm, dd: 4.440 ppm, d: 6.092, s: 8.233, s: 8.333); 4) AMP (dd: 4.371 ppm, dd: 4.516 ppm, d: 6.146 ppm, s: 8.233 ppm, s: 8.578 ppm); 5) Hypoxanthine (s: 8.192, s: 8.208), 6) ATP (s: 8.509 ppm); 7) ADP (s: 5.519 ppm); 8) IMP (s: 8.549 ppm). Abbreviations for multiplicities are: s = singlet, dd = doublet of doublets, d = doublet, t = triplet and m = multiplet (denotes complex pattern).

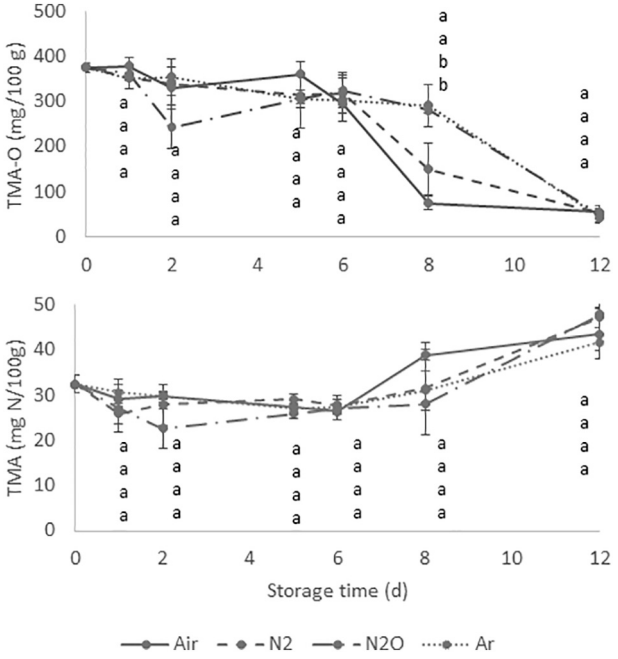

Fig. 4. Changes in TMAO-N (A) and TMA-N (B) concentrations of the sardine fillets in MAP during storage at 2–4 °C.

greatest reduction of TMAO-N values was observed for the air samples among the 6th and 8th day, which showed at the 8th of storage a value by 74.6 mg/kg, significantly lower ($p < 0.05$) than the MAP samples. This fact could be correlated with the high load of *Pseudomonas* spp. in air samples at 8th of storage. However, at the end of storage no significant differences were observed between the samples.

At the beginning of the storage period, no significant changes in TMA-N content was observed for all samples. The initial mean TMA-N content of sardine muscle was 32.4 ± 2.0 mg N/kg, slightly higher compared to the value observed by Erkan et al. (2006). For all samples, TMA-N content remained fairly stable until the 6th day, then a slight increase was observed for the Air sample. Samples packaged with the different MAP conditions showed an increase after the 8th day. At the end of the storage period of 12 days, the values were similar in all the samples, between 41 and 47 mg N/kg with no significant differences. In general, the upper limit for TMA-N before consumer rejection of fish is usually 50 to 100 mg/kg (Ocaño-Higuera et al., 2011). Although the microbial load of all samples reached the threshold for spoilage, TMA-N levels of all samples were below the acceptability level for the whole storage period considered.

The TMA-N reducing capacity strongly depends on the microbial species. Besides, according to Erkan et al. (2006) in different fatty fish and shellfish, the concentration of TMA-N never reaches the value of 50 mg/kg.

3.4.2. Nucleotide degradation

The amount of ATP-degradation products is considered as a good indicator of fish freshness (Shumilina, Ciampa, Capozzi, Rustad, & Dikiy, 2015). In the early post mortem stages, the nucleotides produced by the ATP decomposition, such as ADP and AMP, Ino and IMP, as well as Hx, are considered useful indicators of fish freshness (Mendes, Quinta, & Nunes, 2001) and their amount is combined to define the K-value parameter (Ocaño-Higuera et al., 2011).

Hx accumulation in fish tissue reflects the initial phase of autolytic deterioration as well as subsequent contribution through bacterial spoilage and has been related to loss of freshness and taste (Ocaño-Higuera et al., 2011). Changes in the Hx content of sardines stored under different modified atmosphere packaging conditions at 2–4 °C are shown in Fig. 5.

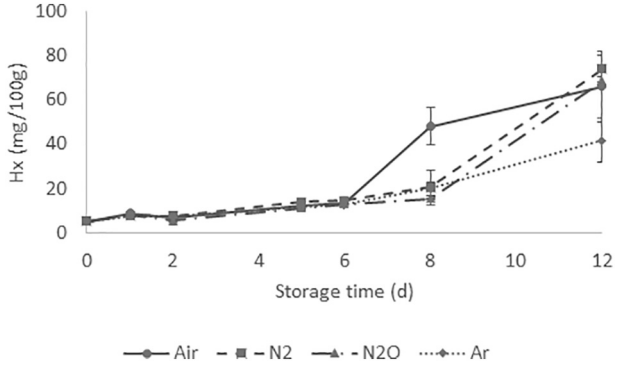

Fig. 5. Changes in Hx content of sardine fillets in MAP during storage at 2–4 °C.

In this study, Hx concentration increased with storage period, as reported for sardines (Özoğul, Özoğul, & Kuley, 2007) and for other species (Alasalvar et al., 2001; Özogul, Taylor, Quantick, & Özogul, 2000). At day 8, air packed samples showed significantly higher ($p < 0.05$) Hx content (5.00 mg/100 g) compared to the rest of the MAP samples (20.0 mg/kg). At the end of the storage period of 12 days, while Air, N_2 and N_2O samples were characterized by similar values (around 60–70 mg/kg), the sample packed in Ar gas mixture showed a significant lower value ($p < 0.05$) (around 40 mg/kg). This result is in agreement with the lower microbial load found in Ar samples at the end of the storage.

Freshness or spoilage indicators related to the breakdown of nucleotides are based on the autolysis of ATP in the muscle. Indeed, while ATP, ADP and AMP generally remain fairly constant, the increase of K is mainly due to the degradation of IMP to Ino and Hx that reflects a loss of desirable compounds characteristic of fresh fish (Alasalvar et al., 2001). The initial increase of K is at first due to enzymatic degradation and only later to microbial action (Sikorski, Kolakowska, & Burt, 1990).

The changes in the K value of the sardine fillets were quite similar in samples stored in air and in MAP samples (Fig. 6A). After an initial value of about 12%, a constant increase was observed throughout the

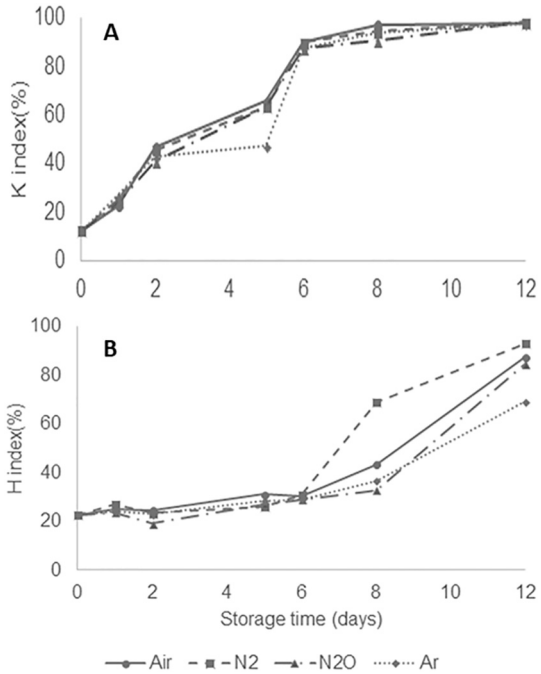

Fig. 6. Changes in k index (A) and H index (B) of sardine fillets in MAP during storage at 2–4 °C.

storage time, reaching values close to 90% by the 12th day. These results are similar to those previously reported for sardines stored in air by Özogul et al. (2004). However, while these authors found a decrease in samples stored in MAP, in the present study, no effect of the atmosphere composition on the K value was observed. Also López-Gálvez, De La Hoz, Blanco, and Ordonez (1998) found no effect of the atmosphere on the K value of sole fillets stored aerobically in ice or CO_2-enriched atmospheres despite differences observed in the microbial load. Thus, the changes in the K-index may not always be representative of microbial activity.

The initial level of H-index was about 22% and increased fairly slowly and in a similar way up to 29% in all samples until the 6th day (Fig. 6B). In the second part of the storage, a sharp increase was observed in all samples, in particular for N_2 at the 8th day and at the 12th day for the other samples. However, Ar sample showed a lower value compared to other, confirming the trend observed for Hx formation.

Results were fairly similar to the ones observed by Özogul et al. (2004) in sardine fillets stored in different packaging conditions. Their MAP sample (that correspond to the present N_2 sample) showed a higher increase of H index in the last part of the storage that was attributed to a rapid decrease of inosine.

4. Conclusions

The effect of the use of innovative gases for modified atmosphere packaging of sardine fillets was studied for the first time.

The removal of oxygen from the packages confirmed to reduce the lipid oxidation in a similar way in all samples. While the use of N_2O instead of N_2 did not show any advantage in terms of the investigated parameters, the use of Ar allowed to increase sardine shelf-life up to 12 days, showing an inhibitory effect on bacterial spoilage and on the development of hypoxanthine during storage.

The promising results obtained with Ar should be confirmed by extending the investigation to other fish species.

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Paper II

Effects of novel modified atmosphere packaging on lipid quality and stability of sardine (Sardina pilchardus) fillets.

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Original article

Effects of novel modified atmosphere packaging on lipid quality and stability of sardine (*Sardina pilchardus*) fillets

Ana Cristina de Aguiar Saldanha Pinheiro,[1] Silvia Tappi,[1,2*] Francesca Patrignani,[1,2] Rosalba Lanciotti,[1,2] Santina Romani[1,2] & Pietro Rocculi[1,2]

1 Department of Agricultural and Food Science, Alma Mater Studiorum, University of Bologna, Campus of Food Science, Piazza Goidanich 60, Cesena, FC, Italy

2 Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Via Quinto Bucci 336, Cesena, FC, Italy

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Summary Modified atmosphere packaging (MAP) is an efficient method to increase shelf-life of fishery products by inhibiting bacterial growth and oxidative reactions. Beside the traditional gases used for MAP, novel gases such as argon (Ar) and nitrous oxide (N_2O) were approved for food use in the European Union. The present research investigates the effect of MAP with unconventional gas mixtures, that previously positively affected microbial shelf-life, on colour, lipid oxidation and sensorial characteristics of sardine fillets during storage. Four atmosphere conditions were tested: Air (20.8% O_2/79.2% N_2), N_2 (30% CO_2/70% N_2), N_2O (30% CO_2/70% N_2O) and Ar (30% CO_2/70% Ar). Samples were stored for 12 days at 3 °C. Results showed that the removal of oxygen significantly inhibited the oxidation process; however, most of the investigated parameters related to fat oxidation did not show any improvement, except for a slight decrease in lipid hydrolysis and improvement in sensory properties in the packaging containing Ar.

Keywords Argon, modified atmosphere packaging, nitrous oxide, oxidation, sardine.

Introduction

Seafood products play an important role in a nutritionally balanced diet; however, fresh products are highly perishable due to endogenous enzymes and metabolic activities of microorganisms (Sivertsvik *et al.*, 2002). One of the major deteriorative processes that occurs during the storage of fish and which has a major influence on quality is lipid oxidation. Lipid oxidation may affect both odour and flavour of fish and, in severe cases, its nutritional value (Pacetti *et al.*, 2015). Fish and shellfish are excellent sources of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5v3) and docosahexaenoic acid (DHA, 22:6v3). PUFAs are known to be markedly susceptible to peroxidation and to be readily incorporated into the mechanism of lipid peroxidation to yield free radicals and lipid peroxy radicals. Therefore, their protection against oxidation by use of novel packaging technologies as well as storage conditions is essential.

Modified atmosphere packaging (MAP) can extend the shelf-life of most fishery products by inhibiting bacterial growth and oxidative reactions. The achievable extension of shelf-life depends on species, fat content, initial microbial population, gas mixture, the ratio of gas volume to product volume and storage temperature (Sivertsvik *et al.*, 2002). Traditional MAP uses different concentrations of O_2, CO_2 and N_2, generally removing or reducing oxygen and increasing the concentration of CO_2 (Erkan *et al.*, 2006), while N_2 is used as filler gas. In fish products, the aim is to inhibit lipid oxidation and microbial growth.

However, since Directive 95/2/CE and further amendments the EU has also approved argon (Ar) and nitrous oxide (N_2O) for food use. They are generally used to replace N_2, an inert gas, in packaging, hence as fillers. However, both Ar and N_2O were observed to increase the sensitivity of different microorganisms to anti-microbial compounds (Qadir & Hashinaga, 2001). Moreover, some reports indicate that Ar is able to interfere with receptor sites of enzymatic oxygen (Spencer & Humphreys, 2003; Rocculi *et al.*, 2005). Randell *et al.* (1977) and Choubert *et al.* (2008) found contrasting results regarding the increase of shelf-life due to the use of Ar in rainbow trout and herring fillets packaging.

*Correspondent: E-mail: silvia.tappi2@unibo.it

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On the other side, positive effects of N_2O have been reported to mainly for fruit packaging, on account of its ability to partially inhibit respiration and senescence (Rocculi *et al*., 2005).

Sardine (*Sardina pilchardus*) is an important Mediterranean commercial fish species; its high fat content makes it very susceptible to oxidation. In a previous study of our group, we investigated the effect of Ar and N_2O in MAP of sardine fillets on microbiological quality and freshness indicators during storage. Results indicated that Ar allowed to inhibit bacterial spoilage and freshness decay, increasing the shelf-life of the product (Pinheiro *et al*., 2019).

The present research is focused on the evaluation of the effect of MAP with unconventional gas mixtures (Ar and N_2O) on the colour and different lipid oxidation indicators of sardine fillets during refrigerated storage.

Materials and methods

Fish samples

Sardines (*S. pilchardus*), fished in the Adriatic Sea (Cesenatico, Italy), were mechanically filleted (8.5 $\pm$ 0.5 cm average length and 10 $\pm$ 1 g average weight). A total of 35 kg were packed in different MAP conditions.

Modified atmosphere packaging

Two hundred fifty gram of fillets was placed in polypropylene trays and sealed with a PET/PP high barrier film (permeability to O_2 < 123.2 cm^3 m^{-2} day bar and water vapour transmission rate, WVTR < 4.8 g m^{-2} day) with a volume ratio product:-gas of 1:1. A gas quaternary mixer mod. KM100-4 (Witt-100 Gasetechnik, Witten, Germany) combined to a gas-flushing welding machine mod. Multiple 315 (Orved Srl, Venezia, Italy) was used to obtain different combinations of selected gases: Air (20.8% O_2 + 79.2% N_2), N_2 (30% CO_2 + 70% N_2), N_2O (30% CO_2 + 70% N_2O) and Ar (30% CO_2 + 70% Ar). For each atmosphere, 24 packages were prepared and stored at 3 $\pm$ 1 °C for 12 days. For the analytical determinations, four trays for each MAP condition were collected after 0, 1, 2, 5, 6, 8, 12 days of storage. Sampling times were chosen to be representative of the shelf-life of the fillets according to previous experiments.

Physico-chemical analyses

pH values were assessed in samples homogenised with distilled water (1:2 sample:water ratio) with a pH meter (Crison, Barcellona, Spain).

Colour parameters lightness (L^*), redness (a^*) and yellowness (b^*) were measured with a spectrophotocolorimeter mod. ColorFlex™ (Hunterlab, Reston, VA, USA). The tristimulus L^*, a^*, b^* measurement mode (CIE, 1987) was used. The hue angle (H°) was calculated as follow:

$$H = \arctan\frac{b^*}{a^*}$$

For each sample and storage time, the average of at least 15 measurements was calculated.

Lipid oxidation indexes

Total Lipid extraction

Lipids were extracted with a method previously described by Bligh & Dyer (1959) according to the modification of Vernocchi *et al*. (2007).

Peroxide value (PV), conjugated diene (CD) and trienes (CT) measurements were used for the determination of primary lipid oxidation. Thiobarbituric acid-reactive substances (TBARS) was used to determine the secondary oxidative products and lipid hydrolysis was measured by free fatty acid (FFA) contents. Moreover, fatty acid composition was determined by gas chromatography (GC).

Peroxide value

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

Conjugated diene and triene hydroperoxides

Conjugated diene and CT were measured according to the method of Abdalla & Roozen (1999). Results were expressed in terms of absorbance (232 and 268 nm for dienes and trienes, respectively) relative to 50 mg of fat. The measurement was performed in duplicate for each extract.

Thiobarbituric acid-reactive substances

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

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

Fatty acid composition

Lipid purification and methyl ester synthesis. The lipid fractions were obtained using an aminopropyl bonded sorbent columns (SPE-NH2) ISOLUTE (Biotage, Milan, Italy). Columns were equilibrated with 9 mL of hexene and loaded with 20 mg of total lipid extract. FFAs fractions were recovered using 2% formic ethyl conveyed in diethyl ether. After purification samples were dried at room temperature under nitrogen flux and the methyl ester synthesis was carried out using diazomethane. Fatty acid methyl esters were dried under nitrogen flux, resuspended into hexane and stored at −40 °C until the GC-MS analysis.

GC-Mass spectrometry analysis and fatty acid identification. GC-MS analysis was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionisation voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm i.d., 1.2 μm df) was used (Chrompack, Middelburg, The Netherlands). The temperature program was 50 °C for 2 min, then programmed at 1 °C min^{-1} to 65 °C and finally at 5 °C min^{-1} to 220 °C, which was maintained for 22 min. Injector, interface and ion source temperatures were 250, 250 and 230 °C, respectively. Injections were performed in triplicate with a split ratio of 1:30 and helium (1 mL min^{-1}) as the carrier gas.

Free fatty acids methyl esters samples were prepared by adding 100 ppm of undecanoic acid methyl ester as internal standard. Injections were performed in triplicate. FFAs were identified using the NIST library. The amount of each FFA (mg kg^{-1}) was determined by the comparison of each peak area with the peak area of the internal standard (C11:0). The results are means of three independent experiments.

Sensory evaluation

Sensory evaluation was carried out according to the quality index method (QIM) described in detail by Stamatis & Arkoudelos (2007) for sardine fillets. The attributes examined were as follows: (i) the development of slime on the surface of the fillet; (ii) muscle incision and firmness; (iii) odour; and (iv) overall appearance of the fish fillet. Each assessment was carried out by a minimum of six trained panellists with a long-term training and experience in fish evaluation. Four categories were ranked: highest quality or excellent (E), good quality (A), fair quality (B) and unacceptable quality (C).

Statistical analysis

Significant differences (P-level < 0.05) between means at different storage times were explored by the analysis of variance (ANOVA with *post hoc* Tukey HSD); Kruskal–Wallis test was used if significant differences emerged by the Levene's test.

Results and discussion

Physico-chemical parameters

The initial pH values of sardine samples were 6.24 and, after 12 days of storage, a slight increase up to 6.52 was observed for all samples. Similar results were

Table 1 Changes in the colour parameters of sardine fillets under different MAP conditions at 3 °C

	Storage time (days)						
Samples	**0**	**1**	**2**	**5**	**6**	**8**	**12**
L^*							
Air	46.56 ± 1.5	45.52 ± 0.5^{a}	43.71 ± 2.8^{a}	43.68 ± 2.6^{a}	45.07 ± 2.7^{a}	43.94 ± 2.6^{a}	45.55 ± 2.4^{a}
N_2	–	45.47 ± 1.8^{a}	43.98 ± 3.1^{a}	43.15 ± 1.74^{a}	43.43 ± 2.2^{a}	42.03 ± 2.6^{a}	44.33 ± 1.6^{a}
N_2O	–	42.85 ± 1.3^{b}	43.63 ± 2.9^{a}	41.91 ± 1.95^{a}	41.25 ± 2.5^{a}	41.95 ± 2.8^{a}	42.66 ± 2.7^{b}
Argon	–	43.78 ± 1.2^{ab}	45.30 ± 2.0^{a}	42.61 ± 1.92^{a}	43.50 ± 2.1^{a}	42.66 ± 2.4^{a}	43.70 ± 1.6^{ab}
a^*							
Air	1.22 ± 0.9	1.96 ± 2.1^{a}	3.07 ± 1.8^{a}	2.35 ± 1.1^{b}	4.41 ± 1.8^{a}	4.36 ± 2.7^{b}	3.89 ± 2.5^{b}
N_2	–	0.99 ± 1.5^{a}	3.52 ± 1.6^{a}	5.05 ± 2.4^{a}	5.71 ± 1.3^{a}	6.53 ± 2.3^{a}	5.94 ± 2.6^{a}
N_2O	–	1.45 ± 1.0^{a}	4.36 ± 2.2^{a}	4.40 ± 2.8^{a}	5.89 ± 1.6^{a}	6.64 ± 1.4^{a}	4.96 ± 2.9^{ab}
Argon	–	2.05 ± 1.4^{a}	2.94 ± 2.3^{a}	4.55 ± 2.6^{a}	5.89 ± 1.3^{a}	6.24 ± 1.5^{a}	6.26 ± 2.6^{a}
H°							
Air	80.21 ± 11.2	79.02 ± 19.7^{a}	70.29 ± 13.4^{a}	75.68 ± 12.3^{a}	62.91 ± 11.2^{a}	65.88 ± 13.8^{a}	54.45 ± 13.3^{b}
N_2	–	83.89 ± 11.6^{a}	66.59 ± 13.6^{a}	59.84 ± 11.6^{b}	54.12 ± 5.0^{b}	51.48 ± 9.1^{b}	56.20 ± 11.1^{a}
N_2O	–	77.95 ± 8.4^{a}	63.91 ± 10.7^{a}	62.99 ± 14.6^{b}	52.68 ± 6.1^{b}	50.54 ± 5.6^{b}	54.19 ± 14.2^{a}
Argon	–	74.25 ± 10.4^{a}	71.22 ± 14.4^{a}	62.37 ± 13.2^{b}	54.18 ± 5.2^{b}	52.56 ± 6.4^{b}	56.73 ± 10.9^{a}

Different superscript letters in the same column indicate significant differences (P < 0.05).

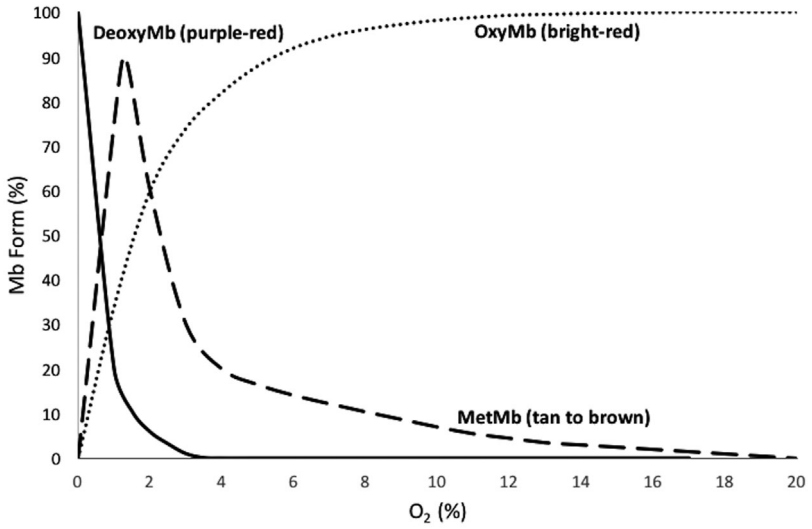

Figure 1 Relationship of oxygen concentration on myoglobin chemical state (modified from Thippareddi & Phebus, 2002).

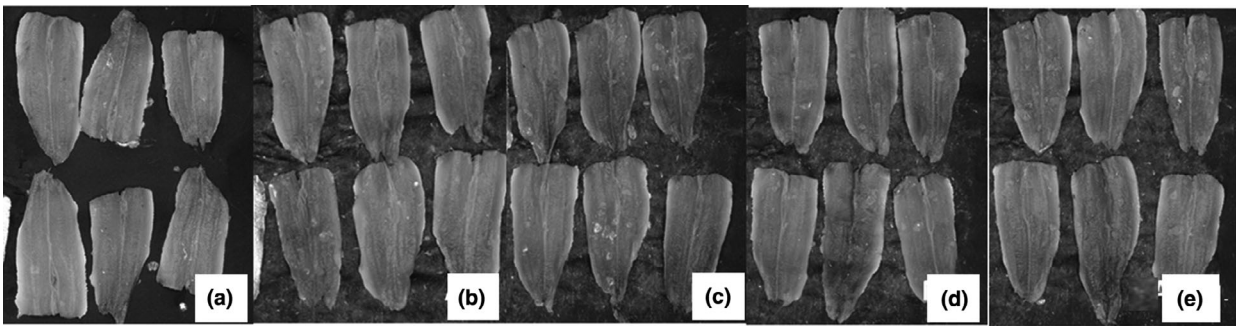

Figure 2 Images of fresh sample (a) and of samples air (b), N_2 (c), N_2O (d) and Ar (e) after 12 days of storage at 3 ± 1 °C.

previously reported for sardine fillets (Erkan *et al.*, 2006). However, the observed changes were not statistically significant (data not reported).

Changes in the L^*, a^* and H° colour parameters of sardine fillets packed in air and MAP during storage are shown in Table 1. During storage, L^* (lightness) showed a slight decrease in all samples, but at the end, sardine fillets packaged in N_2O showed a significantly lower L^* value compared to the N_2 and air samples ($P < 0.05$). On the contrary, a^* (redness) values increased in all samples but after the fifth day until the end of storage, in fillets packed in N_2 and Ar, it was significantly higher compared to samples in air.

The hue angle (H°) decreased during storage showing differences among the tested atmospheres. From day 5 until day 8, hue values in Air samples were significantly higher than the MAP samples. However, at the end of the storage, no significant differences were observed among the different MAP samples ($P > 0.05$).

As shown by Thippareddi & Phebus (2002), the concentration of oxygen in the package determines the oxidative state of the myoglobin in fresh meats (Fig. 1). From a macroscopic point of view, this phenomenon is evident considering the images of sardine fillets acquired in conditions of standardised illumination reported in Fig. 2. After 12 days of storage, while sample packed in air (b) evidenced a yellow/brown colour, the N_2, N_2O and Ar samples (c, d, e) evidenced an evident purple-red colour component, even more evident also respect to the fresh sample (a). The yellow-brown tint and the inhomogeneous distribution of colour in the surface of the sample packed in air is indicating an advanced state of oxidation. On the contrary, the absence of oxygen or an oxygen concentration very close to 0% promotes deoxymyoglobin

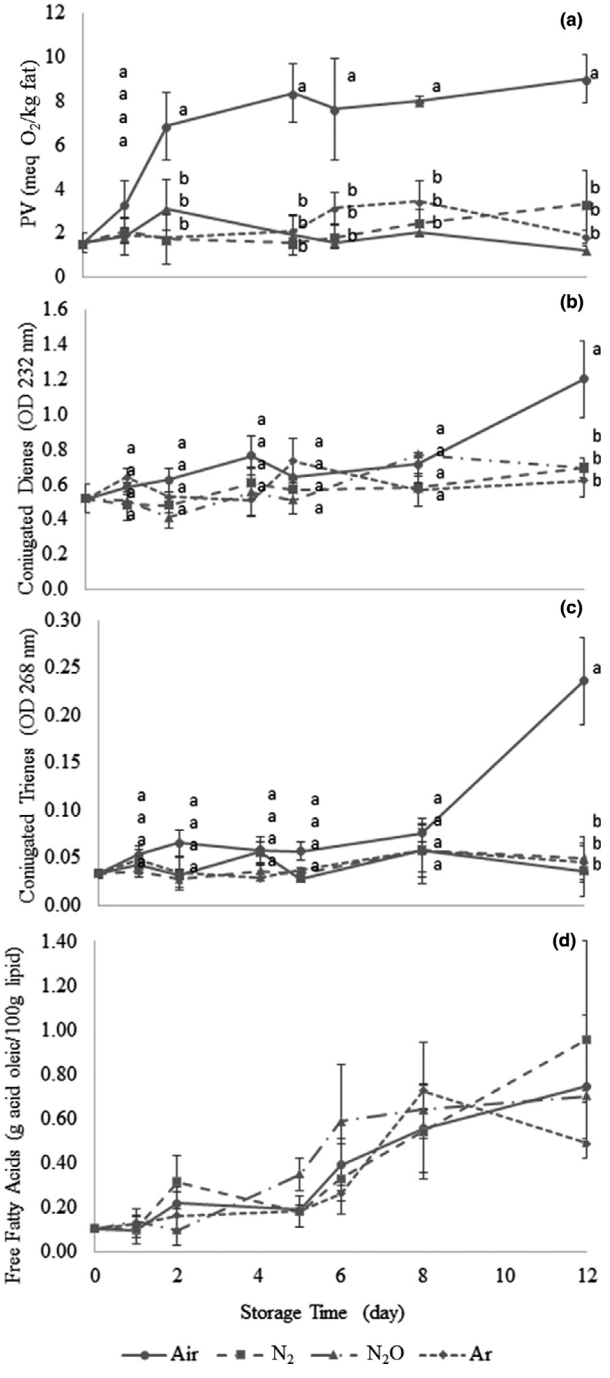

Figure 3 Peroxide values (a), conjugated dienes (b) and conjugated trienes (c) and free fatty acids (d) content measured in sardine fillets in MAP during storage at 3 °C. Different letters indicate significant differences among samples at the same storage time ($P < 0.05$).

(DMb) development, that is probably the reason for the increase of the redness observed in fillets packed in N_2, N_2O and Ar.

Lipid oxidation indexes

Primary lipid oxidation was evaluated by PV. Evolution in the PV during storage is shown in Fig. 3a. At the beginning, the value was rather low (1.56 meq O_2 kg^{-1} fat), indicating a good oxidative quality of the raw material. In the sample packed in air, the increase in PV began just on day one, following the typical trend of peroxide formation. At the end of the 12 days, PV was significantly greater (8.34 meq O_2 kg^{-1} fat) in Air samples compared to the MAP samples, in which PV remained almost constant during storage with values that never exceeded 4 meq O_2 kg^{-1} fat.

The low peroxide values in the MAP samples are probably due to the low concentration of this gas in the headspace of the packages. According to the classification reported by Pinheiro *et al.* (2019), samples in air could be considered 'spoiled' (PV = 8–10) at the end of the storage, while the other samples were still 'good' (PV = 2–5).

Figure 3b,c shows the absorbance at 232 and 268 nm of the conjugable oxidation products, dienes and trienes, respectively. For all samples, no difference in CD and CT of sardine fillets was observed within the first 8 days of storage ($P > 0.05$). Similarly, Chaijan *et al.* (2006) reported that in the sardine muscle (*Sardinella gibbosa*), no difference in CD was observed within the first 12 days of iced storage.

