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# Substâncias naturais e processamento por alta pressão para conservar robalo

# Natural substances and high pressure processing to preserve sea bass

**Universidade de Aveiro** Departamento de Química
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica da Doutora Maria Leonor Martins Braz de Almeida Nunes, Investigadora Principal e Coordenadora da Divisão de Aquacultura e Valorização do Instituto Português do Mar e da Atmosfera, e do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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**o júri**

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**palavras-chave**

robalo, *Dicentrarchus labrax*, pescado, filetes, extractos de plantas, óleos essenciais, películas de proteínas, conservação de pescado, tempo de vida útil, qualidade de pescado, processamento por alta pressão, actividade antioxidante, actividade antibacteriana, enzimas, análise sensorial, microbiologia.

**resumo**

Esta tese teve como objectivo estudar estratégias de conservação de pescado fresco, recorrendo ao uso de extractos e óleos essenciais de plantas e do processamento por alta pressão (HPP), usando filetes de robalo como um caso de estudo modelo.

Relativamente aos extractos e óleos essenciais, avaliaram-se as suas propriedades antibacterianas e antioxidantes. Os extractos aquosos quente de poejo e de orégão e o óleo essencial de cravinho apresentaram a maior actividade antioxidante. Os óleos essenciais foram mais eficientes do que os extractos para inibir o crescimento das estirpes bacterianas testadas, tendo-se observado os menores valores de concentração mínima inibitória nos óleos essenciais de orégão, citronela, alho e orégão Espanhol. De seguida, estudou-se o efeito dos óleos essenciais de orégão Espanhol e de limão na conservação de filetes de robalo fresco tendo em conta critérios microbiológicos, químicos, físicos e sensoriais. A aplicação do óleo essencial de orégão Espanhol aumentou o tempo de vida útil dos filetes sob o ponto de vista bacteriano, mas não em termos sensoriais. A combinação dos óleos essenciais de orégão Espanhol e de limão melhorou o efeito antioxidante e reduziu a intensidade do odor e a sua eficácia em relação às Enterobacteriaceae, comparando com o tratamento com óleo essencial de orégão Espanhol *per se*.

No sentido de reduzir o odor conferido pelos óleos essenciais realizou-se um estudo de conservação para avaliar o efeito de películas com óleos essenciais (citronela, alho e tomilho) em filetes de robalo, recorrendo a uma teste de desafio bacteriano. As películas sem óleos essenciais aumentaram o tempo de vida útil sob o ponto de vista bacteriano, mas este efeito não foi observado com a incorporação dos óleos essenciais nas películas.

Em relação ao HPP, testaram-se diversas condições (nível de pressão, tempo de pressurização e taxa de pressurização) e avaliaram-se os efeitos na actividade enzimática, na qualidade global e na conservação de filetes de robalo fresco. Em geral, o aumento do nível de pressão e do tempo de pressurização diminuiu a actividade da fosfatase ácida e das enzimas proteolíticas, a carga bacteriana e a capacidade de retenção de água, enquanto que os filetes ficaram mais brancos. O HPP revelou potencial para o desenvolvimento de novos produtos: mais brancos, não translúcidos, mais firmes e com maior tempo de frescura e estabilidade microbiológica.

Em conclusão, os óleos essenciais e o HPP têm potencial para conservar pescado fresco, devido aos seus efeitos na qualidade bacteriana. Ainda assim, mais esforços devem ser feitos no sentido de reduzir a transferência de odor dos óleos essenciais para o pescado e os efeitos do HPP no aspecto do pescado e na oxidação lipídica.

**keywords**

sea bass, *Dicentrarchus labrax*, seafood, fillets, plant extracts, essential oils, protein films, fish preservation, shelf life, fish quality, high pressure processing, antioxidant activity, antibacterial activity, enzymes, sensory analysis, microbiology.

**abstract**

This thesis aimed to study extracts and essential oils from plants and high pressure processing (HPP) to preserve fresh fish, using sea bass fillets as a case study model.

Considering extracts and essential oils, their antibacterial and antioxidant properties were evaluated. The hot water extracts of European pennyroyal and oregano and the essential oil of clove showed the highest antioxidant activities. The essential oils were more efficient than the extracts to inhibit the growth of the bacterial strains tested, being oregano, citronella, garlic, and Spanish oregano essential oils those with the lowest values of minimum inhibitory concentration. Then, the effect of essential oils (Spanish oregano and lemon) in the preservation of fresh sea bass fillets was studied taking into account microbiological, chemical, physical, and sensory criteria. The application of Spanish oregano essential oil increased the microbiological shelf life of sea bass fillets, but not the sensory shelf life. The combination of Spanish oregano and lemon essential oils enhanced the antioxidant effect and reduced the odour intensity and its efficacy against Enterobacteriaceae, compared to the treatment with Spanish oregano essential oil *per se*.

To reduce the essential oils odours imparted to fillets, a preservation study was carried out to evaluate the effect of films with essential oils (citronella, garlic, and thyme) in sea bass fillets, using a microbiological challenge test. Films without essential oils increased the shelf life of sea bass fillets taking into account microbiological criteria, but the incorporation of essential oils in films did not.

Regarding HPP, several conditions (pressure level, pressure holding time, and pressurization rate) were tested and its effects were evaluated in the activity of enzymes, in the overall quality, and in the preservation of fresh sea bass fillets. In general, the increase in pressure level and holding time decreased the activities of acid phosphatase and proteolytic enzymes, bacterial load, and water holding capacity, while fillets became whiter. HPP showed potential to create new products: whiter, not translucent, firmer, and with increased freshness period and microbiological stability.

In conclusion, essential oils and HPP showed potential to preserve fresh fish, due to their effects in microbiological quality. Still, more efforts should be done to reduce the essential oils odour imparted to fish and the effects of HPP in fish appearance and lipid oxidation.

# GENERAL INDEX

# ABBREVIATIONS AND LATIN EXPRESSIONS

| | |
|---|---|
| A | actin |
| *a** | red-green value |
| AAI | antioxidant activity index |
| Act | actinin |
| ADP | adenosine diphosphate/adenosine 5'-diphosphate |
| AMP | adenosine monophosphate/adenosine 5'-monophosphate |
| AP | sample application point |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate/adenosine 5'-triphosphate |
| AU | absorbance units |
| *b** | yellow-blue value |
| BHT | butylated hydroxytoluene |
| Bis-Tris | 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol |
| C | control treatment |
| *C** | chroma |
| *ca.* | *circa*/approximately |
| CECT | Colección Española de Cultivos Tipo/Spanish Type Culture Collection |
| CFU | colony forming unit |
| CIE | Commission International de l'Eclairage |
| C-Pt | C protein |
| D | desmin |
| DMSO | dimethylsulfoxide |
| DPPH | α,α-diphenyl-β-pycrylhydrazyl |
| DTT | dithiothreitol |
| *e.g.* | *exempli gratia*/for example |
| $EC_{50}$ | concentration providing 50 % of inhibition |
| EDTA | ethylenediaminetetracetic acid |
| EGTA | ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid |
| FAO | Food and Agriculture Organization of the United Nations |
| FRAP | ferric reducing antioxidant power |
| FU | fluorescence units |

| | |
|---|---|
| h | hour/hours |
| *h** | hue |
| HPP | processamento por alta pressão/high pressure processing |
| Xa | xanthine |
| Hx | hypoxantine |
| HxR | inosine |
| *i.e.* | *id est*/that is/in other words |
| IEF | isolectric focusing |
| IMP | inosine monophosphate/inosine 5'-monophosphate |
| *in vitro* | in glass/processes or reactions taking place in a test tube, culture dish, or elsewhere outside a living organism |
| *in vivo* | within the living/processes or reactions studied in a living organism |
| IOD | integrated optical density |
| *L** | lightness |
| log | logarithmic |
| MDA | malondialdehyde |
| meq | milliequivalents |
| MHC | myosin heavy chains |
| MIC | minimum inhibitory concentration |
| min | minute/minutes |
| MLC | myosin light chains |
| M-Pt | M protein |
| N.D. | inhibition or band not detected |
| n.d. | not described |
| nd | not determined |
| O | treatment with Spanish oregano essential oil |
| OL | treatment with Spanish oregano and lemon essential oils |
| P | parvalbumin |
| *p* | probability of the statistic test |
| *per se* | by itself |
| PF | puncture deformation |
| pH | the negative logarithm (base 10) of the molar concentration of hydrogen ions |
| pI | isoelectric point |
| *post mortem* | after death |
| *post rigor* | after *rigor mortis* |

| | |
|---|---|
| *pre rigor* | before *rigor mortis* |
| PS | treatment with potassium sorbate |
| r | Pearson product-moment correlation coefficient |
| RI | retention index |
| *rigor mortis* | stiffness of death |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel |
| spp. | specific epithet of species not identified/different species within a genus |
| TBA | 2-thiobarbituric acid |
| TEP | 1,1,3,3-tetraethoxypropane |
| TM | tropomyosin |
| TMA | trimethylamine |
| TMA-N | trimethylamine nitrogen |
| TPTZ | 2,4,6-tris(2-pyridyl)-*s*-triazine |
| tr | traces |
| Tris-HCl | trizma hydrochloride |
| TVB | total volatile bases |
| TVB-N | total volatile basic nitrogen |
| W | whiteness |
| WHC | water holding capacity |
| WHO | World Health Organization |
| WVP | water vapour permeability |

# CHAPTER 1. General introduction and Objectives

## INDEX

# General introduction

The demand towards fish products has increased in the last decades, not only due to the high number of available species, but also as a result of its role in human health. In fact, fish is known to prevent coronary heart diseases, hypertension, and cancer, due to the presence of high protein content and quality, (*n*-3) polyunsaturated fatty acids, liposoluble vitamins, and essential elements content, and low levels of cholesterol and saturated fatty acids [1]. However, it is also known that fish is highly perishable due to autolytic reactions from endogenous enzymes, chemical, and microbiological deterioration [2]. Thus, it is important to process and preserve adequately such products in order to maintain good nutritional and organoleptic attributes, and to extend the shelf life.

## 1. Challenges in fresh fish preservation

Many preservation methods have been successfully developed to preserve fish products for long periods, *e.g.* freezing or canning. However, several characteristics of fresh fish, which are greatly appreciated by consumers, are lost with these processing conditions.

Chilling and/or refrigeration reduce the rate of biochemical and microbiological changes, and hence extend the shelf life of fresh foods, causing minimal changes to sensory characteristics and nutritional properties of foods [3]. However, fish even when held under chilled conditions quickly deteriorates, and considerable amounts are wasted due to spoilage, representing important economic losses. Accordingly to FAO, fish losses caused by spoilage are estimated as 10 to 12 million tonnes per year, accounting for around 10 % of the total production from capture fisheries and aquaculture [4].

Food additives, *e.g.* as antimicrobials and antioxidants, are considered to be without potential adverse effects and some are classified as generally recognized as safe [5]. Nevertheless, it is known that consumers privilege the use of substances from natural origin. Additionally, consumers are increasingly demanding for high quality, convenient, fresh-like appearance, long shelf life, and minimally processed fish products.

Many attempts have been done to search new technologies to preserve fresh fish, especially those able to extend quality over longer shelf life periods, in order to meet consumer demands, contributing simultaneously to waste

reduction. In this context, the subjects addressed in this thesis are the use of natural substances with bioactive properties extracted from plants and high pressure processing (HPP) to preserve fresh fish, namely sea bass.

## 1.1. *Sea bass Dicentrarchus labrax*

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species belonging to the family Moronidae, widely distributed in the Mediterranean Sea and Atlantic Ocean, and is one of the main marine fish species farmed in Mediterranean countries, *e.g.* Greece, Spain, and Italy [6-7] (Figure 1.1). This species production was almost 126 thousand tonnes in 2010, accordingly to recent published results of global aquaculture production [8].

**Kingdom:** Animalia (animals)
**Phylum:** Chordata (chordates)
**Subphylum:** Vertebrata (vertebrates)
**Superclass:** Osteichthyes (bony fishes)
**Class:** Actinopterygii (ray-finned fishes)
**Subclass:** Neopterygii
**Infraclass:** Teleostei
**Superorder:** Acanthopterygii
**Order:** Perciformes
**Suborder:** Percoidei
**Family:** Moronidae
**Genus:** *Dicentrarchus*
**Species:** *Dicentrarchus labrax* (Linnaeus, 1758)

**Synonyms:** *Morone labrax* (Linnaeus, 1758)
**Common names:** sea bass
European seabass
white salmon

**Length at first maturity:** 23-46 cm
**Common length:** 50 cm
**Minimum size for catch:** 36 cm in European Union waters
**Conservation status:** Least concern

**Figure 1.1.** Taxonomic classification, photo, and general data of sea bass *Dicentrarchus labrax*. Source: Integrated Taxonomic Information System and FishBase; photo taken by Fabio Crocetta.

Sea bass is highly appreciated because of its excellent organoleptic properties and high nutritional quality [6-7]. The protein content is in the range 17.6-23.4 %, while fat, moisture and ash vary between 1.2-9.2 %, 64.4-77.9 %, and 1.1-1.9 %, respectively, based on a review on compositional and organoleptic quality of sea bass done by Grigorakis [9]. It is also known that feeding characteristics (composition, quality, and amount), season, photoperiod, species size, and life cycle phase contribute to different compositions in fish muscle [9].

For many fish species, consumers prefer wild specimens than farmed counterparts. However, differences in the organoleptic characteristics between farmed and wild fish were not described for sea bass, and this is probably due to the fact that artificial diets are closer to the sea bass nutritional needs [9].

Usually, sea bass is sold in retail stores as whole or gutted, and after preparation (*e.g.* scaling) it is packed in cheap plastic bags that offer no more than just a convenient way to transport products. Fillets in pre-packed trays also appear in some supermarkets and chain stores, but it is not so frequent.

Many efforts have been done to study alternative methods to extend the shelf life of fresh ungutted and gutted sea bass, including the use of melting ice [10], flake ice [11], pretreatments with slurry ice [11], gamma radiation [12], and modified atmosphere packaging [13], all combined with refrigerated storage (0-4 ºC; Table 1.1).

According to recent data, whole sea bass presents a shelf life period of 19 days [10]. However, more convenient presentations to consumers, such as gutted fish and fillets, reveal shelf life periods of 7-13 days (Table 1.1). The shorter shelf life reflects bacterial contamination during processing, which is also enhanced by the enzymatic and oxidative reactions in the exposed fish flesh [7].

The combined use of $CO_2$ solubilisation, followed by packaging in air [14] or under vacuum [15] and refrigeration storage is an example of the innovative methods tested to preserve fresh sea bass fillets. It was also referred that packaging under vacuum increased the overall shelf life of fresh sea bass fillets by four additional days, compared with atmospheric air packaging [14-15] or with fillets just covered with polyethylene films and stored in flake ice [7], all combined with refrigeration.

In the study of Chéret and co-authors [16], similar values of shelf life (14 days taking into account microbiological analysis) were obtained in pressurized (500 MPa) vacuum packed fillets and stored under refrigerated conditions. These results seem to indicate that HPP does not offer an important improvement to extend the shelf life of sea bass fillets, compared with the use of vacuum packaging only. However, the initial total microbial counts of fillets was higher in the fish treated with HPP (6 log CFU.g$^{-1}$) [16] than in the study with vacuum packed fillets (2 log CFU.g$^{-1}$) [15].

The use of natural substances from plants revealed potential to preserve fresh fish [17]. Thus, treatments of fresh sea bass fillets with thyme essential oil (0.2 % v/w) further stored under modified atmosphere conditions (10 % $O_2$, 60 % $CO_2$, 30 % $N_2$) increased the shelf life to 17 days [17].

