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Innovative technologies for packaging, preservation, shelf-life extension and traceability of the aquaculture and fishery products

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1. Messina, C. M., Arena, R., **Ficano, G.,** Randazzo, M., Morghese, M., La Barbera, L., Sadok, S., Santulli, A. Effect of Cold Smoking and Natural Antioxidants on Quality Traits, Safety and Shelf Life of Farmed Meagre (*Argyrosomus regius*) Fillets, as a Strategy to Diversify Aquaculture Products. *Foods*. 2021, 10, 2522. https://doi.org/10.3390/foods10112522. **(Chapter 3)**

2. Messina, C. M., Manuguerra, S., Arena, R., Renda, G., **Ficano, G**., Randazzo, M., Fricano, S., Sadok, S., Santulli, A. In Vitro Bioactivity of Astaxanthin and Peptides from Hydrolisates of Shrimp (*Parapenaeus longirostris*) By-Products: From the Extraction Process to Biological Effect Evaluation, as Pilot Actions for the Strategy "From Waste to Profit". *Marine Drugs*. 2021, 19(4), 216. https://doi.org/10.3390/md19040216. **(Chapter 6)**

3. Messina, C. M., Arena, R., Manuguerra, S., Renda, G., Laudicella, V.A., **Ficano, G.**, Fazio, G., La Barbera, L., Santulli, A. Farmed Gilthead Sea Bream (*Sparus aurata*) by-Products Valorization: Viscera Oil ω-3 Enrichment by Short-Path Distillation and In Vitro Bioactivity Evaluation. *Marine Drugs*. 2021, 19(3),160. https://doi.org/10.3390/md19030160. **(Chapter 5)**

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Summary

As it was described in the **Chapter 1** of the present PhD thesis, the fish production sector is among the main food production sectors as it meets the global growing demand for seafood products.

Seafood consumption increased from 1961 to 2017 at an average annual rate almost twice that of annual world population growth (1.6%) for the same period (FAO, 2020). So, the fish production sector has grown over the last decades in order to satisfy the global growing demand for seafood, and it is foreseen to increase in the future (FAO, 2020).

In this context, capture fisheries production has been relatively static since 1990s. Specifically, in order to meet the increasing global demand for seafood, fishing effort on natural resources has been increasing over the years bringing global catches to over 90 million tons in 1990s (Pontecorvo & Schrank, 2012). At this point captures stabilized, suggesting that oceanic fish stocks are generally being exploited at their maximum capacity. Nowadays, overexploitation affects 34.2% of global stocks while 59.6% of marine stocks are classified as being maximally sustainably fished stocks (FAO, 2020). In terms of landings, it is estimated that 78.7% of current marine fish landings come from biologically sustainable stocks (FAO, 2020).

The aquaculture production contributed for 46% of the total fish production in 2018. In the next years, global growth in fish production will be driven by the aquaculture sector, whose production is foreseen to increase (FAO, 2020). Indeed, based on which predicted, in the 2030, the aquaculture production will provide 109 million tons out of the 204 million tons produced at global level, i.e. the 53% of the total production.

Therefore, to support this increasing sector, at both global and European levels, technological innovation is necessary and must be driven by the market, with adaptation of the products to consumer preferences, by sustainability issues and by the diversification of the seafood production chain through the development of new products from new or traditional species. In this context, technological innovation plays the major role in increasing competitiveness, environmental sustainability and profitability of the seafood production sector, for instance paying attention to the production of processed products through exploitation of underutilized fishery species with high nutritional value and species coming from the aquaculture sector. Among the other things, these strategies would allow to satisfy consumers preferences in the food sector, who prefer products of high nutritional value, whose availability is more sustainable and whose origin and quality are guaranteed.

Moreover, the considerably high volumes of production in the seafood industry result in the production of equally high volumes of by-products derived from fish processing (70% of fish processed depending on the type of processing and fish) (FAO, 2020), which is both a social and environmental problem that needs to be solved.

Therefore, the current project was aimed to increase the environmental and economic sustainability, competitiveness, profitability and safety of the aquaculture and fisheries sectors through product differentiation and extension of product shelf life, by improving new and traditional species and developing new value-added products. In particular, through:

1. Development of new value-added products from new and traditional species of the aquaculture sector.

2. Development of value-added products and shelf-life extension of commercially important fishery species, considering that loss or wastage between landing and consumption accounts for 27% of landed fish. Therefore, it is necessary to develop preservative technologies that help maintain freshness and quality of fish for longer period.

These objectives were pursuied by applying innovative techniques tested on both fisheries and aquaculture species: cold smoking used alone or in combination with natural antioxidants and cold technologies such as slurry ice.

3. Valorization of fish by-products that represent high percentage of the processed products and a resource rich in bioactive compounds.

The by-products are usually represented by heads (9–12% of total fish weight), viscera (12–18%), skin (1–3%), bones (9–15%) and scales (about 5%) (FAO, 2020). These by-products are a very huge source of bioactive compounds that can be exploited in a lot of sectors, such as nutraceutics, pharmaceutics, cosmeceutics. In particular, I focused on both exploiting farmed Gilthead sea bream viscera to obtain omega 3-enriched oil and on the setting-up of pilot-scale protocol for the reutilization of by-products of processed shrimp. Indeed, non-edible parts of crustaceans could be a rich source of valuable bioactive compounds such as the carotenoid astaxanthin and peptides, which have well-recognized beneficial effects. These compounds are widely used in nutraceutics, pharmaceutics and feed industry, and their market is rapidly growing, suggesting the need to find alternative sources.

Regarding objective 1: development of value-added products from new or traditional species in the Mediterranean aquaculture sector, the part 1 of the present PhD thesis focuses on the development of new value-added seafood products by exploiting European sea bass (*Dicentrarchus labrax*) a well-known aquaculture species, and meagre (*Argirosomus regius*) another aquaculture species whose production has been increasing in Europe. As described in the **Chapter 2,** the effectiveness of cold smoking on European sea bass fillets was investigated. The aptitude of frozen and fresh *D. labrax* fillets for cold smoking was investigated by processing both fresh and thawed fillets kept previously at −20°C for 15, 30, 60 and 90 days. Moreover, to develop a low-salt product, fillets were immersed in low-sodium or standard brine.

It was seen that the quality of the raw material was affected by the time of frozen storage and that these starting conditions consequently impaired the quality of the smoked fillets. However, cold smoking was proven to be an effective process to develop a valuable product contributing to the growth of the aquaculture sector.

Cold smoking coupled with natural antioxidants was also applied to develop a new value-added seafood product by processing meagre (*Argyrosomus regius*) fillets, as shown in the **Chapter 3**. In the present work, the effectiveness of cold smoking combined with antioxidants (SA), compared to cold smoking alone (S), on quality and shelf life of meagre fillets was investigated. Sensory, biochemical, microbiological and physical-chemical analyses were performed on the smoked fillets during vacuum packaged storage for 35 days at 4 ± 0.5 °C. Results showed positive effects of the SA treatment on biochemical parameters of meagre fillets. The total volatile basic nitrogen (TVB-N) in smoked meagre fillets was significantly lower in the SA treatment at the end of storage, compared to the S treatment. Moreover, SA had a positive effect on lipid peroxidation. Lower values of malondialdehyde (mg MDA/kg) were observed in the SA treatment during preservation compared to S treatment.

As far as the second objective is concerned, the **Chapter 4** focuses on a study aimed at investigating the effectiveness of an innovative cold technology, specifically Slurry Ice, to extend fish shelf life of an important commercially fishery species such as anchovy (*Engraulis encrasicolus*), answering the call to develop preservative technologies that help maintain freshness and quality of fish for longer period. Sensory, biochemical, and physical-chemical analyses were performed on specimens stored under Slurry Ice (SI) and Flake Ice (FI) at each sampling time during 12 days of preservation (0, 1, 3, 6, 9 and 12 days). The sensory evaluation of specimens of *E. encrasicolus* showed that the specimens stored in SI showed better sensory characteristics, during the shelf life, compared to specimens stored in FI. Moreover, the samples stored in SI showed lower values of pH during the preservation period, highlighting better control of both endogenous and microbial activity in the fish muscle. As far as biochemical parameters related to the fish shelf life were concerned significantly lower values were recorded in samples stored under SI, compared to the samples preserved in FI, highlighting that the innovative technique would seem to reduce the spoilage of anchovy.

The third part of the present PhD thesis focuses on the third objective of my PhD thesis, i.e. exploitation and valorization of seafood by products to increase sustainability, competitiveness and profitability of the seafood production sector. Briefly, in the **Chapter 5**, farmed Gilthead Sea Bream (*Sparus aurata*) viscera were processed to obtain omega 3-enriched oil. This oil was tested *in vitro* for bioactivity, attesting to the possibility to turn waste into profit. The quality of the oil, in

terms of requirements for animal and human consumption, was assessed by determining some chemical parameters, both in crude viscera oil (CVO) and refined viscera oil (RVO). Among the extraction conditions, the higher CVO yields were obtained at 60 °C for 10 min (57.89%) and at 80 °C for 10 min (67.5%), and the resulting oxidation levels were low when utilizing both extraction conditions. RVO, obtained from CVO extracted at 60 °C, showed the highest quality. The ethyl esters of the total fatty acid (TFA) contents extracted from RVO were enriched in the ω-3 polyunsaturated fatty acid fraction (PUFAE) up to almost 56% via short path distillation (SPD). Antioxidant activities and adipogenic properties were tested in vitro. PUFAE protected 3T3 L1 cells from oxidative stress and exerted an anti-adipogenic effect in *Dicentrarchus labrax* pre-adipocytes, attesting to the beneficial properties for both farmed fish and human health.

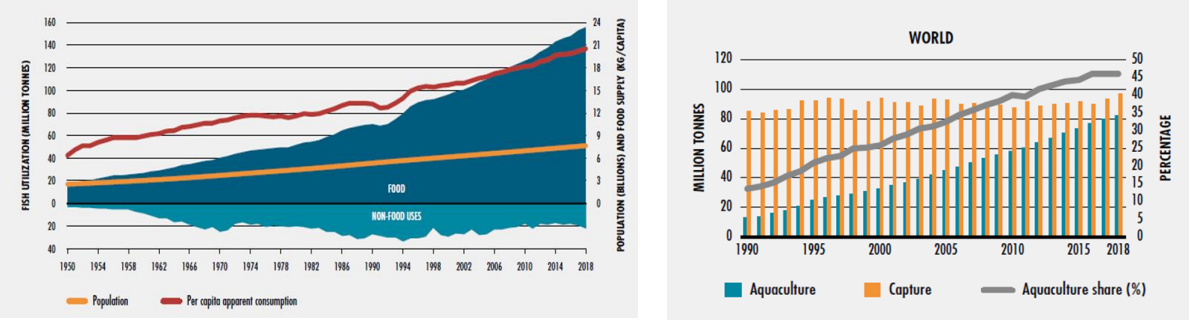
The **Chapter 6** is focused on the set-up of pilot-scale protocol for the reutilization of by-products of processed shrimp, addressing the utilization of this valuable biomass for nutraceutical and pharmaceuticals application, through the extraction of astaxanthin-enriched oil and antioxidant-rich protein hydrolysates. Astaxanthin (AST) was obtained using "green extraction methods," such as using fish oil and different fatty acid ethyl esters as solvents and through supercritical fluid extraction (SFE), whereas bioactive peptides were obtained by protease hydrolysis. Both astaxanthin and bioactive peptides exhibited bioactive properties in vitro in cellular model systems, such as antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities (IA). The results showed higher astaxanthin yields in ethyl esters fatty acids (TFA) extraction and significant enrichment by short-path distillation (SPD) up to 114.80 ± 1.23 µg/ml. Peptide fractions of <3 kDa and 3–5 kDa exhibited greater antioxidant activity while the fraction 5–10 kDa exhibited a better ACE-IA. Lower-molecular-weight bioactive peptides and astaxanthin extracted using supercritical fluids showed protective effects against oxidative damage in 142BR and in 3T3 cell lines.

The techniques mentioned above will be useful for the fish production sector, considering that they could allow to exploit new and traditional species for producing value-added seafood products that would allow to diversify the fish production sector and satisfy the consumer preferences in terms of healthy and ready to eat/cook products whose availability does not determine a problem for the environment. Moreover, in order to increase sustainability, profitability and competitiveness of the fish production sector, innovation must consider reutilization of by-products for making value-added seafood products and implementation of cold technologies for extending fish shelf life in order to prevent fish spoilage between landing and consumption and assure consumer safety.

Chapter 1. General introduction

Fisheries and aquaculture are among the major food production sectors as they meet the ever-increasing global demand for seafood products, considering that, the average consumption rate of these products has exceeded the growth rate of the world's population (FAO, 2020). Global food fish consumption increased from 1961 to 2017 at an average annual rate of 3.1%, almost twice that of annual world population growth (1.6%) for the same period (FAO, 2020) (Figure 1). The report says that by 2030, the combined production of capture fisheries and aquaculture will grow reaching 204 million tons, expected to still expand over the next decade, and leading to an 14.5% increase from the production level of 179 million tons recorded in 2018 (FAO, 2020). In the next years, global growth in fish production will be driven by the aquaculture sector whose production is foreseen to increase (FAO, 2020). Indeed, the aquaculture production contributed for 46% of the total fish production in 2018, i.e., of 179 million tons produced in the world, the aquaculture production represented 82 million tons (Figure 1). In the 2030, it is foreseen that the aquaculture production will increase of 32.2% so that of the 204 million tons of total food fish produced in the 2030 the aquaculture sector will provide 109 million tons, i.e., the 53% of the total fish production.

Of the total million tons produced in 2018, 156 million tons were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita, increasing from 18.4 kg per capita recorded in the period 2006-2015. The increase in the consumption of fish products, recorded over the years, has also been determined by the fact that fish is increasingly considered a healthy and nutritious food. The nutritional properties of fish provide 3.2 billion people worldwide with nearly 20% of their average *per capita* animal protein needs. Fish products are important from a nutritional and health point of view because they provide high quality proteins, micronutrients, minerals and polyunsaturated fatty acids, while providing low amounts of saturated fatty acids, cholesterol and carbohydrates. In developing countries, the importance of fish products in meeting nutritional needs is far greater. In Europe it is recommended the consumption of at least 2 portions of fish per week which can be satisfied only with an increase in aquaculture production.



a) **b)**

Figure 1. a) World fish utilization and apparent consumption. b) Contribution of aquaculture in total production of aquatic animals. (FAO, 2020)

The increase in fish consumption determined an increase in fishing pressure in order to increase production and meet the demand, with negative effect on the state of the marine fishery resources. According to The State of World Fisheries and Aquaculture (SOFIA) report (FAO, 2020), it was found that the fraction of marine fish stocks fished within biologically sustainable levels exhibited a decreasing trend from 90% in 1974, to 65.8% in 2017 (a 1.1% decrease since 2015) (Figure 2), with 59.6% classified as being maximally sustainably fished stocks and 6.2% underfished stocks. In contrast, the percentage of fish stocks fished at biologically unsustainable levels (overfished) increased from 10% in 1974 to 34.2% in 2017, with the largest increase occurring between the late 1970s and 1980s. In terms of landings, it is estimated that 78.7% of current marine fish landings come from biologically sustainable stocks.

In 2017, the Mediterranean and Black Sea had the highest percentage of stocks fished at unsustainable levels (62.5%) followed by the Southeast Pacific (54.5%), and Southwest Atlantic (53.3%) while the Eastern Central Pacific, Southwest Pacific, Northeast Pacific and Western Central Pacific had the lowest proportion of stocks fished at biologically unsustainable levels (FAO, 2020).

This situation, if not properly addressed, can lead not only to negative ecological consequences but also to social and economic ones, since it would reduce fish production in the long term.

Therefore, considering the problem, efforts have been made by various countries to increase the percentage of stocks fished at sustainable levels. As an example, 57% of the stocks of the main marketable tuna species were fished at sustainable levels in 2015, increasing to 66.6% in 2017, an increase of about 10% from 2015.

The Sustainable Development Goal 14 (2030 Agenda) calls on the international community to undertake policies to use in a sustainable way oceans, seas and marine resources, for example through regulating fishing practices to stop destructive, unregulated, illegal and unsustainable fishing practices, and then also, through the implementation of evidence-based management plans,

restoring fish stocks as soon as possible, at least to levels that can produce the maximum sustainable yield (MSY), determined by their biological characteristics.

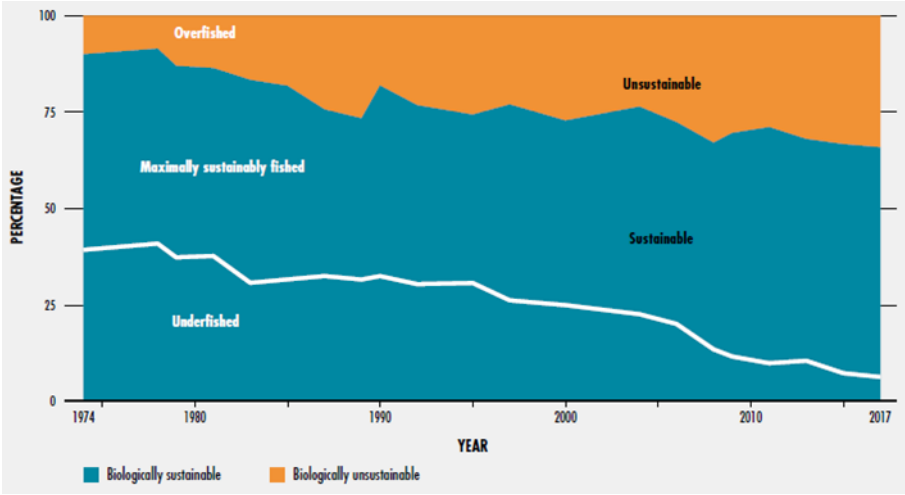


Figure 2. Global trends in the state of the world's marine fish stocks, 1974–2017 (FAO, 2020)

In view of this, the Common Fisheries Policy (CFP) of the European Union aims at increasing the sustainability of the fishery sector by promoting exploitation of underutilized fishery species that do not create sustainability problem and present some ideal characteristics to plan targeted actions: seasonality, important for the environmental sustainability point of view, product diversification, territory promotion and high nutritional value of the product (Messina et al., 2015, 2016, 2019). The increased sustainability of the fish production sector is also due to the implementation of production from the aquaculture sector that can help meet the growing demand for fish products.

The European Strategy through the European Maritime and Fisheries Fund (EMFF) which ran from 2014 to 2020, and now through the European Maritime, Fisheries and Aquaculture Fund (EMFAF) 2021-2027, includes among its objectives the promotion of socially responsible, competitive, profitable and environmentally sustainable fishing and aquaculture, aimed at a balanced and inclusive territorial development of fisheries and aquaculture sectors. It promotes also the improvement of packaging and preservation techniques in order to extend the shelf-life of fisheries and aquaculture products, assuring sustainable, profitable, healthy and safe seafood products. These objectives are also pursuied by the FARNET project of the European Union, that aims to increase the sustainability, competitiveness and profitability of the fish production sector by adding value to fishery resources through the diversification of products available for the consumers and implementing labeling of the products.

Promoting the sustainable development of fishing and aquaculture with responsible, innovative and good practices is also at the base of the blue economy model that the Fisheries and Blue Growth District extends to countries in the Mediterranean, Africa and the Middle East. In recent years, the District has promoted a shared approach to the use of sea resources, providing useful tools to

integrate international regulations with the development of maritime policies. The "Blue Strategy", from the point of view of the circular economy, promotes the valorization of production waste to allow rationalized use of resources.

Moreover, concerning the National Smart Specialization Strategy (NSSS), in the context of the European Strategy, the thematic area “Health, food and quality of life” through the development trajectory “Systems and technologies for packaging, preservation, traceability and safety of food production, Nutraceutics, Nutrigenomics and Functional Foods” promotes the definition of new innovative technologies for packaging, preservation and shelf life extension of aquaculture and fishery products that can help enhance sustainability of production as well as assure safety and health of consumers.

Talking about sustainability of the seafood production sector means also thinking about new innovative tools that can allow to valorize the huge amounts of by-products generated by the processing sector and that amount for 70% of the seafood processed (FAO, 2020). The NSSS by means of the development trajectory “Systems and technologies for water and waste treatment” related to the thematic area “Smart and sustainable industry, power and environment” aims also at developing innovative technologies for recovering important and beneficial compounds from by-products such as polyunsaturated fatty acids of the n-3 series or antimicrobial compounds such as chitosan, that also can help extend seafood shelf life (Kulawik et al., 2020; Messina et al., 2013).

Aquaculture: an ever-increasing food production sector

In 2018, global fish production amounted to 179 million tons, and aquaculture accounted for 52% of the total production used for human consumption. World aquaculture production of farmed aquatic animals grew on average at 5.3% per year in the period 2001–2018. Specifically, in 2018, aquaculture produced 82.5 million tons of fish and other organisms, 32.4 million tons of plant organisms, and good amount of products not intended for consumption (26000 tons) (FAO, 2020). In 2018, aquaculture fish production was dominated by finfish (54.3 million tons – 47 million tons from inland aquaculture and 7.3 million tons from marine and coastal aquaculture), while a smaller percentage was represented by shellfish (17.7 million tons) and crustaceans (7.9 million tons) (FAO, 2020).

The aquaculture contribution to world fish production has increased, reaching 46.0% in 2016–18, from 25.7% in 2000. In 2018, 39 countries, located across all regions except Oceania, produced more aquatic animals from farming than fishing. Mariculture and coastal aquaculture collectively produced 30.8 million tons of aquatic animals in 2018. Despite technological developments in marine finfish aquaculture, marine and coastal aquaculture produce currently many more molluscs than finfish and crustaceans.

In 2018, shelled molluscs (17.3 million tons) accounted for 56.2% of the production of marine and coastal aquaculture. Finfish (7.3 million tons) and crustaceans (5.7 million tons) were responsible for 42.5%.

Aquaculture accounted for 16–18% of total fish production in Europe. Overall European Union (EU) production has been stable since 2000, whereas global production has been growing between 5% and 7% per year. The main aquaculture-producing EU countries in terms of volume are Spain, France, Italy and Greece. European aquaculture is based on a few species: mussels, salmon, sea bream, sea bass, oysters, rainbow trout and carp. In addition, two more species need to be mentioned: meagre (*Argyrosomus regius*) and the Senegalese sole (*Solea senegalensis*) (EUMOFA, 2020). In the EU, their production rose progressively over the decade: in 2018, the production of meagre reached 6827 tons (+270%) and that of Senegalese sole reached 893 tons (+1318%). Both fish species are farmed predominantly in Spain: in 2018, Spanish production covered 59% of meagre's EU production, and 100% of that of Senegalese sole. Other producers of meagre are Greece and Croatia (EUMOFA, 2020).

In 2018, Italy represented 11% of production volume and 9% of value. In Europe, it was the main producer country of clams (*Ruditapes philippinarum*): 77% in volume and 66% in value.

In Italy, the most represented sector is that of shellfish farming; in addition to clams, Italy produces mussels (*Mytilus galloprovincialis*). Other species farmed: sea bream, sea bass and meagre. Nevertheless, the production of sea bass and sea bream resulted to be unprofitable because of the Greek and Turkish products more competitive on the market, leading farmers to look at other rearing species to diversify their production.

Among freshwater species, trout is one of the main species farmed, of the 35% of fish farmed, the rearing of trout represents 39%, while sea bass and sea bream represent 20% and 18% respectively.

In Italy, in the period 2014-2030 it has been estimated a growth in production from aquaculture equal to +38%, thanks to investments aimed at implementing facilities and the creation of new ones.

The expected increase in the value of productions will be a positive consequence of investments aimed at increasing also the added value of productions, through the production of processed, and ready-to-cook/eat products.

In 2010, the income of the Sicilian aquaculture sector was equal to about 27 million Euros of which more than 77% was achieved by the fish farming segment (I.R.E.P.A. onlus, 2010). The production of the "euryhaline species" (Gilthead Bream, European Sea Bass) in 2010 was about 3100 tons, representing, in volume, about 15% of the national production. In 2010, the regional distribution of enterprises that farmed as main species sea bass and sea bream were in the provinces of Agrigento, Syracuse and Trapani and only one enterprise in the province of Messina as shown in the IREPA

report (I.R.E.P.A. onlus, 2010). Among the Sicilian aquaculture plants, those in extensive farming in the "cold tanks" of the saltworks of Trapani and Marsala should be mentioned. This production, represented by sea bass, sea bream, mugilids, eels, is small but undoubtedly of high value than the traditional intensive ones. Thanks to policies of the Sicilian Region, and through the European Maritime and Fisheries Fund 2014/2020, it was possible to develop aquaculture facilities of freshwater species. In addition to trout farming (*Oncorhynchus mykiss* and *Salmo cettii*), in order to increase the regional aquaculture production contributing to meet the European and national demand for fish products, largemouth bass (*Micropterus salmoides*) has been recently reared, adding to the size-commercial fish production the culture of plant species by using water rich in nutrient coming from the rearing tank (www.mangroviaproject.com).

So, in order to fulfil the objectives of the European Strategy aimed at implementing the seafood production sector in a sustainable and profitable way, it is important the role played by research, for instance in the identification of new species for the diversification of production, as well as the development of innovative techniques to valorize seafood products and, finally, valorization of by-products in the circular economy point of view.

Consumption of seafood products at global, European and national level

Between 1961 and 2017, the average annual increase in global seafood consumption (3.1%) outpaced population growth (1.6%) and exceeded that of all other animal protein foods (2.1%). Globally, per capita seafood consumption grew from 9 kg in 1961 to 20.5 kg in 2018, by about 1.5% per year. Live, fresh or chilled fish still represented the largest share (44%) of fish utilized for direct human consumption as being often the most preferred and highly priced form of fish. It was followed by frozen (35%), prepared and preserved fish (11%) and cured at 10%. Freezing represents the main method of preserving fish for food, accounting for 62% of all processed fish for human consumption (i.e., excluding live, fresh or chilled fish) (FAO, 2020).

In 2018, the European per capita seafood consumption was around 24.36 kg, showing a slight decrease compared to the per capita seafood consumption recorded in 2017 (EUMOFA, 2020). According to the EUMOFA report, Spain, Italy and France are the top three consumers, accounting for close to 80% of the total volume of fresh fishery and aquaculture products consumed by households in 12 EU Member States analyzed. In Italy, total per capita consumption increased in 2017 compared to 2016 by 4%, and it increased further by 1% from 2017 to 2018. Tuna is confirmed to be the most consumed species in the EU (99% caught and 1% farmed) with a per capita consumption of 3.05 kg. EU consumption of tuna is largely supported by imports, but there is also internal production, mainly consisting of Spanish and French catches. However, a significant share of these catches is landed abroad close to fishing areas, further processed there and then re-

exported. An increase in per capita consumption was observed for Alaska pollock (+9%), shrimps (+7%) and hake (+6%). Salmon is by far the most-consumed farmed species in the EU. In 2018, it accounted for 36% of the total apparent consumption of aquaculture products followed by mussels supplied by Spanish production.

In 2019, out-of-home consumption of processed fishery and aquaculture products in the EU was 734800 tons, which was the highest level in more than ten years.

Shelf-stable products had the highest consumption, followed by frozen and chilled products. Nevertheless, shelf-stable products consumption varied a lot among countries in 2019, ranging from 7% in Sweden, where frozen products were preferred, to 81% in Spain.

In order to meet the internal demand for seafood products a lot of products are imported from extra-EU countries. The extra-EU trade balance deficit in 2019 was worth 21 billion euros, slightly higher than the previous year (EUMOFA, 2020). The deficit in real terms grew by 33% from 2010 to 2019. Almost half of the 2019 deficit (49%), corresponding to 10.2 billion euros, was due to frozen products. As for fresh and prepared-preserved products, “fresh” with a value of 6.1 billion euros accounted for 29% of the 2019 deficit, while prepared-preserved with a value of 3.6 billion euros accounted for 17%. Compared with 2018, all the three major preservation categories showed a worsening trade balance.

More than one-quarter of extra-EU imports originates from Norway, the main supplier. China is second, accounting for less than 10% of the total, while Iceland, Ecuador, Morocco, Vietnam and the United States account for 5%. The most imported products are salmon, mainly from Norway, shrimps from Ecuador and Vietnam, cod from Norway and Iceland, skipjack tuna from Ecuador, Alaska pollock from China and the US, and fishmeal not destined for human consumption from Norway.

Tunas imported in the EU in 2019 amounted to 787613 tons worth 3.17 billion euros. This consisted almost entirely of processed products, of which 72% was canned and 28% was frozen. In terms of species, skipjack tuna accounted for more than half of EU imports of tuna, followed by yellowfin tuna.

Almost all skipjack tuna is imported as prepared-preserved products. Ecuador is the main supplier, followed by the Philippines and Mauritius. EU imports of prepared-preserved skipjack tuna have been increasing since 2015 from all the three main origins in both volume and value terms.

Therefore, considering that Europe is the biggest market in the world for fish consumption, in terms of value, Axis 4 of the European Fisheries Fund proposed by the European Commission has promoted the increase of the added value of fishery and aquaculture products to promote the development of the territory through the development of local production. Innovation plays the major rule in increasing competitiveness, sustainability and profitability of the local seafood

production chain, paying attention to the production of processed products by exploiting local underutilized fishery species with high nutritional value and species coming from the aquaculture sector. Among the other things, this strategy would allow to satisfy consumers preferences in the food sector, who prefer products of high nutritional value, whose availability is more environmentally sustainable and whose origin and quality are guaranteed (Bonanomi et al., 2017).

For pursuing this objective, it is necessary to know the market, considering the change in consumer lifestyles, which have become much more dynamic with change in consumption habits, in terms of decreasing interest in whole fresh fish, in favor of easy-to-prepare products, ready-to-eat fish products, gastronomic specialties, fillets and slices.

In Italy, seafood products were sold much better also in the pandemic period showing a positive trend compared to the previous year. In 2020, in Italy it was recorded an increase of 7% in the consumption of fresh, preserved and processed seafood products compared to the year 2019 (ISMEA, 2021). Moreover, in the first quarter of 2021, the seafood sector has been one of the most dynamic sectors showing an increase in consumer expenditure of 15% on annual basis. Fresh and smoked seafood products, in this first part of 2021, enjoy a growing preference by consumers, with increases in volumes purchased of 30% and 20% respectively.

Utilization of by-products to extract bioactive compounds and produce value added products: from waste to profit

The considerably high volumes of production in the fish industry in order to meet the increasing consumption of seafood products, result in the production of equally high volumes of waste derived from fish processing (70% of fish processed depending on the type of processing and fish) (FAO, 2020), which is both a social and environmental problem that needs to be solved.

Fish by-products can be used for several purposes. Heads, frames and fillet cut-offs and skin can be used directly as food or processed into fish sausages, cakes, snacks, gelatin, sauces and other products for human consumption (FAO, 2020). By-products are also used in the production of feed, biodiesel and biogas, dietetic products (chitosan), pharmaceuticals (including oils), natural pigments, cosmetics and constituents in other industrial processes. Fish collagens are used in cosmetics and in extraction of gelatin. Fish bones, in addition to be a source of collagen and gelatin, are also an excellent source of calcium and other minerals such as phosphorus, which can be used in food, feed or food supplements (FAO, 2020). Calcium phosphates present in fish bone, such as hydroxyapatite, can help hasten bone repair after major trauma or surgery. Fish skin, in particular from larger fish, provides gelatin.

A possible solution to the problem is therefore the implementation of methodologies that lead to the production of value-added fish products by implementing extraction techniques of bioactive compounds whose by-products are rich.

Among possible uses mentioned above, an example of valorization of fish by-products is the production of omega-3 enriched-oils exploiting matrices that are particularly rich in n-3 PUFA, such as DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid), compounds that are extremely beneficial for the human health (Arab-Tehrany et al., 2012).

One possible alternative, for fish oils production for human consumption, could be the use of by-products from the processing sector of the aquaculture species. Indeed, these by-products represent a cost to farmers as they represent resources for disposal. Moreover, they have interesting properties for an industrial application, starting from the low cost; a highly controlled chain that certifies high organoleptic properties and the absence of major environmental contaminants. Moreover, the production chain within the plants ensures a constant yield and production of raw material for industrial processing, giving significant economic returns to the companies also in terms of the quality of the farmed product. Moreover, it is important to identify new sources for the industrial production of PUFA-rich oils and ω-3 PUFA concentrates, considering the high pressure of fishing on fatty species belonging to the families such as Scomberesocidae, Gadidae, and Clupeidae, with a consequent depletion of wild fish stocks (Šimat et al., 2019).

Moreover, as the production and processing of crustaceans and bivalves have increased, efficient use of their shells has become important, not only to maximize financial return, but also to address waste disposal problems because of their slow natural degradation rate (FAO, 2020). Chitosan, produced from shrimp and crab shells, has shown a wide range of applications, for example in water treatments, cosmetics, food and beverages and pharmaceuticals. Chitosan is a natural polymer obtained by alkaline deacetylation of chitin, a natural compound entracted mainly from the exoskeletons of crabs and shrimps, by chemical or biological methods. It has several beneficial properties, such as being antimicrobial, anti-inflammatory, anti-tumor with wound-healing activity. Moreover, it can be used in the packaging and preservation of seafood because of its antimicrobial and antioxidant properties in order to make biofilm that can help mantain seafood quality and enhance sustainability of production. It has been reported that applications of chitosan-based films or coatings or treatments have resulted in shelf life extension of a lot of food products not only fish (Kulawik et al., 2020).

Crustacean wastes also yield pigments (carotenoids and astaxanthin) for use in the pharmaceutical industry. Moreover, as fish cannot synthesize their own colouring pigments *de novo,* the colouring agents extracted form seafood by-products can be used in the aquaculture industry to be incorporated into the fish (salmon, ornamental fish species) diet in order to impart color for the fish

muscle and skin. Indeed, pigmentation is a very important factor to consider for consumer acceptability. Moreover, the consumer concern about using synthetic additives, such as synthetic pigments, into the fish diet of rearing fish has led the feed industry towards the use of compounds of natural origin to be included in the fish feed, such as compounds nauturally present in microalgae (Lorenz & Cysewski, 2000).

Aims of the PhD thesis

The development of innovative technologies for packaging, preservation and shelf life extension of aquaculture and fishery products was aimed at increasing the competitiveness, profitability and sustainability of the seafood production sector, one of the main food production sector, taking into account the sustainability needs and the consumer preferences. In order to address these issues, strategies applied could be the diversification of the available products, exploiting new species from aquaculture and fishery sectors, as well as traditional species, whose supply often exceeds the demand, to produce new value-added seafood products.

Therefore, the current project was aimed to increase the environmental and economic sustainability, competitiveness, profitability and safety of the aquaculture and fisheries sectors through product differentiation and extension of product shelf life, by improving new and traditional species and developing new products. Through:

1. Development of new value-added products from new and traditional species of the aquaculture sector.

2. Development of value-added products and shelf-life extension of commercially important fishery species, considering that loss or wastage between landing and consumption accounts for 27% of landed fish. Therefore, it is necessary to develop preservative technologies that help maintain freshness and quality of fish for longer period.

These objectives were pursuied by applying innovative techniques tested on both fisheries and aquaculture species: cold smoking used alone or in combination with natural antioxidants and cold technologies such as slurry ice.

3. As the considerably high volumes of production in the seafood industry result in the production of equally high volumes of by-products derived from fish processing (FAO, 2020), both a social and environmental problem that needs to be solved, third objective of my PhD thesis concerned the exploitation and valorization of fish by-products to increase sustainability and profitability of production. Through the definition of protocols for recovery of bioactive compounds (omega 3 long-chain polyunsaturated fatty acids, astaxanthin, protein hydrolysates) from seafood by-products.

The techniques developed in the present work can contribute to:

- Extension of shelf life.
- Traceability and Differentiation of the products.
- Lowering of production costs.
- Reduction of the environmental impact by maintaining at the same time profitability.
- Consumers' safety and acceptability.

The effectiveness of the innovative techniques on maintaining quality and prolonging seafood shelf life was evaluated by means of a multidisciplinary approach, i.e., by means of sensory, physical-chemical, and biochemical analysis.

Multidisciplinary approach to the evaluation of quality and freshness of fish products

In order to detect levels of seafood product freshness and quality, it is important that physical-chemical, microbiological and biochemical-nutritional parameters are investigated, considering that they undergo alterations from the moment of the animal's death and during preservation and therefore have to be properly monitored to be sure of the suitability of the product for human consumption.

In the determination of freshness and quality of fish products, it is necessary to carry out also the evaluation of the sensory characteristics, i.e., color, odor, taste, juiciness and texture evaluation of the product, intended to be marketed, in order to evaluate the degree of product acceptability for consumers. These sensory parameters also change in relation to the type of product analyzed and therefore, must be species-specific, with appropriate evaluations adapted to the type of product.

The EU scheme, for quality assessment in the seafood sector, is a classification system by categories, according to which three classes of product quality are defined, namely E (Extra), A, B and below B if the product is very fresh, fresh, bad quality but suitable for consumption and not suitable for marketing respectively. This scheme is based on sensory evaluation of fish and marine invertebrates (crustaceans, cephalopods) by trained panels. The individual observations are considered to define an overall judgment of product quality by assigning one of the above-mentioned categories (Regolamento (CE) n. 2406/96). Nevertheless, the sensory evaluation carried out through the EU scheme does not consider the species-specific characteristics, being too much generic. Characteristics that the Quality Index Method (QIM) allows to consider.

QIM, initially developed by the Tasmanian Food Research Unit (Bremner, 1985), is used for the sensory evaluation of many fish species, based on the description of parameters such as gills, skin, mucus, eye etc. According to this method, demerit points are assigned to qualitative parameters, considered independent from each other, ranging from 0 to 3 (from very fresh to unfit for consumption) based on standardized indicators of a certain degree of freshness (Knowles et al.,

2007). Some parameters are not considered as much as others, therefore some parameters, less important, will have a lower range of values assigned compared to others. The sum of the individual scores obtained gives the total score which makes it possible to establish the number of days still available for conservation on ice, since there is a positive linear relationship between the demerit score obtained and the days of conservation on ice, where the score 0 corresponds to a fixed point in the calibration curve (0.0), i.e. the day the fish was caught or harvested, while the maximum demerit score corresponds to the maximum time of conservation on ice.

Physical-chemical parameters

Biochemical parameters related to shelf life

Evaluations of specific chemical parameters allow to define the level of deterioration of the product as well as sensorial parameters, therefore they must be correlated to these ones.

Total volatile basic nitrogen (TVB-N), one of the most widely used indicators of fish spoilage, is formed by the degradation of nitrogen compounds by endogenous and bacterial enzymes, which produce ammonia and volatile amines. Trimethylamine (TMA), a volatile nitrogen compound, is formed by bacterial action due to the reduction of an alkylamine widely present in the tissues of marine vertebrates and invertebrates, Trimethylamine N-oxide (TMAO), leading to the formation of TMA which causes the characteristic odor of spoiled fish at concentrations between 4-6 mg/100 g. The production of TMA depends on the action of so-called spoilage bacteria, such as *Photobacterium phosphoreum* and *Shewanella putrefaciens* (Dalgaard, 1995). The bacteria that generate this compound are facultative anaerobes and use TMAO in their metabolism, making this compound a good indicator of an advanced stage of alteration. On the other hand, another nitrogenous compound that develops by the action of endogenous enzymes is dimethylamine (DMA), which originates from TMAO by the action of a TMAO demethylase present in fish tissues, under freezing conditions of the product. DMA production is also associated with the formation of formaldehyde, which causes denaturation of myofibrillar proteins, a phenomenon much more pronounced in gadiforms (Atlantic cod) (Gill et al., 1979).

The content of lipids, basically phospholipids and triglycerides, can be very high in fish, varying from one species to another; so, depending on the muscle fat, fish are classified into lean (<5%), medium-fat (5-15%) and fat (>15%) fish species (Tang et al., 2001). Fish is characterized by high quantities of polyunsaturated fatty acids (PUFAs), beneficial for human health but also extremely susceptible to peroxidation. Lipid peroxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially PUFAs. Their oxidation generates free radicals that trigger chain reactions that eventually lead to

the generation of compounds responsible for extremely unpleasant odors as well as the loss of compounds of very high nutritional value (Arab-Tehrany et al., 2012). In particular, in the early stages of initiation and propagation, free radicals are generated from the oxidation of lipids (Figure 3) leading to the formation of hydroperoxides, which are odorless and therefore not correlated to sensory evaluations, but quantifiable by appropriate methods (e.g., peroxide value, PV) (Wang et al., 2017). The breakdown of hydroperoxides results in the formation of short-chain side products such as ketones, aldehydes, and fatty acids, substances responsible for off flavours (Figure 3) (Azhar & Nisa, 2006; Wang et al., 2017). Cells have enzymes that reduce the amounts of hydroperoxides produced such as glutathione peroxidase, which, however, requires reduced glutathione for the reaction, which is lost with the death of the animal. In addition, cell membranes contain natural antioxidants such as α-tocopherol (Vitamin E), considered one of the most important, which, by reacting with free radicals generated by lipid oxidation causes their removal (Arab-Tehrany et al., 2012).

Current assays to assess food oxidative rancidity involve the measurement of hydroperoxides (PV) for the determination of primary oxidation products and low-molecular-weight aldehydes for secondary products (e.g. Malondialdehyde, MDA) (Ayala et al., 2014). MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its reaction with thiobarbituric acid (TBA) (Esteribauer & Cheeseman, 1991).

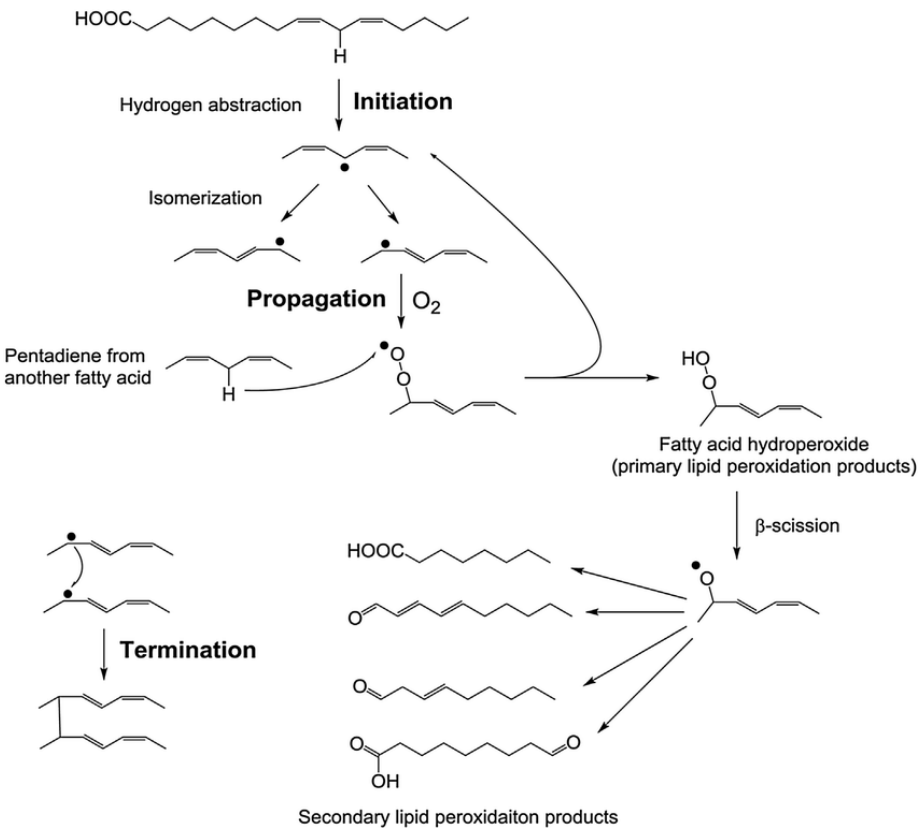


Figure 3. A simplified scheme of lipid peroxidation (Wang et al., 2017)

Color

In order to assess the quality and freshness of fish products, color is an important attribute, indicative of chemical components and sensory attributes of food (Cheng et al., 2015). Among the color parameters measured in the evaluation of seafood quality and freshness there are lightness (L*), red-green chromaticity (a*), yellow-blue chromaticity (b*) the color saturation or intensity (Chroma, C*) and Hue angle (h) (Robertson et al., 1977).

It has been seen how these parameters change in relation to product quality loss. Yagiz et al. (2007) treated *Coryphaena hippurus* fillets with high pressure (HP) and reported that L* value increased slightly as a function of increased pressure and general loss of quality. An increase in lightness during preservation was associated with a loss of quality in cold smoked salmon (Birkeland & Bjerkeng, 2005). Regarding redness (a*), it has been seen that a* tended to decrease as a function of product quality loss in *C. hippurus* fillets when not subjected to appropriate treatments such as storage in modified atmosphere packaging combined with antioxidants (Messina et al., 2015). Supporting the use of these parameters in seafood quality analysis, Lee et al. (2003) reported a decrease in a* in relation to an increase in thiobarbituric acid reactive substances (TBARS) in cold stored yellowfin tuna. In Messina et al. (2015), loss of red color correlates with peroxide value (PV). An increase in b* (yellowness) may be the result of the browning bloodline due to heme protein oxidation, corresponding to the reduction in a*(Richards et al., 2002).

Water Holding Capacity (WHC)

WHC is another parameter that contributes to making a product appreciable by consumers (Fuentes et al., 2010). The water holding capacity measures the ability of the muscle to retain water. Most free water, that can be easily released, lies between the actin and myosin filaments of myofibrils in living or pre-rigor muscles. This water, lost as drip during postmortem storage, is also known as drip loss. Both WHC and drip loss can affect surface appearance and texture, thereby the sensory quality of food. A low WHC is related to postmortem changes in the muscle such as myofibril shrinkage, and a high drip loss is usually related to greater protein denaturation (Duun, 2008). These are undesirable as they lead to greater water and nutrients loss, and directly result in lower seafood quality and sale value (Chan et al., 2020). Therefore, maintaining a high WHC and low drip loss is a common aim for fish producers.

A more pronounced reduction in WHC was seen during the last period of preservation of fillets of *C. hippurus* that were not preserved in modified atmosphere packaging. On the other hand, in the MAP-preserved fillets, drip loss reached approximately 2% (Messina et al., 2016). Similar results consisting of a less pronounced reduction in WHC under modified atmosphere packaging have been

reported for Atlantic cod, salmon, sea bream, and sea bass (Dalgaard et al., 1993; Goulas & Kontominas, 2007; Sivertsvik et al., 2002; Torrieri et al., 2006). On the other hand, particular attention must be paid to the gas mixture used when applying modified atmosphere storage; in particular, high quantities of CO_2 can cause a marked reduction in muscle pH, which then affects the WHC with a consequent increase in drip loss (Sivertsvik et al., 2002).Water loss around 3-5% maintains an acceptable degree of juiciness (Torrieri et al., 2006), so applying absorbent pads in the packages can mitigate the negative impact of drip loss on both quality and consumer acceptability (Messina et al., 2016; Torrieri et al., 2006).

Moreover, as far as smoking is concerned, it was seen that WHC increased after smoking in sea bass fillets as the result of the increment in salt content positively correlated with the WHC (Fuentes et al., 2012). Previous studies reported that brining allows proteins to retain more moisture, as lower salt concentrations cause a lower degree of protein denaturation and induces swelling of muscle fibers, leading to a higher WHC (Offer & Trinick, 1983). This occurs due to the repulsive electrostatic forces of Cl^- anions weakly attached to the myofibrillar and sarcoplasmic proteins, causing protein to coagulate and entrap free water. At higher salt concentrations, protein denaturation increases and myofibrillar proteins lose water, causing muscle dehydration and eventually yield loss (Thorarinsdottir et al., 2004).

Moreover, drip loss could be affected by the rate of superchilling. Rapid superchilling results in the formation of both intra- and extracellular ice-crystals. Though the cells are dehydrated due to the formation of extracellular crystals, due to the rapid rate of superchilling, intracellular water freezes before it diffuses out of the cells. Moreover, formation of crystals cannot keep pace with the rate of heat removal leading to activation of more nucleation sites, increase in the number of small ice-crystals, minimum damage to muscle structure, less drip loss and other quality parameters during thawing (Banerjee & Maheswarappa, 2019).

Texture

As above-mentioned, texture of fish muscle is an additional quality parameter of fish products strictly related to WHC and muscle water loss.

Immediately after the death of the animal, the muscle is soft and elastic. With the onset of rigor mortis, the muscle changes considerably, becoming extremely rigid. During this phase the muscle becomes harder and then becomes soft again. Tenderness continues to increase as proteolytic processes proceed during the storage period and at certain freezing temperatures; moreover, freezing can induce changes in muscle proteins modifying the texture of the product.