After day 8, the CD and CT values in the Air samples increased significantly ($P < 0.05$) compared to the MAP samples that instead remained almost constant. At the end of storage, the CD and CT values were highest in the Air samples compared with the MAP samples.

Conjugated diene and CT are oxidation indexes for commonly used vegetable oils and for olive oils EU regulation (EC 796/2002) sets specific threshold values. However, for different oils and fats UV absorbance values should be evaluated individually due to their own chemical and physical characteristics. The CD and CT values in all MAP samples remained very low and did not increase with the storage time, indicating a protective effect of MAP on lipid oxidation.

According to Pinheiro *et al.* (2019), fish quality can be considered good until TBARS levels are below 5 mg malonaldehyde kg^{-1}, while the threshold for consumption is 8 mg malonaldehyde kg^{-1}.

Thiobarbituric acid reactive substances values measured during storage of packed sardine fillets are reported in Table 2. Fresh samples showed values of about 0.33 mg MA kg^{-1}, similarly to values reported by other authors (Méndez *et al.*, 2017), that increased during storage in all samples. However, for MAP samples, values were far lower compared to sample packed in Air and were maintained low until the end of the

Table 2 Changes in the TBARS values (mg malondialdehyde kg^{-1}) of sardine fillets under different MAP conditions at 4 °C

	Storage time (days)						
Samples	**0**	**1**	**2**	**5**	**6**	**8**	**12**
Air	0.33 ± 0.0	1.34 ± 0.1[a]	0.58 ± 0.2[a]	1.21 ± 0.5[a]	2.62 ± 1.1[a]	2.00 ± 0.7[a]	3.39 ± 0.9[a]
N_2	–	0.61 ± 0.4[b]	0.61 ± 0.3[a]	0.62 ± 0.2[a]	0.55 ± 0.1[b]	1.11 ± 0.3[a]	1.19 ± 0.1[b]
N_2O	–	0.59 ± 0.1[b]	0.58 ± 0.3[a]	0.73 ± 0.1[a]	0.63 ± 0.1[b]	1.61 ± 0.7[a]	1.01 ± 0.1[b]
Argon	–	0.44 ± 0.2[b]	0.45 ± 0.0[a]	1.08 ± 0.3[a]	0.71 ± 0.0[b]	1.34 ± 0.1[a]	1.09 ± 0.4[b]

Different letters in the same column indicate significant differences ($P < 0.05$).

storage. These results are in agreement with previous researches (Caglak *et al*., 2014; Messina *et al*., 2015). After 12 days, a value of 3.39 mg MA kg^{-1} was recorded for the Air sample, while in MAP samples, the higher value was 1.09 mg MA kg^{-1}. Both values are lower than the reported critical values.

Moreover, values are also lower compared to the ones reported by Erkan *et al*. (2006) for sardine fillets after 5 days of storage in two different MAP conditions. This difference may be explained by the fact that in the mentioned research the atmosphere composition included 5% of O_2 that probably allowed a faster lipid oxidation.

While Choubert *et al*. (2008) observed an improvement using Ar instead of N_2, that was attributed to its higher density that allows it to remove oxygen better than nitrogen, inhibiting the initiation and progression of oxidation, in the present research, although the three different tested MAP (containing N_2, N_2O and Ar) were effective in minimising TBARS development, no significant difference ($P < 0.05$) was observed among traditional and innovative gases.

Hydrolysis of ester bonds in lipids by enzymatic action or heating in the presence of water liberates FFAs (Bernárdez *et al*., 2005). In general, hydrolysis, induced by lipases and phospholipases, produces FFAs that can undergo further oxidation and production of low molecular weight compounds responsible for the rancid off-flavour of fish products (Chaijan *et al*., 2006). Furthermore, FFAs and their oxidation products could impact muscle texture and functionality due to their ability to interact with myofibrillar proteins and to promote protein aggregation (Pacheco-Aguilar *et al*., 2000).

The evolution of lipid hydrolysis is presented in Fig. 3d. The initial fresh fillets value (0.09 ± 0.01 g oleic acid/100 g lipids) was similar to that obtained by Chaijan *et al*. (2006) for another sardine specie (*S. gibbosa*). FFAs values for all samples increased slightly with storage time. In Ar samples, the higher value was observed at the day 8 (0.73 ± 0.22 g/100 g lipids) followed by a decrease on day 12. At the end of the storage period, the samples packed on Ar gas mixture were lower than all samples. Chaijan *et al*. (2006) reported a high lipid hydrolysis (>6 g FFA/100 g lipid) in iced sardines at the end of the storage (15 days), probably because the authors used whole fish in which hydrolytic enzymes can derive also from internal organs. In this study, the highest FFAs value (0.96 ± 0.44 g/100 g lipids) were found on N_2 samples on day 12 and was similar to the values reported by Fagan *et al*. (2004) for mackerel and salmon fillets packed in MAP (60% N_2/40% CO_2) combined with freeze-chilling. However, no significant differences were found among Air, N_2 and N_2O samples throughout the storage period.

In order to understand the effect of the packaging atmosphere on the qualitative release of FFAs, gas-chromatographic analyses were performed, results are reported in Table 3. The data showed that the patterns of fat hydrolysis were different according to the atmosphere considered. The raw material was characterised by the release of mainly saturated FFAs such as C16:0, C18:0 and, to a minor extent, C14:1, respectively, 92% and 8% of the total. After 8 days of storage, all samples were characterised by an increase of the lipolysis although slightly higher in the Ar samples with a major release of saturated and monosaturated fatty acids such including stearic (C18:0) and oleic (C18:1) ones. On the other hand, the sardine fillets packed in Ar, after 8 days of storage, presented high total amount of polyunsaturated FFAs, related in particular to an increase content of 22:6 ($n - 3$). However, this sample was characterised by the absence of C18:2n6c and C20:4n6, present both in the Air and N_2 samples. The highest amount of PUFA, after 8 days, was detected in Air samples, characterised also by the highest amount of TBARS (Table 2). The Air sample was characterised by the presence of C18:2n6c, C20:4n6, C20:5n3, 22:6 ($n - 3$). Similar data were found by Chaijan *et al*. (2006) who found that the FFAs released in sardines were prone to oxidation as shown also by the marked increase in TBARS.

After 12 days of storage at 3 °C, the release of saturated and polyunsaturated fatty acids increased in all samples with the exception of the Ar sample that showed a slight decrease.

Table 3 Release of FFAs (mg kg^{-1}) in *Sardina pilchardus* fillets during storage at 3 °C in relation to the packaging atmosphere

	Raw material	Air – 8 days	Air – 14 days	N_2 – 8 days	N_2 – 14 days	N_2O – 8 days	N_2O – 14 days	Ar – 8 days	Ar – 14 days
C13:0	–*	–	4.28 ± 0.34	–	–	6.52 ± 2.39	–	–	–
C14:0	–	3.24 ± 0.13	3.25 ± 0.48	3.47 ± 0.05	3.53 ± 0.40	3.96 ± 0.52	4.49 ± 0.78	1.56 ± 2.21	4.10 ± 0.40
C16:0	37.25 ± 5.92	26.94 ± 2.62	36.23 ± 1.23	36.49 ± 1.90	30.90 ± 2.38	35.58 ± 5.33	49.26 ± 18.76	38.78 ± 5.63	38.16 ± 1.35
C17:0 iso	–	9.99 ± 4.13	–	–	–	–	–	–	–
C18:0	29.81 ± 2.25	8.74 ± 0.18	12.09 ± 1.2	13.88 ± 0.72	9.98 ± 0.88	9.07 ± 1.25	10.79 ± 1.10	16.39 ± 3.25	12.37 ± 1.14
C22:0	–	–	–	–	30.15 ± 2.63	–	–	–	
Σ saturated	67.06	48.91	55.85	53.84	74.56	55.13	64.54	56.73	54.63
% Saturated	91.93	49.70	51.77	52.73	60.84	51.28	53.37	50.39	51.45
C14:1n9	5.89 ± 1.67	–	–	–	–	–	–	3.63 ± 0.87	–
C16:1n9	–	5.00 ± 0.26	4.63 ± 0.1	4.97 ± 0.57	5.45 ± 1.30	5.56 ± 0.25	9.33 ± 1.74	4.94 ± 1.00	6.54 ± 0.17
C18:1n9c	–	3.07 ± 0.82	–	3.14 ± 1.00	2.55 ± 0.37	2.55 ± 0.05	–	5.14 ± 0.10	2.80 ± 0.76
C18:1n9t	–	2.02 ± 0.01	–	2.23 ± 0.39	-	2.53 ± 0.37	–	–	2.89 ± 0.42
Σ monounsaturated	5.89	10.09	4.63	10.34	8.00	10.64	9.33	13.71	12.23
% Monounsaturated	8.07	10.25	4.29	10.13	6.53	9.90	7.72	12.18	11.52
C18:2n6c	–	1.82 ± 0.16	3.62 ± 1.10	1.34 ± 0.60	2.64 ± 0.22	1.39 ± 0.64	6.55 ± 0.89	–	–
C18:3n3	–	–	2.59 ± 0.2	2.26 ± 0.40	2.12 ± 0.37	–	2.86 ± 0.98	–	–
C20:4n6	–	1.70 ± 0.16	–	1.27 ± 0.13	–	1.39 ± 0.64	–	–	–
C20:5n3	–	14.05 ± 1.63	13.68 ± 2.14	12.53 ± 0.90	13.91 ± 4.33	14.71 ± 0.04	20.50 ± 9.00	13.67 ± 1.40	16.10 ± 1.59
22:6 (*n* − 3)	–	21.85 ± 7.77	27.51 ± 5.61	20.53 ± 1.75	21.43 ± 5.53	24.24 ± 2.84	17.14 ± 2.56	28.47 ± 0.48	23.22 ± 7.90
Σ polyunsaturated		39.42	47.4	37.93	40.1	41.73	47.05	42.14	39.32
% Polyunsaturated	0.00	40.05	43.94	37.15	32.72	38.82	38.91	37.43	37.03
Total	72.95	98.42	107.88	102.11	122.56	107.5	120.92	112.58	106.18

*Not detected.

Regarding the monounsaturated fatty acids, for each considered sample, the total amounts found after 12 days was lower with respect the samples analysed at 8 days of storage.

Considering the relative quantities (%) of the three fractions on the total amount of FFA for each sample, it is possible to notice how in the Air sample, the release of PUFA was higher compared to the other samples at both considered sampling times, probably causing the higher oxidation level as shown by the previously considered indexes.

According to Choubert *et al*. (2008), the positive effect of replacing N_2 with Ar is related to its physical properties, particularly to its higher density, that allow for better inhibition of lipid oxidation. However, the authors did not specifically investigate lipid hydrolysis.

The accumulation of FFAs in fish muscle is mainly due to the enzymatic activity of lipase and phospholipase found in muscle, in digestive organs when present, but also deriving from microorganisms (Chaijan *et al*., 2006). In previous research, we showed that Ar was able to reduce microbial growth in sardine fillets compared to N_2; however, the effect on enzymatic activity was not investigated. It could be possible that the observed differences in FFAs release are related to an effect of enzymatic reactions in the fish tissue; however, specific research should be carried out to clarify this aspect.

Sensory evaluation

Samples packed in air reached the unacceptable condition (C) at the 5th day of storage (data reported in Table S1), while for samples packed in MAP the sensorial shelf-life was significantly improved. N_2 and N_2O samples were considered unacceptable at the 8th day, while sample packed in Ar maintained the 'fair' score (B) until the end of the storage. These results are in agreement with previous data on microbial spoilage (Pinheiro *et al*., 2019), confirming that the use of Ar allowed to increase shelf-life of sardine fillets because of improved microbiological status and sensorial characteristics.

Conclusions

As extensively known, the use of modified atmosphere in fish packaging with the removal of oxygen has confirmed to significantly inhibit the oxidation process during refrigerated storage.

Although previous studies have shown that Ar allowed to increase microbial shelf-life of sardine fillets most of the investigated parameters related to fat oxidation did not show any improvement during storage, except for a slight decrease in lipid hydrolysis in the packaging containing Ar. However, sensorial analysis showed that Ar sample was considered acceptable until the end of the storage.

Considering that the two investigated novel gases are at present more expensive compared to nitrogen, before a possible utilisation at industrial level, a careful consideration of benefits vs. costs should be carried out. Moreover, further studies are in due course in our laboratory in order to consider the reversibility of the visual quality improvement promoted by MAP after packaging opening, and in simulated preparation and consumption operations.

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Author contributions

Ana Cristina de Aguiar Saldanha Pinheiro: Formal analysis (lead); methodology (equal); writing-original draft (equal). **S. Tappi:** Conceptualization (equal); formal analysis (supporting); investigation (equal); methodology (equal); writing-review & editing (lead). **Francesca Patrignani:** Formal analysis (supporting); investigation (equal); supervision (equal). **Rosalba Lanciotti:** Resources (equal); supervision (equal); writing-review & editing (supporting). **Santina Romani:** Conceptualization (equal); supervision (equal). **Pietro Rocculi:** Conceptualization (equal); project administration (equal); resources (equal); writing-review & editing (supporting).

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethics approval was not required for this research.

Peer review

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Data availability statement

Research data are not shared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sensory acceptance* during sardine storage under different MAP conditions at 3 °C.

Paper III

Quality Changes during Frozen Storage of Mechanical-Separated Flesh Obtained from an Underutilized Crustacean.

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Quality Changes during Frozen Storage of Mechanical-Separated Flesh Obtained from an Underutilized Crustacean

Silvia Tappi [1,2,*], Ana Cristina De Aguiar Saldanha Pinheiro [1], Dario Mercatante [3], Gianfranco Picone [1], Francesca Soglia [1], Maria Teresa Rodriguez-Estrada [2,3], Massimiliano Petracci [1], Francesco Capozzi [1,2] and Pietro Rocculi [1,2]

1 Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, 47521 Cesena, Italy; anacristin.deaguiar2@unibo.it (A.C.D.A.S.P.); gianfranco.picone@unibo.it (G.P.); francesca.soglia2@unibo.it (F.S.); m.petracci@unibo.it (M.P.); francesco.capozzi@unibo.it (F.C.); pietro.rocculi3@unibo.it (P.R.)

2 Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum—University of Bologna, (FC) 47521 Cesena, Italy; maria.rodriguez@unibo.it

3 Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, 40127 Bologna, Italy; dario.mercatante2@unibo.it

* Correspondence: silvia.tappi2@unibo.it; Tel.: +39-0547-338103

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Abstract: Despite their high nutritional value, high quantities of fish caught in the Adriatic Sea are underused or discarded for their insignificant economic value. Mechanical separation of flesh represents an opportunity for developing innovative semi-finished products, even if it can promote an increased quality degradation rate. The aim of this study was to evaluate physico-chemical modifications of mechanically separated mantis shrimp flesh during deep-freezing storage. Flesh samples obtained using a belt-drum separator, frozen and vacuum-packed, were stored at 3 temperatures (industrial: −26 °C; domestic: −18 °C and abuse: −10 °C) for 12 months. During storage, qualitative (color, water content, pH, fatty acids (FA) and lipid oxidation) were evaluated. Fish freshness parameters (e.g., trimethylamine (TMA), dimethylamine (DMA) and amino acids) were assessed using nuclear magnetic resonance (^{1}H-NMR). The mechanical separation process accelerated the initial oxidation phenomena, promoting color alterations, compared to manual separation. The main degradation phenomena during storage were significantly affected by temperature and were related to changes in luminosity, oxidation of n-3 polyunsaturated fatty acids (PUFA), increased lipolysis with release of free FA, production of TMA and DMA by residual enzymatic activity, and changes in amino acids due to proteolysis. The inter-disciplinary approach permitted important findings to be made, in terms of the extent of different degradative phenomena, bound to processing and storage conditions of mechanically separated mantis flesh.

Keywords: *Squilla mantis*; mantis shrimp; mechanically separated flesh; frozen storage; lipolysis; lipid oxidation; proteolysis; quality changes

1. Introduction

Despite its high nutritional value, a high quantity of fish caught in the Adriatic Sea is underused or discarded mainly due to its insignificant local economic value or for regulatory reasons [1]. In the Adriatic Sea, bottom trawling represents 40% of total landings and the mean discard rate of this fishing gear ranges between 20% and 67% of total catches [2]. Moreover, many seafood products are characterized by significant changes in quality and abundance throughout the year. Therefore,

it would be convenient, from both economic and sustainability standpoints, to promote strategies for their valorization, which lead to the development of innovative, high-added value products with prolonged shelf-life and that are available throughout the year, thereby reducing waste.

Mechanical separation is a technology that is successfully applied in the fish sector, even though the loss or modification of the normal structure of the muscle fiber often occurs during this operation [3]. Mechanical separation of fish flesh could represent an opportunity for the development of fish-based innovative products from seafood that would otherwise be discarded.

Moreover, the by-products of mechanical separation of crustaceans (shells and carapaces) could be further exploited for the extraction of valuable compounds (such as chitin and chitosan) that have several uses in the food industry (i.e., anti-microbial, antioxidant, and anti-inflammatory agents), as well as in the non-food sector [4], contributing to increase the sustainability and the economic value of the overall food chain.

However, this type of preparation process could lead to an increased quality degradation rate of an already highly perishable product. Indeed, besides microbial proliferation, fish are also highly susceptible to lipid and protein oxidation, which can promote the production of biogenic amines and other compounds considered hazardous compounds [5]. Storage under deep-freezing conditions greatly increases the shelf-life of seafood products by arresting microbial growth and, in general, slowing down all other chemical and enzymatic degradation reactions. Freezing could help provide high-quality product constantly throughout the year and prevent product waste. However, freezing temperatures are only able to slow down enzymatic activity and oxidation. The main limiting factor of the shelf-life of frozen fish products is, in fact, represented by lipid degradation, due to both oxidative and hydrolytic reactions which affect their nutritional and sensory profile.

From an economical point of view, the whole process mainly requires a mechanical deboner and a temperature blast chiller. While many companies working in the seafood sector generally possess a blast chiller, the mechanical deboner, often used in the poultry sector, may represent the main initial cost of investment. However, it can be considered relatively inexpensive processing equipment that does not require big changes in production lines and that is also characterized by limited energy consumption, confirming its suitability to valorize underutilized or low-value species.

The mantis shrimp (*S. mantis*) is a common specie along the coast of the Mediterranean Sea and it is one of the most important resources in the northern and central Adriatic Sea, due to its easy capture and commercial/economic value, where it represents 66% of the demersal fisheries mainly caught by bottom trawlers [6]. Among the crustaceans it is characterized by a low market value, nevertheless, is can be considered a good source of n-3 and n-6 polyunsaturated fatty acids (PUFAs). Moreover, the high levels of essential amino acids can make it an alternative source of proteins for the populations of developing countries [7]. However, it is characterized by a marked seasonality, with the highest values occurring in winter and the lowest in April–July [8] and a large discarded amount due to its small size.

The aim of this study was to evaluate the modifications of some qualitative indices of mechanically separated mantis flesh during deep-freezing storage. The mantis shrimp was separated, deep-frozen, vacuum-packed and stored at three different temperatures that represented domestic storage (−18 °C), industrial preservation (−26 °C) and thermal abuse condition (−10 °C). Different qualitative indices (pH, dry substance, color, main lipid classes, fatty acid (FA) composition, thiobarbituric acid reactive substances (TBARs), trimethylamine-N (TMA-N), trimethylamine-O (TMA-O), dimethylamine (DMA), lysine, alanine and sarcosine) were determined during storage.

2. Materials and Methods

2.1. Chemicals

All chemicals and solvents were of analytical grade. Methanol and *n*-hexane were purchased from Merck (Darmstadt, Germany); anhydrous sodium sulfate was supplied by BDH (Poole, England) The

standard mixture of fatty acid methyl esters (GLC 463) was purchased from Nu-Chek (Elysian, MN, USA), whereas the tridecanoic acid methyl ester was supplied by Steraloids (Newport, RI, USA). All the other chemicals and standards were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Raw Materials

Mantis shrimps (*S. mantis*) were fished in February 2017 in the Adriatic Sea (Food and Agriculture Organization (FAO) Major Fishing Area 37, Subarea 37.2.1) and subsequently kept in ice for 24 h before processing. Mantis shrimp flesh was obtained by either manual separation (FF, control group) or using a belt-drum separator (MSF). In the mechanical deboner model 600 (Baader, Germany), flesh was forced by means of a rubber conveyor belt through a perforated drum (holes diameter 3 mm) and collected from the inside of the drum, while carapaces were discarded on the outside. Flesh was divided into polypropylene (PP) trays of about 100 g each. 57 trays were frozen in a cooling system until reaching −26 °C at the sample core. Temperature was monitored by inserting thermocouples at the core of 4 trays. Once the samples were already frozen, the trays were vacuum-packed in a high barrier PP film.

2.3. Storage

The frozen and packaged samples were divided into three freezers (18 trays each) at temperatures of −26 °C, −18 °C and −10 ± 0.5 °C to simulate industrial, domestic and thermal abuse conditions, respectively. During storage, three trays per each temperature were sampled at different time intervals (0, 1, 2, 4, 6, 9 and 12 months), thawed at 4 °C for 16 h and analyzed.

2.4. Analytical Determinations

2.4.1. Physico-Chemical Parameters

pH was measured by a pH meter (Crison, Barcelona, Spain). For each sampling time, the measurement was performed in triplicate. Water content was evaluated with a gravimetric method, measuring the weight difference before and after drying, until constant weight was reached in an oven at 70 °C. The measurement was performed in triplicate on each sample at each storage time. Color was evaluated with a portable tristimulus spectrum-photocolorimeter (Hunterlab ColorFlex™, Reston, VA, USA) using the CIELab scale with L*, a* and b* as color parameters. In the present study, the L* value (brightness indicator, with values between 0 and 100) and the red index a*, were considered. The color was measured in triplicate.

2.4.2. Lipid Oxidation

Thiobarbituric acid reactive substances (TBARs) were determined according to Bao and Ertbjerg [9] and used as a lipid oxidation indicator. Samples (5 g) were homogenized in ice using a IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) at 13,000 rpm for 30 sec in 15 mL of 5% trichloroacetic acid (TCA) (w/v) and 0.5 mL of butyl hydroxytoluene (BHT) (4.2% w/v in ethanol). After filtering the homogenate through filter paper (Whatman 42), a 2 mL aliquot was taken and added with 2 mL of thiobarbituric acid (0.02 M). Thereafter, after incubating the solution at 100 °C for 40 min and cooling down the samples in an ice bath, the absorbance was measured at 532 nm with an ultraviolet (UV)-visible spectrophotometer (mod. UV-1800; Shimadzu, Kyoto, Japan). To calculate the amount of malondialdehyde (MDA) produced, a standard 1,1,3,3-tetraethoxypropane curve was used in the concentration range of 0.1 to 2.0 mM. Finally, TBARs content was expressed in mg MDA/kg sample. The measurement was performed in triplicate for each storage interval and for each temperature.

2.4.3. Lipid Extraction

Lipid fraction was extracted from 50 g of samples using chloroform and methanol according to the modified Bligh and Dyer method [10]. The lipid content was determined gravimetrically, and the

results were expressed as g lipid/100 g of the sample. The extraction was performed twice on each sample. Extracted lipids were stored at −40 °C until analyzed.

2.4.4. Total Lipid Profile

The determination of the main lipid classes (free fatty acids (FFA), monoacylglycerols (MAG), free sterols (STE), diacylglycerols (DAG), esterified sterols (EST) and triacylglycerols (TAG)) were determined by gas chromatography-flame ionization detection (GC-FID) [9]. About 20 mg of lipid matter to which were added 0.251 mg of 5α-cholestane (internal standard) were dissolved in 1 mL of n-hexane: One µL of the solution was injected into a GC-FID (Shimadzu GC 2010 PLUS, Kyoto, Japan) under the same analytical conditions as Gallina Toschi et al. [11] (2014) and Luise et al. [12]. A fused silica capillary column (SE-52 MEGA, 10 m × 0.25 mm i.d. × 0.1 µm film thickness; Legnano, MI, Italy), coated with 95% dimethyl and 5% diphenyl polysiloxane, was used. The temperature was programmed from 100 to 355 °C at a rate of 5 °C/min and the final temperature was kept for 20 min. The injector and FID temperatures were set at 355 °C. Helium was used as carrier gas at a flow of 2.02 mL/min and a split ratio of 1:25. The different lipid classes were identified using diverse commercial standards (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). The amount of each lipid class was determined using the internal standard method with the response factor of each main lipid class (estimated using suitable commercial standards), as reported by Luise et al. [12]. Two independent replicates were analyzed.

2.4.5. Total Fatty Acid Profile

About 20 mg of lipid extract were methylated with 200 µL of diazomethane, added with 0.6 mg of tridecanoic acid methyl ester (C13, internal standard), transmethylated with 40 µL of 2 N potassium hydroxide (KOH) in methanol [13], vortexed for 1 min, left standing for 5 min, and centrifuged at 1620× *g* for 5 min. One microliter of supernatant was injected into a GC-FID (GC8000 series, Fisons Instruments, Milan, Italy), interfaced with a data acquisition system (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A Restek RTX 2330 fused-silica column (30 m × 0.25 mm × 0.2 µm film thickness) (Bellefonte, PA, USA) coated with 90% biscyanopropyl and 10% cyanopropyl-phenyl polysiloxane, was used. Oven temperature was programmed from 100 °C to 240 °C at 5 °C/min, and kept at 240 °C for 20 min. Both injector and detector temperatures were set at 250 °C. Helium was used as carrier gas at a constant pressure of 75 KPa and a split ratio of 1:30. Peak identification was performed by comparing the retention times with those of the GLC 463 FAME standard mixture. Tridecanoic acid methyl ester was used as internal standard for FA quantification; the GC response factor of each FA was calculated by using the GLC 463 FAME standard mixture and the internal standard. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as signal-to-noise ratios equal to 3:1 and 10:1, respectively.

FAME quantification was performed according to the following formula:

$$Qi = (Ai \times Qis)/(Ais \times W \times Kris)$$

where Qi is the FA concentration (mg/100 mg), Ai is the FA peak area, Qis is the concentration of the internal standard (C13 methyl ester, mg), Ais is the internal standard peak area, W is the weight of the lipid sample (mg) and Kris is the response factor.

2.4.6. Nuclear Magnetic Resonance (^{1}HR-NMR) Metabolomics for Quality Indexes

Samples were prepared as reported by Picone et al. [14]. Briefly, for each sample, an extraction with 7% of perchloric acid solution was performed in triplicate. The acid mixtures were neutralized to pH 7.8 using 9 M KOH and then centrifuged at 14,000 rpm for 10 min at 4 °C in order to remove potassium perchlorate precipitate. We added to 720 µL of supernatant 80 µL of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM and then centrifuged it one

more time at 14,000 rpm for 10 min at 4 °C. Samples were transferred to a 5 mm NMR tube and spectra were acquired and processed using parameters reported in previous researches [15,16].

Signals were assigned by using a multimedia library included in Chenomx NMR Suite 8.2 professional software (Chenomx, Edmonton, AB, Canada) and the concentration of trimethylamine-N (TMA-N), trimethylamine-O (TMA-O), dimethylamine (DMA), lysine, alanine and sarcosine were determined as indicators of fish freshness.

2.5. Statistical Analysis

Differences between mean values of manually and mechanically separated flesh were analyzed by a *t*-test ($p < 0.05$). Storage data were analyzed using two-way analysis of variance (ANOVA) including storage time (St) and temperature (T) and their interaction (St T) as factors. Means were separated by Tukey's honest significance test ($p < 0.05$). Pearson's analysis (p-level < 0.05) was performed to evaluate the correlation between data. Principal component analysis (PCA) was used as explorative technique to discriminate the samples and to display the correlation between the parameters. Statistical analysis of the data was performed by using the software Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA)

3. Results and Discussion

3.1. Comparison between Manually and Mechanically Separated Fresh Flesh

In Table 1, the physico-chemical parameters and fatty acid (FA) composition of manually and mechanically separated flesh, are compared. No significant differences were observed for water content and pH. On the contrary, the color parameter L* showed a significant variation; indeed, after mechanical separation, the flesh appeared darker. Secci et al. [17] detected a similar color variation in horse mackerel after mechanical separation. The initial TBARs value was similar to that found by Sundararajan et al. [18] in frozen shrimp. However, a significant increase of this parameter was observed, indicating that the mechanical separation process promoted lipid oxidation; similar results were detected by Secci et al. [3] in two different fish species.

Table 1. Physico-chemical parameters, thiobarbituric acid reactive substances (TBARs), main lipid classes and fatty acid classes of fresh mantis flesh obtained by manual (FF) and mechanical separation (MSF).

	FF	MSF
Water content (%)	85.19 ± 0.71 ^{a}	86.19 ± 0.14 ^{a}
pH	6.71 ± 0.03 ^{a}	6.74 ± 0.01 ^{a}
L*	41.33 ± 0.05 ^{a}	40.93 ± 0.09 ^{b}
a*	4.77 ± 0.14 ^{a}	5.56 ± 0.08 ^{a}
TBARs (mg MDA/kg)	0.67± 0.03 ^{b}	1.72 ± 0.31 ^{a}
Total lipid content (%)	3.01 ± 0.42 ^{a}	2.85 ± 0.35 ^{a}
FFA (% of total lipid)	12.74 ± 2.28 ^{a}	9.46 ± 0.18 ^{a}
MAG (% of total lipids)	5.10 ± 0.78 ^{a}	6.75 ± 0.13 ^{a}
DAG (% of total lipids)	29.94 ± 0.91 ^{a}	31.24 ± 0.19 ^{a}
TAG (% of total lipids)	13.78 ± 8.48 ^{a}	17.56 ± 0.03 ^{a}
EST (% of total lipids)	28.50 ± 1.32 ^{a}	28.47 ± 0.09 ^{a}
STE (% of total lipids)	5.42 ± 1.38 ^{a}	4.97 ± 0.26 ^{a}
SFA (% of total FA)	31.27 ± 3.09 ^{a}	30.17 ± 0.77 ^{a}
MUFA (% of total FA)	55.72 ± 3.27 ^{a}	57.87 ± 0.48 ^{a}
PUFA (% of total FA)	13.00 ± 0.63 ^{a}	11.95 ± 0.29 ^{a}
PUFA n-3 (% of total FA)	10.18 ± 0.04 ^{a}	9.35 ± 0.39 ^{a}
PUFA n-6 (% of total FA)	2.82 ± 0.27 ^{a}	2.78 ± 0.11 ^{a}
PUFA n-6/PUFA n-3	3.64 ± 0.56 ^{a}	3.69 ± 0.30 ^{a}

Different letters (a–b) indicate significant differences ($p < 0.05$) between values for each considered parameter. L*, Luminosity; a*, red index; DAG, diacylglycerols; EST, esterified sterols; FFA, free fatty acids; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAG, triacylglycerols; STE, sterols; TBARs, thiobarbituric acid reactive substances.