**Table 1.1.** Shelf life of fresh sea bass *Dicentrarchus labrax* as influenced by processing and storage conditions, reported in different studies.

| Sample details and processing/packaging conditions | | | Storage conditions | Shelf life | Reference |
|---|---|---|---|---|---|
| whole fish | | | melting ice + refrigeration (0-4 ºC) | 19 days | [10] |
| whole fish | control (flaked ice) | atmospheric air; packed (not specified) | ice (1:1 fish:ice) + refrigeration (4 ºC) | 13 days | [11] |
| | slurry ice (2 h pre-treatment with 40 % of ice and 60 % of seawater with 3.3 % of salinity) | | | 15 days | |
| whole fish | control (non-irradiated) | polystyrene boxes | plastic bags with ice + refrigeration (4 ºC) | 13 days | [12] |
| | gamma radiation 2.5 kGy (0.02 $kGy.min^{-1}$) | | | 15 days | |
| | gamma radiation 5 kGy (0.02 $kGy.min^{-1}$) | | | 17 days | |
| not specified | | | ice + refrigeration (4 ºC) | 6 days | [18] |
| | | | ice + refrigeration (1 ºC) | 10 days | |
| whole fish | | polystyrene boxes; fish covered with polyethylene film | flaked ice (3:1 ice:fish) + refrigeration (2 ºC) | 12-13 days | [7] |
| fillets | | | | 8-9 days | |
| whole fish | | polystyrene boxes; fish covered with polyethylene film | flaked ice | 13 days | [19] |
| gutted fish | | | | 8 days | |
| whole fish | | wrapped with stretch films | refrigeration (4 ºC) | 10 days | [20] |
| scale-less-gutted fish | | | | 8 days | |
| scale-less fillets | | | | 8 days | |
| gutted fish | modified atmosphere (0-40 % $O_2$, 0-70 % $CO_2$, 0-79 % $N_2$) | polystyrene trays sealed with a film of polyamide / ethylene vinyl alcohol / polyethylene | refrigeration (3 ºC) | 7-9 days* | [13] |
| fillets with skin | control (without soluble gas stabilization treatment) | atmospheric air; polyamide / polyethylene bag | refrigeration (1 ºC) | 9 days | [14] |
| | soluble gas stabilization ($CO_2$; 2 bars, 30-60 min) | | | 9 days | |
| fillets with skin | control (without soluble gas stabilization treatment) | vacuum; polyamide / polyethylene bag | refrigeration (1 ºC) | 13 days | [15] |
| | soluble gas stabilization ($CO_2$; 2 bars, 30-60 min) | | | 13 days | |
| fillets | control (not pressurized) | vacuum; polyethylene bag | refrigeration (4 ºC) | 7 days** | [16] |
| | high pressure processing (100-400 MPa; 10 ºC; 5 min) | | | 7 days** | |
| | high pressure processing (500 MPa; 10 ºC; 5 min) | | | 14 days** | |
| fillets | atmospheric air | low density polyethylene / polyamide barrier pouches | refrigeration (4 ºC) | 6 days | [17] |
| | thyme essential oil (0.2 % v/w); atmospheric air | | | 8 days | |
| | modified atmosphere (10 % $O_2$, 40-60 % $CO_2$, 30-50 % $N_2$) | | | 12 days* | |
| | thyme essential oil (0.2 % v/w); modified atmosphere (10 % $O_2$, 60 % $CO_2$, 30 % $N_2$) | | | 17 days | |

Farmed sea bass was used in all studies.
* shelf life of the best treatment tested.
** shelf life considering only microbiological analysis.

## 2. Fish *post mortem* changes

Immediately after fish death, several biochemical reactions start, which are of main importance for the quality and shelf life of seafood [21]. Postharvest biochemical events in fish can be classified into two phases: metabolic and microbial [22]. The metabolic changes result from the activity of enzymes present in the fish flesh, while microbial changes result from the activity of microorganisms from fish microflora, environment, or other external contamination [22].

These reactions depend on several factors, such as fish species, physiological condition, season, and the environmental influence to which the living fish has been exposed to (*e.g.* water temperature and salinity), which may have a determinant effect on the quality loss and on the spoilage rate [21]. In addition, catching and harvesting methods, as well as slaughtering procedures and performance have also a great effect on the biochemical reactions related to changes on the fish muscle [21]. In general, fish spoil more slowly in larger, lean, and bony fish, than in smaller, fatty, and cartilaginous fish species, respectively [23].

Freshness, the loss of freshness, and the development of spoilage characteristics in fish can be monitored by means of objective measurements (microbiological, biochemical, chemical, and physical methods) and also by sensory evaluation. Most often, quality refers to the aesthetic appearance and freshness or degree of spoilage which the fish has undergone, but it may also involve nutritional, convenience, and safety aspects, and it's a term that may imply different things for different people [24]. Since the consumer is the ultimate judge of quality, it is of utmost importance that results of chemical, physical, and microbial methods are correlated with sensory findings [24].

### 2.1. *Rigor mortis*

Immediately after death, fish muscle is relaxed, soft, and can easily be flexed, and at this time the fish is said to be in *pre rigor* phase [24-25]. Shortly after death, if the fish was starved or stressed, glycogen reserves are depleted, the muscle begins to get stiffen and harden, and the muscle is said to be in *rigor mortis* [23-25]. After some hours or days, depending on the species, the muscle gradually begins to soften and become limp again, and the muscle is said to be in *post rigor* phase [25].

Fish *rigor mortis* results from a series of complex biochemical changes in the muscle after death [25]. Thus, even after fish death, some of the enzymes in the flesh act on those substances that normally keep the muscle pliable and life-like with a limp elastic texture that usually persists for some hours [24-25]. For so long as the muscle contains a reserve of energy, substances that keep the muscle pliable are transformed or replaced by one set of enzymes as fast as they are destroyed by another; thus the muscle stay pliable for a time after death [25]. Once the energy reserves are used up, the replacement stops because adenosine triphosphate (ATP) is no longer sufficient to remove the connection between thick (myosin) and thin (actin) filaments of the muscle myofibrils [21; 25-26]. The connection remains in a contracted state and gets rigid [21; 25-26].

The resolution of *rigor mortis* is a process still not completely understood, but results in the subsequent softening (relaxation) of the muscle tissue and is thought to be related to the activation of one or more of the naturally-occurring muscle enzymes, digesting away components of the *rigor mortis* complex [25]. The physical condition of the fish at death, its size and species, the method used for stunning and killing the fish, and the temperature at which it is kept after death are some of the factors that can markedly affect the time a fish takes to go into, and pass through *rigor mortis* [23; 25].

*Rigor* condition can affect the quality of fish muscle by causing gaping (*i.e.* weakening of the connective tissue and rupture of the fillet), toughness, and excessive drip loss in fish [25]. As an example, if the fish is cooked in *pre rigor* phase the texture will be very soft and pasty, if it is cooked in *rigor mortis* the texture is tough but not dry, and if cooking occurs in the *post rigor* phase the flesh will become firm, succulent, and elastic [23-24].

### 2.2. *Nucleotide catabolism*

In live fish, muscle contraction is powered by the release of energy resulting from the breakdown of ATP [27]. The enzyme involved in this process is a calcium-activated ATPase, and when intracellular calcium levels are higher than 1 μM, this enzyme reduces the amount of free ATP in the sarcoplasm and the muscle contracts due to cyclic nature of the formation of actin/myosin non-covalent cross bridges [27-28].

The ATP breakdown products most commonly formed in fish after death and the enzymes involved are illustrated in Figure 1.2. Such products result from a chain reaction in which metabolites typically rise and then fall as the next metabolite in the degradative chain begins to rise [27]. ATP is converted to

adenosine diphosphate (ADP), ADP to adenosine monophosphate (AMP), and AMP to inosine monophosphate (IMP) and usually this process takes place within 24 hours [27-28]. IMP has been recognized as having flavour-enhancing properties, and these changes are thought to be mainly autolytic since insufficient time has elapsed to allow the proliferation of spoilage organisms [27; 29]. On the other hand, degradation of inosine (HxR) to hypoxanthine (Hx) is due to the presence of both autolytic and bacterial enzymes (5' nucleotidase and acid phosphatase), and Hx contributes to the off-flavours typical of spoiled fish [27]. The degradation of Hx to xanthine and uric acid are perhaps of less importance in the catabolic breakdown of nucleotides, since the fish quality has been already judged unacceptable [27].

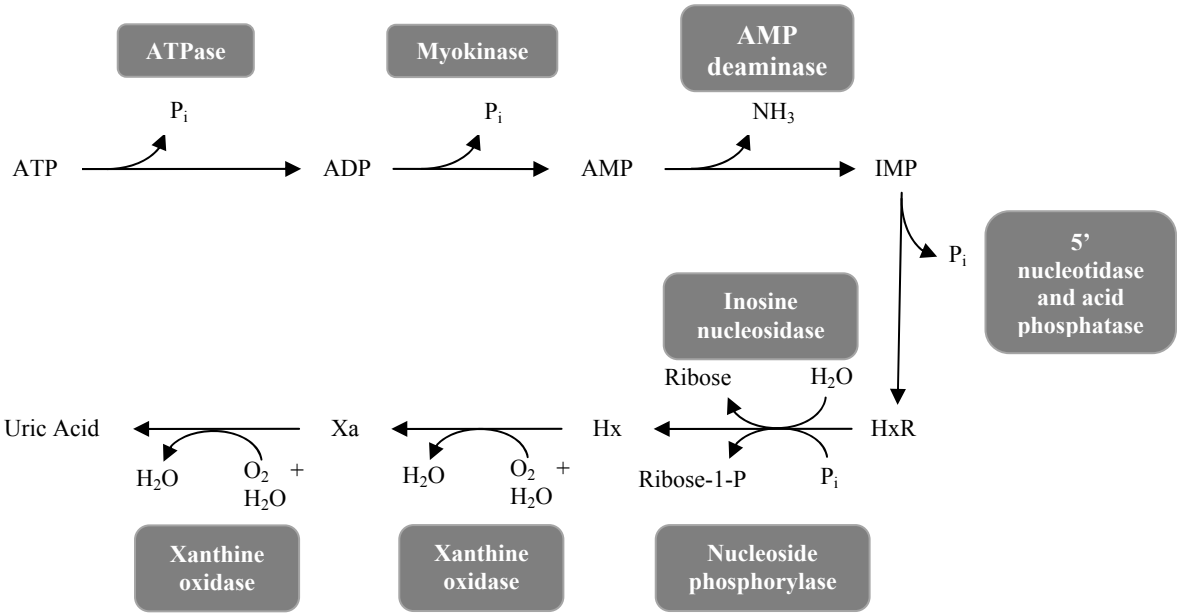


**Figure 1.2.** *Post mortem* ATP degradation in fish. *Abbreviations*: ADP – adenosine diphosphate; AMP – adenosine monophosphate; IMP – inosine monophosphate; HxR – inosine; Hx – hypoxanthine; Xa – xanthine. The enzymes involved in the reactions are indicated in boxes with gray backgrounds. Adapted from Gill [27].

Adenosine nucleotides and their degradation products have been used to evaluate freshness of fish muscle [30-31], but the rate of *post mortem* degradation is strongly influenced by species condition, muscle type, and temperature [22]. Whitin the freshness indexes, K-value is commonly used and expresses the relative degree of decomposition. It was defined by Saito and co-authors [32] as the ratio of the final breakdown products of ATP catabolism, namely HxR and Hx, to the total amount of ATP and related compounds (ADP, AMP, IMP, HxR, and Hx) in the fish muscle:

$$K = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx}$$

Taking into account that adenosine nucleotides rapidly decrease and are negligible within 25 h *post mortem*, the K-value can be simplified as the $K_I$-value, which was defined by Karube and co-authors [33], that is estimated accordingly to the following equation:

$$K_I = \frac{HxR + Hx}{IMP + HxR + Hx}$$

**2.3. *Total volatile bases accumulation***

Total volatile bases (TVB) comprise amines with one atom of nitrogen per molecule, and are one of the most widely used measurements of spoilage evaluation of fishery products [34]. TVB include trimethylamine (TMA), produced by spoilage bacteria; dimethylamine, produced by autolytic enzymes; ammonia, produced by the deamination of amino acids and nucleotide catabolites; and other volatile basic nitrogenous compounds associated with seafood spoilage [24; 34]. TVB and TMA are estimated as total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N), respectively. TVB-N values do not reflect the mode of spoilage, bacterial or autolytic, and are considered unreliable for the measurement of spoilage during the first days of storage of most fish species, still TVB-N value reflects later stages of advanced spoilage [24; 35]. However, when the results of sensory evaluation are not conclusive, the European Commission [36] has established regulation for TVB-N values for some fish species, and values cannot exceed 25-35 mg.100 $g^{-1}$ of fish muscle depending on the species.

Trimethylamine oxide is naturally present in the living tissue of many marine fish species, it makes part of the non-protein nitrogen fraction, and is an important compound for maintenance of several physiological functions in fish [37]. Trimethylamine oxide reduction by the action of bacterial activity forms TMA, which is often associated with the typical fishy odour of spoiled fish [24]. The correlation between TMA-N and bacterial numbers is often not very good, because only specific spoilage bacteria are capable of producing large amounts of TMA compounds, representing a small number of the total bacterial microflora [24].

Certain types of fish, such as gadoid species, contain the enzyme trimethylamine oxide dimethylase, which converts trimethylamine oxide into equimolar quantities of dimethylamine and formaldehyde, especially in frozen

gadoid fish [24]. Dimethylamine has little or no effect on the flavour or texture of fish *per se,* but is an indirect indicator of protein denaturation which is often traceable to improper handling before and/or during frozen storage [24].
Ammonia is formed by the bacterial deamination of proteins, peptides, and amino acids and is also produced by the autolytic breakdown of AMP [24]. Although ammonia has been identified as a volatile component in a variety of spoiling fish, and has potential as an objective quality indicator for fish which degrades autolytically rather than primarily through bacterial spoilage, few studies reported the quantification of this compound [24].

## 2.4. *Protein degradation*

The sensory quality of fish depends largely on their texture characteristics, which in turn are related to their structural features [38]. The flesh of teleost fish is divided into blocks of muscle cells separated into flakes (myotomes) by connective tissue called myocommata, and each muscle cell is surrounded with connective tissue which attaches to the myocommata by means of fine collagenous fibrils [24]. The main protein components in the fish muscle tissue are the myofibrillar proteins, which form the myofibrils, the basic contractile element of the musculature, the sarcoplasmic proteins mostly composed of enzymes, and the connective tissue proteins, mainly consisting of collagen [22; 24; 38].
Endogenous proteolytic enzymes that cleave muscle proteins under physiological conditions and at neutral pH can be a factor in the resolution of *rigor mortis* [28]. Autolytic degradation of myofibrillar and connective tissue proteins during *rigor mortis*, resolution of *rigor mortis*, and storage are believed to cause textural changes in the fish muscle, resulting in the softening of the muscle tissue [22; 28]. This reduces the cohesiveness of the muscle segments in fillets, which promotes gaping or breakdown of the myotome [28].
Many proteases have been isolated from fish muscle, including cathepsins and calpains. The cathepsins are acid proteases usually found in lysosomes, and are believed to be responsible for protein breakdown at sites of injury in living tissues, and become released into the cell juices upon physical abuse or upon freezing and thawing of *post mortem* muscle [24; 28]. Cathepsin D is considered to be the most important enzyme in *post mortem* degradation [39], since other cathepsins have a relatively narrow pH range of activity far too low to be of physiological significance [24]. Calpains also remain active at the slightly acidic

*post mortem* pH, exist principally in the cytosol, and are capable of degrading myofibrillar proteins [24; 28; 40].

### 2.5. *Lipid degradation*

Lipids occur mainly in fish as triacylglycerols, which are the main form of stored energy and are localized in the flesh and liver, and also as phospholipids and cholesterol that are essential components of cell membranes and mitochondria [41]. The reactions in fish lipids of importance for quality deterioration are hydrolysis (Figure 1.3) and oxidation (Figure 1.4), and result in production of substances among which some have unpleasant (rancid) taste and smell [24]. Fish that contain high levels of lipids, are particularly susceptible to lipid degradation [26].

The various reactions are either catalyzed by microbial enzymes or by intracellular or digestive enzymes from the fish themselves [24]. An important response with regard to lipid oxidation is the cellular membranes' inability to maintain their integrity after death, and lysosomal enzymes, such as phospholipase and lipase, may be released, affecting the susceptibility of lipids [26].

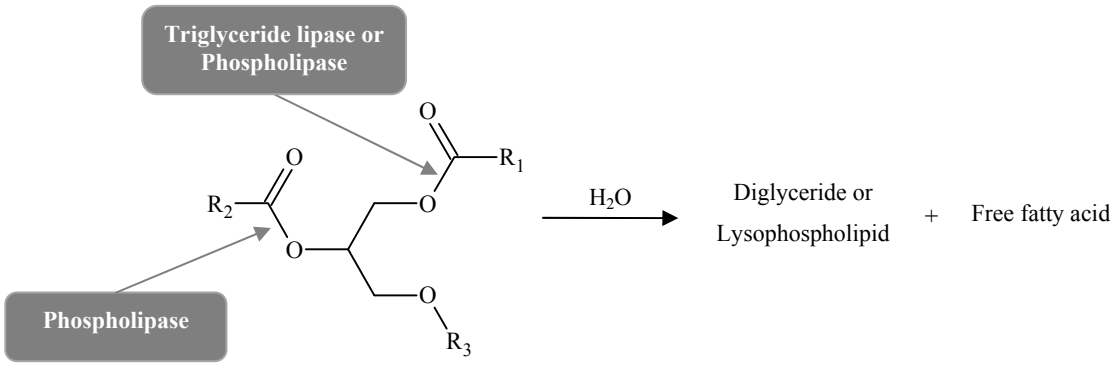


**Figure 1.3.** Hydrolytic reactions of triglycerides and phospholipids. The enzymes involved in the reactions are indicated in boxes with gray backgrounds. Adapted from Huss [24].