As mentioned above, freezing of the product can affect the texture of the muscle depending on the rate at which ice crystals form at extracellular and intracellular levels and on the muscle state, i.e.,

post or pre-rigor, which result in a conspicuous loss of intracellular water. At slow freezing and with muscle in post-rigor, crystallization occurs extracellularly and causes an increase in extracellular osmotic pressure that leads to leakage of intracellular water with an increase in the concentration of solutes in intracellular water, a condition that lowers the freezing point, getting freezing to occur at very low temperatures; cellular dehydration and the ionic strength of the intracellular aqueous solution can then interfere with the hydrogen bonds and secondary forces that determine the three-dimensional structures of myofibrillar proteins, leading to their denaturation and aggregation (Love, 1997; Shenouda, 1980). In contrast, faster freezing in the post-rigor phase ensures that, although water begins to freeze extracellularly, the temperature falls so rapidly that prevents much of water from going out cells. As the freezing rate increases, the size of intracellular ice crystals decreases and their number increases (Bevilacqua et al., 1979). Independent of freezing rate, however, is the pre-rigor phase, in which, intracellular water associated with proteins prevents diffusion from inside out, resulting in crystallization. In Chen & Pan (1997) was seen how the reduction of freezing rate in tilapia muscle resulted in an increase in extracellular space due to the formation of larger crystals in this area. Therefore, if proper freezing and maintenance procedures are not implemented the texture of the fish muscle may be compromised due to a reduction in WHC and increase in water loss during thawing, resulting in a hard and dry product.

Therefore, instrumental evaluation of muscle texture is important for evaluation of fish quality. Hardness (N) and Young Modulus (N/mm2), i.e., compactness, are among variables considered in the texture analysis of seafood product, (i.e., the force and the slope of the curve at 50% compression, respectively) (Messina et al., 2019; Orban et al., 1997).

Microbiological parameters

In live fish and in freshly caught or harvested fish, the microbial flora tends to be concentrated in gills, surface mucus, and intestinal contents, with extremely strong variations in bacterial load among species, conditioned by several factors related to the fish's habitat and biological cycle (Austin, 2006).

In fish from temperate or warm waters, the microbial flora consists mostly of Gram-negative bacteria, such as *Pseudomonas* spp., *Moraxella, Acinetobacter, Shewanella, Flavobacterium*, Vibrionaceae, and Gram-positive bacterial species (*Bacillus, Corynebacterium, Micrococcus, Clostridium, and Lactobacillus*) (Gram & Huss, 1996). In fish caught in cold water, however, the predominant microflora consists of Gram negative psychrotrophic or psychrophilic species in the surface mucus (essentially *Pseudomonas, Alteromonas, and Shewanella*) and Gram-positive species in the intestinal contents (*Clostridium* spp.) (Gram & Huss, 1996). In addition to the essentially autochthonous microflora, i.e., linked to the ecological niche of the fish, there is also the microflora

resulting from external microbial contamination of microorganisms of fecal origin or belonging to the terrestrial sphere, regarding fish that move or live in waters contaminated by urban sewage and brackish waters, or as a result of fishing, landing and product handling processes. Such microorganisms are:

- Human pathogens (*Salmonella* spp., *Listeria monocytogenes*, *Vibrio* spp., *Clostridium botulinum*, enteric viruses, strains of *Escherichia coli*, *Shigella* spp., *Aeromonas* spp., enterotoxic strains of *Staphylococcus aureus* and *Bacillus cereus*);
- Spoilage bacteria or indicators of fecal pollution, such as *Enterobacteriaceae*, total and fecal coliforms, *Pseudomonas* spp., *Shewanella* spp., *Photobacterium* spp., enterococci.

In addition to the extrinsic factors mentioned above, i.e., the ecological niche of the fish, secondary contamination and water quality, there are several intrinsic factors that determine the survival and microbial growth such as pH, water activity (aw), the connective tissue content and the redox potential.

The pH is an ecological factor that can support the survival and growth of bacteria in fish because of the composition of the muscle characterized by a low glucidic content, which therefore cannot support the fermentation and production of lactic acid with no acidification of the medium (Gram & Huss, 1996). In this regard, it has been seen that the pH tends to increase during fish storage as an effect of the deterioration process induced by bacterial activity leading to the production of basic amines (Messina et al., 2016; Torrieri et al., 2006).

Regarding water activity (aw), ratio between the vapor pressure of the water present in the food and that of pure water at the same temperature, which ranges from 0 to 1, most of the pathogens bacteria and many spoilage microorganisms do not develop at values of aw < 0.94, except for halotolerants and halophiles that continue to multiply at aw < 0.91 and up to 0.83; in fresh unprocessed fish, this rather high factor may favor microbial growth.

Another factor potentially favorable to bacterial invasion and growth is the low content in connective tissue of fish muscle (0.3-3%). The smaller the connective tissue in the meat, the faster the penetration of the bacteria from the skin, gills and intestines towards the depths of the muscle.

In addition, the amount of non-protein nitrogen in fish is sufficiently high in the form of free amino acids and Trimethylamine N-oxide (TMAO). TMAO represents one of the non-protein nitrogenous components of muscle that imposes on meat a tendentially positive oxidation-reduction potential that favors the multiplication of strict aerobic or facultative aerobic microorganisms. *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Vibrio* spp. reduce TMAO when a condition of anaerobiosis is established, producing substances that alter the sensory characteristics of the product.

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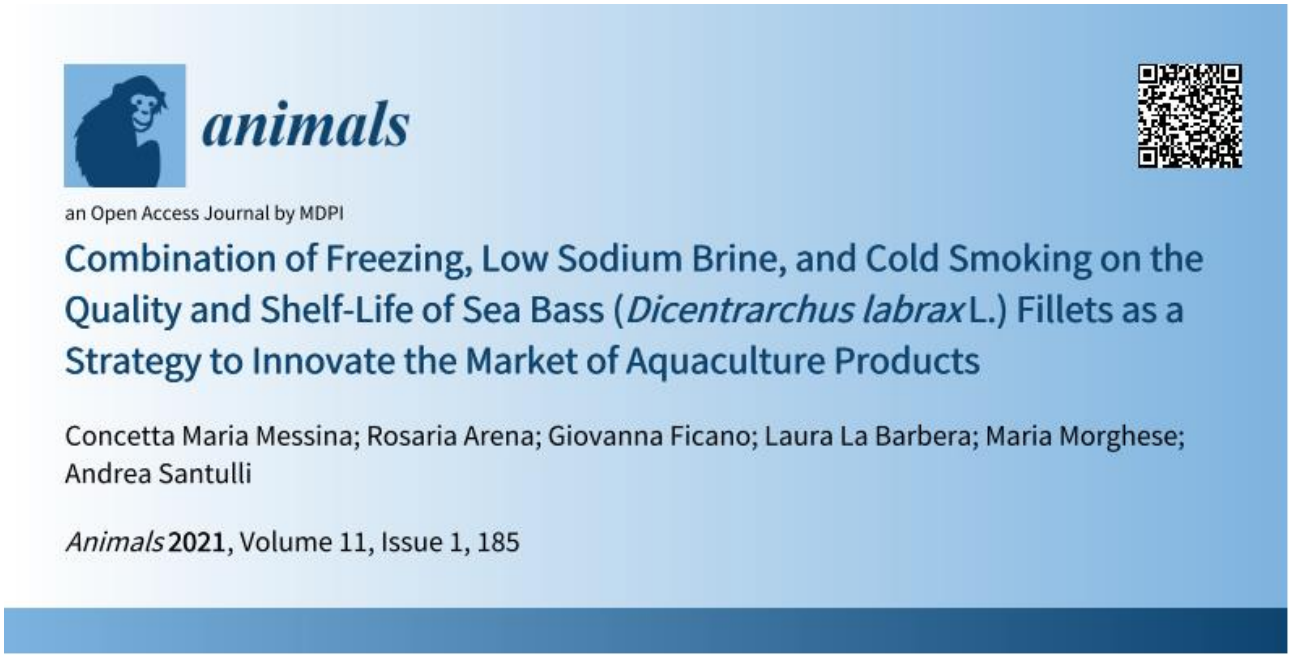
Part 1.
Production of value-added products from traditional or novel species of the aquaculture sector

Chapter 2



Simple summary

The growing fish consumption driven by the increased production, the concomitant reduction in wastage, and the huge amount of fish food traded globally, makes it important to address the sustainability, profitability, security, and safety issues related to the seafood production sector. In this direction, innovative methods extending shelf-life, maintaining seafood quality, safety and nutritional characteristics and that open new market opportunities, satisfy consumer preferences, and improve product traceability are required. Answering this call, this study aimed to develop a new value-added product exploiting a species largely cultured in Italy (i.e., European sea bass). In particular, sea bass fillets were cold smoked using both fresh and frozen fillets to understand the effectiveness of this kind of processing on developing a new fish product and whether the quality of the raw material that could be affected by freezing and thawing could change the quality of the final product. It was seen that the quality of the raw material was affected by the time of frozen storage and that these starting conditions consequently impaired the quality of the smoked fillets. However, cold smoking was proven to be an effective process to develop a valuable product contributing to the growth of the aquaculture sector.

Abstract

Aquaculture is playing a leading role in both meeting the growing demand for seafood and increasing the sustainability of the fish production sector. Thus, innovative technologies that improve its sustainability, competitiveness, and safety are necessary for growth in the sector. This study aimed to develop cold smoked sea bass fillets from aquaculture. The aptitude of frozen and fresh fillets for cold smoking was investigated by processing both fresh and thawed fillets kept previously at −20 °C for 15, 30, 60, and 90 days. Moreover, to develop a low-salt product, fillets were immersed in low-sodium or standard brine. Sensory, biochemical, and physical-chemical analyses were performed on both the raw fillets and the smoked fillets during vacuum packaged storage for 35 days at 1 ± 0.5 °C. Young modulus values, representative of texture and sensory evaluation, showed that the quality of fresh fillets was better compared to the thawed ones, thus affecting the quality of the final product as the correlation between parameters showed (principal component analysis). Cold smoking was effective in both maintaining the total volatile basic nitrogen (TVB-N) below the threshold for spoilage and preventing lipid peroxidation. Moreover, partial sodium replacement by potassium did not alter the sensory attributes of smoked fillets, which maintained high scores up to 21 days.

Introduction

Fish has always been considered an important part of human consumption for both its good taste and the high nutritional value of its flesh. According to the FAO (2020), aquaculture as a food production system has largely grown over the last decades due to the increasing demand for seafood, function of the growing world population, and the increased awareness of the beneficial effects on human health related to fish consumption. Among the most exploited species in the aquaculture sector is European sea bass (*Dicentrarchus labrax*) (Grigorakis, 2007), which is widely cultured in Mediterranean areas, with Greece, Turkey, Italy, Spain, Croatia, and Egypt the biggest producers (FAO, 2020). Specifically, the Italian production, which can rely on good nutritional and safety standards (Parisi et al., 2014; Trocino et al., 2012), has to satisfy the general need to enlarge production with innovative products. To do so, it is important to assess all parameters previously related to the quality evolution during shelf-life in order to support the production and contribute to the growth of the sector.

Indeed, given that growing fish consumption has been driven by the increased production, the concomitant reduction in wastage, and the huge amount of fish food traded globally (FAO, 2020), important issues have to be addressed with regard to the environmental and economic sustainability, profitability, security, and safety of the seafood production sector. Toward this direction, innovative methods that extend shelf-life, maintain seafood quality, safety, and its own nutritional

characteristics and that open new market opportunities, satisfy consumer preferences, and improve product traceability are required.

Cold smoking is among the techniques widely used for adding value to seafood products as well as to extend their shelf-life, maintaining high quality and sensory features of the product during storage (Arvanitoyannis & Kotsanopoulos, 2012; Gomez-Guillen et al., 2009). The effectiveness of the method is due to both smoke components such as phenols, aldehydes, ketones, hydrocarbons, esters, ethers, alcohols and the drying and salting phases that lower water activity, a parameter that influences microbial growth. Moreover, chloride ions are toxic for some microorganisms, inhibiting enzymatic systems (Leroi et al., 2000). In addition, the antioxidant activity of smoke components prevents healthy biochemical compounds like omega-3 polyunsaturated fatty-acids to be oxidized, for instance, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which exert a strong positive influence on human health, for example, by playing a significant role in preventing cardiovascular and cognitive diseases and reducing the symptoms in rheumatoid arthritis (Arab-Tehrany et al., 2012). It is also important to consider the synergistic effect of cold smoking with appropriate storage conditions (i.e., preservation of the processed product under vacuum-packaging or modified atmosphere packaging on extending the shelf-life of fish products by retarding microbial growth). Combination of these techniques with natural antioxidants was proven to extend dolphinfish shelf-life and prevent the loss of high beneficial compounds like EPA and DHA (Messina et al., 2015, 2016, 2019).

Several studies have reported the effectiveness of cold smoking on salmon (*Salmo salar*) by dealing with several technological aspects related to the technique and the quality of the raw material in order to improve this technique, which is able to prolong fish shelf-life and produce a value-added seafood product (Birkeland et al., 2004; Birkeland & Bjerkeng, 2005; Montero et al., 2003; Rora & Einen, 2003; Sigurgisladottir et al., 2000a). Moreover, it has been used over the years in many other fish species in order to make available all year round seasonal species as in the case of dolphinfish (*Coryphaena hippurus*) (Gómez-Estaca et al., 2010; Gomez-Guillen et al., 2009; Messina et al., 2019), but even to enhance fishery species with excess catches such as sardines (*Sardina pilchardus*) (Gómez-Estaca et al., 2010; Gomez-Guillen et al., 2009) and herring (*Clupea harengus*) (Cardinal et al., 2006).

To deal with fluctuations in cold-smoked salmon demand (Montero et al., 2003), the aptitude of fresh and frozen salmon fillets for cold smoking has been investigated by several authors (Martinez et al., 2010; Montero et al., 2003; Rora & Einen, 2003; Sigurgisladottir et al., 2000a). For this purpose, several quality features need to be studied because the frozen storage may affect physicochemical and sensory aspects of the processed product. Sigurgisladottir et al. (2000a)

studied the effect of freezing and thawing of the raw material on the microstructure and texture of the smoked product where it was seen that these previous conditions determined the muscle fiber shrinkage and the increase of the extracellular space, which could determine the liquid leakage of the smoked product. Nevertheless, a reduction in the yield during the smoking process was not observed. Freezing salmon fillets before smoking and keeping them frozen for up to 24 h before consumption increased the shelf-life of the smoked product compared to refrigerated smoked fillets and improved several flesh properties such as color intensity and firmness, which can make the product more acceptable to consumers (Martinez et al., 2010). Another study showed that the characteristics of raw salmon for cold smoking affected the smoked product in terms of yield and sensory features. Indeed, smaller, leaner individuals appeared to be more sensitive to freezing before smoking (Cardinal et al., 2001).

The aptitude of frozen fish for cold-smoking was investigated in other fish species, for instance, to make the smoked product available all year round (Gómez-Estaca et al., 2010). It was seen that both sardines and dolphinfish fillets could be frozen for up to two/four and twelve months, respectively, to gain high quality smoked products as expressed by biochemical parameters, protein functionality, and sensory scores. It was also noticed that few studies had dealt with the effect of freezing/thawing of the fish muscle on the quality of the smoked product.

Salt is commonly used for fish processing as well as in the cold smoking process because it helps to increase shelf-life. Salt has an important effect on water retention capacity (WHC), fat bonding, color, taste, and texture (Fuentes et al., 2011). However, high salt (sodium) intake is associated with raised blood pressure, hypertension, and cardiovascular diseases, the first cause of death and disability in adults worldwide. Clinical guidelines do not treat the majority of individuals with the tendency of high blood pressure with drugs, therefore, a population-approach through nonpharmacological measures (diet and lifestyle) is the most feasible option, as recommended by the World Health Organization (WHO) (2012) and adopted under a UN Resolution of the 66th World Health Assembly in 2013 (World Health Assembly, 2013).

High salt intake, as above-mentioned, is associated with high blood pressure and a moderate reduction in salt consumption can cause a significant reduction in blood pressure (Aburto et al., 2013) and reduced cardiovascular events. The possibility of creating products with low sodium content could generate new value-added functional foods for new markets.

Salt substitutes with similar properties and whose consumption is not deleterious for humans have also been considered in fish products (Barat et al., 2013). Potassium chloride (KCl) is the most popular inorganic salt replacer, but is characterized by acrid, metallic, and bitter tastes. Moreover, considering that sodium chloride (NaCl) substitution may lead to changes in several physical-

chemical parameters, it is important to investigate how NaCl replacement could affect the final product quality and which percentage is the best one to consider in the process, also considering that the moisture content and the water activity need to be very similar to the traditional product (Fuentes et al., 2010). Fuentes et al. (2010, 2011, 2012) investigated the effect of partial sodium replacement with potassium on European sea bass smoked with liquid smoke solutions in order to develop a healthier product. It was seen that a substitution percent higher than 50% negatively affected the taste of the smoked product (Fuentes et al., 2010), inferring the sensory acceptability of the smoked product. Moreover, it was seen that the type of vacuum and modified atmosphere packaging increased the shelf-life of smoked sea bass compared to air, highlighting the importance of using appropriate storage conditions to prevent product spoilage and extend the shelf-life (Fuentes et al., 2012). Moreover, the feasibility of partial replacement of NaCl with KCl has been investigated on other fish species such as salmon and herring (Giese et al., 2019; Osheba, 2013). It has been shown that in smoked herring, the best substitution percent of NaCl with KCl was not higher than 40% (Osheba, 2013).

In order to develop a new value-added product by using the traditional aquaculture species *D. labrax*, thus contributing to the diversification of seafood products and satisfying the consumer's preferences and sustainability needs, the aim of the current study was to develop cold smoked sea bass fillets by replacing sodium chloride with potassium chloride in the salting phase. In particular, the fillets were cold smoked by using either a NaCl brine solution or a NaCl/KCl (1:1 *w/w*) brine solution in order to provide evidence on the effectiveness of the partial sodium replacement with potassium on the quality of the cold smoked product. In addition, the present study provides evidence to the production sector on the suitability of both frozen and fresh sea bass fillets for cold smoking by applying the process to both fresh and thawed fillets.

Materials and methods

Fish Sampling and Processing

A total of 195 fillets of farmed sea bass (*D. labrax*) were sampled immediately after production at the processing sector of the aquaculture facility located in Sicily (Italy), stored in ice, and brought to the laboratory under cold storage. The fillets were divided into five lots, each one with 39 fillets.

The first batch of 39 fillets was immediately used for the smoking process, in particular, three fresh fillets of this lot were used for the analysis at T0 (i.e., the time right before processing), while the remaining fresh fillets of this lot were immediately smoked (N = 36), as shown in Figure 1.

The other four batches (each of 39 fillets) were stored under vacuum in Foodsaver (HDPE) and nylon bags (http://www.gopack.it), subjected to rapid freezing at −80 °C, and maintained at −20 °C for 15, 30, 60, and 90 days before processing. As far as the frozen fillets were concerned, they were placed in air-permeable low-density polyethylene (LDPE) bags and thawed at 4 °C for 8 h before processing.

Salting, Smoking, Storage, and Sampling

The whole process was represented by four steps: brine salting, first drying, cold smoking, and second drying (Figure 1).

For all batches, 18 fillets were immersed in standard brine (STD) composed of 15% (*w/v*) NaCl solution, and the remaining 18 fillets of the same batch were immersed in a 15% (*w/v*) NaCl/KCl solution (1:1 *w/w*). For both treatments, a fillet:brine ratio equal to 1:4 was used (Gómez-Estaca et al., 2011; Messina et al., 2019). The brine salting lasted 90 min, after which, the samples were dried for 30 min at a temperature that never exceeded 30 °C. Then, all treatments were cold smoked for the same time and at the same temperature as described in Messina et al. (2019). Briefly, cold smoking was performed using a Moduline oven model FA082E (Scubla srl, Remanzacco (Ud), Italy) for 30 min at 30 °C by employing a mixture of the hacks pane (*Fagus* spp.) (Scubla srl, Remanzacco (Ud) Italy) and smoke shavings and Segamehl smoked flour (Scubla srl, Remanzacco (Ud) Italy).

As a final step, the experimental fillets were dried again for 30 min at 30 °C. At the end of the process, fillets were packaged under vacuum and stored at 1 ± 0.5 °C for 35 days. Samples were analyzed at regular intervals defined previously (1, 7, 14, 21, 28, and 35 days after smoking), during which they were monitored through a multidisciplinary approach (i.e., by biochemical, sensory and physical-chemical analyses).

Each lot of fillets was treated as described above in order to carry out five tests: the first smoking (I) was carried out by treating fresh fillets; in the second (II), third (III), fourth (IV), and fifth (V) smoking, fillets kept frozen for 15, 30, 60 and 90 days, respectively, were treated.

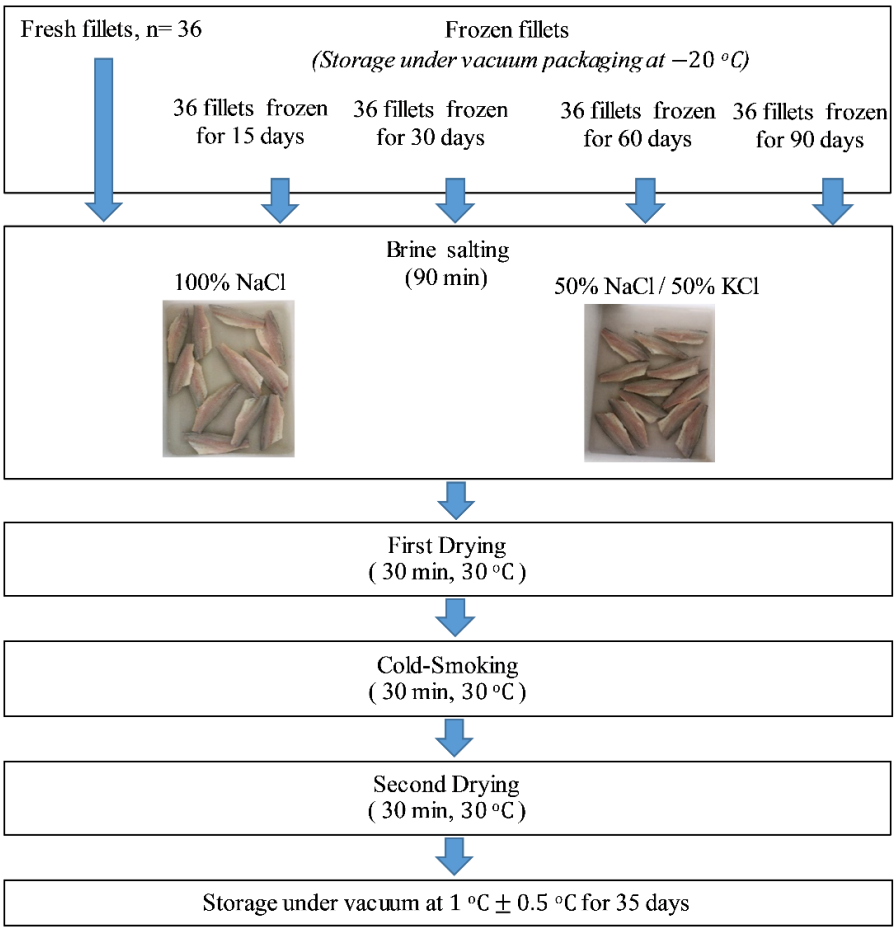


Figure 1. Flowchart of the experimental design.

Physical-Chemical Parameters

Color

The analysis was carried out using a Konica Minolta colorimeter (Osaka, Japan), and the results were reported according to the CIE system (CIE, 1977). Lightness (L*), redness index (a*), yellowness index (b*), Hue, and Chroma were recorded, whereas the numerical total color difference (ΔE) between samples was calculated by Equation (1):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where ΔL*, Δa*, and Δb* are the differentials among the color parameters of the samples over the course of shelf-life and the color parameters of the samples at T0.

To perform this kind of analysis, first, the instrument was calibrated, and the instrument reading head was placed on the surface of the fillet. The color evaluation was performed on the fillet in two dorsal regions along the cephalo-caudal direction. The analysis was performed in triplicate. With regard to the kind of observer, it was 2° while the source of light was D65.

Texture Profile Analysis

Two small fragments (1.8 cm Ø), obtained from the same portions of each fillet, were used. The analysis was performed at room temperature using an Instron Texture Analyzer Mod. 3342 (Turin, Italy).

The measured parameters were Hardness (*N*) and the Young Modulus or modulus of deformability (N/mm^2) (i.e., the force and the slope of the curve at 50% compression, respectively) (Messina et al., 2019; Orban E., 1997). The analysis was performed in triplicate. For each replicate as above-mentioned, two fragments per fillet along the dorsal margin were considered. The samples were maintained in ice before the analysis.

Water Holding Capacity

Water holding capacity was determined using the method described by Teixeira et al. (2014) with some modifications. Briefly, chopped muscle (2 g; *Ws*) was placed in a centrifuge tube along with filter paper (also weighted, *Wi*) and centrifuged at 3000 g for 10 min at 20 °C. After centrifugation, the sample was removed, and the filter paper weighted (*Wf*). The moisture in the samples was determined according with the AOAC method (AOAC, 1990). WHC was calculated following

Equation (2) and expressed as gram of water in the sample after centrifugation per 100 g of water initially present in the sample. The analysis was performed in triplicate.

$$WHC = \frac{Ws * \frac{Moisture\%}{100} - (Wf - Wi)}{Ws * \frac{Moisture\%}{100}} \quad 2)$$

Muscular pH

The muscular pH of the fillet was measured at three points along the lateral line with a Crison pH meter (Barcelona, Spain) equipped with a BlueLine pH 21 Schott Instruments (Weilheim, Germany) combined electrode.

Determination of the NA, K, and Salt Content

Na, K, and salt were determined according to the technique described by Greiff et al. (2014) with modification. Briefly, about 2 g of sample were homogenized in distilled water (1:4 *w/v*) using an Ultraturrax T25 (IKA, Labortechnik, Staufen, Germany) and centrifuged at 5000 g for 5 min. The extract was filtered on cotton wool. Na and K content was directly measured with a Horiba B-722 (Na^+) and B-731 (K^+) LAQUAtwin Compact Ion Meter (Horiba Instruments, Inc., Kyoto, Japan). A conductivity meter (Model 131 Analytical Control, Milano, Italy) was used to measure the salinity. The obtained value expressed in µS cm^{-1} was converted into salinity (expressed in ‰). The values obtained were expressed in g/100 g of product. The tests were carried out on the newly processed product. All analyses were performed in triplicate.

Water Activity Determination

Water activity (aw) was measured with a fast water activity meter (HP23-AW Rotronic, AG, Bassersdorf, Switzerland). The temperature at which the water activity was measured was equal to 21 °C. The analysis was performed in triplicate.

Biochemical Parameters Related to the Shelf-Life

After the physical-chemical and sensory analyses, in order to carry out further analysis, the fillets were cold homogenized.

The ash (ignition at 600 °C for 5 hours) and moisture (drying at 105 °C for 24h) contents (% ash and moisture) were assessed according to the AOAC method (AOAC, 1990). The protein content (% protein) was determined according to the AOAC method (AOAC, 1992). The total lipids (TL) were determined according to Folch, Lees, and Stanley (1953) and the fatty acid (FA) methyl esters

were determined by the method of Lepage & Roy (1984); gas-chromatography was carried out following the operating conditions described in Messina et al. (2013).

The production of thiobarbituric acid reactive substances (TBARS) was determined using the method described by Botsoglou et al. (1994). The total volatile basic nitrogen (TVB-N) was measured by direct distillation of the homogenized samples according to the EU Commission Decision 95/149/EC (E.C.C., 1995).

All analyses were performed in triplicate.

Sensory Analysis

Sensory analysis of raw fillets with skin was conducted by a panel of six trained judges in accordance with an adapted version of the EU scheme (E.C.C., 2005; Poli et al., 2006). The analysis consisted of the evaluation of skin odor and flesh texture, brightness, and color, as illustrated in Table 1. The numeric scores 3, 2, 1 and lower than 0.5 were assigned, respectively, to the E class (very fresh, extra quality), A class (fresh, good quality), B class (bad quality but still fit for sale), and unfit for sale, in order to obtain a quantitative evaluation of parameters (Poli et al., 2006). The analysis was performed in triplicate.

Table 1. Evaluation of the raw fillets with skin (Poli et al., 2006).

Object of Assessment	**Criteria**			
	Freshness Category			
	Extra Score 3	**A Score 2**	**B Score 1**	**Not Admitted Score 0**
	External evaluation			
Texture of fillet with skin	Very firm, rigid, elastic	Fairly rigid, firm, slightly less elastic	Slightly soft, less elastic	Soft (flaccid)
Skin odor	Fresh seaweed	Neutral	Slightly sour	Sour
	Flesh			
Flesh color	Translucent, smooth, bluish Color around the spinal column: absent	Slightly modified and waxy Color around the spinal column: slightly pink	Slightly opaque and waxy Color around the spinal column: pink	Opaque, yellow Color around the spinal column: red

As far as the smoked fillets were concerned, the sensory assessments were performed on fillets by a six-member panel according to the protocol developed by Fuentes et al. (2010) in which attributes such as odor, color, appearance, taste were assessed by using a 5-point descriptive scale (1 = very unpleasant; 2 = unpleasant; 3 = neutral; 4 = less typical; 5 = very typical). The analysis was performed in triplicate.

Statistical Analysis

Statistical differences were evaluated for each parameter with analysis of variance (ANOVA). The differences among the mean values were assessed using the Student–Newman–Keuls test. The degree of heterogeneity was measured by the Cochran test (Underwood, 1997). Principal component analysis was performed including eight variables on an individual basis. Data were processed for one-way ANOVA and principal component analysis (PCA) by Statistica (version 8.0, Statsoft, Inc., Tulsa OK, USA).

Results and discussion

Color

Color is one of the main qualitative aspects of interest to the consumer before purchase (Fuentes et al., 2012). CIE L* a* b*, hue (h*), chrome (C*) and ΔE appear in the Supplementary Materials (Supplementary Table S1 and Table S2). Regarding the L* parameter, differences were observed between the thawed fillets compared to the fresh fillets (Supplementary Table S1). These findings agreed with those obtained in Atlantic salmon fillets (Regost et al., 2004; Rora & Einen, 2003) in which L* increased significantly with frozen storage, showing that this parameter is affected by freezing.

This study showed that L* in smoking I remained stable during the storage time for both samples with significant increase ($p < 0.05$) at T35 (see Supplementary Table S1 in the Supplementary Materials). This trend was observed in all subsequent smoking processes. Birkeland et al. (2004) observed a reduction in lightness after cold smoking of salmon fillets; the lightness was more reduced in dry-salted than injection-salted fillets, highlighting that the difference could be due to a difference in the water content. Moreover, an increase in lightness and reduction in redness was associated with a loss of quality in cold smoked salmon influenced by the duration of the brine salting treatment (Birkeland & Bjerkeng, 2005). Yagiz et al. (2007) treated *C. hippurus* fillets with high pressure (HP) and reported that the L* value increased slightly as a function of increased pressure and general loss of quality.

A positive a* value indicates redness. As far as this parameter was concerned, a constant trend for smoking I was observed, where no significant differences ($p > 0.05$) were observed between the two treatments (Supplementary Table S1), highlighting the preservative effect of smoke on lipid oxidation as shown by other authors (Messina et al., 2019). From smoking II, the a* value tended to decrease compared to the thawed fillets (Supplementary Table S1). This difference was significant ($p < 0.05$) after seven days of storage in all smoking processes.

The same pattern related to the increase of L* (lightness) in the thawed fillets compared to fresh fillets was observed for b* (yellowness), which was significantly different ($p < 0.05$) between the fresh fillets and frozen fillets after thawing, which tended to increase in the latter ones (Supplementary Table S1). The same results were obtained in Atlantic salmon fillets, which showed an increase in yellowness due to freezing (Regost et al., 2004; Rora & Einen, 2003).

In all smoking processes, the b* value decreased significantly after processing ($p < 0.05$), which then increased during storage (Supplementary Table S1). This increase was more significant ($p < 0.05$) in smoking V, where b* values of 6.48 ± 2.17 and 4.89 ± 1.29 for NaCl and NaCl/KCl samples, respectively, were observed at T35. This pattern may be indicative of an increased yellowness of fish muscle as a result of the browning of the bloodline due to heme protein oxidation and a corresponding reduction in a* (Richards & Hultin, 2000). No significant differences ($p > 0.05$) were observed between the two treatments.

The smoking process caused significant variation to the color parameters ($p < 0.05$) and the effect on the color variation after smoking was well observable from the values shown in the ΔE (Supplementary Table S2). The first smoking, performed on fresh samples, was the one that showed a lower ΔE for both treatments, a value that remained constant during the shelf-life. Samples that were frozen and subsequently smoked showed a higher ΔE value.

Texture

The ability of the smoking process to preserve fish is due to the synergistic action of salt incorporation, the preservative effect of smoke compounds, and dehydration. This also causes a significant increase in all textural parameters, as demonstrated on other fish species (Birkeland et al., 2004; Fuentes et al., 2010, 2012; Gómez-Guillén et al., 2000; Messina et al., 2019; Regost et al., 2004; Sigurgisladottir et al., 2000b). In our study, as shown in Supplementary Table S3, textural parameters (Young Modulus and hardness) increased significantly ($p < 0.05$) after smoking in both treatments. This trend could also be observed in all subsequent smoking processes.

In smoking I, differences in Young Modulus and hardness were observed in the NaCl samples during storage, and these parameters increased to 0.76 ± 0.11 and 24.83 ± 1.23 on day 14 ($p < 0.05$), respectively. This then decreased and stabilized in the following days of shelf-life (Supplementary Table S3), while in the NaCl/KCl samples, the textural parameters remained constant throughout the shelf-life.

These results highlight that the degradation processes, related to autolytic phenomenon and to the denaturation of protein in the muscle tissue, were more accentuated in the NaCl samples than the NaCl/KCl samples (Liu et al., 2010).

No significant differences ($p > 0.05$) were observed between the different smoking processes in the early shelf-life times. It was from T21 that differences were observed, in particular, smoking III and V showed significantly higher ($p < 0.05$) hardness values than the other smoking processes.

Water Holding Capacity (WHC)

The water holding capacity measures the ability of the muscle to retain water and it increased significantly ($p < 0.05$) in the smoked fillets compared to the untreated samples without differences between the two treatments (Supplementary Table S4), which agrees with results obtained in other studies (Fuentes et al., 2012; Messina et al., 2019).

Significant differences ($p < 0.05$) were found between the fresh fillets and the thawed fillets, with the second ones showing lower values than the first ones, perhaps as a result of the damage due to freezing/thawing (Sigurgisladottir et al., 2000a). The WHC increased after smoking in all smoking processes and remained stable throughout the storage time without differences between the two treatments in accordance with Fuentes et al. (2012), as the result of the increment in salt content positively correlated with the WHC.

Muscular pH

The pH value of the fresh fillets was significantly different ($p < 0.05$) from those of the thawed fillets as shown in Supplementary Table S5. Soto-Valdez et al. (2015) found an increase in the pH during ice storage, probably due to the endogenous proteolytic activity or microbial action that determines an accumulation of alkaline compounds.

Regarding the smoked samples, the pH was reduced significantly by the smoking process ($p < 0.05$). Indeed, a significant difference between the untreated samples and the smoked samples was observed and this difference remained over the storage time ($p < 0.05$). Regarding smoking I, the samples treated with NaCl tended to show lower pH than those of NaCl/KCl samples at the end of the storage time ($p < 0.05$), as shown by Fuentes et al. (2012).

These findings, in agreement with those shown in previous studies on smoked fish like dolphinfish, salmon, and sea bass (Fuentes et al., 2012; Leroi et al., 2000; Messina et al., 2019), were probably due to the salting and smoking treatments that determine an increase in the ionic strength. The difference found in the present study between the NaCl and NaCl/KCl samples could have been due to the salt composition, which affected the ionic strength of the intracellular solution. Moreover, pH may be affected by the lack of production of volatile basic components by spoilage bacteria observed in this study that help maintain a constant low value of pH during storage after processing (Fuentes et al., 2012; Osheba, 2013).

NA, K, and Salt Content

Significant differences ($p < 0.05$) in the sodium content were found between smoking I and smoking V at 1 g/100 g and 2 g/100 g, respectively, in the NaCl samples, and significant differences ($p < 0.05$) were found between the two treatments in all smoking processes, as shown in Figure 2.

The potassium content increased significantly ($p < 0.05$) from 1 g/100 g in smoking I to around 1.5 g/100 g in smoking V regarding the NaCl/KCl samples, and significant differences ($p < 0.05$) were found between the two treatments in all smoking processes.

The fillets in smoking V were significantly saltier ($p < 0.05$) than the fresh/smoked fillets, and significant differences ($p < 0.05$) were found between the two different treatments.

The fish processing led to a reduction in the moisture content and to an increase in the mineral content as observed in previous studies (Fuentes et al., 2010, 2012; Messina et al., 2019). In the present study, smoking V showed a higher salt content ($p < 0.05$) compared to the previous ones as far as the NaCl samples were concerned, probably because of the pH and textural changes that inferred the capacity of the muscle to retain water.

These findings confirmed that salt replacement with KCl may contribute to healthier products, considering that they may also be a source of potassium compared to the traditional product, providing around 30% of the recommended allowances for adults (4.7 g) when both the fresh fillets and the frozen fillets were processed (Fuentes et al., 2012; Giese et al., 2019).

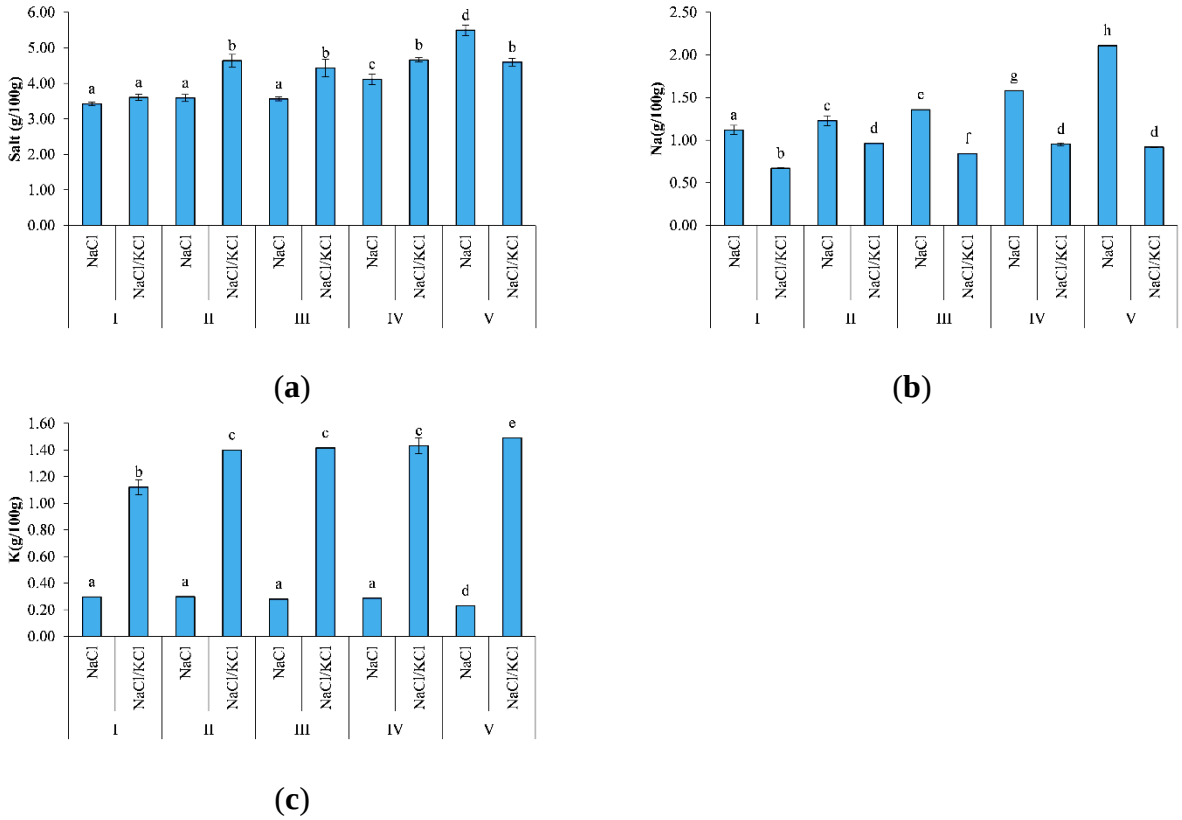


Figure 2. Salt, Na, and K determined in the smoked fillets of *D. labrax*, in the five smoking processes (I, II, III, IV, V). Different letters (a, b, c...) indicate significant differences ($p < 0.05$). (**a**) Salt content; (**b**) Na content; (**c**) K content.

Biochemical Parameters Related to the Shelf-Life

The proximate composition and water activity of fresh and smoked fillets are shown in Table 2. The lipid content showed that *D. labrax* is a medium-fat fish species (Tang et al., 2001). The nutritional values remained unchanged following smoking, except for the contents of ash and water, which varied due to water loss. These features agreed with those obtained in previous studies for the same fish species (Fuentes et al., 2010). Moreover, as fish processing led to a decrease in aw (Fuentes et al., 2010, 2012; Messina et al., 2019), even in the present study, aw was reduced after smoking compared to unprocessed products (Table 2) and did not show any difference between all smoking processes carried out regarding both NaCl and NaCl/KCl samples throughout the storage time (data not shown).

Table 2. Proximate composition in the fresh and smoked fillets of *D. labrax*.

		Smoked Fillets	
	Fresh Fillets	**NaCl**	**NaCl/KCl**
Lipids	6.16 ± 2.34 ^{AB}	5.93 ± 0.31 ^{A}	6.58 ± 0.86 ^{B}
Moisture	74.13 ± 1.78 ^{A}	72.79 ± 0.3 ^{B}	71.3 ± 0.55 ^{B}
Ash	1.42 ± 0.21 ^{A}	4.14 ± 0.19 ^{B}	4.86 ± 0.19 ^{B}
Protein	18.29 ± 4.33 ^{A}	17.14 ± 1.52 ^{B}	17.26 ± 1.6 ^{B}
aw	0.987 ± 0.001 ^{A}	0.942 ± 0.004 ^{B}	0.95 ± 0.004 ^{B}

Data within a row, showing different superscript letters (A,B) are statistically different ($p < 0.05$).

Fatty acid (FA) composition of European sea bass fillets showed no significant difference among salting treatments up to the end of the storage time (T35) (Table 3). This finding confirms that the cold smoking process and the type of salt used in the brining phase of the process do not alter the biochemical and nutritional properties of the sea bass fillets. The mean values (± SD) of the data recorded on the day after smoking (T1) and on day 35 of the storage period related to both salting treatments are shown in Table 3. All lots were characterized by high levels of monounsaturated FAs followed by saturated FAs, omega-6 (*N*-6) polyunsaturated fatty acids (PUFAs), and *N*-3 polyunsaturated fatty acids (PUFAs) (Table 3).

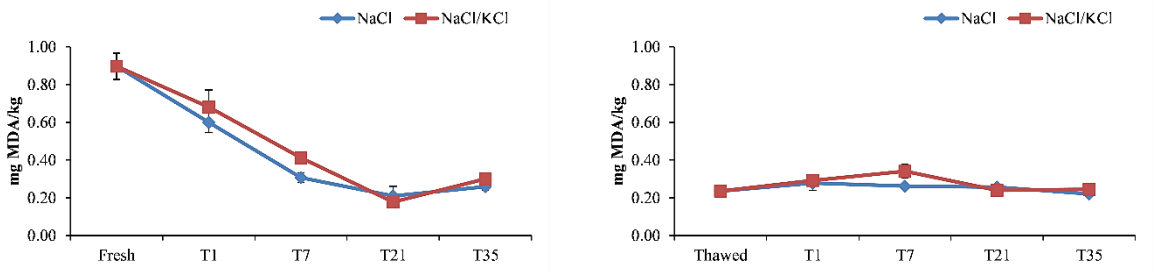
Table 3. Fatty acid composition in the fresh and smoked fillets of *D. labrax* at the beginning and at the end of storage time.

		Smoked fillets			
		T1		T35	
	Fresh Fillets	NaCl	NaCl/KCl	NaCl	NaCl/KCl
SFA	21.29 ± 0.32 ^{A}	21.14 ± 0.48 ^{A}	20.82 ± 1.59 ^{A}	19.96 ± 1.94 ^{A}	19.73 ± 1.39 ^{A}
MUFA	45.79 ± 2.67 ^{A}	45.33 ± 0.43 ^{A}	46.11 ± 0.35 ^{A}	46.68 ± 2.09 ^{A}	46.40 ± 0.96 ^{A}
PUFA	32.91 ± 2.99 ^{A}	33.54 ± 0.91 ^{A}	33.07 ± 1.24 ^{A}	33.36 ± 0.15 ^{A}	33.87 ± 2.35 ^{A}
Tot n-3	15.26 ± 2.74 ^{A}	15.75 ± 0.52 ^{A}	15.88 ± 0.54 ^{A}	15.70 ± 0.86 ^{A}	16.05 ± 2.87 ^{A}
Tot n-6	16.96 ± 0.23 ^{A}	17.04 ± 0.45 ^{A}	16.47 ± 0.65 ^{A}	16.91 ± 0.65 ^{A}	17.08 ± 0.56 ^{A}

Data within a row showing the same superscript letter (A) were not statistically different ($p < 0.05$).

The smoking process was effective on preventing lipid oxidation and the loss of high beneficial compounds such as n-3 PUFAs considering that no difference ($p > 0.05$) was observed between the first day after processing (T1) and the last day of storage. Moreover, no difference was observed between the two salting treatments applied (Table 3).

Regarding the effectiveness of the smoking process on preserving fish fillets on the loss of high beneficial compounds, the malonaldehyde (MDA) content reduced significantly ($p < 0.05$) after smoking with similar patterns between the NaCl and NaCl/KCl samples (Figure 3).



(**a**) (**b**)

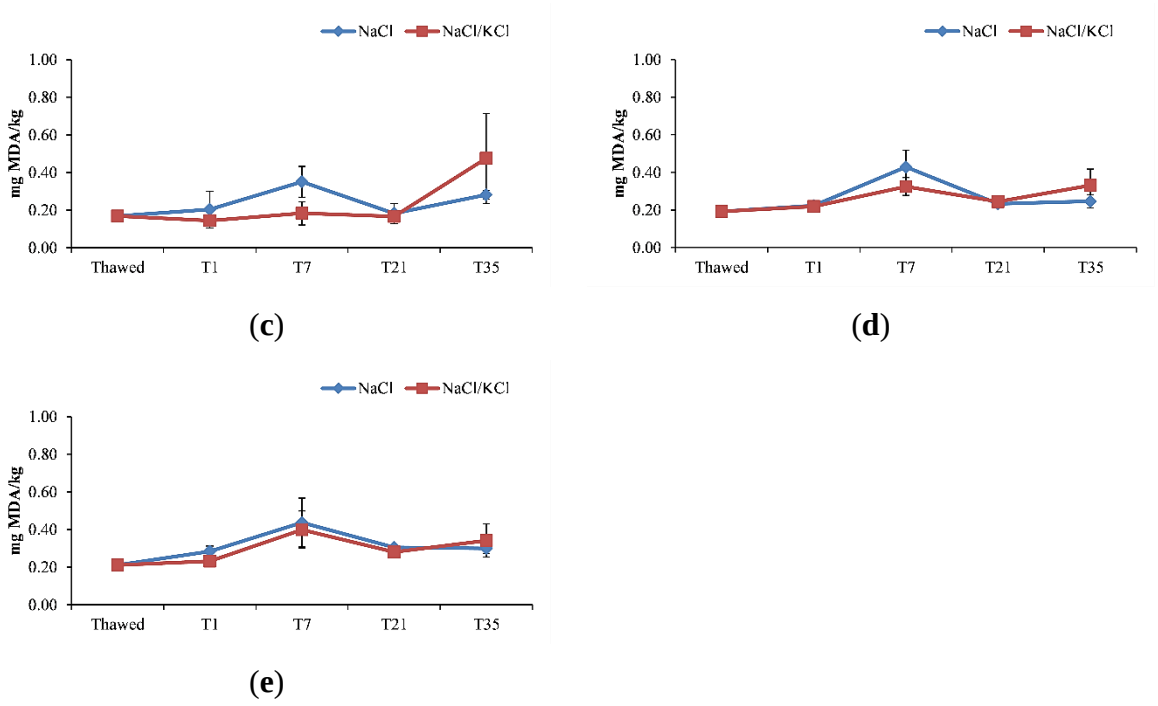


Figure 3. Malondialdehyde (MDA) levels (mg/kg) in the smoked fillets of *D. labrax* during the 35 days of preservation, in the five smoking processes. (**a**) smoking I; (**b**) smoking II; (**c**) smoking III; (**d**) smoking IV; (**e**) smoking V.