The fat content of the flesh varied from 2.8% to 3%. This value is in agreement with literature results [7–19] and did not change according to the different separation method.

Regarding lipid classes, no significant differences were observed between the manually and mechanically separated fresh flesh. It must be noted that DAG, EST and TAG were the most abundant lipid classes in both types of mantis flesh, evidencing already a hydrolytic process of lipids. Concerning the total FA composition (Supplementary material—Table S1), it remained practically unchanged in both manually and mechanically separated fresh flesh, even though mechanical separation led to higher oxidation which was reflected in a significant decrease of some unsaturated FA (C16:1 n-7 and C20:3); similar results were obtained by Secci et al. [17] on horse mackerel flesh. The main FA were oleic acid (C18:1 n-9, 16.9 ± 0.2% of total FA) and palmitic acid (C16:0, 16.4 ± 0.5% of total FA), followed by palmitoleic acid (C16:1 n-7, 14.6 ± 0.4% of total FA) and nervonic acid (C24:1 n-9, 14.6 ± 0.5% of total FA). Eicosapentaenoic (C20:5 n-3, EPA) and docosahexaenoic (C22:6 n-3, DHA) acids were present as 5.26 ± 0.36% and 0.88 ± 0.02% of total FA, respectively. Among FA categories, monounsaturated FA (MUFA) were the more abundant, followed by saturated FA (SFA) and PUFA. In particular, SFA represented about 30% of total lipids and were mainly composed by C16:0 and C18:0; in the case of MUFA, C18:1 n-9 and C16:1 n-7 represented about 60% of this FA class. On the other hand, PUFA accounted for 13% of total lipids and, while more than 90% was constituted by PUFA n-3 (in particular EPA and DHA), only 2.8% was represented by PUFA n-6. These values are within the ranges of FA percentage distribution reported by Mili et al. [7] for *S. mantis* fished in Tunisian waters in different seasons, as well as those found by Passi et al. [19] for Mediterranean mantis shrimp. In fact, it is well known that FA composition of fish lipids can be affected by species, genetic, physiological, morphological, dietary, seasonal and environmental factors, among others [20,21]. The PUFA n-6/PUFA n-3 ratio, suitable index to compare the nutritional value of food, was around 3.6 and no significant differences were observed with respect to the separation process used. According to Simopoulos [22], a low PUFA n-6/PUFA n-3 ratio (< 4) is desirable for a healthy human diet. This result confirms the importance of Mediterranean mantis shrimp as a rich dietary source of PUFA n-3.

3.2. Variation of Quality Indices during Frozen Storage

During the storage at three different temperatures, pH varied from 6.49 to 6.71, while moisture content ranged from 85.59% to 86.62% (pH and moisture data not reported); in both cases, no significant differences were observed across storage at the different temperature conditions.

Figure 1 shows the evolution of the colorimetric parameters of luminosity L* (Figure 1A) and red index a* (Figure 1B) measured in the mechanically separated *S. mantis* flesh during frozen storage at the three selected temperatures.

Evolution of color during storage can be associated with structural changes [23], as well as variations in pigments concentrations and their oxidative status [24]. While at the temperatures of −18 °C and −26 °C the L* values were roughly constant (37–42) throughout the 12-months storage, the sample stored at −10 °C showed a significant increase during the entire storage period, reaching values of 56. This parameter was significantly influenced by storage temperature, time and their interaction (Table 2). By contrast, the red index was significantly affected only by storage time and by the interaction between time and temperature. Although some significant variations were observed among samples during storage, there was not a clear trend and values remained between 5 and 7.

Sundararajan et al. [18] observed an increase in a* value for peeled frozen shrimp stored at –21 °C for 180 days, while no significant changes in L* values were observed. These authors suggested that the decrease in a* values could be mainly attributed to the degradation of astaxanthin and lipid oxidation.

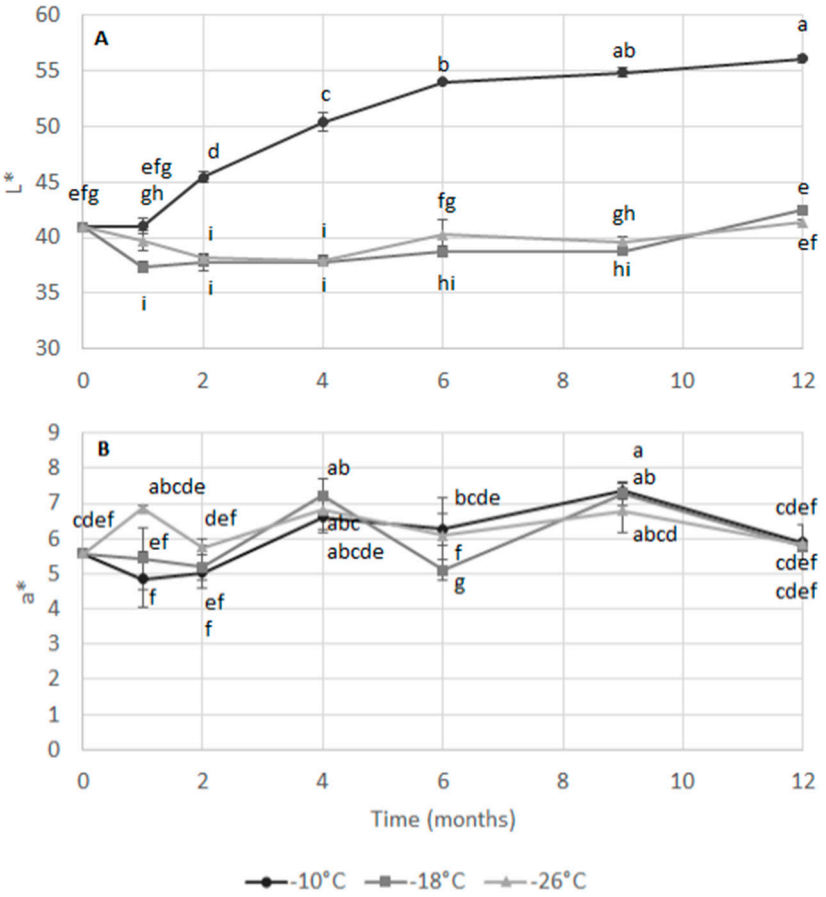

Figure 1. Colorimetric parameters of luminosity (L*) (**A**) and red index (a*) (**B**) of mechanically separated mantis shrimp flesh during frozen storage at −10, −18 and −26 °C. Different letters indicate significant differences (at $p < 0.05$) among samples.

Table 2. F value and relative significance of the influence of storage time (St) and temperature (T) and their interaction (St T) on color parameters (L* and a*) and lipid oxidation (TBARs) data of fresh mantis flesh obtained by manual and mechanical separation.

	Parameter		
	L*	**a***	**TBARs**
St	267.82 ***	48.77 ***	5.91 ***
T	2933.21 ***	0.65 ns	1.64 ns
St T	160.82 ***	13.41 ***	2.01 *

* $p < 0.05$; *** $p < 0.001$; ns: not significant. TBARs, thiobarbituric acid reactive substances.

To the best of our knowledge, there are no previous reports about the storage of mechanically separated flesh obtained from crustaceans, hence it is impossible to directly compare our results. Changes in the mechanically separated fish flesh obtained from horse mackerel [16], as well as from gilted sea bream, sea bass and rainbow trout [3], were evaluated during frozen storage, showing that color variations depended on the species considered. Shrimp flesh is highly perishable and normally high product quality can be obtained when immediately frozen after capture [25]. Generally, results showed that the main color differences occurred during processing rather than during storage and that white flesh led to lower changes, proving to be more suited for the development of fish processed products [3]. However, color fading, lipid oxidation, protein denaturation, and dehydration can occur during the frozen storage of shrimp and other crustaceans [25]. Color variations observed in the sample stored at −10 °C may be related to enzymatic and non-enzymatic reactions that result in degradation

of myofibrillar proteins and disorganization of myofibrils. Chéret et al. [23] and Torres et al. [26] observed a similar change upon high hydrostatic pressure processing of sea bass fillets and horse mackerel, respectively.

Figure 2 reports the TBARs values found in the *S. mantis* flesh during the frozen storage, which varied from 1.4 to 2.4 mg MDA/kg for all the considered period, without significant variations in all storage conditions. Despite the secondary lipid oxidation initially induced by the mechanical separation process, TBARs did not show a steady increase during storage as expected, being thus in disagreement with data reported by various authors for oxidative stability of crustacean flesh and minced fish during frozen storage [3,27–30]. Sundararajan et al. [18] found a value of 0.47 mg MDA/kg in shrimp that increased progressively during frozen storage up to 2.96 after 180 days. Tsironi et al. [25] observed an increased rate of TBARs formation with increasing storage temperature in frozen shrimp. However, in the present research, after the initial increase of TBARs during processing, no further oxidation was detected by means of this index.

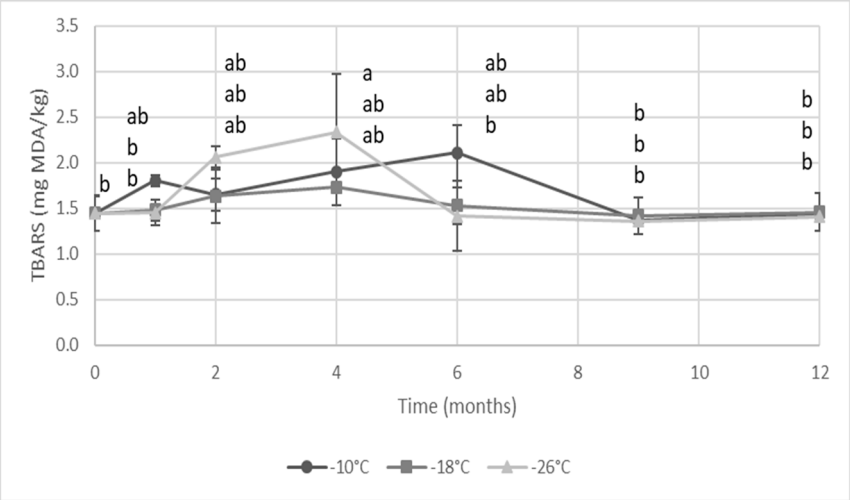

Figure 2. Thiobarbituric acid reactive substances (TBAR) values of mechanically separated mantis shrimp flesh during frozen storage at −10, −18 and −26 °C. Different letters indicate significant differences (at $p < 0.05$) among samples.

Besides the data dispersion observed, other factors could have also contributed too, such as the type of packaging, the presence and amount of lipophilic (such as vitamin E) and enzymatic (i.e., GSH) antioxidants in mantis shrimp [19]. On the other hand, aldehydes deriving from lipid oxidation could have also interacted with other matrix components (such as proteins, amines and peptides) [30], thus leading to the formation of compounds (i.e., Schiff bases) that cannot be determined as TBARs. In fact, lipid and protein oxidations can occur independently or in parallel, but they can also interact with each other [30].

Table 3 reports the distribution of the main lipid classes (expressed as % of total lipids) and the main total FA classes (expressed as % of total FA) in mechanically separated *S. mantis* flesh, as related to storage conditions. The total fat content (% on flesh) was significantly affected only by storage time (St); however, no significant differences were observed among all the determined values.

Concerning the distribution of the main lipid classes, FFA was found to be influenced by both storage temperature and time, increasing from 9% up to around 40% in samples stored at −10 °C after 6 months. Similarly, MAG rose by increasing storage time and storage temperature, while TAG and DAG content showed the opposite trend. These results evidence the occurrence of lipid hydrolysis during frozen storage, being more intense at storage temperatures above −26 °C. The accumulation of FFA in frozen marine species is related to some extent with lack of acceptability. FFA, in fact, are known to cause deterioration of seafood products through their interaction with proteins and have been

reported to exert a great effect on lipid oxidation development [26]. FFA have also been shown to oxidize faster than higher molecular-weight lipids, i.e., TAG and phospholipids, due to their higher accessibility caused by their lower steric hindrance to oxygen and other prooxidant molecules [27].

Table 3. Composition of lipid classes (expressed as % of total lipids) and the fatty acid classes (expressed as % of total fatty acids) of the mechanically separated mantis flesh, after 0, 6 and 12 months storage at different temperatures.

Storage Temperature	Fat Content (%)	FFA	MAG	DAG	TAG	STE	EST
				% of Total Lipids			
				T0			
-	2.9 ± 0.4	9.5 ± 0.2 ^{d}	6.8 ± 0.1 ^{de}	33.5 ±0.2 ^{a}	17.6 ± 0.0 ^{a}	4.9 ± 0.3 ^{a}	27.9 ± 0.9 ^{ab}
				6 months			
−10 °C	1.7 ± 0.1	41.3 ± 0.5 ^{a}	42.3 ± 0.5 ^{a}	1.9 ± 0.1 ^{e}	1.4 ± 0.2 ^{d}	1.3 ± 0.2 ^{cd}	11.9 ± 0.4 ^{de}
−18 °C	1.9 ± 0.1	22.2 ± 1.7 ^{c}	18.0 ± 1.8 ^{c}	20.8 ± 1.4 ^{c}	12.1 ± 0.3 ^{b}	2.7 ± 0.1 ^{b}	24.2 ± 1.8 ^{b}
−26 °C	1.8 ± 0.8	12.8 ± 0.8 ^{d}	9.1 ± 0.3 ^{de}	26.7 ± 0.2 ^{b}	18.6 ± 0.5 ^{a}	4.4 ± 0.0 ^{a}	28.4 ± 0.5 ^{a}
				12 months			
−10 °C	2.3 ± 1.0	47.6 ± 5.1 ^{a}	39.0 ± 3.8 ^{a}	0.4 ± 0.1 ^{e}	1.1 ± 0.7 ^{d}	0.9 ± 0.2 ^{d}	11.0 ± 0.6 ^{e}
−18 °C	2.1 ± 0.2	31.7 ± 2.4 ^{b}	26.1 ± 1.6 ^{b}	16.2 ±0.3 ^{d}	6.3 ± 1.0 ^{c}	2.0 ± 0.0 ^{bc}	15.2 ± 0.2 ^{cd}
−26 °C	2.6 ± 0.3	21.5 ± 0.9 ^{c}	12.0 ± 1.4 ^{d}	31.4 ± 2.2 ^{a}	13.6 ± 1.4 ^{b}	4.4 ± 0.5 ^{a}	17.1 ± 2.1 ^{c}
Factor				**F value**			
St	7.04 *	223.79 ***	254.23 ***	777.70 ***	407.13 ***	167.80 ***	287.78 ***
T	0.16 ns	223.38 ***	246.77 ***	692.27 ***	50.82 ***	118.70 ***	96.44 ***
St T	0.26 ns	31.96 ***	68.18 ***	187.70 ***	98.24 ***	31.11 ***	40.01 ***
	SFA	**MUFA**	**PUFA**		**n-3**	**n-6**	**n-6/n3**
			% of total fatty acids				
				T0			
-	30.2 ± 0.8 ^{b}	57.9 ± 0.5 ^{a}	12.0 ± 0.3 ^{a}		9.4 ± 0.4 ^{ab}	2.8 ± 0.1	3.5 ± 0.3
				6 months			
−10 °C	31.8 ± 0.6 ^{ab}	55.9 ± 0.2 ^{ab}	12.3 ± 0.4 ^{a}		9.6 ± 0. 3 ^{ab}	2.7 ± 0.1	3.5 ± 0.1
−18 °C	30.5 ± 1.5 ^{b}	57.6 ± 0.4 ^{a}	12.0 ± 1.1 ^{a}		9.4 ± 1.1 ^{ab}	2.6 ± 0.0	3.5 ± 0.4
−26 °C	29.8 ± 1.0 ^{b}	57.4 ± 0.9 ^{a}	12.7 ± 0.1 ^{a}		10.0 ± 0.0 ^{ab}	2.7 ± 0.1	3.5 ± 0.1
				12 months			
−10 °C	27.5 ± 5.1 ^{b}	58.2 ± 2.9 ^{a}	14.4 ± 2.1 ^{a}		11.2 ± 1.1 ^{a}	3.2 ± 1.1	3.6 ±0.9
−18 °C	40.2 ± 1.2 ^{a}	51.3 ± 1.9 ^{b}	7.5 ± 0.7 ^{b}		5.0 ±1.5 ^{b}	2.5 ± 0.6	2.9 ± 0.1
−26 °C	30.2 ± 1.5 ^{b}	58.6 ± 0.6 ^{a}	11.2 ± 0.8 ^{a}		8.6 ± 0.7 ^{ab}	2.6 ± 0.1	3.4 ± 0.1
Factor				**F value**			
St	2.57 ns	3.25 ns	3.29 ns		1.29 ns	0.19 ns	1.02 ns
T	6.95 *	5.71 *	10.59 **		5.39 *	0.57 ns	0.60 ns
St T	8.34 **	8.26 **	9.07 **		4.78 *	0.52 ns	0.72 ns

Different letters indicate significant differences ($p < 0.05$) among samples for each considered index. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant. DAG, diacylglycerols; EST, esterified sterols; FFA, free fatty acids; MAG, monoacylglycerols; TAG, triacylglycerols; STE, sterols; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Regarding the total FA composition (expressed as % of total FA), significant differences were observed in all FA classes as related to both storage temperature and the interaction between time and temperature (St T), except for PUFA n-6 and the n-6/n-3 ratio. Among the single FA (Table S2), docosapentaenoic acid (DPA) was noticeably affected by the storage temperature. On the other hand, storage time did not show any significant effect on the main FA classes, but some single FA (such as linolenic acid) varied to a relevant extent depending on the time of storage. However, it must be noted that there was not a clear trend of the concentration of most single FA with respect to the temperature and time of storage (Table S2), which could depend on a dynamic equilibrium between their accumulation and conversion into other compounds (i.e., oxidized fatty acids, volatile compounds).

In general, the impact of the storage method and duration on FA content varies according to the fish species and seems to greatly depend on their total lipid content. In fact, Rudy et al. [31] observed that the effect of storage conditions was greatest in fish species whose lipid content was around 10–19%, while species with lower lipid content (< 10%), like *S. mantis* (2.9–3.1%) in the present study, are usually less affected. The lipid content relates to the taxonomic classification, environment (freshwater or

marine), season and/or geographic location (warm or cool waters), and lipid storage; all these factors influence the FA content of fish tissue and their susceptibility to degradation under diverse storage conditions. Although long-chain PUFA are usually more prone to oxidation, Rudy et al. [31] observed that FA degradation was more a function of fish species rather than FA type, suggesting species-specific FA dynamics during storage, probably related to the total lipid content in the fish species. In our work, as well as in that of Rudy et al. [31], no specific FA or FA class consistently and preferentially underwent a change in quantity with increasingly poor handling and storage conditions, even though a decrease in the amount of some FA was observed over time. Besides the influence of lipid content on FA alterations in marine species, they may also depend on other factors like size, sex, diet, season, state when captured, microbial load, genuineness, presence of natural antioxidants, tissue type, number of lipases and their location in cells [31].

Figure 3 shows the concentration of some selected compounds present in the *S. mantis* flesh during storage and analyzed by ^{1}HR-NMR. In Table 4, results of multivariate analysis show that all parameters were significantly influenced by storage time, temperature, and their interaction. TMA-O breakdown can occur via bacterial enzymes that release TMA [32], or by the activity of trimethylamine oxide demethylase (TMAOase) that leads to the formation of DMA and formaldehyde [33]. The production of TMA during refrigerated storage is considered an index of fish freshness as it is strongly correlated with microbial spoilage and it is characterized by a pungent, often associated with the typical "fishy" smell of seafood undergoing spoilage [34]. During frozen storage, bacterial activity should be absent; however, as mentioned earlier, TMA has also been reported to be a product of enzymatic degradation of TMA-O.

In the present study, TMA-O (Figure 3A) decreased rapidly in the first month and then remained fairly constant in samples stored at −18 °C and −26 °C. In the sample stored at −10 °C, instead, lower values were observed during the rest of the storage; in fact, at the end of the 12-month storage period, TMAO was half as much the initial value. In parallel to the decrease of TMAO, both TMA (Figure 3B) and DMA (Figure 3C) increased in samples stored at −18 and −10 °C, while roughly the same values were observed in the sample at −26 °C. In particular, TMA increased from the 4th month in both samples stored at −10 °C and −18 °C proportionally to the storage temperature. DMA started to increase after the first month for samples stored at –10 °C, whereas in samples kept at −18° C it rose just after 4 months and to a lower extent.

Sotelo et al. [35] found an increase of TMA during storage at −5 °C, but not at −12 °C. These authors suggested that some residual bacterial activity could still be found at temperatures slightly below zero. However, the TMA increase observed at −10 °C and −18 °C in the present study is probably related to enzymatic degradation. According to García-Soto et al. [27], the formation of TMA during frozen storage of crustaceans can also be due to biochemical breakdown of proteins and non-protein nitrogen (NPN) compounds.

Free amino acids in fish are the main components of non-protein nitrogen and, since some of them are precursors of aromatic components, they are directly responsible for the development of flavor and taste during cooking [36]. Amino acids have also been used as quality indices for various fish and crustacean species [37]. Some of them are precursors of biogenic amines obtained by decarboxylation, which are very important from the toxicity standpoint, and as quality control indices for fish spoilage.

During storage, changes in amino acids are caused by muscle autolysis and the concentration of the single components depends on a dynamic balance between their production and destruction, this balance being associated with muscle enzymes [37].

In the present study, starting from the 4th month, lysine (Figure 3D) and alanine (Figure 3F) began to increase more rapidly in sample at −10 °C and to a lesser extent in samples stored at −18 °C. In samples stored at –26 °C, by contrast, it remained constant. These results indicate a high level of proteolysis at storage temperatures above −26 °C. On the contrary, sarcosine (Figure 3E) displayed a variable trend. After a rapid decrease in the first months, it started to increase slowly in all samples until the 8th month and, thereafter, it decreased notably in all three samples.

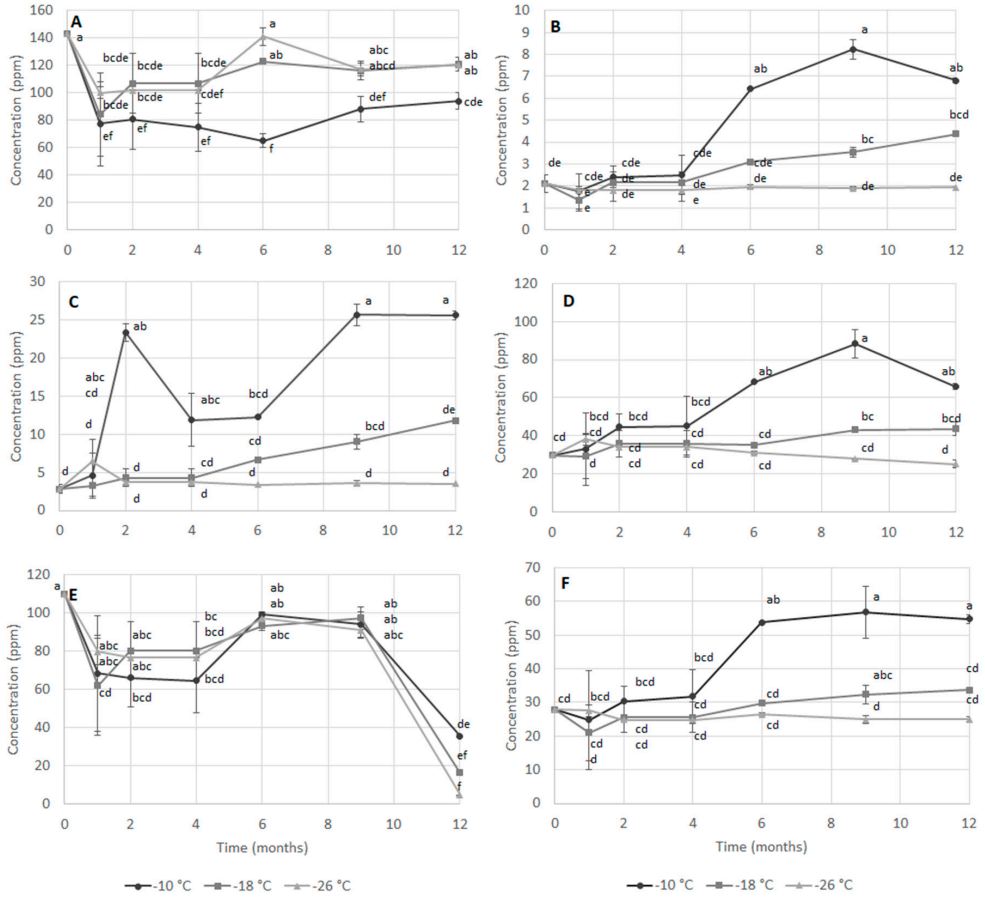

Figure 3. Concentration (ppm) of trimethylamine-O (TMA-O) (**A**), trimethylamine (TMA) (**B**), dimethylamine (DMA) (**C**), lysine (**D**), sarcosine (**E**) and alanine (**F**) measured by ^{1}HR-NMR in extracts of mantis shrimp mechanically separated flesh during frozen storage at −10, −18 and −26 °C. Different letters indicate significant differences (at $p < 0.05$) among samples.

Table 4. F (Fisher) values and relative significance of the influence of storage time (St) and temperature (T) and their interaction (St T) on quality parameters of mechanically separated mantis flesh, evaluated by ^{1}HR-NMR.

Factor	Parameter					
	TMA-O	TMA	DMA	Lys	Sarc	Ala
St	32.58 ***	21.24 ***	16.61 ***	8.02 ***	81.18 ***	7.84 ***
T	58.10 ***	41.77 ***	68.93 ***	35.67 ***	0.35 ns	30.04 ***
St T	45.17 ***	6.30 ***	7.66 **	4.15 ***	2.17 *	3.27 **

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant. Ala, alanine; Dimethylamine, DMA; Lys, lysine; Sar, sarcosine; TMA, trimethylamine-N; TMA-O, trimethylamine-O.

3.3. Data Correlation

The Pearson correlation matrix of all data obtained during frozen storage of samples is reported in Table 5. Luminosity showed high positive or negative correlation with the majority of the other tested parameters, proving to be a valid indirect parameter for the quality determination of frozen flesh. By contrast, the red index (a*) was not correlated to any other parameters.

Table 5. Correlation matrix among measured quality parameters of mechanically separated mantis flesh, after storage at different temperatures.

	L*	a*	TBARs	TMA-O	TMA	DMA	Lys	Sarc	Ala	FFA	MAG	TAG	SFA	MUFA	PUFA	n-6	n-3	n-6/n-3
L*	-																	
a*	0.570	-																
TBARs	0.530	0.429	-															
TMAO	−0.858 *	−0.478	−0.803 *	-														
TMA	0.921 *	0.377	0.561	−0.873 *	-													
DMA	0.846 *	0.237	0.158	−0.642	0.903 *	-												
Lys	0.939 *	0.462	0.647	−0.890 *	0.986 *	0.847 *	-											
Sarc	−0.099	−0.096	0.384	0.083	−0.121	−0.323	0.009	-										
Ala	0.971 *	0.456	0.610	−0.888 *	0.976 *	0.863 *	0.990 *	−0.004	-									
FFA	0.882 *	0.367	0.458	−0.874 *	0.958 *	0.909 *	0.914 *	−0.367	0.911 *	-								
MAG	0.894 *	0.412	0.638	−0.928 *	0.984 *	0.845 *	0.972 *	−0.160	0.953 *	0.969 *	-							
TAG	−0.858 *	−0.335	−0.545	0.894 *	−0.966 *	−0.864 *	−0.924 *	0.312	−0.909 *	−0.984 *	−0.981 *	-						
SFA	−0.228	0.020	0.067	0.037	0.038	−0.074	−0.023	−0.331	−0.136	0.061	0.091	−0.167	-					
MUFA	0.021	−0.139	−0.167	0.110	−0.255	−0.136	−0.206	0.266	−0.087	−0.241	−0.290	0.339	−0.963 *	-				
PUFA	0.443	0.128	0.096	−0.221	0.211	0.282	0.286	0.408	0.381	0.143	0.147	−0.041	−0.951 *	0.832 *	-			
n-6	0.513	0.155	0.088	−0.250	0.310	0.399	0.380	0.392	0.465	0.229	0.233	−0.125	−0.896 *	0.742	0.986 *	-		
n-3	0.715	0.322	−0.067	−0.344	0.574	0.767 *	0.583	−0.047	0.649	0.551	0.480	−0.430	−0.592	0.387	0.747	0.829 *	-	
n-6/n-3	0.286	−0.043	0.167	−0.136	0.103	0.111	0.193	0.630	0.273	−0.015	0.041	0.081	−0.917 *	0.820 *	0.954 *	0.927 *	0.565	-

* indicates significant correlation between parameters ($p < 0.05$). Ala, alanine; DAG, diacylglycerols; Dimethylamine, DMA; EST, esterified sterols; FFA, free fatty acids; Lys, lysine; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Sar, sarcosine; SFA, saturated fatty acids; TAG, triacylglycerols; STE, sterols; TBARs, thiobarbituric acid reactive substances; TMA, trimethylamine-N; TMA-O, trimethylamine-O.

The use of TBARs for the determination of the oxidation level during storage only showed a significant correlation with TMA-O, confirming its low suitability for the discrimination of samples as described above.

The concentration of TMA-O, TMA and DMA were highly correlated to the content of lysine and alanine indicating that these components are strictly related to the protein breakdown occurring during shelf-life. They showed also highly significant correlation to some lipid classes, in particular FFA, MAG and TAG. This may indicate that the variation of all these indexes during storage at −10 °C is related to the same cause, probably a residual enzymatic activity. The relative content of the different FA classes instead did not show significant correlation with other quality parameters.

PCA was developed considering all parameters evaluated in this study and the score plot is reported in Figure 4. Table S3 reports the contribution of the variables to each component. Along PC1 (45.58%), samples stored at −10 °C after 6 and 12 months are clearly separated from the rest. Both samples stored at −26 °C and sample stored for 6 months at −18 °C were very close to the initial sample (0), while after 12 months of storage at −18 °C a separation occurred along the PC2 (33.79%), confirming the faster degradation rate due to increased storage temperature.