In enzymatic hydrolysis, phospholipids and triglycerides are cleaved by lipases, originated from the digestive tract or excreted by certain microorganisms, forming free fatty acids [22; 24] (Figure 1.3). However, a correlation between the activity of these enzymes and the rate of appearance of free fatty acids has not been firmly established yet [24]. Free fatty acids may be responsible for the formation of off-flavours or off-odours, the destruction of some vitamins and

aminoacids, changes in water holding capacity of muscle proteins, resulting in a detrimental effect on both the textural properties and flavour of fish [42]. Hydrolysis is more profound in ungutted than in gutted fish probably because of the involvement of digestive enzymes, and in fatty fish, but production of free fatty acids also occurs in lean fish species, even at low temperatures [24].

In the oxidation process, oxygen from the atmosphere is captured by unsaturated fatty acids, creating unstable intermediates that eventually breakdown to form unpleasant flavour and aroma compounds [26].

The most common and important process by which unsaturated fatty acids and oxygen interact is a free radical mechanism characterized by three main phases: initiation, propagation, and termination (Figure 1.4). Initiation occurs as hydrogen is abstracted from an unsaturated fatty acid, resulting in a lipid free radical, which in turn reacts with molecular oxygen to form a lipid peroxyl radical, which will then proceed to abstract hydrogens from unsaturated fatty acids resulting in a lipid hydroperoxide and a new lipid free radical [24; 26]. This propagation continues until one of the radicals is removed by reaction with another radical or with an antioxidant, whose resulting radical is much less reactive [24; 26].

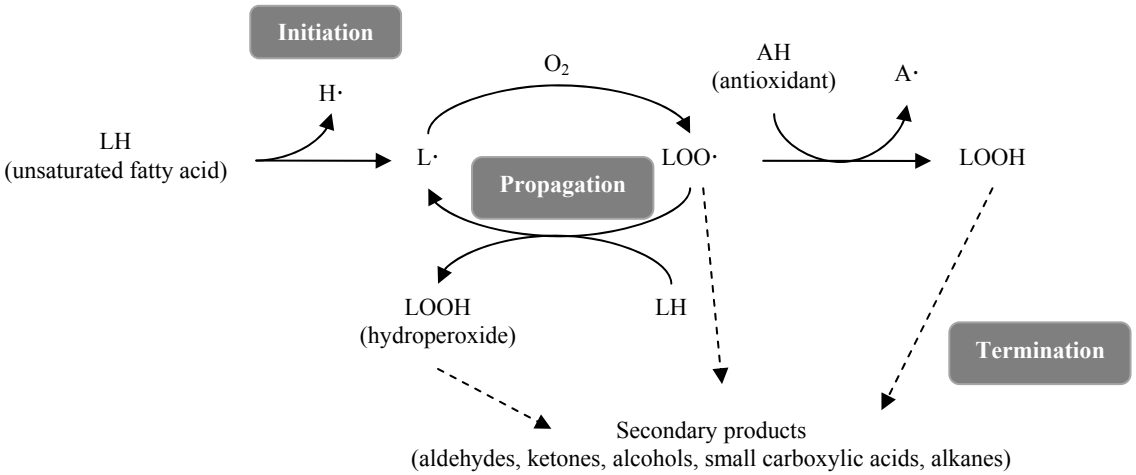


**Figure 1.4.** Autoxidation of polyunsaturated fatty acids. Adapted from Huss [24].

Fatty acid hydroperoxides may also be formed enzymatically, catalyzed by the enzyme lipoxygenase, which is unstable and is probably important for lipid oxidation only in fresh fish, since cooking or freezing/thawing destroys the enzyme activity [24].

By themselves, lipid hydroperoxides are not considered harmful to food quality and are tasteless, and therefore measurements of peroxide value usually correlates poorly to sensory properties [24; 26]. However, the hydroperoxides are readily broken down, a reaction catalyzed by heavy metal ions, to secondary autoxidation products of shorter carbon chain-length, and these compounds are responsible for off-flavours [24; 26].

The secondary products (mostly aldehydes, ketones, alcohols, small carboxylic acids, and alkanes) give rise to a very broad odour spectrum and in some cases to a yellowish discoloration, and the low flavour threshold of most aldehydes formed during lipid oxidation means they are easily perceived by the consumer, and therefore reduce the acceptability of the products [24]. Several of the aldehydes can be determined as thiobarbituric acid reactive substances.

### 2.6. *Microbiological*

In live fish, microorganisms are found on the outer surface (skin and slime), and also in some inner surfaces like gills and gastro-intestinal tract, while the remaining inner tissues of a healthy fish are sterile [43-44]. The most susceptible part of the fish is the gills region, and if the caught fish is not gutted, bacteria can spread into the flesh [44].

The initial contamination may include a wide variety of microorganisms. Temperate water fish species show a microflora dominated by psychrotrophic Gram-negative bacteria such as *Pseudomonas*, *Shewanella*, *Acinetobacter*, Aeroemonadaceae, *Flavobacterium*, Vibrionaceae, *and Moraxella,* but Gram-positive organisms such as *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus*, and *Corynebacterium* can also be found [43]. However, only a selection of these is able to colonize and contaminate fish products [43].

Spoilage organisms first utilize the simpler compounds, and microbiological spoilage, which is a consequence of microbial activity and/or growth, can result in off-odours and off-flavours production caused by specific bacteria, or even the result of microbial growth *per se* that becomes evident as visible growth [43-44].

The time between the catch and spoilage depends mainly on handling procedures, storage temperature, and fish species, but other important factors can greatly influence this period, like the fish aquatic environment, a high *post mortem* pH in the flesh, and the presence of large amounts of non-protein nitrogen [43]. Changes in processing, packaging, and storage conditions of fish

cause changes in the development and composition of the spoilage association and a complete different type of spoilage microorganisms can be found [43; 45].
The most common microbiological methodologies, used in studies whereas fish is stored under refrigerated temperatures, include the determination of psychrophiles and mesophiles microorganisms, *Pseudomonas*, hydrogen sulfide producing bacteria, Enterobacteriaceae, and lactic acid bacteria, but more specific analyses may also be performed, especially to determine the presence of pathogenic microorganisms. An example of the dominant microflora in fish is shown in Table 1.2, depending on storage and packaging conditions.

**Table 1.2.** Dominating microflora at spoilage of fresh fish (cod) (adapted from Huss [24]).

| Storage conditions | Packaging atmosphere | Dominant microflora |
|---|---|---|
| 0 ºC | aerobic | Gram-negative psychrotrophic, non-fermentative rods (*Pseudomonas*, *S. putrefaciens*, *Moraxella*, *Acinetobacter*) |
| 0 ºC | vacuum | Gram-negative rods; psychrotrophic or with psychrophilic character (*S. putrefaciens*, *Photobacterium*) |
| 0 ºC | modified atmosphere | Gram-negative fermentative rods with psychrophilic character (*Photobacterium*)<br>Gram-negative non-fermentative psychrotrophic rods (1-10% of flora; *Pseudomonas*, *S. putrefaciens*)<br>Gram-positive rods (lactic acid bacteria) |
| 5 ºC | aerobic | Gram-negative psychrotrophic rods (Vibrionaceae, *S. putrefaciens*) |
| 5 ºC | vacuum | Gram-negative psychrotrophic rods (Vibrionaceae, *S. putrefaciens*) |
| 5 ºC | modified atmosphere | Gram-negative psychrotrophic rods (Vibrionaceae) |
| 20-30 ºC | aerobic | Gram-negative mesophilic fermentative rods (Vibrionaceae, Enterobacteriaceae) |

Seafood should not contain microorganisms, their toxins, or metabolites in quantities that present a risk for human health [46]. The European Commission [46] established regulation for microbiological criteria of foods, including fish products, and the permissible limits for several bacteria are expressed in this legislation. However, it is not established in legislation the maximum permissible levels of total viable counts in seafood. Accordingly to International Commission for the Microbiological Specifications of Foods [47], the maximum recommended microbiological limit for raw seafood is $10^5$ colony forming unit (CFU) per g to be considered as having good quality, and $10^7$ CFU.g$^{-1}$ as the upper limit of acceptability.

### 2.7. *Sensory*

In sensory evaluation, appearance, odour, texture, and flavour are evaluated using the human senses. However, the response to the same level of stimuli can vary between assessors, and therefore could contribute to a non-conclusive answer of the test [24]. Different types of tests have been developed for sensory

evaluation, and information about sensory evaluation of fish to be used by pannelist can be consulted in the guidelines proposed by FAO & WHO [48]. In this context, sensory evaluation should be carried out in adequate rooms, by trained people, who evaluate a specialized range of products, and use one sensory methodology in order to receive objective responses, which describe features of the fish being evaluated [24; 48].

During fish storage, the intensity of sensory attributes change [23]. Generally, the quality deterioration of fresh fish is characterized by a initial loss of the intrinsic fresh fish flavour with a sweet, seaweedy, and delicate taste which can be slightly metallic, to a final phase, in which fish is spoiled and putrid, and is rejected [24; 43]. As the fish deteriorates, the texture is usually still kept pleasant, but there is a loss of the initial odour and taste, as the flesh becomes neutral, and progressively signs of spoilage appear and volatile and unpleasant-smelling substances are produced, resulting in a very characteristic fishy smell [24]. At the beginning, the off-flavours may be slightly sour, fruity, and slightly bitter, especially in fatty fish, and during the later stages sickly sweet, cabbage-like, ammoniacal, sulphurous, and rancid smells develop, and the texture becomes either soft and watery or tough and dry [24].

## 3. Preservation methods

### 3.1. *Natural substances*

#### 3.1.1. *General characteristics of natural substances*

Food additives are very often used for preservation and technological purposes, and the characteristics and objectives of such substances are defined in regulation. Food additives are substances not normally consumed as a food in itself and not normally used as a characteristic ingredient [49]. Additives are added to food intentionally for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport, or storage, such as the preservation of food [49]. The European Commission has established regulation on food additives. To be included in the approved list, substances must meet the following conditions: does not, on the basis of the scientific evidence available, pose a safety concern to the health of the consumer at the level of use proposed; there is a reasonable technological need that cannot be achieved by other economically and technologically practicable means; its use does not mislead the consumer; and must have other advantages for consumers like preserving the nutritional quality of the food and providing necessary ingredients or

constituents for foods manufactured for groups of consumers with special dietary needs [49].

The increase consumer demands for food products free of additives from synthetic origin resulted in a growing interest in the investigation of natural substances for the discovery of active compounds that can potentially be used by the food industry. Aromatic plants have been known for long to possess bioactive properties, such as antimicrobial and antioxidant activities [50-51], and research on this field increased greatly worldwide due to its potential for food preservation.

Plants belonging to the genus *Origanum, Thymus*, *Rosmarinus*, *Salvia*, *Mentha*, *Citrus*, and *Coriandrum* are among the most studied ones, but in the last years the number of plant species studied in this field increased greatly. In several research works, the antimicrobial and antioxidant properties of plant extracts and essential oils have been studied *in vitro* [52-54]. Essential oils constituents had also been the focus of attention, both for antioxidant [55-56] and antimicrobial [57-60] properties.

Natural substances extracted from plants can be used to prolong the storage stability of food, by inhibiting the growth of foodborne spoilage or pathogenic microorganisms and protecting food from oxidative stress damage. However, changes in the relative concentrations of constituents may affect its efficacy. A plant extract or an essential oil with a consistent chemical composition is required for application in the food industry [61]. In general, a higher concentration (between 2 and 100 times higher, depending on the food) of essential oil is required to achieve an antibacterial effect in food, than indicated in preceding *in vitro* trials [62].

### 3.1.2. *Extraction of bioactive compounds*

Plant secondary metabolites are compounds of interest, some with known bioactive properties that need to be extracted and isolated from plants. Secondary metabolites are chemical compounds synthesized in several plant organs, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, or bark, and stored in secretory cells, cavities, canals, epidermic cells, or glandular trichomes [50; 61; 63]. These compounds have diverse ecological functions, acting as defensive substances against microorganisms and herbivores, but can also be important to attract insects for the dispersion of pollens and seeds [50].

The extraction of secondary metabolites starts with the preparation of plants. In general, after the plants are harvested, they are cleaned, and the desired plant parts are dried without using high temperatures, under controlled conditions to minimize the occurrence of chemical changes [64-65]. The reduction in water content allows the plant to be stored [65]. Then, and for an efficient extraction, it is necessary to cause cells rupture, which is done mechanically [65].

The non-volatile secondary metabolites are isolated from plants by solvent extraction, and the choice of solvent depends on the characteristics of the compounds to be extracted [65]. In some cases, water may be a suitable solvent, but organic solvents may be required [61]. If plant extracts are intended for food preservation non-toxic solvents must be used [65]. Non-volatile compounds include amino acids, lectins, glycoproteins, flavonoids, tannins, quinines, coumarins, terpenoids, steroids, and alkaloids as some of the most important bioactive compounds [61].

The volatile compounds are usually isolated from plant material through steam- or hydro-distillation methods, and the complex mixture with strong odour obtained is referred as an essential oil [50; 61]. Expression is also a method for essential oils extraction, used almost exclusively for the production of *Citrus* essential oils [66]. Supercritical carbon dioxide extraction is also used to extract compounds from plants, and the final product is normally recognized as an essential oil, although some authors do not defined them as such [66]. Essential oils can contain about 20-60 components at quite different concentrations, and are characterized by two or three major components at rather high concentrations [50]. The components extracted are mainly terpenes, terpenoids, phenol-derived aromatic components, and aliphatic components [50].

Several factors influence the chemical composition of plant extracts and essential oils, including the species, part of the plant [67], season of harvesting [68-69], geographical origin [70], and also the extraction method [71].

### 3.1.3. *Antibacterial properties*

A review on antibacterial properties of essential oils was done by Burt [62]. Studies showed that essential oils inhibit several food spoilage bacteria and human pathogenic bacteria, including both Gram-positive and Gram-negative bacteria [62]. The minimum inhibitory concentration (MIC) is a measure of the essential oil performance (although its definition differs between publications) and reported results showed that the MIC of essential oils from different plants

varied, in general, from 0.1 to 100 μL.mL$^{-1}$ [62]. The antibacterial activity of essential oils may be explained due to the presence of compounds, like carvacrol, thymol, and geraniol, with known antibacterial activity [62].
The methodologies that have been employed to evaluate the antibacterial activity of essential oils are based in different principles (*e.g.* diffusion and dilution) [62]. Changes in culture medium, bacteria growth phase, emulsifier, and essential oils volume are also frequent between studies [62]. All these modifications can affect the MIC value and thus comparison of published data is difficult and may not be conclusive.

### 3.1.4. *Antioxidant properties*

The antioxidant properties of medicinal and aromatic plants were reviewed by Miguel [72]. In general, the methods used to evaluate the antioxidant activity measure the lipid peroxidation or the free radical scavenging ability either by hydrogen atom transfer or by single electron transfer [72]. It is frequent to compare the essential oils antioxidant activity with that of commercial antioxidants (*e.g.* ascorbic acid and butylated hydroxytoluene – BHT). In particular, the essential oils of oregano *Origanum vulgare*, thyme *Thymus vulgaris*, and basil *Ocimum basilicum* were able to reduce α,α-diphenyl-β-pycrylhydrazyl (DPPH) radical even better than BHT [73]. The presence of phenolic compounds (*e.g.* carvacrol, thymol, and eugenol) in essential oils composition is normally pointed out to explain the antioxidant activity [72]. Still, the antioxidant activity is dependent on the chosen method, and also in essential oils origin and chemical composition [72].

### 3.1.5. *Natural substances application in the fresh fish preservation*

Previous studies reported the potential of natural substances to preserve fresh fish, including Asian sea bass (*Lates calcarifer*) [74-75], bluefish (*Pomatomos saltatrix*) [76], swordfish (*Xiphias gladius*) [77-78], rainbow trout (*Oncorhynchus mykiss*) [79-81], sea bream (*Sparus aurata*) [82], cod (*Gadus morhua*) [83], and sea bass [17] (Table 1.3).
In general, the essential oils of oregano and thyme species (from *O. vulgare* and *T. vulgaris*, respectively, or other species not specified) were chosen and tested in concentrations up to 1 % (v/w) of fish weight. The highest shelf life extension (7 days) was obtained for rainbow trout with the combined use of oregano essential oil (0.4 % v/w), salting (final concentration of NaCl in fish muscle was 13 g.kg$^{-1}$), and vacuum packaging [80], as a result of antibacterial activity of oregano essential oil.