Indeed, it reduced significantly at T1 from 0.9 mg MDA/kg measured in the raw material, which approached the value of 1 mg MDA/kg, considered as an indicator of the onset of oxidative rancidity in the fresh fish (Sweet, 1973). This value reduced progressively during the storage time (Figure 3). In the other smoking processes, the MDA content was significantly lower in the thawed fillets compared to the fresh fillets and the smoking process contributed to maintain that low value. In general, in the frozen/smoked samples, the MDA content tended to increase slightly at T7, being constant enough during the storage time, and at T35, it was not significantly different between the smoking processes, maintaining well below the 4.5 mg MDA/kg value considered acceptable for smoked fish in the literature (Osheba, 2013). As observed in previous studies (Fuentes et al., 2011; Gómez-Estaca et al., 2010; Sweet, 1973), even in this study, the salting/smoking treatment did not exert any effect on lipid oxidation, as revealed by the MDA content, and could not be used as an indicator of fish spoilage. This should be attributed to the antioxidant activity of the phenolic fraction of the smoke, despite the potential prooxidant effect of salting (Fuentes et al., 2011). The right salting concentration and salting time can also be decisive in avoiding lipid oxidation (Osheba, 2013). Moreover, in the present study, no difference was observed ($p > 0.05$) between NaCl samples and NaCl/KCl samples in terms of MDA content during the 35 days of storage, thus confirming the effectiveness of the partial sodium replacement on preventing lipid oxidation, as shown in other studies (Osheba, 2013). Moreover, vacuum packaging might be effective in

preserving frozen fillets and processed products from lipid oxidation, providing more evidence on the effective use of this method of conservation (Gómez-Estaca et al., 2010).

The TVB-N content in the fresh fillets was 16.41 mg/100 g. No difference was observed in the TVB-N content between the fresh fillets and the thawed fillets kept frozen for 30 days (Figure 4).

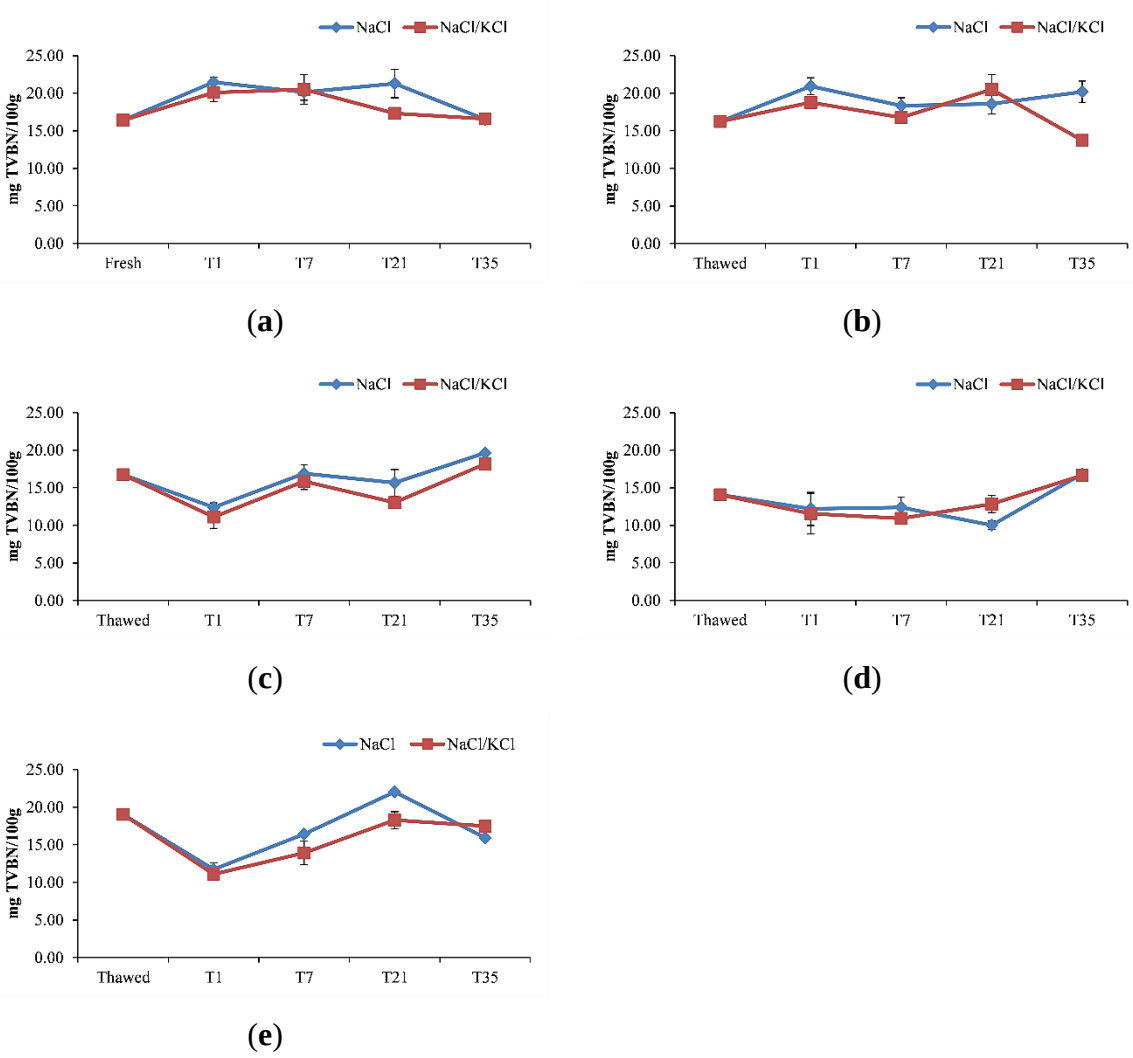


Figure 4. Total volatile basic nitrogen (TVBN) (mg/100 g) in the smoked fillets of *D. labrax* during the 35 days of preservation, in the five smoking processes. (**a**) smoking I; (**b**) smoking II; (**c**) smoking III; (**d**) smoking IV; (**e**) smoking V.

The TVB-N content increased at T1 as far as the first two smoking processes were concerned, but it reduced significantly after smoking at T1 in the fillets kept frozen for 30, 60, and 90 days, without significant differences between these last three frozen/smoked samples, and after that time, it increased again. As reported in previous studies where the aptitude of frozen fish fillets for cold smoking was investigated (Gómez-Estaca et al., 2010), a reduction in the level of TVB-N in frozen fillets after salting/smoking was observed most likely because part of the volatile bases produced during frozen storage were dissolved in the brine or volatilized during smoking. The acceptability

limit of 30–35 mg *N*/100 g (E.C.C., 1995) was not exceeded in any of the smoking processes, at any time of the storage period, agreeing with the results obtained previously on vacuum packaged smoked sea bass, which showed values of TVB-N below the above-mentioned limit of up to 42 days of storage (Fuentes et al., 2011). Moreover, the TVB-N may be affected by the reduction in both the spoilage bacteria and the activity of endogenous enzymes due to the salting/smoking processes, which agrees with another study dealing with the effect of different smoking flavorings on the quality of farmed European sea bass (Vidal et al., 2017). The results of this study provide evidence that for up to 30 days of storage, the smoking carried out in the three smoked samples greatly inhibited the formation of spoilage oxygenated metabolites and therefore the growth of the microorganisms responsible for it. Nevertheless, sulfur derivatives were detected in samples smoked with the smoke flavoring with the higher phenol content after 25 days of vacuum packaging at refrigeration temperature.

It has to be highlighted that the presence and proliferation of TMA-N producing bacteria, for instance, Enterobacteriaceae, were prevented by strictly observing the hygiene conditions before and during treatments; moreover, the temperature was monitored during refrigerated storage at 4 °C. Lactobacilli have also been identified as spoilage bacteria that produce sulfuric acid that can cause unpleasant odors, which was not found in this work. In Rizo et al. (2016), it was shown that TVB-N reached values that considered the smoked cod product not acceptable after 21 days of storage although bacterial counts led to the consideration that the product was acceptable after 40 days of storage. Another study by Messina et al. (2019) therefore took into consideration both the biochemical and microbiological parameters in assessing the effectiveness of cold smoking in extending the shelf-life of smoked dolphinfish fillets to evaluate how cold smoking, carried out under the same conditions observed in the present work, significantly ($p < 0.05$) prevented the proliferation of mesophilic and psychrophilic bacteria, which remained within the acceptable limits ($< 10^7$ CFU/g) until the end of its shelf-life, although in this case, the concentration of TVB-N also remained within the acceptable limits up to 21 days of refrigerated storage. Therefore, this evidence supports the fact that despite the microbiological risk due to the low temperatures used in the process, cold smoking, if carried out with appropriate measures (the same ones observed in the present study) has an effective bacteriostatic action that combined with the appropriate storage conditions such as temperature and vacuum packaging does not cause microbial proliferation as indicated by the TVB-N values used among the quality indicators of the smoked product as described above. Moreover, another study by Gómez-Estaca et al. (2010) aimed at evaluating the effect of freezing sardine and dolphinfish fillets on the quality of the cold-smoked product did not consider the microbiological parameters among those used.

In the present study, the partial replacement of NaCl with KCl maintained, in general, the TVB-N content during the storage period, which in all the smoking processes was significantly lower compared to 100% NaCl samples, highlighting the effectiveness of using KCl along with NaCl on maintaining low values of this parameter during the shelf-life.

Sensory Analysis

In order to gain good results by a processing technique that is useful to add value to a seafood product such as cold smoking, it is necessary that the raw material be of good quality. If this assumption is not observed, the quality of the final product can be impaired regardless of the effectiveness of the technique used (Sampels, 2015).

The sensory evaluation of raw and treated fillets provides important evidence about the quality of the raw material and the degree of consumer acceptability by analyzing attributes of the product that are directly evaluated by consumers that in the case they were altered, they could compromise their choice and satisfaction. These features, for example, are taste, texture, odor, and color, which could be affected by the smoking process, so the physicochemical parameters of the finished products that would need to be regulated.

The quality of raw fresh fillets was also of good quality, except in smoking V, where the sensory parameters of the thawed fillets were on average evaluated to be of bad quality (Table 4). The sensory evaluation of the raw material showed that the quality of fillets was impaired by freezing, as reported in the literature for salmon (Regost et al., 2004), and the main parameter altered by freezing was texture, as shown in the present study.

Table 4. Sensory analysis measured in fresh and thawed *Dicentrachus labrax* fillets.

Raw Fillet (Smoking)	Texture	Odor	Color	Average
Fresh (I)	3.00 ± 0.00 [a]	2.00 ± 0.00	1.67 ± 0.47	2.22 ± 0.69
Thawed (II)	2.00 ± 0.00 [b]	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Thawed (III)	1.50 ± 0.71 [c]	2.00 ± 0.00	1.50 ± 0.71	1.67 ± 0.29
Thawed (IV)	1.00 ± 0.00 [d]	2.00 ± 0.00	2.00 ± 0.00	1.67 ± 0.58
Thawed (V)	1.00 ± 0.00 [d]	2.00 ± 0.00	1.25 ± 0.35	1.42 ± 0.52

3–2.5 score: Extra class (extra quality fish); <2.5–1.5 score: A class (good quality fish); <1.5–0.5 score: B class (bad quality fish); <0.5 score ¼ unfit for human consumption. Data within a column showing different superscript letters (a, b, c…) are statistically different ($p < 0.05$).

The findings of this study related to the sensory assessment of cold smoked fresh fillets (Supplementary Table S6) showed that the partial replacement of NaCl by KCl did not significantly alter the odor, color, appearance, and taste of the smoked fillets. These results were in accordance

with those obtained in previous studies where 50% of sodium replacement by potassium was shown to be feasible for smoked sea bass based on the sensory evaluation (Fuentes et al., 2010). There were significant differences between the smoking processes at T1 ($p < 0.05$). In smoking V, the sensory attributes were considered neutral, unlike the previous smoking processes where they were evaluated with higher scores (4–5) that corresponded to less typical and very typical.

Moreover, it has to be highlighted that the odor maintained high scores that ranged from neutral to typical during refrigerated storage under vacuum packaging, providing that no sulfur compounds were produced, so shows that the treatment was effective on preventing the growth of microorganisms responsible for their formation, like, for instance, *Shewanella putrefaciens* (Vidal et al., 2017).

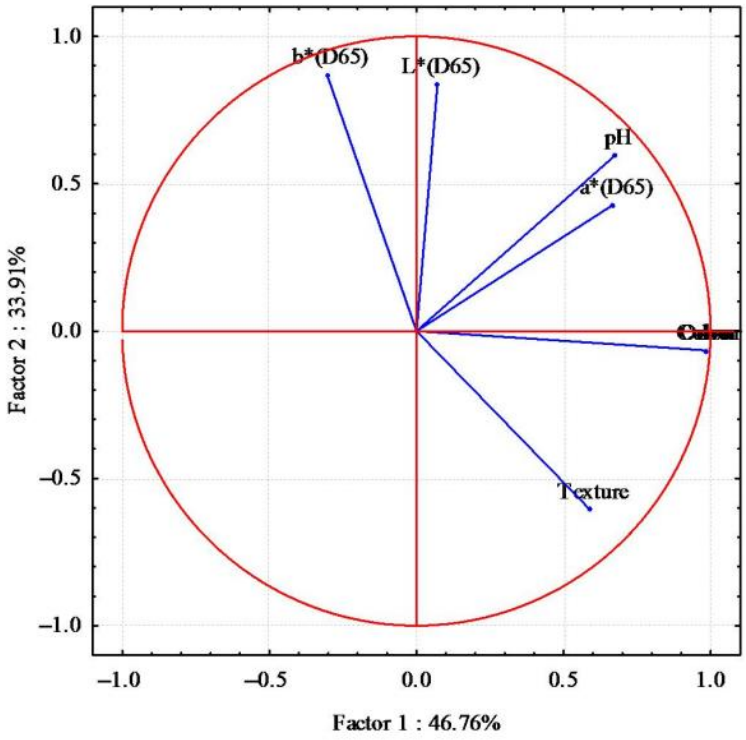
There were significant differences between the sensory attributes scores of the smoked samples at several times of the storage period considered. It was observed that during the storage time, the fillets of the fifth batch, which were treated in smoking V, showed lower scores compared to the other ones. However, the attributes of the fillets treated in the previous smoking processes maintained higher scores up until 21 days.

In general, no significant differences were found for any of the evaluated attributes depending on the type of salt.

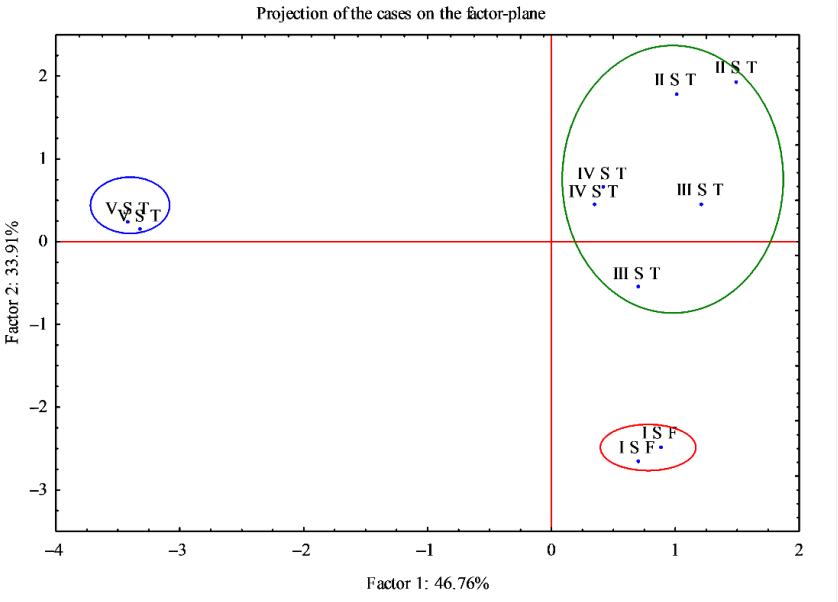
Correlation among Quality Parameters of Raw and Smoked Fillets by Principal Component Analysis

The differences observed in sensory and instrumental analysis (color and pH) between fresh and thawed fillets and between smoked fillets were further analyzed through analysis of the main components (PCA) (Figures 5 and 6).

Figure 5 shows the graph obtained from the PCA. Seven variables (sensory analysis: odor, color and texture; color: L*, a* and b*; pH) and 10 cases were analyzed. The analysis generated a small number of linear combinations on the seven variables and only two main components were identified with an eigenvalue greater than 1. These two main components explain 80.67% of the variance of the original variables.



(**a**)



(**b**)

Figure 5. Principal component analysis (PCA) obtained from correlation of seven components (sensory analysis, pH, and color (L*, a*, b*) and 10 cases determined on fresh and thawed fillets of D. labrax. (a) Correlation circle; (b) Factor-plan.

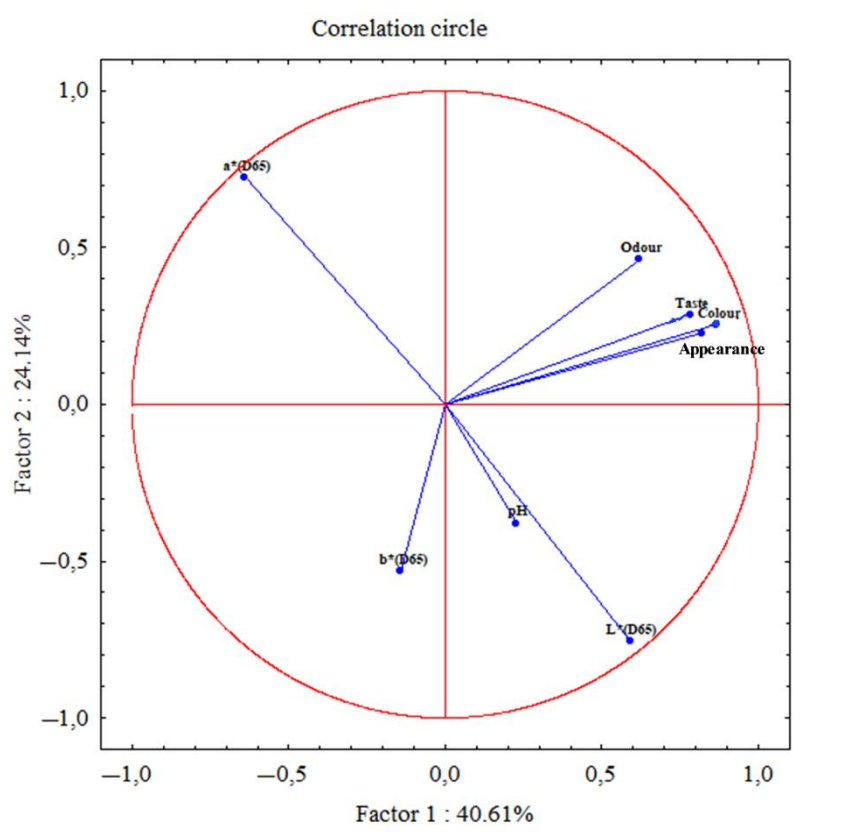
The first main component (Factor 1) explains 46.76% of the variance and the second component 33.91%. The circular correlation between the first two main components is shown in Figure 5a. Figure 5b shows the factorial map of the samples, identifying three very distinct groups; in particular, it was observed that the samples of the last thawing were arranged in another quadrant of the map and were different from the other fresh and thawed samples (Figure 5b).

The fresh samples (I S F) were arranged in the lower quadrant, where the variable that mainly affected their characteristics was the texture (Figure 5a, b). In fact, due to thawing, the thawed samples had a lower score (Table 4), losing their tonicity; moreover, fresh fillets showed greater compactness, recording higher Young Modulus values (Table 4).

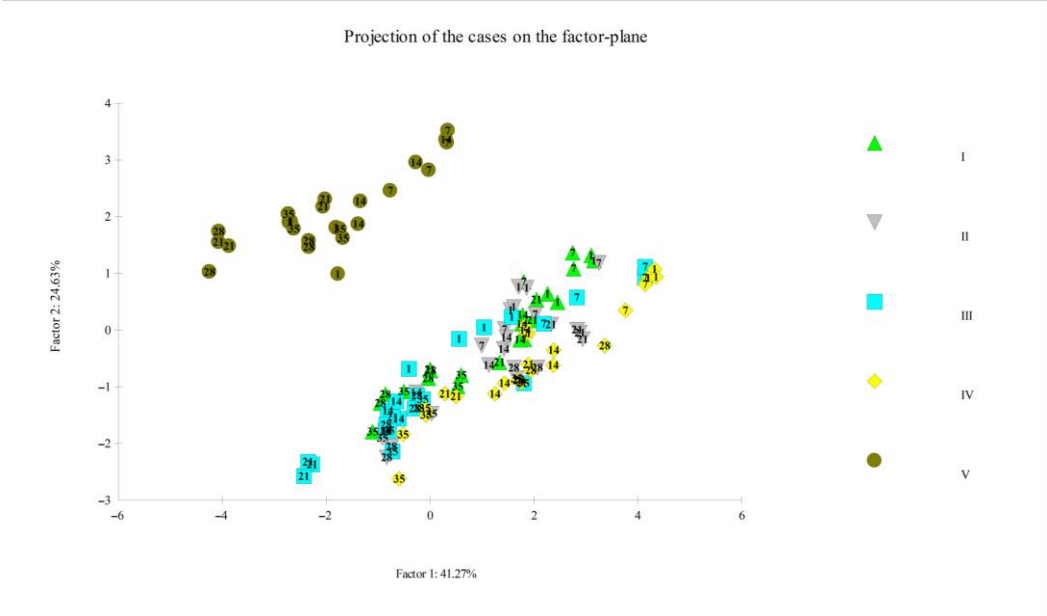
The PCA was also performed on smoked fillets for all five smoked products; eight variables were identified (sensory analysis: odor, color, appearance and taste; color: L*, a* and b*; pH) and 120 cases. The analysis generated a small number of linear combinations on the eight variables and only three main components with an eigenvalue greater than 1 were identified. These three components explain 79.28% of the variance of the original variables.

The first main component (Factor 1) explains 41.27% of the variance, the second component 24.63%, and the third component 13.38%. The circular correlation has been realized considering the first two main components (Figure 6a); this graph shows how the eight variables were arranged in the experimental case examined. Figure 6b shows the factorial map of the samples, identifying two very distinct groups, where smoking V is clearly separated from the other smoking processes (Figure 6b).

This reflects the result of the previous PCA (Figure 5a, b) where the thawed fillets, used in smoking V, were of inferior quality compared to other fillets, thus affecting the smoking and the final product (Sampels, 2015).



(**a**)



(**b**)

Figure 6. Principal component analysis obtained from the correlation of eight components (sensorial analysis, pH, and color) and 120 cases, determined in the smoked fillets *D. labrax*. (**a**) Correlation circle; (**b**) Factor-plan. In Figure 6b, different symbols, from I to V, indicate the smoking cycles.

Conclusions

Freezing affected the texture of the sea bass fillets, in particular, the fresh fillets were of better quality in terms of compactness (i.e., Young Modulus) compared to the thawed fillets, and the worst ones were fillets thawed after 90 days of frozen storage. The quality of the raw fillets consequently affected the quality of the cold smoked fillets, based on the sensory attributes scores and color evaluation, indicating that sea bass fillets could not be frozen for more than 60 days if they were meant to be treated by cold smoking.

Cold smoking was effective on preventing lipid peroxidation and in maintaining TVB-N values below the threshold for spoilage. Moreover, the type of salt used in the process did not alter the quality of the smoked fillets, providing evidence about using potassium as a sodium replacement when cold smoked sea bass fillets are produced, in order to develop a low-salt fish product.

The findings of the present study provided important evidence on developing a new value-added fish product from aquaculture that can contribute to diversifying the seafood products available to consumers given that the supply of European sea bass fillets often exceeds the demand. In this case, a ready-to-eat product could be developed with important practical applications for the sector considering that the pace of modern life makes this kind of product greater in demand.

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Supplementary materials

Table 1. Parameters related to color—L* a*, and b*— measured in the smoked fillets *D. labrax* during the 35 days of preservation in the five smoking processes (I, II, III, IV,V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**			**L*(D65)**		
Fresh/thawed		41,86±0,86$^{ab/A}$	51,39±2,48$^{a/B}$	44,49±3,12^{AC}	46,19±1,31$^{a/C}$	46,67±2,57$^{ab/C}$
1	NaCl	39,95±1,70$^{a/A}$	45,53±2,85$^{b/B}$	44,38±2,96^{B}	40,70±2,15$^{c/A}$	48,31±1,01$^{ab/B}$
	NaCl/KCl	42,18±0,96$^{ab/A}$	49,82±3,76$^{ab/B}$	43,29±2,89^{A}	41,26±0,47$^{bc/A}$	45,09±2,54$^{a/A}$
7	NaCl	41,26±0,80$^{ab/A}$	46,60±2,75$^{ab/AB}$	44,60±2,02^{AB}	41,25±2,37$^{bc/A}$	46,80±2,64$^{ab/B}$
	NaCl/KCl	39,89±1,03$^{a/A}$	45,23±2,25$^{b/C}$	44,59±1,80^{BC}	41,69±1,31$^{bc/AB}$	46,93±2,28$^{ab/C}$
14	NaCl	42,31±1,78^{ab}	45,28±1,10^{b}	43,11±3,17	44,28±1,22^{abc}	46,42±2,99^{ab}
	NaCl/KCl	43,23±0,95^{b}	46,29±2,76^{ab}	45,24±3,01	44,03±2,09^{abc}	46,10±3,07^{ab}
21	NaCl	44,14±2,12$^{bc/AB}$	47,09±1,46$^{ab/B}$	42,95±2,01^{A}	45,74±2,32$^{ab/AB}$	47,32±1,44$^{ab/B}$
	NaCl/KCl	43,59±1,58^{b}	45,60±2,40^{b}	45,56±1,42	43,71±3,51^{abc}	46,51±0,92^{ab}
28	NaCl	43,41±1,61^{b}	45,37±1,50^{b}	45,75±1,56	44,79±2,47^{abc}	47,53±3,01^{ab}
	NaCl/KCl	42,29±0,63^{ac}	48,55±1,06^{ab}	46,94±1,91	47,39±1,26^{a}	48,84±3,02^{ab}
35	NaCl	46,28±1,20$^{c/A}$	45,10±1,46$^{b/A}$	47,21±1,08^{A}	46,81±1,30$^{a/A}$	51,15±2,41$^{b/B}$
	NaCl/KCl	44,29±1,76^{bc}	46,89±2,58^{ab}	46,16±1,52	46,49±2,71^{a}	48,37±1,94^{ab}
				a*(D65)		
Fresh/thawed		-1,77±0,40	-1,01±0,31^{a}	-0,39±1,38^{a}	-1,30±0,61^{ab}	-1,62±1,28^{a}
1	NaCl	-0,95±0,56^{A}	-2,20±0,28$^{b/B}$	-1,84±0,18$^{ab/B}$	-1,41±0,49$^{ab/AB}$	-3,13±0,66$^{ab/C}$
	NaCl/KCl	-1,02±0,63^{A}	-3,73±0,48$^{bc/B}$	-2,09±0,59$^{ab/AB}$	-0,95±1,43$^{a/A}$	-2,43±1,27^{abAB}
7	NaCl	-1,28±0,97^{A}	-3,19±1,06$^{bc/CD}$	-2,70±0,81$^{b/BC}$	-2,05±0,69$^{abc/AB}$	-4,12±0,53$^{b/D}$
	NaCl/KCl	-0,90±1,01^{A}	-3,55±0,65$^{bc/BC}$	-3,06±0,40$^{b/BC}$	-1,99±0,89$^{abc/AB}$	-3,97±0,45$^{ab/C}$
14	NaCl	-1,72±1,49	-3,55±0,88^{bc}	-2,79±0,87^{b}	-1,79±1,33^{abc}	-3,34±0,46^{ab}
	NaCl/KCl	-2,03±1,05	-3,56±1,01^{bc}	-2,84±0,67^{b}	-1,86±0,77^{abc}	-3,64±1,23^{ab}
21	NaCl	-1,80±1,45	-3,96±1,01^{bc}	-1,79±0,80^{ab}	-2,23±1,10^{abc}	-3,89±0,72^{ab}
	NaCl/KCl	-1,20±1,59^{A}	-3,23±1,02$^{bc/B}$	-3,44±0,72$^{b/B}$	-3,17±0,44$^{bc/B}$	-3,91±0,77$^{ab/B}$
28	NaCl	-1,38±1,53	-3,95±0,20^{bc}	-2,38±1,66^{b}	-2,06±0,71^{abc}	-2,81±1,81^{ab}
	NaCl/KCl	-1,67±1,43^{A}	-4,17±0,67$^{c/B}$	-3,36±0,60$^{b/B}$	-2,95±1,02$^{abc/AB}$	-4,35±0,36$^{ab/B}$
35	NaCl	-2,98±0,63	-3,35±0,58^{bc}	-3,59±0,77^{b}	-3,30±0,81^{bc}	-2,91±1,37^{ab}
	NaCl/KCl	-1,46±0,95	-3,32±0,71^{bc}	-2,81±1,75^{b}	-3,81±0,61^{c}	-3,13±1,06^{ab}
				b*(D65)		
Fresh/thawed		-4,78±0,55$^{ac/A}$	-2,02±1,40$^{ac/B}$	-1,27±0,92$^{ac/B}$	-1,71±0,86$^{ad/B}$	-0,48±0,90$^{ab/B}$
1	NaCl	-5,79±0,68$^{a/A}$	-4,86±0,79$^{b/AB}$	-3,47±1,12$^{a/BC}$	-5,05±1,00$^{b/AB}$	-2,46±0,79$^{a/C}$
	NaCl/KCl	-7,75±1,72$^{b/A}$	-6,53±1,20$^{b/AB}$	-5,91±0,69$^{b/AB}$	-4,30±1,03$^{bc/BC}$	-2,49±1,24$^{a/C}$
7	NaCl	-5,89±0,51$^{ac//A}$	-2,03±1,24$^{ac/B}$	-2,14±0,99$^{ac/B}$	-3,48±1,05$^{abc/B}$	2,07±1,33$^{cd/C}$
	NaCl/KCl	-5,18±1,95$^{ac/A}$	-2,61±0,62$^{a/B}$	-3,21±1,00$^{a/AB}$	-3,46±0,85$^{abc/AB}$	1,17±1,87$^{bc/C}$
14	NaCl	-4,63±0,68$^{ac/A}$	-0,74±0,57$^{acd/B}$	-2,49±0,85$^{ac/C}$	-1,98±1,02$^{ad/C}$	2,47±0,36$^{cde/D}$
	NaCl/KCl	-4,16±0,57$^{acd/A}$	-0,34±1,66$^{acd/BD}$	-1,67±1,53$^{ac/BC}$	-2,59±0,84$^{acd/C}$	1,13±0,70$^{bc/D}$
21	NaCl	-3,31±0,81$^{acd/A}$	0,87±1,07$^{d/C}$	-1,15±1,28$^{ac/B}$	-1,51±0,59$^{ad/B}$	4,15±0,44$^{def/D}$
	NaCl/KCl	-4,63±1,00$^{ac/A}$	0,16±1,78$^{cd/B}$	-2,51±1,21$^{ac/C}$	-1,54±0,98$^{ad/BC}$	4,27±1,57$^{def/D}$
28	NaCl	-1,90±0,62$^{d/A}$	1,09±1,10$^{d/B}$	-1,62±0,40$^{ac/A}$	-0,73±0,90$^{d/A}$	6,83±1,52$^{g/C}$
	NaCl/KCl	-3,52±1,32$^{acd/A}$	1,18±0,72$^{d/B}$	-0,55±0,72$^{cd/BC}$	-1,47±1,75$^{ad/C}$	3,43±0,93$^{cde/D}$
35	NaCl	-2,78±0,93$^{cd/A}$	0,67±1,46$^{d/B}$	-1,35±0,72$^{ac/AB}$	-1,12±1,06$^{ad/AB}$	6,48±2,17$^{fg/C}$
	NaCl/KCl	-3,53±1,54$^{acd/A}$	0,51±1,22$^{d/B}$	0,88±1,77$^{d/B}$	-0,64±1,66$^{d/B}$	4,89±1,29$^{efg/C}$

Data within a column, showing different superscript letters (a, b, c…), are statistically different (P<0.05). Data within a row, showing different superscript letters (A, B, C…), are statistically different (P<0.05)

Table 2. Parameters related to color—C*, h, and ΔE — measured in the smoked fillets *D. labrax* during the 35 days of preservation in the five smoking processes (I, II, III, IV, V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**			**C*(D65)**		
Fresh/thawed		5,10±0,60$^{ab/A}$	2,42±1,04$^{a/B}$	1,65±1,20$^{a/B}$	2,16±1,01$^{a/B}$	1,89±1,23$^{a/B}$
1	NaCl	5,88±0,72^{bc}	5,34±0,82^{b}	3,94±1,07^{ac}	5,28±0,91^{b}	4,00±0,91^{bc}
	NaCl/KCl	7,83±1,76$^{c/A}$	7,54±1,05$^{c/A}$	6,28±0,83$^{b/AB}$	4,55±1,13$^{ab/BC}$	3,54±1,61$^{b/C}$
7	NaCl	6,09±0,47$^{bc/A}$	3,84±1,43$^{ab/AB}$	3,46±1,21$^{ac/B}$	4,06±1,17$^{ab/B}$	4,74±0,66$^{bc/AB}$
	NaCl/KCl	5,37±1,81^{ab}	4,42±0,78^{ab}	4,49±0,76^{c}	4,01±1,15^{ab}	4,45±0,25^{bc}
14	NaCl	5,05±1,07^{ab}	3,65±0,92^{ab}	3,76±1,16^{ac}	2,85±1,22^{a}	4,16±0,54^{bc}
	NaCl/KCl	4,70±0,69^{ab}	3,84±1,13^{ab}	3,45±1,13^{ac}	3,27±0,80^{ab}	3,91±1,01^{bc}
21	NaCl	3,95±0,95$^{ab/A}$	4,19±0,82$^{ab/A}$	2,32±1,05$^{ac/A}$	2,77±1,00$^{a/A}$	5,69±0,80$^{bcd/B}$
	NaCl/KCl	4,94±1,22$^{ab/AB}$	3,64±0,69$^{ab/A}$	4,33±1,05$^{ac/AB}$	3,58±0,78$^{ab/A}$	5,96±0,60$^{cd/B}$
28	NaCl	2,54±1,23$^{ab/A}$	4,21±0,21$^{ab/A}$	3,04±1,26$^{ac/A}$	2,36±0,51$^{a/A}$	7,59±1,29$^{d/B}$
	NaCl/KCl	4,03±1,57^{ab}	4,38±0,58^{ab}	3,46±0,60^{ac}	3,53±1,40^{ab}	5,59±0,57^{bcd}
35	NaCl	4,11±0,93$^{ab/A}$	3,60±0,86$^{ab/A}$	3,86±0,95$^{ac/A}$	3,59±0,91$^{ab/A}$	7,30±1,65$^{d/B}$
	NaCl/KCl	3,83±1,76^{a}	3,52±0,72^{ab}	3,27±1,86^{ac}	4,11±0,68^{ab}	5,96±0,57^{cd}
				h (D65)		
Fresh/thawed		249,77±3,68^{ab}	232,97±30,28^{a}	270,47±45,03^{a}	232,30±9,48^{abc}	211,97±55,35^{a}
1	NaCl	260,82±4,90$^{b/A}$	245,55±2,22$^{a/BC}$	240,94±5,44$^{abc/C}$	253,75±7,06$^{bc/AB}$	217,74±6,78$^{a/D}$
	NaCl/KCl	262,72±3,56$^{b/A}$	239,83±6,14$^{a/AB}$	250,88±3,78$^{ab/A}$	261,41±19,60$^{c/A}$	226,26±15,49$^{a/B}$
7	NaCl	257,83±9,45$^{ab/A}$	209,68±12,81$^{abc/B}$	216,46±9,01$^{bcd/B}$	239,52±6,85$^{abc/C}$	154,45±15,64$^{bc/D}$
	NaCl/KCl	257,87±13,53$^{ab/A}$	216,23±5,80$^{ab/C}$	225,33±10,61$^{bcd/BC}$	241,17±6,32$^{abc/AB}$	163,52±24,76$^{b/D}$
14	NaCl	251,72±14,40$^{ab/A}$	191,39±7,75$^{bcd/B}$	221,83±6,20$^{bcd/A}$	235,50±31,73$^{abc/A}$	143,53±3,36$^{bc/C}$
	NaCl/KCl	244,56±12,40$^{ab/A}$	180,04±24,60$^{cd/CD}$	206,12±21,54$^{bcd/BC}$	233,83±15,47$^{abc/AB}$	160,58±14,85$^{b/D}$
21	NaCl	242,59±20,50$^{ab/A}$	165,58±17,47$^{d/B}$	208,77±29,84$^{bcd/A}$	217,03±15,62$^{abd/A}$	132,91±2,31$^{bc/C}$
	NaCl/KCl	258,09±17,83$^{ab/A}$	173,54±32,77$^{cd/B}$	214,57±12,$^{70bcd/C}$	204,32±11,67$^{ad/C}$	133,58±16,20$^{bc/D}$
28	NaCl	242,99±30,18$^{ab/A}$	164,85±15,05$^{d/}$	224,44±31,23$^{bc/Ad}$	199,35±25,71$^{ad/AB}$	112,92±15,28$^{c/C}$
	NaCl/KCl	249,82±21,30$^{ab/A}$	163,64±10,19$^{d/BD}$	189,63±12,13$^{d/BC}$	198,08±30,44$^{ad/C}$	142,16±8,34$^{bc/D}$
35	NaCl	222,28±8,77$^{a/A}$	171,96±21,43$^{cd/B}$	199,78±6,51$^{cd/A}$	198,30±15,28$^{ad/A}$	116,51±14,41$^{bc/C}$
	NaCl/KCl	248,78±6,11$^{ab/A}$	173,78±21,10$^{cd/B}$	158,26±31,27$^{e/B}$	186,54±24,14$^{d/B}$	123,59±15,32$^{bc/C}$
				ΔE		
Fresh/thawed		0,00±0,00^{a}	0,00±0,00^{a}	0,00±0,00^{a}	0,00±0,00^{a}	0,00±0,00^{a}
1	NaCl	2,70±1,01$^{bc/A}$	6,87±2,09$^{b/B}$	3,65±1,29$^{b/A}$	6,64±1,44$^{b/B}$	3,21±0,43$^{b/A}$
	NaCl/KCl	3,33±1,48^{bc}	6,38±1,33^{b}	5,60±1,35^{b}	5,79±0,32^{bc}	3,38±2,01^{b}
7	NaCl	1,75±0,46$^{b/A}$	5,56±2,45$^{b/C}$	3,18±0,62$^{b/AB}$	5,35±2,55$^{bc/C}$	4,36±0,83$^{b/B}$
	NaCl/KCl	2,86±1,15^{bc}	6,78±2,02^{b}	3,75±0,50^{b}	4,93±1,59^{bcd}	3,86±0,42^{b}
14	NaCl	2,09±0,59$^{b/A}$	6,78±1,24$^{b/B}$	3,88±1,93$^{b/AB}$	2,60±0,77$^{ef/C}$	4,24±0,98$^{b/B}$
	NaCl/KCl	1,97±0,54$^{bc/A}$	6,37±2,07$^{b/B}$	3,91±0,63$^{b/A}$	2,82±1,64$^{def/AB}$	3,85±0,99$^{b/A}$
21	NaCl	3,37±1,37$^{bc/A}$	6,16±1,00$^{b/B}$	2,59±1,77$^{b/A}$	2,28±1,21$^{f/A}$	5,35±0,74$^{bc/B}$
	NaCl/KCl	2,54±1,37$^{bc/A}$	6,89±2,02$^{b/B}$	3,87±0,19$^{b/A}$	4,05±2,10$^{cdef/A}$	5,47±1,00$^{bc/B}$
28	NaCl	3,83±0,51$^{c/A}$	7,49±1,10$^{b/B}$	3,09±0,43$^{b/A}$	2,64±1,67$^{ef/A}$	8,04±1,65$^{d/B}$
	NaCl/KCl	1,90±1,32$^{bc/A}$	5,41±0,84$^{b/B}$	4,24±1,01$^{b/B}$	2,81±0,91$^{ef/A}$	5,76±1,57$^{bc/B}$
35	NaCl	5,15±0,84$^{d/A}$	7,33±1,64$^{b/B}$	4,34±0,92$^{b/A}$	2,69±0,36$^{ef/C}$	8,62±2,61$^{d/B}$
	NaCl/KCl	3,15±1,80^{bc}	6,03±1,68^{b}	4,25±1,45^{b}	3,82±1,01^{cde}	6,14±1,29^{c}

Data within a column, showing different superscript letters (a, b, c…), are statistically different (P<0.05). Data within a row, showing different superscript letters (A, B, C…), are statistically different (P<0.05)

Table 3. Parameters related to texture—Young Modulus (N/mm2) and Hardness (N)— measured in the smoked fillets *D. labrax* during the 35 days of preservation in the five smoking processes (I, II, III ,IV ,V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**	**Young Modulus (N/mm^2)**				
Fresh/thawed		0,27±0,01$^{a/A}$	0,28±0,03^{A}	0,27±0,03$^{a/A}$	0,22±0,09$^{a/AB}$	0,20±0,02$^{a/B}$
1	NaCl	0,55±0,00^{b}	0,39±0,09	0,39±0,08^{ab}	0,38±0,05^{b}	0,60±0,20^{b}
	NaCl/KCl	0,39±0,03^{ab}	0,38±0,04	0,48±0,02^{ab}	0,41±0,04^{b}	0,41±0,10^{bc}
7	NaCl	0,43±0,13^{ab}	0,44±0,03	0,48±0,01^{ab}	0,46±0,12^{b}	0,45±0,03^{bc}
	NaCl/KCl	0,55±0,26^{b}	0,50±0,31	0,54±0,14^{b}	0,45±0,03^{b}	0,49±0,04^{bc}
14	NaCl	0,76±0,11$^{c/A}$	0,48±0,12^{B}	0,47±0,00$^{ab/B}$	0,36±0,04$^{ab/B}$	0,42±0,01$^{bc/B}$
	NaCl/KCl	0,39±0,01^{ab}	0,63±0,18	0,46±0,05^{ab}	0,36±0,03^{ab}	0,55±0,08^{bc}
21	NaCl	0,34±0,03$^{ab/A}$	0,37±0,00^{A}	0,46±0,01$^{ab/B}$	0,33±0,02$^{ab/A}$	0,38±0,02$^{c/A}$
	NaCl/KCl	0,38±0,01$^{ab/AB}$	0,42±0,00^{B}	0,37±0,07$^{ab/AB}$	0,31±0,03$^{ab/A}$	0,40±0,00$^{bc/AB}$
28	NaCl	0,39±0,00$^{ab/A}$	0,29±0,03^{B}	0,31±0,08$^{a/AB}$	0,31±0,06$^{ab/AB}$	0,35±0,03$^{c/AB}$
	NaCl/KCl	0,42±0,00$^{ab/A}$	0,37±0,05^{BC}	0,35±0,02$^{ab/CD}$	0,31±0,01$^{ab/D}$	0,39±0,00$^{c/AB}$
35	NaCl	0,37±0,01$^{ab/A}$	0,33±0,04^{AB}	0,28±0,01$^{a/B}$	0,33±0,04$^{ab/AB}$	0,44±0,04$^{bc/C}$
	NaCl/KCl	0,41±0,00$^{ab/AB}$	0,33±0,00^{A}	0,37±0,06$^{ab/AB}$	0,34±0,03$^{ab/A}$	0,47±0,01$^{bc/B}$
				Hardness (N)		
Fresh/thawed		7,70±1,68^{a}	9,78±0,80^{a}	8,69±1,70^{a}	7,11±4,53^{a}	8,30±1,40^{a}
1	NaCl	16,99±3,11^{bc}	19,58±0,18^{b}	15,79±1,31^{ab}	18,00±2,27^{cde}	20,55±1,46^{bc}
	NaCl/KCl	10,84±1,96$^{b/A}$	17,11±2,24$^{ab/B}$	18,37±0,23$^{b/B}$	20,11±1,13$^{de/B}$	17,98±2,97$^{bc/B}$
7	NaCl	17,87±3,68^{c}	18,12±1,26^{b}	24,01±1,54^{b}	22,19±3,94^{e}	18,77±2,73^{bc}
	NaCl/KCl	17,18±3,41^{bc}	18,18±6,60^{b}	22,92±6,10^{b}	19,02±2,00^{cde}	23,10±2,39^{b}
14	NaCl	24,83±1,23^{d}	20,80±2,04^{b}	18,67±2,80^{b}	19,62±3,79^{cde}	19,15±0,14^{bc}
	NaCl/KCl	15,85±2,72$^{bc/A}$	18,15±1,14$^{b/A}$	23,01±3,40$^{b/A}$	18,70±0,36$^{cde/AB}$	26,64±3,70$^{c/B}$
21	NaCl	14,53±1,91$^{bc/A}$	12,67±0,59$^{ab/A}$	19,98±0,78$^{b/B}$	10,39±0,89$^{ab/C}$	17,07±0,49$^{b/D}$
	NaCl/KCl	17,35±1,30$^{bc/AC}$	15,40±0,44$^{ab/A}$	17,51±0,46$^{b/AC}$	12,44±2,55$^{abc/B}$	20,06±1,57$^{bc/C}$
28	NaCl	14,73±0,12^{bc}	16,02±1,89^{ab}	15,57±3,21^{ab}	14,98±1,29^{cde}	18,19±1,40^{bc}
	NaCl/KCl	18,99±0,10$^{c/AB}$	18,67±1,33$^{b/AB}$	16,24±0,87$^{ab/A}$	19,75±0,42$^{cd/B}$	18,13±0,24$^{bc/AB}$
35	NaCl	12,87±0,26$^{bc/A}$	15,22±0,20$^{ab/A}$	14,57±1,11$^{ab/A}$	14,18±4,07$^{bcd/A}$	20,53±0,73$^{bc/B}$
	NaCl/KCl	14,50±3,10$^{bc/A}$	14,47±0,82$^{ab/A}$	15,05±0,62$^{ab/A}$	18,31±0,64$^{bcde/B}$	19,26±0,90$^{bc/B}$

Data within a column, showing different superscript letters (a, b, c…), are statistically different (P<0.05). Data within a row, showing different superscript letters (A, B, C…), are statistically different (P<0.05)

Table 4. Water holding capacity (WHC) variations measured in the smoked fillets *D. labrax* during the 35 days of preservation in the five smoking processes (I, II, III, IV, V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**	**WHC**				
Fresh/thawed		0,88±0,00$^{a/A}$	0,80±0,03$^{a/B}$	0,74±0,04$^{a/C}$	0,82±0,02$^{a/B}$	0,78±0,03$^{a/B}$
1	NaCl	0,96±0,02^{b}	0,96±0,01^{b}	0,96±0,01^{b}	0,97±0,00^{bc}	0,96±0,00^{bc}
	NaCl/KCl	0,98±0,00$^{b/A}$	0,95±0,01$^{b/A}$	0,92±0,01$^{b/B}$	0,95±0,04$^{c/A}$	0,98±0,00$^{c/A}$
7	NaCl	0,97±0,00$^{b/A}$	0,97±0,01$^{b/A}$	0,94±0,04$^{b/B}$	0,98±0,00$^{c/A}$	0,98±0,00$^{c/A}$
	NaCl/KCl	0,96±0,00^{b}	0,95±0,00^{b}	0,95±0,01^{b}	0,98±0,00^{bc}	0,98±0,00^{c}
14	NaCl	0,97±0,01^{b}	0,96±0,00^{b}	0,94±0,08^{b}	0,97±0,01^{bc}	0,98±0,00^{c}
	NaCl/KCl	0,97±0,01^{b}	0,96±0,00^{b}	0,95±0,02^{b}	0,98±0,01^{bc}	0,98±0,01^{c}
21	NaCl	0,94±0,01^{b}	0,93±0,02^{b}	0,97±0,01^{b}	0,95±0,02^{b}	0,97±0,00^{bc}
	NaCl/KCl	0,96±0,01^{b}	0,89±0,05^{b}	0,90±0,04^{b}	0,97±0,01^{bc}	0,97±0,01^{bc}
28	NaCl	0,86±0,08^{a}	0,92±0,06^{b}	0,93±0,03^{b}	0,98±0,01^{bc}	0,96±0,00^{b}
	NaCl/KCl	0,94±0,04^{b}	0,91±0,06^{b}	0,92±0,04^{b}	0,96±0,02^{bc}	0,96±0,00^{b}
35	NaCl	0,95±0,00^{b}	0,95±0,01^{b}	0,95±0,02^{b}	0,96±0,00^{bc}	0,97±0,02^{bc}
	NaCl/KCl	0,93±0,02^{b}	0,92±0,09^{b}	0,91±0,04^{b}	0,95±0,01^{b}	0,96±0,00^{b}

Data within a column, showing different superscript letters (a, b, c…), are statistically different (P<0.05). Data within a row, showing different superscript letters (A, B, C…), are statistically different (P<0.05)

Table 5. pH variations measured in the smoked fillets *D. labrax* during the 35 days of preservation in the five smoking processes (I, II, III, IV, V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**			**pH**		
Fresh/thawed		6,29±0,00$^{a/A}$	6,53±0,11$^{a/B}$	6,45±0,06$^{a/B}$	6,44±0,04$^{ab/B}$	6,24±0,00$^{a/A}$
1	NaCl	5,94±0,13$^{bc/A}$	5,49±0,07$^{b/B}$	5,64±0,01$^{a/A}$	6,04±0,02$^{ab/A}$	5,69±0,06$^{a/C}$
	NaCl/KCl	6,02±0,03$^{bc/A}$	5,50±0,05$^{b/B}$	6,04±0,02$^{a/A}$	6,05±0,04$^{ab/A}$	5,93±0,10$^{a/C}$
7	NaCl	5,70±0,02$^{e/A}$	5,39±0,11$^{b/B}$	5,78±0,05$^{d/C}$	5,96±0,05$^{a/D}$	5,62±0,01$^{c/E}$
	NaCl/KCl	6,07±0,14$^{b/A}$	5,39±0,07$^{b/B}$	5,76±0,05$^{d/C}$	6,00±0,01$^{ab/A}$	5,77±0,01$^{b/C}$
14	NaCl	5,89±0,11$^{d/A}$	5,45±0,02$^{b/B}$	5,90±0,00$^{bc/A}$	6,02±0,01$^{ab/C}$	5,61±0,04$^{c/D}$
	NaCl/KCl	5,83±0,04^{d}	5,56±0,01^{b}	5,91±0,12^{ab}	6,03±0,02^{abc}	5,83±0,05^{b}
21	NaCl	5,68±0,10$^{e/A}$	5,66±0,01$^{b/A}$	5,93±0,06$^{ab/B}$	5,97±0,13$^{a/B}$	5,65±0,03$^{c/A}$
	NaCl/KCl	5,92±0,04$^{cd/A}$	5,66±0,02$^{b/B}$	5,93±0,01$^{ab/A}$	6,11±0,03$^{c/C}$	5,65±0,19$^{c/B}$
28	NaCl	5,64±0,06$^{e/A}$	5,77±0,01$^{b/B}$	5,89±0,07$^{bc/C}$	5,96±0,09$^{a/D}$	5,43±0,07$^{d/E}$
	NaCl/KCl	5,82±0,02^{d}	5,76±0,01^{b}	5,81±0,02^{cd}	6,04±0,05^{abc}	5,82±0,02^{b}
35	NaCl	5,89±0,00$^{d/A}$	5,71±0,12$^{b/B}$	5,89±0,05$^{bc/A}$	5,99±0,01$^{ab/A}$	5,42±0,18$^{d/C}$
	NaCl/KCl	6,00±0,03$^{bc/A}$	5,72±0,02$^{b/B}$	5,86±0,05$^{bc/C}$	6,07±0,05$^{bc/D}$	5,71±0,02$^{b/B}$

Data within a column, showing different superscript letters (a, b, c…), are statistically different ($P<0.05$). Data within a row, showing different superscript letters (A, B, C…), are statistically different ($P<0.05$)

Table 6. Sensorial analysis measured in the smoked fillets *D. labrax* during the 35 days of preservation, in the five smoking processes (I, II, III, IV, V).