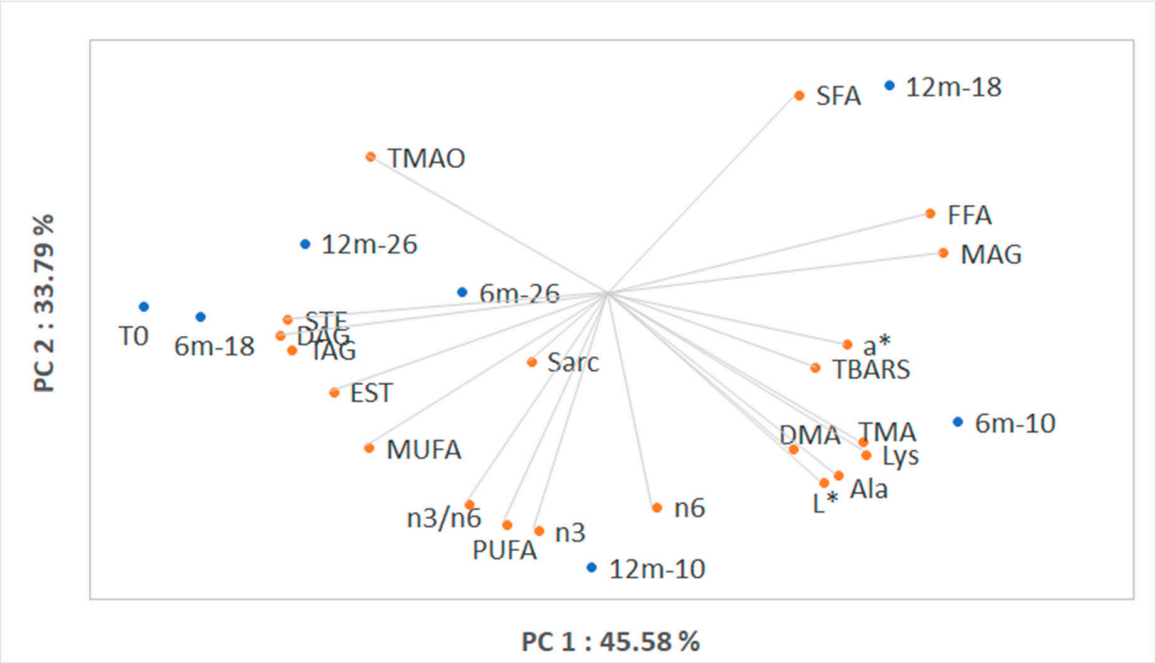

Figure 4. Scores and loadings biplot of data obtained from samples at the beginning of the storage (0), after 6 months (6 m) and 12 months (12 m) of storage at −10, −18 and −26 °C. PC, Principal Component; a*, red index; Ala, alanine; DAG, diacylglycerols; Dimethylamine, DMA; EST, esterified sterols; FFA, free fatty acids; L*, luminosity; Lys, lysine; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Sar, sarcosine; SFA, saturated fatty acids; TAG, triacylglycerols; STE, sterols; TBARs, thiobarbituric acid reactive substances; TMA, trimethylamine-N; TMA-O, trimethylamine-O.

The loading plot of the variables shows that the discrimination is related mainly to the transformation of TMA-O in TMA and DMA and to lipolysis leading to the release of FFA and MAG from TAG. The different FA classes were separated mainly along PC2. The colorimetric parameter of lightness (L*) showed a higher influence on the PC1 compared to the red parameter (a*). Among the considered amino acids, alanine and lysine concentration were highly correlated to the quality degradation, while sarcosine, being close to zero, showed a weak influence.

These results confirm that the quality of mechanically separated mantis flesh subjected to proper industrial and domestic frozen storage is preserved, thus representing a suitable processing and

storage technology for obtaining a valuable alternative source of n-3 and n-6 PUFA and essential amino acids for the development of innovative fish-based products addressed for human consumption. Attention should be paid in particular to avoiding the abuse of storage temperature (−10 °C) as it was demonstrated that it promoted an extensive lipid and protein degradation, due to both hydrolytic and oxidative reactions which affected the overall product quality. Whenever the frozen chain is abused, degraded frozen stored mechanically separated mantis flesh could be instead utilized for non-food sectors (animal feeding, pet food, pharmaceutical, cosmetic, etc.), thus contributing in any case to increasing the sustainability and the economic value of the overall food chain.

4. Conclusions

Mechanical separation and freezing of mantis shrimp flesh was carried out with the aim of valorizing an underutilized fish species from the Mediterranean characterized by high seasonality. This study demonstrated that the obtained product had a high content of PUFA, PUFA n-3 and a good PUFA n-6/PUFA n-3 ratio (< 4), confirming its high nutritional value. The separation process was shown to accelerate the initial oxidation phenomena in the flesh and to mainly promote color changes.

During frozen storage, the degradation rate was proportional to storage temperature and time, with very slight or absent quality changes at the lowest temperature (−26 °C) and a fast quality degradation at the abuse storage temperature (−10 °C). The main degradation phenomena observed were related to changes in the flesh luminosity, increase in lipid hydrolysis with the release of FFA and production of TMA and DMA probably ascribable to residual enzymatic activity and changes in amino acids concentration due to proteolytic activity.

The inter-disciplinary approach of this study permitted important findings, in terms of the extent of different degradative phenomena, related to processing and storage conditions of mechanically separated mantis flesh.

The current finding may help to develop a frozen product based on the *S. mantis* flesh characterized by a high added value aimed at the valorization of this seafood product, keeping in mind the problems related to its storage. Considering the high susceptibility of this product, in order to increase its value and its shelf-life at domestic refrigeration temperatures a possible solution could be the use of natural antioxidants added to the flesh and a strict control of the temperature during processing and storage.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/10/1485/s1: Table S1: Total fatty acid composition (expressed as % of total lipids) of mechanically separated mantis flesh (MSF) and fresh mantis flesh obtained by manual (FF), Table S2: Total fatty acid composition (expressed as % of total lipids) of mechanically separated mantis flesh, after 0, 6 and 12 months of storage at different temperatures, Table S3: Factor coordinates of the variables, based on correlations.

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Paper IV

Quality and stability of different seafood products treated with high hydrostatic pressure (HPP) intended for raw consumption.

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Quality and stability of different seafood products treated with high hydrostatic pressure (HPP) intended for raw consumption

Ana Cristina de Aguiar Saldanha Pinheiro[a], Silvia Tappi[a,b*], Giacomo Braschi[a,b], Jessica Genovese[a], Francesca Patrignani[a,b], Pietro Rocculi[a,b]

[a]Department of Agricultural and Food Science, *Alma Mater Studiorum*, University of Bologna, Campus of Food Science, Piazza Goidanich 60, Cesena (FC), Italy

[b]Interdepartmental Centre for Agri-Food Industrial Research, *Alma Mater Studiorum*, University of Bologna, Campus of Food Science, Via Ravennate 933, Cesena (FC), Italy.

* Corresponding author: silvia.tappi2@unibo.it

Abstract

The consumption of raw fish has rapidly increased in recent years, but being a highly perishable product, is characterized by a very short microbiological shelf-life. High hydrostatic pressure (HPP) processing is a non-thermal technology recently emerged as a promising alternative to thermal processing for food pasteurization capable of maintaining fresh-like characteristics and nutritional value. However, the induced changes on product quality should be carefully assessed. The present research aimed to investigate the effect of HPP on different seafood products, namely grey mullet, tiger prawn and rose shrimp, intended for the raw consumption. Three pressure levels (400, 500 and 600 MPa) were applied for 10 min. During refrigerated storage, microbiological quality, chemical parameters, colour and texture and fat oxidation were analysed.

Results showed that the application of the lower pressure was able to inactivate *E. coli*, *Pseudomonas* and/or positive coagulase staphylococci, however they were able to recover during storage. On the other side, the application of 600 MPa allowed to extend the microbiological shelf life up to 30 days. For all samples, a general whitening occurred while the texture was affected in a different way for the three considered species. Fat oxidation was only minimally affected and remained quite low during storage.

Keywords: grey mullet, tiger prawn, rose shrimp, microbiological inactivation, shelf-life

1. Introduction

The consumption of raw fish has rapidly increased in recent years, also in areas where it was not a traditional habit, due to changes in food taste but also to the adoption of culinary traditions of other countries. Sushi and sashimi that are typically oriental specialities are becoming increasingly popular also in European countries. Moreover, the use of low temperature cooking and processing such as cold smoking is spreading fast (Brutti et al. 2010).

These new habits have increased the microbiological risk for fish product consumption. Moreover, seafood products are highly perishable, their microbiological shelf-life is very short and, in order to sell a fish product to be consumed raw, a strategy to increase its shelf-life could increase its marketability. Therefore, a non-thermal technology able to reduce the microbial load is highly necessary.

High pressure processing (HPP) is a non-thermal technology that has recently emerged as a promising alternative to thermal processing for food pasteurization capable of maintaining fresh-like characteristics and nutritional value. The application of pressure higher than 300 MPa for few minutes at room temperature has shown to significantly reduce the initial microbial load in many fish species (Truong et al. 2015). The extent of microbial inactivation depends on treatment parameters such as pressure level, holding time, temperature, but also on the characteristics of the microflora in the product (Truong et al. 2015). The inactivation of microorganisms by HPP is the result of a combination of factors including changes in the cell membranes, cell walls, proteins and enzyme-mediated cellular functions (Campus 2010).

Cell membranes are the primary site of pressure-induced damage, with consequent alterations of cell permeability, transport systems, loss of osmotic responsiveness, organelle disruption and inability to maintain intracellular pH.

In general, Gram-negatives and cells in the growth phase are more sensitive than Gram positives and cells in the stationary phase. Nevertheless, investigations have shown that cell disruption is more depending on the geometry of the bacteria rather than to the Gram type. For example, morphological changes for the rod-shaped *Escherichia coli* and *Pseudomonas aeruginosa* were observed whereas *Staphylococcus aureus* (cocci) was more resistant to pressure.

However, in complex matrices like food the desired effect on microbial inactivation may also produce physical and biochemical changes which may affect the product properties in a negative manner.

The denaturation of proteins in fish muscle could cause significant changes of important parameters for consumer acceptability. In particular, the application of high pressures is known to lead to a cooked appearance (Matser et al. 2000), that can be specifically detrimental in products intended for raw consumption. Moreover, the effect on protein structure and on enzymatic activity can lead to variation of textural properties of seafood products, not only after the treatment but also during the refrigerated storage. The effect of HPP has been studied in a variety of fish and seafood matrices, but results are very variable depending on process parameters but also on specific product characteristics (Truong et al. 2015).

In a product aimed to the raw consumption, the microbiological quality is of paramount importance throughout the shelf life, however, the effect of HPP on quality might lead to undesirable changes.

The aim of the present research was to investigate the effect of HPP treatment on different types of seafood products, i.e grey mullet, tiger prawn and rose shrimp, intended for the raw consumption. Because the EU legislation requires seafood products to be frozen for at least 24 h before raw consumption, HPP was applied to frozen-thawed products. Three pressure levels were applied, and microbiological quality, safety, chemical parameters, colour and texture were analysed during the refrigerated storage.

2. Materials and methods

2.1 Fish samples preparation

Grey mullet (*Mugil cephalus*), striped prawn (*Melicertus kerathurus*) and deep-water rose shrimp (*Parapenaeus longirostris*) were fished in the Adriatic Sea. Products were fast frozen in an industrial blast chiller at a temperature of -18°C and kept for 24 h by the company Economia del Mare (Cesenatico, Italy). Thawing was carried out at 4°C for 16 h, then seafood samples were subjected to mechanical deboning and shell removal. Flesh was manually cut into pieces and packed in polypropylene (PP) trays containing 6 monoportions of about 15-20 g each that were packed under vacuum with a PP film.

2.2. HPP treatment

Vacuum packed samples were subjected to HPP treatments performed by the company HPP Italia s.r.l (Parma, Italy). Samples were placed in 350 L chamber, filled with water and subjected to 400, 500 or 600 MPa for a total time of 10 min. Untreated sample was used as control. Samples were coded using the initial of the specie (M for grey mullet, P for striped prawn and S for rose shrimp) and the pressure level applied (0, 400, 500 and 600). For each treatment 24 packages were prepared.

2.3 Storage

After treatment, samples were carried to the laboratories of the Campus of Food Science of the University of Bologna, where they were stored at 2±1°C. During storage, samples were subjected to analytical determinations after 0, 1, 6, 9, 14, 21, 28 and 35 days. Storage duration was determined for each sample on the basis of the results of microbiological analysis, considering the end of the shelf life when reaching a microbial load of 6 log cfu/g referred to total mesophilic bacteria.

For each HPP treatment, at each storage time, 3 different packages were used.

2.4 Analytical determinations

2.4.1 Microbiological analysis

Microbiological analyses were performed on untreated grey mullet, striped prawn and red shrimp and samples treated at 400, 500 and 600 MPa. All the samples, immediately after HPP treatments and along the storage time, were investigated for the presence of *Salmonella spp.* and *Listeria monocytogenes* according to EN ISO 6579-1:2017/A1:2020 and ISO 11290-1:2017, respectively. Microbial groups considered in this research were total mesophilic bacteria (TMB), *Lactobacillus spp.*, *Pseudomonas*, sulfite reducing anaerobic bacteria, total *Coliforms, E. coli* and coagulase positive staphylococci. 10 g of samples were serially diluted using physiological saline solution (0.9% NaCl) and appropriate inoculum was included or spread in different selective colture media such as plate count agar-PCA (Oxoid-Thermofisher, Milan, Italy) for TMB; De Man, Rogosa and Sharpe agar MRS (Oxoid-Thermofisher, Milan, Italy) supplemented with cycloheximide (0.2% p/v) for *Lactobacillus* spp.; Selective *Pseudomonas* Agar Base-PAB (Oxoid-Thermofisher, Milan, Italy) for *Pseudomonas* spp; Reinforced Clostridial Agar-RCA (Oxoid-Thermofisher, Milan, Italy) for sulfite reducing anaerobic bacteria, Violet Red Bile Agar-VRBA (Oxoid-Thermofisher, Milan, Italy) supplemented with 4-Methylumbelliferyl-β-D-glucuronide (MUG, Oxoid-Thermofisher, Milan, Italy) for total Coliforms and *E.coli*, respectively, and Baird Parker agar (BP) for coagulase positive Staphylococci. Plates were incubated for 24/48 h at 30°C for *Pseudomonas* spp. (PA) and 37 °C for Lactobacilli, sulfite reducing anaerobic bacteria, total Coliform, *E. coli* and coagulase positive staphylococci. Sulfite reducing anaerobic bacteria were incubated in anaerobic conditions using gas generating kit (Oxoid-Thermofisher, Milan, Italy).

2.4.2 pH and moisture content

pH values were assessed in samples homogenized for 60 s with an Ultraturrax (T-25, Ika, Germany) with distilled water (1:2 sample:water ratio w/w) with a pH meter (Crison, Barcellona). Moisture

was evaluated with gravimetric method by drying samples in an oven at 70°C until constant weight. The analysis has been performed at least in triplicate for each sample.

2.4.3 Colour

Colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured with a spectrophotocolorimeter mod. ColorFlex™ (Hunterlab, Reston, Virginia). The tristimulus L*, a*, b* measurement mode (CIE, 1976) was used. The hue angle (H^0) was calculated as follow:

$$h° = \left(\left(\tan^{-1}\frac{b*}{a*}\right)/2\pi\right) \times 360$$

For each sample and storage time, the average of at least 15 measurements was calculated.

2.4.4 Texture

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 sample were inserted in a cylindrical cup (diameter of 3 cm) and a piston was used to compress the sample up to 50% of its height. The pressure was held for 60 s. Maximum force was considered as hardness (F_1, N) and the force at the end of the compression was considered as index of resistance to compression (F_2, N). Texture was evaluated on at least 6 replicates for each sample.

2.4.5 Peroxide value (PV)

Lipids were extracted from 25 g of sample with a method previously described by Bligh & Dyer (1959). 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. The analysis has been performed at least in triplicate for each sample.

2.5 Statistical analysis

Significance of differences was tested by the analysis of variance (ANOVA) using Tukey HSD as post-hoc test ($p<0.05$). Statistical analysis was carried out with the software STATISTICA 8.0 for Windows.

3 Results and discussion

Microbial inactivation

The effects of HPP treatments (400, 500 and 600 MPa), compared to the untreated controls, on the microbiological quality of packaged grey mullet, striped prawn and rose shrimp are reported in **Tables 1, 2 and 3**, respectively. In all the tested conditions, *Salmonella* spp. and *Listeria monocytogenes* were never detected during the shelf-life of the considered products.

Table 1 Evolution of microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas* spp., total Coliforms, sulfite reducing anaerobic bacteria (AB), *E. coli* during the refrigerated storage of packaged grey mullet (*Mugil cephalus*) flesh in relation to the High Hydrostatic Pressure (HPP) treatments applied (400, 500, 600 MPa).

	Mullet	Log CFU/g			
		M-0	M-400	M-500	M-600
Days of storage 0d	TMB	4.67 ± 0.43	<1	<1	<1
	Lactobacillus spp.	3.18 ± 0.33	<1	<1	<1
	Sulfite reducing AB	4.20 ± 0.54	<1	<1	<1
	Pseudomonas spp.	4.45 ± 0.22	<1	<1	<1
	Total Coliforms	2.56 ± 0.38	<1	<1	<1
	E. coli	1.12 ± 0.10	<1	<1	<1
2d	TMB	5.14 ± 0.44	<1	<1	<1
	Lactobacillus spp.	3.30 ± 0.29	<1	<1	<1
	Sulfite reducing AB	5.15 ± 0.51	<1	<1	<1
	Pseudomonas spp.	5.20 ± 0.45	<1	<1	<1
	Total coliforms	1.80 ± 0.12	<1	<1	<1
	E. coli	1.22 ± 0.10	<1	<1	<1
6d	TMB	6.22 ± 0.52	<1	<1	<1
	Lactobacillus spp.	4.00 ± 0.28	<1	<1	<1
	Sulfite reducing AB	5.54 ± 0.46	<1	<1	<1
	Pseudomonas spp.	5.27 ± 0.35	<1	<1	<1
	Total coliforms	1.85 ± 0.16	<1	<1	<1
	E. coli	1.50 ± 0.20	<1	<1	<1
12d	TMB	-*	5.67 ± 0.31	<1	<1
	Lactobacillus spp.	-	<1	<1	<1
	Sulfite reducing AB	-	<1	<1	<1

	Pseudomonas spp.	-	2.46 ± 0.29	<1	<1
	Total coliforms	-	<1	<1	<1
	E. coli		<1	<1	<1
19d	TMB	-	7.52[a] ± 0.43	4.22 [b] ± 0.41	2.20 [c] ± 0.23
	Lactobacillus spp.	-	1.06 ± 0.32	<1	<1
	Sulfite reducing AB	-	<1	<1	<1
	Pseudomonas spp.	-	5.11 ± 0.29	<1	<1
	Total coliforms	-	<1	<1	<1
	E. coli		<1	<1	<1
32d	TMB	-	-	5.55[a] ± 0.42	3.10 [b] ± 0.33
	Lactobacillus spp.	-	-	<1	<1
	Sulfite reducing AB	-	-	<1	<1
	Pseudomonas spp.	-	-	<1	<1
	Total coliforms	-	-	<1	<1
	E. coli	-	-	<1	<1

-[*]: not analyzed since the microbiological threshold fixed at 6 log CFU/g was reached in the previous time of analysis

In the same row, values with different letter are significantly different ($p < 0.05$).

Also, coagulase positive staphylococci were never found in untreated and HPP treated samples of grey mullets and striped prawn. In general, the application of the HPP treatments increased the microbiological shelf-life of the considered products and the inactivation effect became more severe as the pressure increased. The microbiological threshold to define product shelf-life was fixed at the attaining of 6 log CFU/g for total mesophilic bacteria (TMB) even if, according to the Regulation 2073/2005, other important microbiological criteria such as the cell load of *Escherichia coli* and positive coagulase staphylococci were considered in the data discussion.

More specifically, the detected microbiological data for grey mullets, untreated and in relation to the pressure applied and time of storage, are reported in **Table 1**. As clearly showed, the control sample spoiled within 6 days of storage at 2°C, reaching a cell load of total mesophilic bacteria of 6.22 log CFU/g with a corresponding level of total coliforms of 1.8 log CFU/g. In contrast, the application of treatments of 400, 500 and 600 MPa prolonged the product shelf-life to 12, 19 and 32 d, respectively. The application of HPP treatments ranging between 400 and 600 MPa decreased the cell loads of *E. coli,* compared to the untreated sample, under the detection limit (1 log CFU/g). *Pseudomonas* spp were strongly affected by the level of HPP applied since cell loads of 2.4 CFU/g

Table 2. Evolution of microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas* spp., total Coliforms, sulfite reducing anaerobic bacteria (AB), *E. coli* during the refrigerated storage of packaged striped prawn in relation to the High Hydrostatic Pressure (HPP) treatments applied (400, 500, 600 MPa).

Days of storage	Striped prawn	Log CFU/g			
		P-0	P-400	P-500	P-600
0d	TMB	4.65[a] ± 0.44	2.45[b] ± 0.43	<1	<1
	Lactobacillus spp.	3.95 ± 0.29	<1*	<1	<1
	Sulfite reducing AB	3.83 ± 0.39	<1	<1	<1
	Pseudomonas spp.	4.63 ± 0.56	<1	<1	<1
	Total coliforms	4.60[a] ± 0.48	1.30[b] ± 0.33	<1	<1
	E. coli	2.10 ± 0.33	<1	<1	<1
2d	TMB	4.50[a] ± 0.46	2.96[b] ± 0.43	<1	<1
	Lactobacillus spp.	3.50 ± 0.36	<1	<1	<1
	Sulfite reducing AB	3.20 ± 0.51	<1	<1	<1
	Pseudomonas spp.	5.25 ± 0.48	<1	<1	<1
	Total coliforms	4.75 ± 0.49	<1	<1	<1
	E. coli	2.80 ± 0.15	<1	<1	<1
6d	TMB	6.30[a] ± 0.52	2.88[b] ± 0.55	<1	<1
	Lactobacillus spp.	5.82 ± 0.42	<1	<1	<1
	Sulfite reducing AB	4.42 ± 0.49	<1	<1	<1
	Pseudomonas spp.	5.35 ± 0.35	<1	<1	<1
	Total coliforms	3.75 ± 0.46	<1	<1	<1
	E. coli	2.95 ± 0.15	<1	<1	<1
12d	TMB	7.20[a] ± 0.39	4.61[b] ± 0.23	2.00[c] ± 0.15	1.50[c] ± 0.50
	Lactobacillus spp.	6.80[a] ± 0.55	5.12[b] ± 0.34	<1	<1
	Sulfite reducing AB	5.84 ± 0.51	<1	<1	<1
	Pseudomonas spp.	5.30[a] ± 0.45	3.88[b] ± 0.39	<1	<1
	Total coliforms	3.72[a] ± 0.43	1.48[b] ± 0.46	<1	<1
	E. coli	3.20 ± 0.23	<1	<1	<1
19d	TMB	-	8.35[a] ± 0.48	6.18[b] ± 0.35	4.80[c] ± 0.39
	Lactobacillus spp.	-	6.56[a] ± 0.30	4.59[b] ± 0.33	<1
	Sulfite reducing AB	-	<1	<1	<1
	Pseudomonas spp.	-	7.83[a] ± 0.37	5.72[b] ± 0.47	<1
	Total coliforms	-	1.95 ± 0.34	<1	<1
	E. coli	-	<1	<1	<1
32d	TMB	-	-	-	6.51 ± 0.39
	Lactobacillus spp.	-	-	-	<1
	Sulfite reducing AB	-	-	-	<1
	Pseudomonas spp.	-	-	-	<1
	Total coliforms	-	-	-	<1
	E. coli	-	-	-	<1

-*: not analyzed since the microbiological threshold fixed at 6 log CFU/g was reached in the previous time of analysis

In the same row, values with different letters are significantly different ($p < 0.05$).

were detected at 400 MPa after 12d from the treatment to further increase on the 19th day of storage. In all the other treated samples, and for each time of sampling considered, *Pseudomonas* spp was always under the detection limit (< 1 Log CFU/g).

Regarding untreated striped prawn (**Table 2**), the total mesophilic bacteria reached the threshold level of 6.0 log CFU/g after 6 days of refrigerated storage, while the application of HPP pressure levels of 500 and 600 MPa determined a significant increase of the product shelf-life to 19 and 32 d, respectively, and a cell load of *E. coli* always under the detection limit. However, for the sample treated at 500 MPa, after 19 days, Lactobacilli and *Pseudomonas* spp were able to recover and reach cell load of 4.49 and 5.72, respectively, differently from the samples treated at 600 MPa, where these microbial groups were found to always be under the detection limit.

The application of pressure level of 400 MPa determined a shelf-life of about 12 days. Similar trends were observed for shrimp products (**Table 3**) for which the application of pressure level of 600 MPa allowed to reach the threshold shelf-life after 28 days. Differently, the samples treated at 500 MPa reached the spoilage threshold after 21 days. It is interesting to evidence that the treatment at 400 MPa was not able to completely inactivate coagulase positive staphylococci, which were able to recover, but only after the shelf-life threshold.

The rationale for the use of HPP for fish and fish products is based on its ability to inactivate pathogenic and spoilage microorganisms and microbial enzymes, resulting in an increased shelf-life (de Alba et al. 2019) and also, an increased yield of the shucking process of bivalves and crustaceans (Patterson 2014). In general, according to the literature data, the HPP treatments on fish samples are commonly applied between 150 and 450 MPa (Perez-Won et al. 2020) since higher pressures, aimed to increase the microbial inactivation, are generally associated with significant changes in physicochemical, texture and sensory properties such as increase in discoloration, cooked appearance or lipid oxidation (Truong, Buckow, Stathopoulos, & Nguyen, 2015). However, the data resulting from this research have evidenced that treatments at 400 MPa are not able to significantly increase the shelf-life of the considered products. Although this level of pressure

seemed adequate to inactivate E. coli during the shelf-life period of the considered fish samples, other microbial groups, having a role in the spoilage and safety issue, such as Pseudomonas or positive coagulase staphylococci, were able to recover during the storage, highlighting the critical issue of viable but not culturable (VBNC) cells. In fact, although the efficiency of microbial inactivation is influenced by various factors, including the food matrix characteristics and food processing parameters, also the physiological diversity within a microbial population has to be taken into consideration, especially in the validation of the effectiveness of a treatment on a specific food product (Patrignani et al. 2019).

Table 3. Evolution of microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas* spp., total Coliforms, sulfite reducing anaerobic bacteria (AB), *E. coli,* positive coagulase (PC) *staphilococci* during the refrigerated storage of packaged rose shrimp in relation to the High Hydrostatic Pressure (HPP) treatments applied (400, 500, 600 MPa).

Days of storage	Rose shrimp	Log CFU/g			
		S-0	S-400	S-500	S-600
0d	TMB	5.04[a] ± 0.47	4.46[a,b] ± 0.56	3.78[b,c] ± 0.53	3.34[c] ± 0.45
	Lactobacillus spp.	5.34 ± 0.44	<1	<1	<1
	Sulfite reducing AB	4.53[a] ± 0.51	2.48[b] ± 0.35	2.38[b] ± 0.43	<1
	Pseudomonas spp.	5.16 ± 0.37	<1	<1	<1
	Total Coliforms	3.53 ± 0.46	<1	<1	<1
	E. coli	2.26 ± 0.45	<1	<1	<1
	PC staphilococci	2.20 ± 0.20	<2	<2	<2
7d	TMB	6.48[a] ± 0.49	5.18[b] ± 0.46	4.35[b,c] ± 0.39	3.63[c] ± 0.47
	Lactobacillus spp.	6.05 ± 0.55	<1	<1	<2
	Sulfite reducing AB	6.08 ± 0.36	<2	<2	<1
	Pseudomonas spp.	5.32 ± 0.42	<1	<1	<1
	Total coliforms	4.70 ± 0.20	<1	<1	<1
	E. coli	3.60 ± 0.15	<1	<1	<1
	PC staphilococci	3.40 ± 0.25	<2	<2	<2
14d	TMB	-*	8.15[a] ± 0.35	4.31[b] ± 0.51	4.20[b] ± 0.56
	Lactobacillus spp.	-	<2	<1	<1
	Sulfite reducing AB	-	<2	<2	<1
	Pseudomonas spp.	-	3.56 ± 0.44	<1	<1
	Total coliforms	-	<1	<1	<1
	E. coli	-	<1	<1	<1
	PC staphilococci	-	2.60 ± 0.41	<2	<2
21d	TMB	-	-	5.64 ± 0.42	3.6 ± 0.41
	Lactobacillus spp.	-	-	<1	<1
	Sulfite reducing AB	-	-	<2	<1

	Pseudomonas spp.	-	-	<1	<1
	Total coliforms	-	-	<1	<1
	E. coli	-	-	<1	<1
	PC staphilococci	-	-	<2	<2
	TMB	-	-	8.43 [a] ± 0.37	5.46 [b] ± 0.43
	Lactobacillus spp.	-	-	<1	<1
	Sulfite reducing AB.	-	-	4.32 ± 0.33	<1
28d	*Pseudomonas spp.*	-	-	<1	<1
	Total coliforms	-	-	<1	<1
	E. coli	-	-	<1	<1
	PC staphilococci	-	-	<2	<2
	TMB	-	-	-	7.49 ± 0.39
	Lactobacillus spp.	-	-	-	<1
	Sulfite reducing AB	-	-	-	<1
35	*Pseudomonas spp.*	-	-	-	<1
	Total coliforms	-	-	-	<1
	E. coli	-	-	-	<1
	PC staphilococci	-	-	-	<2

-*: not analyzed since the microbiological threshold fixed at 6 log CFU/g was reached in the previous time of analysis

In the same row, values with different letters are significantly different ($p < 0.05$).

In particular, *Pseudomonas* spp, strict aerobic bacteria, whose growth decreased in vacuum conditions, was able to recover in grey mullet and striped prawn samples treated at 400 MPa during their shelf-life period. *Pseudomonas*, having also a psychrotrophic behavior and being able to produce specific H_2S off-flavors, could have a negative impact also for the production of specific proteases potentially able to affect the textural properties of the food matrix.

Moisture content and pH

Moisture content was in the range of 77.0-78.8% in grey mullet, 76.1-78.2% in tiger prawn and 77.6-85.2% in rose shrimp. No differences were observed after the treatments and, during storage, only few significant differences were found, probably due to the natural variability of the raw material (data not reported).

The initial pH values measured in the samples according to the HPP treatment and during storage are reported in **Table 4** for grey mullet, tiger prawn and rose shrimp. The initial values for the untreated sample were 6.4 ± 0.1, 7.2 ± 0.01, 7.6 ± 0.03 for grey mullet, tiger prawn and rose shrimp,

Table 4. pH and Peroxide values (PV) (meq O_2/kg fat) measured in HPP treated seafood samples during refrigerated storage.