**Table 1.3.** Shelf life of fresh fish preserved with natural substances from plants and stored under refrigerated conditions (0-4 ºC), reported in different studies.

| Sample details and processing/packaging conditions | | | Shelf life | Reference |
|---|---|---|---|---|
| Asian sea bass (*Lates calcarifer*); whole fish | thyme (*Thymus vulgaris*) or oregano (*Origanum vulgare*) essential oils (0.05 % v/v) | | > 33 days | [74] |
| bluefish (*Pomatomos saltatrix*); ungutted | thyme or laurel essential oil (1 % v/w) | High barrier plastic bags | 11 days* (3-4) | [76] |
| swordfish (*Xiphias gladius*); fillets | thyme essential oil (0.1 % v/w); atmospheric air | low density polyethylene / polyamide barrier pouches | 8-9 days (1-2) | [77] |
| | thyme essential oil (0.1 % v/w); modified atmosphere (5 % $O_2$, 50 % $CO_2$, 45 % $N_2$) | | 10-11 days (2-3) | |
| swordfish (*Xiphias gladius*); fillets | oregano essential oil (0.1 % v/w); atmospheric air | low-density polyethylene / polyamide barrier pouches | 6 days (0-1) | [78] |
| | oregano essential oil (0.1 % v/w); modified atmosphere (5 % $O_2$, 50 % $CO_2$, 45 % $N_2$) | | 10 days (0) | |
| rainbow trout (*Oncorhynchus mykiss*); fillets | oregano essential oil (0.4 % v/w) | atmospheric air; low density polyethylene / ethylene vinylalcohol / low density polyethylene barrier pouches | 7-8 days (3) | [79] |
| | oregano essential oil (0.4 % v/w); $O_2$ absorber | | 12-13 days (3) | |
| rainbow trout (*Oncorhynchus mykiss*); fillets | Salting (final concentration of NaCl in fish muscle was 13 $g.kg^{-1}$); oregano essential oil (0.2 % v/w) | vacuum; low density polyethylene / polyamide barrier pouches | 8 days (1) | [80] |
| | Salting (final concentration of NaCl in fish muscle was 13 $g.kg^{-1}$); oregano essential oil (0.4 % v/w) | | 14 days** (7) | |
| sea bream (*Sparus aurata*); fillets | Salting (final concentration of NaCl in muscle was 19 g $NaCl.kg^{-1}$); oregano essential oil (0.4 % v/w) | modified atmosphere (30 % $O_2$, 40 % $CO_2$, 30 % $N_2$); low density polyethylene / polyamide / low density polyethylene barrier pouches | 32-33 days*** (5) | [82] |
| | Salting (final concentration of NaCl in muscle was 19 g $NaCl.kg^{-1}$); oregano essential oil (0.8 % v/w) | | >33 days (> 5) | |
| sea bass (*Dicentrarchus labrax*); fillets | thyme essential oil (0.2 % v/w); atmospheric air | low density polyethylene / polyamide barrier pouches | 8 days (2) | [17] |
| | thyme essential oil (0.2 % v/w); modified atmosphere (10 % $O_2$, 60 % $CO_2$, 30 % $N_2$) | | 17 days (5) | |
| cod (*Gadus morhua*); fillets | bovine-hide gelatine-chitosan film with clove (*Syzygium aromaticum*) essential oil (0.75 $mL.g^{-1}$ of biopolymer) | vacuum | 11 days (8) | [83] |
| rainbow trout (*Oncorhynchus mykiss*); fillets | fillets immersed (2 × 30 seconds) in a coating of chitosan (2 % w/v) with cinnamon essential oil (1.5 % v/v) and dried (5 h; 10 ºC) | | 16 days (0) | [81] |
| Asian sea bass (*Lates calcarifer*); slices | unicorn leatherjacket (*Aluterus monoceros*) gelatine films with lemongrass (*Cymbopogon citratus*) essential oil (0.25 $g.g^{-1}$ of protein) | polystyrene trays wrapped with extensible polypropylene film | > 12 days (> 8) | [75] |

Values in brackets, in the shelf life column, represent the shelf life extension (in days) compared with the control treatment without natural substances from plants.
* stored in ice and refrigeration
** sensory analysis did not contribute for the shelf life.
*** microbiological analysis did not contribute for the shelf life.

Aqueous extracts from plants have also been studied to preserve fresh fish. In a previous report, treatments with ice prepared from aqueous extracts (oregano

*O. vulgare* or rosemary *Rosmarinus officinalis*) showed lower values of TVB-N and oxidation, compared with traditional ice treatments in Chilean jack mackerel *Trachurus murphyi* [84]. Films represent another form of antimicrobial and antioxidant carriers, which is the focus of the following section.

## 3.2. *Films*

### 3.2.1. *General characteristics of films*

The terms film and coating are generally used to indicate any type of material with a thin thickness (< 0.3 mm) used to cover the surface of a food, being the film a stand-alone wrapping material, whereas a coating is a particularly case of film, being it applied and formed directly on food surface itself [85].

Factors contributing to the interest in films development include: consumers demand for high quality foods and natural preservatives; food processors' needs for new preservation techniques; environmental concerns over disposal of non-renewable food packaging materials; and opportunities for creating new market outlets for under-utilized film-forming ingredients [86]. Additionally, films could hold in juices, prevent dripping, enhance product presentation, and eliminate the need for placing absorbent pads at the bottom of trays [86].

Films have been developed to protect food from dehydration, deterioration, and loss of appearance, flavour, and nutritional value, which take place due to time-consuming steps involved in handling, storage, and transportation of food items between producers and consumers [85].

Choice of a film forming substance and/or active additive depends on the desired objective, nature of the food product, and specific application, while the functional efficiency of films strongly depends on the nature of components and physical structure [87]. Films must meet a number of specific functional requirements, including colour, appearance, barrier properties to moisture and gases, mechanical, and rheological characteristics [88]. Additionally, films can control flavour and aroma transfer between food components and the atmosphere surrounding the food [89].

The composition of films to be in contact with food should be recognized as being safe under conditions of its intended use [85]. The definition of its edibility goes behind its legality and safety to use on food products, as they have to be acceptable by consumers; and might even have nutritional value [90]. Both edible and non-edible films have the potential to function as environmental-friendly packages [91].

Proteins, carbohydrates, and lipids can be used to prepare films. As a general rule, lipids are used to reduce water vapour permeability, while polysaccharides are used to control oxygen and other gas permeability [85]. Films primarily composed of proteins usually have suitable mechanical and optical properties [88], and most of them provide excellent barriers to oxygen [91]. This characteristic has been utilized to effectively protect high fat foods, which are known to form rancid off-flavours due to oxidation [91]. On the other hand, protein based films show poor water vapour barrier properties because of their hydrophilic nature [88].

### 3.2.2. *Preparation of protein films*

Protein based films are prepared from solutions comprised of three main components: protein, solvent, and plasticizer. Most protein sources are mixtures of various proteins comprising a considerable range of molecular weights [85]. Lower molecular weight components are generally more easily solubilised, though they exhibit higher permeabilities than higher molecular weight entities within films [85]. If they are used in solution rather than in emulsion, the solution will contain different protein fractions than the emulsion, unless all protein fractions are equally dissolved [85].

Water is the most common solvent chosen, because of its safety for consumption [91]. Plasticizers are compounds that can be added to film forming solution to improve the flexibility and mechanical properties of the film matrix [91]. Plasticizers reduce brittleness, increase flexibility and extendibility, and soften the films structure, by decreasing intermolecular forces between polymer chains [89]. The amount of plasticizer added varies widely within the range of 10-60 % by protein weight [89].

Solvent casting is the preferred method used to form protein films for research [91]. Because it is effective and cost-efficient, the most commonly used method for forming protein film samples for research is by manually spreading dilute film solutions (usually 5-10 % solids) of protein and plasticizer into levelled plates, and then drying them under ambient conditions or controlled relative humidity [91].

#### 3.2.2.1. *Fish protein films*

Protein films have been successfully prepared using fish collagen, myofibrillar, and sarcoplasmic proteins. Fish protein films studied were prepared with a considerable diversity of species, including round scad (*Decapterus maruadsi*) [92], bigeye snapper (*Priacanthus tayenus* and *Priacanthus*

*macracanthus*) [93-94], sardine (*Sardina pilchardus*) [95], Nile tilapia (*Oreochromis niloticus*) [96], blue marlin (*Makaira mazara*) [97], Allaska pollack (*Theragra chalcogramma*) [98], brownstripe red snapper (*Lutjanus vitta*) [94], and tuna (*Thunnus tynnus*) [99]. Most studies done so far focused on the optimization conditions for films preparation, including the influence of pH [93], components quality [100], and protein composition [92]. In general, films were characterized in terms of thickness, colour, transparency, mechanical properties, films solubility, and water vapour permeability.

To prepare films, proteins from fish muscle need to be purified and concentrated. This involves successive washing treatments to remove undesirable compounds like fat and pigments, in a process known as surimi (or minced fish) and it results in semipurified protein fractions [101-102]. Then, myofibrillar proteins are solubilised by adjusting the pH of the film forming solution [101].

The interest in fish protein films production is related not only with the demand for environmental-friendly and renewable food packaging materials, but also with the upgrading of by-products from the fish industry.

In the fish processing industry, the production of fillets and fish portions generates a large volume of by-products that include heads, tails, fins, viscera, backbones, and trimmings (*i.e.* muscle joined to the skin and bones) [102-103]. In particular, when fish is processed for fillets, the by-products account for 60-70 % of fish weight [104]. These by products are usually used to produce fish meals or just dumped [102]. The dump procedure is considered as an irresponsible utilization of natural resources, especially at a time where environmental concerns are high. These rejections can be used to recover proteins for several applications, including the preparation of films, representing a valuable alternative for the upgrading of by-products from fish processing industry [102].

### 3.2.3. *Films as active packaging*

Active packaging are systems with more than the conventional barrier properties, able to extend the shelf life of food products by maintaining their quality longer, increase their safety by securing foods from pathogens, inhibit the growth of spoilage microorganisms extending its shelf life, and enhance the convenience of food processing, distribution, retailing, and consumption [105]. Preservation, convenience, and other basic functions of packaging are certainly

important, but its disposal is also a matter of major concern of packaging development [105].

The use of films can be an application of active food packaging [105]. Films can act as vehicles for active compounds like antimicrobials and antioxidants, to increase the shelf life of foods, but nutrients, nutraceuticals, flavours, and pigments can also be incorporated to increase the nutritional value and enhance food quality [87; 89; 91]. The biodegradability and edibility of films are also extra functions that are not present in conventional packaging systems [105]. A review on films application as packaging was done by Debeaufort and co-authors [106].

A great variety of films incorporated with essential oils and plant extracts were studied. Films based on milk protein [107-108], gelatine from pigskin [109], and cassava starch-chitosan [110] incorporated with essential oils or water extracts of oregano (*O. vulgare*, *Origanum minutiflorum*, or other species), rosemary (*R. officinalis*), garlic (*Allium sativum*), and pimento are some examples of films studied. The concentration of essential oils or water extracts varied up to *ca*. 55 $\mu L.cm^2$ of film or 20 % of film forming solution. Films physical and mechanical characteristics were evaluated, as well as antimicrobial and antioxidant properties by performing *in vitro* tests. In some cases, microbiological and oxidative changes were followed during storage of several food items.

The incorporation of active compounds, like essential oils, in films can improve their functional properties, by reducing water vapour permeability, as well by providing antimicrobial and antioxidant properties, and thus extend food shelf life [107-109; 111].

### 3.2.4. *Films application in the fresh fish preservation*

Only few studies tested films incorporated with natural substances from plants to preserve fresh fish (Table 1.3). Cod (*G. morhua*) [83], rainbow trout (*O. mykiss*) [81], and Asian sea bass (*L. calcarifer*) [75] were preserved with films prepared with gelatine (from bovine or fish) and/or chitosan, and incorporated with the essential oils of clove (*Syzygium aromaticum*), lemongrass (*Cymbopogon citratus*), or cinnamon. Results showed a slower growth of fish spoilage bacteria in treatments with films incorporated with essential oils, during refrigerated storage of cod and Asian sea bass. However, the incorporation of cinnamon essential oil in a chitosan coating did not increase

the microbiological shelf life of rainbow trout, as the chitosan coating did by itself [81].

### 3.3. *High pressure processing*

#### 3.3.1. *General characteristics of high pressure processing*

High pressure processing (HPP) is a non-thermal food processing technology whereby foods are subjected to pressure levels generally in the range 100-600 MPa, but higher levels of pressure, up to 900 MPa, may also be used [112-113]. A typical modern high pressure system consists of a pressure vessel and a pressure-generating device [112; 114]. A schematic representation of a high pressure device is shown in Figure 1.5. Most pressure vessels are made from a single piece of material of high tensile strength which can withstand pressures of 400-600 MPa, and for higher pressure levels a pre-stressed multi-layer or wire-wound vessels are used [112]. The wall thickness of the vessel is determined by the maximum working pressure, the vessel diameter, and the number of cycles for which the vessel was designed to [112].

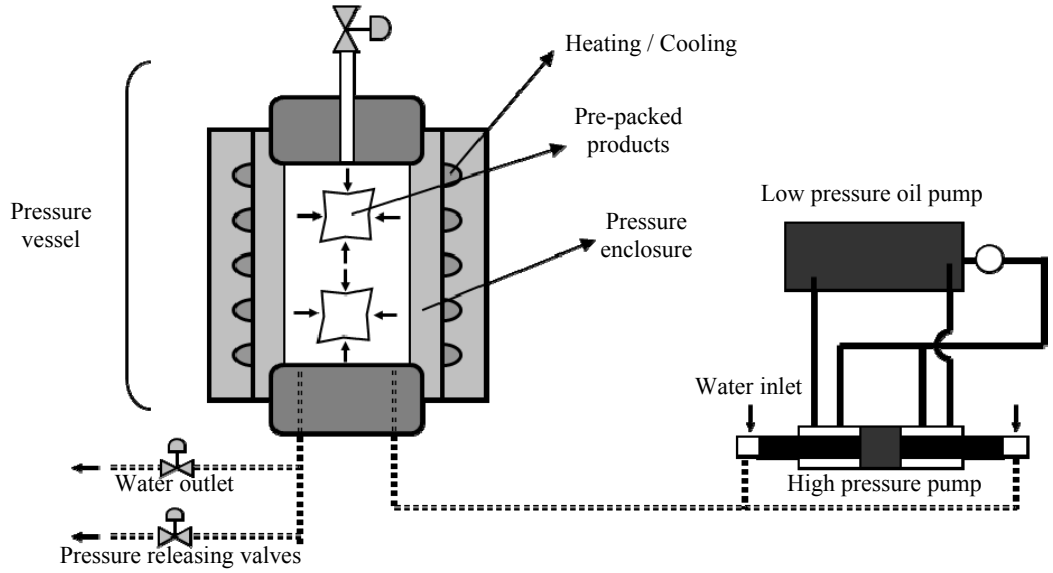


**Figure 1.5.** Typical high pressure processing system to treat pre-packed foods. Adapted from Singh [115].

A fundamental principle underlying HPP is that it is applied in an isostatic manner, in such a way that all regions experience a uniform pressure level unlike heat processing, where temperature gradients are established [114]. The uniform application of HPP to all parts of a food, irrespective of its size or shape is a significant advantage over other methods of processing, because the

food is treated evenly throughout [3], which makes easier its application in an industrial scale.

The second key feature of HPP, arising from Le Chatelier's principle, indicates that any phenomenon that results in a volume decrease is enhanced by an increase in pressure [116]. Thus, hydrogen bond formation is favoured by the application of pressure while some of the other weak linkages found in proteins are destabilised [116]. However, covalent bonds are unaffected [116].

In general, food packages are loaded in the pressure vessel, the pressure vessel is closed, and after all air is removed, a pressure transmitting medium (*e.g.* water or water-oil mixture) is pumped from a reservoir into the pressure vessel using a pressure intensifier until the desired pressure level is reached [3; 114]. Then, valves are closed, the pumping is stopped, and the pressure level can be maintained for the desired period [114].

With increasing pressure, the food reduces in overall size in proportion to the pressure applied (*e.g.* water reduces in volume by approximately 15 % at 600 MPa) but retains its original shape [3; 116]. The package used in the HPP treatments needs to be suitable to the pressures used, due to reduction in volume, and therefore a considerable stress and distortion of the package and the seal takes place [3].

The work of compression during HPP treatments also increases the temperature through a process known as adiabatic heating, and the extent of the temperature increase varies with the composition of the food (normally 3-9 ºC per 100 MPa) [114].