		I	II	III	IV	V
Day of storage	**Type of brine solution**			**Odor**		
1	NaCl	$4.00\pm0.00^{ab/A}$	$4.00\pm0.00^{ab/A}$	$4.00\pm0.00^{ab/A}$	$4.67\pm0.00^{a/B}$	$3.00\pm0.00^{a/C}$
	NaCl/KCl	$4.50\pm0.71^{bc/A}$	$4.00\pm0.00^{ab/A}$	$4.33\pm0.00^{b/A}$	$4.67\pm0.00^{a/A}$	$3.00\pm0.00^{a/B}$
7	NaCl	5.00 ± 0.00^{c}	4.50 ± 0.00^{ac}	5.00 ± 0.00^{c}	4.33 ± 0.00^{ab}	5.00 ± 0.00^{c}
	NaCl/KCl	$4.75\pm0.35^{c/A}$	$3.75\pm0.35^{b/B}$	$5.00\pm0.00^{c/A}$	$4.00\pm0.00^{abc/B}$	$4.00\pm0.00^{b/B}$
14	NaCl	$4.00\pm0.00^{ab/A}$	$4.00\pm0.00^{ab/A}$	$3.00\pm0.00^{d/B}$	$3.75\pm0.35^{bc/A}$	$5.00\pm0.00^{c/C}$
	NaCl/KCl	$4.00\pm0.00^{ab/A}$	$3.50\pm0.71^{b/B}$	$3.00\pm0.00^{d/C}$	$3.00\pm0.00^{d/C}$	$4.00\pm0.00^{b/A}$
21	NaCl	$5.00\pm0.00^{c/A}$	$5.00\pm0.00^{c/A}$	$2.83\pm0.24^{de/B}$	$4.00\pm0.00^{abc/C}$	$4.00\pm0.00^{b/C}$
	NaCl/KCl	$3.00\pm0.00^{de/A}$	$5.00\pm0.00^{c/B}$	$2.50\pm0.71^{e/C}$	$4.00\pm0.00^{abc/D}$	$4.00\pm0.00^{b/D}$
28	NaCl	$3.00\pm0.00^{de/A}$	$4.17\pm0.24^{ab/B}$	$4.00\pm0.00^{ab/B}$	$4.00\pm0.00^{abc/B}$	$3.75\pm0.35^{b/B}$
	NaCl/KCl	$3.00\pm0.00^{de/A}$	$3.00\pm0.00^{d/A}$	$4.00\pm0.00^{ab/B}$	$4.00\pm0.00^{abc/B}$	$3.00\pm0.00^{a/A}$
35	NaCl	$3.50\pm0.71^{ad/A}$	$3.67\pm0.00^{b/A}$	$4.00\pm0.00^{ab/A}$	$4.00\pm0.00^{abc/A}$	$3.00\pm0.00^{a/B}$
	NaCl/KCl	$2.75\pm0.35^{e/A}$	$3.83\pm0.24^{ab/B}$	$3.50\pm0.71^{a/AB}$	$3.50\pm0.24^{c/AB}$	$3.00\pm0.00^{a/AB}$
				Colour		
1	NaCl	$4.00\pm0.00^{a/A}$	$4.00\pm0.00^{ab/A}$	$3.33\pm0.00^{ab/B}$	$4.83\pm0.24^{a/C}$	$3.00\pm0.00^{a/B}$
	NaCl/KCl	$4.00\pm0.00^{a/A}$	$4.00\pm0.00^{ab/A}$	$3.00\pm0.00^{bc/B}$	$4.67\pm0.00^{ab/C}$	$3.00\pm0.00^{a/B}$
7	NaCl	4.00 ± 0.00^{a}	4.00 ± 0.00^{ab}	4.50 ± 0.00^{d}	4.33 ± 0.00^{bc}	4.00 ± 0.00^{b}
	NaCl/KCl	4.00 ± 0.00^{a}	4.00 ± 0.00^{ab}	4.25 ± 0.35^{d}	4.17 ± 0.24^{c}	4.00 ± 0.00^{b}
14	NaCl	$4.00\pm0.00^{a/A}$	$3.50\pm0.71^{ade/A}$	$3.00\pm0.00^{bc/B}$	$4.00\pm0.00^{c/A}$	$3.75\pm0.35^{b/A}$
	NaCl/KCl	$4.00\pm0.00^{a/A}$	$3.25\pm0.35^{de/B}$	$3.00\pm0.00^{bc/B}$	$4.00\pm0.00^{c/A}$	$3.00\pm0.00^{a/B}$
21	NaCl	$3.00\pm0.00^{b/A}$	$4.50\pm0.71^{b/B}$	$2.33\pm0.00^{ce/C}$	$3.00\pm0.00^{d/A}$	$3.00\pm0.00^{a/A}$
	NaCl/KCl	$3.33\pm0.47^{b/A}$	$5.00\pm0.00^{c/B}$	$2.17\pm0.24^{e/C}$	$4.00\pm0.00^{c/D}$	$2.00\pm0.00^{d/C}$
28	NaCl	$3.00\pm0.00^{b/A}$	$4.00\pm0.00^{ab/B}$	$3.00\pm0.00^{bc/A}$	$4.00\pm0.00^{c/B}$	$2.00\pm0.00^{d/C}$
	NaCl/KCl	$3.00\pm0.00^{b/A}$	$3.00\pm0.00^{d/A}$	$3.00\pm0.00^{bc/A}$	$4.17\pm0.24^{bc/B}$	$2.50\pm0.71^{c/A}$
35	NaCl	$3.00\pm0.00^{b/A}$	$3.00\pm0.00^{b/A}$	$3.75\pm0.35^{a/B}$	$3.00\pm0.00^{d/A}$	$3.00\pm0.00^{a/A}$
	NaCl/KCl	3.00 ± 0.00^{b}	3.67 ± 0.00^{ab}	3.50 ± 0.71^{ab}	3.00 ± 0.00^{d}	3.00 ± 0.00^{a}
				Appearance		
1	NaCl	$4.00\pm0.00^{a/A}$	$4.00\pm0.00^{ab/A}$	$3.17\pm0.24^{a/B}$	$4.67\pm0.00^{a/C}$	$3.00\pm0.00^{ab/B}$
	NaCl/KCl	$4.00\pm0.00^{a/A}$	$4.00\pm0.00^{ab/A}$	$3.00\pm0.00^{a/B}$	$4.67\pm0.00^{a/C}$	$4.00\pm0.00^{c/A}$
7	NaCl	4.00 ± 0.00^{a}	4.00 ± 0.71^{ab}	4.50 ± 0.00^{b}	4.33 ± 0.00^{ab}	3.50 ± 0.71^{bc}
	NaCl/KCl	4.00 ± 0.00^{a}	4.50 ± 0.71^{bc}	4.25 ± 0.35^{b}	3.83 ± 1.18^{ab}	3.75 ± 0.35^{c}
14	NaCl	$4.00\pm0.00^{a/A}$	$3.50\pm0.71^{ab/AB}$	$3.00\pm0.00^{ac/B}$	$4.00\pm0.00^{ab/A}$	$3.50\pm0.00^{bc/AB}$
	NaCl/KCl	$4.00\pm0.00^{a/A}$	$3.75\pm0.35^{ab/A}$	$3.50\pm0.71^{a/AB}$	$4.00\pm0.00^{ab/A}$	$3.00\pm0.00^{ab/B}$
21	NaCl	$4.00\pm0.00^{a/A}$	$4.50\pm0.71^{bc/B}$	$2.33\pm0.00^{c/C}$	$3.00\pm0.00^{c/D}$	$3.00\pm0.00^{ab/D}$
	NaCl/KCl	$3.17\pm0.24^{bc/A}$	$5.00\pm0.00^{c/B}$	$2.83\pm0.24^{ac/A}$	$4.00\pm0.00^{ab/C}$	$2.00\pm0.00^{d/D}$
28	NaCl	$3.25\pm0.35^{bc/AB}$	$3.67\pm0.00^{ab/B}$	$2.83\pm0.24^{ac/A}$	$3.50\pm0.71^{bc/B}$	$2.50\pm0.71^{a/A}$
	NaCl/KCl	$3.50\pm0.71^{ab/A}$	$3.50\pm0.71^{ab/A}$	$3.00\pm0.00^{ac/A}$	$4.17\pm0.24^{ab/B}$	$3.00\pm0.00^{ab/A}$
35	NaCl	$4.00\pm0.00^{a/A}$	$3.00\pm0.00^{a/B}$	$3.25\pm0.35^{a/B}$	$3.00\pm0.00^{c/B}$	$3.00\pm0.00^{ab/B}$
	NaCl/KCl	$3.00\pm0.00^{c/A}$	$3.50\pm0.24^{ab/AB}$	$3.50\pm0.71^{a/AB}$	$3.00\pm0.00^{c/A}$	$4.00\pm0.00^{c/B}$
				Taste		
1	NaCl	$5.00\pm0.00^{a/A}$	$4.00\pm0.00^{a/B}$	$3.67\pm0.00^{ad/B}$	$4.67\pm0.00^{a/A}$	$3.00\pm0.00^{a/C}$
	NaCl/KCl	$5.00\pm0.00^{a/A}$	$4.00\pm0.00^{a/B}$	$4.00\pm0.00^{a/B}$	$4.67\pm0.00^{a/A}$	$3.00\pm0.00^{a/C}$
7	NaCl	$5.00\pm0.00^{a/A}$	$5.00\pm0.00^{b/A}$	$4.50\pm0.00^{b/AB}$	$4.33\pm0.00^{a/AB}$	$4.00\pm0.00^{b/B}$
	NaCl/KCl	$3.75\pm1.06^{b/A}$	$4.00\pm0.00^{a/A}$	$5.00\pm0.00^{c/B}$	$4.33\pm0.47^{a/AB}$	$4.00\pm0.00^{b/A}$
14	NaCl	$4.00\pm0.00^{b/A}$	$4.00\pm0.00^{a/A}$	$3.00\pm0.00^{de/B}$	$4.50\pm0.71^{a/C}$	$4.00\pm0.00^{b/A}$
	NaCl/KCl	$4.00\pm0.00^{b/A}$	$3.75\pm0.35^{a/A}$	$3.00\pm0.00^{de/B}$	$4.00\pm0.00^{a/A}$	$4.00\pm0.00^{b/A}$
21	NaCl	$5.00\pm0.00^{a/A}$	$3.00\pm0.00^{d/B}$	$2.67\pm0.47^{e/B}$	$4.00\pm0.00^{a/C}$	$3.00\pm0.00^{a/B}$
	NaCl/KCl	$4.00\pm0.00^{b/A}$	$2.25\pm0.35^{c/B}$	$2.50\pm0.71^{e/B}$	$4.00\pm0.00^{a/A}$	$2.00\pm0.00^{c/B}$
28	NaCl	$3.00\pm0.00^{c/A}$	$4.00\pm0.00^{a/B}$	$3.00\pm0.00^{de/A}$	$4.00\pm0.00^{a/B}$	$2.00\pm0.00^{c/C}$
	NaCl/KCl	$3.00\pm0.00^{c/A}$	$3.00\pm0.00^{d/A}$	$3.00\pm0.00^{de/A}$	$4.00\pm0.00^{a/B}$	$3.00\pm0.00^{a/}$
35	NaCl	3.00 ± 0.00^{c}	3.67 ± 0.00^{a}	3.50 ± 0.00^{ad}	3.00 ± 0.00^{b}	3.00 ± 0.00^{a}
	NaCl/KCl	$3.00\pm0.00^{c/A}$	$3.83\pm0.24^{a/B}$	$3.50\pm0.71^{ad/AB}$	$3.00\pm0.00^{b/A}$	$3.00\pm0.00^{a/a}$

Data within a column, showing different superscript letters (a, b, c…), are statistically different ($P < 0.05$). Data within a row, showing different superscript letters (A, B, C…), are statistically different ($P < 0.05$).

Chapter 3

Abstract

Aquaculture has been playing a leading role over the years to satisfy the global growing demand for seafood. Moreover, innovative techniques are necessary to increase competitiveness, sustainability and profitability of the seafood production chain, exploiting new species from aquaculture, such as meagre (*Argyrosomus regius)* to develop value-added products and diversify the production. In the present work, the effectiveness of cold smoking combined with antioxidants (SA), compared to cold smoking alone (S) on meagre fillets quality and shelf life was investigated. Sensory, biochemical, physical-chemical and microbiological analyses were performed on the smoked fillets during vacuum packaged storage for 35 days at 4 ± 0.5 °C. Results showed positive effects of the SA treatment on biochemical parameters of meagre fillets. The total volatile basic nitrogen (TVB-N) in smoked meagre fillets was significantly lower in the SA treatment at the end of storage, compared to the S treatment. Moreover, SA had a positive effect on lipid peroxidation. Lower values of malondialdehyde (mg MDA/kg) were observed in the SA treatment during preservation compared to S treatment. This work will contribute to the growth of the fish production chain producing a value-added fish product by exploiting meagre whose production has been increasing over decades.

Keywords: meagre; *Argyrosomus regius*; cold smoking; natural antioxidants; halophyte; aquaculture; value-added food product; shelf-life; fillets; fish quality.

Introduction

Global fish consumption has been increasing over the years due to the increasing world population as well as the awareness of the beneficial effects of fish inclusion in a balanced diet on the human health. It has been reported that the average annual rate at which global fish consumption increased, from 1961 to 2017, accounted for 3.1%, almost twice that of the annual growth of the global population (FAO, 2020).

In this context, the fish production sector has grown over the years in order to satisfy the global growing demand for seafood, and it is foreseen to increase in the future. Specifically, production of capture fisheries has not been increasing since the late 1980s, while aquaculture has been playing a leading role in satisfying the seafood demand over the years. In this scenario, the aquaculture contribution to fish production has constantly increased at global level, reaching 46.0% in the period 2016-2018. In 2018, aquaculture accounted for 52% of the total production of seafood products that were used for human consumption. Specifically, in 2018, aquaculture produced 82.5 million tons of fish and other organisms, 32.4 million tons of plant organisms, and a good amount of products not intended for consumption (26000 tons) (FAO, 2020).

Innovation plays the major role in increasing competitiveness, sustainability and profitability of the seafood production chain, paying attention to develop value-added processed products by exploiting new species coming from aquaculture, an ever-increasing food production sector. This strategy would allow to satisfy consumers' preferences in the food sector, who prefer ready-to-eat products with high nutritional value, whose availability does not determine negative effect on the environment and whose origin and quality are guaranteed (Bonanomi et al., 2017). In this context, meagre (*Argyrosomus regius*) represents an aquaculture species that must be considered and valorized, considering that its production in Europe has been increasing over the years. Specifically, meagre production has been rising progressively in Europe over the decades; in 2018, the production of meagre reached 6827 tons (+270%), out of which 59% was covered by Spain (EUMOFA, 2020).

Meagre, which belongs to the *Sciaenidae*, is a euryhaline fish, spread in the Mediterranean Sea. Thanks to some characteristics of *A. regius*, such as its high adaptability to several environmental conditions and high resilience to stressors, it has been considered suitable for the diversification of the Mediterranean aquaculture (Parisi et al., 2014). In addition to these aspects, *A.regius* grows rapidly and reaches the commercial size (700-1200 g) only after 12 months and 2-2.5 kg after 24 months under the optimal conditions (Parisi et al., 2014). Moreover, meagre is characterized by high nutritional value thanks to its flesh, lean and with high lipid quality (Poli et al., 2003).

Fish is the main dietary source of omega-3 polyunsaturated fatty acids (PUFAs) highly beneficial for human health but also extremely prone to peroxidation, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). In particular, their oxidation generates free radicals that trigger chain reactions that eventually lead to the generation of compounds responsible for extremely unpleasant odors as well as the loss of compounds of very high nutritional value (Arab-Tehrany et al., 2012; Mei, Ma, & Xie, 2019). Considering the characteristics of the raw material processed, highly perishable, preservatives should be used to preserve fish quality and extend shelf life, indeed even if fish is preserved by freezing or cold storage to extend its shelf life, this could not be sufficient to prevent fish spoilage, lipid oxidation and rancidity with negative effects in terms of consumer acceptability.

Chemical preservatives have been used in order to prevent fish spoilage and extend fish shelf life, such as sodium acetate, sodium lactate, and sodium citrate, whose effects were investigated on extending shelf life of salmon (*Onchorhynchus nerka*) during refrigerated storage (Sallam, 2007), highlighting that using these preservatives was effective on extending seafood shelf life. Sodium bisulfite is also used as chemical preservative to reduce enzyme activity and microbial degradation in shrimps (*Litopenaeus vannamei*) for 4-6 days, nevertheless it has been reported that consuming food treated with sodium-bisulfite for long time can lead to serious health crisis (Lalithapriya et al., 2019).

Therefore, the food industry has been prompted about consumers' concerns and preference for natural preservatives to the use of natural antioxidants and antimicrobials instead of synthetic ones (Mei, Ma, & Xie, 2019). Marine and terrestrial species have thus been used for the purpose of extracting antioxidant compounds such as ascorbic acid, glutathione, carotenoids, terpenes and phenolic compounds (Anaëlle et al., 2013; Burritt, Larkindale, & Hurd, 2002; Herrero, Cifuentes, & Ibañez, 2006; Liau et al., 2010; Mei, Ma, & Xie, 2019; Yoshie-Stark & Hsieh, 2003).

Among plant-derived compounds, there are essential oils, a complex mixture of volatile compounds produced as secondary metabolites in plants and that give them their characteristic odor. Essential oils have been used as natural preservatives for preserving fish quality and extending shelf life due to their well-recognized antimicrobial and antioxidants potentials (Hassoun & Emir Çoban, 2017) Plant extracts could also been used for application in fish preservation for their antimicrobial, antifungal activities, as well as their capacity to inhibit lipid oxidation (Kharchoufi et al., 2018; Mei, Ma, & Xie, 2019).

Salicornia strobilacea (*Halocnemum strobilaceum*) is a plant that belongs to halophytes and grows along salt marshes, salt lakes and saltworks all over the world. Due to the extreme environmental conditions under which this kind of plant grows it is characterized by powerful antioxidant systems

that employ several components among which secretion and accumulation of polyphenols. These bioactive compounds were obtained from the plant (*H. strobilaceum*) and tested in vitro, showing strong antioxidant and antibacterial power (Messina et al., 2019a).

The positive effects of antioxidants from *H. strobilaceum* when combined with other preservative techniques such as modified atmosphere packaging and cold smoking was shown previously on sensory, physical–chemical, nutritional, biochemical and microbiological properties of dolphinfish (*Coryphaena hippurus*) fillets (Messina et al., 2015; Messina et al., 2019b).

Cold smoking is a preservation technique also used to create new value-added products. Specifically, in cold smoking, after an initial treatment with salt the product is smoked in order to give the product organoleptic characteristics appreciated by consumers as well as to transfer to it antimicrobial and antioxidant compounds (aldehydes, ketones, alcohols, phenols), with a significantly longer shelf-life than fresh product. During the different phases of the process, the temperature is never higher than 30 or 33°C (Arvanitoyannis & Kotsanopoulos, 2012; Sampels, 2015). Up to date, several fish species have been processed by this technique, such as farmed European sea bass (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*) (Birkeland and Bjerkeng, 2005; Messina et al., 2021). Moreover, fishery species have been valorized by applying cold smoking, in order to make available all year round seasonal species as in the case of dolphinfish (*Coryphaena hippurus*), but even to enhance fishery species with excess catches such as sardines (*Sardina pilchardus*) (Gómez-Estaca et al., 2010; Gómez-Guillén et al., 2009; Messina et al., 2019b) and herring (*Clupea harengus*) (Cardinal et al., 2006).

The effectiveness of combining traditional techniques such as cold-smoking with addition of natural preservatives on maintaining quality and prolonging shelf life of new value-added seafood products has been investigated (Gómez-Estaca, Gómez-Guillén, & Montero, 2013; Messina et al., 2019b). Combining an antioxidant treatment with oregano extract and cold-smoking produced a reduction in sardine (*S. pilchardus*) lipid oxidation, compared to traditional smoking, highlighting the effectiveness of the combined treatments on improving the shelf life of smoked fish (Gómez-Estaca, Gómez-Guillén, & Montero, 2013).

The combination of cold smoking and natural preservatives was also investigated on dolphinfish (*C. hippurus*) fillets (Messina et al., 2019b). Overall, combination of antioxidants with cold smoking showed positive effects on the quality of dolphinfish fillets, improving biochemical, microbiological, and sensory aspects of the product, and consequently enhancing the marketability of *C. hippurus* and contributing to costal fisheries sustainability.

The present work was aimed at studying how the combination of cold smoking and natural antioxidants affects the sensory, biochemical, physical–chemical and microbiological properties of

meagre (*Argyrosomus regius*) fillets. As a source of antioxidants, *H. strobilaceum* extracts were used.

The present study will contribute to increase competitiveness, profitability and sustainability of the seafood production sector, by developing a new value-added product by using an aquaculture species (*A. regius*) of high nutritional value whose production has been increasing over the years, contributing to diversify seafood production, meeting consumers preferences and reducing pressure on overexploited fishery resources.

Materials and Methods

Fish sampling and processing

A total of 20 specimens of *A. regius* (average size 27.17±1.91 cm and average weight 347.72±62.85 g) were processed in a Sicilian (Italy) aquaculture plant to obtain forty fillets (mean weight: 73.48 ± 22.49 g), that were stored on ice. While in laboratory (less than 30 minutes) the fillets were stored under vacuum in Foodsaver bags (HDPE) and nylon bags (http://www.gopack.it, accessed on 1 September 2021), and subjected to rapid freezing at -35°C (AB 2/3 ALLFORFOODD (PU), Italy) maintained in this condition for 24 hours to inhibit the bacterial growth (Puke and Galoburda, 2020).

The next day, all samples were removed from the bags, placed in air-permeable LDPE bags, and thawed at 4°C for 8 h before processing.

Salting and Smoking

Thawed *A. regius* fillets were processed as follows: four thawed fillets were used for the analyses of the untreated product, the remaining 36 fillets underwent a smoking process consisting of four steps: salting, first drying, smoking, and second drying (Messina et al., 2021; Messina et al., 2019b), as shown in Figure 1.

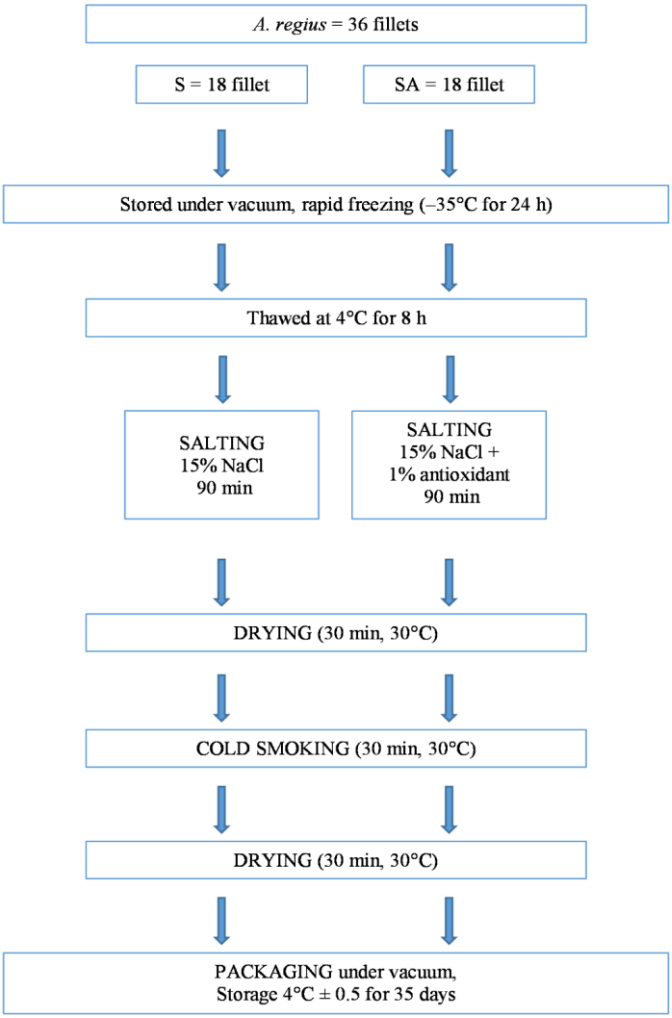


Figure 1. Experimental design flowchart. Cold smoking (S); Cold smoking combined with antioxidants (SA)

Thawed fillets were separated in 2 batches: in the first batch, 18 fillets were immersed in standard brine (S) consisting of 15% NaCl solution (w/v); in the second batch 18 fillets were treated with the same standard brine with 1% antioxidants (SA). For both treatments, a fillet: brine ratio of 1:4 was used (Messina et al., 2021; Messina et al., 2019b).

The antioxidant solution was prepared as described by Messina et al. (2015). Briefly, dried and pulverized *Halocnemum strobilaceum* was extracted with distilled water (1:10 w/v) for 24 hr. The sample was then filtered and lyophilized (Sung et al., 2009). The final solution of *H. strobilaceum* was prepared by dissolving 10 g of freeze-dried extract in 1 ml of distilled water, with a polyphenol content equal to 500 mg gallic acid equivalent (GAE)/L (Messina et al., 2015; Messina et al., 2019b).

Brine salting was performed for 90 min, then samples were dried for 30 min at a temperature of 30°C. Fillets were cold smoked using a Moduline oven model FA082E (Scubla srl, Remanzacco (Ud), Italy) for 30 min at 30°C as described by Messina et al. (2019b, 2021).

After cold smoking, fillets were dried as described previously.

At the end of the process, all the fillets were sealed in vacuum bags and stored at 4 ± 0.5°C for 35 days.

Three fillets from each treatment were analyzed at regular intervals (1, 7, 14, 21, 28, and 35 days after smoking). The effects of the smoking process combined with natural antioxidants on the quality of *A. regius* fillets were evaluated through a multidisciplinary approach involving sensory, physicochemical, biochemical, and microbiological parameters.

Following sampling for the microbiological analysis and the sensory and instrumental analyses, the fillets were cold homogenized for the analysis of biochemical and nutritional parameters related to shelf life.

Physical-Chemical Parameters

Color

Color readings were taken in the L*, a*, b* color space (CIELAB color space, D65 standard illumination and a 2° observer) and repeated 3 times using a Konica Minolta colorimeter (Osaka, Japan). The evaluated color parameters were lightness (L*), red-green chromaticity (a*), yellow-blue chromaticity (b*) the saturation or intensity of a color Chroma (C*) and Hue angle (h) as recommended by the International Commission on Illumination (CIE, 2004; CIE, 1977; Robertson, 1990). Analyses were performed in triplicate for each sample. The color evaluation was carried out in two dorsal regions of the fillets along the cephalo-caudal direction.

Texture Profile Analysis

Texture analysis was conducted as described by Messina et al. (2021). Two small fragments (1.8 cm Ø), obtained from the same portions of each fillet, were used. The analysis was performed at room temperature using an Instron Texture Analyzer Mod. 3342 (Turin, Italy). The measured parameters were Hardness (N) and the Young Modulus or modulus of deformability (N/mm^2) (i.e., the force and the slope of the curve at 50% compression, respectively) (Messina et al., 2019b; Orban, Sinesio, & Paoletti, 1997). The analysis was performed in triplicate. For each replicate as above-mentioned, two fragments per fillet along the dorsal margin were considered. The samples were maintained in ice before the analysis.

Water Holding Capacity (WHC)

Water holding capacity (WHC) was determined using the method described by Teixeira et al. (2014) with some modification reported by Messina et al. (2021). Analyses were performed in triplicate for each sample. The results were expressed in percentage (% WHC).

Muscular pH

The muscular pH of the fillet was measured at three points along the lateral line with a Crison pH meter (Barcelona, Spain) equipped with a BlueLine pH 21 Schott Instruments (Weilheim, Germany) combined electrode.

Water Activity Determination

Water activity (aw) was measured with a fast water activity meter (HP23-AW Rotronic, AG, Bassersdorf, Switzerland). The temperature at which the water activity was measured was equal to 21 °C . The analysis was performed in triplicate.

Proximate composition and Biochemical Parameters Related to the Shelf-Life

Proximate composition

After the physical-chemical and sensory analyses, in order to carry out further analysis, the fillets were cold homogenized.
The ash (ignition at 600 °C for 5 hours) and moisture (drying at 105 °C for 24h) contents (% ash and moisture) were assessed according to the AOAC method (AOAC, 1990) The protein content (% protein) was determined according to the AOAC method (AOAC, 1992).
The total lipids (% lipids) were determined according to Folch et al. (1957) and the fatty acid (FA) methyl esters (%) were determined by the method of Lepage & Roy (1984); gas-chromatography was carried out following the operating conditions described in Messina et al. (2013).

Biochemical Parameters Related to the Shelf-Life

The production of thiobarbituric acid reactive substances (TBARS) was determined using the method described by Botsoglou et al. (1994) and the results expressed in mg MDA

(Malondialdehyde)/kg. The total volatile basic nitrogen (TVB-N) was measured by direct distillation of the homogenized samples according to the EU Commission Decision 95/149/EC (Commissione delle Comunità Europee, 1995) and the values expressed in mg/100 g of product. All analyses were performed in triplicate.

Microbiological analyses

Microbiological analysis was performed as described by Messina et al. (2019b).

Sensorial analysis

Sensory analysis on cold-smoked meagre fillets was conducted by a group of six trained judges according to an adapted version of the scheme proposed by Bilgin & Değirmenci (2019) on hot-smoked meagre.
The parameters evaluated were appearance, odor, flavor, texture and color. The six judges rated the overall acceptability of the samples using a 10-point descriptive scale. A score of 10 to 9 indicates a perfect product, 8 to 7 good, 6 to 5 medium, 4 to 3 the limit of acceptability. The product is considered unacceptable when scored less than 3 (Bilgin & Değirmenci, 2019).

Statistical analysis

Results are expressed as mean ± standard deviation. Homogeneity of variance was analyzed by Levene's test. Data were analyzed by one-way analysis of variance (ANOVA), and Student-Newman-Keuls and Games-Howell post hoc tests were performed to make multiple comparisons between experimental groups. Differences were statistically significant when $p < 0.05$. All data were analyzed by the SPSS for Windows® application (version 15.0, SPSS).

Results and discussions

Physical-Chemical Parameters

Smoking is a process that alters the physical and chemical properties of the raw material, such as color, texture, pH, aw, water holding capacity (WHC), etc. It is important to control the physical

and chemical parameters in the processed product, as they affect shelf life and sensory characteristics (Fuentes et al., 2010).

Color

Color is an important attribute of fish quality and freshness and also indicative of chemical components and sensory attributes of food (Cheng et al., 2015).

The results of the instrumental color analysis, in terms of L*, a* and b*, C* and *h* coordinates, obtained from *A. regius* fillets, are shown in Table 1.

Table 1. Results of color of cold smoked meagre samples during storage at 4 ± 1 °C.

	Storage Time (Days)	**S**	**SA**
L*(D65)	Thawed	45.48±1.43^{c}	44.65±1.41^{d}
	0	40.86±1.26^{a}	39.87±1.13^{b}
	1	40.52±0.57^{a}	39.46±0.18^{b}
	7	39.17±0.67^{a}	38.87±0.10^{ab}
	14	40.96±1.03$^{a/B}$	37.96±0.41$^{a/A}$
	21	40.14±0.18$^{a/B}$	38.36±0.07$^{a/A}$
	28	39.90±0.76^{a}	39.84±0.59^{b}
	35	42.72±0.33$^{b/B}$	41.38±0.39$^{c/A}$
a*(D65)	Thawed	-2.12±0.39^{d}	-2.31±0.43^{c}
	0	-2.35±0.28^{cd}	-2.72±0.31^{bc}
	1	-2.57±0.07$^{c/B}$	-2.85±0.10$^{bc/A}$
	7	-3.47±0.36^{b}	-3.55±0.12^{ab}
	14	-3.29±0.03^{b}	-2.63±0.79^{b}
	21	-4.31±0.02$^{a/A}$	-3.50±0.10$^{ab/B}$
	28	-3.61±0.06$^{b/A}$	-3.09±0.09$^{b/B}$
	35	-3.31±0.39$^{b/B}$	-4.25±0.10$^{a/A}$
b*(D65)	Thawed	-3.56±0.83^{b}	-2.61±0.75^{a}
	0	-6.67±0.59$^{a/A}$	-3.90±0.68$^{a/B}$
	1	-5.62±0.63$^{b/A}$	-2.80±0.58$^{a/B}$
	7	-3.41±0.96$^{c/A}$	-1.05±0.01$^{b/B}$
	14	-2.20±0.13$^{cd/A}$	0.37±0.37$^{c/B}$
	21	-0.75±0.64^{d}	0.43±0.22^{c}
	28	-0.14±0.54^{d}	1.41±0.59^{c}
	35	-1.68±0.79$^{cd/A}$	1.51±0.08$^{c/B}$
C*(D65)	Thawed	4.21±0.84^{a}	3.53±0.82^{ab}
	0	7.09±0.62$^{b/B}$	4.78±0.69$^{b/A}$

	1	$6.25\pm0.53^{b/B}$	$4.04\pm0.47^{ab/A}$
	7	4.91 ± 0.92^{a}	3.82 ± 0.05^{ab}
	14	4.16 ± 0.23^{a}	2.81 ± 0.67^{a}
	21	$4.53\pm0.16^{a/B}$	$3.68\pm0.16^{ab/A}$
	28	3.80 ± 0.01^{a}	3.50 ± 0.31^{ab}
	35	$3.93\pm0.01^{a/A}$	$4.60\pm0.14^{b/B}$
h (D65)	Thawed	238.17 ± 4.55^{d}	226.24 ± 5.82^{cd}
	0	$250.57\pm1.60^{e/B}$	$234.51\pm3.37^{d/A}$
	1	$244.05\pm2.92^{d/B}$	$222.83\pm5.22^{c/A}$
	7	$222.51\pm5.36^{c/B}$	$195.21\pm0.43^{b/A}$
	14	$211.64\pm0.05^{bc/B}$	$167.86\pm10.09^{a/A}$
	21	188.24 ± 7.93^{a}	172.47 ± 3.32^{a}
	28	180.02 ± 8.95^{ab}	156.66 ± 8.67^{a}
	35	$206.35\pm13.97^{bc/B}$	$161.40\pm0.16^{a/A}$

The means with different letters (abc...) in the same column are significantly different ($p < 0.05$)

The means with different letters (ABC...) in the row are significantly different ($p < 0.05$)

As for the L* parameter (Table 1), it was observed that the cold-smoking process significantly affected lightness, as both S and SA fillets showed a significant decrease ($p < 0.05$) of this parameter immediately after smoking. This is because of smoking which induces a loss of water, that could have led to an increase in carotenoid concentration and a decrease in hue and lightness (Birkeland et al., 2004; Cardinal et al., 2001; Choubert, Blanc, & Courvalin, 1992; Messina et al., 2021).

During shelf life, the L* parameter remained constant and increased significantly ($p < 0.5$) in the last days of storage (Table 1). This significant increase was also observed in other species such as sea bass (Messina et al., 2021).

Significant differences ($p < 0.05$) were observed between the two treatments (S and SA) starting from the 14th day of storage; S samples showed significantly higher L* values than SA samples probably due to a higher moisture content in the sample. In fact, the higher percentage of water contributes to the creation of refractive indices in the food matrix, which leads to greater luminosity (Bekhit et al., 2009; Offer et al., 1989).

The parameter a*decreased significantly ($p < 0.05$) during shelf life in both treatments (S and SA). This decreased redness, mainly caused by the smoking process, has also been reported in salmon (Chan et al., 2020a), in fact it is observed a general tendency in cold-smoked salmon to be darker and less red (Chan et al., 2020a).

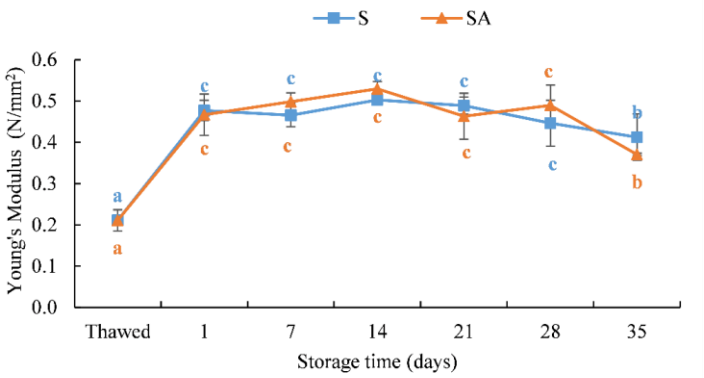
Regarding the parameter b*, it decreased significantly after smoking ($p < 0.05$), then increased during storage (Table 1).

This yellowness of the muscle may be due to the resulting darkening of bloodline due to heme-protein oxidation, associated with a corresponding reduction in a* (Messina et al., 2021; Richards & Hultin, 2000).

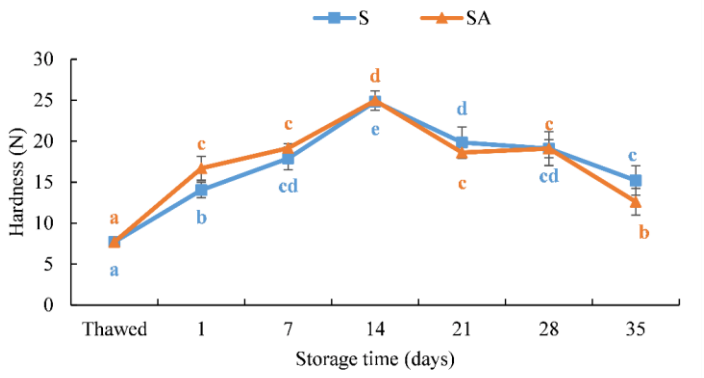
As shown in the present study, a general trend in cold-smoked meagre fillets was observed, i.e. they were darker and less red, but more yellowish than unprocessed fillets. This yellowness was more evident in fillets treated with the natural antioxidant added during salting. Probably the coloration of the antioxidant affected the coloration of the final product.

Texture Profile Analysis

The synergistic action of salt incorporation, the preservative effect of smoke compounds and dehydration, during the smoking process, can preserve fish. This leads to an increase in tissue parameters, such as Hardness (Birkeland et al., 2004; Fuentes et al., 2012; Fuentes et al., 2010; Gómez-Guillén et al., 2000; Messina et al., 2021; Messina et al., 2019b; Regost, Jakobsen, & Rørå, 2004; Sigurgisladottir et al., 2000). The results of the texture analysis (Young's Modulus and Hardness) are shown in Figure 2.



(**a**)



(**b**)

Figure 2. Results of texture of cold smoked meagre samples during storage at 4 ± 1 °C. (a) Young's Modulus; (b) Hardness. Means with different letters (a, b, c and d) indicate significant differences ($p < 0.05$) during shelf life for each single treatment.

Young's Modulus (or modulus of deformability) values (Figure 2a) increased significantly ($p < 0.05$) after cold smoking and remained constant until T28; a significant decrease ($p < 0.05$) was observed at T35. No significant differences were observed between the two treatments.

The Hardness value (Figure 2b) increased significantly until the 14th day of storage (Table 1), but no significant differences were observed between treatments.

The increase in hardness is due to the synergistic effect of salting and drying causing meat hardening (Fuentes et al., 2010).

From the 21st day of storage, Hardness decreased significantly ($p < 0.05$) (Figure 2b), this decrease was probably due to the initiation of degradation processes, mainly related to autolytic phenomena and protein denaturation in muscle tissue, resulting in a progressive reduction in tissue hardness (Hernández, Martínez, & García García, 2001; Liu et al., 2010; Messina et al., 2019b).

Water Holding Capacity (WHC)

WHC, is considered one of the most important parameters for preserving fish quality and can influence the appearance and texture of fresh and processed fish products and thus the sensory quality of food (Chan et al., 2020a; Chan et al., 2020b; Huff-lonergan & Lonergan, 2005).

Changes in WHC detected in fresh and smoked meagre during shelf life are shown in Figure 3.

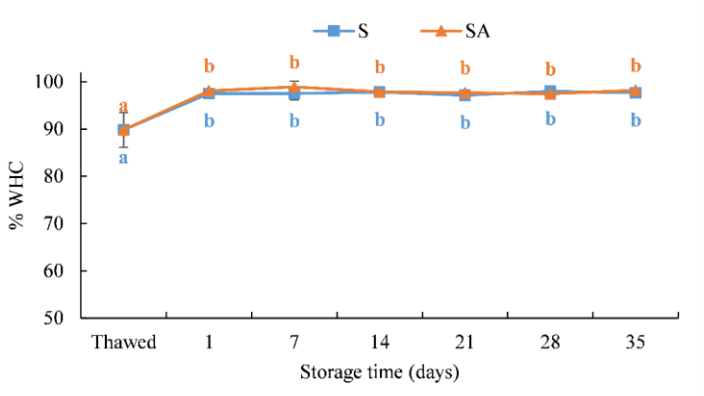


Figure 3. Results of WHC% of cold smoked meagre samples during storage at 4 ± 1 °C. (Means with different letters (a, b) indicate significant differences ($p < 0.05$) during shelf life for each single treatment.

The smoking process resulted in a significant ($p < 0.05$) increase in WHC in both treatments (Figure 3). This finding is in accordance with the results obtained in other cold-smoked species (Fuentes et

al., 2012; Messina et al., 2021; Messina et al., 2019b). As observed by some authors (Fuentes et al., 2012; Messina et al., 2021; Messina et al., 2019b), the increase in WHC in smoked products is the result of the increase in salt content. Indeed, as it is shown in Table 2 after cold smoking an increase in the percentage of ash in the muscle was measured as a consequence of the salt uptake in the fish muscle.

WHC remained stable throughout the storage time with no significant differences between the two treatments.

Muscular pH

Changes in pH values of cold smoked meagre fillets during shelf life are shown in Figure 4.

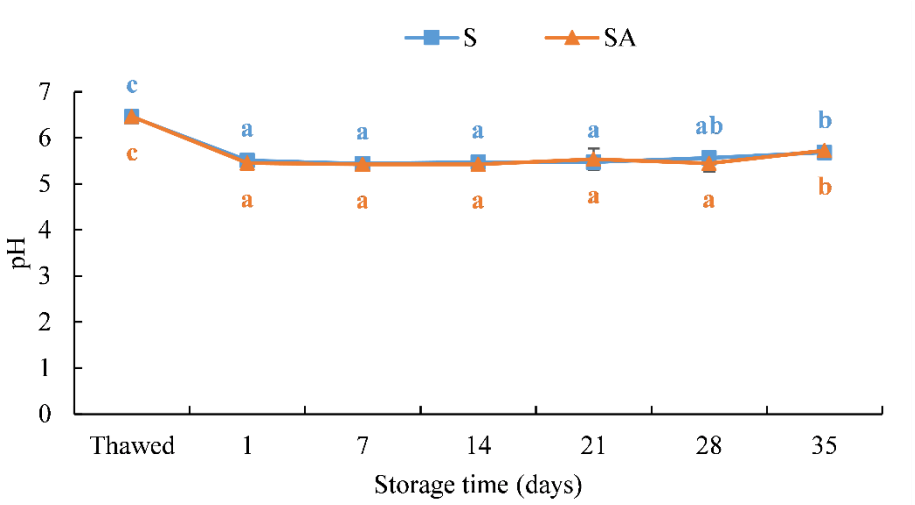


Figure 4. Results of pH of cold smoked meagre samples during storage at 4 ± 1 °C. (Means with different letters (a,b and c) indicate significant differences ($p < 0.05$) during shelf life for each single treatment.

The smoking process significantly reduced pH ($p < 0.05$). In fact, a significant difference was observed between untreated and smoked samples ($p < 0.05$) (Figure 4).

The reduction in pH after smoking is related to the absorption of smoke acids, moisture loss, and the reaction of phenols, polyphenols, and carbonyl compounds with protein and amine groups (Fuentes et al., 2012; Hassan, 1988). In addition, the presence of salt, which causes an increase in the ionic strength of the solution within the cells, also contributes to the decrease in pH (Fuentes et al., 2012; Leroi et al., 2000).

During the shelf life, it was observed that the pH remained constant in both treatments (Figure 4) and increased significantly on the last day of storage (day 35) with values of 5.69±0.14 (S) and 5.73±0.19 (SA).

The increase in pH can be attributed to the production of basic volatile components such as ammonia, trimethylamine and total volatile nitrogen by fish spoilage bacteria (Messina et al., 2021; Osheba, 2013; Ruiz-Capillas & Moral, 2005).

Water Activity Determination

The smoking process led to a decrease in aw values detected in meagre fillets (Figure 5), also related to moisture reduction, an increase in ash and mineral content as also observed in other species (Fuentes et al., 2012; Messina et al., 2021).