	Storage time (days)						
	1	6	9	14	21	28	35
Sample				pH			
M-0	6.41 ± 0.13^{a}	6.34 ± 0.03^{b}	6.46 ± 0.05^{a}	6.23 ± 0.03^{b}			
M-400	6.44 ± 0.06^{a}	6.55 ± 0.06^{a}	6.52 ± 0.04^{a}	6.47 ± 0.02^{a}	6.34 ± 0.07^{c}		
M-500	6.44 ± 0.01^{a}	6.50 ± 0.04^{a}	6.45 ± 0.01^{a}	6.46 ± 0.01^{a}	6.51 ± 0.04^{a}	6.31 ± 0.10^{a}	6.51 ± 0.01^{a}
M-600	6.46 ± 0.01^{a}	6.50 ± 0.03^{a}	6.41 ± 0.02^{a}	6.45 ± 0.01^{a}	6.41 ± 0.03^{b}	6.40 ± 0.03^{a}	6.51 ± 0.01^{a}
P-0	7.18 ± 0.01^{b}	7.20± 0.01^{c}	7.54 ± 0.12^{a}	7.44 ± 0.06^{b}			
P-400	7.45 ± 0.01^{a}	7.29 ± 0.03^{b}	7.27 ± 0.01^{b}	7.25 ± 0.01^{c}	7.06 ± 0.01^{c}		
P-500	7.40 ± 0.01^{a}	7.45 ± 0.01^{a}	7.32 ± 0.01^{b}	7.24 ± 0.03^{c}	7.47 ± 0.02^{a}	7.27 ± 0.04^{b}	7.17 ± 0.01^{b}
P-600	7.50 ± 0.02^{a}	7.44 ± 0.08^{a}	7.26 ± 0.02^{b}	7.54 ± 0.02^{a}	7.36 ± 0.03^{b}	7.36 ± 0.01^{a}	7.37 ± 0.01^{a}
S-0	7.59 ± 0.04 ^{b}	7.68 ± 0.08^{a}		7.73 ± 0.03^{a}			
S-400	7.65 ± 0.05 ^{a}	7.44 ± 0.03^{b}		7.47 ± 0.03^{b}	7.46 ± 0.01^{a}		
S-500	7.55 ± 0.11 ^{b}	7.52 ± 0.02^{b}		7.50 ± 0.01^{b}	7.39 ± 0.03^{a}	7.37 ± 0.01^{b}	
S-600	7.74 ± 0.02 ^{a}	7.61 ± 0.01^{ab}		7.48 ± 0.01^{b}	7.51 ± 0.04^{a}	7.48 ± 0.01^{a}	7.41 ± 0.02
Sample				PV			
M-0	0.88 ± 0.09^{b}	0.55 ± 0.08^{b}	1.40 ± 0.05^{a}	0.33 ± 0.10^{b}			
M-400	1.00 ± 0.06^{b}	0.68 ± 0.10^{b}	0.77 ± 0.06^{b}	1.50 ± 0.11^{a}	1.52 ± 0.07^{a}		
M-500	0.99 ± 0.05^{b}	0.90 ± 0.14^{a}	1.00 ± 0.22^{ab}	1.25 ± 0.11^{a}	1.10 ± 0.06^{a}	1.51 ± 0.14^{a}	0.79 ± 0.04^{b}
M-600	1.28 ± 0.05^{a}	0.92 ± 0.07^{a}	0.92 ± 0.05^{b}	1.14 ± 0.05^{a}	1.57 ± 0.10^{a}	1.06 ± 0.17^{a}	1.75 ± 0.15^{a}
P-0	1.88 ± 0.03^{ab}	1.36 ± 0.15^{b}	1.56 ± 0.16^{b}				
P-400	2.03 ± 0.02^{a}	2.03 ± 0.11^{a}	1.37 ± 0.02^{c}	1.30 ± 0.14^{a}	1.30 ± 0.14^{a}		
P-500	1.54 ± 0.14^{b}	2.5 ± 0.07^{a}	2.68 ± 0.41^{a}	1.57 ± 0.11^{a}	1.92 ± 0.31^{a}	1.89 ± 0.07^{a}	1.78 ± 0.2 ^{a}
P-600	1.52 ± 0.11^{b}	0.70 ± 0.07^{c}	1.96 ± 0.11^{b}	1.44 ± 0.16^{a}	1.82 ± 0.12^{a}	0.89 ± 0.03^{b}	1.66 ± 0.12^{a}
S-0	0.91 ± 0.14 ^{a}	0.43 ± 0.02^{b}		0.59 ± 0.01^{b}			
S-400	0.63 ± 0.12 ^{a}	0.82 ± 0.13^{a}		0.59 ± 0.02^{b}	0.68 ± 0.11^{a}		
S-500	0.66 ± 0.08 ^{a}	0.86 ± 0.04^{a}		0.82 ± 0.05^{a}	0.72 ± 0.01^{a}	0.62 ± 0.04^{a}	
S-600	0.98 ± 0.10 ^{a}	0.67 ± 0.03^{ab}		0.85 ± 0.08^{a}	0.57 ± 0.13^{a}	0.49 ± 0.02^{a}	0.65 ± 0.04

Different letters indicate significant differences ($p<0.05$) among samples of the same specie at the same storage time.

respectively. While for grey mullet values were consistent with the literature (Tsogas et al. 2019), for prawn and shrimp they were slightly higher compared to previous data (Kaur et al. 2013; Bindu et al. 2013).

In the present study, despite no difference was observed among grey mullet samples just after treatment, after 6 days, pH was slightly higher in HPP treated samples compared to the untreated one. During storage, a slight decrease was observed for M-0, while for the samples subjected high pressure, values showed little variability until the end of the storage period.

On the other hand, for tiger prawn, after HPP all samples showed higher values compared to the control of about 7.2-7.5, without differences in relation to the different pressure levels applied.

However, while for the control sample an increase of pH was observed during storage, treated samples showed an opposite trend (Figure 1S). In sample P-400 values decreased of about 0.4 points, while in P-500 and P-600 they showed little variability. In rose shrimp, only S-600 showed a significant increase compared to the untreated sample just after treatment. During storage, the control samples showed a slight increase, while for all treated samples the values decreased progressively.

The increase of pH after pressurization of fish and seafood tissues has been observed by many authors (Briones-Labarca et al. 2012; Bindu et al. 2013; Kaur et al. 2013; Angsupanich and Ledward 1998) and has been explained with the induction of protein unfolding by pressure and the following ionisation of denatured proteins.

On the other side, the evolution of pH during storage can be attributed mainly to the activity of spoilage microorganisms that can produce various compounds, both basic (e.g., ammonia, trimethylamine, and other biogenic amines) and acidic (e.g., lactic acid in the case of Lactobacillus).

Color

L* and h° values of the three seafood species are reported in **Figure 1**. Luminosity (L*) of the flesh was significantly increased for all the three considered species of about 20 units straight after the treatment. This effect has been largely observed in many fish species and it has been attributed to protein denaturation. Considering that 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 level adopted, it can be assumed that protein denaturation occurred in all samples to a similar extent for 400 to 600 MPa. During storage, all L* values were very close to the initial ones, for the control and the pressure treated samples.

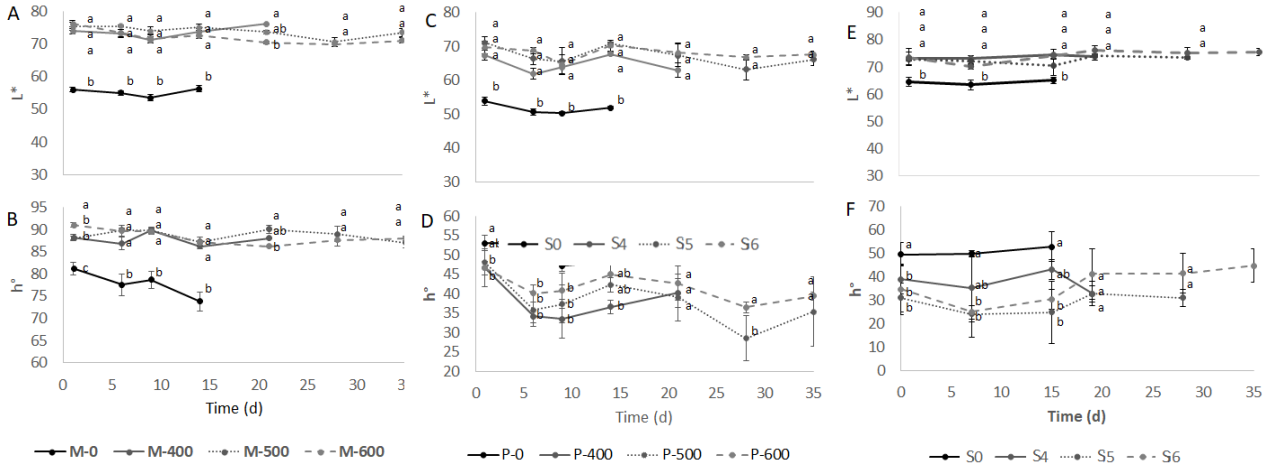

Fig. 1 Colour coordinates of luminosity (L*) and hue angle (h°) of mullet (A and B), Shrimp (C and D) and rose shrimp (E and F). Different letters indicate significant differences among samples ($p<0.05$).

Similarly, just after treatment, significant differences between control and treated samples were observed for the hue angle (h°). However, while for grey mullet h° was increased by HPP, for shrimp and prawn, the values of this chromatic parameter were decreased. h° is calculated using both red and yellow index; in all samples a* was remarkably decreased, while the changes in b* were higher in shrimp and prawn as compared to grey mullet. According to the literature, the variation of colour during storage can depend on the degradation of myofibrillar proteins and the disorganisation of myofibrils caused by enzymatic and non-enzymatic reactions, but also on the possible oxidation of pigments (Yagiz, Kristinsson, Balaban, & Marshall, 2007). Hence, colour change occurring during storage in seafood products subjected to high pressures can differ significantly according to the species and the adopted treatment conditions. In the present study, the final effect for all samples was a general whitening and the occurrence of a cooked appearance, that is typical for muscle food subjected to pressurization (Figure 2S).

Considering that the visual quality is a very important parameter for consumer acceptability, and that these products are intended for raw consumption, the cooked appearance might represent a problem that could be probably addressed with a marketing and/or informative strategy.

Texture

Texture is a very important parameter for seafood products appreciation. Even if, generally, on fish fillets the Texture Profile analysis (TPA) test is carried out, considering the specific characteristics of the investigated products, in the present study a compression with following application of steady pressure was applied. Results of the two parameters analysed (hardness (F1) and the index of resistance to compression (F2), Figure 3S), are reported in **Figure 2** for all the three considered species. In the present work, the observed effect was different for the three considered species.

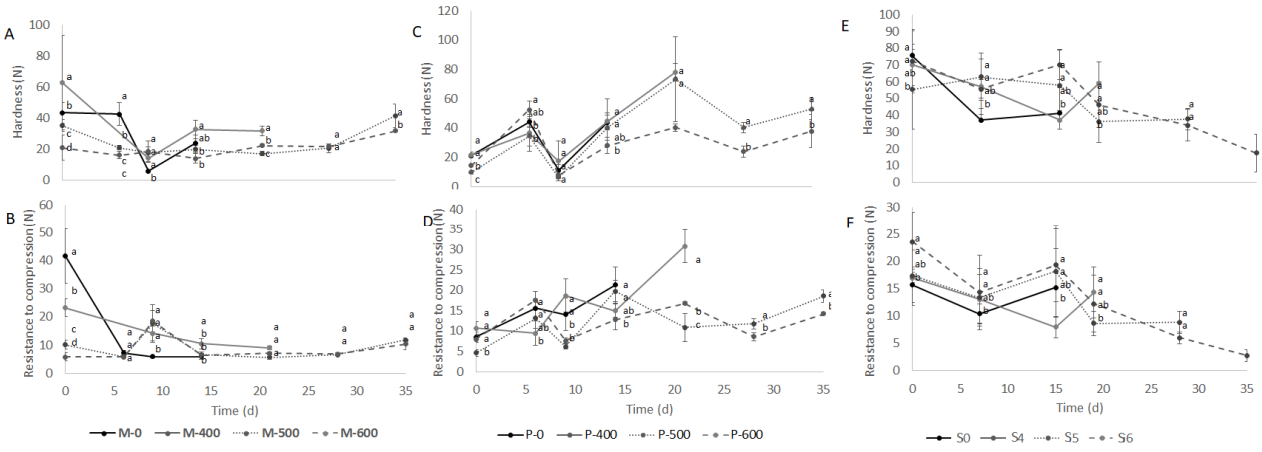

Fig. 2 Texture parameters of Hardness (N) and Resistance to compression (N) of mullet (A and B), Shrimp (C and D) and rose shrimp (E and F). Different letters indicate significant differences among samples ($p<0.05$).

For grey mullet (**Figure 2A-B**), a reduction in the initial hardness was observed for the higher applied pressures (500 and 600 MPa) just after the treatments. Moreover, the increasing pressure

promoted a decrease in the resistance to compression of the tissues (**Figure 2B**) proportionally to the applied pressure level. For both parameters, in the control sample a decrease was observed during storage, as reported by Chéret, Chapleau, Delbarre-Ladrat, Verrez-Bagnis, & Lamballerie (2005) for seabass. The softening of the tissue during refrigerated storage can be attributed to enzymatic activity of proteases that can act on both myofibrillar proteins and connective tissue bringing about myofibrillar fragility and gaping. A similar behaviour can be observed for the M-400 sample, but with a more gradual decrease of both values. Indeed, after nine days, the resistance to compression was significantly higher compared to the control, possibly for a partial enzymatic denaturation due to pressure. The M-500 and M-600 instead showed quite constant values for both parameters for all storage period with few differences between them.

In striped prawn (**Figure 2C-D**), a slight but significant reduction of hardness was observed for the 500 and 600 MPa treatments, while the resistance parameter was reduced only for the 600 MPa. During storage, hardness showed a fluctuating behaviour, but tended to increase compared to the initial value for the P-0, P-400 and P-500 samples, while it remained practically unchanged for the P-600 one. Resistance was shown to increase during storage only in the P-0 and P-400 samples, although the storage was shorter due to microbiological spoilage.

On the contrary, in rose shrimp (**Figure 2E-F**), hardness was not influenced by any pressure applied while the resistance to compression was found significantly higher after the 600 MPa treatment. During storage, both parameters showed a decreasing trend for all samples. Values were highly variables and very few significant changes were observed.

These results are in contrast with the increase in hardness measured after high pressure application by Bindu et al. (2013) in Indian white prawn and by Jantakoson, Kijroongrojana, & Benjakul (2012) and Kaur et al. (2013) in black tiger shrimp. Moreover, the behaviour of the two considered parameters during storage is different between the two crustaceans. A tendency to increase is found in prawn, while in shrimp values progressively decreased for the considered period. Decrease of hardness during storage was also observed by Kaur et al. (2013) that attributed it to the effect of

proteolytic enzymes. However, values were mostly constant for hardness in the P-600 sample and for resistance for the P-500 and P-600. This effect might be explained by a partial inactivation of such enzymes.

Generally, after HPP an increase in hardness has been observed by many authors on different fish species such as rainbow trout and mahi mahi (Yagiz et al., 2007), cod (Angsupanich and Ledward 1998) tuna (Zare 2004) that was explained by the unfolding of actin and sarcoplasmic proteins and the formation of new hydrogen bonded networks and by an increase in protein–protein interactions and bond formation. On the other hand, Briones-Labarca et al. (2012) found no differences in red abalone treated with HPP up to about 500 MPa compared to the control. (Chéret et al. 2005) observed a softening effect after subjecting seabass to pressures up to 300 MPa, while 400 and 500 MPa treatments did not promote changes in the hardness.

Beside the modification to the myofibrillar proteins, HPP also promotes pH changes and modification of hydrogen and hydrophobic bonds that result in changes in the structural characteristics of proteins. Moreover, an effect on collagen and connective tissue of red abalone was observed by Briones-Labarca et al. (2012) through scanning electric microscope, that confirmed a significant change in the microstructure of the flesh upon high pressure application. Hence, the effect of HPP on fish texture is the resultant of all the modifications to water bonding and holding capacity, activity of enzymes such as proteases that can be inhibited or enhanced, and structural modification of myofibrillar and sarcoplasmic protein.

The results obtained in the present study confirmed that the effect on texture is strictly dependent on the considered specie of the raw material and the specific tissue structure. Moreover, the effect observed during storage probably depends on the possible inactivation of proteolytic enzymes that again, is probably matrix dependent.

The effect of HPP on proteolytic enzymes in fish has been studied on many species by different authors, but results appear to be quite variable depending on seafood species, pressure level and holding time and type and structure of the enzyme.

Low pressure levels of about 100 MPa showed to enhance enzymatic activity of calpain in sea bass muscle treated at 100 MPa and 10 °C for 5 min (Chéret et al. 2005), while (Teixeira et al. 2013) observed that pressurization rate could also play a role in the activation of the same enzyme in sea bass fillets. Generally, increasing pressure level and holding times were shown to inactivate enzymes. However, although seafood muscles have been shown to be more sensitive to pressure compared to bovine ones, the food matrix can strongly influence the effect on enzymes (Truong et al. 2015). The application of pressure causes a variation in protein structure that can lead to the breakdown of cell membrane and release of proteolytic enzymes in the cytoplasm, favouring their denaturation.

Lipid oxidation

Table 4 reports the PV measured in the three considered species subjected to HPP treatment during refrigerated storage. A slight but significant increase of this parameter was observed after treatment in grey mullet samples pressurized at 600 MPa. However, while no differences were observed for rose shrimp, lower values were measured in prawn samples treated at the higher pressures. Moreover, PV values remained very low for all the storage period considered (below 1.75, 2.7 and 0.98 for grey mullet, prawn and shrimp respectively).

Generally, an enhancement of lipid oxidation has been observed in the literature for many seafood product in particular when pressures above 300 MPa were used (Truong et al. 2015). This increase was mainly attributed to the presence of haemoglobin and myoglobin, that containing iron in their structure that is released as a consequence of pressurization, can act as oxidation promoters. However, (Yavuz Yagiz et al. 2009) found that a treatment of 300 MPa helped to inhibit lipid oxidation of Atlantic salmon compared to the untreated sample, and to the sample subjected to 150 MPa during storage. The authors suggested that the perturbation to changes to cell membrane structure brought about by the pressure actually made phospholipids less susceptible to oxidation

even in a fish that is considered fatty. Moreover, the presence of astaxanthin is believed to have a powerful antioxidant effect.

The effect of pressure on the activity of enzymes responsible for lipid oxidation are still scarce. The reduced oxidation level showed by the evolution of its primary index in the present study is probably due to different reasons. Firstly, the considered seafood species are characterized by a low fat content (about 2.5, 0.8 and 1.1 for grey mullet, prawn and shrimp respectively) and are therefore not very susceptible to lipid oxidation in the first place. Then, we can assume that further reduction of the susceptibility to oxidation might have been brought about by the modification of the cell membranes induced by pressure. The very low presence of oxygen in the packages has also surely contributed to inhibit oxidative reactions during storage.

4. Conclusions

The investigation of the effect of HPP treatment on the different types of considered seafood products, intended for raw consumption, highlighted a significant microbiological shelf-life increase at the highest applied pressure levels (500 and 600 MPa), for all the considered species.

Even if lower pressure (400 MPa) seemed adequate to inactivate *E. coli*, *Pseudomonas* and/or positive coagulase staphylococci, they were able to recover during fish product storage, stressing the issue of VBNC cells. On the other side the application of 600 MPa allowed to extend the microbiological shelf life up to around 30 days.

In terms of visual quality, the final effect for all samples was a general whitening and the occurrence of a cooked appearance, that is typical for muscle food subjected to pressurization. The texture response was strictly dependent on the considered species of the raw material and the specific tissue structure. Fat oxidation was only minimally affected and remained quite low during storage.

Further studies are in due course in our laboratories, in order to better clarify the physico-chemical and biological causes of the detected differences also through microstructural assessment, and their overall sensorial impact.

However, considering the effect on microbiological shelf life, for all the three considered species, reaching a pressure over 500 MPa seems necessary, particularly because the effect on quality did not show particular changes at the higher pressures. It is important to underline that in the optic of the commercialization of HPP treated seafood intended for raw consumption, a specific marketing/informative strategy is strictly needed, evidencing to the final consumer the important advantages of this non-thermal technology in terms of nutritional and sensorial food properties.

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Supplementary material

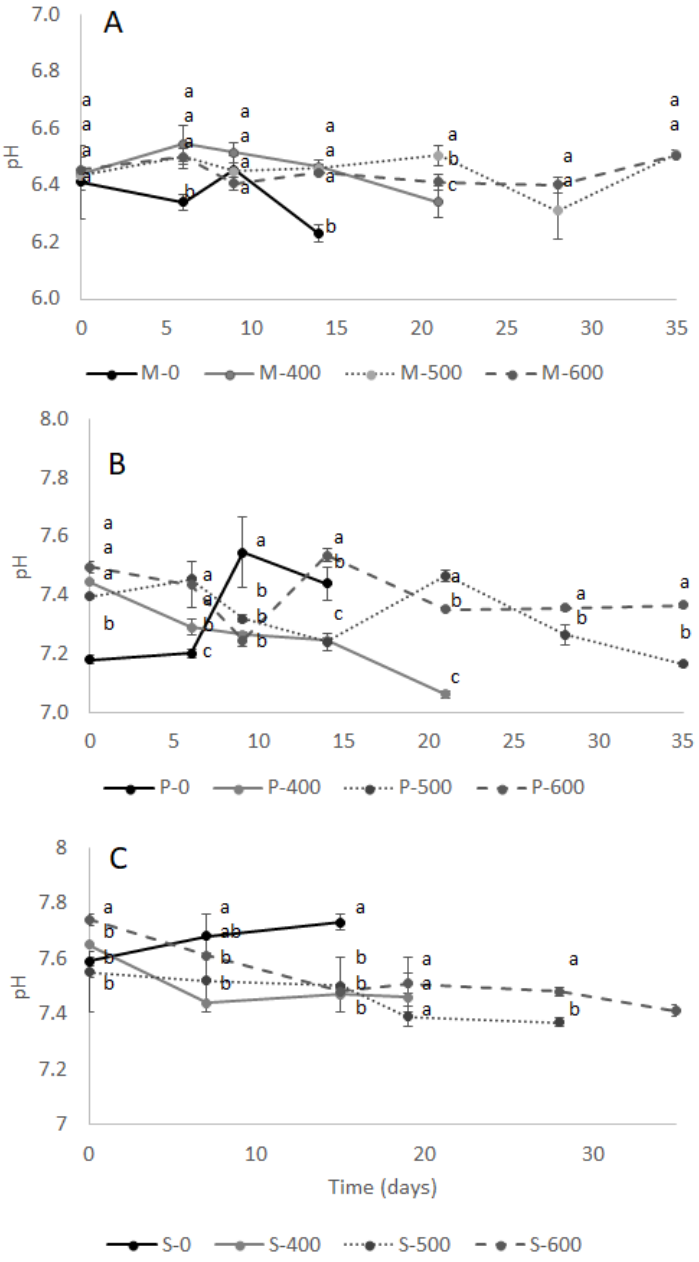

Fig 1S pH measured in HPP treated seafood samples during refrigerated storage**.**

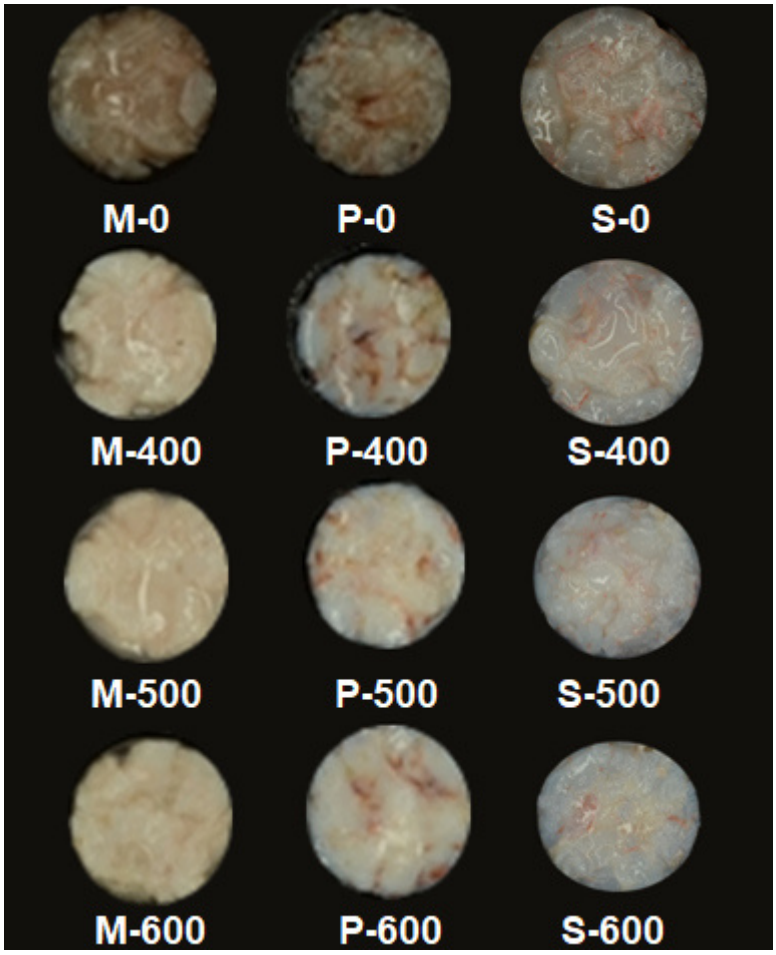

Fig 2S Digital images of grey mullet (M), striped prawn (P) and rose shrimp (S) samples treated at 400, 500 and 600 MPa compared to the untreated ones.

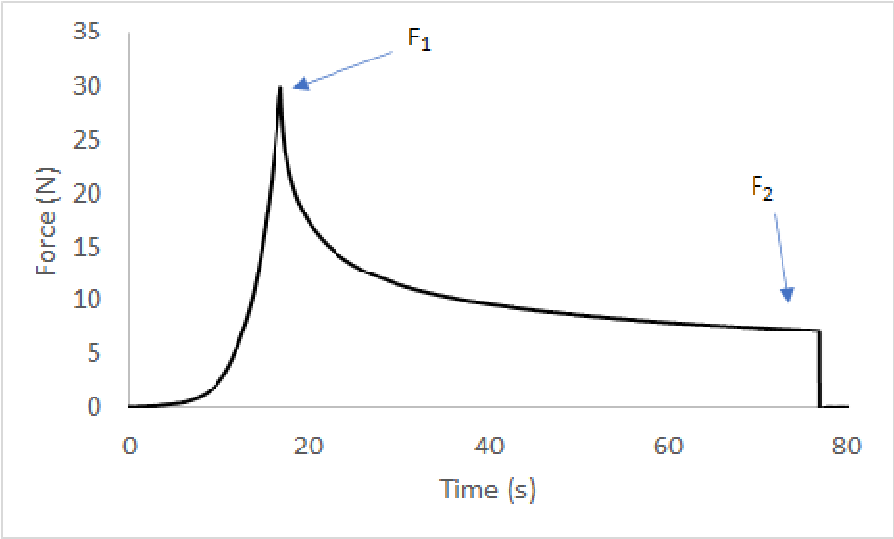

Fig 3S Results of the two parameters analysed (hardness (F1) and the index of resistance to compression (F2).

Paper V

Innovative Non-Thermal Technologies for Recovery and Valorization of Value-Added Products from Crustacean Processing By-Products—An Opportunity for a Circular Economy Approach.

Foods, 10(9), 2030

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Review

Innovative Non-Thermal Technologies for Recovery and Valorization of Value-Added Products from Crustacean Processing By-Products—An Opportunity for a Circular Economy Approach

Ana Cristina De Aguiar Saldanha Pinheiro [1], Francisco J. Martí-Quijal [2], Francisco J. Barba [2,*], Silvia Tappi [1,3] and Pietro Rocculi [1,3]

[1] Department of Agricultural and Food Science, Campus of Food Science, Alma Mater Studiorum, University of Bologna, Piazza Goidanich, 60, 47522 Cesena, FC, Italy; anacristin.deaguiar2@unibo.it (A.C.D.A.S.P.); silvia.tappi2@unibo.it (S.T.); pietro.rocculi3@unibo.it (P.R.)
[2] Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine, Faculty of Pharmacy, Universitat de València, Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot, València, Spain; francisco.j.marti@uv.es
[3] Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Via Quinto Bucci, 336, 47521 Cesena, FC, Italy
* Correspondence: francisco.barba@uv.es; Tel.: +34-963544972

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Abstract: The crustacean processing industry has experienced significant growth over recent decades resulting in the production of a great number of by-products. Crustacean by-products contain several valuable components such as proteins, lipids, and carotenoids, especially astaxanthin and chitin. When isolated, these valuable compounds are characterized by bioactivities such as anti-microbial, antioxidant, and anti-cancer ones, and that could be used as nutraceutical ingredients or additives in the food, pharmaceutical, and cosmetic industries. Different innovative non-thermal technologies have appeared as promising, safe, and efficient tools to recover these valuable compounds. This review aims at providing a summary of the main compounds that can be extracted from crustacean by-products, and of the results obtained by applying the main innovative non-thermal processes for recovering such high-value products. Moreover, from the perspective of the circular economy approach, specific case studies on some current applications of the recovered compounds in the seafood industry are presented. The extraction of valuable components from crustacean by-products, combined with the development of novel technological strategies aimed at their recovery and purification, will allow for important results related to the long-term sustainability of the seafood industry to be obtained. Furthermore, the reuse of extracted components in seafood products is an interesting strategy to increase the value of the seafood sector overall. However, to date, there are limited industrial applications for this promising approach.

Keywords: chitosan; carotenoids; astaxanthin; non-thermal technologies; valuable compounds

1. Introduction

During the previous decade, the commercial production of processed fish and seafood products has significantly expanded with a consequent increase in by-product generation. Crustacean by-products represent a significant kind of by-product from seafood processing plants. Every year, approximately 6–8 million tons of waste is produced around the world following crustacean processing [1], mainly related to the recovery and conditioning of the edible parts of various crustaceans such as crabs, shrimps, and lobsters.

The major components of crustacean by-products (head and shells) are proteins (25–50%), followed by chitin (25–35%), minerals (15–35%), lipidic components (0.2–17%), and pigments [2,3]. Considering the increasing volumes generated and the length of the nat-

ural degradation process of shells, their efficient use is of paramount importance. The valorization of these residues, rather than their disposal or incineration, introduces the concept of circular economy to the seafood processing sector. As discussed by Ruiz-Salmón et al. [4] and Jacob et al. [5], among the challenges for increasing the sustainability of the European seafood sector, various approaches are being undertaken. The circular economy approach includes ensuring significant material savings throughout value chains and production processes but also generating extra value and unlocking economic opportunities.

Currently, crustacean by-products are mainly used for the recovery of chitin and chitosan, which is its deacetylated form. These compounds have been correlated to important biological activities, such as antioxidants, antimicrobial, and various other properties that could be exploited for food formulation to improve safety, quality, and shelf-life [6]. Moreover, other valuable components could be applied in the food and pharmaceutical industries, in particular, crustacean by-products can be exploited for the extraction of enzymes, products of protein hydrolysis (hydrolysates), lipids rich in polyunsaturated fatty acids (PUFA), and carotenoids could be also recovered from crustacean by-products [7,8].