The HPP treatment can be used in combination with temperature, by controlling the temperature by heating or cooling the entire vessel at the outside, or by internal temperature control whereby the heat or cold source is place inside the vessel [112]. Even though, the effects of temperature variation on pressure, through expansion or shrinking of the pressure medium or the load, have to be taken into account [112].

### 3.3.2. *Effects of high pressure processing in food*

HPP is a technology of growing interest for processing and preservation of food, namely due to its ability to reduce microorganisms, to improve food functional properties, and to better retain food's nutritional and organoleptical characteristics [114; 117]. HPP is known to affect non-covalent chemical bonds of molecules, thus inducing modifications in water, proteins, polysaccharides, and lipids [39].

The main aim of treating food with HPP is to reduce or eliminate the relevant microorganisms that may be present, keeping the quality and safety of food [118]. The effectiveness in destroying microorganisms depends on several factors that must be taken into account when optimizing HPP treatments [114]. In HPP there is a threshold value, specific to each microorganism, below which no inactivation occurs, and above it, the lethal effect of the process tends to increase as pressure level increases, but not necessarily as pressure holding time increases[116]. High pressures cause collapse of intracellular vacuoles, and damage to cell walls and cytoplasmic membranes, and bacteria in the log phase of growth are, in general, more barosensitive than cells in the stationary or dormant phases [3].

The effects of pressure in vegetative microorganisms led already to several commercial pasteurized and extended shelf life food products, such as oysters, fruit juices, and sauces [113]. However, bacterial spores are the most resistant group and they cannot be significantly inactivated by pressure alone [113]. To kill bacterial spores initial temperatures above 70 ºC may be required to achieve sterile and shelf-stable foods [114]. A review on this subject was done by Patterson [113], and more recently by Yaldagard and co-authors [119].

The application of HPP may also be used to modify the functionality of food proteins, control enzymes related to food deterioration [120-121], or change the physical properties of the food material [122]. Enzymes vary in their barosensitivity: some can be inactivated at room temperature by pressures of a few hundred MPa, whereas others can withstand 1000 MPa, or even have their activity enhanced [3]. Both activation or inactivation is strongly dependent on pH, substrate composition, and temperature [3]. Furthermore, HPP affects cellular membranes, which when ruptured, may permit reactions between released intracellular enzymes and their substrates [3]. A review on these subjects was done by Messens and co-authors [120].

Pressure vessels used in research were generally regarded as costly and had a very small usable vessel volume, and hence the prospect of scaling up the design to a full production system was commercially difficult [116]. The desire to produce delicate, fresh, quality, and long shelf life products for a niche market, that could not be fully satisfied by other technologies, led to the appearance of HPP food products in a commercial scale [116].

### 3.3.3. *High pressure processing application in the fresh fish preservation*

Several research works studied the effect of HPP treatments to preserve fresh fish, including salmon (*Salmo salar* or other species) [123-126], sea bass [16], sea bream (*S. aurata*) [127-128], red mullet (*Mullus surmuletus*) [129], and yellowfin tuna (*Thunnus albacares*) [130] (Table 1.4). The pressure conditions applied varied between studies: pressure levels between 100 and 800 MPa, pressure holding times from 30 seconds until 60 minutes, and pressurization rates of 3 to 50 $MPa.s^{-1}$ were used. Furthermore, in some cases refrigeration temperatures were used, and in others the temperature was not controlled or processing was performed at room temperature.

**Table 1.4.** Shelf life of fresh fish treated with high pressure processing (HPP) and stored under refrigerated conditions, reported in different studies.

| Sample details and processing/packaging conditions | | | Storage conditions | Shelf life | Reference |
|---|---|---|---|---|---|
| Atlantic salmon; fillets | HPP (150 MPa; 1-5 ºC; 10 min) | vacuum; polyester / polyethylene bags | refrigeration (5 ºC) | 7-8 days (1) | [123] |
| | | modified atmosphere (50 % $O_2$; 50 % $CO_2$); polyester / polyethylene bags | | 14 days (2) | |
| salmon (*Salmo salar*); fillets | HPP (150-300 MPa; 15 min) | vacuum; bags | refrigeration (4 ºC) | > 6 days (> 1) | [124] |
| salmon (*Oncorhynchus kisutch*); fillets | HPP (135-200 MPa; 30 seconds; 15 ºC; 17 $MPa.s^{-1}$ ramp rate) | polyethylene bags | flake ice + refrigeration (4 ºC) | 17 days* (0) | [126] |
| sea bass (*Dicentrarchus labrax*); fillets | HPP (100-400 MPa; 10 ºC; 5 min; 3 $MPa.s^{-1}$ ramp rate) | vacuum; polyethylene bag | refrigeration (4 ºC) | 7 days* (0) | [16] |
| | HPP (500 MPa; 10 ºC; 5 min; 3 $MPa.s^{-1}$ ramp rate) | | | 14 days* (7) | |
| gilthead sea bream (*Sparus aurata*); fillets | HPP (250 MPa; 3-15ºC; 5 min; 25-50 $MPa.s^{-1}$ ramp rate) | bags | refrigeration (4 ºC) | 18 days (3) | [127] |
| red mullet (*Mullus surmuletus*) | HPP (220-330 MPa; 3-25 ºC; 5 min; 25-50 $MPa.s^{-1}$ ramp rate) | bags | refrigeration (4 ºC) | 14-15 days (2-3) | [129] |
| yellowfin tuna (*Thunnus albacares*); fillets | HPP (150-30 min; 150-220 MPa-15 min; < 20ºC) | vacuum; plastic pouches | refrigeration (4 ºC) | 15 days** (5) | [130] |
| | HPP (220 MPa-15 min; 200-220 MPa-30 min; < 20ºC) | | | 21 days** (11) | |

Values in brackets, in the shelf life column, represent the shelf life extension (in days) compared with the control treatment without HPP treatments.
* shelf life considering only microbiological analysis.
** shelf life considering only sensory analysis.

The shelf life of fresh fish treated with HPP and the processing conditions used are summarized in Table 1.4. The highest shelf life extension (11 days) was obtained in yellowfin tuna treated with HPP at 200 (30 min) and 220 MPa (15 and 30 min).

In general, HPP treatments caused a reduction in bacterial load, but an increase in the microbiological shelf life was not always observed. The effects of HPP in lipid oxidation was also studied, but reported results are not always in agreement, possibly due to differences in the species under study, package, storage, and HPP conditions.

## Objectives

This thesis aimed to study extracts and essential oils from plants and high pressure processing (HPP) to preserve fresh fish, using sea bass fillets as a case study model (see thesis work outline in Figure 1.6).

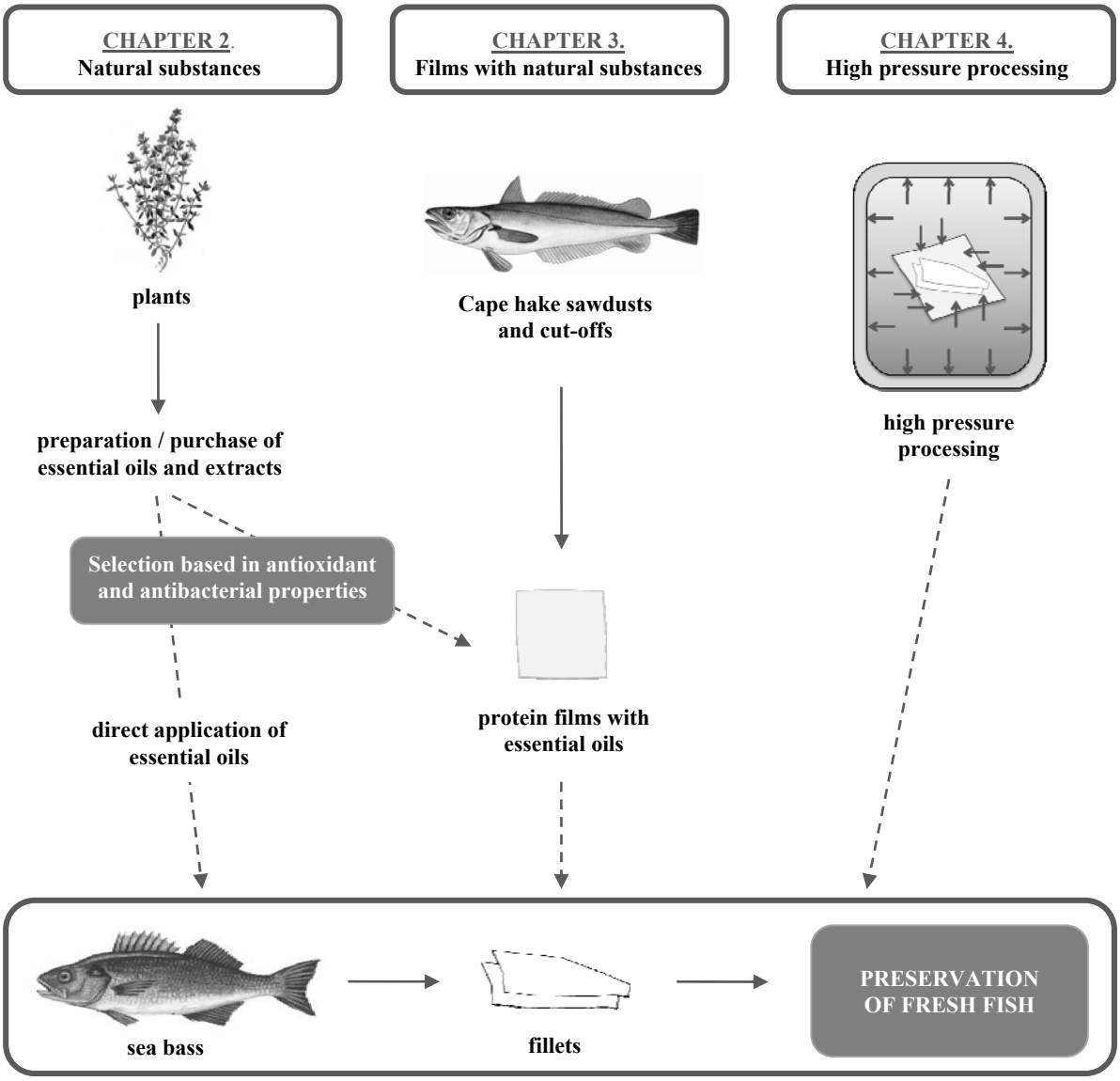


**Figure 1.6.** Thesis work outline. Plants from Master Gardeners of Hamilton County; Cape hake illustration from http://www.richardsbrothersseafoods.com.au; sea bass illustration from http://www.flyfishingireland.net.

Considering natural substances from plants, several extracts and essential oils were characterized in terms of antibacterial and antioxidant properties (Chapters 2.1, 2.2, and 2.3). Then, the effect of natural substances from plants in the preservation of fresh sea bass fillets was studied, and the quality changes

evaluated taking into account microbiological, chemical, physical, and sensory criteria (Chapter 2.4).

An alternative approach for preservation of sea bass fillets was performed incorporating natural substances from plants in films (Chapter 3). These films, prepared with fish proteins, were characterized for physical, mechanical, antioxidant, and antibacterial properties (Chapter 3.1). Then, the effect of films with natural substances in the preservation of fresh sea bass fillets was studied performing a microbiological challenge test (Chapter 3.2).

Regarding HPP, several pressure conditions were employed in sea bass fillets to study their effects in the activity of degradative enzymes and in the quality of sea bass fillets, immediately after HPP treatments (Chapters 4.1 and 4.2). Then, the effect of HPP treatments in the preservation of fresh sea bass fillets was studied, and the quality changes evaluated taking into account microbiological, chemical, physical, and sensory criteria (Chapter 4.3).

Finally, in Chapter 5 are discussed the main results obtained in all chapters, namely by applying natural substances from plants and HPP in fresh sea bass fillets.

# CHAPTER 2. Development of methods to preserve sea bass fillets: natural substances

# CHAPTER 2.1.

# European pennyroyal (*Mentha pulegium*) from Portugal: chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil

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**Abstract**

There is a growing interest of industry to replace synthetic chemicals by natural products with bioactive properties from plant origin. The aim of this study was to determine the chemical composition of European pennyroyal (*Mentha pulegium*) essential oil and to characterize the *in vitro* antioxidant and antimicrobial activities of its water (hot and cold) and ethanolic extracts and of the essential oil. The essential oil revealed menthone, pulegone and neo-menthol as the main constituents, comprising 35.9, 23.2 and 9.2 % of the essential oil, respectively. The hot water extract exhibited the highest antioxidant activity and phenol content. In contrast, the extracts were not very effective to inhibit the growth of the seven foodborne spoilage and pathogenic bacteria tested, but the essential oil showed antibacterial activity against all bacterial strains. In conclusion, extracts and essential oil of *M. pulegium* from Mediterranean origin have huge potential as an alternative to chemical additives for the food industry.

**Keywords**

Antimicrobial activity; antioxidant activity; essential oil; ethanol and aqueous extract; European pennyroyal; *Mentha pulegium*

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## 1. Introduction

There has been growing interest in the investigation of natural products for the discovery of active compounds with antimicrobial and antioxidant properties that can be applied to the food industry. As consumers are avoiding the consumption of products with synthetic additives or preservatives, natural active compounds could be an alternative to the employ of synthetic chemicals. Such compounds can be used to prolong the storage stability of food, by inhibiting the growth of foodborne spoilage or pathogenic microorganisms and protecting food from oxidative stress damage. Several authors have tested essential oils of aromatic plants to prolong the shelf life of food [17; 131-132], while others have focused on the antioxidant and antimicrobial properties of plant extracts and essential oils [133-134].

The European pennyroyal (*Mentha pulegium*) is an aromatic herb that belongs to the family Lamiaceae, is naturalized in America and thrives in Western, Southern and Central Europe, Asia, Iran, Arab countries and Ethiopia [135]. Its essential oil and dry parts have been traditionally used in medicine (digestive, liver and gallbladder disorders, amenorrhea, gout, colds, increased micturition, skin diseases and abortifacient), gastronomy (culinary herb), aromatherapy and cosmetics [135-136].

Most studies performed so far on *M. pulegium* were carried out with its essential oil in different regions of the world, including Iran [137], Greece [138], Turkey [68] and Portugal [139], and focused mainly its chemical composition.

Currently, there is a lack of information concerning the bioactive properties of *M. pulegium* extracts. Recently, Mata and co-authors [140] characterized antioxidant properties of *M. pulegium* essential oil and extracts, yet no information is available on antimicrobial properties of this species. In this context, the aim of this study was to study the chemical composition of *M. pulegium* essential oil from Portuguese origin, and to evaluate the antioxidant activity, total phenol contents and antimicrobial potential of water (hot and cold) and ethanolic extracts and essential oil against foodborne spoilage and pathogenic bacteria.

## 2. Material and methods

### 2.1. *Preparation of extracts and essential oil*

*M. pulegium* was collected in Santarém (Ribatejo, Portugal) (39° 21' 37.44" N, 8° 45' 41.04" W) in summer 2008. Voucher specimens were deposited in the Herbarium of the Portuguese National Institute of Biological Resources, I.P. The dried aerial parts were used to prepare three extracts (hot water, cold water and ethanolic) and the essential oil. The extracts were obtained by maceration of dry plant material (150 g) in: boiling water for 3 h (hot water extract); water for 3 days at room temperature (cold water extract); or ethanol for 3 days at room temperature (ethanolic extract). The macerates were filtered under vacuum through a Buckner funnel with filter paper (Whatman #4), whereas the ethanolic extract was dried in a rotary evaporator under vacuum (40 ºC, 178 mbar). The extracts were freeze-dried at -50 ºC (Heto Powerdry, LL3000, Mukarov, Czech Republic). The essential oil was obtained from dry plant material (100 g) by hydrodistillation using a modified Clevenger system, for 3 h. Afterwards, the essential oil was dried through sodium sulfate anhydrous. The extracts and essential oil were stored at -20 ºC and their final concentrations were 32.3 $mg.mL^{-1}$ (hot water extract), 23.1 $mg.mL^{-1}$ (cold water extract), while the essential oil had a density of 927.4 $mg.mL^{-1}$ and the dry weight of the ethanolic extract was 8.2 g. Yields of the extractions were 21.5 % (hot water extract), 12.3 % (cold water extract), 5.4 % (ethanolic extract) and 0.9 % (essential oil).

### 2.2. *Chemicals*

Phosphate buffer, trichloroacetic acid, ferric chloride, ascorbic acid and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Fluka (Buchs, Germany); Folin-Ciocalteu reagent, gallic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH), potassium hexacyanoferrate III and dimethylsulfoxide (DMSO) from Sigma-Aldrich (Steinheim, Germany); ferrous sulphate, sodium acetate, potato dextrose agar, tryptic soy agar and plate count agar from Merck (Darmstadt, Germany); sodium carbonate and butylated hydroxytoluene (BHT) from BDH (Poole, England); sodium sulphate anhydrous from Panreac Quimica S.A.U. (Barcelona, Spain); brain heart infusion broth from Oxoid (Basingstoke, Hampshire, England); ethanol had a purity grade of 99 % and the water used was Milli-Q purified and distilled.