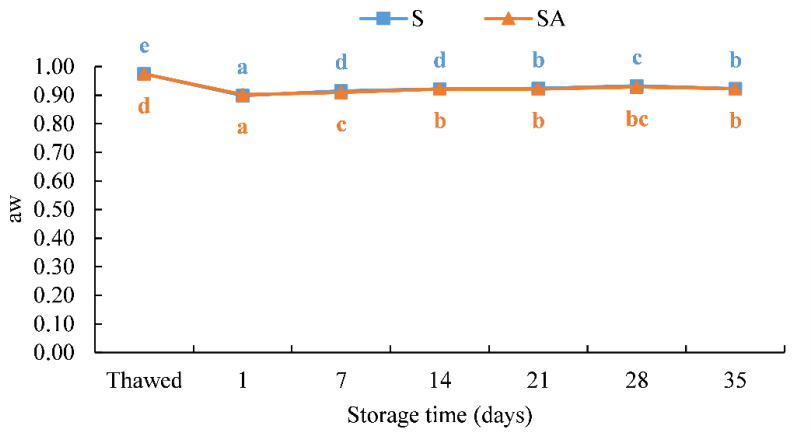


Figure 5. Results of Water Activity (aw) of cold smoked meagre samples during storage at 4 ± 1 °C. (Means with different letters (a, b, c, d and e) indicate significant differences ($p < 0.05$) during shelf life for each single treatment.

Normally, the aw factor in fish is close to 1, a value that was also observed in thawed meagre fillets (Figure 5). The fish processing can determine the reduction in aw values, reaching even values of 0.8 and 0.7 after heavy salting and drying (Sampels, 2015).

The decrease in aw, due to osmotic pressure, results in lower activity of bacteria and enzymes (Oliveira et al., 2012).

Proximate composition and Biochemical Parameters Related to the Shelf-Life

Proximate composition

The proximate composition of untreated and smoked meagre fillets is shown in Table 2.

Table 2. Proximate composition (g/100g) of Thawed and cold smoked meagre samples during storage at 4 ± 1 °C.

	Storage Time (Days)	S	SA
Total lipid	Thawed	0.85±0.06[a]	0.88±0.12[a]
	1	1.72±0.26[b]	2.15±0.23[b]
	35	2.25±0.26[b]	2.51±0.39[b]
Moisture	Thawed	79.57±1.74[b]	80.57±1.79[b]
	1	74.96±1.54[a]	74.19±1.05[a]
	35	73.03±1.17[a]	73.08±1.71[a]
Protein	Thawed	18.06±1.24	17.04±1.18
	1	18.18±0.10	18.02±0.10
	35	18.84±0.53	18.84±0.81
Ash	Thawed	1.26±0.07[a]	1.23±0.07[a]
	1	5.13±0.88[b]	5.22±0.68[b]
	35	5.10±0.15[b]	4.67±0.88[b]

The means with different letters (abc...) in the same column are significantly different ($p < 0.05$)
The means with different letters (ABC...) in the row are significantly different ($p < 0.05$)

The lipid content showed that *A. regius* is a low-fat fish species (Tang et al., 2001) (Table 2).
In fact, farmed meagre has much lower muscle fat than more commonly farmed Mediterranean fish species, including sea bream and European sea bass (Grigorakis et al., 2011).
The relative contents of ash, water and lipids changed following smoking, due to water loss. Salting and smoking determined a reduction in moisture and increase in ash and mineral contents, as observed in previous studies on other fish species, such as European sea bass and dolphinfish (Fuentes et al., 2012; Messina et al., 2021; Messina et al., 2019b).
Figure 6 shows the fatty acid profile of smoked meagre fillets.

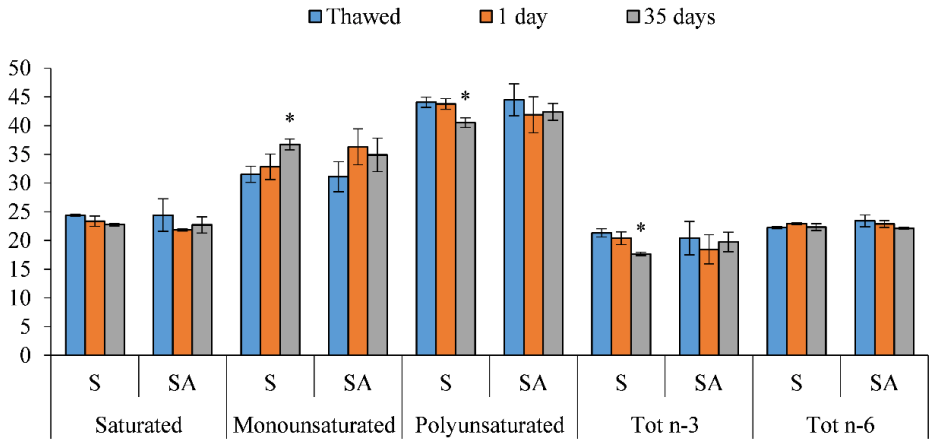


Figure 6. Fatty acid classes of thawed and cold smoked meagre samples during storage at 4 ± 1 °C. * ($p < 0.05$)

The role played by the natural antioxidant in preserving the oxidation of the fatty acids was evident. In fact, S fillets (Figure 6) showed a significant reduction in polyunsaturated fatty acids ($p < 0.05$) at the end of smoking (35 days), due to a decrease in polyunsaturated fatty acids of the n-3 series (Tot n-3) ($p < 0.05$) and a simultaneous increase in monounsaturated fatty acids (Figure 6). In SA smoked sea bass fillets, Tot n-3 remained unchanged during shelf life, as did the other classes of fatty acids.

Biochemical Parameters Related to the Shelf-Life

Antioxidant treatment of fillets of different species has been shown to prevent lipid oxidation (Messina et al., 2019b).

Malondialdehyde (MDA) is a secondary lipid oxidation product. As observed in other fish species, such as dolphinfish (*C. hippurus*), a lower lipid content, combined with high polyunsaturated fatty acid content, could lead to greater susceptibility to peroxidation than other fish species, such as sardine (*S. pilchardus*), which have a higher lipid content but a higher saturated fatty acid content, which are less susceptible to oxidation (Gómez-Estaca, Gómez-Guillén, & Montero, 2013).

As shown in Figure 7, the SA treatment determined a marked reduction in MDA content (mg MDA/kg) in the smoked meagre fillets, compared to S treatment, that produced higher MDA content after 7 days of refrigerated storage. The levels of MDA resulted statistically different between treatments ($p< 0.05$) (Figure 7) from 7 days to the end of the trial.

Low MDA content was detected in sous vide meagre (*A. regius*) fillets treated with natural antioxidants during cold storage, and MDA values did not exceed the limit value all over the storage period (Bozova & İzci, 2021). In that study, low values of MDA (0.52±0.13 mg MDA/kg) were detected in the raw fillets, and lower values of MDA were found during cold storage in fillets treated with antioxidants compared to control, highlighting that application of natural antioxidants in the meagre sous vide process could have had positive effects on lipid peroxidation process (Bozova & İzci, 2021).

Moreover, the results obtained in the present study in terms of the low MDA content detected in smoked fillets during storage agreed with those obtained in another farmed species, i.e., European sea bass (*D. labrax*), whose fillets were treated by cold smoking, highlighting the preservative effect of the process on this biochemical parameter related to fish shelf life (Messina et al., 2021).

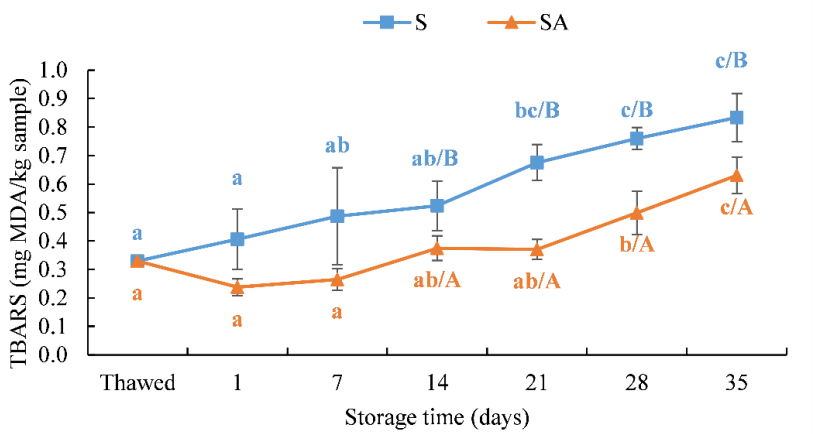


Figure 7. Results of TBARS (determined as mg MDA/kg samples) of cold smoked meagre samples during storage at 4 ± 1 °C. (Means with different letters (a, b and c) indicate significant differences ($p < 0.05$) during shelf life for each single treatment; Means with different letters (A, B) indicate significant differences ($p < 0.05$) between the two treatments at the same storage time.

The results obtained in the present study also agreed with what obtained in previous studies in which the antioxidant treatment combined with smoking resulted effective on preventing the lipid peroxidation (Gómez-Estaca, Gómez-Guillén, & Montero, 2013; Messina et al., 2019b). Indeed, the combined treatments reduced markedly the MDA content in dolphinfish fillets up to 35 days of storage compared to cold smoking used alone as preservative treatment (Messina et al., 2019b). Moreover, as in the case of sardines (*S. pilchardus*), a fatty fish species, the lipid oxidation results showed that, the combined application of oregano extract and smoking, significantly reduced oxidation ($p < 0.05$) in sardines, in comparison with the batch that was only smoked (Gómez-Estaca, Gómez-Guillén, & Montero, 2013).

It has to be highlighted that in both cases, cold smoked fillets did not exceed 4-5 mg MDA/kg value considered acceptable for smoked fish in the literature (Osheba, 2013). Moreover, vacuum packaging might be effective in preserving processed products from lipid oxidation, in the hurdle technology point of view, providing more evidence on the effective use of these methods of preservation (Hussain et al., 2021; Leistner & Gorris, 1995).

Total volatile basic nitrogen (TVB-N) values detected in smoked meagre fillets during storage are shown in Figure 8. European legislation has established an upper limit for TVB-N, ranging from 25 to 35 mg TVB-N/100 g (Commissione delle Comunità Europee, 1995). Nevertheless, considering that a TVB-N threshold for processed fish products has not been established so far, the limit equal to 35 mg TVB-N/100 g has been considered for smoked fish products (Gómez-Guillén et al., 2009; Messina et al., 2019b). In the present study, this threshold was not exceeded during the preservation period and up to 35 days of storage in both batches (SA and S) (Figure 7). Nevertheless, it has to be

highlighted that at T35 the level of TVB-N content in the SA batch was lower than that observed in the S batch, with values significantly different ($p < 0.05$).

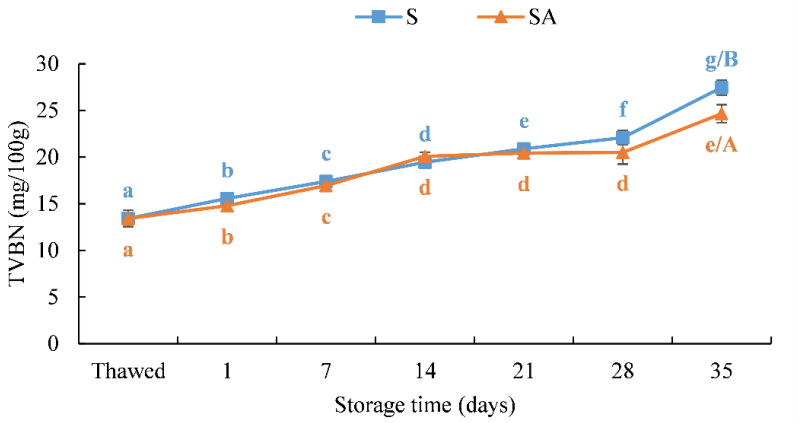
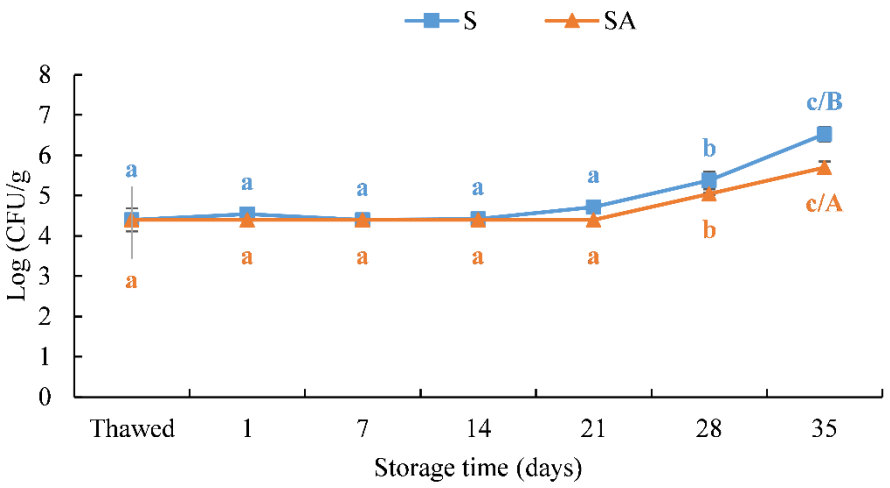


Figure 8. TVB-N contents in cold smoked meagre fillets during storage at 4 ± 1 °C. (Means with different letters (a,b, c, d, e, f, and g) indicate significant differences ($p < 0.05$) during shelf life for each single treatment; means with different letters (A, B) indicate significant differences ($p < 0.05$) between the two treatments at the same storage time.

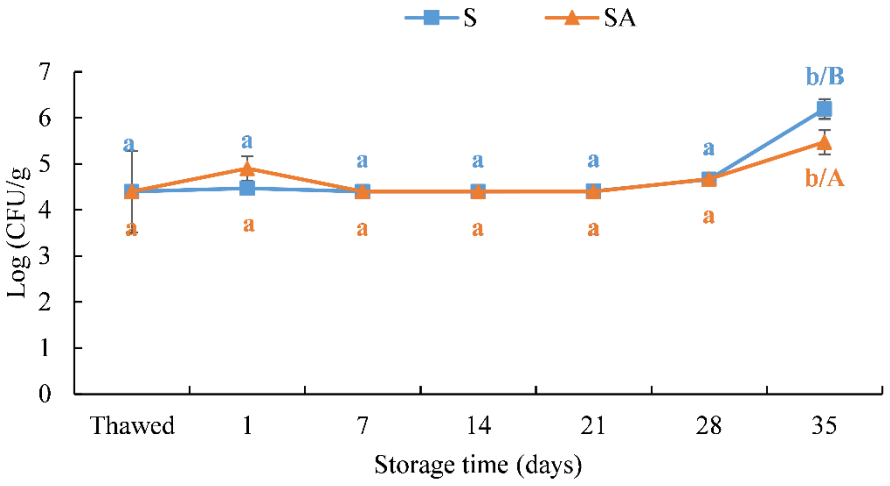
The TVB-N may be affected by the reduction in both the spoilage bacteria and the activity of endogenous enzymes (Özyurt et al., 2009). The findings obtained in the present study agreed with what obtained in previous studies, which highlighted the effectiveness of the salting/smoking processes on maintaining the TVB-N content below the threshold for spoilage during refrigerated storage under vacuum or modified atmosphere packaging (Fuentes et al., 2011; Messina et al., 2021; Messina et al., 2019b).

Microbiological analyses

Microbiological analyses highlighted the bacteriostatic effect of cold smoking combined with the natural antioxidant. This effect is due to the synergistic action of the salt uptake during the brining phase and the polyphenols deposition during the smoking phase (Gómez-Guillén et al., 2009; Messina et al., 2019b; Oueslati et al., 2012). In thawed meagre fillets, the bacterial load was 4.40± 0.29 log (CFU/g) for mesophilic bacteria and 4.40± 0.89 log (CFU/g) for psychrophilic bacteria (Figure 9). During storage, the bacterial load remained constant in both treatments up to 21 and 28 days of storage for mesophilic bacteria and psychrophilic bacteria respectively. At the end of storage, a significant increase in both mesophilic and psychrophilic bacteria was observed (Figure 9) in the two treatments although the values did not exceed 10^7 CFU/g, according to the current standard (AFSSA, 2008; D.S. Reg.Piemonte, 2013; Decreto Ministeriale del 18/09/2002; FCD, 2009; Healt Protection Agency, 2009).



(**a**)



(**b**)

Figure 9. Microbiological evaluation during the shelf life of cold smoked meagre fillets, stored at 4 ± 1 °C. (**a**): mesophilic (37°C); (**b**): psychrophilic (6°C). Means with different letters (a, b and c) indicate significant differences ($p < 0.05$) during shelf life for each single treatment; Means with different letters (A, B) indicate significant differences ($p < 0.05$) between the two treatments at the same storage time.

Sensorial analysis

Results from sensory evaluation are strictly related to those of the physical-chemical and biochemical parameters considered to determine fish quality and freshness during shelf life trials and give important findings about the consumer perception and acceptability (Gómez-Guillén et al., 2009; Messina et al., 2016; Messina et al., 2019b). In the present study, both S and SA batches did not show any differences for any of the sensory attributes evaluated, showing that the method of processing and the use of antioxidants from the halophyte *H. strobilaceum* effectively preserved the

quality and shelf life of meagre (Messina et al., 2019b). Sensory quality evaluation showed that the smoked fillets of meagre were of good quality until 35 days of refrigerated storage under vacuum packaging (Table 3).

Table 3. Results of sensory analysis of cold smoked meagre samples during storage at 4 ± 1 °C.

	Days	S	SA
Appearance	1	9.00±0.82^{bcd}	9.00±0.00^{bcd}
	7	10.00±0.00^{d}	9.25±0.50^{d}
	14	8.25±0.50^{abc}	8.75±0.50^{bcd}
	21	8.00±1.15^{abc}	7.75±0.50^{ab}
	28	8.00±0.89^{abc}	8.17±0.41^{abc}
	35	7.17±0.75^{a}	7.00±0.89^{a}
Colour	1	9.25±0.96^{cd}	8.75±0.50^{bc}
	7	10.00±0.00^{d}	9.25±0.50^{c}
	14	8.50±0.58^{bc}	8.75±0.50^{bc}
	21	8.50±0.58^{bc}	8.25±0.96^{b}
	28	8.17±0.98^{bc}	8.50±0.55^{b}
	35	7.33±0.52^{ab}	7.00±0.89^{a}
Odour	1	9.50±0.58^{d}	8.00±0.00^{b}
	7	9.50±0.58^{d}	9.25±0.50^{b}
	14	8.50±0.58^{bcd}	9.00±0.82^{b}
	21	8.50±0.58^{bcd}	8.00±0.82^{b}
	28	7.83±0.75^{bc}	8.17±0.41^{b}
	35	7.17±0.75^{ab}	6.67±1.03^{a}
Texture	1	9.75±0.50^{c}	7.50±0.58^{a}
	7	9.75±0.50^{c}	9.00±0.00^{bc}
	14	9.00±0.00^{bc}	9.75±0.50^{c}
	21	8.50±0.58^{b}	8.25±0.96^{b}
	28	9.00±0.00^{bc}	9.00±0.00^{bc}
	35	7.33±0.52^{a}	7.00±0.89^{a}
Taste	1	9.50±0.58^{b}	8.25±0.50^{bc}
	7	9.50±0.58^{b}	9.25±0.50^{c}
	14	8.50±0.58^{ab}	9.00±0.82^{c}
	21	8.25±0.96^{ab}	7.25±0.96^{ab}
	28	7.83±0.75^{a}	8.17±0.41^{bc}
	35	7.17±0.75^{a}	6.67±1.03^{a}

The means with different letters (abc...) in the same column are significantly different ($p < 0.05$)

As far as odor and taste were concerned, the sensory evaluation performed confirmed what obtained from the biochemical analyses in terms of TVB-N and MDA contents. In particular, even if

significant differences were observed between batches in terms of MDA contents, the minimum value of 1.44 mg MDA/kg detectable by the panelists (Ruiz-Capillas & Moral, 2001), was not exceeded in both batches up to the end of storage (Figure 6).

Conclusions

A. regius is today one of the new frontiers of aquaculture. Our work has demonstrated the validity of processing this species through cold smoking with the use of natural antioxidants, which could therefore represent a means of obtaining an innovative yet safe product.

In fact, the combined treatment with cold smoking and antioxidant resulted in an overall improvement in quality characteristics and shelf life of meagre fillets; the antioxidant treatment prevented lipid peroxidation and degradation of nitrogen components.

While from a sensory and technological point of view, the presence of antioxidants of natural origin did not lead to detectable changes in the final product.

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Part 2.
Shelf life extension and development of new value-added products from commercially important fishery species

Chapter 4. Innovation in the Sicilian salted fish value-chain: effect of slurry ice on quality and shelf life of purse-seine anchovies (Engraulis encrasicolus)

Abstract

The introduction of innovative cold techniques could meet the high demand for fish products and contribute to reduce fish losses in between landings and consumption, bringing to both economic and environmental benefits, and contributing to the growth of the fish production sector. In the present work, the effectiveness of storage under a slurry ice (SI) system on quality and freshness of a commercial important fishery species, i.e. European anchovy (*Engraulis encrasicolus*), compared to flake ice (FI) was investigated. Sensory, biochemical, and physical-chemical analyses were performed on specimens stored under SI and FI at each sampling time during 12 days of preservation (0, 1, 3, 6, 9 and 12 days). The sensory evaluation of specimens of *E. encrasicolus* showed that the specimens stored in SI showed better sensory characteristics, during the shelf life, compared to specimens stored in FI. As far as pH values were concerned, significant differences ($p < 0.05$) were highlighted between the two treatments starting from the sixth day of storage with lower values for the samples stored in SI. A lower pH probably indicated better control of both endogenous and microbial activity in the fish muscle stored under SI. The sodium content was found to be higher in samples stored in SI compared to samples stored in FI ($p < 0.05$), probably due to the quantity of salt contained in the ice, which was added to the water in order to decrease the freezing point. Malondialdehyde (MDA) values showed significant differences ($p < 0.05$) between the two treatments during shelf life with significantly lower values in samples stored in SI, compared to samples stored under FI. Concerning the total volatile basic nitrogen (TVB-N) content, significantly lower values were recorded in samples stored under SI, highlighting that the innovative technique would seem to reduce the spoilage of anchovy. Overall, this pilot study indicated that the use of SI could be effective on maintaining fish freshness and extending the product shelf life compared to the traditional technique, i.e., FI. competing effectively with other cooling methods used in industry and the fishing sector.

Introduction

Nowadays, in order to meet the increasing global demand for seafood, fishing effort on natural resources has been increasing over the years bringing global catches from around 20 million tons in 1950s to over 90 million tons in 1990s (Pontecorvo & Schrank, 2012). At this point, regardless of the increment in the effort, captures stabilized, suggesting that oceanic fish stocks are generally

being exploited at their maximum capacity. Nowadays, overexploitation affects 34.2% of global stocks, and only 6.2% of marine stocks remain underfished, while 59.6% of marine stocks are classified as being maximally sustainably fished stocks. In terms of landings, it is estimated that 78.7% of current marine fish landings come from biologically sustainable stocks (FAO, 2020a). In 2017, the Mediterranean Sea, a hotspot of biodiversity (Coll et al., 2010), had, together with the Black Sea, the highest percentage of stocks fished at unsustainable levels (62.5%) (FAO, 2020a), constituting an evident anthropogenic pressure that often overlaps with areas of high biodiversity. Total landings in the Mediterranean and Black Sea have been about 1.3 million tons per year since 2015. Many stocks of anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) show biomass levels below biologically sustainable levels, unlike commercial stocks of hake (*Merluccius merluccius*) and turbot (*Scophthalmus maximus*), which show particularly high fishing pressure (FAO, 2020a)

In the Mediterranean Sea, European anchovy (*Engraulis encrasicolus*) and European sardine (*Sardina pilchardus*) represent around 40% of the total fish catch (FAO, 2020b). Indeed, purse seiners and pelagic trawlers continue to be the fleet segment groups responsible for the largest share of total landings.

European anchovy is a coastal small pelagic fish. It occurs in the Eastern Atlantic from southern Norway to South Africa, and in the Mediterranean and the Black Sea (Whitehead, 1985). It spawns from April to November with peaks usually in the warmest months. European anchovy is a serial spawner. Eggs are ellipsoidal to oval, floating in the upper 50 m and hatching in 24-65 hours. The larval stage is planktonic and lasts for about 37 days. Marketed fresh, dried, smoked, canned and frozen; made into fish meal.

This species is rich in polyunsaturated fatty acids (PUFAs), among which are the omega 3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), very beneficial for the human health (Arab-Tehrany et al., 2012; Tufan et al., 2011). However, the fat content and the Fatty Acids (FA) composition of the fish are not constant. They are related to the life cycle of the fish and external factors, seasonal variation like temperature, salinity and FA composition of their food (Gökçe et al., 2004)

Anchovy is an economic important fishery species in the Mediterranean region. Italy is the world's fourth-largest producer of anchovy with 37511 tons caught in 2015. Spain and Italy account for 71% of EU anchovy consumption. In Italy, anchovy is generally eaten fresh or processed. The processed products are salted anchovies, preserved anchovy in oil and marinated anchovy. Salted anchovy may be considered as a final product and as a raw material to produce preserved anchovy in oil (EUMOFA, 2018)

Fresh anchovy is mainly imported in Spain, from intra-EU origins. Fresh anchovy imported to Spain comes from Portugal, Italy and France. The next largest importers are Italy (3.591 t in 2015 and 2.772 t in 2016), Germany and France. In 2016, salted anchovy imported to Spain mainly came from Argentina and to a lesser extent from Peru and Morocco; salted anchovy imported to Italy mainly comes from Spain, Morocco, Croatia, Albania and Tunisia. Imports of dried anchovy are significant only in the UK, Italy and Spain (EUMOFA, 2018). The main exporters are Spain, Italy, Croatia, France and Greece. Three products are highly exported: salted anchovy, fresh anchovy and preserved or prepared anchovy. Salted anchovy is the most exported anchovy product. Spain, Croatia and Italy are all important exporters of salted anchovy. Most of the exports are targeted to third countries (mainly Albania, Morocco and Tunisia). Prepared and preserved anchovy is one of the main products exported in terms of volume in 2016: the main exporters are Spain, Italy, France, Croatia and Greece. Prepared and preserved anchovy is mainly exported intra-EU from Spain, Croatia, France and Greece. Only Italy is more oriented toward third countries (EUMOFA, 2018).

Moreover, 10% of the total Italian production of anchovies in 2017 was provided by Sicily, which is also an important producer at national level of salted and prepared-preserved anchovies highly marketed like the fresh product (BMTI S.C.p.A., 2017).

Fish products are known to be highly perishable. Quality and freshness decline rapidly after death, leading to a rapid decrease in their commercial value. In fact, spoilage begins immediately after capture or slaughter and continues at a rate that is directly dependent on storage and processing conditions (Yu et al., 2019). The perishability of seafood products is mainly related to a number of characteristics such as high water content and non-protein nitrogen content, poorly firm muscle and skin structure, and low collagen content, with a significant content of PUFAs, fatty acids that are particularly prone to oxidation. The perishability of these products, if not controlled, leads to losses in sensory and nutritional quality (Yu et al., 2019).

Anchovies, subjected to chemical phenomena and postmortem pH variations, are very susceptible to deterioration thus facilitating microbial growth (Ólafsdóttir et al., 1997). In particular, the hydrolytic and oxidative activities are the main cause of a shorter shelf life of the product, generally due to the oxidation of free fatty acids which, producing aldehydes and ketones, cause unpleasant odors and flavors of fish.

The sensitive rancidity of anchovies was traditionally contrasted with low storage temperatures, antioxidants, or chemical products. However, recent studies on innovative conservation techniques have shown that the use of ozonated slurry ice or slurry ice could reduce the deterioration and rancidity of fish, such as anchovies, by increasing the shelf life (Bensid et al., 2014; Chun-hua et al., 2014; Yu et al., 2019; Ntzimani et al., 2021).

The introduction of innovation in this area, therefore, can help to increase the added value of products and, thus, the competitiveness of the sector. Indeed, anchovy is one of the most important fish species for the Mediterranean Sea, consumed as both fresh and processed products.

Developing cold technologies that prevent fish spoilage is very important talking about sustainability, because of the huge amount of fish that deteriorates between landing and consumption (27%) (FAO, 2020a). Moreover, it is important to develop new techniques effective on preserving fish whose availability in the future could be compromise considering that anchovies are among small fish species that might not be able to cope with climate change. Indeed, a recent study revealed that with sea temperatures rising faster than ever, fish will very quickly get left behind in evolutionary terms and struggle to survive. This has serious implications for all fish and our food security, as many of the species we eat could become increasingly scarce or even non-existent in decades to come (Avaria-Llautureo, Venditti and Rivadeneira, 2021).

Slurry ice, or fluid ice, consisting of a suspension of spherical microparticles of ice in water, shows interesting qualities for the preservation of fish or other products, for example in reducing the temperature by a couple of degrees below 0 and prevent damage to the product (Kauffeld & Gund, 2019), unlike flake ice, which allows cooling to higher temperatures and can damage fish because of the sharp edges (Chen et al., 2016). The combined use of slurry ice and ozone for the conservation of fish could also improve the quality and extend the shelf life of this species. In particular, the use of ozone derives from the ability to be a powerful antimicrobial agent (Chen et al., 2016).

The slurry ice is advantageous in many applications as it maintains a constant low-temperature level during the cooling process and provides a higher heat transfer coefficient than water or other single-phase liquids (Chen et al., 2016). Another benefit for seafood storage is the complete coverage of the fish surface insulating fish and preventing fish damage like those caused by oxygen (Chen et al., 2016). Optimal conservation of fish depends on the immediate cooling at moment of capture which must involve a temperature slightly above the freezing point and the maintenance of this temperature throughout the cold chain.

It has been shown that the combined use of slurry ice and ozone for the conservation of sardine (*Sardina pilchardus*) could improve the quality and extend shelf life of this fishery species (Campos et al., 2005). Moreover, the croaker (*Johnius dussumieri*) stored in slurry ice showed longer shelf life in terms of sensory, microbiological and biochemical parameters analyzed (Annamalai et al., 2018).

The effectiveness of ozonated slurry ice and superchilling (-1°C) on preventing microbial deterioration of two pelagic fishery species, sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*), was also shown (Bono et al., 2017).

Moreover, the technique was effective on reducing lipid oxidation and maintaining sensory quality of farmed turbot (*Psetta maxima*) preserved in slurry ice combined with ozone (Campos et al., 2006).

Considering the economic importance of anchovy, one of the most important species for fishery and processing industries all over the Mediterranean basin, especially Sicily, and the need to develop new cold technologies that can prevent fish spoilage and assure longer shelf life, the goal of the study was to evaluate the effectiveness of an innovative slurry ice system, compared to traditional flake ice, on extending the shelf life of *Engraulis encrasicolus*.

Materials and methods

Harvesting, preservation and samplings for analyses

Anchovies harvested by purse seine in November 2020 in western Sicily (Italy), were immediately stored in polystyrene boxes filled with traditional flake ice (FI) or slurry ice (SI) (Figure 1). Twelve boxes for each type of ice were put in cold chamber at the harbor, and two boxes of each were delivered to the Laboratory of Marine Biochemistry and Ecotoxicology in Trapani at each sampling time, during 12 days of preservation (0, 1, 3, 6, 9 and 12 days).

On each day of sampling, from each box, an average of 18 fish were collected in order to assess sensory analysis, instrumental evaluation of color and texture, temperature and water holding capacity (WHC); after these individual evaluations, fish from each box, were homogenized in 3 pools, to determine, for each pool, in three replicates: water activity (aw), pH, sodium content, biochemical parameters related to the shelf life, such as total volatile basic nitrogen (TVB-N) and Malondialdehyde (MDA) and, only at T0 and T12, the proximate composition (moisture, ash, lipids and protein contents) as well as the fatty acids profile.

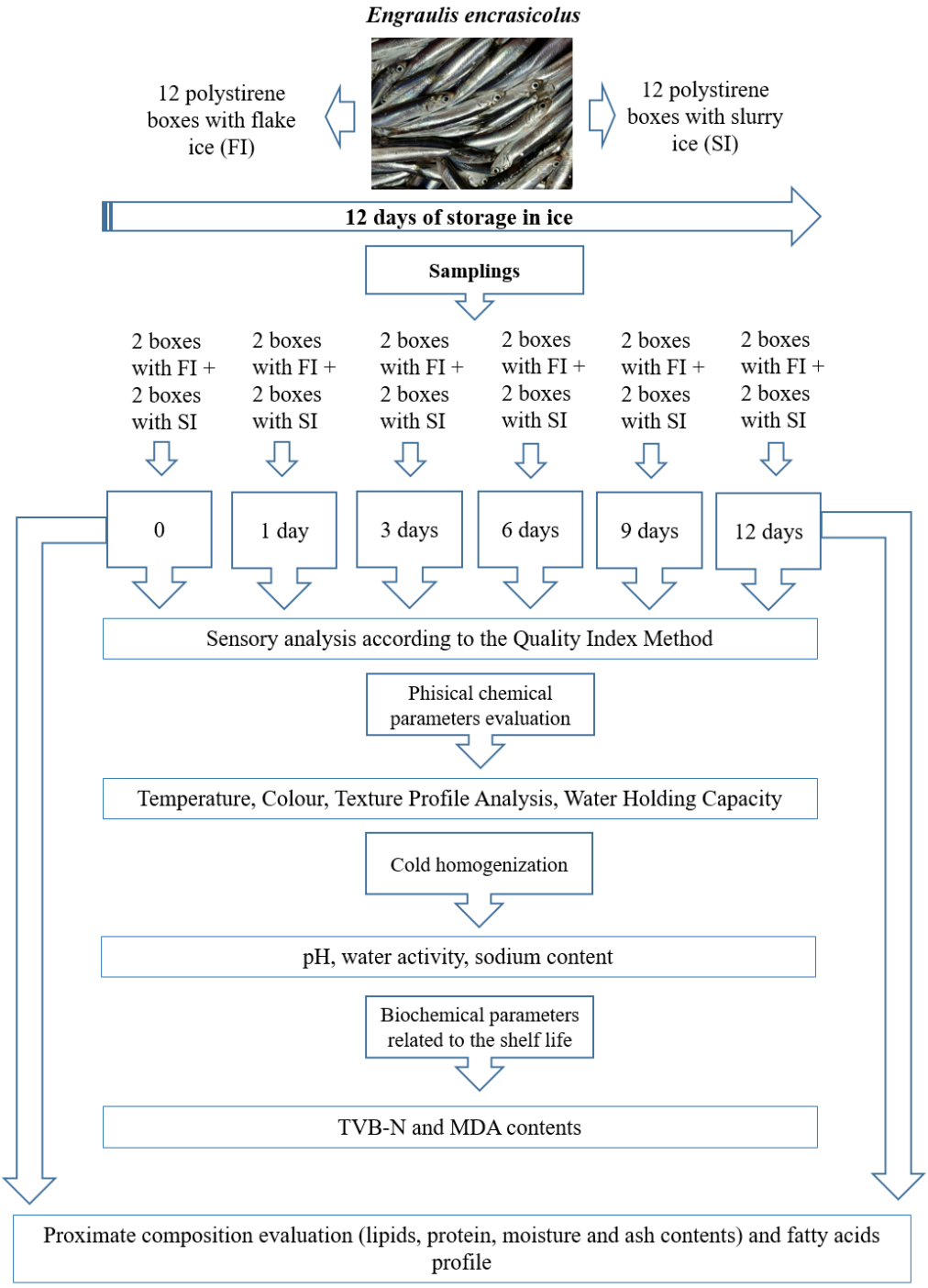

Figure 1. Experimental flow chart and analyses performed

Sensory Analysis

The freshness of the fish was assessed by sensory analysis according to the QIM (Quality Index Method) which allows a quick and easy assessment of the attributes of raw fish during storage, adapted to the species (Pons-Sánchez-Cascado et al., 2006).

According to some authors, with the QIM method, is possible to estimate the shelf life with an error of about 1-2 days. It represents a classification system for categories based on the sensory attributes

considered most significant for a specific species with evaluation through a demerit score. The fish is inspected by recording the demerit score for each evaluated attribute to provide an overall sensory score that represents the quality index.

This method was used for the sensory evaluation at each sampling time following the scheme in Table 1 (Pons-Sánchez-Cascado et al., 2006) for the raw anchovy (*Engraulis encrasicolus*). The analysis was performed independently by four trained technicians, assigning each attribute of the parameters analysed a demerit score from 0 (best quality) to 3 (lowest quality). The quality index (QI) was the ratio between the sum of the demerit points of each attribute assessed and the total score that can be achieved (23 points).

Table 1. Quality index method for raw anchovy (*E. encrasicolus*) (Pons-Sánchez-Cascado et al., 2006)

Parameter	Descriptor	Demerit points
General appearance		
Surface appearance	Very bright, iridescence bluish-violet	0
	Less bright, iridescence not bluish	1
	Slightly dull, not bright	2
Slime	Transparent watery mucus	0
	Slightly cloudy mucus, abundant	1
	Plentiful (slippery), yellowish-brownish mucus	2
Skin	Intact	0
	Slightly broken or easy to break	1
	Torn and damaged	2
Eyes		
Clarity (cornea)	Clear, transparent	0
	Slightly cloudy	1
	Opaque	2
Pupil	Bright black	0
	Dull black, not so circular	1
	Grey	2
	Grey and distorted	3
Shape	Convex	0
	Flat	1
	Concave	2
	Sunken	3
Gills		
Colour	Bright red	0
	Dull-red	1
	Brownish-red	2
	Discoloured	3
Smell	Seaweedy	0
	Slightly seaweedy, neutral	1
	Slightly acrid, rancid or sweet, metallic	2
Abdomen		
Postgill (belly-burst)	Firm	0
	Burst (<50%), and soft	1
	Burst, very soft	2
Flesh		
Appearance and colour	Valvety, translucent	0
	Waxy pink	1
	Wax-like matt, dark red colour	2
Range of score		0-23

Physical-chemical parameters

Temperature

Internal temperature of specimens was measured manually using a digital thermometer at each sampling time.

Color

The analysis was carried out using a Konica Minolta colorimeter (Osaka, Japan), and the results were reported according to the CIE system (CIE, 1977). Lightness (L*), redness index (a*), yellowness index (b*), Hue, and Chroma were recorded, whereas the numerical total color difference (ΔE) between samples was calculated by Equation 1*:*

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad 1)$$

where ΔL*, Δa*, and Δb* are the differentials among the color parameters of the samples over the course of shelf life and the color parameters of the samples at T0.

To perform this kind of analysis, first, the instrument was calibrated, and the instrument reading head was placed on the surface of the fillet. The color evaluation was performed on the fillet in two dorsal regions along the cephalo-caudal direction. With regards to the kind of observer, it was 2° while the source of light was D65.

Texture Profile Analysis

Two small fragments (1 cm Ø), obtained from the same portions of each fillet, were used. The analysis was performed at room temperature using an Instron Texture Analyzer Mod. 3342 (Turin, Italy).

The measured parameters were Hardness (N) and the Young Modulus or modulus of deformability (N/mm2) (i.e., the force and the slope of the curve at 50% compression, respectively) (Messina et al., 2019, 2021; Orban et al., 1997). For each replicate as above-mentioned, two fragments per fillet along the dorsal margin were considered. The samples were maintained in ice before the analysis.

Water holding capacity (WHC)

Water holding capacity was determined using the method described by Teixeira et al. (2014) with modification. Briefly, chopped muscle (1 g; Ws) was placed in a centrifuge tube along with filter paper (also weighted, Wi) and centrifuged at 3000 g for 10 min at 20 °C. After centrifugation, the

sample was removed, and the filter paper weighted (Wf). The moisture in the samples was determined according with the AOAC method (AOAC, 1990). WHC was calculated following Equation 2 and expressed as gram of water in the sample after centrifugation per 100 g of water initially present in the sample.

$$WHC = \frac{Ws * \frac{Moisture\%}{100} - (Wf - Wi)}{Ws * \frac{Moisture\%}{100}} \qquad 2)$$

After the abovementioned analyses, in order to carry out further analysis, the fillets were cold homogenized.

Water activity (aw)

Water activity (aw) was measured with a fast water activity meter (HP23-AW Rotronic, AG, Bassersdorf, Switzerland). The temperature at which the water activity was measured was equal to 21 °C.

pH

The muscular pH of the fillet was measured in the omogenate with a Crison pH meter (Barcelona, Spain) equipped with a BlueLine pH 21 Schott Instruments (Weilheim, Germany) combined electrode.

Sodium content

The sodium content was determined according to the technique described by Greiff et al. (2014) with modification. Briefly, about 2 g of sample were homogenized in distilled water (1:4 w/v) using an Ultraturrax T25 (IKA, Labortechnik, Staufen, Germany) and centrifuged at 9000 rpm for 5 min. The extract was filtered on cotton wool. Na^+ content was directly measured with a Horiba B-722 (Na+) LAQUAtwin Compact Ion Meter (Horiba Instruments, Inc., Kyoto, Japan). The values obtained were expressed in g Na^+/100 g of product.

Proximate composition and evaluation of the biochemical parameters related to the shelf life

The ash (ignition at 600 °C for 5 hours) and moisture (drying at 105 °C for 24h) contents were assessed in the minced muscle (AOAC, 1990). The crude protein content was determined according to the AOAC method (AOAC, 1992). The total lipids (TL) were determined according to Folch, Lees, & Stanley (1957). The fatty acid (FA) methyl esters were determined by the method of Lepage & Roy (1984) and analyzed by gas chromatography (Messina et al., 2013). The instrument was a Perkin Helmer autosystem XL equipped with a highly polar phase (polyethylene glycol) capillary column (30 m × 0.32 mm × 0.25 µm Omegawax 320, Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). Air and hydrogen flows were set at 450 ml/min and 45 ml/min, respectively. Helium was used as the carrier gas (25 cm s^{-1}). The column temperature was programmed at 200°C, injector and detector were maintained at 250°C and 300°C, respectively. Separation took place under isothermal conditions for approximately 40 minutes. Individual FAMEs were identified by comparison of known standard (mix PUFA of fish oil, Supelco, Bellefonte, PA, USA).

The production of thiobarbituric acid reactive substances (TBARS) was determined using the method described by Botsoglou et al. (1994). The total volatile basic nitrogen (TVB-N) was measured by direct distillation of the homogenized samples according to the EU Commission Decision 95/149/EC.

Statistical analysis

Data are reported as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed and Student–Newman–Keuls (SNK) was used as post hoc test for comparison of the means among samples. Significance was accepted at probabilities $p < 0.05$. Analyses were performed using the SPSS software (IBM SPSS Statistics Version 20).

Results and Discussion

The effect of slurry ice on anchovy (*Engraulis encrasicolus*) quality parameters was evaluated through sensory, physico-chemical and biochemical analysis and compared with samples stored in flake ice. The results obtained showed changes in relation to the different parameters not only during shelf life but also between the two treatments.

Sensory Analysis

Sensory analysis is the most effective tool for rapidly estimating the shelf life of fish products in order to measure the qualitative properties and the degree of deterioration of fish (Loutfi et al., 2015). The sensory attributes of fish quality are also related to the chemical and biochemical composition of the fish, hence sensory evaluation is related to their variations over the shelf life (Messina et al., 2016). The shelf life and organoleptic properties of fish species, such as anchovy, depend above all on the storage temperature and processing methods (Messina et al., 2016).

Changes in the sensory attributes of anchovy specimens were assessed through the QIM (Quality Index Method), a reliable sensory analysis method for assessing fish freshness, using a demerit points system from 0 to 23 (Pons-Sánchez-Cascado et al., 2006).

The results of the sensory evaluation of *Engraulis encrasicolus* stored in FI and SI are shown in Table 2.

Table 2. Sensory parameters assessed by QIM method, on specimens of *Engraulis encrasicolus* during the storage period in Flake ice (FI) and Slurry ice (SI).

	T0	T1	T3	T6	T9	T12
FI	$0.58\pm0.69^{a/A}$	$0.50\pm0.58^{a/A}$	$5.67\pm0.98^{b/B}$	$8.17\pm2.81^{c/A}$	$14.42\pm1.26^{d/B}$	$15.67\pm0.86^{d/A}$
SI	$0.25\pm0.50^{a/A}$	$1.92\pm0.83^{b/B}$	$3.67\pm0.82^{c/A}$	$9.67\pm0.67^{d/A}$	$11.83\pm0.64^{e/A}$	$14.83\pm0.79^{f/A}$

Different lowercase letters (abcdef) per line indicate significant differences ($p < 0.05$) within the same treatment during shelf life. Different capital letters (AB) per column indicate significant differences ($p < 0.05$) between treatments on the same day of storage. Values are expressed as mean±standard deviation. N=36

It is possible to note that no significant differences were found between the two treatments at the initial time T0, the final time T12 and the intermediate time T6. Significant differences ($p < 0.05$) between samples stored in SI and samples stored in FI were found at T3 and T9 (Table 2). In particular, at these intervals of time, samples stored in SI showed lower demerit points, suggesting a better conservation of anchovies under SI up to 9 days of shelf life. Samples stored in SI undewent increasing sensory losses during the shelf life even if they showed lower values compared to samples stored in FI at the end of the storage time considered.

Studies in the literature show that also for other species the sensory qualities were maintained for a longer period in fish preserved in SI rather than in FI: specimens of bighead croaker (*Collichthys niveatus*), preserved in slurry ice maintained their sensory characteristics for up to 9 days and were considered acceptable until day 15, unlike specimens in FI, which were unacceptable by day 9 (Chen et al., 2016); specimens of cantang grouper (*Epinephelus sp.*) preserved in SI were considered acceptable up to day 14 (Putra et al., 2020); Bombayduck (*Harpodon nehereus*) stored in SI for 12 days showed a higher score for overall acceptability, higlighting the improvement of sensory attributes that can be achieved by employing slurry ice (Jeyakumari et al., 2020);

Specimens of Croaker (*Johnius dussumieri*) stored in SI had better scores for appearance, odor, consistency and acceptability, unlike the specimens stored in flake ice which were rejected on day 10 due to the unpleasant odor (Annamalai et al., 2018); Sardines (*Sardina pilchardus*) stored in ozonised slurry ice had a shelf life of 19 days, while those stored in traditional flake ice had a shelf life of 8 days (Campos et al., 2005).

In the present study, the findings from the sensory evaluation of *E. encrasicolus* specimens stored under SI and FI for 12 days showed that, for both types of ice storage, after 9 days of storage, the acceptability limits suggested in the literature were exceeded. Nevertheless, the specimens stored in SI showed better sensory characteristics, during the shelf life, compared to specimens stored in FI.

Temperature

The storage temperature, certainly, represents one of the fundamental parameters along the shelf life of fish species.

Temperature values of *E. encrasicolus* stored in FI and SI are shown in Figure 2.

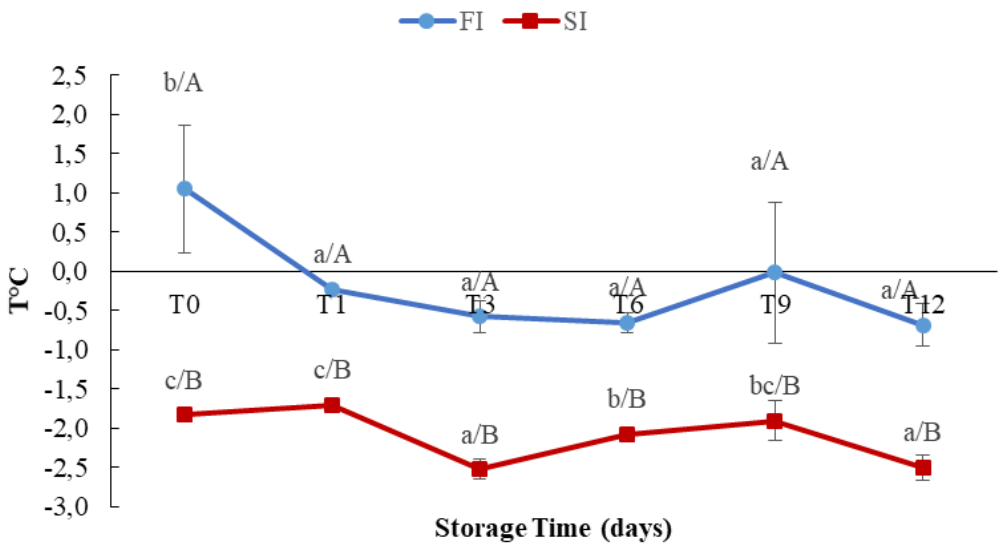

Figure 2. Temperature detected on specimens of *Engraulis encrasicolus* during the period of conservation in Flake ice (FI) and Slurry ice (SI). Different lowercase letters (abc) indicate significant differences ($p < 0.05$) within the same treatment during shelf life. Different capital letters (AB) indicate significant differences ($p < 0.05$) between treatments on the same day of storage. Values are expressed as mean±standard deviation. N=36

Significant differences ($p < 0.05$) (Figure 2) were found between the two treatments on each sampling day as shown in Figure 2, according to the results of several studies which investigated the effectiveness of slurry ice on prolonging fish shelf life (Annamalai et al., 2018; Campos et al., 2005; Gallardo & Aubourg, 2006; Losada et al., 2006; Putra et al., 2020)

The samples preserved in FI did not show significant differences starting from T1 with values just below 0, unlike the T0 which showed a significantly higher value probably given by harvesting that took place on the same day. Samples stored in SI, on the other hand, showed significant differences along the shelf life with lower values at T3 and T12.