The most common strategy to recover chitin and chitosan from crustacean by-products is still the use of chemical treatments (mainly involving strong alkali and acid), resulting, however, in negative economic and environmental consequences due to high costs and the production of harmful effluent wastewater [9]. Lately, the sustainable development of the environment and economy has gained increasing political and social interest, privileging the development of "green technologies" and the use of "green products" over conventional industrial ones. Moreover, concepts such as circular economy have been regarded as leading principles for eco-innovation, that aims a "zero waste" society and economy, in which waste and by-products are exploited as raw material for the development of new products and applications [10].

Innovative food processing technologies, based on non-thermal methods (i.e., ultrasound, high-pressure processing, pulsed electric fields, cold plasma, supercritical fluid extraction) have been proposed for use within the food industry including the extraction of valuable components from wastes and by-products [11]. The extraction of valuable compounds from crustacean by-products, combined with the development of novel technological strategies aimed at their recovery and purification, will allow for important results related to the long-term sustainability of the seafood industry to be obtained.

This review aims at providing a literature summary of the major crustacean by-products, the main emerging non-thermal process for their recovery, and the current applications in the seafood industry. First, the main potential valuable components recovered from crustacean processing by-products are described. Then, a summary of the most relevant research for the optimization of innovative non-thermal extraction technologies is reported regarding biomolecules from crustacean by-products obtained from their industrial processing. Finally, examples of the potential use and applications of the extracted compounds for quality improvement and shelf-life extension of the seafood products are summarized.

2. Crustacean By-Products as a Source of Valuable Compounds

Crustacean processing by-products (heads, shells, pleopods, and tails) contain several valuable compounds such as chitin, chitosan, carotenoids, lipids, and proteins, (Figure 1). These compounds show important biological activities, for instance, antioxidant, antimicrobial, and various other effects which can be exploited by the food industry with the aim of improving safety, quality, and shelf-life [12].

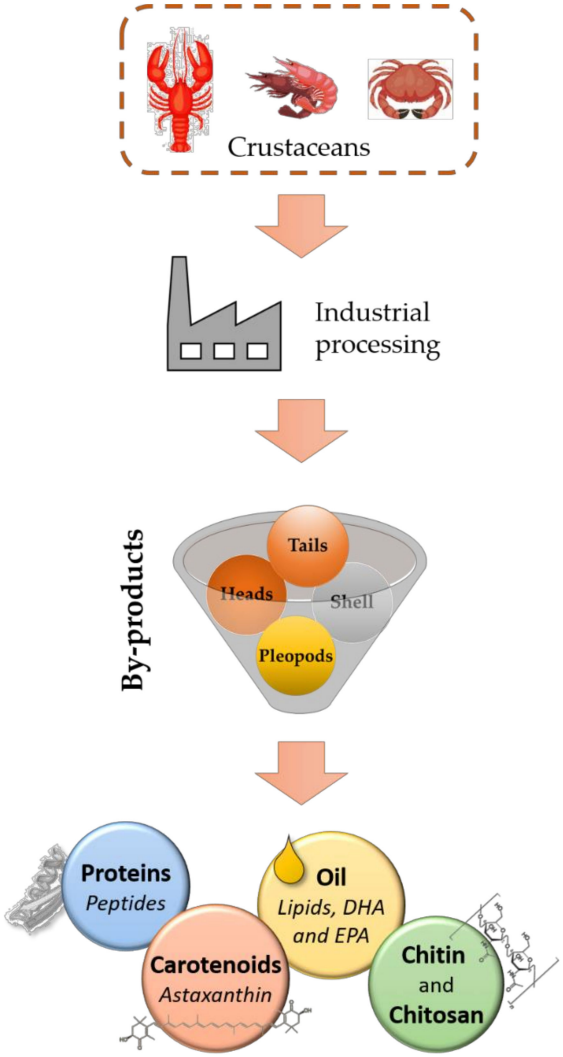

Figure 1. Valuable compounds derived from crustacean processing by-products.

2.1. Proteins

The crustacean processing industry generates by-products rich in high-quality proteins and amino acids. Shrimp heads are characterized by high amounts of protein (50–65% dry weight) and are a very good source of essential amino acids, which is the reason they are used in aquatic animal feeds and are also included in livestock and poultry diets [13]. Lobster by-products are also extremely rich in protein and are characterized by an amino acid profile comparable to red meat, although higher in non-protein nitrogen (in the range from 10–40%) [14]. In lobster liver, proteins represent up to 41% of the dry matter [15], while the head retaining fleshy parts (body, breast, and leg) is approximately 20% of the total weight [16]. Additionally, the nutritional value of the lobster protein is greatly enhanced by its natural binding with a large amount of astaxanthin (295 µg/g), a powerful antioxidant to form a protein–carotenoid complex known as carotenoprotein [17]. Carotenoprotein isolated from shrimp by-products has shown high antioxidant activity, as well as being a rich source of essential amino acids and carotenoids [18,19], and has the potential for use as an additive to enrich foods and promote human health benefits [20]. With respect to other seafood species, proteins obtained from crustaceans are characterized by a higher content of some amino acids such as glycine, glutamic acid, arginine, and alanine, resulting in increased palatability compared to finfish proteins [14]. Moreover, on account of its optimal essential amino acid profile, the nutritional value of crustacean protein is similar or even higher compared to red meat [21] or soya bean [22]. For this reason, protein hydrolysates from shrimp by-products have been used for the fortification of different types of food

products, such as biscuits [23] and bread [24]. Moreover, the functional properties of protein extracts from crustaceans have also been investigated for the production of an edible film [25].

The extraction efficiency of protein from crustacean by-products varies depending on the processing methods [26]. Hydrolysis is a common strategy for processing fish and shrimp waste with the aim of producing highly nutritive protein hydrolysates and recover bioactive molecules. Traditionally, protein hydrolysates from crustacean by-products have been obtained through the application of chemicals, microbial fermentation, and/or commercial enzymes [26]. However, chemical extraction leads to protein hydrolysates characterized by a higher degree of hydrolysis and lower efficiency of recovery, if compared to those obtained by enzymatic methods.

Enzymatic hydrolysis allows proteins to break down, altering their functional, chemical, and sensorial characteristics but maintaining the nutritional value [27].

Proteins can also be recovered from wash waters, e.g., from the washing process used to obtain surimi and from the peeling of shellfish and krill [28], but also from industrial cooking of crustaceans such as shrimp [29]. Apart from sarcoplasmic proteins and other water-soluble substances, a significant amount of functional myofibrillar protein can be found in waste waters. The recovery of these compounds is useful to reduce the amounts of contaminants and pollutants but also to valorize the by-product of industrial crustacean processing. There are many methods to recover these proteins, such as centrifugation, precipitation, micro- or ultra-filtration, and their combination. Ramyadevi et al. [30] optimized a process of aqueous two-phase system partitioning for the recovery and concentration of proteins obtained from the wash waters of shrimp. The functionality of the recovered proteins offers many possibilities for exploitation in further processes. For example, proteins recovered from shrimp surimi processing have been successfully exploited for the production of edible films [31].

2.2. Lipids

Crustaceans have appreciable proportions of ω-3 (omega-3) long-chain PUFA, particularly eicosapentaenoic and docosahexaenoic acid (EPA and DHA) [14]. The PUFAs are probably the most successful bioactive components isolated from marine sources because they have been widely recognized to be related to excellent health benefits [32].

Lipid content in crustacean by-products may be variable depending on the species, the fishery's geographical location, and the kind of by-products. Recently, Albalat et al. [33] showed that oil recovered from the head waste of the Norway Lobster (*Nephrops norvegicus*) contains a higher proportion of EPA and DHA (15.0% and 8.3% of total neutral lipids, respectively, than krill oil (4.3% of EPA and 2.3% of DHA)). However, the content and profile of the recovered lipid are subjected to considerable variations according to the geographic location of the fishery and the seasonality.

Among crustacean waste products, cephalothorax and hepatopancreas have also been used as an excellent source of lipids with high PUFAs content [34,35] with a yield of approximately 2.7% and 11.6%, respectively. Although, in both waste types, PUFA represented the major lipid class and fatty acid profiles were different. The lipids from cephalothorax showed higher amounts of both DHA and EPA than those from hepatopancreas.

The lipid extract from crustacean cephalothorax processing by-products, containing high levels of PUFAs (including DHA and EPA), α-tocopherol, and astaxanthin, has recently been suggested to be added as a natural ingredient to food formulation where it could exert different effects, as a food coloring and as a functional ingredient [33,36]. Biological activities that have been attributed to lipids derived from shrimp by-products include antioxidant, anti-proliferative, anti-mutagenic, and anti-inflammatory effects [36–38].

Cholesterol may be a significant constituent of the lipid content of crustaceans. In the Pacific white shrimp (*L. vannamei*) by-products (cephalothorax, shells, tails, and pleopods), the lipid extract showed an appreciable amount of cholesterol (65 ± 1 mg/g) [39], in raw shrimp this value is usually greater than 100 mg/100 g of the edible portion of shrimp [40].

Lipids from crustacean by-products are oxidatively unstable, and the processes involved in their extraction may significantly affect their yield, quality, and stability [34]. The presence of astaxanthin and α-tocopherol seems to increase lipidic extract stability on account of their antioxidant properties [39]. However, their content was found to decrease during storage. Therefore, to expand their industrial application and utilization, recovery strategies that can improve yields without causing detriment to the quality of the extracted oil are necessary [41].

The most commonly used method for lipid extraction is based on the use of solvents; however, the high temperatures and the toxicity of solvents have increased the need for alternative extraction technologies. Alternative methods, to enhance efficiency in extraction such as the microwave, ultrasound-assisted extraction, supercritical fluid extraction, etc., represent a more environmentally friendly choice, requiring less use of less toxic chemical compounds (green technologies) [25]. In recent years, encapsulation of shrimp lipid extracts has also been investigated with the aim of increasing their stability and potential applications in food products. Various encapsulation techniques have been described for the oil obtained by crustacean by-products, such as complex coacervation [42], microencapsulation [43], spray-drying [44], and nano-liposomes [45,46]. Gómez-Guillén et al. [36] reported that the encapsulation process improved different functional properties, especially the antioxidant and anti-inflammatory properties and the water solubility, and maximized the bioaccessibility of astaxanthin. Based on the obtained results, the authors suggested the incorporation of the encapsulated extract with bioactive and technological functionalities, in different types of food products, for instance, meat or fishery products, soups, and sauces.

2.3. Carotenoids Pigments

Carotenoids are fat-soluble pigments found naturally in many marine products. Crustacean by-products represent important natural sources of carotenoid, among which astaxanthin (AX) is the major one. AX is composed of beta and beta-carotene-4,4′-dione with two hydroxy substituents in the positions 3 and 3′ (the 3S,3′S diastereomer) (Figure 2), and belongs to the xanthophyll family. In crustaceans, it is found in complexes with proteins and is the pigment that gives the typical animals' color, and it is responsible for many biological properties such as protection from oxidative damage and the stimulation of growth and reproduction [47,48]. The content of AX in crustaceans can vary substantially, the variations observed in different shrimp species were in the range between 24 and 199 μg/g [49]. The observed differences could be due to variations in the amounts of carotenoid available in the feed, environmental conditions, and species, as well as due to the methods used for extraction.

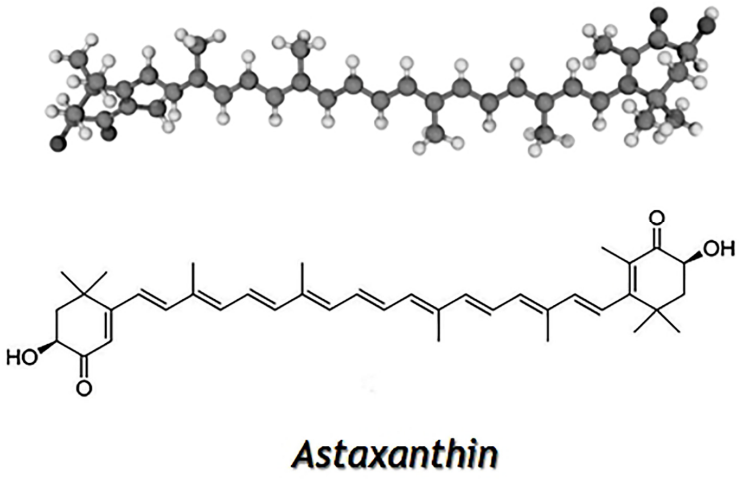

Figure 2. Chemical structure of astaxanthin.

Generally, carotenoids are additives allowed in animal feed but also for food products and health supplements. The main application of AX is as a coloring agent added in the formulation of diets for various aquaculture species, in particular salmon, and it has been used for functional foods development [50], but it also finds various uses in the cosmetic and pharmaceutical industries [48]. The use of microencapsulation has shown great potential for the use of AX as a food ingredient for maintaining its coloring ability and overcoming some of its drawbacks such as odor [51] and improving its bio-accessibility and antioxidant capacity [52]. The antioxidant, activity of AX is ten times higher compared to other carotenoid pigments and approximately 100 times more than α-tocopherol [48].

The most used method for AX recovery is based on solvent extraction from wastes and by-products. A variety of solvents have been used, including hexane, acetone, isopropanol, ethyl acetate, methylethylketone, methanol, and ethanol, however, this method is considered costly, time-consuming, and not environmentally friendly [53]. Recently, other techniques aimed at increasing the process sustainably were investigated for carotenoid recovery, for example, microwave- and enzyme-assisted extraction methods, the use of supercritical fluid, and their combination. However, details regarding costs, efficiency, and environmental aspects related to these proposed strategies need to be carefully assessed [54].

2.4. Chitin, Chitosan, and Derived Compounds

Chitin, poly (β-(1→4)-N-acetyl-d-glucosamine) is a biopolymer, second in abundance only to cellulose. The major component of the exoskeleton of arthropods such as crustaceans and insects can also be found in some bacteria and fungi cell walls. The deacetylated form of chitin, mainly composed of glucosamine, 2-amino-2-deoxy-β-D-glucose, or (1→4)-2-amino-2-deoxy-D-glucose, is known as chitosan, which, contrasting with chitin, which is highly insoluble in most solvents, can be solubilized by decreasing the pH of the solutions.

Chitosan is characterized by the presence of three kinds of reactive functional groups as shown in Figure 3, an amino group in position C-2 and hydroxyl groups in positions C-3 and C-6. As well as the native forms of chitin and chitosan, it is possible to obtain modified forms, and all have a variety of applications [55]. To isolate chitin, first demineralization and deproteinization are applied, for both chemical and enzymatic treatments [56]. For residual pigment removal, it is possible to additionally apply a step of decolorization. Although different techniques have been suggested to purify chitin, a standard method is still lacking [57].

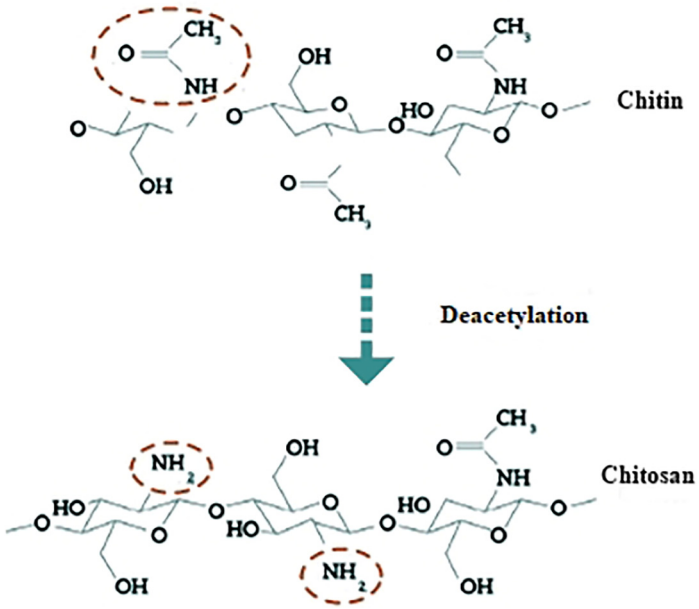

Figure 3. Deacetylation of chitin to chitosan.

Crustacean exoskeletons are the main source of α-chitin aimed at commercial use on account of their high content and easy accessibility. These compounds, chitin, and its derivatives, gained increasing attention in various fields, from the pharmaceutical, biotechnology, biomedical to the food sector [6,57] on account of their various beneficial properties, as they are biocompatible, biodegradable, and safe. However, chitin has a limited application due to its insolubility in water and many solvents. Therefore, water-soluble derivatives are produced, chitosan being the most important. It shows interesting biological activities, such as antimicrobial and antioxidant characteristics that make it attractive for preservation as a possible alternative to chemical preservatives and for food packaging for producing edible antimicrobial films based on its good film-forming properties [58–60].

The deacetylation of chitin into chitosan can be obtained using chemical and enzymatic processes. For commercial purposes, the thermal-assisted chemical process that involves the use of a strong alkali (generally 40–50%, *w*/*w*, NaOH) coupled with high temperature is extensively used, due to the low cost and suitability for large-scale production. However, this process presents some drawbacks, such as a long reaction time, the use of high temperatures, low reproducibility of heterogeneous processes, which, in turn, leads to changes of chitosan characteristics, the possibility of depolymerization reactions caused by the use of highly concentrated alkali, and the production of high amounts of alkali wastewater that represent a potential environmental hazard [61]. The enzymatic method for converting chitin into chitosan is conducted using various chitin deacetylases (CDA) obtained by bacteria and fungi. However, previous studies show that deacetylation using CDA showed a lower degree of deacetylation (DD) than alkali treatment, indicating that these enzymes are not effective on insoluble chitins [62]. The enzymatic deacetylation reaction presents some limitations due to some chitin physical properties like crystallinity, solubility, and molecular weight [26]. Therefore, it is necessary to pretreat chitin before adding the enzyme, for increasing its accessibility to the substrate (acetyl group) and enhancing the yield of deacetylation [57].

To overcome the poor solubility of chitosan in water, hence widening its application, some processes can be exploited. Various polyphenol–chitosan conjugates have been developed, mainly for the development of films for food preservation. However, the study of their effects has been mainly carried out in in-vitro studies [63].

Through a chemical or enzymatic depolymerization process, chitooligosaccharides (COS) can be obtained from chitosan or chitin. They are characterized by shorter chain lengths and the presence of free amino groups in the unit of D-glucosamine; hence, they are soluble in water at a neutral pH, in contrast to chitin and chitosan, and present a low viscosity. These features make chitosan in its oligosaccharide form very attractive for use in the food and nutrition fields to enhance food quality and human well-being. COS industrial production is commonly obtained by acid hydrolysis aimed at cleaving the glycosidic linkages of chitosan. Nevertheless, this method leads to low yields and the production of a large quantity of monomeric D-glucosamine units [64]. On the other side, the use of the enzymatic process, based on non-specific enzymes, like proteases, lipases, and cellulases, and specific ones like chitosanases, is considered safe and easy to control [65]. However, the industrial application is limited by the high costs of enzymes, in particular the specific ones, such as chitinase.

The antimicrobial activity of chitosan is highly variable and depends on many factors. Some are related to the chitosan molecule, e.g., the kind of chitosan, the molecular weight, and the deacetylation degree, while some extrinsic factors include the specific microorganism and the applied medium conditions, like pH, ionic strength, and types of solutes that can interact with chitosan hindering or blocking the reactivity of the active amine group. Considering the information obtained by the published literature, although antimicrobial properties of chitosan are variable, and many conflicting results have been presented, it seems widely accepted that the most sensitive group to chitosan are yeasts and molds, and then bacteria, Gram-positive and Gram-negative [66].

3. Innovative Non-Thermal Technologies for Recovery of Bioactive and Other Valuable Compounds from Crustacean By-Products

Recently, the development of novel technological processes characterized by reduced energy consumption and impact on the environment increased the quality and safety of the final products, that can be applied for by-product valorization, have gained growing interest [67]. For these reasons, various modern non-thermal processes, such as supercritical fluid extraction (SFE), high-pressure processing (HPP), pulsed electric field (PEF), and ultrasound (US), have recently been suggested with the aim of shortening the processing time, increasing recovery yield, improving the product quality, and enhancing the functionality of extracts from crustacean by-products [68]. Figure 4 shows the main compounds extracted using non-thermal methods.

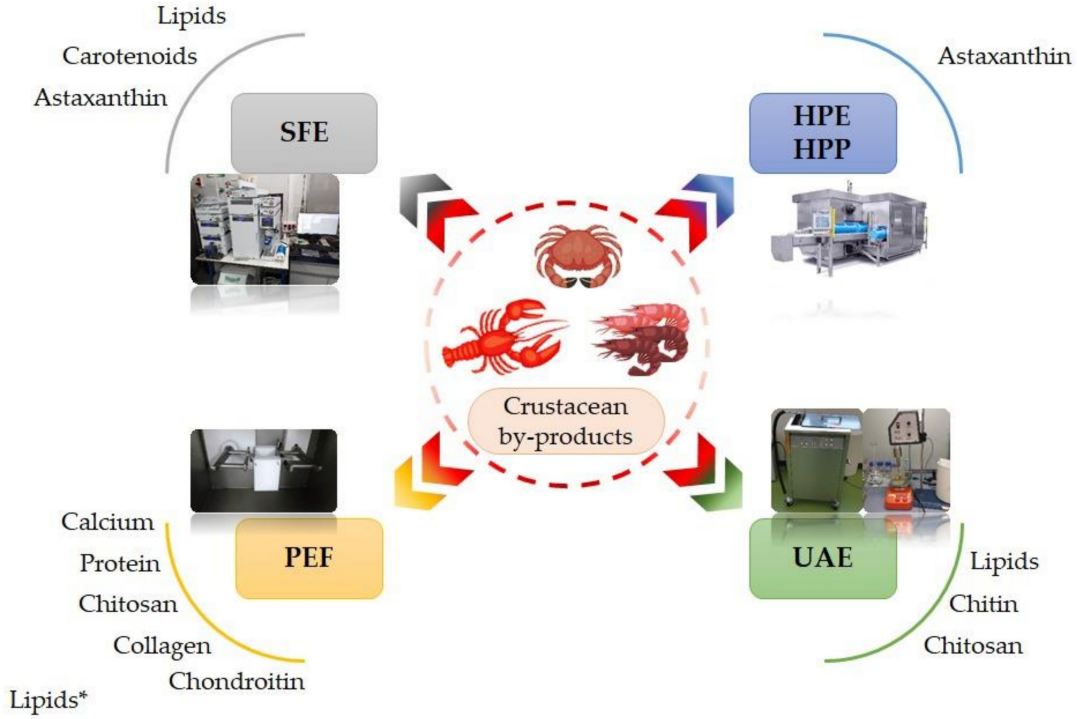

Figure 4. Main compounds extracted using non-thermal technologies. SFE: Supercritical Fluid Extraction; HPE: High-Pressure Extraction; HPP: High-Pressure Processing; PEF: Pulsed Electric Fields; UAE: Ultrasounds Assisted Extraction. * = used as a pre-treatment. The HPE/HPP picture was obtained from HIPERBARIC (Burgos, Spain) and used with permission.

3.1. Supercritical Fluid Extraction (SFE)

SFE technology is based on the separation of one component from a matrix, solid or liquid, using a supercritical fluid. Supercritical fluids are particularly suited for the extraction process because they are characterized by physicochemical properties that fall between those of a liquid and those of a gas, for instance, low viscosity, high diffusivity, and low surface tension [69]. An extensive variety of solvents can be used for SFE, such as carbon dioxide (CO_2), nitrous oxide, ethane, propane, n-pentane, ammonia, fluoroform, sulfur hexafluoride, and water. However, CO_2 represents the ideal solvent for application in the food industry, being inert, non-toxic, non-flammable, and cheap, therefore, it is the most used (conditions to obtain the critical state = 30.9 °C and 73.8 bar). Moreover, supercritical CO_2 (SC-CO_2) extraction is carried out at relatively low temperatures; hence, it is suited to heat-labile compounds, like carotenoids and lipids [70]. SC-CO_2 shows good solvent characteristics for non-polar or slightly polar compounds and shows great affinity with oxygenated organic compounds of medium molecular weight [32]. Furthermore, CO_2 creates a non-oxidizing atmosphere, hindering the oxidative degradation of compounds

during extraction. Alternatively, it shows low affinity to polar compounds; hence, the use of co-solvents is suggested for their extraction as they increase their solubility in SC-CO_2.

SFE is a modern technology for extracting bio-compounds from various matrices that can be applied in the pharmaceutical and food industries. SFE has shown various advantages compared with traditional extraction processes, such as high yields, reduced processing times, and the use of solvents generally recognized as safe (GRAS), which make it a very popular green extraction method [71].

SFE has also been largely investigated for the valorization of food industry by-products [72]. For seafood by-products, much research is focused on the recovery of components producing high added value products, principally lipids and lipophilic components, such as carotenoids [73]. For the SFE of carotenoids, the five most critical parameters are processing temperature, pressure, time, CO_2 density (solvent power) and flow rate, and entrainers concentration [54].

Table 1 reports examples of SC-CO_2 for the recovery of lipids and astaxanthin from by-products derived from crustacean processing. Most of the published results showed that, when SC-CO_2 was used alone, pressure and temperature did not impact the yield of oil extraction leading to the low quantity of recovered lipids and astaxanthin [70,74]. However, some authors have reported that adding co-solvents, generally ethanol or methanol, improved the extraction yields of both lipids and astaxanthin from by-products of crustacean processing. In this sense, Radzali et al. [75] investigated the use of different concentrations of different co-solvents (ethanol, water, methanol) for SFE of astaxanthin from the by-products of the shrimp *Penaeus monodon*. Lyophilized samples were extracted at a temperature of 60 °C and pressure of 20 MPa. The presence of ethanol maximized the yield (97.1% recovery compared to 100% with solvent extraction) of the total carotenoid (84.02 ± 0.8 μg/g) dry weight (DW). Sánchez-Camargo et al. [76] observed that at the conditions of 300 bar and 50 °C and using a 300 mL extractor with a constant solvent/feed (S/F) mass ratio (71.4), increasing the concentration of ethanol from 5 to 15%, allowed for the enhancement of the extraction of total lipid from freeze-dried shrimp by-products (*Farfantepenaeus paulensis*) by up to 136%. Considering the initial content in the waste material, lipids and astaxanthin were recovered up to 93.8% and 65.2%, respectively. Lipid recovery was significantly higher compared to other methods; 67% was recovered using only hexane as the solvent and 44.7% under the same conditions of temperature and pressure but without the use of a co-solvent.

Mezzomo et al. [77] evaluated carotenoid concentration through SFE from processing by-products of pink shrimp (*P. brasiliensis and P. paulensis*), taking into account the technical and the economic viability of the process. By-products were heat-treated, oven-dried, and milled, and 16 g were extracted in a 100 cm^3 cell. Different parameters were investigated, such as the moisture content of the raw material (11.21% and 46.30%), temperature (40 °C and 60 °C), pressure (10–30 MPa), the solvent flow rate (8.3 g/min and 13.3 g/min), and nature of the co-solvent. The optimal conditions that allowed for astaxanthin yield maximization were the use of CO_2 with the addition of 2% hexane: isopropanol solution (50:50) as a modifier, at 300 bar/60 °C. The cost analysis suggested the application of an SFE unit with 2 × 400 L vessels for 25 min extraction as the most lucrative process design.

Amiguet et al. [78] evaluated the SFE efficiency on the recovery of PUFAs from the processing by-products of Northern shrimp (*Pandalus borealis*). They used a 100 mL extraction vessel for the processing of 10 g of an air-dried sample with a flow rate of 3–5 L/min. SC-CO_2 extraction at 35 MPa and 40 °C resulted in deep red oil, with a high content of ω-3 PUFAs, in particular 7.8 ± 0.06% EPA and 8.0 ± 0.07% DHA.

Table 1. Supercritical fluid extraction (SFE) of crustacean by-products for the recovery of valuable compounds.

Species	By-Products	Compounds	Extraction Conditions	Optimum Condition	Yield and Characteristics of Products'	References
Australian Rock Lobsters (*Jasus edwardsii*)	Livers	Lipids	P: 25, 30, 35 MPa T: 50 °C CO_2 flow rate: 0.434 kg/h Time: 240 min	35 MPa and 50 °C for 4 h:	94% of lipid yield, 4 and 7 times higher content of DHA and EPA, respectively, compared to those obtained by Soxhlet extraction	[15]
Tiger shrimp (*Penaeus monodon)*	Head and shells	Astaxanthin and other carotenoids	T: 60 °C, P: 20 MPa Co-solvents: ethanol, water, methanol, 50% (v/v) ethanol in water, 50% (v/v) methanol in water, 70% (v/v) ethanol in water, and 70% (v/v) methanol in water.	50% (v/v) ethanol in water	Carotenoid yield: 84.02 ± 0.8 µg/g dry weight (DW), Extracted astaxanthin complex: 58.03 ± 0.1 µg/g DW free astaxanthin content: 12.25 ± 0.9 µg/g DW	[75]
Pink shrimp (*Penaeus brasiliensis* and *Penaeus paulensis*)	Head, shell, and tail	Astaxanthin and other carotenoids	Moisture content (11.21–46.30%), solvent flow rate (8.3–13.3 g/min), T: 40–60 °C P: 100–300 bar co-solvent (hexane: isopropanol solution, 50:50, and sunflower oil)	Solvent: CO_2 + 2% hexane: isopropanol solution, 50:50 Flow rate: 13.3 g CO_2/min 11.21%: moisture content P: 300 bar T: 333.15 K	Global yield (amount of extract removed by the solvent and related to the solvent power, i.e., to the process temperature and pressure): 4.2 ± 0.2	[77]
Brazilian redspotted shrimp (*Farfantepenaeus paulensis*)	Head, shell and tail	Astaxanthin and ω3 fatty acid (EPA + DHA)	CO_2/Ethanol Etahnol 5, 10 and 15% wt, P: 300 bar, T: 50 °C.	15% wt of ethanol.	93.8% and 65.2% for lipids and astaxanthin Total lipid extraction yield increased to 136% increasing ethanol from 5 to 15% wt.	[76]
Brazilian redspotted shrimp (*Farfantepenaeus paulensis*)	Head, shell and tail	Lipids, astaxanthin	P: 200–400 bar T: 40–60 °C	43 °C and 370 bar	Astaxanthin: 39% recovery Lipids yield similar under different conditions (1.74% to 2.21%) Possibility to fractionate oil	[70]
Northern shrimp (*Pandalus borealis*)	Head, shell and tail	Lipids (EPA+DHA)	Low P: 15 MPa, 50 °C Moderate P: 35 MPa; 40 °C	35 MPa; 40 °C	Total Fatty Acids: 795 mg/g Oil rich in ω-3 PUFAs (EPA:78 mg/g, DHA:79.7 mg/g)	[78]
Louisiana crawfish (*Procambarus clarkii*)	Shell and tail	Astaxanthin	T: 50–60–70 °C P: 13.8–22.4–31.0 MPa, Moisture content: freeze-dried 0–25–50%.	75 °C, 24.1 MPa, and 13% moisture.	Predicted maximum extractable astaxanthin: 207.6 mg/kg	[74]

P: Pressure; T: Temperature.