### 2.3. *GC-MS analysis*

The *M. pulegium* essential oil was analyzed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the split

mode (1:50) at 250 ºC was used, into which a fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 µm film thickness; HP-5MS; 5 % diphenyl 95 % dimethyl polydimethylsiloxane, Agilent Technologies) was installed. The oven temperature was programmed at 45 ºC for 1 min, raised to 250 ºC at 5 ºC.min$^{-1}$ and maintained at 250 ºC for 5 min. Helium was used as carrier gas at 30 cm.s$^{-1}$ and the injection volume was 1 µL. The transfer line, ion source and quadrupole analyzer temperatures were maintained at 280 ºC, 230 ºC and 150 ºC, respectively, and a turbo molecular pump ($10^{-5}$ Torr) was used. In the full-scan mode, electron ionization mass spectra in the range 40-400 Da were recorded at 70 eV electron energy. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The identity of each compound was assigned by comparison of their retention indexes, relative to a standard mixture of n-alkanes [141], as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA). For semi-quantification purposes the normalized peak area abundances without correction factors were used.

## 2.4. *Antioxidant activity assays*

### 2.4.1. *Ferric reducing antioxidant power*

The ferric reducing antioxidant power (FRAP) assay was based on the methodology of Benzie & Strain [142] modified by Deighton and co-authors [143]. Briefly, FRAP reagent was prepared by combining 1 mM TPTZ with 2 mM ferric chloride in 0.25 M sodium acetate (pH 3.6). The sample (0.2 mL) was mixed with FRAP reagent (1.8 mL), stood for 4 min at room temperature and the absorbance was determined at 593 nm (ATI-UNICAN-UV2, Cambridge, UK). *M. pulegium* extracts were diluted in water and the essential oil was diluted in ethanol 50 %. Negative controls with water (aqueous and ethanolic extracts) or ethanol 50 % (essential oil) were also included. All determinations were performed in triplicate.

The difference between the absorbance of sample and the negative control was calculated and the reducing capacity of samples was compared with that of a reaction with a ferrous ion standard solution. The standard was prepared from ferrous sulphate that reacted with the TPTZ reagent, following the same procedure as with samples. The absorbance was plotted against ferrous ion

concentration (0.125-3.500 μM $Fe^{2+}$), and FRAP values were expressed as μmol $Fe^{2+}$ per g of sample.

### 2.4.2. *Reducing power*

The capacity of both *M. pulegium* extracts and essential oil to reduce iron (III) to iron (II) was determined accordingly to the modified method of Oyaizu [144]. Briefly, the sample (1 mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium hexacyanoferrate III (1 % w/v). After 30 min of incubation at 50 ºC in the dark, 2.5 mL trichloroacetic acid (10 % w/v) were added and the mixture kept at room temperature for 10 min. Afterwards, 2.5 mL of this mixture was added to 2.5 mL water and 0.5 mL ferric chloride (0.1 % w/v), vigorously mixed, and the absorbance measured at 700 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Extracts and essential oil were diluted in ethanol. All determinations were performed in triplicate. Negative (ethanol) and positive (ascorbic acid in the 10-40 $\mu g.mL^{-1}$ range) control reactions were performed, in order to plot the absorbance of ascorbic acid against concentration. The results were expressed as μmol of ascorbic acid per g of sample.

### 2.4.3. *Free radical scavenging*

The scavenging effect of DPPH free radical was assessed by the modified method of Kondo and co-authors [145]. Briefly, 0.1 mL of each plant extract or essential oil at different concentrations (in 95 % ethanol) was added to 2 mL DPPH (0.21 mM in 95 % ethanol). The mixture was shaken, left for 60 min at room temperature in the dark, and the absorbance was measured at 517 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). The percentage of DPPH inhibition was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ is the absorbance of the control reaction (blank with 0.1 mL of ethanol and DPPH) and $Abs_{sample}$ is the absorbance of the sample reaction (0.1 mL sample diluted in ethanol and DPPH). The sample concentration (in 1 mL reaction mixture) providing 50 % inhibition ($EC_{50}$) was estimated by plotting percentages of inhibition against concentrations of sample. All determinations were performed in triplicate. $EC_{50}$ was also estimated for the synthetic antioxidant reagent BHT.

To standardize DPPH results, the antioxidant activity index (AAI), proposed by Scherer & Godoy [146], was calculated as follows:

$$AAI = \frac{\text{DPPH concentration in reaction mixture } (\mu g.mL^{-1})}{EC_{50}\ (\mu g.mL^{-1})}$$

Samples were classified as showing poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when 0.5 < AAI < 1.0, strong antioxidant activity when 1.0 < AAI < 2.0, and very strong when AAI > 2.0 [146].

**2.4.4. *Phenol content***

Total phenol content was measured using a modified Folin-Ciocalteu assay [147]. Briefly, water (5 mL), sample (1-3 mL) and Folin-Ciocalteu reagent (0.5 mL) were mixed, allowed to stand for 5-8 min at room temperature, followed by the addition of 1.5 mL sodium carbonate (20 % w/v) together with water to obtain a final volume of 10 mL. The solution was mixed, allowed to stand for 2 h and filtered (0.45 μm poly-tetrafluoroethylene filter, Whatman) prior to absorbance reading at 750 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Aqueous and ethanolic extracts were diluted in water, whereas essential oil was diluted in ethanol 50 %. All determinations were performed in triplicate. Negative controls were performed with water (aqueous and ethanolic extracts) or ethanol 50 % (essential oil). Total phenol content was quantified by comparison of samples absorbance values with those of gallic acid reaction. The calibration curve of gallic acid was prepared in the 5-25 $mg.L^{-1}$ range, and results were expressed as mg of gallic acid per g of sample.

## 2.5. *Antibacterial activity*

**2.5.1. *Strains and growth conditions***

The antibacterial activity tests included foodborne spoilage and pathogenic bacteria purchased from American Type Culture Collection (ATCC) or Spanish Type Culture Collection (CECT): *Brochothrix thermosphacta* (CECT 847), *Escherichia coli* (ATCC 25922), *Listeria innocua* (CECT 910), *Listeria monocytogenes* (CECT 5873), *Pseudomonas putida* (CECT 7005), *Salmonella typhimurium* (ATCC 14028) and *Shewanella putrefaciens* (CECT 5346). These strains kept at -70 ºC in a cryopreservative solution (Microbank, Pro-lab Diagnostics, Richmond Hill, ON, Canada) were inoculated in tryptic soy agar and incubated overnight at 30 ºC, except *L. monocytogenes* that was inoculated in plate count agar. Subsequently, one colony from these cultures was inoculated on brain heart infusion broth and incubated at 30 °C for 18-24 h with shaking (75 rpm), in order to obtain freshly cultured microbial suspensions ($10^8$-$10^9$ $cells.mL^{-1}$) for tests.

### 2.5.2. *Paper disc diffusion method*

The antibacterial activity of *M. pulegium* extracts and essential oil was determined using the paper disc diffusion method [148]. Briefly, the bacterial suspensions were adjusted to $1 \times 10^7$ CFU.mL$^{-1}$ and spread in tryptic soy agar or plate count agar (10-15 mL per 90 mm Ø Petri dishes) using a sterile cotton swab. Subsequently, filter paper discs (6 mm Ø; Whatman #1) were placed in the surface of Petri dishes and impregnated with 20 µL of the extracts or essential oil at different concentrations. Essential oils were diluted in DMSO, while ethanolic and aqueous extracts were diluted in ethanol and water, respectively. Negative controls were prepared using the same solvents employed to dissolve samples.

After staying at 4 ºC for 2 h, Petri dishes were incubated at 30 ºC for 24 h, except *L. monocytogenes* that was incubated for 48 h. All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of the inhibition zones to the nearest millimetre, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of bacteria.

### 2.5.3. *Effect of MIC on liquid medium*

The MIC of plant extracts and essential oil was tested for antibacterial activity using liquid media. The bacterial suspensions were adjusted to $1 \times 10^7$ CFU.mL$^{-1}$ in brain heart infusion broth, and 180 µL were added to each well of 96-well plates. Subsequently, 20 µL of plant extract or essential oil were added, in order to obtain the MIC determined with the paper disc diffusion method. Water extracts were not tested with this assay because it was not possible to concentrate these extracts to the levels required for tests. Negative controls were prepared with the solvents used to dissolve extracts and essential oil, as well as positive controls with solvents and bacteria, but without the plant extract or essential oil. Plates were incubated at 4 ºC (2 h) and 30 ºC (24 h). The absorbance at 595 nm was measured using a micro plate reader (BIO-RAD model 680, Hercules, CA, USA) before and after incubation period, and the microbial concentration was determined by viable counts on tryptic soy agar or plate count agar. All determinations were performed in triplicate.

## 2.6. *Statistical analysis*

Differences between extracts and essential oil were tested with analysis of variance. In order to satisfy analysis of variance assumptions, data were transformed, followed by multiple comparisons tests (Tukey's HSD) to identify differences between groups. If transformed data could not met assumptions,

non-parametric analysis of variance (Kruskall-Wallis) was performed, followed by non-parametric multiple comparisons test (Dunn). All statistical analyses were tested at a 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

### 3.1. *Chemical composition of essential oil*

Volatiles of *M. pulegium* essential oil revealed 53 different compounds accounting for 86.3 % of the essential oil composition that are identified in Table 2.1.1. The essential oil contained a complex mixture consisting mainly by oxygenated monoterpenes (76.8 %), such as menthone (35.9 %), pulegone (23.2 %), neo-menthol (9.2 %) and 8-hydroxy-δ-4(5)-*p*-menthen-3-one (2.1 %). Menthone and pulegone have known antioxidant and antibacterial properties, whereas neo-menthol has also antibacterial activity against several bacteria [55; 149]. Previous studies with the same plant harvested in Iran, India and Turkey reported menthone and pulegone as the major components of the essential oil, but in different proportions (Table 2.1.2) [68; 136-137]. Nonetheless, there is a great variability in the chemical composition of *M. pulegium* essential oil among the studies performed so far, being pulegone, menthone, piperitone, piperitenone and isomenthone among the major components (Table 2.1.2) [137; 149-150]. Such variability may be related with different vegetative phases of the plant, and also with environmental conditions (*e.g.* seasonal and geographical variations, soil composition) [68].

### 3.2. *Antioxidant activity of extracts and essential oil*

The antioxidant activity of *M. pulegium* measured by the FRAP method ranged between 4.7 and 10.3 μmol of $Fe^{2+}$ per g of sample (Figure 2.1.1). The hot water extract had statistically the highest antioxidant activity, followed by essential oil, ethanolic extract and cold water extract.

The ferric reducing power of *M. pulegium* revealed that the hot water extract had statistically the highest antioxidant activity, followed by the ethanolic extract, and the cold water extract (all varying between 116.6 and 154.2 μmol ascorbic acid per g of sample). Yet, the lowest value was observed for the essential oil (2.2 μmol ascorbic acid per g of sample). Previous studies with hot water extracts of *Mentha* species (*M. pulegium* not included) showed slightly higher ferric reducing power (*ca*. 100 to 250 mg ascorbic acid per g of extract) [151].

**Table 2.1.1.** Chemical composition of the *Mentha pulegium* essential oil volatiles.

| Compound | RI | %* | Compound | RI | %* |
|---|---|---|---|---|---|
| ***Monoterpene hydrocarbons*** | | ***0.9*** | Mint furanone 2 | 1590 | 0.1 |
| α-Thujene | 853 | tr | ***Oxygenated sesquiterpenes*** | | ***1.1*** |
| α-Pinene | 861 | 0.2 | (-)-Allo-spathulenol | 1643 | 0.1 |
| Camphene | 877 | tr | Caryophyllene oxide | 1649 | 0.5 |
| Sabinene | 909 | tr | Veridiflorol | 1658 | 0.1 |
| 2-β-Pinene | 912 | 0.2 | Muurolol | 1708 | 0.1 |
| *m*-Cymene | 970 | 0.1 | α-Cadinol | 1723 | 0.1 |
| L-Limonene | 975 | 0.2 | Palustrol | 1794 | 0.2 |
| Carene | 1335 | 0.2 | ***Oxygenated diterpenes*** | | ***0.1*** |
| ***Sesquiterpenes hydrocarbons*** | | ***0.1*** | Epimanoyl oxide | 2040 | 0.1 |
| β-Bourbonene | 1426 | 0.1 | ***Esters*** | | ***0.2*** |
| ***Oxygenated monoterpenes*** | | ***76.8*** | 3-Octyl acetate | 1100 | 0.2 |
| Eucalyptol | 988 | 0.5 | ***Others*** | | ***7.1*** |
| Linalool | 1076 | 0.1 | 1-Ethyl-3-methyl-2-(2-methylpropylidene) imidazolidine | 1282 | 0.8 |
| Menthone | 1138 | 35.9 | 4,6-Diethyl-2-methoxypyrimidine | 1309 | 0.3 |
| neo-Menthol | 1164 | 9.2 | 3-Methyl-cyclopentanone | 771 | 0.1 |
| *trans*-5-Methyl-2-(1-methylethenyl)-cyclohexanone | 1173 | 1.1 | 5-Methyl-3-heptanone | 923 | 0.1 |
| Menthol | 1179 | 0.1 | 3-Octanol | 935 | 0.7 |
| 3-*p*-Menthanol | 1182 | 0.1 | 1-Methyl-3,5-dimethoxy-1*H*-pyrazole | 1394 | 0.6 |
| α-Terpineol | 1189 | 0.1 | 2-Cyclohexen-1-ol | 1404 | 1.1 |
| Berbenone | 1213 | 0.1 | Methyleugenol | 1452 | 0.2 |
| 4-Hydroxy-δ-8-*p*-menthen-3-one | 1221 | 0.9 | 3-(2-Oxocyclopentyl) propanal ethylene ketal | 1463 | 0.5 |
| *trans*-(+)-Carveol | 1223 | 0.1 | 2-Amino-6-chloro-4-(2-ethenylamino) pyrimidine | 1476 | 0.9 |
| Pulegone | 1262 | 23.2 | 2,6-Dimethoxytoluene | 1486 | 0.9 |
| (1*RS*,4*SR*)-8-hydroxy-*p*-menthan-3-one | 1267 | 0.4 | 3-Methyl-hexanedioic acid | 1491 | 0.2 |
| Piperitone | 1271 | 0.4 | 2-*tert*-Butyl-4-Methylphenol | 1552 | 0.1 |
| 3-Menthene | 1295 | 1.6 | Elemicin | 1618 | 0.1 |
| 8-Hydroxy-δ-4(5)-*p*-menthen-3-one | 1315 | 2.1 | β-(3-Thienyl)acrylic acid | 1628 | 0.4 |
| 1-Menthene | 1317 | 0.2 | Hexadecanoic acid | 2005 | 0.1 |
| Piperitenone | 1374 | 0.4 | | | |
| Mint furanone 1 | 1555 | 0.2 | **Total identified** | | **86.3** |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
***** Normalized peak area abundances without correction factors.

The DPPH assay also identified *M. pulegium* hot water extract ($EC_{50}$ = 16.3 ± 0.4 µg.mL$^{-1}$) as having statistically the highest free radical scavenging activity (*i.e.* highest antioxidant activity and lowest $EC_{50}$), followed by ethanolic extract, cold water extract and essential oil ($EC_{50}$ = 6.2 ± 0.2 mg.mL$^{-1}$; Figure 2.1.1). Accordingly to the categories defined by Scherer & Godoy [146], *M. pulegium* essential oil and cold water extract tested in the present study presented poor antioxidant activity, while ethanolic extract and hot water extract were classified as moderate and very strong antioxidants, respectively (AAI values: essential oil = 0.01; cold water extract = 0.45; ethanolic extract = 0.80; hot water extract = 4.83).