Color

Color is the main quality parameter that influences the consumer's choice at the time of purchase (Cheng et al., 2015).

Color values of *E. encrasicolus* stored in flake and slurry ice are shown in Table 3.

Table 3. Color detected on specimens of *Engraulis encrasicolus* during the storage period in Flake ice (FI) and Slurry ice (SI).

			L*			
	T0	T1	T3	T6	T9	T12
FI	$36.6\pm2.56^{a/A}$	$37.4\pm1.5^{a/A}$	$34.6\pm2.1^{a/A}$	$37\pm2.16^{a/A}$	$38.7\pm1.8^{a/A}$	$35.9\pm2.88^{a/A}$
SI	$32.6\pm2.1^{a/B}$	$32.1\pm4^{a/B}$	$34.5\pm0.27^{a/A}$	$39\pm2.66^{b/A}$	$34.6\pm1.87^{a/A}$	$34.5\pm4.41^{a/A}$
			a*			
	T0	T1	T3	T6	T9	T12
FI	$2.82\pm1.07^{a/A}$	$2.17\pm1.02^{a/A}$	$3.89\pm1.97^{a/A}$	$3.29\pm1.42^{a/A}$	$2.81\pm1.5^{a/A}$	$6.74\pm3.21^{a/A}$
SI	$5.1\pm1.73^{a/A}$	$5.98\pm2.12^{a/B}$	$4.5\pm2.28^{ab/A}$	$2.12\pm0.81^{b/A}$	$6.11\pm1.47^{a/B}$	$8.34\pm3.77^{a/A}$
			b*			
	T0	T1	T3	T6	T9	T12
FI	$-1.4\pm0.8^{a/A}$	$-2.7\pm1.3^{a/A}$	$-2.5\pm1.41^{a/A}$	$-1.2\pm0.57^{a/A}$	$-1.3\pm0.49^{a/A}$	$-1.9\pm1.3^{a/A}$
SI	$-2\pm1.44^{ab/A}$	$-1.7\pm1.1^{ab/A}$	$-2.6\pm1.36^{ab/A}$	$-1.1\pm0.87^{a/A}$	$-1.4\pm0.5^{ab/A}$	$-3.9\pm1.89^{b/A}$
			ΔE			
	T0	T1	T3	T6	T9	T12
FI	$0\pm0^{a/A}$	$2.56\pm0.68^{b/A}$	$3.36\pm1.92^{b/A}$	$2.48\pm1.22^{b/A}$	$2.61\pm1.74^{b/A}$	$5.18\pm1.84^{b/A}$
SI	$0\pm0^{a/A}$	$3.39\pm2.45^{b/A}$	$3.46\pm0.95^{b/A}$	$5.77\pm1.82^{b/A}$	$3.02\pm1.41^{b/A}$	$5.95\pm2.62^{b/A}$

Different lowercase letters (ab) per line indicate significant differences ($p < 0.05$) within the same treatment during *shelf life*. Different capital letters (AB) per column indicate significant differences ($p < 0.05$) between treatments on the same day of storage. Values are expressed as mean±standard deviation. N=36

The lightness values (L*) between the two treatments showed significant differences ($p < 0.05$) only at T0 and T1 with lower values for the samples stored in SI. During the shelf life in the samples stored in FI no significant differences were found, unlike those stored in SI that showed a slightly higher value on the sixth day of storage (Table 3).

The redness (a*) and yellowness (b*) values did not show significant differences between the two treatments as well as those related to ΔE. During the shelf life, samples stored in FI did not show significant differences as far as the redness and yellowness values were concerned; the samples preserved in SI showed significant differences for the a* values at T6 and as for the b* values at T12 (Table 3).

The ΔE values did not show significant variations along the shelf life for both samples stored under FI and SI .

It was seen that specimens of croaker (*Johnius dussumieri*) preserved in slurry ice anf flake ice showed a decreasing trend in the L* and a* values during the shelf life. Specimens preserved in slurry ice showed higher values of b* during shelf life, problably due to the salt uptake in the fish muscle that occurred in samples stored in slurry ice. Moreover, the decrease in L* values resulted in the increase in TBA values (Annamalai et al., 2018). In the present study, the slurry ice system did not determine an increase in the yellowness as it was seen in the aforementioned study.

Texture Profile Analysis

Texture represents one of the main quality attributes as it expresses the softness of the fish muscle which increases due to the autolytic activity (Huss, 1999; Tomac et al., 2015).

The softening of the texture is related to the loss of quality of the anchovies (Cabrer et al., 2002; Tomac et al., 2015).

Texture, evaluated by means of the Young Modulus and Hardness, did not reveal any significant difference between the two treatments (Table 4).

Table 4. Texture detected on specimens of *Engraulis encrasicolus* during the period of conservation in Flake ice (FI) and Slurry ice (SI).

	Young Modulus					
	T0	T1	T3	T6	T9	T12
FI	$0.36\pm0.09^{a/A}$	$0.25\pm0.07^{ab/A}$	$0.14\pm0.022^{c/A}$	$0.18\pm0.031^{bc/A}$	$0.12\pm0.022^{c/A}$	$0.13\pm0.011^{bc/A}$
CI	$0.36\pm0.05^{a/A}$	$0.26\pm0.06^{ab/A}$	$0.13\pm0.023^{cd/A}$	$0.17\pm0.037^{bd/A}$	$0.13\pm0.019^{c/A}$	$0.16\pm0.044^{cd/A}$
	Hardness					
	T0	T1	T3	T6	T9	T12
FI	$15.41\pm2.63^{a/A}$	$10.39\pm3.09^{acd/A}$	$6.48\pm2.651^{b/A}$	$6.58\pm1.239^{cd/A}$	$4.99\pm0.829^{b/A}$	$5.17\pm2.046^{bd/A}$
CI	$13.25\pm2.55^{a/A}$	9.39 ± 2.46^{abA}	$3.92\pm1.085^{bc/A}$	$7.29\pm1.56^{bc/A}$	$4.01\pm1.041^{c/A}$	$5.80\pm1.186^{c/A}$

Different lowercase letters (abcd) per line indicate significant differences ($p < 0.05$) within the same treatment during *shelf life*. Different capital letters (AB) per column indicate significant differences ($p < 0.05$) between treatments on the same day of storage. Values are expressed as mean±standard deviation. N=36

The hardness, considered a very important value for commercial purposes, showed a gradual decrease in samples stored in FI and SI during the shelf life, probably due to proteolytic phenomena. The hardness values of the samples stored in SI were not found to be improved, as shown in other fish species, such as *Collichthys niveatus* (Chen et al., 2016) and *Johnius dussumieri* (Annamalai et al., 2018). Even the compactness (Young Modulus) showed a decreasing trend during shelf life in samples preserved in both type of ice, probably as a result of the autolytic phenomenon and to the denaturation of protein in the muscle tissue, that occurred during the preservation period.

Water activity (aw)

Reduced aw values in fish species reduce and inhibit microbial growth (Collignan et al., 2001; Tsironi & Taoukis, 2014).

No significant differences were found between the two treatments as well as along the shelf life of the samples stored in SI and in FI (Table 5).

Water holding capacity (WHC)

Water holding capacity is a fundamental parameter both for industrial processing and for the consumer acceptability. This parameter translates into the ability of the muscle to retain water and depends on protein changes in the muscle, allowing to measure the tenderness and juiciness of the fish meat (Messina et al., 2019).

There were no significant differences either between the two treatments or during the shelf life for each treatment as can be seen in Table 5.

pH

Increase in pH values is related mainly to the accumulation of basic substances, such as ammonia and TMA produced by microbial development in fish muscle (Ruiz-capillas & Moral, 2001).

The pH values and variations during the shelf life depend on the fish species, the season and the diet, the level of activity or stress during capture and the type of muscle (Ocaño-Higuera et al., 2011). This parameter is important for carrying out a comprehensive qualitative analysis of the anchovy.

As far as pH values were concerned, samples stored in FI showed an increase in pH over the shelf life as well as samples stored in SI. Nevertheless, significant differences ($p < 0.05$) (Table 5) were found between the two treatments starting from the sixth day of storage with lower values for the samples stored in SI. A lower pH indicates better control of both endogenous and microbial activity in the fish muscle. Furthermore, similar results were found for European hake (*Merluccius merluccius*) (Rodríguez et al., 2004) and farmed turbot (*Psetta maxima*) (Gallardo & Aubourg, 2006) preserved in SI.

Sodium content

In this study, the sodium content was found to be higher in samples stored in SI compared to samples stored in FI. Starting from T1, significant differences were found between the two treatments ($p < 0.05$) (Table 5) with higher values for the samples stored in SI, probably due to the

amount of salt contained in the ice, which was added to the water in order to decrease the freezing point. During the shelf life of the samples stored in FI, a slight increase in sodium values was found, unlike the samples stored in SI which were subject to significant increases during the 12 days of storage as can be seen in Table 5. This increase was probably due to pH and textural changes, which have inferred the ability of the muscle to retain water (Messina et al., 2021).

The effect of slurry ice, containing NaCl, on the increase in sodium content, observed during the present study, agreed with what observed in the literature (Losada et al., 2007). Specifically, in sardine samples stored with SI containing NaCl, a significantly higher sodium content was observed compared to samples treated with traditional FI.

Table 5. Water activity **(**aw), WHC, pH, Na^+ detected on specimens of *Engraulis encrasicolus* during the period of conservation in Flake ice (FI) and Slurry ice (SI).

	T0	T1	T3	T6	T9	T12
			aw			
FI	$0.974\pm0.003^{b/A}$	$0.974\pm0.002^{b/A}$	$0.973\pm0.001^{b/A}$	$0.977\pm0.003^{b/A}$	$0.973\pm0.002^{b/A}$	$0.977\pm0.001^{b/A}$
SI	$0.974\pm0.005^{a/A}$	$0.974\pm0.004^{a/A}$	$0.976\pm0.001^{a/A}$	$0.976\pm0.000^{a/A}$	$0.973\pm0.001^{a/A}$	$0.973\pm0.001^{a/A}$
			WHC			
FI	$90.6\pm2.01^{a/A}$	$92.3\pm1.28^{a/A}$	$92.1\pm1.47^{a/A}$	$87.4\pm2.52^{a/A}$	$89.5\pm1.71^{a/A}$	$86.8\pm3.42^{a/A}$
SI	$90.5\pm1.15^{a/A}$	$89.8\pm1.9^{a/A}$	$87.1\pm3.45^{a/A}$	$91.9\pm1.38^{a/A}$	$90.9\pm0.68^{a/A}$	$86.7\pm4.37^{a/A}$
			pH			
FI	$6.26\pm0.01^{a/A}$	$6.31\pm0.00^{b/A}$	$6.33\pm0.07^{b/A}$	$6.43\pm0.03^{c/B}$	$6.52\pm0.00^{d/B}$	$6.51\pm0.01^{d/B}$
SI	$6.26\pm0.00^{a/A}$	$6.31\pm0.00^{b/A}$	$6.28\pm0.01^{c/A}$	$6.34\pm0.01^{d/A}$	$6.39\pm0.02^{e/A}$	$6.34\pm0.01^{d/A}$
			Na^+			
FI	$0.18\pm0.00^{a/A}$	$0.12\pm0.01^{b/A}$	$0.14\pm0.01^{b/A}$	$0.17\pm0.02^{c/A}$	$0.17\pm0.01^{c/A}$	$0.17\pm0.01^{c/A}$
SI	$0.59\pm0.01^{a/A}$	$0.58\pm0.01^{b/B}$	$0.91\pm0.01^{c/B}$	$1.03\pm0.05^{d/B}$	$1.00\pm0.07^{d/B}$	$1.50\pm0.12^{e/B}$

Different lowercase letters (abcde) per line indicate significant differences ($p < 0.05$) within the same treatment during *shelf life*. Different capital letters (AB) per column indicate significant differences ($p < 0.05$) between treatments on the same day of storage

Proximate composition and evaluation of the biochemical parameters related to the shelf life

Proximate composition of *E. encrasicolus* stored in FI and SI are shown in Table 6.

Table 6. Proximate composition (g/100g) detected on specimens of *Engraulis encrasicolus* at T0 and T12 in Flake ice (FI) and Slurry ice (SI).

Parameters	Treatment	T0	T12
Lipids	FI	$1.89\pm0.10^{a/A}$	$1.79\pm0.06^{a/A}$
	SI	$2.03\pm0.17^{a/A}$	$1.45\pm0.47^{a/A}$
Moisture	FI	$76.89\pm0.23^{a/B}$	$80.52\pm0.71^{b/B}$
	SI	$75.76\pm0.16^{a/A}$	$79.16\pm0.21^{b/A}$
Ash	FI	$1.37\pm0.2^{b/A}$	$1.13\pm0.1^{a/A}$
	SI	$1.91\pm0^{a/B}$	$2.75\pm0^{b/B}$
Protein	FI	$19.91\pm0.071^{b/A}$	$16.96\pm0.481^{a/A}$
	SI	$19.82\pm0.15^{b/A}$	$16.13\pm0.236^{a/A}$

Different lowercase letters (ab) per line indicate significant differences ($p < 0.05$) within the same treatment during *shelf life*. Different capital letters (AB) per column indicate significant differences ($p < 0.05$) between treatments on the same day of storage.
Values are expressed as mean±standard deviation. N=18

The proximate composition was calculated at the beginning and at the end of the shelf life.

Lipid and protein contents showed no significant changes between the two treatments at both T0 and T12. Significant differences were observed for all parameters ($p < 0.05$) except for lipids, during the shelf life, in both samples preserved in FI and those preserved in SI (Table 6). The protein percentage for both treatments reduced, unlike the percentage of moisture that increased probably due to the preservation period in ice. The percentage of ash, on the other hand, increased for the samples stored in SI and decreased for the samples stored in FI; in particular, the increase in ash, observed in samples stored in SI at the end of the storage period, may be due to the salt uptake in the fish muscle during storage.

In the evaluation of the fatty acids profile, no significant differences were found between the two treatments and along the shelf life (Table 7).

Table 7. Fatty acids composition (%) detected on specimens of *Engraulis encrasicolus* during the storage period in Flake ice (FI) and Slurry ice (SI).

Parameters	T0		T12	
	FI	SI	FI	SI
Saturated	29.25±0.88	29.55±2.72	30.41±0.64	29.88±1.02
Monounsaturated	11.73±1.92	13.18±1.99	12.11±1.36	12.53±1.38
Polyunsaturated	59.02±2.79	57.27±4.71	57.48±0.72	57.60±2.40
Tot n-3	53.39±3.25	50.65±2.45	51.66±0.91	51.13±0.14
Tot n-6	2.56±0.05	3.88±2.06	2.66±0.09	3.80±2.04

Values are expressed as mean±standard deviation. N=18

This confirms that storage in SI does not alter the biochemical and nutritional properties of anchovy fillets.

Slurry ice had no effect on the fatty acids profile, and on the high content of polyunsaturated fatty acids (PUFAs), especially of the n-3 series, which characterize *Engraulis encrasicolus* (Gencbay & Turhan, 2016). PUFAs provide numerous human health benefits; in fact, the self-defense capacity of tissue against oxidative stress depends on its lipid composition (ω-6: ω-3) (Ciriminna et al., 2019).

On the other hand, PUFAs are prone to peroxidation, causing a loss of quality for fishery products. Indeed, the oxidation of PUFAs induces changes in the physical-chemical properties that cause rancidity and deterioration of fish taste. These changes lead to a shortened shelf life and a reduction in nutritional values, resulting in a loss of product quality (Karel et al., 1975; Refsgaard et al., 2000) (Messina et al., 2015). In the present work, malondialheyde content (mg MDA/kg) was measured as an indicator of secondary lipid peroxidation. MDA values showed significant differences ($p<0.05$) (Table 8) between the two treatments at T0, T3, T6 and T9 with significantly lower values for samples stored in SI during the shelf life, compared to samples stored under FI.

Table 8. MDA and TVB-N values detected on specimens of *Engraulis encrasicolus* during the period of conservation in Flake ice (FI) and Slurry ice (SI).

	T0	T1	T3	T6	T9	T12
			MDA			
FI	$0.20\pm0.04^{a/A}$	$1.85\pm0.47^{b/A}$	$8.33\pm1.08^{d/B}$	$12.6\pm1.17^{f/B}$	$10.6\pm0.4^{e/B}$	$5.62\pm2.18^{c/A}$
SI	$0.43\pm0.12^{a/B}$	$1.34\pm0.15^{a/A}$	$2.79\pm0.31^{b/A}$	$4.82\pm0.21^{c/A}$	$6.74\pm1^{d/A}$	$6.15\pm2.42^{cd/A}$
			TVB-N			
FI	$18.9\pm2.05^{a/A}$	$22.4\pm1.27^{b/B}$	$25.7\pm0.76^{c/B}$	$28.1\pm1.19^{d/B}$	$28.7\pm1.39^{d/B}$	$32\pm0.67^{e/B}$
SI	$16.9\pm0.42^{a/A}$	$18.9\pm0.19^{ab/A}$	$19.9\pm2.85^{ab/A}$	$19.4\pm0.33^{ab/A}$	$19.8\pm1.18^{ab/A}$	$21.2\pm1.33^{b/A}$

Different lowercase letters (abcdef) per line indicate significant differences ($p<0.05$) within the same treatment during *shelf life*. Different capital letters (AB) per column indicate significant differences ($p<0.05$) between treatments on the same day of storage. Values are expressed as mean±standard deviation. N=18

Furthermore, the samples stored in FI showed high MDA values not remaining in the acceptable range of 8 mg MDA/kg (Oyarekua, 2014). Values detected in samples stored in SI were significantly lower during the shelf life as shown on other fish species stored under SI (*Collichthys niveatus* and *Johnius dussumieri*) (Annamalai et al., 2018; Chen et al., 2016).

Concerning TVB-N values, samples stored in FI showed significant variations ($p < 0.05$) (Table 8) during shelf life with the highest value reached at T12, i.e., 32 ± 0.67 mg TVB-N/100 g, while those stored in SI maintained constant and significantly lower values from T1 to T9, therefore the innovative technique would seem to reduce the deterioration capacity of the fish under investigation.

Nevertheless, it has to be highlighted that, in any case, for both treatments the values did not exceed the acceptability limit established by European legislation (European Communities Commission (E.C.C.), 1995) between 25 and 35 mg TVB-N per 100 g of muscle, depending on the species.

Similar results were shown on other species such as sardines and *Epinephelus* sp. stored in SI, where the increase of TVB-N was related to sensory variations and deterioration of the fish (Campos et al., 2005; Putra et al., 2020). These increases were probably caused by the breakdown of fish proteins which produce volatile bases (Putra et al., 2020). However, as for the anchovy also for the hake, TVB-N values of the samples stored in SI showed lower values over the shelf life compared to samples stored in FI (Rodríguez et al., 2004).

Multivariate analysis revealed that anchovy storage in FI resulted in significantly higher values for MDA and TVB-N than storage in SI (Table 8), whereby the storage of anchovies in SI significantly slowed down the formation of MDA and TVB-N compared to storage in FI, as observed in sardine (Campos et al., 2005).

The obtained results clearly show a greater conservation efficiency in the SI system compared to FI. Although there were some parameters such as aw that did not show significant differences between the two treatments, other parameters, such as sensory evaluation, showed that SI maintained more similar characteristics to fresh products, for longer time than FI samples.

These attributes are essential for evaluating and ensuring the quality and freshness of fresh fish.

Conclusions

The introduction of innovative cold techniques could meet the high demand for fish products and the reduction of waste, bringing to both economic and environmental benefits, so contributing to the growth of the fish production sector.

During the shelf life of specimens stored under SI and FI (Table 9), slurry ice resulted more effective in ameliorating some key parameters related to evolution of quality during shelf-life, representing the best solution to extend freshness of this species in view of its utilization in the processing industries.

Table 9. Summary of the positive and negative effects, on the different quality parameters of *Engraulis encrasicolus,* stored in FI (Flake ice) and in SI (Slurry ice)

Parameters analyzed	**FI**	**SI**
Sensory analysis	-	+
Physical-chemical paramenters		
Temperature	-	+
Color	-	+
Texture Profile	=	=
Water activity (aw)	=	=
Water holding capacity (WHC)	=	=
pH	-	+
Sodium content	=	=
Proximate composition	=	=
Biochemical parameters related to the shelf life		
MDA	-	+
TVB-N	-	+
Fatty acids profile	=	=

Sensory analysis, color and biochemical parameters related to shelf-life, play a pivotal role in monitoring the quality and deteriorability of anchovy and, in the present study, showed significant differences between the two treatments. Overall, this pilot study indicated that the use of SI could be effective on maintaining fish freshness and extending the product shelf life compared to the traditional technique, i.e., FI. competing effectively with other cooling methods used in industry and the fishing sector.

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Part 3.

Actions to increase profitability and sustainability of production by reutilization of processing by-products to obtain value-added products

Chapter 5

marine drugs

MDPI

Article

Farmed Gilthead Sea Bream (*Sparus aurata*) by-Products Valorization: Viscera Oil ω-3 Enrichment by Short-Path Distillation and In Vitro Bioactivity Evaluation

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Abstract

This study shows a pilot scale protocol aimed to obtain an omega 3-enriched oil after the processing of farmed gilthead sea bream viscera (SBV); this oil was tested in vitro for bioactivity, attesting to the possibility to turn waste into profit. The quality of the oil, in terms of requirements for animal and human consumption, was assessed by determining some chemical parameters, such as peroxide value (PV), thiobarbituric acid reactive substances (TBARS), ρ-anisidine (ρ-AV) content, total oxidation value (TOTOX), and phospholipids and free fatty acid (%), both in crude viscera oil (CVO) and refined viscera oil (RVO). Among the extraction conditions, the highest CVO yields were obtained at 60 °C for 10 min (57.89%) and at 80 °C for 10 min (67.5%), and the resulting oxidation levels were low when utilizing both extraction conditions. RVO, obtained from CVO extracted at 60 °C, showed the highest quality based on the assessed parameters. The ethyl esters of the total fatty acid (TFA) contents extracted from RVO were enriched in the ω-3 polyunsaturated fatty acid fraction (PUFAE) up to almost 56% via short path distillation (SPD). Antioxidant activities and adipogenic properties were tested in vitro. PUFAE protected 3T3 L1 cells from oxidative stress and exerted an anti-adipogenic effect in *Dicentrarchus labrax* pre-adipocytes, attesting to the beneficial properties for both farmed fish and human health. These results could stimulate the adoption of solutions aimed to recover and utilize aquaculture by-products at a higher

scale, turning “waste into profit” and indicating a strategy to reach more sustainable business models in aquaculture resource utilization according to the principles of the circular economy.

Introduction

The increasing pressure on natural resources has resulted in an urgent need to optimize the destiny of the by-products of the main food supply chains. The reduction of food loss and waste, as well as their valorization, is crucial to achieve the goal of “zero waste.” Considering that environmental sustainability is closely linked to economic sustainability, scientific and technical knowledge is essential to outline the road map of more sustainable business models and to optimize the efficiency of aquatic resource utilization (Alfio et al., 2021; Coppola et al., 2021; García-de-Vinuesa et al., 2021), according to the 2030 agenda. In view to target the 14th Sustainable Development Goal (SDG) of the United Nations Development Programme (UNDP) (devoted to the proper utilization of the “resources below the water”) and considering the pivotal role of aquaculture in satisfying the global demand for fish products, the contribution of this industry to the production of by-products must be properly managed at the national and regional levels. It is well-known that marine oils are an excellent source of ω-3 long chain, polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These classes of PUFA are common in marine organisms; in particular, PUFAs are synthesized in microalgae and tend to significantly accumulate in fatty fish and in the oil extracted from these organisms and their by-products (Caruso et al., 2020; Coppola et al., 2021; Lund, 2013; Marsol-Vall et al., 2020; Pateiro et al., 2020; Rimm et al., 2018; Zárate et al., 2017). In recent years, there has been an exponential growth in the market of the ω-3 PUFAs for human consumption, thanks to numerous studies showing the significant beneficial effects determined by fish oil and ω-3 PUFA-rich functional foods daily consumption in terms of dietetic (Mentoor et al., 2019; Zárate et al., 2017) anti-tumor (Field et al., 2007; Omabe et al., 2015; Siddiqui et al., 2008; Tarasiuk et al., 2018; Zárate et al., 2017), and antithrombotic properties (Colussi et al., 2017; Mozaffarian & Wu, 2011; Rimm et al., 2018). In addition, the demand for ω-3 PUFA fish oils for animal feeds, in particular for aquaculture, has also been rapidly growing (Guillen et al., 2019; Shepherd & Jackson, 2013; Turchini et al., 2010) due to the recognized beneficial effects on growth performance, nutritional value (Fountoulaki et al., 2009; Messina et al., 2013a, 2013b; Monge-Ortiz et al., 2018), and immune system of reared fish (Torrecillas et al., 2017). As the industrial production of fish oil is based on the intensive fishing of fatty fish species belonging to the families such as Scomberesocidae, Gadidae, and Clupeidae, with a consequent depletion of wild fish stocks, it is

important to identify new sources for the industrial production of PUFA-rich oils and ω-3 PUFA concentrates (Caruso et al., 2020; Šimat et al., 2019). In this context, the use of fisheries and aquaculture by-products and wastes as raw material can be an important resource that still contains a large amount of components with high nutritional value, such as ω-3 PUFA (Antelo et al., 2012; Caruso et al., 2020; Colussi et al., 2017; Galanakis, 2012; Maschmeyer et al., 2020; Rustad et al., 2011; Stevens et al., 2018; Ucak et al., 2021; Villamil et al., 2017). By-products from processed farmed fish are extremely interesting because they are obtained from a highly controlled processing chain (Shepherd & Jackson, 2013), with a high organoleptic quality (Messina et al., 2013a) and a high content of oils and fats that, if properly stored, processed, and enriched, can provide a high amount of ω-3 PUFA for direct human consumption (Rincón Cervera et al., 2015; Šimat et al., 2020) with significant beneficial effects (Alfio et al., 2021; Colussi et al., 2017; Mentoor et al., 2019; Tarasiuk et al., 2018; Zárate et al., 2017). In the wide range of beneficial effects, several studies have suggested that ω-3 PUFA also has antioxidant, anti-inflammatory, and anti-adipogenic effects. These effects, with important nutritional and nutraceutical implications, have been demonstrated in vitro on different cell lines (Kusunoki et al., 2013; Riera-Heredia et al., 2020; Sakai et al., 2017; Todorčević et al., 2008).

The utilization of by-products from fisheries and aquaculture, according to the principles of the circular economy, will turn "waste into profit," indicating more sustainable business models and optimizing the efficiency of aquatic resource utilization (Coppola et al., 2021). Companies, in fact, will have a direct economic return from the commercialization of bio-products and an indirect return from the reduction waste to be sent to landfills (Hathwar et al., 2011; Šimat et al., 2019; Vázquez et al., 2019). This last aspect will also allow for a positive environmental impact by reducing the pressure on the environment of these productive activities (Pateiro et al., 2020). About 30% of the total marine aquaculture production in Sicily (around 2000 t/year) is minimally processed (gilled, gutted, and fillet) and marketed at local markets or in large-scale distribution. We estimate that, on the regional scale, reared seabass and sea bream processing guarantees a production of 36/40 t/year of viscera, with this component being about 6/7% of the total by-product. In the main Sicilian fish farm, wastes from processed fish are automatically collected during the production cycle, stored, and sent to landfills. In agreement with this farm, as part of an industrial research project, we developed a pilot process for the recovery and valorization of this waste with the aim to turn "waste into profit" and contribute to increasing the economic and environmental sustainability of aquaculture as a paradigm of the circular economy. The aim of this research was to develop and optimize methods for the separation of crude viscera oil (CVO) taken from reared sea bream (*Sparus aurata*) viscera (SBV) and its refinement to obtain refined viscera oil (RVO) by

short path distillation (SPD). After transesterification to produce total fatty acid (TFA) ethyl esters from RVO, SPD was employed to enrich the ω-3 PUFA fraction (PUFAE) and separate the exhausted fatty acid ethyl esters fraction (EFA). On refined oil and separated fractions, bioactive properties were evaluated in vitro to investigate the potential antioxidant effects in the 3T3-L1 cell line and anti-adipogenic effects in primary pre-adipocytes of *Dicentrarchus labrax*.

Results and Discussion

Proximate Composition of Sea Bream Viscera (SBV) By-Product

The proximate composition of SBV (Table 1) showed a high total lipid content (51.79 ± 12.92%) and

moisture, protein, and ash values of 40.81 ± 4.86%, 5.67 ± 0.02%, and 1.43 ± 0.55%, respectively (Table 1).

Table 1. Proximate composition of sea bream viscera (SBV) by-product (mean ± standard deviation values; n = 12).

Parameters	g/100g SBV
Lipid	51.79±12.92
Moisture	40.81±4.86
Protein	5.67±0.02
Ash	1.43±0.55

The total lipid content was higher than the data reported in the literature by Pateiro et al. (2020) (34.11% in the guts and 25.76% in the liver of reared sea bream), Rincón Cervera et al. (2015) (34% in the viscera of sea bream from semi-extensive farming), and Sinanoglou et al. (2017) (29.92 ± 3.5% under organic and 42.61 ± 5.29% under conventional production systems). Kandyliari et al. (2020) showed a total lipid content equal to 43.19% (large size) and 55.12% (small size) in the intestine of sea bream reared in a pilot-scale cage farm. These diverse data confirm that lipid content in fish tissues is significant influenced by rearing conditions. The reared sea bream, also due to the considerable availability of artificial food, usually shows a greater accumulation of fat when compared to wild specimens, and as expected, the fatty acid profile tends to reflect that of the administered diet (Gelibolu et al., 2018; Lenas et al., 2011; Pateiro et al., 2020; Rincón Cervera et al., 2015; Sinanoglou et al., 2017). The fatty acid profile of SBV total lipids is shown in Table 2.

Table 2. Fatty acid profile (%) of the total lipids in sea bream viscera (SBV) (mean ± standard deviation values; n = 12). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acid.

Fatty Acids	%
14:0	4.99 ± 0.17
16:0	15.56 ± 0.18
16:1n−7	10.72 ± 0.18
16:2n−4	0.89 ± 0.03
16:3n−4	0.59 ± 0.02
18:0	2.96 ± 0.06
18:1n−9	16.11 ± 0.40
18:1n−7	6.05 ± 0.15
18:2n−6	2.42 ± 0.16
18:3n−4	0.01 ± 0.00
18:3n−3	0.62 ± 0.04
18:4n−3	1.68 ± 0.06
20:1n−9	11.14 ± 0.27
20:4n−6	0.13 ± 0.03
20:4n−3	0.38 ± 0.08
EPA	8.19 ± 0.12
22:1n−11	4.71 ± 0.17
22:1n−9	0.38 ± 0.02
22:5n−3	1.19 ± 0.08
DHA	11.27 ± 0.30
SFA	23.51 ± 0.28
MUFA	49.12 ± 0.76
PUFA	27.38 ± 0.48

Monounsaturated fatty acids (MUFAs) were the most abundant class of fatty acids (49.13 ± 0.76%), followed by PUFAs (27.38 ± 0.48%) and saturated fatty acids (SFAs) (23.51 ± 0.28%). The predominant fatty acid was oleic acid (18:1n9, 16.11 ± 0.40%) (Table 2). This profile was comparable to results reported by other authors for reared sea bream by-products (Kandyliari et al., 2020; Pateiro et al., 2020; Rincón Cervera et al., 2015; Sinanoglou et al., 2017) and was in accordance with the observation that the relative proportion of oleic acid is not strictly affected by the rearing system (Sinanoglou et al., 2017). For reared species, such as sea bass and sea bream, numerous data in the literature attest to the direct effect of the diets on tissues fatty acid composition and, consequently, on the nutritional, organoleptic and shelf-life properties of the products (Gelibolu et al., 2018; Grigorakis, 2007; Kandyliari et al., 2020; Lenas et al., 2011;

Messina et al., 2013b; Rincón Cervera et al., 2015; Sinanoglou et al., 2017; Yıldız et al., 2018). Increasing levels of ω-6 PUFA are known to result from the supplementation of fish feed with vegetable oils, which increases the proportion of dietary C18:2 n-6 (Benedito-Palos et al., 2011; Grigorakis, 2007; Sinanoglou et al., 2017; Turchini et al., 2010). As expected, this parameter is also strictly subjected to quantitative and qualitative variations in relation to life cycle, diet, and other ecological factors (Benedito-Palos et al., 2011; Grigorakis, 2007; Maschmeyer et al., 2020; Messina et al., 2013b; Sinanoglou et al., 2017; Turchini et al., 2010). Among PUFAs, the predominant fatty acids were EPA and DHA (8.19 ± 0.12% and 11.27 ± 0.30, respectively) (Table 2). Pateiro et al. (2020) showed a lower content of total long-chain PUFA but a higher content of EPA and DHA, while data reported by Rincón Cervera et al. (2015) showed a lower content of EPA and a higher content of DHA in sea bass and sea bream by-products. Moreover, the sum of EPA and DHA content, in our study, was higher when compared to that reported by Maschmeyer et al. (2020) in sea bass and sea bream by-products, probably because of the administered diet.

Yield and Quality of Crude Viscera Oil (CVO)

The monitoring of the extraction process was carried out by evaluating the percentage of oil recovery (%) (Figure 1) and the quality parameters, as shown in Table 3. An increase of CVO yield was observed as the reaction temperature rose (Figure 1). The yield, calculated on the total lipid content of SBV, increased from 38.46 ± 2.43% at 40°C for 10 min to 67.51 ± 2.60% at 80 °C for 10 min (Figure 1). These results confirmed that, as already reported (Chantachum et al., 2000; Honold et al., 2016; Sathivel et al., 2003), higher temperatures are preferential to increase the extraction yield. Under optimal extraction conditions, without the use of any solvents, the procedure we have described allows us to extract and separate about 6.5 L of oil from 20 kg of viscera in about three hours.

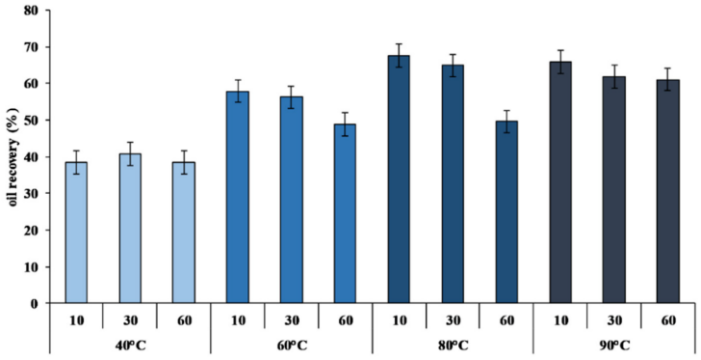

Figure 1. Percentage of crude viscera oil (CVO) recovery calculated on the total lipid content of sea bream viscera (SBV) at different temperatures (40, 60, 80 and 90 °C) and different extraction times (10′, 30′, and 60′) (n = 12).

Table 3. Quality of crude viscera oil (CVO) extracted at different temperatures (40, 60, 80, and 90 °C) and different extraction times (10′, 30′, and 60′), as determined by the evaluation of peroxide value (PV; meq O_2/kg) and thiobarbituric acid reactive substances (TBARS) (MDA μg/g). Commercial cod liver oil was used as the control oil (CO).

Sample		**Parameters**	
Temperatures	**Times**	**PV**	**TBARS**
40	10	6.17 ± 0.47 [b]	16.01 ± 0.02 [b]
	30	6.51 ± 0.22 [b]	15.59 ± 0.03 [b]
	60	10.29 ± 2.37 [c]	21.18 ± 5.85 [bc]
60	10	5.42 ± 0.13 [b]	14.15 ± 0.07 [b]
	30	6.38 ± 0.03 [b]	16.58 ± 0.01 [b]
	60	11.33 ± 0.75 [cd]	14.31 ± 0.22 [b]
80	10	11.01 ± 1.77 [cd]	17.50 ± 2.41 [b]
	30	13.27 ± 2.13 [d]	25.38 ± 8.59 [c]
	60	19.69 ± 0.90 [e]	19.89 ± 2.29 [b]
90	10	10.99 ± 0.53 [cd]	31.41 ± 6.33 [d]
	30	19.67 ± 2.10 [e]	33.09 ± 1.31 [d]
	60	22.74 ± 2.27 [f]	33.43 ± 5.63 [d]
CO		2.10 ± 0.53 [a]	5.51 ± 0.81 [a]

Different superscript letters in the same column indicate significant differences (a, b, c…: $p < 0.05$). The data are reported as mean ± standard deviation (n = 12).

The evaluation of primary and secondary lipid oxidation markers like peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) showed that these compounds were highly influenced by the extraction conditions. It is known that higher temperatures promote lipid oxidation, which leads to a decrease in oil quality (Honold et al., 2016). The peroxide content ranged from 5.42 ± 0.13 meq O_2/kg in CVO extracted at 60 °C for 10 min to a maximum of 22.74 ± 2.27 meq O_2/kg in CVO extracted at 90 °C for 60 min (Table 3). The maximum PVs were recorded in CVO extracted at 60 min, rather than 10 and 30 min, at each temperature (Table 3) ($p < 0.05$). The PV in CVO was higher than values registered in the commercial cod liver oil control oil (CO) for human consumption (2.10 ± 0.53 meq O_2/kg) (Table 3). None of the CVO we extracted from SBV showed a content lower than 5 meq O_2/kg, so it was necessary to refine it for the human consumption and, in any case, for further applications (FAO, 2017; Genç et al., 2015). The increase of oxidation parameters at higher temperatures (Table 3) was comparable to the yield and quality of *Oncorhynchus nerka* (Yin et al., 2011) and *Clarias gariepinus* (Eke-EJiofor & Ansa, 2018). The evaluation of lipid oxidation by TBARS, expressed as the content of malondialdehyde (MDA) in oil (μg/g), showed a comparable trend to the PV content (Table 3). TBARS content, in fact, increased in relation to CVO extraction temperature (Table 3) from 14.15 ± 0.0, at 60 °C for 10 min to a

maximum of 33.43 ± 5.63 at 90 °C for 60 min (Table 3). In any case, the TBARS content of CVO was higher than the TBARS content of the reference oil (CO: 5.51 ± 0.81 MDA μg/g) (Table 3). However, the TBARS content of CVO (Table 3) was lower than the values observed by Šimat et al. (2019, 2020) in crude oil extracted from sea bass and sea bream guts. At higher extraction temperatures, we reported an alteration of CVO color (data not shown). From the CIE Lab coordinates (Stearns, 1988), we observed a variation from yellow (parameter b*) in CVO extracted at 40 and 60 °C to red (parameter a*) in CVO extracted at 80 and 90 °C (data not shown). Furthermore, the increase of extraction temperature also led to a reduction of CVO lightness (parameter L*) (data not shown). In any case, the color of all our CVO was always different from the color of the reference oil (CO) ($p < 0.05$) (data not shown). The high temperature extraction-induced lipid oxidation that determined a variation of the extracted oil color (Franklin et al., 2020; Sabzipour et al., 2019; Shabanpour et al., 2016; Yin et al., 2011). Thus, extracted oil color evaluation could be utilized as quick and cheap oil quality marker. The variation of the CVO's main classes of fatty acids, based on extraction temperature, is shown in Table 4. A significant reduction in PUFA was observed as the temperature increased, with a significantly lower value in the oil extracted at 90° for 60 min ($p < 0.05$). It is known in literature, in fact, that high temperatures lead to the thermal degradation of some polyunsaturated fatty acids (Yves et al., 2016).

Table 4. Main classes of fatty acids (%) extracted from sea bream viscera (SBV) and crude viscera oil (CVO) after extraction at different temperatures (°C) and reaction times (min).

Samples		**SFA**	**MUFA**	**PUFA**	**EPA**	**DHA**
SBV		**23.51±0.28** ^{a}	**49.12±0.76** ^{ab}	**27.38±0.48** ^{e}	**8.19±0.12** ^{eg}	**11.27±0.30** ^{e}
CVO						
Temperature	**Time**					
40	10	24.98 ± 1.03 ^{ab}	50.09 ± 0.66 ^{b}	24.93 ± 0.37 ^{bcd}	7.12 ± 0.52 ^{bcd}	10.15 ± 0.06 ^{cd}
	30	24.26± 0.58 ^{a}	50.28 ± 0.15 ^{b}	25.46 ± 0.58 ^{cde}	7,78 ± 0.18 ^{def}	10.03 ± 0.36 ^{cd}
	60	26.61 ± 1.35 ^{bc}	49.81 ± 0.28 ^{b}	23.58 ±1.64 ^{bc}	6.91 ± 0.51 ^{bc}	9.25 ± 1.00 ^{bc}
60	10	25.28 ± 0.01 ^{ab}	49.84 ± 0.11 ^{b}	24.88 ± 0.10 ^{bcd}	7.54 ± 0.05 ^{cdef}	9.69 ± 0.02 ^{bcd}
	30	25.69 ± 1.41 ^{abc}	49.43 ± 0.06 ^{b}	24.88 ± 1.36 ^{bcd}	7.71 ± 0.56 ^{def}	9.61 ± 0.49 ^{bcd}
	60	25.17 ± 0.79 ^{ab}	48.86 ± 0.49 ^{b}	25.97 ± 1.28 ^{de}	7.92 ± 0.69 ^{ef}	9.85 ± 0.67 ^{cd}
80	10	26.62 ± 1.66 ^{bc}	47.41± 0.69 ^{a}	25.97 ± 2.35 ^{de}	7.56 ± 0.35 ^{cdef}	10.49 ± 1.37 ^{de}
	30	27.23 ± 0.80 ^{c}	49.83 ± 0.42 ^{b}	22.94 ± 0.37 ^{b}	6.73 ± 0.08 ^{b}	8.71 ± 0.37 ^{ab}
	60	24.78 ± 0.77 ^{a}	49.42 ± 0.38 ^{b}	25.79 ± 1.15 ^{cde}	7.38 ± 0.56 ^{bcde}	10.61 ± 0.54 ^{de}
90	10	24.48 ± 0.62 ^{a}	48.54 ± 0.33 ^{ab}	26.98 ± 0.97 ^{de}	8.64 ± 0.42 ^{g}	10.40 ± 0.52 ^{cde}
	30	25.25 ± 0.76 ^{ab}	48.72 ± 0.39 ^{b}	25.76 ± 0.84 ^{cde}	7.44 ± 0.30 ^{cdef}	9.68 ± 0.45 ^{bcd}
	60	26.69 ± 0.21 ^{bc}	52.30 ± 2.75 ^{c}	21.02 ± 1.90 ^{a}	6.09 ± 0.45 ^{a}	7.97 ± 0.43 ^{a}

Different superscript letters in the same column indicate significant differences (a, b, c…: $p < 0.05$). The data are reported as mean ± standard deviation (n = 12).

However, at the highest extraction temperature, the EPA and DHA contents were higher than those observed by Šimat et al. (2019, 2020) in crude oil extracted from sea bass and sea bream by-products under similar conditions (95 °C for 12 min). The evaluation of the yield and quality parameters of the extracted CVO confirmed that the extraction conditions directly affected the oil yield, reaching a maximum value in CVO extracted at 80 °C (Figure 1). In addition, at high temperatures, a higher level of lipid oxidation was observed, as evidenced by the increase in the value of peroxides and TBARS (Table 3), especially at longer reaction times. However, the quality of CVO extracted at 60 °C for 10 minutes and 80 °C for 10 minutes was within the guidelines for the evaluation of primary and secondary lipid oxidation markers of unprocessed fish oils that were recently published by the European Food Safety Agency (EFSA Panel on Biological Hazards (BIOHAZ), 2010). For this reason, we chose to test the refining process on CVO 60 °C 10 min and CVO extracted at 80 °C 10 min because these the extraction conditions that offered the highest quality combined with a good product yield.

Crude Oil Refining: Effects of the Chemical Processes on Oil Quality

Crude fish oil is not suitable for direct human consumption, and further processing is need (Chakraborty & Joseph, 2015; EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2017; Šimat et al., 2019). Phospholipids, water, free fatty acids, mono and diglycerides, pigments, hydrocarbons, sterols, vitamins, pigments, carbohydrates, proteins, and lipid oxidation products may give undesirable flavors and colors (Chakraborty & Joseph, 2015; EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2017; Šimat et al., 2019). The refining process is necessary to improve the quality of crude fish oil. With the aim to remove undesirable components, CVO undergoes a refining process to obtain RVO and stabilize it (EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2017), as described below. The effect of the starting CVO quality, extracted at two temperatures (60 and 80 °C) on the different quality parameters of the final RVO (EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2011, 2017; Kuo et al., 2017; Šimat et al., 2019), are shown in Table 5.

Table 5. Effect of crude viscera oil (CVO) extraction temperature (60 and 80 °C) on the quality of refined viscera oil (RVO), as determined by peroxide value: PV; ρ-anisidine: ρ-AV; thiobarbituric acid reactive substances: TBARS; total oxidation value: TOTOX; phospholipids; % free fatty acid: %FFA. Commercial cod liver oil was used as the control oil (CO).

		Parameters					
Samples	**CVO Extraction Temperature**	**PV ($meqO_2$ /kg)**	**ρ-AV**	**TBARS (MDA µg/g)**	**TOTOX**	**Phospholipids (mg kg^{-1})**	**Acid value (%FFA)**
CVO	60°C	5.41±0.14^{b}	28.98±0.90 ^{d}	14.15±0.07^{d}	39.80±1.18^{c}	24.75±1.61^{d}	8.23±0.82 ^{d}
RVO		3.90±1.15^{b}	13.49±0.20^{b}	6.62±0.39^{b}	21.30±2.50^{b}	8.00±1.10^{a}	1.74±0.33^{b}
CVO	80°C	11.17±1.31^{d}	34.56±2.36^{e}	17.50±2.41^{e}	56.90±0.26^{e}	47.47±14.05^{e}	7.41±0.49 ^{c}
RVO		8.63±0.31^{c}	24.06±1.13^{c}	8.96±0.86^{c}	41.32±0.51^{d}	14.42±3.90^{c}	6.62±0.74^{c}
CO		2.10±0.53^{a}	4.96±0.89^{a}	5.51±0.81^{a}	10.83±0.24^{a}	10.74±2.45^{b}	0.50±0.04^{a}

Different superscript letters in the same column indicate significant differences (a, b, c…: $p < 0.05$). The data are reported as mean ± standard deviation (n = 12).

Similarly, in the present study, after the refining process of SPD, a decrease in the main parameters related to the lipid components oxidation was observed, in accordance with literature (Šimat et al., 2019; Soldo et al., 2019). In fact, both primary oxidation components (like PV) and secondary oxidation products (like ρ-anisidine (ρ-AV) and TBARS) showed a significant decrease in RVO produced from CVO that was extracted at 60 and 80 °C (Table 5). Total oxidation value (TOTOX) also reflected the trend of the previous lipid oxidation markers (Table 5) with a significant decrease in RVO produced from CVO extracted at 60 and 80 °C. These results confirmed the influence of the refining process on oil oxidation status and, in terms of PV and ρ-AV of RVO produced from

CVO extracted at 60 °C, had results comparable to those observed by Šimat et al. (2019). The values of TOTOX and TBARS (Table 5) in RVO produced from CVO extracted at 60 °C were lower than those observed by the same authors (Šimat et al., 2019). The results of this study for the PV, ρ-AV, and TOTOX contents of RVO at 60 °C were comparable to the values observed by Franklin et al. (2020) in oil extracted from yellowtail fish waste by supercritical CO_2 extraction. The contents of phospholipids, which act as emulsifiers and increase oil viscosity (Maschmeyer et al., 2020), have been analyzed to evaluate the effectiveness of the degumming, the first step of the refining process. As already reported (Maschmeyer et al., 2020; Mei et al., 2013; Vaisali et al., 2015), CVO refining determines a reduction of phospholipid content (Table 5) below 10 mg/kg, the optimal suggested value for edible oils (EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2011, 2017; Vaisali et al., 2015). The refining process is also able to remove free fatty acids (FFAs), which are among the most responsible for the characteristic rancid odor as consequence of the oxidation process in the oil (Chaiyasit et al., 2007), thus necessitating the deacidification step in the refining process (Vaisali et al., 2015). Our results showed a significant reduction of FFA in RVO produced from CVO extracted at 60 °C (Table 5), reaching values lower than 3%, which is the threshold recommended for edible oils (EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2017). Colorimetric analyses showed significant differences ($p < 0.05$) among all considered parameters between CVO and RVO (data not shown). These observations were comparable to those reported in the literature for refined fish oils (Afaq et al., 2005; Chakraborty & Joseph, 2015; García-Moreno et al., 2013), confirming that the refining process produces more transparent oil, an increase in the luminosity, and a tendency to be yellow (Afaq et al., 2005; Chakraborty & Joseph, 2015; García-Moreno et al., 2013). It is known that during the refining process, the removal of residues and impurities, such as oxidation products, peroxides, phospholipid, metals, soaps, and organic contaminants, leads to a brightening of the oil, as attested to by an increase of the L* parameter (Afaq et al., 2005; Chakraborty & Joseph, 2015; García-Moreno et al., 2013). Kuo et al. (2017) observed a different trend with the decrease in the a* value (redness) and increase in the b* value (yellowness) as a result of the removal of some pigments during the cobia liver oil refining process. In fact, the bleaching process could remove some pigments and their secondary products, like aldehydes, ketones, trace metals, and sulfurous compounds, to modify the final color of the refined oil (Kuo et al., 2017). SPD allows for the use of low temperatures for the deodorization and refining of fish oils, as well as significant decreases of oxidation molecules and free fatty acid contents in treated oil (Oliveira & Miller, 2014). By utilizing SPD, it is possible to obtain an oil that meet the quality standards for human consumption (EFSA Panel on Biological Hazards (BIOHAZ), 2010; FAO, 2011, 2017). Based on the analyzed quality markers, the RVO produced from CVO

extracted at 60 °C (Table 5) is the most appropriate oil for a direct human consumption, according to the current guidelines for fish oil for human consumption (PV ≤ 5 meqO_2/kg; ρ-AV ≤ 20; TOTOX ≤ 26; and AV ≤ 3 mg KOH/g) (FAO, 2011, 2017; EFSA Panel on Biological Hazards (BIOHAZ), 2010). The ω-3 PUFA fraction of RVO produced from CVO extracted at 60 °C was then enriched by SPD.