Moreover, Nguyen et al. [15] optimized SC-CO_2 lipid recovery with enriched PUFAs from Australian rock lobster (*Jasus edwardsii*) liver by using a 100 mL vessel for the treatment of 10 g of a freeze-dried sample at 35 MPa, 50 °C for 4 h (mass flow rate: 0.434 kg/h). Approximately 94% recovery was obtained and the extracted lipids were particularly rich in PUFAs (31.3% of total lipids), with a content four times higher compared to the one obtained by Soxhlet extraction (7.8%). In particular, DHA and EPA content was seven times higher.

Despite the various advantages of SFE, several concerns have been raised about the environmental and safety impact as well as the high energy consumption of the process. Other disadvantages of SFE include the limited sample size, extraction efficiency affected by matrix type, analyte type and moisture content of the matrix, and the high cost of SFE equipment [79]. Possible solutions investigated to increase its efficiency are the combination with other pre-treatments such as enzymatic treatment or the addition of co-solvents [73].

3.2. High-Pressure Extraction (HPE)

High-pressure processing (HPP) is a non-thermal food processing technique that involves the application of high pressure to solid or liquid foods with the aim of microbial inactivation but also of quality improvement [80]. Recently, the use of high pressure has been suggested for extraction purposes (High-Pressure Extraction—HPE) with the aim of reducing extraction time, solvent consumption, increasing extraction yields, and improving the quality of the obtained extracts [81].

HPE is based on the same principles of HPP (isostatic and Le Chatelier's principles), the applied pressure levels usually range from 100 to 600 MPa, not affecting the covalent bonds, and the use of room or refrigerated temperatures prevents thermal degradation [82]. HPP produces physical damage to the plant tissue, cellular wall, membrane, and organelles, making cells more permeable to solvents, increasing the mass transfer rate, and facilitating the release of extracts. For this reason, HPE can be a useful strategy to valorize by-products facilitating the recovery of bioactive compounds. Indeed, compared to the conventional methods used such as thermal or solvent extraction, HPE is faster, allows for the increase of extraction yields, reduces impurities, and preserves the bioactivity of the extracted compounds, in particular, thermo-sensitive ones [81]. Another important advantage of HPE is its ability to use different solvents (and solvent ratios), with distinct polarities, enabling it to extract different components and to minimize the presence of impurities present [49].

HPE has been actively used to recover some biologically active substances from natural biomaterial; however, few researchers have evaluated the extraction from crustacean by-products (Table 2). Du et al. [82] studied the application of HPP for the extraction of astaxanthin from shrimp (*Litopenaeus vannamei*) by-products (shell and head) at ambient temperature, using ethanol as the extraction solvent, considering different variables such as the liquid-to-solid ratio (10 to 50 mL/g), applied pressure (0.1~600 MPa), and pressure holding time (0~20 min). The highest extraction yield (89.12 μg/g) was obtained by applying a pressure of 210 MPa for 10 min and a liquid-to-solid ratio of 32 mL/g. Similarly, Li et al. [49] studied the effects of pressure, holding time, different solvents (acetone, dichloromethane, and ethanol), and solvent-to-solid ratios for the HPE of astaxanthin from shrimp by-products at ambient temperature. The higher extraction yield (71.1 μg/g) was obtained in 5 min, using ethanol with a solvent/solid rate of 20 mL/g and a pressure range from 200 to 400 MPa. The antioxidant activity of the extracted astaxanthin was also found to be higher (EC_{50} = 81.54%) compared to that of conventional solvent extraction (EC_{50} = 45.31%).

Table 2. High-pressure extraction (HPE) of crustacean by-products for the recovery of valuable compounds.

Species	By-Products	Compound	Extraction Conditions	Optimum Conditions	Yield and Characteristics of Products'	References
Pacific white shrimp (*Litopenaeus vannamei*)	Head and shell	Astaxanthin	P: 0.1–600 MPa, liquid-to-solid ratio (10 to 50 mL/g), and pressure holding time (0–20 min)	P: 210 MPa P holding time:9.2 min, liquid-solid-ratio: 32 mL/g	89.12 μg/g	[82]
Pacific white shrimp (*Litopenaeus vannamei*)	Shells	Astaxanthin	P: 0.1–600 MPa, holding times (0–20 min), different solvents (acetone, dichloromethane, and ethanol), and solvent to solid ratios (10–50 mL/g)	Ethanol, liquid to solid ratio of 20 mL/g and 200 MPa for 5 min.	71.1 μg/g, better antioxidant activity in the extract than conventional solvent extraction	[49]
Rainbow Shrimp (*Parapenaeopsis sculptili*) Bird shrimp (*Metapenaeus lysianassa*) Giant river prawn (*Macrobrachium rosenbergii*) Shrimp (*Metapenaeopsis hardwickii*) Banana shrimp (*Penaeus merguiensis*) Giant tiger prawn (*Penaeus monodon*)	Head, shell, and tail	Astaxanthin	P: 210 MPa, time 10 min, solvent mixture of acetone and methanol (7:3, *v*/*v*).	Higher total carotenoid and astaxanthin yield obtained for *P. monodon*	Total carotenoid: 68.26 μg/mL astaxanthin yield: 59.9744 μg/gdw	[83]

P: Pressure.

Recently, Irna et al. [83] studied the effect of HPE for astaxanthin extraction from six types of shrimp at 210 MPa, for 10 min with a solvent mixture of acetone and methanol (7:3, *v*/*v*), and compared it to conventional chemical extraction. Among the six species, the black tiger (*Penaeus monodon*) was the one with the higher amount with both extraction methods. Moreover, the same authors observed that the astaxanthin from shrimp carapace (*P. monodon*) extracted by HPE was characterized by higher antioxidant activity and a greater zone of inhibition against four bacterial strains (*E. coli*, *E. aerogenes*, *S. aureus,* and *B. subtilis*) compared to the chemically extracted one [84].

3.3. Pulsed Electric Fields (PEF)

PEF processing represents a novel, non-thermal method that has been shown as a potential tool to recover bioactive compounds from agri-food by-products [85]. Compared to conventional techniques, PEF offers several advantages such as non-thermal behavior, high selectivity, less time and energy consumption, and does not require any additional chemicals. PEF technology involves the application of a series of short high voltage pulses to a biological material (plant, animal, or microbial cells) placed between two electrodes. Pulses generally have a duration in the range from microsecond to millisecond, and a pulse amplitude that ranges from 100 to 300 V/cm to 20–80 kV/cm depending on the characteristics of the material. PEF treatment causes a phenomenon known as "electroporation", related to the formation of pores in the cell membrane that facilitates the cell's intracellular content release [86].

PEF treatment may be a promising method for the isolation and extraction of different components from seafood by-products such as calcium, chondroitin, collagen, chitosan, and protein [87–89]. However, the study of this technology for the extraction of compounds from crustacean by-products has been limited.

Luo et al. [89] investigated the effect of the intensity of the electric field strengths up to 25 kV/cm (pulse duration (τ) of 20 µs, pulse frequency (f) of 1000 Hz, pulse number of 12, and flow rate of 100 mL/min) on the degradation of large molecular chitosan. From the traditional deacetylation of chitin, the obtained chitosan is characterized by high molecular weight (over 10^5 Da) and low solubility in an aqueous solvent; hence, its application in food products results limited. The average molecular weight (MW) measured as the intrinsic viscosity value, of the PEF-treated chitosan, was reduced by increasing the intensity of the electric field. After the application of 15, 20, and 25 kV/cm, the MW decreased by 19.57%, 35.23%, and 45.19%, respectively, compared with the initial chitosan. At the same time, the authors observed significant damage to the crystalline region of the sample treated at 25 kV/cm, indicating a possible degradation of high MW. PEF treatments have shown a significant effect on the molecular structure of chitosan which may be responsible for the variation of its physicochemical and biochemical properties.

Gulzar and Benjakul [90] used a PEF pretreatment (to extract lipids from the cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) (electric field strengths in the range from 4–16 kV cm^{-1} and pulse number in the range from 120–240) in combination with an ultrasound-assisted process (UAE) that allowed for the maximization of lipid yield (30.34 g 100 g^{-1}) and the reduction of lipid oxidation. Indeed, lipids from PEF-pretreated samples extracted using the UAE process showed an increased content of PUFAs and carotenoids, but peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were decreased. The authors suggested that the negative effects on lipid quality due to UAE might have been, to some degree, mitigated by PEF pretreatment; however, they did not put forward a possible mechanism for this observed phenomenon.

3.4. Ultrasound-Assisted Extraction (UAE)

The application of ultrasound (US) has proven to be a powerful method in food technology for processing, preservation, and extraction. US offers a significant advantage in productivity, yield, selectivity, reduced processing time, improved quality, the reduced

presence of chemical and physical hazards, being considered environmentally friendly overall [91].

The major effects obtained by the application of US in a liquid medium are related to the cavitation phenomena and compression and decompression of molecules leading to the creation, enlargement, and implosion of microbubbles of gases dissolved in the liquid. The mechanical effects of US promote an increased penetration of solvent into the cellular material, an improved mass transfer thanks to micro-streaming, and the release of cell content due to the disruption of the biological cell walls [92].

The application of UAE in food processing technology improves the extraction of compounds from plant and animal tissues. The advantages of UAE include the reduction of extraction time and solvent consumption, improved reproducibility, simplified manipulation and work-up, and improved purity of the final product [91].

In recent years, several studies have demonstrated that UAE is a powerful method for extracting lipids from crustacean processing by-products due to its cavitation effect (Table 3). UAE increases the extraction yield of lipids and carotenoids; however, in some cases, it can lead to degradative processes such as lipid oxidation and hydrolysis that can be explained with the incorporation of oxygen and mechanical effects and with increased exposure of substrates to enzymes [93,94].

Table 3. Ultrasound-assisted extraction (UAE) of crustacean by-products for the recovery of valuable compounds.

Species	By-Products	Compounds	Extraction Conditions	Optimum Conditions	Yield and Characteristics of Products	References
Pacific white shrimp (*Litopenaeus vannamei*)	Head	Lipids and carotenoids	Pulse and continuous Mode, sonication time (15, 20, 25, and 30 min), amplitudes of 50– 90%, 4 °C	80% amplitude with continuous mode, for 25 min.	50% yield, Extract richer in free fatty acids and higher oxidation level	[93]
Pacific white shrimp (*Litopenaeus vannamei*)	Head	Lipids and carotenoids	Frequency: 20 kHz, Power: 750 W, amplitudes: 60–100%	80% amplitude for 10 min at 4 °C	Lipid yield: 10–11 g/100 g, carotenoids yields: 8.6–8.8 mg/g lipid, higher lipid oxidation and hydrolysis	[94]
Giant river prawn (*Macrobrachium rosenbergii*)	Shell	Chitin	Demineralization in 0.25 M HCl (1:40 solid-to-solvent, *w*/*v*) at 40 °C, sonicated for 0, 1, and 4 h. Deproteinization in 0.25 M NaOH (1:15 solid-to-solvent, *w*/*v*) at 40 °C, sonicated for 0, 1, and 4 h	4 h	Lower content of proteins (7.45%) and deacetylation degree (61.4%)	[96]
Pacific white shrimp (*Litopenaeus vannamei*)	Shell	Chitosan	Deacetylation: NaOH (35%–65%, *w*/*w*), ratio of chitin (1:15, *w*/*v*), 80 °C, 360 min., frequency of 37 kHz and power of 300 W	Deacetylation rate improved with concentration of NaOH below 45% (*w*/*w*)	Higher solubility of chitosan	[95]

Several authors observed that UAE significantly improved the extraction yield of lipids and carotenoids from Pacific White shrimp (*Litopenaeus vannamei*) [90,93,94]. However, the UAE process caused, and increased, the lipid oxidation of lipids shown by higher peroxide values (PVs) and thiobarbituric acid reactive substances (TBARS), which was further increased using UAE with a continuous mode compared to the pulsed one.

The addition of an antioxidant combined with UAE is a potential approach to reduce the disadvantages brought along by cavitation, in particular, the accelerated oxidation. As observed by Gulzar and Benjakul [90], pre-heating, along with 0.1% tannic acid addition, reduced lipid oxidation during the UAE of Pacific white shrimp.

The yield of lipids extracted from the lipid-containing solid residue (LSR) obtained from the protein hydrolysis of Pacific white shrimp cephalothorax was increased from 7.2 to 12% dry basis and carotenoid content increased from 5.7 to 8.6 mg/g of lipid when UAE was used at 80% amplitude for 10 min with a 30 s on-and-off pulse mode.

Currently, UAE is widely used for the recovery of chitin and chitosan from crustacean by-products (Table 3). The ultrasound-assisted deacetylation (USAD) has been reported as an efficient process to produce chitosan. Birolli et al. [61], investigated the conversion of α-chitin from the cephalothoraxes of freshwater prawn (*Macrobachium Rosenbergii*) into chitosan applying the USAD. It was shown that the treatment of α-chitin suspended in 40% aqueous sodium hydroxide with high-intensity ultrasound irradiation strongly favored the N-deacetylation reaction, favoring the production of fully acid-soluble chitosan at high yield (>95%). Additionally, the USAD process allowed for the preparation of chitosan exhibiting a lower average degree of acetylation.

Ngo and Ngo [95] evaluated the effects of low-frequency US on the heterogeneous deacetylation of chitin from the shell of white shrimp (*Penaeus vannamei*). At a low concentration of sodium hydroxide, below 45% (*w*/*w*), results showed that the US enhanced the deacetylation rate and, therefore, reduced the time of the reaction and improved the solubility of the chitosan.

Kjartansson et al. [96] investigated the effect of sonication during chitin extraction from freshwater prawn (*Macrobrachium rosenbergii*) shells on the yield, purity, and crystallinity of chitin. Dry shells were suspended in 0.25 M HCl at 40 °C and sonicated for 0, 1, and 4 h at 41 W/cm^2. Demineralized shells were suspended in 0.25 M NaOH at 40 °C and the samples were sonicated for 0, 1, and 4 h. It was found that the crystallinity indices and extraction yield of chitin decreased as the sonication time increased. The decrease in extraction yield was attributed to the leaching of depolymerized chitin during the washing step. The application of ultrasound enhanced the removal of proteins. Additionally, the degree of acetylation of chitin was unaffected by sonication, but the degree of acetylation of chitosan produced from sonicated chitin decreased from 70.0 to 61.4% after 4 h of sonicating the samples.

Furthermore, some authors confirmed that the sonication of chitosan significantly reduces the MW of this polymer and has become an alternative method for degrading chitosan into low-molecular-weight chitosan (LMWC), chitosan oligomers, and glucosamine [97–99]. Intrinsic viscosity and average MW decreased exponentially with increasing sonication time, which is often desirable for its increase in antimicrobial activity and its use in pharmaceutical and biological applications.

3.5. Comparison of Technologies

Table 4 shows the main advantages and disadvantages of the considered innovative technologies used for the recovery of bioactive compounds in comparison with traditional ones. Generally, they all respond to the requirements of reduced processing times and reduced environmental impact. However, despite the numerous publications on the recovery of compounds from crustacean by-products, to the best of our knowledge, there are no publications comparing these new non-thermal technologies (SFE, HPE, PEF, and UAE) applied specifically for this aim. This is one reason it is not possible to select one of the investigated technologies as the optimal one, but others are because of the many processing parameters affecting the results and the numerous types of by-products derived from crustacean processing as described in the above paragraph. Indeed, as reported by Aoude et al. [100], a generic solution in terms of the recovery of high-value compounds from food waste does not exist; therefore, in each case, the optimal solution should be identified after individual study and optimization.

Table 4. Advantages and disadvantages of the considered technologies in comparison with traditional ones.

Technology	Advantages	Disadvantages	References
SFE	- High yields for carotenoids extraction - Reduced processing times - Use of solvents generally recognized as safe (GRAS), CO_2 is inert, non-toxic, non-flammable, and cheap, high affinity for apolar compounds - Short extraction time and minimal usage of organic solvents	- Limited sample size - Extraction efficiency affected by matrix type, analyte type, and moisture content of the matrix - Difficult to optimize conditions - High cost of SFE equipment - Few commercial plants available	[71,101]
HPE	- Environmentally friendly - Rapid and highly efficient extraction - Reduced presence of impurities - Preservation of the bioactivity of the extracted compounds, in particular, thermo-sensitive ones - Ability to use different solvents (and solvent ratios) with distinct polarities	- High initial investment and capital costs - Batch or semi-continuous operation - No selectivity	[49,81]
PEF	- Non-thermal behavior - High selectivity - Less time and energy consumption - High yields for carotenoids extraction - Does not require any additional chemicals - Can be used in continuous mode	- Limitedly studied for the extraction of compounds from crustaceans by-products - Use can be limited due to the conductivity of matrix - High initial investment of PEF equipment - Limited extraction of lipophilic compounds	[85,87]
UAE	- Higher extraction yield or rate - Opportunity to use alternative (GRAS) solvents - Enhancing yield extraction of heat-sensitive components - Increase the yield of lipids	- Scale-up to industrial applications still needs to be explored and optimized - Can lead to degradative processes such as lipid oxidation and hydrolysis - No selectivity	[91,94]

Tsiaka et al. [102] compared the use of UAE with Microwave-Assisted Extraction (MAE) for the recovery of carotenoids from *Aristeus antennatus* shrimp. Both technologies obtained higher yields compared to traditional ones, but although using different solvents, results were similar for both. Moreover, the possibility to combine different techniques should also be considered.

4. Re-Use of Ingredients from Crustacean By-Products in Seafood and Food Products

The main compounds obtained from crustacean by-products are proteins and protein hydrolysates, oil-rich in PUFA, carotenoids, and in particular, astaxanthin and chitin derivatives, namely chitosan and COS. The recovery of flavors has also been investigated using the membrane filtration of seafood cooking effluents [103]. All these compounds find many applications in different sectors. The utilization of compounds obtained from shrimp processing by-products has been investigated in-depth, considering many applications in foods and feeds [104]. However, in the circular economy concept, their use in seafood-based products will increase the overall value of the seafood sector. In this section, some

examples of the use of ingredients or compounds obtained from crustacean by-products for the formulation of seafood-based products are reported in Table 5.

Proteins recovered from shrimp can possess different functionalities that can be exploited in various food applications. The film-forming properties allows for the production of an edible coating [31] that can be applied to different kinds of products. Protein extracted and isolated from the muscles of *L. vannamei* were used for developing a coating that showed good potentiality to be applied for extending the shelf-life of fish-based products [105].

The protein recovered in the form of hydrolysates can be used as a flavoring and incorporated into fish-based foods or feed for aquaculture [106]. Moreover, the hydrolysates are also sources of biologically active peptides, with considerable potential in functional foods, nutraceuticals, and possibly supplements and/or as growth-stimulating agents in animal feeds [107]. Peptides derived from shrimp processing by-products, in particular, cephalothorax, shell, and tail have been demonstrated to exhibit antioxidative and cryoprotective effects in seafood [106,108,109] emphasizing their potential as alternative natural and safe preservatives with bi-functions, antioxidative, and cryoprotective effects, which can be used for maintaining the quality of seafood [108].

Lipid extract from shrimp waste could be used as a food ingredient due to its coloring capacity and antioxidant properties. Recent works have shown that the lipid extract obtained from shrimp (*Litopenaeus vannamei)* by-products is a promising food ingredient with multiple technological applications [25,42,52]. However, currently, there are a small number of studies that have focused on lipid extraction and practical application in food from this source. White shrimp (*Litopenaeus vannamei*) lipid extract rich in astaxanthin was encapsulated by ultrasonic atomization [110], achieving high encapsulation efficiency, antioxidant activity, and sensory acceptance when incorporated in the formulation of yogurt.

Chitosan and its derivatives are noted to have a wide range of functional properties that can be used for processing, preservation, and as a food additive to improve the safety, quality, and shelf-life of seafood. In recent years, chitosan has been researched extensively and shown to be effective in preserving the quality of various seafood products [58,60,111]. Several studies show that chitosan from crustacean by-products is characterized by an antimicrobial activity against a wide range of target micro-organisms on seafood products (Table 6). The effectivity of chitosan use as an antioxidant and antimicrobial on seafood products depends on the application method, type of seafood, concentration, and chitosan properties such as viscosity, particle size, molecular weight, and the degree of deacetylation [112,113].

Chitosan from crustacean by-products has also been used to improve the gelling properties of fish surimi products, the effect depending on the quality of the surimi, the type of chitosan, the concentration, and the gelling treatment [114,115]. Some studies suggest that the enhancing effect of chitosan on the gel formation of surimi could be due to the modification of the activity of the endogenous transglutaminase [116,117]. Chitosan films have been successfully applied as edible films and coatings for the packaging and protection of different seafood products [118]. The addition of protein concentrates rich in antioxidants like astaxanthin obtained from shrimp (*L. vannamei*) by-products has also been investigated [119,120]. The application of a film obtained by chitosan and added with a shrimp concentrate obtained by the cooking juice, achieved a novel product based on fish sausages and to extend its shelf-life up to 42 days [120]. Coating is the most popular application technique for chitosan followed by dipping, vacuum tumbling, spraying, and direct addition to the batter [58,111].

Table 5. Application of bioactive compounds or products obtained from crustacean by-products in seafood products.

By-Product	Compond	Function	Seafood Product	Application	Findings	References
Shrimp Shell	Chitosan	Antioxidant	Rohu (*Labeo rohita*) fish sticks	Addition of 0.5%, 1%, 1.5%, and 2% of chitosan gel in batter for fish stick coating	Increase in chitosan gel concentration reduced oil absorption from 65–78%. Reduced total volatile basic nitrogen (TVBN), PV, and TBARS. Lipid oxidation decreased as the chitosan inclusion in batter increased.	[121]
Shrimp shell (*Metapenaeus dobsoni*)	Chitosan	Gelling	Surimi from Pangasius (*Pangasianodonhypophthalmus*)	Three different formulations by incorporating corn starch (10%) and chitosan (0.75%). A formulation containing only cornstarch (10%) was used as a control.	Reduction of total volatile basic nitrogen (TVBN), free fatty acids (FFA), peroxide value (PV), 2-thiobarbituric acid reactive substances (TBARS), and microbial count of the product during chilled storage. Extended the shelf-life of 17 days in comparison with the control of 10 days.	[122]
Shrimp cephalothorax, shell, and tail (*Penaeus monodon* and *Penaeus sindicus*)	Shrimp protein hydrolysate (SPH)	Antioxidant	Whole Croaker fish (*Johnius gangeticus*)	Dipping in various concentrations of SPH Solution (0.1%, 0.2%, and 0.5% (*w*/*v*) of 5 mg/mL concentration SPH solution)	Lowered TBA values of fillet and maintained yellowishness of skin color during 10 days of refrigerated storage at 4 °C and limited the increase of PV and FFA values.	[106]
Shrimp head (*Pandalus eous, Metapenaeus endeavouri, Penaeus monodon*)	Shrimp protein hydrolysate (SPH)	Cryoprotectant	Lizardfish (*Saurida spp.*) surimi.	Lizardfish surimi with 5% (dried matter) of any of the three SPH	Stabilized freeze-induced denaturation of myofibrillar protein and enhance gel-forming ability of surimi during frozen storage. Decreased the whiteness of all kamaboko.	[109]
Shrimp Shell (*Penaeus monodon), (Metapenaeus endeavouri) (Macrobrachium rosenbergii*)	Shrimp Chitin Hydrolysate (SCH)	Cryoprotectant	Lizardfish (*Saurida spp.*) surimi.	Lizardfish surimi with 5% (dried matter) of shrimp chitin hydrolysates	Delayed freeze-induced protein denaturation and increased the amount of unfrozen water in surimi stored at 25 °C for 6 months.	[123]

Table 5. *Cont.*

By-Product	Compond	Function	Seafood Product	Application	Findings	References
Crab shells	Chitosan	Antioxidant	Cooked comminuted flesh of herring (*Clupea harengus*)	Solutions with 50, 100, and 200 ppm of chitosan with a viscosity of 14, 57, and 360 cP, added directly on the minced fish	PV and TBARS were both reduced following treatment of the fish before cooking with 50, 100, and 200 ppm of chitosan 14, 57, and 360 cP. Inhibition of oxidation was concentrated-dependent and highest for chitosan 14 cP.	[112]
Prawn shell	Chitin and Chitosan	Gelling	Surimi from barred garfish (*Hemiramphus far*)	Chitin or chitosans with different degrees of deacetylation 65%, 83%, 88%, 99% DD) and concentrations were added to the surimi	Chitosan with 65.6% DD at 15 mg/g resulted in the maximum increases in both breaking force and deformation of suwari and kamaboko gels.	[114]

Table 6. Antimicrobial activity of chitosan from crustacean by-products in seafood products.

Species	By-Products	Microorganism Reduced and/or Inhibited	Application	References
Tuna fillets (*Euthynnus affinis*)	Shrimp shell	Aerobic plate count (APC) *Pseudomonas* spp. *Aeromonas hydrophila* *Salmonella enteritidis* *Klebsiella* sp. *Bacillus firmus* *Bacillus cereus* *Micrococcus* sp. *Escherichia coli* *Salmonella paratyphi* *Vibrio cholera* *Salmonella typhi* *Staphylococcus aureus*	Fillets were dipped in edible chitosan coatings (chitosan conc. 1%)	[124]

Table 6. *Cont.*

Species	By-Products	Microorganism Reduced and/or Inhibited	Application	References
Smoked European eel (*Anguilla Anguilla*) stored under vacuum packaging (VP) at 4 °C	Crab shell	*Pseudomonas* spp. *Shewaella* spp. and yeasts/molds	Fillets were dipped in chitosan solution (2.0% *w*/*v*).	[125]
Pacific white shrimp (*Litopenaus vannamei*)	Shrimp processing by-products	Total bacterial counts (TBC) H_2S-producer organisms Luminescent bacteria Total aerobic mesophiles *Pseudomonas* spp. *Enterobacteriaceae* *Lactic acid bacteria*	Chitosan coatings (chitosan conc. 2% *w*/*w*)	[126]
Fresh swordfish steaks (*Xiphia gladius*)	Crab shells	Total Viable Counts (TVC) *Pseudomonas* spp. H2S-producing bacteria Lactic acid bacteria Enterobacteriaceae	Chitosan with a concentration of 0.045% *w*/*w*, added by spraying it directly onto the product.	[127]
Salmon fillets (*Oncorhynchus nereka*)	Shrimp shells	Mesophiles, Psychrotrophs, coliforms, *Aeromonas* spp., and *Vibrio* spp.	Soaked in various concentrations of chitosan solutions (0.2%, 0.5%, or 1.0% in 0.1 N HCl, adjusted to pH 6.0 with 1 N NaOH)	[128]

Chitooligosaccharide derivatives (COS) and chitosan nanoparticles possess potential applications in the seafood industry, due to their ability to protect food products against oxidative degradation, as well as preventing and/or treating free radical-related diseases [129,130]. Additionally, chitosan nanoparticles appear to be a promising agent for further improvement of chitosan coating efficiency. In a recent study, coatings containing chitosan nanoparticles were more effective in inhibiting microbial growth on silver carp (*Hypophthalmicthys molitrix*) fillets during refrigerated storage, than coating with normal chitosan [131]. Regarding the effect of chitosan nanoparticles from shrimp shells on the physicochemical properties of seafood-based products, it was reported that coating fish fingers with chitosan nanoparticles compared to commercial edible coating, reduced oil absorption by 11.86%, and increased moisture content by 18.09% during frozen storage at 18 °C [132].

5. Conclusions

The crustacean processing industry is a large source of by-products that can be a valuable source of nutraceuticals, bioactives, and functional compounds beneficial for human health. The recovery of by-products for beneficial health products offers economic and environmental benefits, thus, contributing to the concept of the circular economy in the seafood processing industry. This review has shown that innovative food processing technologies based on non-thermal concepts have the potential to be applied to the extraction of several biocompounds from crustacean by-products. These techniques are eco-friendly and safe and can increase the extraction yield reducing the processing time.

However, many of these techniques are poorly developed or tailored for crustacean by-product application and are lacking in standardization at the industrial scale. Moreover, crustacean by-products are diverse and complex. Considering these aspects, it is essential to define the appropriate extraction technology that allows for minimizing processing and maximizing quality for the target compounds.

The re-use of the extracted components in seafood products is a promising strategy to increase the value of the seafood sector overall. However, to date, there are limited industrial applications of this virtuous approach, particularly for chitin and chitosan valorization.

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Paper VI

Pulsed Electric Fields (PEF) and Accelerated Solvent Extraction (ASE) for valorization of Red shrimp (Aristeus antennatus) and Camarote shrimp (Melicertus kerathurus) by-products: Recovery of astaxanthin and antioxidant extracts

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Pulsed Electric Fields (PEF) and Accelerated Solvent Extraction (ASE) for valorization of Red shrimp (*Aristeus antennatus*) and Camarote shrimp (*Melicertus kerathurus*) by-products: Recovery of astaxanthin and antioxidant extracts.

Ana Cristina De Aguiar Saldanha Pinheiro* [1], Francisco J. Martí-Quijal [2], Francisco J. Barba[2], Silvia Tappi [1,3] and Pietro Rocculi [1,3]

[1] Department of Agricultural and Food Science, Campus of Food Science, Alma Mater Studiorum, University of Bologna, Cesena, Italy.

[2]Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine, Faculty of Pharmacy, Universitat de València, Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot, València, Spain.

[3]Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Via Quinto Bucci, 336, 47521 Cesena, FC, Italy

* Correspondence: anacristin.deaguiar2@unibo.it

Abstract

Shrimp by-products represent important natural sources of astaxanthin. The optimization of the extraction process of astaxanthin from shrimp by-products is of great importance for the valorization of crustacean by-products and the development of astaxanthin-related products. Pulsed electric field (PEF) treatment may be a promising technology for the isolation and extraction of several components from seafood by-products and accelerated solvent extraction (ASE) is considered a green technique to recover bioactive and nutritional compounds in plants and food matrices. In this study, combined and independent effects of the emerging technologies PEF and ASE using two different solvents (EtOH and DMSO) on astaxanthin extraction of two shrimp species (*M. keranthurus and A. antennatus*) were evaluated. ASE (50 °C, 15 min,103.4 bars) and PEF (3 kV/cm, 100 kJ/kg, 74 pulses) were applied as extraction technologies. The antioxidant capacity of the extracts was evaluated by and Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays. The results showed that ASE and PEF increased the astaxanthin content in the extracts for both shrimp species and solvent used, and the higher recovery was obtained using their combination, while the increase of antioxidant capacity depended on the solvent used. Both technologies seem to be an effective tool to recover astaxanthin and antioxidant extracts from shrimp by-products.