**Table 2.1.2.** Major constituents of *Mentha pulegium* essential oils reported in different studies.

| Origin | Sample period | Extraction method details | Compounds and compounds concentration in the essential oil | Reference |
|---|---|---|---|---|
| Bandar-Anzeli (Iran) | summer 2000 | 4 h hydrodistillation (Clevenger apparatus) | pulegone (37.8 %); menthone (20.3 %); piperitone (6.8 %) | [137] |
| Jammu region and Kashmir valley (India) | July 2001 and 2003 | 3 h hydrodistillation (Clevenger apparatus) | pulegone (65.9-83.1 %); menthone (8.3-8.7 %); isomenthone (3.8-4.0 %) | [136] |
| Algeria | n.d. | Clevenger apparatus | pulegone (4.4-87.3 %); piperitenone (0.1-26.7 %); isomenthone (trace-22.6 %); β-pinene (0.4-20.9 %) | [152] |
| Spanish market | n.d. | 2 h simultaneous distillation-extraction | pulegone (41.1-42.3 %); piperitone oxide (14.9-16.9 %); piperitenone (4.6-6.1 %); piperitone (5.4-6.0 %) | [153] |
| Ksour Essef (Tunisia) | n.d. | 3 h hydrodistillation (Clevenger apparatus) | pulegone (61.1 %); isomenthone (17.0 %); menthone (5.9 %) | [154] |
| Gilan province (Iran) | spring 2008 | Hydrodistillation (Clevenger apparatus) | pulegone (40.5 %); menthone (35.4 %); piperitone (5.2 %) | [155] |
| Kazeron (Iran) | August 2007 | hydrodistillation | piperitone (38.0 %); piperitenone (33.0 %); α-terpineol (4.7 %); menthone (3.0 %); pulegone (2.3 %) | [150] |
| Portuguese market | summer 2004 | hydrodistillation | pulegone (35.1 %); piperitenone (27.4 %) | [140] |
| Monastir (Tunisia) | May, July | 3 h hydrodistillation (Clevenger apparatus) | menthol (40.6-51.6 %); menthone (7.3-20.0 %); 1,8-cineole (11.1-18.5 %); pulegone (3.9-7.0 %) | [156] |
| Akzu (Turkey) | summer 1993 | 3 h steam-distilled | pulegone (205.5 $mg.mL^{-1}$); 1,8-cineole (34.7 $mg.mL^{-1}$); borneol (13.8 $mg.mL^{-1}$); menthone (5.4 $mg.mL^{-1}$) | [157] |
| Antalya, Termessus, Aksu, Düden and Kalkan (Turkey) | April-August 1994 | 3 h steam-distilled (Clevenger apparatus) | pulegone (39.6-419.6 $mg.mL^{-1}$); menthone (12.2-166.0 $mg.mL^{-1}$); borneol (16.7-47.6 $mg.mL^{-1}$); 1,8 cineole (19.8-40.1 $mg.mL^{-1}$) | [68] |
| Tunisia | n.d. | 12 h maceration in hexane | pulegone (17.5-70.2 %; carvone (trace-55.7 %); isomenthone (2.9-34.2 %); menthol (0.1-21.2 %); menthofuran (0.7-10.0 %) | [158] |
| Island of Samos, Argos, Evia, Island of Samothraki and Kalamata (Greece) | summer 2007, 2008 | 4 h hydrodistillation (Clevenger apparatus) | pulegone (61.3-77.9 %); iso-menthone (10.6-18.5 %); menthone (0.6-8.3 %); piperitone (0.3-3.2 %); *cis*-isopulegone (0-1.7 %) | [138] |
| Island of Crete (Greece) | summer 2007, 2008 | 4 h hydrodistillation (Clevenger apparatus) | piperitone (69.3 %); iso-menthone (24.8 %); limonene (1.8 %); menthone (1.6 %) | [138] |
| Sintra (Portugal) | July | 3 h hydrodistillation (Clevenger apparatus); supercritical $CO_2$ | pulegone (78.3-80.9 %); menthone (8.5-9.2 %) | [139] |
| Island of Paros and Vermio Mountains (Greece) | n.d. | 2 h hydrodistillation | pulegone (44.7-50.4 %); piperitone (1.9-13.4 %); isomenthone (0.2-4.5 %); menthone (1.0-1.7 %) | [149] |
| Pangaio Mountain (Greece) | n.d. | 2 h hydrodistillation | isomenthone (77.5 %); menthone (10.3 %); pulegone (1.0 %) | [149] |

*Abbreviations*: n.d. – not described.

Previous studies reported a wide variation in the antioxidant activity of *M. pulegium* extracts or essential oil. For example, the ethanolic extract had identical antioxidant activity (AAI = 0.8) [140] or higher (AAI = 1.9) [159], compared to the values obtained in the present study, the essential oil of *M. pulegium* exhibited higher antioxidant activity (AAI = 1.6) [154], the opposite was also observed (AAI = 0.003) [155], and variability also occurred with hot water extracts [140; 155; 160]. The antioxidant activity of hot water extract was even

higher than that found for BHT ($EC_{50}$ = 20.2 ± 2.6 µg.mL$^{-1}$; AAI = 3.95), and AAI value for BHT was within values obtained by previous authors, *i.e.* between 0.26 [161] and 5.96 [162].

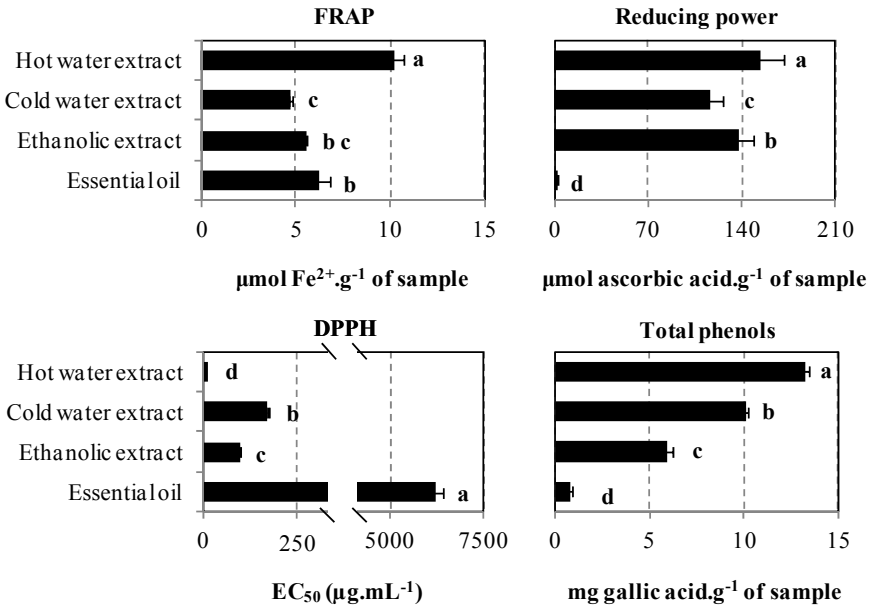


**Figure 2.1.1.** Antioxidant activity (FRAP, reducing power and DPPH) and total phenols of *Mentha pulegium* extracts and essential oil. The bars represent the average, and the error bars represent the standard deviation. In each graphic, different letters denote significant differences ($p < 0.05$) between extracts and essential oil. *Abbreviations*: FRAP – ferric reducing antioxidant power; DPPH – α,α-diphenyl-β-picrylhydrazyl; $EC_{50}$ – sample providing 50 % inhibition.

Phenols are organic compounds that contain a hydroxyl group bound directly to the aromatic ring, and the H-atom of the hydroxyl group can trap peroxyl radicals, preventing other compounds to be oxidized [163]. In this way, antioxidant activity may be partially due to the presence of phenols. In this study, total phenol contents varied between 0.7 and 13.3 mg gallic acid per g of sample (Figure 2.1.1). The essential oil had statistically the lowest phenol content, followed by ethanol extract, cold water extract, and hot water extract. These values were rather low compared to those obtained with hot water extract of other *Mentha* species, where values varied between 128.1 to 230.8 mg gallic acid per g of sample [151]. In the present study, hot water was more efficient to extract phenol compounds from *M. pulegium* than the remaining extraction methods. In contrast, Mata and co-authors [140] observed that total phenol contents of *M. pulegium* was higher in ethanolic extract (71.7 mg pyrogallol per g of sample) than in hot water extract (57.9 mg pyrogallol per g of sample). The differences found between the results obtained in this study and those reported

by previous authors might be related with the duration of the extraction procedure, or with intrinsic characteristics of plants, like origin, not specified in the study of Mata and co-authors [140], and season.

In this study, hot water extract of *M. pulegium* showed simultaneously the highest antioxidant activity and phenol content. The tendency was not so clear for the remaining extracts and essential oil, indicating that other compounds may also play an important role in their antioxidant activity.

### 3.3. *Antibacterial activity*

The antibacterial activity of *M. pulegium* extracts and essential oil suggested that almost all tested bacteria were resistant to hot and cold aqueous extracts (Table 2.1.3), except *S. putrefaciens* (MIC = 323 $mg.mL^{-1}$ hot water extract). The ethanolic extract did not expose inhibition against all Gram-negative bacteria (*S. typhimurium*, *E. coli*, *S. putrefaciens* and *P. putida*), but all Gram-positive bacteria (*L. innocua*, *L. monocytogenes* and *B. thermosphacta*) were sensitive to this extract. The resistance of Gram-negative bacteria could be due to the complexity of their double layer cell membrane in comparison with the simpler cell membrane of Gram-positive bacteria [164].

The essential oil of *M. pulegium* was very effective to inhibit the growth of all bacteria tested, as shown by the low MIC (Table 2.1.3). The antibacterial activity of this essential oil might be due to the presence of pulegone, menthone and neo-menthol. Duru and co-authors [165] have demonstrated the strong antimicrobial activity of pulegone against a set of bacteria, including *S. typhimurium* and *E. coli*. Cytotoxicity of this essential oil appears to include a bacterial membrane damage that occurs when the essential oil passes through the cell wall and cytoplasmic membrane, and disrupts the structure of their different layers of polysaccharides, fatty acids and phospholipids [50].

The lowest MIC was found for *P. putida*, while the highest values were found for *S. typhimurium* and *L. innocua*. Previous authors highlighted strong antimicrobial activity of *M. pulegium* essential oil against *L. monocytogenes* [150] and *E. coli* [156], but the opposite was observed in others' studies with no activity against *E. coli* and *S. typhimurium* [150]. Such differences can be due to distinct plant origins and essential oil chemical composition (Tables 2.1.1 and 2.1.2).

**Table 2.1.3.** Antibacterial activity of *Mentha pulegium* extracts and essential oil against pathogenic and spoilage bacteria.

| | ***Salmonella typhimurium*** | ***Escherichia coli*** | ***Listeria innocua*** | ***Listeria monocytogenes*** | ***Shewanella putrefaciens*** | ***Brochothrix thermosphacta*** | ***Pseudomonas putida*** |
|---|---|---|---|---|---|---|---|
| **Disc diffusion method** | | | | | | | |
| *Inhibition zone radius* (mm) | | | | | | | |
| Hot water extract (32.3 $mg.mL^{-1}$) | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Cold water extract (23.1 $mg.mL^{-1}$) | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Ethanolic extract (30.5 $mg.mL^{-1}$) | N.D. | N.D. | 2 | 2 | N.D. | 2 | N.D. |
| Essential oil (927.4 $mg.mL^{-1}$) | 3 | 4 | 3 | 11 | 32 | 10 | 4 |
| *MIC* ($mg.mL^{-1}$) | | | | | | | |
| Hot water extract | N.D. | N.D. | N.D. | N.D. | 323 | N.D. | N.D. |
| Cold water extract | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Ethanolic extract | N.D. | N.D. | 15.3 | 15.3 | N.D. | 30.5 | N.D. |
| Essential oil | 9.3 | 2.3 | 9.3 | 2.3 | 2.3 | 4.6 | 1.2 |
| **Effect of MIC in liquid medium** | | | | | | | |
| *Logarithmic reductions* | | | | | | | |
| Ethanolic extract | - | - | 0 | 0 | - | 1.8 | - |
| Essential oil | 3.8 | 3.2 | 1.8 | 2.6 | - | 0.8 | 1.3 |
| *Absorbance change* | | | | | | | |
| Ethanolic extract | - | - | +0.044 | -0.067 | - | -2.536 | - |
| Essential oil | +0.015 | +0.206 | -0.445 | -0.255 | +0.037 | +0.169 | +0.080 |

*Abbreviations*: MIC – minimum inhibitory concentration; N.D. – not detected inhibition zone radius or MIC.
The radius of paper disc (3 mm) was not included in the values of inhibition zone radius.
The values of absorbance change with "+" and "-" indicate an increase and decrease in the absorbance values, respectively.

The effect of MIC determined by the disc diffusion method on viable counts of bacteria was also tested in liquid medium (Table 2.1.3). The assay revealed different sensitivities depending on the bacterial strain, and generally viable cells were considerably reduced, except with the ethanolic extract tested against both *Listeria* species. The ethanolic extract caused only a 1.8 log reduction on viable cells of *B. thermosphacta*, whereas the essential oil reduced viable cells of almost all the bacteria tested, except for *S. putrefaciens*. This bacterial strain was very sensitive to the solvents used to dilute the essential oil and ethanolic extract (DMSO and ethanol), and consequently, it was impossible to determine their MIC effect. The highest inhibition was observed for *S. typhimurium* (3.8 log reductions), whereas the lowest value was obtained with *B. thermosphacta* (0.8 log reductions). These results indicate that the use of microdilution methods to determine MIC might reveal lower values, as the concentrations obtained with the disc diffusion method were able to reduce the bacterial population.

The relationship between the bacterial counts and the absorbance measured for the effect of MIC on viable cells was analysed to evaluate the accuracy of using

the absorbance and visual inspection in the determination of MIC of *M. pulegium* extract and essential oil. As shown in Table 2.1.3 no relationship was obtained between the variables, thus indicating that future studies with microdilution methods should focus on bacterial counts for MIC evaluation rather than absorbance or visual inspection.

## 4. Conclusions

This study allowed concluding that *M. pulegium* hot water extract exhibited the highest antioxidant activity and phenol content. In contrast, the essential oil showed strong antibacterial properties. In this way, *M. pulegium* extracts and essential oil have a huge potential as alternatives to synthetic preservatives in food industry. Further studies should evaluate the safety and toxicity of *M. pulegium* extracts and essential oil to human consumption before considering their use for food preservation or medicinal purposes.

# CHAPTER 2.2.

# Chemical composition and bioactivity of different oregano (*Origanum vulgare*) extracts and essential oil

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**Abstract**

There is a growing interest in industry to replace synthetic chemicals by natural products with bioactive properties. Aromatic plants are excellent sources of bioactive compounds that can be extracted using several processes. As far as oregano is concerned, studies are lacking addressing the effect of extraction processes in bioactivity of extracts. This study aimed to characterize the *in vitro* antioxidant and antibacterial properties of oregano (*Origanum vulgare*) essential oil and extracts (in hot and cold water, and ethanol), and the chemical composition of its essential oil. The major components of oregano essential oil were carvacrol, β-fenchyl alcohol, thymol, and γ-terpinene. Hot water extract had the strongest antioxidant properties and the highest phenolic content. All extracts were ineffective in inhibiting the growth of the seven tested bacteria. In contrast, the essential oil inhibited the growth of all bacteria, causing greater reductions on both *Listeria* strains (*L. monocytogenes* and *L. innocua*). In conclusion, *O. vulgare* extracts and essential oil from Portuguese origin are strong candidates to replace synthetic chemicals used by the industry.

**Keywords**

Oregano; antibacterial activity; antioxidant activity; ethanol extracts; aqueous extracts; essential oil

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## 1. Introduction

Synthetic preservatives are widely used for different applications. Yet, since the safety of some synthetic preservatives is questionable, consumers tend to prefer preservative-free products. Therefore, there is a growing interest in assessing the antimicrobial and antioxidant properties of substances from natural sources that can potentially be used by the food and pharmaceutical industries. Essential oils from aromatic and medicinal plants have been known to possess biological activity, such as antimicrobial and antioxidant properties [50-51].

Oregano (*Origanum vulgare*) is an aromatic herb belonging to the Lamiaceae family, and commonly occurs throughout Asia, Europe, and northern Africa [135]. In folk medicine, *O. vulgare* is used to treat respiratory disorders, dyspepsia, painful menstruation, rheumatoid arthritis, scrofulosis, and urinary tract disorders [135]. It is also used a culinary herb in gastronomy [135]. Previous studies reported the potential of oregano essential oil to preserve food, such as fresh chicken breast meat [166], swordfish [78], and octopus [131].

Previous studies on the isolation of *O. vulgare* essential oils, from different regions of the world, including Greece [167], Lithuania [168], India [169], Poland [170], and Italy [171], focused mainly on the chemical composition, although the antioxidant and antimicrobial properties were also explored [172]. Yet, there was no information on the bioactive properties of water and ethanol extracts of *O. vulgare*. Therefore, this study aimed to identify the chemical composition of *O. vulgare* essential oil of Portuguese origin, and to evaluate the total phenolic content, antioxidant activity, and antibacterial properties of the essential oil and water (hot and cold) and ethanolic extracts.