PUFA Enrichment

RVO transesterification showed an average yield of 65.5 ± 3.5% of TFA. The obtained TFAs were submitted to SPD to enrich the PUFA content, in the PUFAE fraction, via the elimination of the fraction containing short chain and EFAs. Fatty acid profiles, determined by gas chromatography, are reported in Figure 2

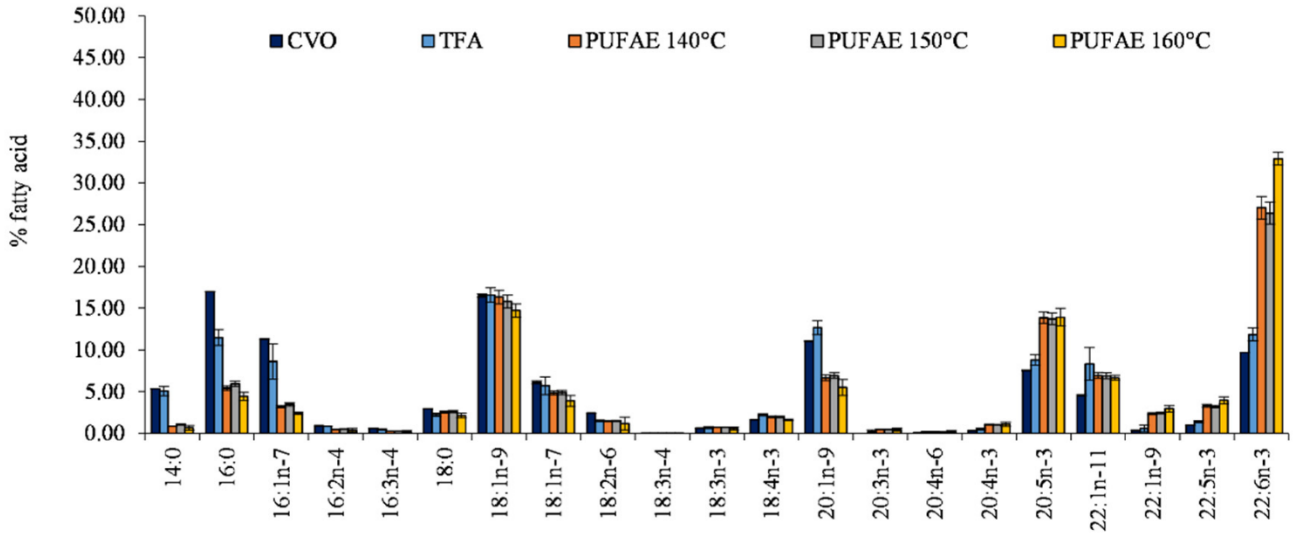

Figure 2. Fatty acid profile (% fatty acids) of total methyl esters obtained by crude viscera oil (CVO), the total ethyl esters of total fatty acid (TFA), and the fraction enriched in PUFA (PUFAE) by short path distillation (SPD) at the three utilized distillation temperatures of 140, 150, and 160 °C. The data are given as an average of the fatty acid profile from 3 distillations.

Differences among CVO, TFA, and PUFAE fatty acid profiles (Figure 2) confirmed the enrichment effect in PUFA induced by SPD (Figure 2). After increasing the distillation temperature, an increase of the percentage content of PUFA in PUFAE was observed (Figure 2). In particular, EPA and DHA increased from 8.80% and 11.84% in TFA to 13.85% (EPA) and 27.03% (DHA) in PUFAE separated at 140 °C, as well as up to 13.92% (EPA) and 32.90% (DHA) in PUFAE distillated at 160 °C (Figure 2). The enrichment of PUFA by SPD is a consequence of the elimination of the short-chain and saturated fatty acids that, under the operation conditions, were distillated in the light phase (Oliveira & Miller, 2014; Solaesa et al., 2016). In fact, after analyzing the percentage fatty

acid contents of TFA, PUFAE, and EFA, separated at the distillation temperature of 160 °C that showed the best yield (Table 6), this enrichment of PUFAE and the depletion of EFA was evident.

Table 6. Fatty acid profile (% fatty acids) and enrichment indexes of the ethyl esters of the total fatty acid (TFA), the fraction enriched in PUFA (PUFAE), and the fraction exhausted in fatty acid ethyl esters (EFA), as obtained by short path distillation (SPD) at 160 °C. The data are reported as mean ± standard deviation. The parameter R is defined as the concentration of EPA plus DHA to that of 16:0 plus 18:1 (Liang & Hwang, 2000).

Fatty acids	**TFA**	**PUFAE**	**EFA**
14:0	5.07 ± 0.57 ^{b}	0.66 ± 0.28 ^{a}	4.35 ± 0.05 ^{b}
16:0	11.47 ± 0.96 ^{b}	4.45 ± 0.46 ^{a}	11.26 ± 0.03 ^{b}
16:1n−7	8.63 ± 2.10 ^{b}	2.43 ± 0.16 ^{a}	7.48 ± 0.02 ^{b}
16:2n−4	0.86 ± 0.02 ^{b}	0.38 ± 0.21 ^{a}	1.01 ± 0.06 ^{b}
16:3n−4	0.48 ± 0.06 ^{b}	0.22 ± 0.12 ^{a}	0.65 ± 0.08 ^{b}
18:0	2.24 ± 0.19	2.15 ± 0.28	2.36 ± 0.23
18:1n−9	16.58 ± 0.87 ^{b}	14.72 ± 0.77 ^{a}	18.32 ± 0.27 ^{b}
18:1n−7	5.71 ± 1.07	3.90 ± 0.66	5.27 ± 0.57
18:2n−6	1.48 ± 0.13	1.17 ± 0.75	1.78 ± 0.06
18:3n−4	0.01 ± 0.01 ^{a}	0.02 ± 0.01 ^{a}	0.17 ± 0.00 ^{b}
18:3n−3	0.69 ± 0.11 ^{a}	0.60 ± 0.19 ^{a}	0.95 ± 0.02 ^{b}
18:4n−3	2.23 ± 0.11 ^{b}	1.61 ± 0.10 ^{a}	2.48 ± 0.14 ^{c}
20:1n−9	12.67 ± 0.83 ^{b}	5.51 ± 0.95 ^{a}	11.44 ± 0.05 ^{b}
20:3n−3	0.22 ± 0.19	0.48 ± 0.18	0.43 ± 0.00
20:4n−6	0.16 ± 0.08	0.20 ± 0.14	0.26 ± 0.00
20:4n−3	0.53 ± 0.11 ^{a}	1.09 ± 0.25 ^{b}	0.97 ± 0.01 ^{b}
EPA-20:5n−3	8.80 ± 0.63 ^{a}	13.92 ± 1.05 ^{b}	9.28 ± 0.03 ^{a}
22:1n−11	8.33 ± 1.95 ^{b}	6.67 ± 0.28 ^{a}	8.31 ± 0.19 ^{b}
22:1n−9	0.59 ± 0.39 ^{a}	2.96 ± 0.38 ^{b}	1.03 ± 0.05 ^{a}
22:5n−3	1.39 ± 0.11 ^{a}	3.97 ± 0.42 ^{b}	1.40 ± 0.05 ^{a}
DHA-22:6n−3	11.84 ± 0.78 ^{a}	32.90 ± 0.76 ^{b}	10.78 ± 0.19 ^{a}
SFA	18.78 ± 1.29 ^{a}	7.27 ± 0.17 ^{b}	17.97 ± 0.19 ^{a}
MUFA	52.51 ± 0.95 ^{b}	36.19 ± 0.88 ^{a}	51.84 ± 0.24 ^{b}
PUFA	28.71 ± 2.11 ^{a}	56.55 ± 0.93 ^{b}	30.19 ± 0.14 ^{a}
R (Fatty acid ratio)	0.74 ± 0.05 ^{a}	2.45 ± 0.20 ^{b}	0.68 ± 0.01 ^{a}
EPA Enrichment Factor	1.00 ± 0.07 ^{a}	1.58 ± 0.12 ^{b}	1.05 ± 0.00 ^{a}
DHA Enrichment Factor	1.00 ± 0.07 ^{a}	2.78 ± 0.06 ^{b}	0.91 ± 0.02 ^{a}
PUFA Enrichment factor	1.00 ± 0.07 ^{a}	1.97 ± 0.03 ^{b}	1.05 ± 0.00 ^{a}
PUFA/SFA	1.54 ± 0.21 ^{a}	7.79 ± 0.26 ^{b}	1.68 ± 0.02 ^{a}

Different superscript letters in the same row indicate significant differences (a, b, c…: $p < 0.05$). The data are reported as mean ± standard deviation, n = 12

The observed decrease in short-chain and saturated fatty acid percentage contents in PUFAE was a consequence of their distillation in the light fraction, EFA (Table 6). The concentration of the main short-chain and saturated fatty acid percentage content in TFA, 5.07% (14:0), 11.47% (16:0), and 2.24% (18:0), decreased in PUFAE distillated at 160 °C, 0.66% (14:0), 4.45% (16:0), and 2.43% (18:0) (Table 6). To reduce thermal damage to the long chain PUFA, the highest temperatures used was 160 °C. In fact, although the SPD technique is considered ideal for the separation of highly thermolabile components with minimal thermal degradation (Solaesa et al., 2016), operation conditions that include extremely high temperatures that could facilitate the separation of high molecular weight molecules are not recommended (Liang & Hwang, 2000).

This temperature, in addition, granted the highest PUFA enrichment. PUFA increased in PUFAE (from 28.71% to 56.55%), while in the EFA fraction, it was depleted (30.19%) (Table 6). Short-chain and saturated fatty acid percentage contents showed a significant decrease ($p < 0.05$) in PUFAE separated at 160 °C by SPD respect to TFA (from 18.78% to 7.27%), though it remained almost constant in EFA (17.97%). The total MUFA showed a significant decrease between TFA (52.51%) and PUFAE (36.19%), while no significant variation was observed in EFA (51.84%) (Table 6). The parameter R, defined as the concentration of EPA plus DHA to that of 16:0 plus 18:1 (Liang & Hwang, 2000), confirmed the increase in PUFAE from 0.74 to 2.45 in TFA, while it was constant in EFA (0.68) ($p < 0.05$) (Table 6). Similarly, the ratio between PUFA and the short-chain and saturated fatty acid percentage contents also increased in PUFAE respect to TFA (from 1.54 to 7.79) (Table 6). Therefore, respect to TFA, the content of EPA and DHA increased ($p < 0.05$) in PUFAE from 1.58 to 2.78, respectively. The PUFA trend showed a general enrichment of 1.97 (Table 6). Breivik et al. (1997), applying SPD at 125 °C on fatty acid ethyl esters from sardine oil, obtained an enrichment of 1.77 folds in EPA and of 1.6 folds in DHA. More recently, Valverde et al. (2013) obtained an enrichment of 1.8 folds in EPA at 200 °C. Our data, according to those reported for different oils (Oliveira & Miller, 2014; Solaesa et al., 2016; Valverde et al., 2013; Wang et al., 2012; Zhang et al., 2018), confirmed that SPD is an effective separation technology that can be used to concentrate PUFAs, in particular EPA and DHA, as ethyl esters from fish oil.

In Vitro Bioactive Properties of the Refined Oil

Many studies have underlined the beneficial effects of fish oils in cellular homeostasis, oxidative stress, and cardiovascular disease prevention and general health (Dong et al., 2018; Ghasemi Fard et al., 2019), thus rendering the test of these properties for new produced oils very useful in view of its commercialization. An in vitro test is an ideal and consolidated experimental model system that can confirm in reliable and fast way some bioactive properties exerted by natural compounds, such as

refined oils, in view of its further industrial applications (Cheng & Chen, 2015; Kusunoki et al., 2013; Riera-Heredia et al., 2020). In view of these considerations, toxicity, antioxidant properties, and modulation of adipogenesis were the main issues we considered in our work to address these oils in human consumption and fish meal inclusion. The effects of CVO, RVO, PUFAE, and EFA on the viability of 3T3 L1 cells exposed to an oxidative stress induced by hydrogen peroxide (H_2O_2; HP) treatment are reported in Figure 3. Oxidative stress induced by hydrogen peroxide (control plus HP) determined a significant reduction of viability ($p < 0.05$) with respect to untreated cells (control minus HP) (Figure 3) in 3T3 L1 cells. Compared to the controls not exposed to oxidative stress (control minus HP), treatments with CVO (CVO minus HP), RVO (RVO minus HP), and PUFAE (PUFAE minus HP) in cells not exposed to oxidative stress did not undergo a variation in cell viability. In 3T3 L1 cells exposed to EFA without oxidative stress induction (EFA minus HP), a significant reduction of viability ($p < 0.05$) was observed compared to both controls and CVO, RVO, and PUFAE treatments without oxidative stress induction (Figure 3).

Oxidative stress induction by hydrogen peroxide determined a viability reduction in control cells (control plus HP) and cells treated with CVO, RVO, and EFA (CVO plus HP, RVO plus HP, and EFA plus HP), compared to the controls not exposed to oxidative stress (control minus HP) (Figure 3). On the contrary, the preliminary treatment of 3T3 L1 cells with PUFAE exerted a marked protective effect. Furthermore, viability was higher in PUFAE and HP with respect to the control plus HP and all other treatments exposed to oxidative stress (CVO plus HP, RVO plus HP, and EFA plus HP) ($p < 0.05$) (Figure 3).

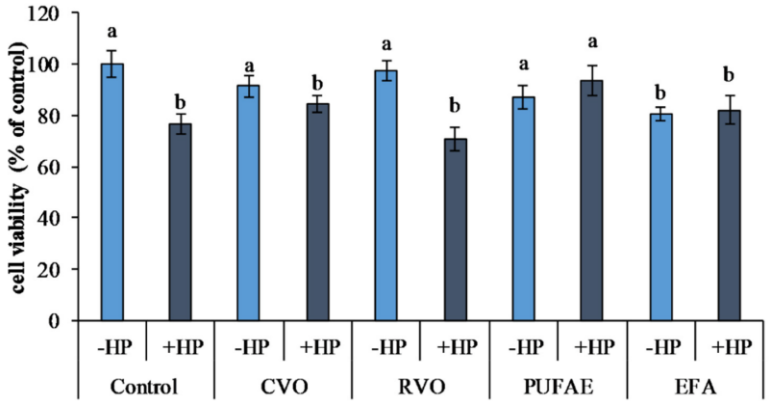

Figure 3. Effects of hydrogen peroxide (HP) (50 μM) induced oxidative stress and treatment with crude viscera oil (CVO), refined viscera oil (RVO), PUFA-enriched fraction (PUFAE), and short chain and unsaturated fatty acid-enriched fraction exhausted in fatty acid ethyl esters ((EFA) on the viability of 3T3 L1 cells. Different letters indicate significant differences ($p < 0.05$).

The reported results (Figure 3) suggested that treatment with PUFA exerted a protective effect against oxidative stress, as described by Sakai et al. (2017) on human aortic endothelial cells, where a significant reduction of cellular mortality after oxidative stress induced by hydrogen peroxide after EPA and DHA treatment was reported (Sakai et al., 2017). Similarly, Kusunoki et al. (2013) observed a protective effect of ω-3 PUFA on 3T3-L1 against hydrogen peroxide-induced oxidative stress, confirming the well-known beneficial properties of these bioactive molecules for human health (Kusunoki et al., 2013). The bioactive properties of the PUFAE were also studied in fish cell line adipogenesis by evaluating morphology variation during the differentiation of *D. labrax* pre-adipocytes induced by PUFAE and EFA. *D. labrax* pre-adipocytes started to differentiate after cell culture confluence (sixth day), in presence of factors-stimulating adipogenesis in the culture medium (L15); the differentiation was attested by the accumulation of lipid drops (Figure 4b); on the contrary, *D. labrax* pre-adipocytes, deprived of the adipogenic differentiation inducers in the culture medium, did not differentiate (Figure 4a). In *D. labrax* pre-adipocyte culture, the presence of PUFAE, after the initial steps of differentiation, stopped the increases volume and number of lipid droplet (Figure 4c), and the resulting undifferentiated adipocytes were almost similar to the undifferentiated controls (Figure 4a) in both form and size, indicating the anti-adipogenic effect of this treatment. On the contrary, EFA treatment promoted a significant lipid accumulation (Figure 4d). The accumulation of cytoplasmatic lipid droplets was moderate during the first three days, but it increased significantly on the fourth day up to a total adipocyte hypertrophy on the seventh day (Figure 4d), thus suggesting that EFA may stimulated fat uptake and fat cytoplasm accumulation (Riera-Heredia et al., 2020).

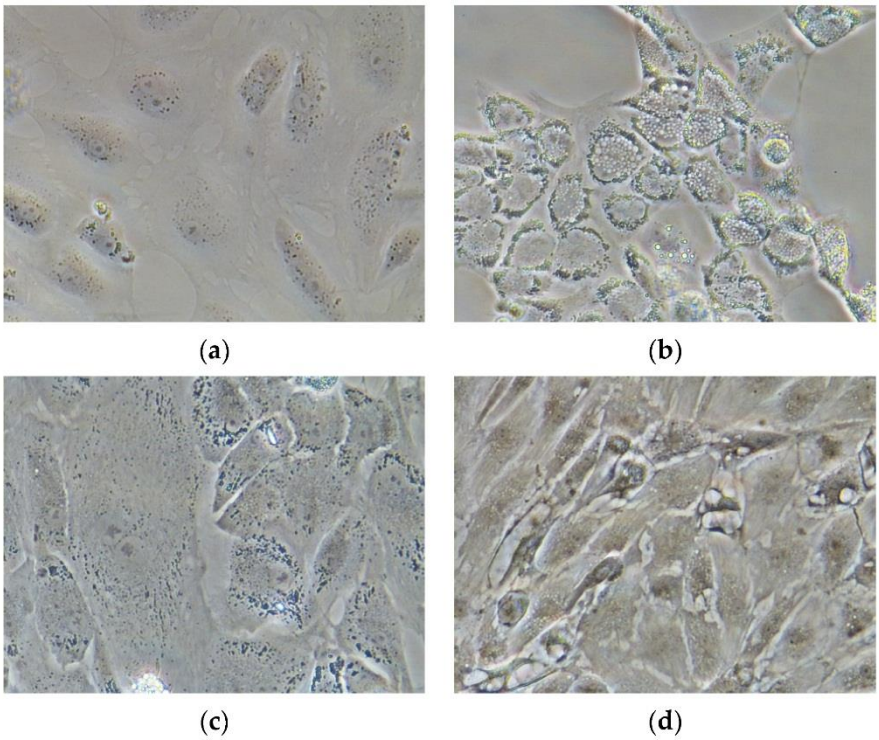

Figure 4. *D. labrax* pre adipocytes under differentiation (representative phase-contrast images at 40X magnification): (a) control undifferentiated cells; (b) differentiated adipocyte, induced by standard differentiation medium; (c) Differentiated adipocyte-induced by medium supplemented with PUFAE; (d) differentiated adipocytes, induced by medium supplemented with EFA.

These findings agreed with results describing the anti-adipogenic effects of EPA and DHA during induced pre-adipocyte differentiation in cobia (*Rachycentron canadum*) (Cheng & Chen, 2015), Atlantic salmon (Vegusdal et al., 2003), and rainbow trout (Riera-Heredia et al., 2020). It is well-known that the fatty acid composition of the diet influences the fatty acid composition of fish (Benedito-Palos et al., 2011; Lenas et al., 2011; Rincon Cervera et al., 2015; Sinanoglou et al., 2017), and that this well-recognized property represents the basis of the artificial diet formulations in aquaculture that are aimed to increase growth performance while maintaining high quality. Growth performance and quality are mainly influenced by the energy content of the artificial diets, integrated by the addition of vegetal oils, rich in monounsaturated and omega-6 fatty acids, by patterns of lipid distribution and metabolic management (Grigorakis, 2007; Lenas et al., 2011; Santulli et al., 1997). These formulations and the consequent high caloric contents are often responsible of the excessive fat deposition in farmed fish compared to the wild (Grigorakis, 2007; Lenas et al., 2011), which is recognized as the principal issue related to oxidative stress and consequent peroxidation due to its negative effects on fish welfare, quality, and consumer perception. In this sense, research actions aimed to preliminarily evaluate the effects of oil composition on antioxidant prevention and lipid deposition are useful to assess the nutritional

properties of new formulations. Our in vitro observations confirmed that PUFAs have significant effects on lipid metabolism and can influence the deposition of lipids in adipocytes, suggesting a possible effect on fat deposition in fish fillet. Therefore, regulating the composition of fatty acids in the diet could strategically change the lipid deposition in various tissues and, consequently, the lipid profile of the edible parts of fish. However, further studies are needed to understand how, through dietary manipulation, it is possible to modulate the adiposity in fish fillets in order to obtain a higher quality product.

Materials and Methods

Sampling

SBV was sampled at the ″Acqua Azzurra s.r.l.″ intensive aquaculture and fish processing farm (Pachino, SR, Italy), immediately placed on ice, transported to the laboratory, grinded, divided into aliquots of 500 g, and stored in zip-lock polyethylene bags at −80 °C, pending further analysis; treatments as summarized in Figure 5.

Proximate Composition and Fatty Acid Profile

The SBV proximate composition was evaluated by determining water and ash (AOAC International, 2005), crude protein (AOAC, 1992), and total lipid (Folch et al., 1957) contents. The fatty acid methyl ester profile of viscera lipids was determined, after transesterification, by GC using a Perkin Elmer Clarus ® 580 gas chromatograph (Perkin Elmer, Shelton, CT, USA) under previous conditions (Messina et al., 2013a).

Extraction of Crude Oil

CVO was extracted from 20 kg SBV batches by wet extraction (Sathivel et al., 2003). Preheated distilled water was added to ground SBV at a 1:2 w/v ratio, and the mixture was incubated in a 50 L steel reactor equipped with an internal heating coil. Extraction trials were performed under constant agitation at different temperatures (40, 60, 80, and 90 °C) and for different reaction periods (10, 30, and 60 min).

Extraction mixtures were filtered on a 125 μm mesh sieve to remove the coarse particulates. The filtrate liquid phase was centrifuged at a centrifugal force of 40,000× g by a continuous tubular centrifuge (CEPA, Carl Padberg, Zentrifugenbau GmbH, Lahr/Schwarzwald, Germany) equipped with a separating cylinder (type TR). The extraction mixture was fed at the bottom of the cylinder

by a Masterflex L/S peristaltic pump equipped with tubing L/S 18 (Cole-Parmer s.r.l., Mi, Italy), with a through-puts of 0.03 L min^{-1}. This configuration allowed us to separate contemporary and continuously solids (retained in the cylinder), a heavy liquid phase (containing protein and cellular end tissue debris), and a light liquid phase (containing CVO) that exited the cylinder in two separate fluxes. CVO samples were stored at −20 °C in 2.5 L dark bottles under nitrogen.

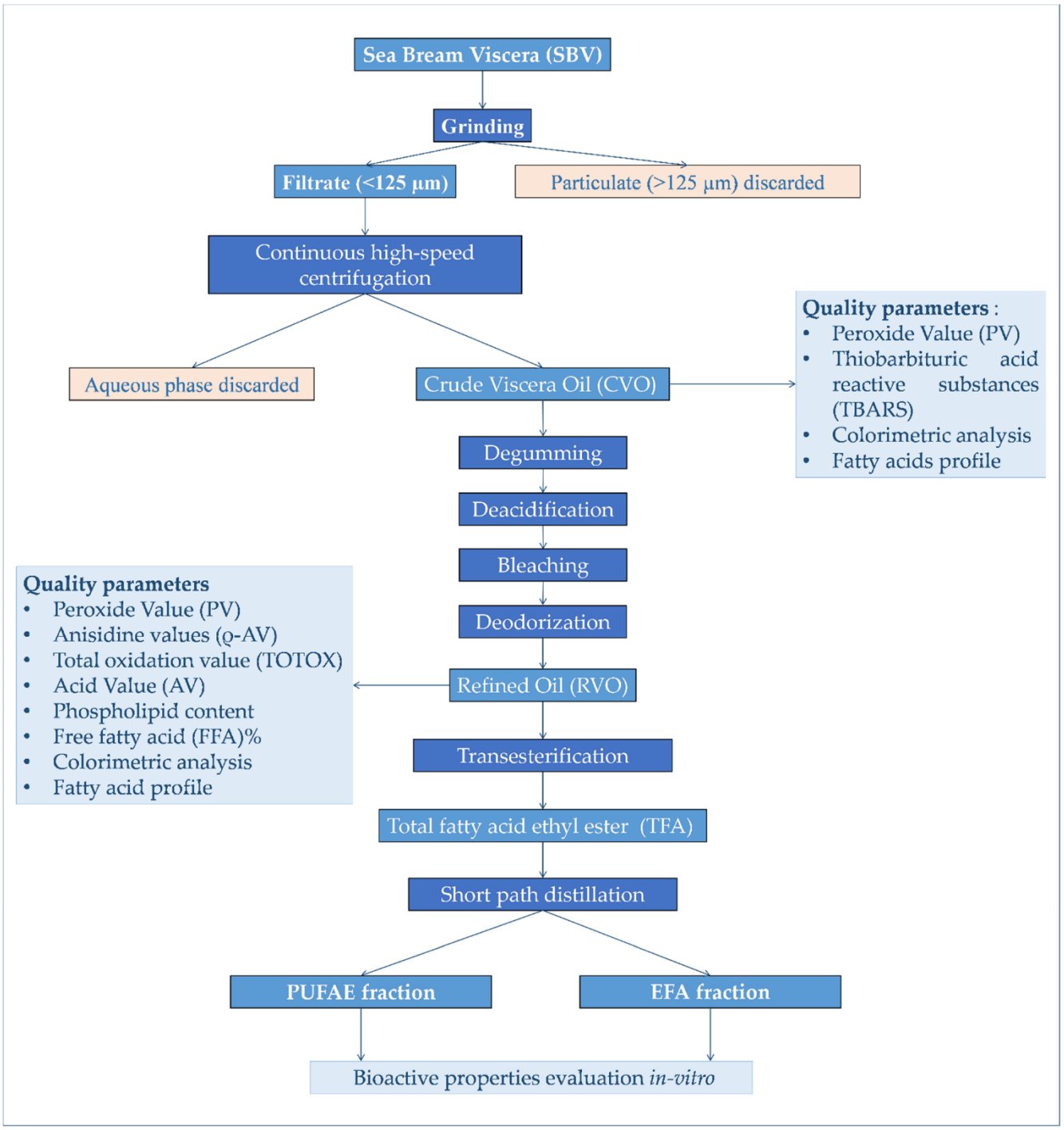

Figure 5. Experimental design adopted for the processing and production of ω-3-enriched-oil, as well as the evaluation of its bioactivity in vitro.

Chemical Refining Process of CVO

The CVO refining process (Figure 5) was carried out in 5 L batches. During the refining process, when required, all liquid phase separation was carried out by a continuous tubular centrifuge equipped with a separating cylinder, as described above. CVO was degummed and neutralized following the procedure of Chakraborty and Joseph (Chakraborty & Joseph, 2015). After degumming and neutralization, CVO bleaching was done to remove color compounds by treatment through a column of activated charcoal powder (5% w/w of CVO). Bleached CVO was deodorized at low temperatures by a VLK 70-4 short path distillation unit (VTA Gmbh; Niederwinkling, Germany). Bleached CVO was kept at 60 °C during the process and continuously fed into the feed vessel by a peristaltic pump (feeding rate 3 L/h) at an evaporator temperature of 120 °C, a condenser temperature of 25 °C, a vacuum of 5 mbar, and a rotor speed of 400 rpm. At the end of the refining process, RVO was collected as the residual phase of SPD and stored at −20 °C in 2.5 L dark bottles under nitrogen, pending further analyses and processing.

Assessment of Oil Quality

The refining process was evaluated using commercial cod liver oil as the CO (Pearson, Campo Ligure, Ge, Italy). The quality of CVO and RVO was evaluated during the various refining steps by monitoring PV, TBARS, ρ-AV, TOTOX, phospholipid content, and free fatty acid (FFA)%, as well as through colorimetric analysis.

Peroxide Value (PV)

Aliquots (1.0 g) of oil samples were used for PV determination. PV, expressed in milliequivalents of active oxygen per kg of oil (meqO_2/kg), was evaluated by iodometric titration with a standard solution of sodium thiosulphate (AOCS, 1998).

Thiobarbituric Acid Reactive Substances Analysis (TBARS)

TBARS analysis was carried out on 0.1 g aliquots of oil following the work of Botsoglou et al. (1994). Spectrophotometric quantification was performed at 532 nm with a UV–Vis spectrophotometer (Cary 50, Varian Inc., Palo Alto, California, US,) using a standard curve of MDA (0.001–0.5 μg/mL 5% trichloroacetic acid), and the results are expressed as content of MDA μg/g.

Content of ρ-Anisidine (ρ-AV)

The ρ-AV was assessed according to the official AOCS method (AOCS, 1993) slightly modified by Honold et al. (2016). Spectrophotometric quantification was performed by assessing the absorbance of each sample (0.5 g) at 350 nm (As) using a UV–Vis spectrophotometer (Cary 50, Varian Inc., Palo Alto, CA, USA) against a blank (Ab) (5 mL Chloroform with 1 mL ρ-anisidine solution) (Sigma, Aldrich).

The ρ-AV calculation is given by Equation (1):

$$\rho - AV = \frac{25x(1.2As - Ab)}{sample\ weight(g)} \qquad 1)$$

Total Oxidation Value (TOTOX)

TOTOX was determined according to Holm (1972) and calculated using Equation (2):

$$TOTOX = (2\ x\ PV) + \rho - AV \qquad 2)$$

Phospholipid Content

The phospholipids, in crude and refined oil, were separated from the total triglycerides by siliceous matrix columns (HF Bond Elut SI, 100 mg, 1 mL, Varian, Palo Alto, CA, USA) by adding 0.1 g of CVO and RVO diluted in 1 mL N-hexane to the top of column and flushing the column with 20 mL of chloroform to separate triglycerides in the eluate.

The column was then flushed with 20 mL of methanol and the eluate to recover phospholipids, the contents of which were then determined gravimetrically.

Acid Value

Acid value was evaluated on aliquots of 1 g of oil sample according to the official AOCS method (AOCS, 1999) by acid–base titration using an ethanol solution of potassium hydroxide (KOH 0.1 N in ethanol 96%) as the titrant and phenolphthalein as the indicator. The acid value was expressed as % oleic acid equivalent according to Equation (3) (Aryee et al. 2011):

$$\%FFA\ (oleic\ acid) = \frac{(Vc - Vb)\ x\ N\ x\ 28.8}{sample\ weight\ (g)} \qquad 3)$$

where Vc is volume of the titrant solution used for the sample, Vb is the volume of the titrant solution for the blank, and N is KOH concentration.

Colorimetric Analysis

The color analysis was performed following the work of Sathivel et al. (2003) on three replicates of each sample (1 mL) by a Konika Minolta CR 400 spectrophotometer (Konica Minolta Chroma Co., Osaka, Japan). Results are expressed by the parameters of L* a*, and b* and by derived variables of color saturation (C*), hue angle (h), and total color variation (ΔE) (Stearns, 1988).

PUFA Enrichment

Batches of 2.5 L of RVO were trans esterified to obtain ethyl esters TFAs without the use of any solvent other than ethanol (Vázquez & Akoh, 2010). During transesterification, all liquid phase separations were carried out by a continuous tubular centrifuge equipped with a separating cylinder, as described above. Batches of 2 L of TFA were distilled by SPD using the VLK 70-4 molecular distillation unit (VTA Gmbh, Niederwinkling, Germany) with an evaporating surface of 4.8 dm^2.
Before PUFA enrichment, to remove impurities and any solvent traces, TFA underwent a degassing step.
TFA samples, preheated to 40 °C, were loaded into the feed vessel (at 40 °C) by a peristaltic pump. Distillation trials were run utilizing the following operating conditions: feeding vessel at 40 °C, condenser at 25 °C; evaporator at 140, 150, or 160 °C (120 °C for degassing); feeding rate of 300 mL/h (500 mL/h for degassing); roller speed of 400 rpm; and vacuum of <0.01 mbar (5 mbar for degassing).
PUFAE (heavy phase) and EFA (distilled phase) were collected, and yields were determined gravimetrically.
In order to evaluate the enrichment process every 1.0 L TFA feed and at the end of distillation, aliquots of the two separated phases were diluted at 1% in c-hexane to analyze fatty acid profiles by GC. Based on the fatty acid profile, the following indices were calculated:

- EPA and DHA%.
- Fatty acid ratio (R), according to Equation (4) (Liang & Hwang, 2000):

$$R = \frac{([EPA] + [DHA])}{[16{:}\,0] + [18{:}\,1\ \omega 9]} \qquad 4)$$

- Enrichment factor for EPA, DHA, and PUFA.
- Ratio of total PUFA to total saturated fatty acids (PUFA/saturated).

In Vitro Bioactive Properties

3T3 L1 mouse cell lines (ECACC n. 86052701, Sigma® (Sigma-Aldrich, Saint Louis, MO, USA) were cultured in 75 cm 2 plastic flasks (Nunc, Darmstadt, Germany) in Dulbecco's Modified

Eagle's Medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, and 100 μg/mL of penicillin–streptomycin (all reagents from Sigma-Aldrich, Saint Louis, MO, USA); they were incubated in a humidified atmosphere (CO_2 5%). Cells were seeded in 96-well plates at a concentration of 7×10^3 cells/well and incubated for 24 h. After 24 h, the cells were treated (three replicates) with CVO, RVO, PUFAE, and EFA dissolved in ethanol at a concentration of 5 μg/mL in the medium, with a final solvent concentration of 0.1% (v/v) and left to incubate for 24 h.

As attested by internal routine procedures (Messina et al., 2019a), ethanol did not exert any detrimental effects when used as vehicle.

After a preliminary test, aimed to assess the dose/dependent toxicity of the oils in a concentration range of 1–5 μg/mL, a final concentration of 5 μg/mL was selected for the bioactivity test.

After 24 h of incubation, control cells and cells incubated with CVO, RVO, PUFAE, and EFA were exposed to oxidative stress by 50 μM hydrogen peroxide, according to a previous standardized protocol (Abbes et al., 2013; Manuguerra et al., 2018; Messina et al., 2019a; 2019b) and incubated at 37 °C for 2 h. The viability was determined by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) method according to Mosmann (1983).

The optical densities (ODs) at 570 nm with background subtraction at 690 were determined in a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA).

The percentage of viability was determined by Equation (5):

$$Viability\ (\%) = \left(\frac{\text{OD of the test sample}}{\text{OD of the control sample}}\right) x\ 100 \qquad 5)$$

OD measurements were performed in triplicate.

D. labrax pre-adipocytes were maintained in an L-15 Leibowitz medium (Sigma, London, UK) supplemented with 10% fetal bovine serum (FBS, Sigma, UK), 2 mM L-glutamine (Sigma, UK), 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES; Sigma, UK), and penicillin–streptomycin solution (Sigma, UK), and they were seeded in 25 cm^2 plastic tissue culture flasks (Nunc, Germany). The cells were kept at 20 °C, and the growth medium was changed every 2–3 days. The cell culture reached confluence after approximately 1 week.

The differentiation of *D. labrax* pre-adipocytes was induced by the slightly modified differentiation-inducing medium described by Todorčević et al. (2008).

The L-15 medium was supplemented with 1 μM dexamethasone, 1 μM isobutylmethylxanthine, 20 μg/mL of insulin, and 0.2 μL/mL of lipid mixture, corresponding to 45 mg/mL of cholesterol and 100 mg/mL of cod liver oil ethyl esters instead of methyl esters (Todorčević et al., 2008).

To study its effect on pre-adipocyte differentiation, the confluent pre-adipocyte culture was incubated as follows: (1) L15 medium deprived of adipocyte differentiation inducers (control), (2) pre-adipocyte standard differentiation medium, (3) pre-adipocyte standard differentiation medium supplemented with 0.2 μL/mL of PUFAE instead of 0.2 μL/mL of lipid mixture, (4) pre-adipocyte standard differentiation medium supplemented with 0.2 μL/mL of EFA instead of 0.2 μL/mL of lipid mixture.

The medium was changed every three days until the cells reached the final step of differentiation (the morphology of mature adipocytes) (21 days) (Ljubojević et al., 2014; Todorčević et al., 2008).

Image Acquisition

Cells in the culture were observed daily using a Nikon Eclipse Ti-S inverted microscope (Nikon Instrument Inc., Melville, NY, USA), and images were captured by a Nikon DS-L3 digital camera (Nikon Corporation, Tokyo, Japan) and the DS-L3 Digital Camera Controller acquisition software. Images represent pre-adipocytes cells observed with phase contrast microscopy at 40X magnification.

Statistical Analysis

Results are reported as mean ± standard deviation. Observed differences among the effects of temperature and extraction time during the extractions were analyzed by an ANOVA (Underwood, 1977). Differences observed among results of the refining process were analyzed by Student t tests. Cochran′s C test was used to test the assumption of variance homogeneity. Student–Newman–Keuls (SNK) post hoc tests were conducted for all significant interaction terms (Underwood et al., 1997). The differences were considered significant for $p < 0.05$. All elaborations were performed using STATISTICA 7.0 (Statsoft Inc., Tulsa, OK, USA).

Conclusions

There is a wide and extensive body of literature attesting to the presence and properties of the bioactive compounds contained in marine discard, wastes, and processed by-products and that stimulate their utilization in diverse industrial sectors; moreover, there is a discrepancy between laboratory and industrial applications, as most results are valid only at the lab scale. This situation is responsible for the delay in innovation in the sector of marine biotechnology and blue economy, which needs the implementation of procedures protocols with high technological readiness levels (TRLs) in order to boost productivity and competitiveness. The present paper attests to the validity

of the pilot protocol for processing high volumes of industrial aquaculture by-products in high volumes of enriched fish oil in view of its application in the real world. This example could stimulate the adoption of solutions aimed to recover and utilize aquaculture by-products at a higher scale, turning “waste into profit” and indicating a strategy to reach more sustainable business models in aquaculture resource utilization according to the principles of the circular economy.

We started from an extensive study on the chemical and nutritional characterization of sea bream by-products (crude and enriched oil extracted from SBV), followed by bio-activity evaluation.

The obtained results showed that SBV is a very suitable source among sea bream waste because it is very rich in lipids and therefore an excellent matrix for production of fish oils. Crude oils showed good characteristics, and refined oils were found to be adequate for a direct human consumption according to the current European Food Safety Authority (EFSA) guidelines. In addition, SPD was found to be a simple, economical, and environmentally sustainable technique that resulted in a product containing up to 56% long-chain PUFAs. Furthermore, the in vitro approach represents a fast and reliable way to appreciate the effects of oil in cells in view of its application for nutraceutical uses and feed formulations in aquaculture to ensure an optimal degree of reared fish adiposity.

Our study confirms the potential recycling of fish by-products for conversion into products of higher value and the reduction of the impact of the aquaculture sector, improving its economic performance and conforming to zero waste strategies.

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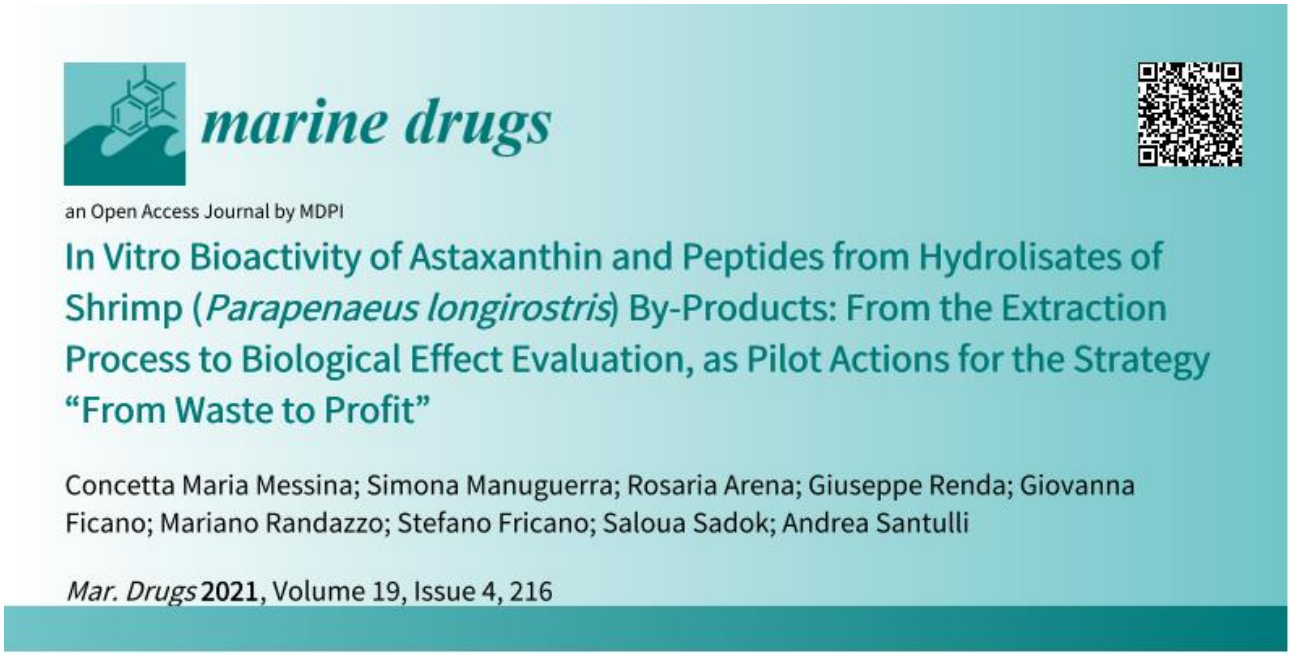
Chapter 6



Abstract

Non-edible parts of crustaceans could be a rich source of valuable bioactive compounds such as the carotenoid astaxanthin and peptides, which have well-recognized beneficial effects. These compounds are widely used in nutraceuticals and pharmaceuticals, and their market is rapidly growing, suggesting the need to find alternative sources. The aim of this work was to set up a pilot-scale protocol for the reutilization of by-products of processed shrimp, in order to address the utilization of this valuable biomass for nutraceutical and pharmaceuticals application, through the extraction of astaxanthin-enriched oil and antioxidant-rich protein hydrolysates. Astaxanthin (AST) was obtained using "green extraction methods," such as using fish oil and different fatty acid ethyl esters as solvents and through supercritical fluid extraction (SFE), whereas bioactive peptides were obtained by protease hydrolysis. Both astaxanthin and bioactive peptides exhibited bioactive properties in vitro in cellular model systems, such as antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities (IA). The results show higher astaxanthin yields in ethyl esters fatty acids (TFA) extraction and significant enrichment by short-path distillation (SPD) up to 114.80 ± 1.23 µg/mL. Peptide fractions of < 3 kDa and 3–5 kDa exhibited greater antioxidant activity while the fraction 5–10 kDa exhibited a better ACE-IA. Lower-molecular-weight bioactive peptides and astaxanthin extracted using supercritical fluids showed protective effects against oxidative damage in 142BR and in 3T3 cell lines. These results suggest that "green" extraction

methods allow us to obtain high-quality bioactive compounds from large volumes of shrimp waste for nutraceutical and pharmaceutical applications.

Keywords: shrimp by-products; supercritical fluid extraction; astaxanthin; fish oil; SPD; PUFA; proteolytic enzymes; protein hydrolysates; antioxidant activity

Introduction

The global demand for fish and marine ingredients is growing rapidly, highlighting the need for sustainable management of marine resources, with actions aimed to better understand the intrinsic biodiversity of the marine environment and to preserve it (Caruso et al., 2020). The recovery of fishery discards, as well as processing waste and marine by-products (Ucak et al., 2021), is necessary so as to save biological resources and to apply the circular economy principle "waste to profit." This principle consists in a commitment from fishery, aquaculture, and fish processing value chains to develop high-value bio-based marine products with a reduced environmental footprint and to contribute to the United Nations' Sustainable Developmental Goals.

Among the wide range of marine bioactive compounds, antioxidant carotenoids are a large group of organic and lipophilic pigments, known for their biological activities, which are produced by plants, algae, various bacteria, and fungi, and are present in huge amounts in crustacean wastes (Caruso et al., 2020; Fathalipour et al., 2020; Giannaccare et al., 2020; Rivera-Madrid et al., 2020). Several studies have been conducted on the beneficial effects of carotenoids in the prevention and management of a large number of diseases, including cancer, cardiovascular diseases, diabetes, osteoporosis, ophthalmic diseases, Alzheimer's disease, and infectious diseases (Fathalipour et al., 2020; Giannaccare et al., 2020; Rivera-Madrid et al., 2020). They can also be used as nutritional supplements in nutraceuticals and pharmaceuticals (Bhatt & Patel, 2020; Fathalipour et al., 2020).

Astaxanthin (AST), contained in the exoskeleton and cephalothorax of crustaceans, is a cheto-carotenoid (3,3′-dihydroxy-β, β-carotene-4,4′-dione) belonging to the family of xanthophylls, derived from the oxidation of β-carotene. Due to its peculiar molecular structure, it has antioxidant, anti-tumor, anti-inflammatory, anti-diabetic, immunomodulatory, and neuroprotective effects (Bhatt & Patel, 2020; Fathalipour et al., 2020; Harnedy & Fitzgerald, 2011; Hussein et al., 2006; Messina et al., 2019a; Radzali et al., 2014; Sila et al., 2015). that suggest important applications in functional foods, cosmetics, and the food industry (Guerin, Huntley, & Olaizola 2003; De Holanda & Netto 2006).

This pigment shows higher antioxidant activity than other carotenoids such as α and β-carotene, lutein, lycopene, canthaxanthin, and vitamin E (Radzali et al., 2014). Due to its cytoprotective and antioxidant capacity, AST has been presented as a promising therapeutic strategy in various ocular diseases (Giannaccare et al., 2020).

It has been suggested that carotenoids prevent, delay, and improve retinopathy in diabetes (Fathalipour et al., 2020). Baccouche et al. (2017) studied the effect of AST, extracted from shrimp waste, on adult retinal cells of the type-2 diabetic model *Psammomys obesus* in hyperglycemic conditions. Their results revealed that AST decreased cell apoptosis, improved mitochondrial function, and improved neurons and the viability of glial cells. Other studies reported that doses of 3 mg/kg, over an eight-week treatment period, reduced retinal oxidative stress and inflammatory mediators in rats with streptozotocin-induced diabetes (Yeh et al., 2016). The utilization of AST, as a promising therapeutic strategy in ocular disease and in particular in diabetic retinopathy, requires the use of AST extracted using "green" methods, as an alternative to traditional chemical methods that use toxic solvents (Razi Parjikolaei et al., 2017). The "green techniques" are based on the discovery and design of extraction processes that reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product (Chemat, Vian, & Cravotto, 2012; Yara-Varón et al., 2017).