Key words: crustacean by-products; valorization; bioactive compounds; extraction; emerging technologies.

1.Introduction

An important category of by-products from seafood processing includes crustacean ones. Approximately 6-8 million tons of crustacean waste is produced worldwide every year (FAO, 2014) Shrimp and prawns are one of the most important internationally traded seafood products, and one of the few that can be considered a "commodity", with a value of US$10 billion (or 16% of world fishery exports) (Gillett, 2008). The deep-sea red shrimp *Aristeus antennatus* is one of the most important target species of trawl fisheries in the Mediterranean Sea (Gorelli et al., 2016). The Camarote shrimp (*Melicertus kerathurus*) is caught along the Mediterranean and Atlantic coasts and is an important target species in terms of landings and value. Shrimp by-products represent important natural sources of carotenoid, among which astaxanthin (ASX) is the major one. ASX content in crustaceans could vary substantially, the observed differences could be due to variations in the amounts of carotenoid available in the feed, environmental conditions, and species, as well as due to the methods used for extraction (Pinheiro et al., 2021). The most popular method for ASX recovery is based on solvent extraction and most studies focused on the screening of conventional solvents, but the extraction yield is low. Recently, other techniques to increase the process in a sustainable way have been investigated for carotenoid recovery, for example, oil-soluble method, ultrasound, microwave and enzyme-assisted, the use of supercritical fluid and their combination. The optimization of extraction process of ASX in from shrimp shells is of great importance for the valorization of crustacean by-products and the development of astaxanthin-related products.

Currently, PEF technology is extensively employed in the food industry as new non-thermal processing technology (Nowosad et al., 2021). Pulsed electric field (PEF) treatment may be a promising method for the isolation and extraction of several components from seafood by-products such as calcium, chondroitin sulphate, collagen, chitosan, and protein (Bruno et al., 2019). Accelerated solvent extraction (ASE) is considered a green technique to recover bioactive and nutritional compounds in plants and food matrices (Sun et al., 2012). Recently, some studies have investigated the application of ASE to obtain aqueous protein extracts with in vitro antioxidant capacity from fish by-products of rainbow trout, sole, sea bass, sea bream and salmon (de la Fuente, Pallarés, Barba, et al., 2021; de la Fuente, Pallarés, Berrada, et al., 2021; Wang et al., 2021). Despite the numerous publications on the recovery of compounds from crustacean by-products, the

application of both technologies for this aim has been very limited (Pinheiro et al., 2021), and, to our knowledge, there are no publications combining PEF and ASE specifically for this purpose. The main objective of the present study was to apply PEF and ASE using two organic solvents (dimethyl sulfoxide, DMSO and ethanol, EtOH) to recovery ASX from shrimp by-products and evaluate the effects of these technologies used independently or in combination on the ASX content and antioxidant activities of the extracts.

2. Materials and Methods

2.1 *Sample preparation*

Fresh Red shrimp (*Aristeus antennatus*) and Camarote prawn (*Melicertus kerathurus*) samples were obtained in a local market in Valencia (Spain) during different days of May 2021, and then transported to the University of Valencia under refrigerated conditions. Shrimp were processed by removing head and body shell (carapace). The by-products (head and shells) were weighted and frozen at -40 °C for 48 h. Afterwards, they were freeze-dried (LABCONCO, 2.5. FREE ZONE, USA) for 72 h. Freeze-dried samples were grounded and frozen at -25 °C until the extraction processes. A portion of fresh shrimp by-products were refrigerated at 4 °C until PEF treatment, after the treatment were frozen and freeze-dried as described above for the other samples.

2.2 *Chemicals and Reagents*

AAPH (2,20-azobis-2-methyl-propanimidamide), Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid), fluorescein sodium salt and potassium persulfate (K2S2O8), ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid), diatomaceous earth (Hyflo®Super Cel®), astaxanthin standard, were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Ethanol absolute ≥99.5% (HPLC grade) and Dimethyl sulfoxide (DMSO) were provided by VWR International Eurolab S.L. (Barcelona, Spain).

2.3 Process description

Dimethyl sulfoxide (100% DMSO) or Ethanol absolute (EtOH) were used as organic solvents, and seven different extraction processes were evaluated for ASX extraction from by-products for each shrimp specie:

1. ASE with EtOH (ASE-EtOH)
2. ASE with DMSO (ASE-DMSO)

3. ASE using samples pre-treated with PEF and EtOH solvent (PEF+ASE-EtOH)
4. ASE using samples pre-treated with PEF and DMSO solvent (PEF+ASE-DMSO)
5. PEF assisted extraction with EtOH (PEF-EtOH)
6. PEF assisted extraction with DMSO (PEF-DMSO)
7. Conventional extraction (Control)

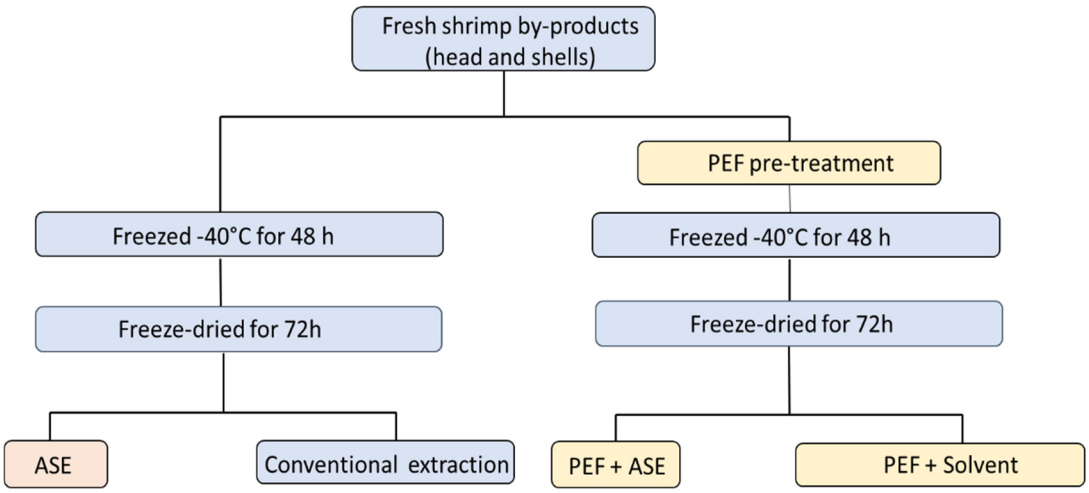

Figure 1. Experimental design of astaxanthin extraction processes from shrimp by-products.

2.4 Solvent extraction

Solvent extraction was carried out following the method proposed by (Kokkali et al., 2020).

In parallel, ASX from shrimp by-products (without PEF treatment) were also extracted with solvent using a conventional solid-liquid extraction in order to compare it with the emerging extraction process (ASE, PEF, and PEF+ASE). For the conventional extraction, samples were added into the solvent EtOH or DMSO (1:10 w/v) and stirring at 400 rpm at room temperature. After that, the samples were centrifuged for 10 min at 4000 rpm using a 5810R centrifuge (Eppendorf AG). The supernatant was collected and kept frozen at -25 °C. Each sample was processed in duplicate.

2.5 Pulsed electric filed (PEF) treatment

PEF treatment conditions were previously selected at the laboratory (data not shown) using a PEF-Cell crack III (German Institute of Food Technologies (DIL) equipment (ELEA, Quakenbrück, Osnabrück, Germany). PEF treatment was carried out between two plate electrodes with 10 cm distance between them, and the corresponding electric field strength *E* was 3 kV/cm. The specific energy input was 100 kJ/kg; the number of pulses was 74 pulses. Fresh by-products (30g) were placed

in the processing chamber, and a 300 ml of tap water was added. Before and after treatment, the temperature and conductivity were measured in the sample with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). After the PEF treatment, the samples were frozen and freeze-dried as described previously. Samples pre-treated with PEF were extracted by solvent extraction (PEF + Solvent EtOH or DMSO) and ASE (PEF + ASE-EtOH or DMSO) (Figure 1).

2.5 Accelerate solvent extraction (ASE) process

Similarly, ASE optimal extraction conditions were previously selected at the laboratory (data not shown). The accelerated solvent extractor ASE 200 Dionex (Sunnyvale, CA, USA) equipped with a solvent controller was used for the extraction of astaxanthin from shrimp by-products. Nitrogen (145 psi) was applied to assist the pneumatic system and to purge the cells. DMSO or EtOH was used as extracting solvent. The standard operating conditions were as follows: preheating period (1 min), heating period (5 min), flush volume (60%), nitrogen purge (60 s), and extraction pressure (1500 psi) for 15 min. Dried samples were mixed with diatomaceous earth (DE) before the extraction process, ratio 1:2 (sample:DE, w/w)). The extractions were performed in 22 mL pressure-resistant stainless-steel cells with a glass fiber filter placed in the end part. The extracts obtained were, divided into several replicates, and stored at -25 °C for subsequent analyses.

2.7 Determination of Astaxanthin content

The determination of the carotenoids in the extracts, reported as astaxanthin, was evaluated by reading the absorbance of the extracts, appropriately diluted, at 470 nm using a spectrophotometer Perkin-Elmer UV/Vis Lambda 2 (Perkin-Elmer, Rodgau-Jügesheim, Germany).

The carotenoids content was calculated as astaxanthin (Simpson & Haard, 1985) using the following equation:

$$\text{C } (\mu g/g \text{ samples}) = \frac{A470\ nm \times V\ \text{extract} \times dilution\ factor}{0.2\ \times W\ \text{sample}} \quad (1)$$

where A470 nm is the maximum absorbance, *V* extract is the volume of the extract, 0.2 is the A470 nm value of 1 µg/ml astaxanthin standard and *W* sample is the weight of the sample. A standard curve of astaxanthin was prepared following the method described by (Tolasa et al., 2005) with slight modifications. Each extraction was performed in duplicate and analyzed twice.

2.8 *Antioxidant Capacity*

2.8.1 Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The TEAC assay measures the reduction of the radical cation ABTS+ by antioxidant compounds, using the method previously reported by (Re et al., 1999). The ABTS+ radical cation stock solution was formed by chemical reaction with 7 Mm ABTS and 140 mM $K_2S_2O_8$ overnight in darkness at room temperature. Next, it was diluted in ethanol until an absorbance of 0.700 ±0.020 at 734 nm to obtain the ABTS+ working solution. Trolox standard solutions were prepared in a range of 0 to 400 µM. The absorbance was measured at a wavelength of 734 nm in a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Rodgau-Jügesheim, Germany). All analyses were performed in triplicate and the results were express as µM Trolox Equivalents.

2.8.2 Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay measures the capacity of the antioxidant compounds to scavenge peroxyl radicals. The fluorimetric method was applied as previously described by (Barba et al., 2013). The reaction was carried out at 37 °C in an automated Multilabel Plate Counter VICTOR3 1420 (PerkinElmer, Turku, Finland) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Sodium fluorescein and AAPH solutions were used at a final concentration of 0.015 and 120 mg/mL, respectively. Trolox (100 µM) was used as antioxidant standard and samples were properly diluted. All of them were prepared with phosphate buffer (75 mM, pH 7). The final reaction consisted of 50 µL of diluted sample, Trolox standard or phosphate buffer (blank), 50 µL of fluorescein, and 25 µL of AAPH. The fluorescence was recorded every 5 min over 60 min (until the fluorescence in the assay was less than 5% of the initial value). The results were calculated considering the differences of areas under the fluorescence decay curve (AUC) between the blank and the sample over time and were expressed as µM Trolox Equivalents.

2.9 Statistical Analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significant differences among samples. Tukey HSD (Honestly Significant Difference) multiple range test, at a significance level of $p < 0.05$ was applied.

3. Results and Discussion

In this study, combined and independent effects of the two emerging technologies PEF and ASE using two different solvents (EtOH and DMSO) on astaxanthin extraction of two shrimp species (*M.*

kerathurus and A. antennatus) were evaluated. The ASE technique combined with PEF treatment allowed us to obtain, for the first time, astaxanthin extracts with high antioxidant capacity in vitro from shrimp by-products.

3.1 Astaxanthin content

The ASX content in extracts derived from shrimp by-products are shown in Figure 2(a) for *M. Kerathurus* and Figure 2(b) for *A. antennatus*. In this study, shrimp by-products showed from 47.32 ± 1.5 μg/g on a dry weight basis (dw) (EtOH) to 85.8 ± 1.9 μg/g dw (DMSO) and from 132.8 ± 1.2 μg/g dw (EtOH) to 169.6 ± 2.6 μg/g dw (DMSO) the content of astaxanthin for *M. Kerathurus* for *A. antennatus* respectively. The content of ASX in crustaceans can vary substantially, due to variations in the amounts of carotenoid available in the feed, environmental conditions, species, and body parts as well as due to the methods and solvent used for extraction (Nakkarike M. Sachindra et al., 2005; Su et al., 2018). Ogawa et al. (2007) reported that the total carotenoid content in the heads of Brazilian shrimp (*Litopenaeus vannamei*) was 47.1 μg/g waste dry wt. In Atlantic shrimp (*Pandalus borealis)* by-products the astaxanthin content was 284.48 μg/g, when extracted with hexane/isopropanol (3:2 (v:v)) (Dave et al., 2020). Takeungwongtrakul et al., (2015) reported that the highest carotenoid content of 378.95 mg/kg was extracted from hepatopancreas of *Litopenaeus vannamei* by using isopropanol:hexane (50:50 (v/v)). ASX content among the two shrimp species was significantly different ($p<0.05$), independent of the extraction process and solvent. *A. antennatus* showed higher astaxanthin content regardless of the method and solvent extraction, ranging from 132.8 ± 1.2 μg/g dw to 585.9 ± 6.9 μg/g dw, while the maximum value for *M. kerathurus* was 213.1 ± 3.4 μg/g dw. However, considering the values from the literature, the content found in both species can be considered high and it indicates the potential of valorizing shrimp processing by-products into high value astaxanthin product. Moreover, the results obtained showed that both the extraction procedures and the solvents used had significant effects on the recovery of astaxanthin content. In the food industry, various organic solvents, such as hexane, isopropanol, acetone, methanol, and ethanol, have been widely used for astaxanthin extraction (Prameela et al., 2017). DMSO was reported to facilitate the extraction of carotenoids from microalgae (Boussiba & Vonshak, 1991; Seely et al., 1972). Accordingly, in the present study, the astaxanthin content in the extracts with DMSO was higher than with EtOH for both species in all extraction processes, including the control. However, regardless of the shrimp species, PEF and ASE technologies used independently or in combination resulted in a significant improvement in ASX extraction for both solvents (EtOH and DMSO) compared to control.

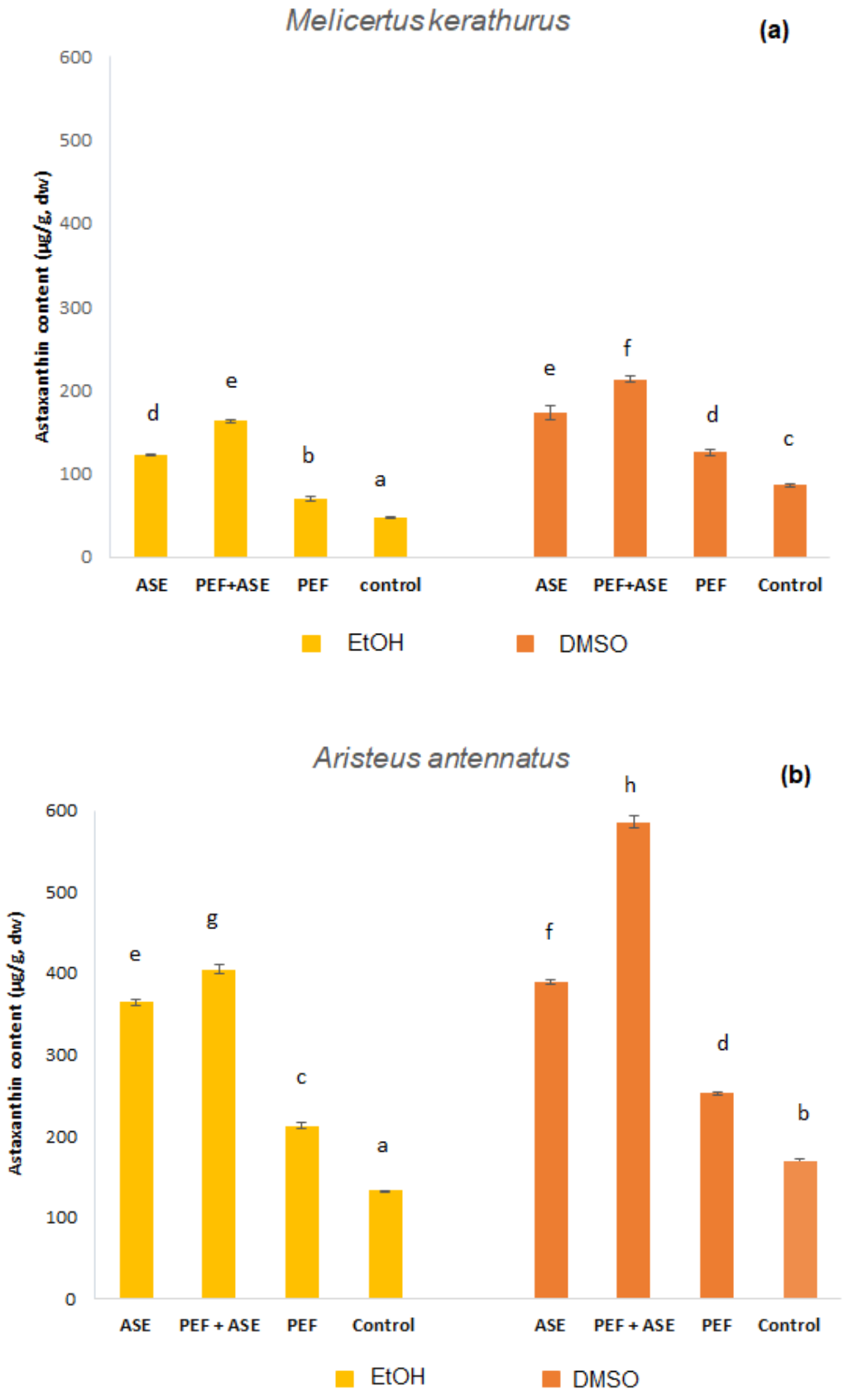

Figure 2. Astaxanthin content in *M. kerathurus* (a) and *A. antennatus* (b) by-products. Different letters above the bars indicate statistically significant differences between treatment averages ($P < 0.05$).

PEF treatment followed by solvent extraction resulted in an increase in extracted astaxanthin in the range of 46-47% and 48%-59% (depending on solvent) in *M. kerathurus* and *A. antennatus*, respectively, compared to the respective control.

In both shrimp species, ASE extracted significantly higher carotenoid content than PEF in combination with solvent extraction, regardless of the solvent used ($p < 0.05$). For the shrimp species with the lowest carotenoid content (*M. kerathurus)*, the application of ASE showed an increase in the amount of astaxanthin extracted from 2.0 (DMSO) to 2.6 (EtOH) fold compared to control.

Gulzar & Benjakul (2020), used a PEF pretreatment in combination with an ultrasound-assisted process (UAE) to extract lipids and carotenoids from the cephalothorax of shrimp (*Litopenaeus vannamei)* (electric field strength in the range of 4-16 kV cm^{-1} and number of pulses in the range of 120-240) the results showed that the treatments maximized the lipid yield (30.34 g/100g) showing an increase in the content of PUFAs and carotenoids. Recently, a response surface design was applied to investigate the effects of temperature (46–114 °C), pressure (43–77 bar) and extraction time (7–24 min) on the recovered astaxanthin from shrimp by-products. The result showed that maximum astaxanthin yield of 24 mg kg^{-1} shrimp by-products was achieved with an extraction temperature, pressure and extraction time of 87 °C, 49 bar and 14 min, respectively (Quan & Turner, 2009).

Furthermore, ASE combined with PEF pretreatment, has been found to be the best process for the extraction of astaxanthin from shrimp by-products, increasing this carotenoid in the extracts independently on the solvent used. This improvement can be attributed to the generation of pores in the matrix after PEF treatment, thus facilitating deep solvent penetration and increasing pigment extraction especially when ASE was applied. In the ASE process, the strong interaction force between the solute and the matrix can be considerably reduced at high temperature and pressure. As a result of using these particular pressure and temperature conditions, there is a change in the physicochemical properties of the solvent. For example, mass transfer, while at the same time, the surface tension and viscosity of the solvent decrease and the solubility of the analyte increases. This allows the solvent to penetrate easier and deeper into the solid matrix to be extracted, increasing extraction yields significantly compared to conventional extractions (Alvarez-Rivera et al., 2019). The highest carotenoid content observed in *M. kerathurus* (213.1 ± 3.4 µg/g dw) and *A. antennatus* (585.9 ± 6.9 µg/g dw) was achieved using PEF pre-treatment combined with ASE process and DMSO.

3.2 *Antioxidant Capacity*

Trolox equivalent antioxidant capacity assay (TEAC) and oxygen radical absorbance capacity (ORAC) of shrimp by-product extracts were used to evaluate the antioxidant capacity of the extracts rich in astaxanthin, and the results are shown in Figures 3 and 4.

3.2.1 Trolox Equivalent Antioxidant Capacity Assay (TEAC)

In this study, the extracts from shrimp by-products exhibited notable ABTS+ radical scavenging activity. Anyway, the radical scavenging in shrimp by-products extracts showed significant differences ($p < 0.05$) among the extraction process for each specie.

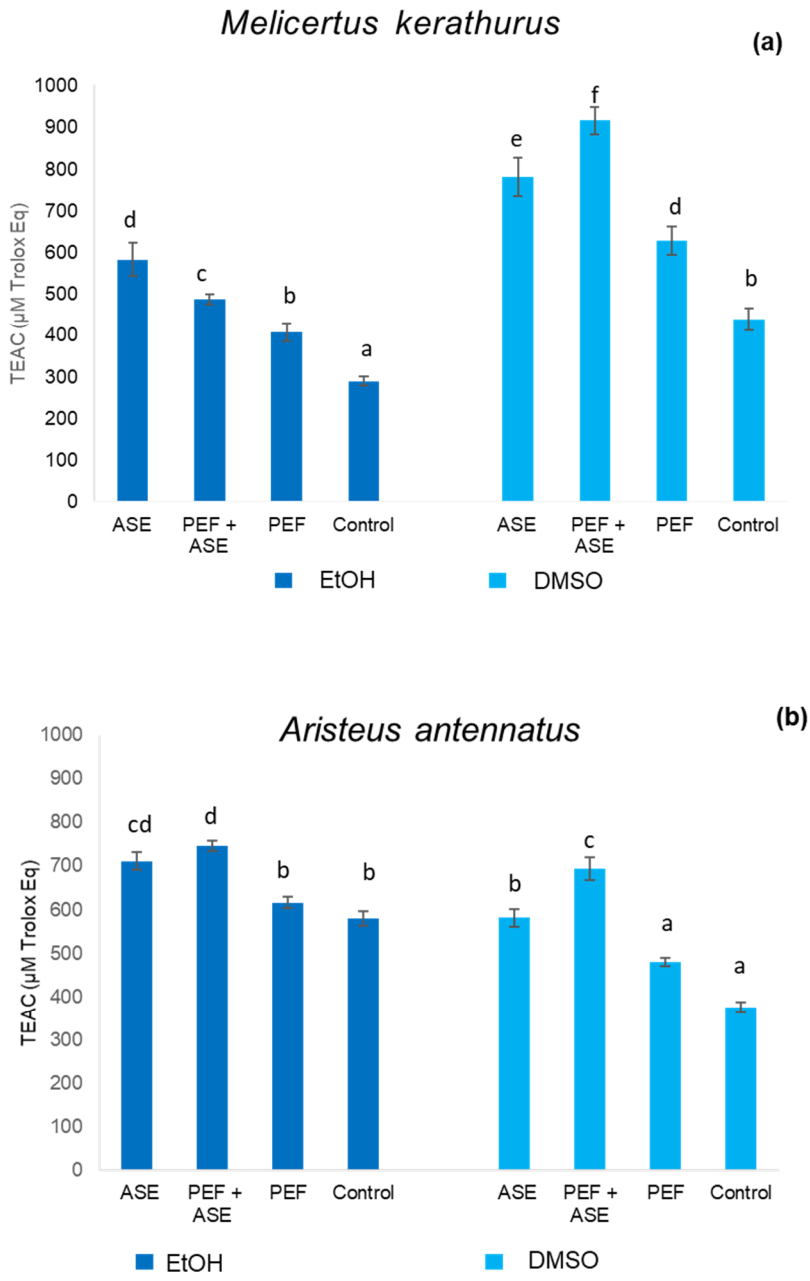

Figure 3. Trolox equivalent antioxidant capacity (TEAC) of the extracts from *M. kerathurus* (A) and *A. antennatus* (B) by-products. Different letters above the bars indicate statistically significant differences between treatment averages ($P < 0.05$).

For *M. kerathurus*, the TEAC values were in the range of 398.2 ± 11.5 to 915.4 ± 52.4 µM TE/g dw (Figure 3a) and the highest value was found in samples pre-treated with PEF and submitted to ASE using DMSO (PEF + ASE-DMSO). While, for *A. antennatus*, TEAC values ranged from 475.8 ± 12.4 and 746.0 ± 12.5 µM TE/g dw, reaching the maximum value for samples pre-treated with PEF and extracted by ASE using EtOH (PEF + ASE-EtOH). ASE and PEF processes have significantly increased the TEAC values ($p < 0.05$) for *M. kerathurus* independently of the solvent used, while for *A. antenntus* PEF processes had no significant effect ($p > 0.05$) compared with control.

3.2.3 Oxygen Radical Absorbance Capacity Assay (ORAC)

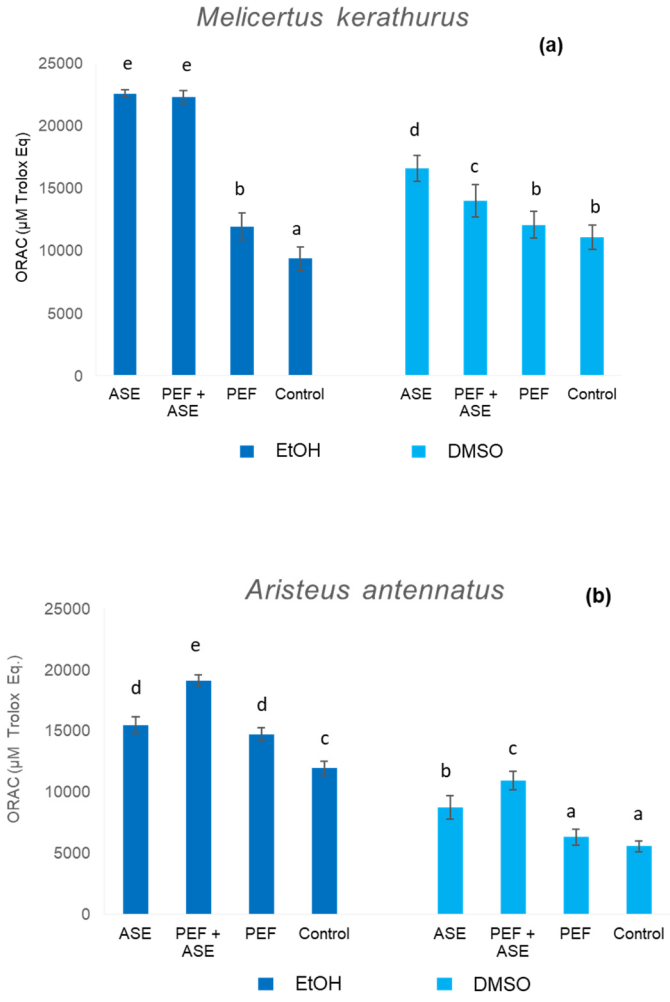

Figure 4. Oxygen Radical Absorbance Capacity Assay (ORAC) of the extracts from *M. kerathurus* (A) and *A. antennatus* (B) by-products. Different letters above the bars indicate statistically significant differences between treatment averages ($P < 0.05$).

The oxygen radical absorbance capacity, or ORAC assay, is one of the most common methods for assessing peroxyl radical ROO• scavenging capacity (Magalhães et al., 2008). The ORAC values

varied from 5567 ± 424 μM TE/g dw to 22600 ± 306 μM TE/g dw. The highest ORAC value was observed in the extracts of *M. keranthurus* extracted by ASE and ASE + PEF-EtOH. On the same line, for *A. antennatus* extracts, the highest ORAC value (19130 ± 459 μM TE/g) was for the extraction processes ASE + PEF-EtOH. The higher ORAC value of the *A. antennatus* extracts found in samples extracted by ASE + PEF-EtOH was in accordance with the higher ABTS+ scavenging activity. In particular, it was observed that *M. kerathurus* extracts showed a higher ORAC value than *A. antennatus* extracts although this species showed significantly lower ($p<0.05$) astaxanthin values than *A. antennatus*. Shrimp by-products contains astaxanthin and its esters as the major pigments (Sachindra et al., 2006), their antioxidant activity is well documented. However, shrimp by-products extract contains other antioxidants, such as phenolics, in addition to carotenoids (Seymour et al., 1996). Moreover, crustaceans are rich of several other lipophilic antioxidants, such as tocopherol and ubiquinol (Passi et al., 2002). The presence of other antioxidants at the same time also affects the antioxidant potential of the extracts, as antioxidants are known to have synergistic action (Milde et al., 2007; Shixian et al., 2005). In this study, it is possible that components other than carotenoids have influenced the radical-scavenging activity of the extracts. Moreover, TEAC and ORAC are based on different antioxidant activity mechanism. Despite both assays using Trolox as a reference antioxidant and expressing results based on Trolox equivalents, results obtained for TEAC and ORAC can lead to different conclusions, in agreement with the data reported in the literature (Zuluaga et al., 2017). Therefore, to better understand the obtained results, a more comprehensive characterization of the extract should be carried out.

4. Conclusion

For the first time it was studied the effect of the application of innovative technologies ASE and PEF alone or combined to recover astaxanthin from shrimp-by-products. This study highlights that the application of the proposed technologies increased the ASX content in the extracts for both shrimp species despite the solvent used and their effect was maximized when used in combination. Both techniques are eco-friendly and safe and seem to be an effective tool to recover rich astaxanthin extracts with strong antioxidant activity from shrimp by-products. However, these techniques are poorly developed and tailored for shrimp by-products application, lacking in standardization at industrial scale. These promising results should be confirmed by extending the investigation to other valuable compounds derived from crustacean by-products.

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