It has been reported that carotenoids can be extracted from shrimp waste using various vegetable oils and their methyl esters (ME) (Parjikolaei et al., 2015, 2016, 2017; Sachindra & Mahendrakar, 2005; Yara-Varón et al., 2017) or cod liver oil (Shahidi & Synowiecki, 1991). Sunflower oil and its ME have been recently indicated as potential "green" solvents that could replace traditional organic solvents (Vian et al., 2017; Yara-Varón et al., 2017). Extraction using sunflower oil methyl ester was the most efficient "green process" studied in terms of production rate and unit cost of concentrated AST (Parjikolaei et al., 2015, 2016, 2017; Sachindra & Mahendrakar, 2005). The use of fish oil, in addition to the advantages already listed regarding the use of vegetable oils and their esters, including excellent solvent properties for the extraction of carotenoids, adds a high content of ω-3 fatty acids (Shahidi & Synowiecki, 1991), which, as reported in the literature, showed, similarly to AST, a beneficial effect in the protection against diabetic retinopathy in obese mice (Dátilo et al., 2018).

The "green extraction" of AST could also be performed using supercritical CO_2 extraction (Ahmadkelayeh & Hawboldt, 2020; Khawli et al., 2019; Messina et al. 2019a; Parjikolaei et al., 2017; Radzali et al., 2014), which is characterized by high solvent power and selectivity of extraction combined with non-toxicity (Herrero et al., 2010; Herrero, Cifuentes, & Ibañez, 2006; Radzali et al., 2014); additionally, it is a non-flammable solvent capable of extracting thermolabile

compounds. From a sustainability point of view, for the nutraceutical industry the extraction of AST from fresh waste (which also involves high disposal costs) represents a great advantage over its chemical synthesis (Radzali et al., 2014).

Shrimp waste, which is mainly made up of the exoskeleton and cephalothorax, makes up from 50 to 70% of its total fresh weight and could contain other components of high biological value apart from AST, such as bioactive peptides obtained from protein hydrolysates, chitin, and chitosan, whose quantities depend on the species and processing conditions (Caruso et al., 2020; De Holanda & Netto, 2006; Maschmeyer, Luque, & Selva, 2020; Nguyen et al., 2020; Sila, Nasri, & Bougatef, 2012). Protein hydrolysates, obtained from shrimp waste by enzymatic reaction, can be integrated in formulated diets for aquaculture as sources of biologically active peptides with a considerable potential in pharmacology and nutraceutical applications and therapies (Doan et al., 2020; Giannetto et al., 2020; De Holanda & Netto, 2006; Jafarpour et al., 2020; Maschmeyer, Luque, & Selva, 2020). The activities reported in the literature for bioactive peptides are very diverse, ranging from antioxidant power to the measurement of angiotensin-converting enzyme inhibition activity (ACE-IA), which is related to the conversion of angiotensin I into angiotensin II (with beneficial side effects on the control of the hypertension), as well as anti-coagulant activity and the regulation of calcium absorption and immune responses (Doan et al., 2020; Kim & Mendis, 2006; Maschmeyer, Luque, and Selva, 2020).

Parapenaeus longirostris is one of the most important commercial shrimp species, distributed and processed throughout the Mediterranean (Sila, Nasri, & Bougatef, 2012), generating a significant amount of by-products (BP), that could represent an important source of AST and bioactive peptides, useful for nutraceuticals and pharmaceuticals application.

The aim of the present study was to define a pilot protocol for the green extraction of AST and bioactive peptides from *P. longirostris* BP, demonstrating their bioactivity, in order to reach the zero-waste goal, by addressing this important biological resource to other applications, avoiding its waste.

Based on reported experiences (Parjikolaei et al., 2015, 2016, 2017; Sachindra & Mahendrakar, 2005) “green procedures” for AST extraction from large amounts of BP, were represented by supercritical fluid extraction (SFE), fish oils, and ethyl esters (ES). The latter solvent allowed to concentrate AST using short-path distillation (SPD) (Othman et al., 2010; Parjikolaei et al., 2017) and CO_2 SFE was also tested as an alternative “green extraction procedure” (Ahmadkelayeh & Hawboldt, 2020; Khawli et al., 2019; Messina et al., 2019a; Parjikolaei et al., 2017; Radzali et al., 2014). Finally, AST extracts and bioactive peptides were tested in vitro for antioxidant capacity and ACE-IA. The definition of the pilot scale protocol for the extraction of AST and PH, together with

the assessment of its bioactive properties, was addressed to support the value-chains of the *P. longirostris* processing plants to exploit its BP at industrial scale, in order to turn wastes to profit, both for the environment and for the economy.

Results and Discussion

Proximate Composition and Fatty Acid Profile of P. longirostris By-Product

The proximate composition of BP (exoskeleton including cephalothorax and abdominal parts), reported in Table 1, fall within the ranges reported in the literature, which showed a large variability according to the species (Arbia et al., 2013; Díaz-Rojas et al., 2006; De Holanda & Netto, 2006; Sánchez-Camargo et al., 2012). Arbia et al. (2013), analyzing the crude exoskeleton composition of *P. longirostris*, reported lower values of ash (25%) and protein (29%) and higher values of chitin (27%) and lipids (15%).

Table 1. Proximate composition (g/100 g DW [a]) of *P. longirostris* by-products (BP).

	g/100 g
Moisture	3.43 ± 0.16
Ash	36.40 ± 0.61
Lipid	4.96 ± 0.17
Protein	38.47 ± 0.46
Chitin	16.75 ± 1.06

[a] sample dry weight.

Considering the possible effects of thermal treatment on fatty acid composition, this parameter was evaluated on both wet (WBP) and dry *P. longirostris* by-products (DBP). It is known, in fact, that high temperatures, during the drying phase, can alter the fatty acid profile, leading to a reduction in PUFA content (Fox et al., 1994; Routray et al., 2017; Yves et al., 2016).

In WBP the predominant class was PUFA of n-3 series with a percentage of 36.08 ± 2.94% (Table 2). Within this series, docosahexaenoic acid, DHA (22:6 n3), showed the highest percentage value (21.66 ± 1.73%), followed by eicosapentaenoic acid, EPA (20:5 n3) (11.97 ± 1.07%) (Table 2). DBP showed values of 16.45 ± 0.90 and 9.69 ± 0.43% for DHA and EPA, respectively (Table 2). The high content of n-3 fatty acids, especially EPA and DHA, suggests the potential use of these matrices as a source of n-3 fatty acids (Sánchez-Camargo et al., 2012).

Table 2. Fatty acid profile (% of total fatty acids) of wet (WBP) and dry (DBP) *P. longirostris* by-products. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

	WBP	**DBP**
14:0	1.47 ± 0.06	0.83 ± 0.08
16:0	17.67 ± 0.20	13.10 ± 0.61
16:1n-7	3.40 ± 0.22	2.46 ± 0.13
16:2n-4	0.38 ± 0.05	0.29 ± 0.03
16:3n-4	0.70 ± 0.06	0.67 ± 0.04
18:0	6.61 ± 0.18	6.02 ± 0.13
18:1n-9	18.25 ± 1.91	22.84 ± 0.55
18:1n-7	4.03 ± 0.18	3.81 ± 0.16
18:2n-6	4.00 ± 1.67	15.24 ± 0.51
18:3n-4	0.23 ± 0.05	0.19 ± 0.03
18:3n-3	0.52 ± 0.04	0.36 ± 0.04
18:4n-3	0.29 ± 0.05	0.16 ± 0.02
20:1n-9	1.54 ± 0.07	1.34 ± 0.04
20:4n-6	4.68 ± 0.39	4.32 ± 0.33
20:4n-3	0.38 ± 0.05	0.23 ± 0.06
20:5n-3 (EPA)	11.97 ± 1.07	9.69 ± 0.43
22:1n-11	0.68 ± 0.06	0.53 ± 0.04
22:1n-9	0.31 ± 0.08	0.26 ± 0.06
22:5n-3	1.26 ± 0.27	1.04 ± 0.14
22:6n-3 (DHA)	21.66 ± 1.73	16.45 ± 0.90
Saturated	25.74 ± 0.31	19.95 ± 0.74
Monounsaturated	28.21 ± 1.72	31.24 ± 0.45
Tot n-3	36.08 ± 2.94	27.92 ± 1.47
Tot n-6	8.67 ± 1.34	19.56 ± 0.51
DHA/EPA	1.81 ± 0.04	1.70 ± 0.05

Enzymatic Hydrolysis

The hydrolysis degree (DH) obtained by Protamex®, during the production of protein hydrolysates (PH), from dry and wet *P. longirostris* BP, is shown in Figure 1.

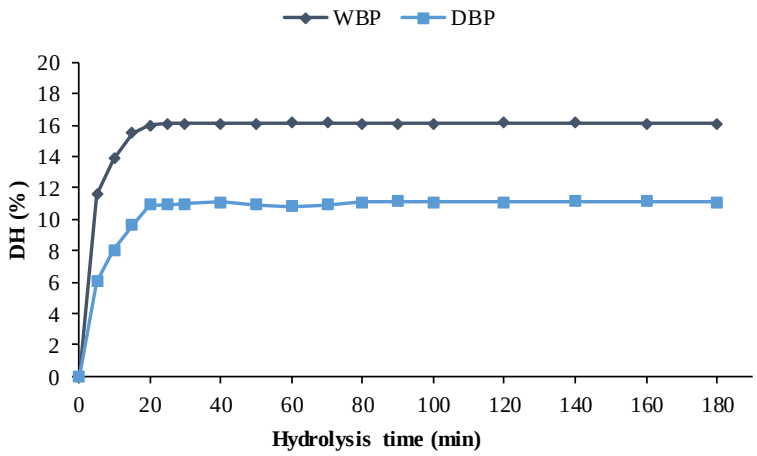


Figure 1. Degree of hydrolysis (DH%) determined in WBP and DBP during reaction with Protamex®.

The maximum value of DH (16.07 ± 1.60%) was obtained in WBP after 25 min of reaction, and this value remained constant till the end of the monitoring of the reaction (Figure 1). In DBP the best result was obtained after 20 min of reaction (10.90 ± 1.12%). Results concerning this aspect are diverse and variable in the literature: Dumay et al. (2006) reported lower DH using Protamex® and Alcalase® (3.1 and 3.3%, respectively) on viscera of *Sardina pilchardus*, while the 15% DH was obtained, after 15 min of reaction, in by-products of *Xiphopenaeus kroyeri* (De Holanda & Netto, 2006), with the enzyme Alcalase®.

Hydrolysates characterization and bioactive properties of the protein fractions

SDS PAGE

Sodium dodecyl sulphate polyacrilamide-electrophoresis (SDS-PAGE) of PH, obtained with Protamex®, showed, in accordance with DH variation, a progressive reduction of the relative molecular mass of proteins, obtained at the different reaction times.

In Figure 2, a decreasing intensity of the bands from T5 to T30 can be observed, confirming the hydrolysis of the native proteins. Similar results were obtained by Tkaczewska et al. (2020) in *Cyprinus carpio* skin gelatin, attesting the ability of the enzyme Protamex® to produce low-molecular-weight peptides at high DH.

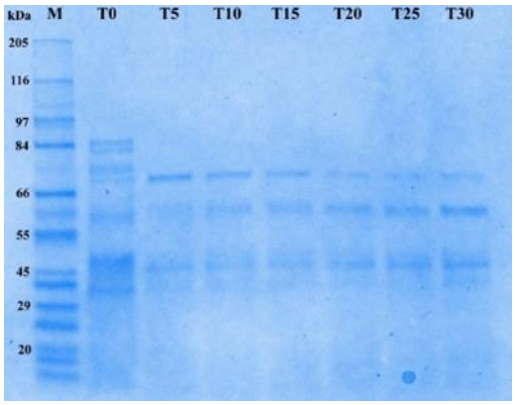


Figure 2. Sodium dodecyl sulphate poly-acrilamide-electrophoresis (SDS PAGE) of *P. longirostris* protein hydrolysates (PH) obtained with Protamex® from T0 to T30 min of reaction. Standard molecular weight marker (M).

Antioxidant Power of Protein Hydrolysates

The evaluation of the antioxidant power on peptide fractions (Pep) isolated from PH showed a significant 1,1-diphenyl-2-picryhydrazyl (DPPH) radical inhibition (>50%) at the maximum tested concentration (4 mg protein/mL) (Figure 3).

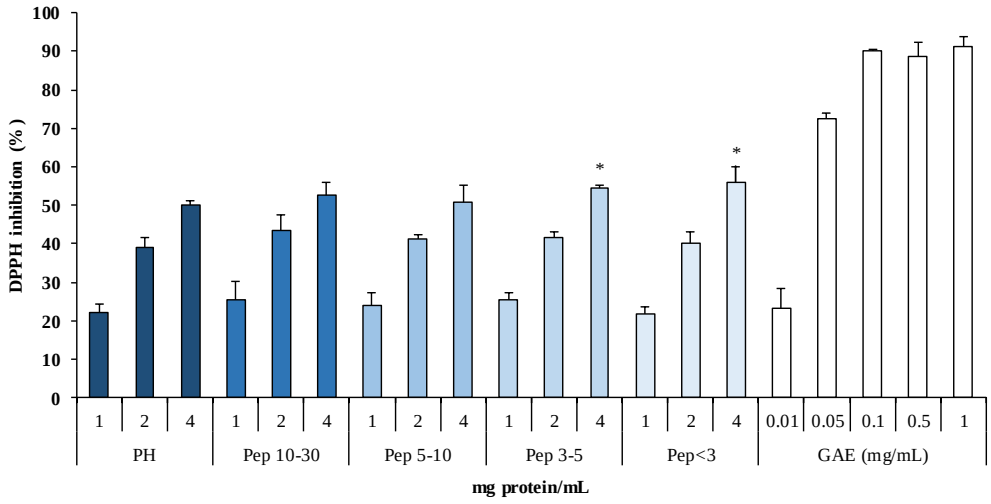


Figure 3. 1,1–diphenyl–2–picryhydrazyl (DPPH) radical inhibition (%) of peptide fractions (Pep) at different concentrations (1, 2, and 4 mg protein/mL) obtained by hydrolysis with Protamex®. Gallic acid equivalents (GAE 0.01–1 mg/mL) (* $p < 0.05$) compared to activity of PH.

Showed results indicate that smaller peptides, such as Pep < 3 kDa (55.8% inhibition) and Pep 3–5 kDa (54.5%), had greater antioxidant activity compared to PH.

Similar results were previously reported: protein hydrolysates fractions < 3 kDa from cod, showed the highest antioxidant power in correspondence of the reduction of the peptides size (Farvin et al., 2014). In the work of Taheri et al. (2014), protein hydrolysate fractions, between 10 and 1 kDa, were found to have higher antioxidant power than the fractions at higher molecular weight. This is

in accordance with the results of Picot et al. (2010), who concluded that the increase in antioxidant power in peptide fractions, compared to PH, may be due to bioactive peptide concentration during the various filtration steps.

ACE-IA Determined by Protein Hydrolysates

Figure 4 shows the ACE-IA exhibited by PH. The highest IA was evident in Pep 5–10 kDa (66%) and the lowest among the isolated fractions was evident in Pep 10–30 kDa (50%) (Figure 4).

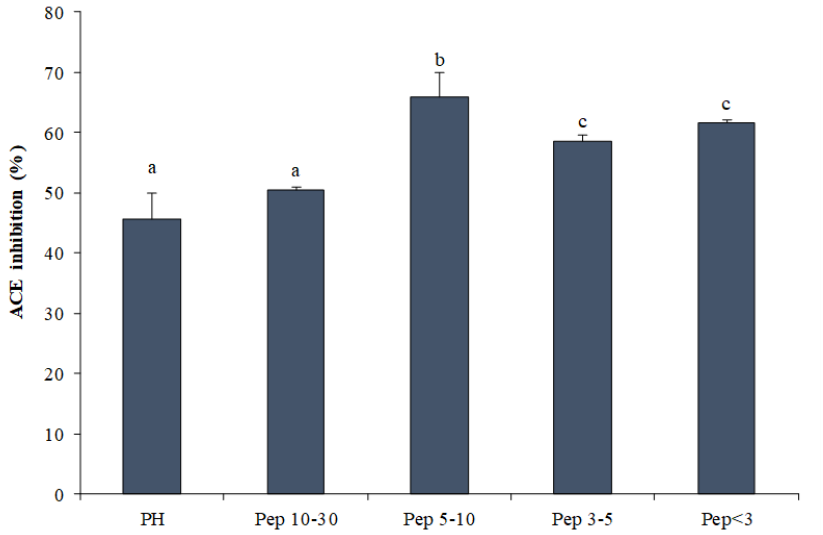


Figure 4. Angiotensin-converting-enzyme inhibition activity (ACE–IA) (expressed in percentage), exerted by peptide fractions obtained by Protamex® and ultrafiltration. Lowercase letters (a–c) indicate significant differences vs. PH ($p < 0.05$).

Figure 4 shows that lower-molecular-weight Pep have higher IA than PH, in accordance with Krichen et al. (2018) results, which indicated a higher activity in peptide fractions from protein hydrolysates of shrimp waste (*P. longirostris*) than in whole hydrolysates.

These results provide further support for the use of PLPD to produce hydrolysates with bioactive peptides.

Extraction, Enrichment and Determination of AST

AST Yields Extracted with Crude Viscera Oil (CVO) and Ethyl Esters

Table 3 shows the AST yields after extraction using CVO, including total fatty acids ethyl esters obtained from CVO (TFA), polyunsaturated fatty acid ethyl esters enriched by SPD (PUFAE), and exhausted fatty acid ethyl esters (EFA) from WBP and DBP, at different extraction ratios (ER: 0.5,

1.0, and 2.0 solvent volume/waste weight). The obtained yields were always higher when increasing the extraction ratio, for both WBP and DBP, reaching the highest values with the 2.0 ratio.

For all extraction ratios with the CVO, TFA, and EFA solvents, WBP showed significantly higher yields ($p < 0.05$) than DBP, whereas no significant differences were observed for PUFAE (Table 2). Utilizing the extraction ratio of 2.0, in WBP, the higher AST yield was reached with TFA (160.06 ± 8.91 µg/g of dry weight (d.w.)) followed by CVO (149.06 ± 0.82 µg/g d.w.), PUFAE (59.06 ± 4.06 µg/g d.w.), and finally EFA (50.50 ± 0.91 µg/g d.w.) ($p < 0.05$). Consequently, the highest AST concentration was found in the extract obtained from WBP with TFA (20.64 ± 0.40 µg/mL); it was significantly higher than that obtained with the CVO (18.86 ± 0.21 µg/mL), PUFAE (7.67 ± 0.36 µg/mL), and EFA extracts (5.31 ± 0.18 µg/mL) (Table 2).

Table 3. Astaxanthin (AST) yields (µg/g on a dry basis) obtained with different solvents—crude viscera oil (CVO), ethyl esters of total fatty acid obtained from CVO (TFA), polyunsaturated fatty acid ethyl esters enriched by short path distillation (SPD) (PUFAE), and exhausted fatty acid ethyl esters (EFA)—and different solvent extraction volume/matrix weight ratios (ER: 0.5; 1.0; 2.0), using WBP and DBP.

	ER	WBP	DBP	$p < 0.05$
CVO	0.5	80.21 ± 2.0 ^{e}	52.56 ± 0.74 ^{e}	*
TFA		86.14 ± 1.88 ^{f}	47.81 ± 3.16 ^{d}	*
PUFAE		31.78 ± 4.19 ^{a}	31.89 ± 1.18 ^{b}	-
EFA		27.17 ± 3.54 ^{a}	19.02 ± 3.74 ^{a}	*
CVO	1.0	97.99 ± 1.30 ^{g}	64.22 ± 2.05 ^{g}	*
TFA		105.23 ± 3.15 ^{h}	58.41 ± 2.88 ^{f}	*
PUFAE		38.83 ± 6.81 ^{b}	38.96 ± 5.54 ^{c}	-
EFA		33.20 ± 2.30 $^{a, b}$	23.23 ± 4.08 ^{a}	*
CVO	2.0	149.06 ± 0.82 ^{i}	97.68 ± 1.51 ^{h}	*
TFA		160.06 ± 8.91 ^{l}	88.85 ± 7.34 ^{h}	*
PUFAE		59.06 ± 4.06 ^{d}	59.26 ± 3.78 $^{f, g}$	-
EFA		50.50 ± 0.91 ^{c}	35.34 ± 1.60 $^{b, c}$	*

Different lowercase letters in the same column indicate significant differences (a, b, c… $p < 0.05$). * in the same row indicates significant differences between wet and dry. Data are reported as mean ± standard deviation.

These results are in accordance with those of Sachindra & Mahendrakar (2005), who found that the AST yield of *Penaeus indicus* by-products, extracted using vegetable oils, is higher using an extraction ratio equal to 2.0. Chen & Meyers (1982) obtained maximum pigment yield from crawfish by-products incubating the mixture at a temperature of 80–90 °C for 30 min. However, temperatures above 70 °C and times above 150 min significantly reduced AST yields in *P. indicus* (Sachindra & Mahendrakar, 2005). As carotenoids are degraded at higher temperature, Sachindra &

Mahendrakar (2005) suggest using lower temperature for longer time to optimize extraction yield of carotenoids from shrimp by-product (Sachindra & Mahendrakar, 2005). Parjikolaei et al. (2015) also found better extraction efficiencies in *Pandalus borealis* by-products by applying a temperature of 70 °C, also considering a shorter extraction time.

The obtained results agree to Parjikolaei et al. (2015) that using ME from vegetable oil, reported a significantly higher extraction efficiency for a wet matrix compared to a freeze-dried matrix, as freeze-drying leads, to some extent, to the collapse of the solid structure of biological materials (Voda et al., 2012). This probably makes the internal freeze-dried matrix less accessible to solvents, reducing both the mass transport rate and the yield. This could explain similar and relatively lower yields of dried samples with respect to wet samples, for all solvents used for freeze-dried matrix extraction (Table 2). In this work, the matrix was dried in a ventilated oven and may have been affected by similar modifications, or AST could have been partially degraded due to its sensitivity to drying temperatures (Parjikolaei et al., 2015). The yields of WBP obtained using TFA as a solvent were higher than yields obtained utilizing CVO as solvent, in accordance with the results showed by Parjikolaei et al. (2015) in *P. borealis* by-products.

Furthermore, similar values were reported by Shahidi & Synowiecki (1991), who utilized a dry matrix and cod liver oil as a solvent, with a yield of 147 µg/g in *P. borealis* by-products.

The higher extraction efficiency obtained by Parjikolaei et al. (2015, 2017) using TFA can be explained considering the lower viscosity of ME compared to sunflower oil, as well as the different polarity and interactions between AST, ME, and sunflower oil.

Supercritical CO_2 Extraction (SFE)

The AST yields obtained by SFE from DBP (4.84 ± 0.06 µg/g) were like Messina et al. (2019a) and to the data reported by Radzali et al. (2014) but resulted lower than data reviewed by Ahmadkelayeh & Hawboldt (2020).

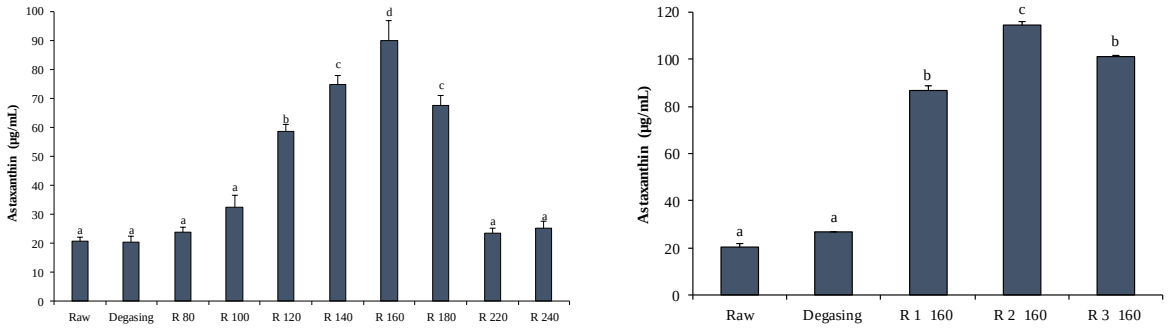
A low recovery of AST and a low selectivity has been reported in samples extracted using supercritical CO_2 compared to traditional chemical methods (Charest et al., 2001). This problem can be overcome by using appropriate co-solvents, at different concentrations, which could improve yield, solubility, and extraction efficiency (Ahmadkelayeh & Hawboldt, 2020; Parjikolaei et al., 2017; Radzali et al., 2014). Although SFE is considered a green method showing several advantages, such as the use of non-toxic solvents, there is a need to optimize the balance among large-scale extraction processes and investment and operating costs (Ahmadkelayeh & Hawboldt, 2020). It is worth stressing that the production costs of AST using sunflower oil or the methyl ester

of sunflower oil extraction (0.06 and 0.16 $/mg of AST, respectively) are lower than those using hexane isopropanol as a solvent or SFE (about 0.6 and 0.82 $/mg of AST, respectively) (Parjikolaei et al., 2017).

AST Enrichment by Short Path Distillation (SPD)

SPD was applied to enrich AST extracted using TFA. In SPD, at a specific temperature and pressure, lighter molecules evaporate and condense in the distillate, while heavy molecules such as AST are recovered and concentrated in the heavy phase. Using SPD, the maximum concentration was reached at evaporation temperature of 160 °C (89.77 ± 7.12 µg/mL) and pressure of 0.002 mbar, with an overall concentration increase of about 4.5 times compared to the initial concentration (Figure 5a). However, maximum AST concentrations were lower than those reported by Parjikolaei et al. (2016, 2017), who obtained an increase from 3.04 to 155 ppm with an evaporation temperature of 140 °C and a pressure of 1000 Pa. A significant decrease at evaporation temperatures above 160 °C was reported by Batistella et al. (2002). This result was attributed to a degradation of the molecule, as an increase in carotenoid decomposition was obtained at temperatures from 150 to 170 °C (Batistella et al., 2002).

To obtain a further concentration of AST, at the evaporation temperature 160 °C, three steps were repeated each time, recycling the previously enriched heavy fraction. The first two steps (R1 and R2) showed a significant AST enrichment present in the heavy phase, of up to 114.80 ± 1.23 µg/mL. The third step (R3) showed a significant decrease compared to the second step (101.33 ± 0.65), probably due to the degradation of the molecule in the evaporator (Batistella et al., 2002) (Figure 5b).



(**a**) (**b**)

Figure 5. (**a**) AST concentrations (µg/mL) obtained in the residue via short path distillation (SPD) at different evaporation temperatures. (**b**) AST concentrations (g/mL) obtained in the residue via SPD by repeating several steps at the same evaporation temperature (160 ºC). Different letters (a,b,c, …) indicate significant differences ($p < 0.05$).

Evaluation of Bioactive Properties of Protein Hydrolysates and AST In Vitro

Antioxidant Activity of Hydrolyzed Fractions in Human Fibroblast (142BR)

The antioxidant properties of the peptide fractions obtained by protein hydrolysates of BP were tested on a human fibroblasts cell line (142BR) against induced oxidative stress, utilizing previous standardized protocol, which employs hydrogen peroxide as an inducer (Messina et al., 2019a).

As expected, the cell viability showed a statistically significant reduction ($p < 0.05$) in cells exposed to hydrogen peroxide (HP) compared to the control (Figure 6).

By contrast, cells pretreated with different concentrations (25–75 µg/mL) of purified peptides (Pep 3–5 kDa) and subsequently exposed to hydrogen peroxide, showed a higher viability than untreated stressed cells (HP), which is comparable to cells treated with the synthetic antioxidant NAC (Figure 6).

The cell viability showed a dose-dependent trend at concentrations of 25 and 50 µg/mL, and then decreased again at 75 µg/mL. Viability was higher at the concentration of 50 µg/mL.

These results suggest that low-molecular-weight peptides have protective effects against oxidative damage, according to the results obtained by Qian et al. (2008) in human embryonic lung fibroblasts cell lines (MRC-5) and mouse macrophages cells (RAW264.7). These authors report that low-molecular weight peptides exhibit high scavenger activity. Peptides with a low molecular weight according to Sabeena Farvin et al. (2014) may act as hydrogen donors and thus convert free radicals into more stable products. In fact, as described above (Figure 3), smaller peptide such as Pep 3–5 kDa exhibited high antioxidant activity and can protect cells from oxidative stress.

These observations confirm that peptides produced via enzymatic hydrolysis from marine resources could represent an alternative source of bioactive compounds with high antioxidant activity that could be used to counteract oxidative stress (Suarez-Jimenez, Burgos-Hernandez, & Ezquerra-Brauer, 2012).

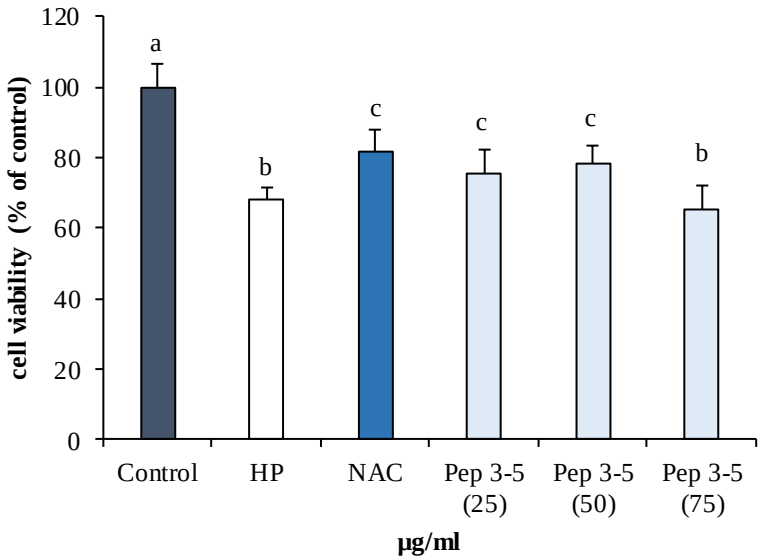


Figure 6. Effect of hydrolyzed fractions on 142RB fibroblast cells exposed to oxidative stress induced by hydrogen peroxide (50 µM). Control–cells maintained in standard culture conditions; HP–hydrogen peroxide treatment; NAC–cells pretreated with the synthetic antioxidant N acetylcysteine. Peptide fractions were obtained by ultrafiltration of BP hydrolysates with Protamex® (Pep). Different letters (a, b, c) indicate significant differences ($p < 0.05$).

In Vitro Effects of AST Extracted Using Fish Oil, Ethyl Esters, and SFE

AST extracted using different "green" solvents was tested on a 3T3 cell line exposed to oxidative stress by the chemical inducer hydrogen peroxide (50 µM).

As expected, the viability underwent a reduction ($p < 0.05$) in cells exposed to hydrogen peroxide alone compared to control cells (Figure 7).

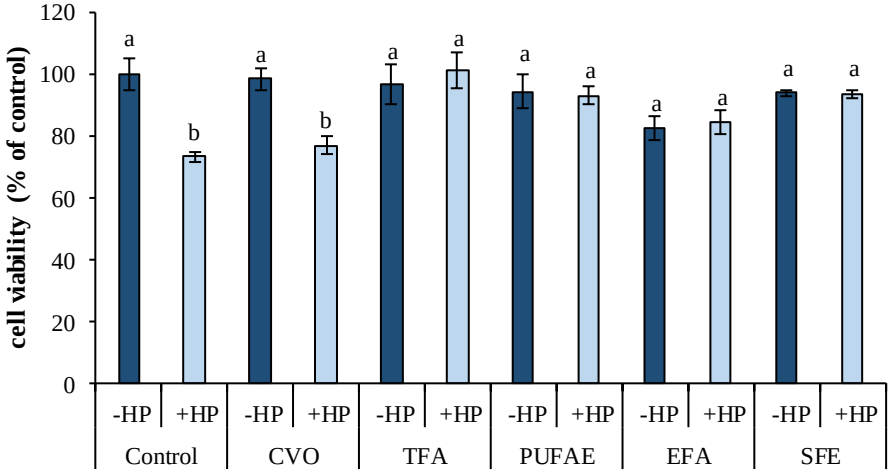


Figure 7. Effect of AST extracted using various methods on the viability of 3T3 cells exposed to oxidative stress with hydrogen peroxide (50 µM). HP-hydrogen peroxide treatment; Control-cells maintained in standard culture conditions; CVO-crude viscera oil; TFA-ethyl esters of total fatty acid obtained from CVO; PUFAE-polyunsaturated fatty acid

ethyl esters enriched by SPD; EFA-exhausted fatty acid ethyl esters; SFE-supercritical fluid extract. Different letters (a,b) indicate significant difference ($p < 0.05$).

Pre-incubation of cells for 24 h with TFA, PUFAE, and SFE significantly inhibited the cytotoxic effects of the pro-oxidant ($p < 0.05$) (Figure 7).

A further confirmation of the antioxidant power was obtained in a preliminary test conducted in a similar cell line and under the same conditions as in previous work (data not shown) (Messina et al., 2019a).

However, the reduction in viability in cells pretreated with CVO shows that the latter is not able to protect cells from oxidative stress (Figure 7). These results indicate that AST exerts an antioxidant effect on oxidative injuries, the extent of which is also dependent on the solvent used for its extraction and dissolution. Results are in accordance with AST's neuroprotective effect demonstrated in primary retinal cells against high glucose-induced retinal damage (Baccouche et al., 2017) and its cytoprotective role in the human neuroblastoma cell line SH-SY5Y (Castelli et al., 2020).

Materials and Methods

Sampling and Sample Processing

By-products of *P. longirostris*, composed of cephalothorax and abdominal parts, were sampled from commercial processing plants located in Mazara del Vallo (Trapani, Italy) and Portopalo (Siracusa, Italy), stored in cold containers, transported to the laboratory, and frozen at −25 °C. The preparation of DBP was carried out in 150 kg batches.

WBP was dried in a ventilated thermostat (volume 540 L) at 60 °C for 72 h and ground. The dried matrix was fractionated on a sieve column and stored under vacuum at 4° C.

Evaluation of Proximate Composition and Fatty acid Profile of *P. longirostris* By Products

The analyses for proximate composition and fatty acid profile were performed in two replicates, to determine the moisture content and ash content (AOAC, 1990), crude protein (AOAC, 1992) and total lipid (Folch, Lees, and Sloane, 1953). Chitin percentage content of was determined by stoichiometric calculation of total nitrogen content (Díaz-Rojas et al., 2006). Fatty acids were

extracted and transesterified according to Lepage and Roy (1984) and determined using gas chromatography (Messina et al. 2013).

Enzymatic Hydrolysis and AST Extraction

Enzymatic hydrolysis was performed on WBP and DBP in two replicates. The reaction was carried out on 10 Kg of WBP or DBP in distilled water (1:1 water volume/waste weight), in 50 L steel reactors under constant agitation and continuous pH value monitoring. To control the reaction temperature of the mixture with high efficiency, the steel reactor was equipped with an internal helical heating coil and connected to a Julabo SE-6 Heating circulator, temperature range −5/350 °C (Seelbach, Germany) filled with Marlotherm® SH (Global Heat Transfer Ltd., Stone, UK). The bacterial protease Protamex® (>1.5 Anson Units (AU)-*N*/g protein) (Sigma-Aldrich, St. Louis, MO, USA) was used for enzymatic hydrolysis.

Protamex® was added (3% *w*/*w* of WBP or DBP), for 180 min at 60 °C, pH at 8 adjusted by 5 *N* NaOH (De Holanda & Netto, 2006; Dumay et al., 2006). The degree of hydrolysis (DH%) was determined by direct evaluation, as described by Adler-Nissen (1982).

To calculate DH%, the value of 7.7 for h_{tot} was used, as reported by De Holanda & Netto (2006) for enzymatic hydrolysis in *X. kroyeri* by-products. We calculated the dissociation factor (α value) using the equation described by De Holanda & Netto (2006). Total of 500 mL aliquots of reaction mixture (collected after 5, 10, 15, 20, 25, 30 until 180 min from enzyme addition) were heated to 90 °C in 4 min and kept at this temperature for 5 min to inactivate enzyme activity (De Holanda & Netto, 2006) in a microwave oven as described by Utne-Palm et al. (2020).

AST was extracted from the hydrolysate by different “green” solvents: crude viscera oil (CVO), total fatty acids ethyl esters obtained from CVO (TFA), polyunsaturated fatty acid ethyl esters enriched by SPD (PUFAE) and exhausted fatty acid ethyl esters (EFA), using extraction ratios of 2.0, 1.0, and 0.5 (oil volume/WBP or DBP weight) (Sachindra & Mahendrakar, 2005), for 150 min at 70 °C, in a steel reactor, as described by Sachindra & Mahendrakar (2005).

The following solvents were added to the hydrolysate in the steel reactor: CVO obtained from sea bream, TFA, PUFAE, and EFA, extracted and enriched as reported by Messina et al. (2021).

Separation of Protein Hydrolysates and AST

Extraction mixtures, obtained after hydrolysis reaction, containing an apolar component (AST) and a polar component (protein hydrolysates) were filtered through a 125 µm sieve and centrifuged

using a continuous high speed tubular centrifuge (CEPA, Carl Padberg, Zentrifugenbau GmbH, Lahr/Schwarzwald, Germany) at room temperature, feed flux of 15 L/h, at 38,454 g, to contemporary and continuously separate the solids (retained in the cylinder), the aqueous phase (containing protein hydrolysates), and the lipid phase (containing AST).

Protein Hydrolysates Characterization

SDS-PAGE

For evaluation of the relative molecular mass of the proteins present in the samples, both total homogenate and hydrolysates from BP obtained in the various hydrolysis steps (5, 10, 15, 20, 25, and 30 min) were separated using SDS-PAGE. Aliquots of hydrolysates (0.5 mL) were centrifuged at 10621 g for 1 min, and 200 μL of supernatant was recovered for protein quantification. The concentration of total proteins in all samples was determined using the method of Lowry et al. (1951), using BSA (bovine serum albumin) (Sigma-Aldrich) as standard.

Aliquots of 100 μg of protein, diluted with a Laemmli buffer (Sigma-Aldrich) and denatured for 5 min at 90 °C, were loaded onto a gradient polyacrylamide minigel (4–15%) (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis at 20 mA for about 2 h (Messina et al., 2019b). A mix of standard proteins with relative molecular masses varying between 250 and 14 kDa (Bio-Rad, Hercules, CA, USA) was run simultaneously into the gel. After the electrophoretic run, the gel was stained with Coomassie Blue (GelCode Blue Stain Reagent, Pierce, Rockford, IL, USA). The image of the gel was acquired and elaborated using the software Image Lab 4.1 (Bio-Rad, Hercules CA, USA).

Fractionation of Proteins

PH, obtained with enzymatic Protamex®, were filtered on a 0.45 μm nylon membrane filter (Pall, Ann Arbor, MI, USA) to remove the coarsest material. The filtrate PH was then fractionated and concentrated according to molecular weight by ultrafiltration on continuous tangential filtration modules (Vivaflow 200, Sartorius, AG, Germany). The fractionation was carried out, in sequence, by membranes of molecular weight cut-offs (MWCOs) of 30, 10, 5, and 3 kDa (Picot et al., 2010).

Protein hydrolyzed fractions (PH), having different molecular mass, in relation to the cut-off of the membranes, (Pep 10–30 kDa, Pep 5–10 kDa, Pep 3–5 kDa, Pep < 3 kDa), were frozen at −80 °C and freeze-dried.

The lyophilized hydrolyzed peptides were reconstituted in distilled water at a concentration of 30 mg/mL (stock solution) and the pH was adjusted to 7.0. The concentration of the obtained fractions was determined with the Lowry assay (1951).

DPPH Radical Scavenging Activity

The total antioxidant power of PH and Pep was measured using the DPPH assay (Arena et al., 2020; Bernatoniene et al., 2011; Messina et al., 2019c). A total of 0.4 mL of different Pep, at concentration of 1, 2, and 4 mg/mL, was prepared by diluting the stock solutions in absolute ethanol (30 mg/mL); they were then mixed with 1.6 mL of 100 μM DPPH in 96% ethanol to start the reaction. The reaction mixture was kept at room temperature and the absorbance was measured at 517 nm after 30 min. Gallic acid (Sigma-Aldrich) was used as a positive control.

Scavenging activity was determined using the following Equation (1):

$$\text{Scavenging activity}\ (\%) = [1 - (\text{Absorbance sample}/\text{Absorbance control})] \times 100 \quad (1)$$

ACE Inhibition Assay

The ACE inhibitory activity (ACE-IA) of PH and Pep was measured using the Cushman and Cheung method (Cushman and Cheung, 1971), with slight modifications (Qian et al., 2007). The sample solution (1 mg protein /mL in 50 mM buffered sodium borate (SBB) pH 8.3, 50 μL) was preincubated with a solution of the angiotensin converting enzyme (ACE, from rabbit lung) (50 μL, 25 U/mL in SBB) (Sigma-Aldrich) at 37 °C for 10 min and then mixed with the enzyme substrate N-ippuryl-L-histidyl-L-leucine (150 μL, 8.3 mM in SBB) for 30 min at the same temperature. The reaction was blocked with HCl 1 M (250 μL). The resulting hippuric acid was extracted from the acidified solution (0.5 mL) of ethyl acetate by vortex mixing for 15 s. After centrifugation (800 g, 15 min), the supernatant (0.2 mL) was dried by evaporation under vacuum conditions for 2 h (Qian et al., 2007). The obtained hippuric acid was dissolved in distilled water and the absorbance was measured at 228 nm with a spectrophotometer. ACE inhibitory activity was calculated according to Equation (2):

$$\text{ACE inhibitory activity}\ (\%) = [(\text{B} - \text{A})/(\text{B} - \text{C})] \times 100 \quad (2)$$

where B is the absorbance of the uninhibited control, C is the absorbance of the inhibited reaction, and A is the absorbance in the presence of PH.

AST Supercritical Fluid Extraction (SFE)

The system used for SFE (Helix System Basic Model, Applied Separation, Allentown, PA, USA) is equipped with an extraction vessel and a separator with volumes of 500 mL that can operate at a maximum pressure of 690 bar and a maximum temperature of 160 °C. The extraction parameters and procedures were described by Messina et al. (2019a). Briefly, the dried and ground matrix was passed on a sieve column in order to obtain the suitable particle size fraction (250–500 µm) for the extraction. The extraction temperature and pressure were set at 40 °C and 350 bar, respectively. After 30 min of static, the dynamic extraction was carried out with a CO_2 flow regulated at 2.5 L/min for 2 h.

Enrichment of AST by Short Path Distillation (SPD)

AST samples extracted using TFA as a solvent were processed by SPD. The molecular distiller with the evaporator type "falling film" (model VLK 70-4 FDRR-SKR-T, VTA Gmbh, Niederwinkling, Germany) has an evaporation surface of 4.8 dm^2, which allows to process up to 1.5 kg h^{-1} of oil at a maximum operating temperature of 350 °C and a vacuum operating pressure of 0.001 mbar (Mazzelli et al., 2019).

The ethyl ester mixture was loaded into the feed vessel by a peristaltic pump preheated to 40 °C and prepared for a first degassing step to remove impurities and solvent traces from the mixture. Subsequently, several distillation cycles were carried out at increasing evaporation temperatures (80, 100, 120, 140, 160, 180, 220, and 240 °C) on the starting ethyl esters (Table 4).

Table 4. Operating conditions used for degassing and molecular distillation.

	Degassing	**Distillation**
Flow (htz)	20	5
T (°C) Feed	40	40
T (°C) Condenser	25	60
T (°C) Residue	60	60
T (°C) Evaporator	80	80–240
Vacuum (mbar)	5	2×10^{-3}

Aliquots of the enriched fraction (heavy phase) and of the distillate (light phase) were sampled, diluted, and analyzed at the spectrophotometer. A second enrichment test was performed by processing the ethyl esters at the evaporation temperature (160 °C) at which the highest AST

concentration was obtained, and three cycles were repeated at the same temperature on the same enriched fraction to obtain a further concentration.

Spectrophotometric Determination of AST

For the determination of AST in the different extracts (CVO, TFA, PUFAE, EFA, and SFE) and enriched fractions (obtained from both WBP and DBP samples), after appropriate dilution, the samples were determined spectrophotometrically at 486 nm applying Equation (3) by Sachindra & Mahendrakar (2005) for vegetable oils but considering the molar extinction coefficient $E_{1cm}^{1\%}$ (2043) calculated by Chen & Myers (1984) for fish oil.

$$\text{Carotenoid as AST } (\mu g/\text{g BP}) = \frac{A \times V \times D \times 10^6}{100 \times E_{1cm}^{1\%} \times W} \qquad (3)$$

where *A* is the absorbance at 486 nm, *V* is the volume of pigmented oil recovered, *D* is the dilution factor, *W* is the weight of BP in grams, and $E_{1cm}^{1\%}$ is the extinction coefficient.

Cell Culture

The antioxidant properties of SFE and AST extracted by utilizing CVO, TFA, PUFAE, and EFA as solvents were determined in two cell lines: human skin fibroblast cell line, 142BR (ECACC n. 90011806, Sigma®) and 3T3 L1 cell lines from mice (ECACC n. 86052701, Sigma®).

142BR cells were grown in minimum essential medium Eagle (MEME) supplemented with 15% fetal bovine serum (FBS, 2 mM glutamine, 1% non-essential amino acids and 100 µg/mL penicillin–streptomycin). 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% calf serum, 2 mM glutamine, and 100 µg/mL penicillin–streptomycin. All reagents were from Sigma-Aldrich.

Assessment of Antioxidant Activity of Pep in 142BR Cells

Confluent cell cultures were trypsinized and seeded in a 96-well plate at a concentration of 1×10^4 cells/well and incubated for 24 h.

Different concentrations of Pep (25, 50, and 75 µg/mL dissolved in sterile distilled water and filtered in 0.22 µm Millipore membrane) (Millex® Merck Millipore, Darmstadt, Germany), were added to cells and incubated for 48 h. After 48 h, all samples except for the control were exposed to

the chemical promoter of oxidative stress, hydrogen peroxide (50 µM) (Carlo Erba reagents, Milano, Italy), according to a previously standardized protocol (Abbes et al., 2013; Arena et al., 2020; Dhouibi et al., 2020; Manuguerra et al., 2018; Messina et al., 2019a, 2019c, 2019d). The viability was measured using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to Mossman's method (Denizot & Lang, 1986), as reported by Messina et al. (2019a). The data were expressed as the mean percentage of viable cells as compared to the respective control culture. Each concentration was tested in three replicates, each consisting of five single determinations.

Protective Effect AST Extracted by CVO, TFA, PUFAE, EFA, and SFE in 3T3 Cells

Cells were seeded in a 96-well plate at a concentration of 7×10^3 cells/well and incubated for 24 h. After 24 h the cells were treated with AST extracted by CVO, TFA, PUFAE, EFA, and SFE, dissolved in ethanol, and utilized at a concentration of 0.2 nM in the medium, with a final solvent concentration of 0.1% (*v/v*), and left to incubate for 24 h, as described by Messina et al. (2019a).
A preliminary toxicity test, aimed to assess the effect of "green solvents" on cell vitality, was carried out in a recent paper, where it was demonstrated that these solvents do not influence cell viability (Messina et al., 2021).
Then all cells, except for the control, were exposed to hydrogen peroxide (50 µM) as described above, to evaluate the protective effect of AST against oxidative stress.

Statistical Analysis

Statistical analysis was performed using the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, IL, USA). All analyses were carried out in triplicate. The results are expressed as mean ± standard deviation. The homogeneity of variance was confirmed by the Levene test. Data were subjected to one-way analysis of variance (ANOVA), and Student–Newman–Keuls or Games–Howell post-hoc tests were performed in order to make multiple comparisons between experimental groups. The significance level was 95% in all cases ($p < 0.05$).

Conclusions

The possibility to obtain bioactive compounds, such as AST and bioactive peptides, from the shrimps processing value-chain, could support the goal to increase the sustainability of marine

resources, avoiding to waste nutrient-rich marine by-products, and could help also to turn waste into profit for the enterprises.

The “green” AST extraction methods, using CVO, TFA, PUFAE, EFA, and SFE, have shown good yields, especially in samples obtained by TFA as a solvent; the AST can be enriched by SPD.

This study demonstrated that different AST extracts have cytoprotective and antioxidant effects in vitro. Obtained results attest to their possible application in pharmaceutics and nutraceutics.

In view of the reported results on the protective effects against retinopathy of both AST (Baccouche et al., 2017; Giannaccare et al., 2020; Ortega, 2021) and fatty acids n-3 (Dátilo et al., 2018; Ortega, 2021), obtained data are particularly interesting because they allow to obtain a product that contains both AST and fatty acids at the same time, and therefore could ensure, through their synergistic effect, a greater protection against ocular diseases.

Bioactive peptides obtained after enzymatic reaction and ultrafiltration show that Peps reveal an increased scavenger activity and shows inhibitory effects on ACE compared to PH.

In addition, “green” methods, used instead of traditional methods that employ chemical solvents, make it possible to obtain high-quality bioactive compounds from large volumes of BP, for application in the pharmaceutical and nutraceutical sectors.

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