## 2. Material and methods

### 2.1. *Preparation of extracts and essential oil*

*O. vulgare* was collected in Santarém (Ribatejo, Portugal) (39° 21' 37.44" N, 8° 45' 41.04" W) in summer 2008. Voucher specimens were identified by Dr Carmo Serrano and deposited in the Herbarium of the Portuguese National Institute of Biological Resources, I.P. (n.º LISU 191009). The dried aerial parts were used to prepare three extracts (hot water, cold water, and ethanolic) and the essential oil. The extracts were obtained by maceration of dry plant material (100 g) in either boiling water for 3 h (hot water extract); or water for 3 days at room temperature (cold water extract); or ethanol (99 %) for 3 days at room temperature (ethanolic extract), without using a successive extraction. The

macerates were filtered under vacuum through a Buckner funnel with filter paper (Whatman #4), whereas the ethanolic extract was dried in a rotary evaporator under vacuum (40 ºC, 178 mbar). The extracts were freeze-dried at -50 ºC (Heto-Powerdry, LL3000, Mukarov, Czech Republic). The essential oil was obtained from dry plant material (75 g) by hydrodistillation for 3 h using a modified Clevenger system. During the boiling process, the dried material soaks up water and the essential oil diffuses through the cell walls by means of osmosis, and then it is vaporized and carried away by the stream of steam [66]. Afterwards, the essential oil was dried through anhydrous sodium sulfate. The extracts and essential oil were stored at -20 ºC. The water extracts concentrations, before the freeze-drying procedure, were 41.7 $mg.mL^{-1}$ (hot water extract) and 23.8 $mg.mL^{-1}$ (cold water extract), while the essential oil had a density of 896.6 $mg.mL^{-1}$, and the total dry weight of the ethanolic extract was 5.4 g.

**2.2. *Chemicals***

Phosphate buffer, trichloroacetic acid, ferric chloride, ascorbic acid, and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Fluka (Buchs, Germany); Folin-Ciocalteu reagent, gallic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH), potassium hexacyanoferrate III, and dimethylsulfoxide (DMSO) from Sigma-Aldrich (Steinheim, Germany); ferrous sulphate, sodium acetate, tryptic soy agar, and plate count agar from Merck (Darmstadt, Germany); sodium carbonate and butylated hydroxytoluene (BHT) from BDH (Poole, England); sodium sulphate anhydrous from Panreac Química S.A.U. (Barcelona, Spain); brain heart infusion broth from Oxoid (Basingstoke, Hampshire, England); ethanol had a purity grade of 99 % and the water used was distilled and Milli-Q purified.

**2.3. *GC-MS analysis***

The *O. vulgare* essential oil was analyzed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto). A vaporization injector operating in the split mode (1:50) at 250 ºC was used, into which a fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 µm film thickness; HP-5MS; 5 % diphenyl-95 % dimethyl polydimethylsiloxane; Agilent Technologies) was installed. The oven temperature was programmed at 45 ºC for 1 min, raised to 250 ºC at 5 $ºC.min^{-1}$, and maintained at 250 ºC for 5 min. Helium was used as carrier gas at 30 $cm.s^{-1}$ and the injection volume was 1 µL. The transfer line, ion

source, and quadrupole analyzer temperatures were maintained at 280 ºC, 230 ºC, and 150 ºC, respectively, and a turbo molecular pump ($10^{-5}$ Torr) was used. In the full-scan mode, electron ionization mass spectra in the range 40-400 Da were recorded at 70 eV electron energy. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The identity of each compound was assigned by comparison of their retention index relative to a standard mixture of n-alkanes [141], as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA). For semi-quantification purposes the normalized peak area abundances without correction factors were used.

### 2.4. *Antioxidant activity assays*

#### 2.4.1. *Ferric reducing antioxidant power*

The ferric reducing antioxidant power (FRAP) assay was based on the methodology of Benzie & Strain [142] modified by Deighton and co-authors [143]. Briefly, FRAP reagent was prepared by combining TPTZ (1 mM) and ferric chloride (2 mM) in sodium acetate (0.25 M; pH 3.6). The sample (0.2 mL) was mixed with FRAP reagent (1.8 mL), allowed to stand for 4 min at room temperature and the absorbance was determined at 593 nm (ATI-UNICAN-UV2, Cambridge, UK). *O. vulgare* extracts were diluted in water and the essential oil was diluted in ethanol 50 %. Negative controls with water (aqueous and ethanolic extracts) or ethanol 50 % (essential oil) were also included. All determinations were performed in triplicate.

The difference between the absorbance of sample and the negative control was calculated and the reducing capacity of samples was compared with that of a reaction with a ferrous ion standard solution. The standard was prepared from ferrous sulphate that reacted with the TPTZ reagent, following the same procedure as with samples. The absorbance was plotted against ferrous ion concentration in the range 0.125-3.500 μM $Fe^{2+}$, and FRAP values were expressed as μmol $Fe^{2+}$ per g of sample.

#### 2.4.2. *Reducing power*

The capacity of *O. vulgare* extracts and its essential oil to reduce iron (III) to iron (II) was determined according to the modified method of Oyaizu [144]. Briefly, the sample (1 mL) was mixed with phosphate buffer (2.5 mL, 0.2 M,

pH 6.6) and potassium hexacyanoferrate III (2.5 mL, 1 % w/v). After 30 min of incubation at 50 ºC in the dark, trichloroacetic acid (2.5 mL, 10 % w/v) was added and the mixture kept at room temperature for 10 min. Afterwards, this mixture (2.5 mL) was added to water (2.5 mL) and ferric chloride (0.5 mL, 0.1 % w/v), vigorously mixed, and the absorbance measured at 700 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Extracts and essential oil were diluted in ethanol. All determinations were performed in triplicate. Negative (ethanol) and a positive (ascorbic acid in the 10-40 $\mu g.mL^{-1}$ range) control reactions were performed. The results were expressed as µmol of ascorbic acid per g of sample.

**2.4.3. *Free radical scavenging***

The scavenging effect of DPPH free radical was assessed by the modified method of Kondo and co-authors [145]. Briefly, each extract or essential oil (0.1 mL) at different concentrations (in 95 % ethanol) was added to DPPH (2 mL, 0.21 mM in 95 % ethanol). The mixture was shaken, left for 60 min at room temperature in the dark, and the absorbance was measured at 517 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). The percentage DPPH inhibition was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ is the absorbance of the control reaction (blank with 0.1 mL ethanol and DPPH) and $Abs_{sample}$ is the absorbance of the sample reaction (0.1 mL sample diluted in ethanol and DPPH). The sample concentration (in 1 mL reaction mixture) providing 50 % inhibition ($EC_{50}$) was estimated by plotting percentages of inhibition against concentrations of sample. All determinations were performed in triplicate. $EC_{50}$ was also estimated for the synthetic antioxidant reagent BHT.

To standardize DPPH results, the antioxidant activity index (AAI), proposed by Scherer & Godoy [146], was calculated as follows:

$$\text{AAI} = \frac{\text{DPPH concentration in reaction mixture } (\mu g.mL^{-1})}{EC_{50}\ (\mu g.mL^{-1})}$$

Samples were classified as showing poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when 0.5 < AAI < 1.0, strong antioxidant activity when 1.0 < AAI < 2.0, and very strong when AAI > 2.0 [146].

**2.4.4. *Phenol content***

Total phenol content was measured using a modified Folin-Ciocalteu assay [147]. Briefly, water (5 mL), sample (1-3 mL) and Folin-Ciocalteu reagent (0.5 mL) were mixed, allowed to stand for 5-8 min at room temperature, followed by the addition of sodium carbonate (1.5 mL, 20 % w/v) together with water to obtain a final volume of 10 mL. The solution was mixed, allowed to stand for 2 h and filtered (0.45 μm poly-tetrafluoroethylene filter, Whatman) prior to absorbance reading at 750 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Aqueous and ethanolic extracts were diluted in water, whereas the essential oil was diluted in ethanol 50 %. All determinations were performed in triplicate. Negative controls were performed with water (aqueous and ethanolic extracts) or ethanol 50 % (essential oil). Total phenol content was quantified by comparison of samples absorbance values with those of gallic acid reaction. The calibration curve of gallic acid was prepared in the 5-25 $mg.L^{-1}$ range, and results were expressed as mg of gallic acid per g of sample.

## 2.5. *Antibacterial activity*

**2.5.1. *Strains and growth conditions***

The antibacterial activity tests included foodborne spoilage and pathogenic bacteria purchased from American Type Culture Collection (ATCC) or Spanish Type Culture Collection (CECT): *Brochothrix thermosphacta* (CECT 847; Gram-positive), *Escherichia coli* (ATCC 25922; Gram-negative), *Listeria innocua* (CECT 910; Gram-positive), *Listeria monocytogenes* (CECT 5873; Gram-positive), *Pseudomonas putida* (CECT 7005; Gram-negative), *Salmonella typhimurium* (ATCC 14028; Gram-negative), and *Shewanella putrefaciens* (CECT 5346; Gram-negative). These strains were kept at -70 ºC in a cryopreservative solution (Microbank, Pro-lab Diagnostics, Richmond Hill, ON, Canada) and were inoculated in tryptic soy agar and incubated overnight at 30 ºC, except *L. monocytogenes*, which was inoculated in plate count agar. Subsequently, one colony from these cultures was inoculated on brain heart infusion broth and incubated at 30 °C for 18-24 h with shaking (75 rpm), in order to obtain freshly cultured microbial suspensions ($10^8$-$10^9$ $cells.mL^{-1}$) for tests.

**2.5.2. *Paper disc diffusion method***

The antibacterial activity of *O. vulgare* extracts and essential oil was determined using the paper disc diffusion method [148]. Briefly, the bacterial suspensions were adjusted to $1 \times 10^7$ $CFU.mL^{-1}$ and spread in tryptic soy agar or plate count agar (10-15 mL per 90 mm Ø Petri dishes) using a sterile cotton

swab. Subsequently, filter paper discs (6 mm Ø; Whatman #1) were placed on the surface of Petri dishes and impregnated with 20 µL of the extracts or essential oil at different concentrations. Essential oils were diluted in DMSO, while ethanolic and aqueous extracts were diluted in ethanol and water, respectively. Negative controls were prepared using the same solvents employed to dissolve samples. After remaining at 4 ºC for 2 h, Petri dishes were incubated at 30 ºC for 24 h, except *L. monocytogenes*, which was incubated for 48 h. All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of inhibition zones to the nearest millimetre, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of bacteria.

### 2.5.3. *Effect of MIC on the liquid medium*

The MIC of extracts and essential oil was tested for antibacterial activity using liquid media. The bacterial suspensions were adjusted to $1 \times 10^7$ CFU.mL$^{-1}$ in brain heart infusion broth, and 180 µL were added to each well of 96-well plates. Subsequently, 20 µL of extract or essential oil were added, in order to obtain the MIC determined with the paper disc diffusion method. Negative controls were prepared with the solvents used to dissolve extracts and essential oil, as well as positive controls with solvents and bacteria, but without the extract or essential oil. Plates were incubated at 4 ºC (2 h) and 30 ºC (24 h). The absorbance at 595 nm was measured using a micro plate reader (BIO-RAD model 680, Hercules, CA, USA) before and after incubation period, and the microbial concentration was determined by viable counts on tryptic soy agar or plate count agar. All determinations were performed in triplicate.

## 2.6. *Statistical analysis*

Differences between extracts and essential oil were tested with analysis of variance. In order to satisfy analysis of variance assumptions data were transformed, followed by multiple comparisons tests (Tukey HSD) to identify differences between groups. If transformed data could not met assumptions, non-parametric analysis of variance (Kruskall-Wallis) was performed, followed by non-parametric multiple comparisons test (Dunn). Statistical analyses were tested at a 0.05 level of probability with the software STATISTICA™ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

### 3.1. *Chemical composition of the essential oil*

Sixty four compounds were identified in *O. vulgare* essential oil, accounting for 92.3 % of the whole composition (Table 2.2.1). The essential oil was mainly composed of oxygenated monoterpenes (53.8 %) and monoterpene hydrocarbons (26.4 %). Within oxygenated monoterpenes, carvacrol (14.5 %), thymol (12.6 %), β-fenchyl alcohol (12.8 %), and δ-terpineol (7.5 %) were the major compounds detected, while γ-terpinene (11.6 %) and α-terpinene (3.7 %) were the most abundant monoterpene hydrocarbons detected. Additionally, 1-methyl-3-(1-methylethyl)-benzene (6.8 %) also represented a substantial fraction of *O. vulgare* essential oil. Carvacrol, thymol, γ-terpinene, and linalool are known to possess strong antioxidant properties [55; 173-174], and carvacrol and thymol also exhibit antibacterial activity against several bacteria [57; 172; 175].

The composition of *O. vulgare* essential oil from different geographical origins has been characterized by several authors, with carvacrol and thymol as the major components, though the proportions vary widely (Table 2.2.2). Other chemotypes have also been reported as important essential oil components, such as *p*-cymene, γ-terpinene, caryophyllene, spathulenol, and germacrene-D (Table 2.2.2). The differences in the chemical composition of *O. vulgare* essential oil may be related to distinct environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, vegetative plant phases, and extraction and quantification methods [169; 171].

### 3.2. *Antioxidant activity of extracts and essential oil*

Ferric reducing antioxidant power (FRAP) analysis revealed essential oil and cold water extract as showing the strongest antioxidant activity, followed by hot water extract and ethanolic extract (Figure 2.2.1). The reducing power analysis showed statistically the highest antioxidant activity for hot water extract, followed by ethanolic extract, cold water extract, and essential oil (Figure 2.2.1). The α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activity followed the same trend of reducing power analysis. *O. vulgare* essential oil (AAI = 0.05) revealed poor antioxidant activity, while cold water extract (AAI = 0.55), ethanolic extract (AAI = 1.23), and hot water extract (AAI = 3.16) were classified as moderate, strong, and very strong antioxidants, respectively, accordingly to the AAI index and antioxidant categories defined by Scherer & Godoy [146]. The AAI values obtained for the essential oil were within those reported by Şahin and co-authors [176] for the

same plant species. The AAI index of the positive control BHT was within the values obtained in previous studies [161-162].

**Table 2.2.1.** Chemical composition of volatiles in the *Origanum vulgare* essential oil.

| Compound | RI | %* | Compound | RI | %* |
|---|---|---|---|---|---|
| ***Monoterpene hydrocarbons*** | | ***26.4*** | ***Oxygenated monoterpenes*** | | ***53.8*** |
| α-Thujene | 853 | 2.2 | Eucalyptol | 988 | 0.3 |
| α-Pinene | 861 | 0.7 | Linalool | 1076 | 2.6 |
| Camphene | 877 | 0.1 | *trans*-1-Methyl-4-(1-methylethyl)-2-cyclohexen-1-ol | 1097 | 0.3 |
| Sabinene | 909 | 1.0 | α-Terpineol | 1118 | 0.2 |
| β-Pinene | 912 | 0.4 | Menthone | 1138 | 0.7 |
| β-Myrcene | 929 | 1.3 | Borneol | 1154 | 0.4 |
| α-Phellandrene | 945 | 0.4 | δ-Terpineol | 1171 | 7.5 |
| $\Delta^3$-Carene | 952 | 0.1 | *trans*-Piperitol | 1193 | 0.1 |
| α-Terpinene | 961 | 3.7 | β-Fenchyl alcohol | 1197 | 12.8 |
| β-Phellandrene | 978 | 0.9 | *cis*-*p*-Menth-1-en-3-ol | 1199 | 0.1 |
| *cis*-β-Ocimene | 990 | 1.6 | *cis*-Piperitol | 1207 | 0.1 |
| *trans*-β-Ocimene | 1002 | 1.5 | Pulegone | 1262 | 1.0 |
| γ-Terpinene | 1016 | 11.6 | Piperitone | 1271 | tr |
| α-Terpinolene | 1053 | 0.9 | Carvacrol | 1330 | 14.5 |
| neo-Allo-ocimene | 1488 | tr | ***Oxygenated sesquiterpenes*** | | ***1.4*** |
| ***Sesquiterpenes hydrocarbons*** | | ***3.6*** | (+)-Spathulenol | 1644 | 0.5 |
| α-Cubebene | 1416 | tr | Caryophyllene oxide | 1649 | 0.6 |
| β-Elemene | 1435 | 0.1 | Viridiflorol | 1658 | tr |
| *trans*-Caryophyllene | 1467 | tr | Isospathulenol | 1704 | 0.1 |
| α-Bergamotene | 1484 | 0.1 | Cadinol | 1709 | 0.1 |
| Allo-aromadendrene | 1489 | 0.1 | α-Cadinol | 1723 | 0.1 |
| Germacrene D | 1537 | 0.3 | ***Oxygenated diterpenes*** | | ***tr*** |
| β-Selinene | 1543 | tr | Epimanoyl oxide | 2040 | tr |
| Ledene | 1552 | tr | ***Others*** | | ***7.1*** |
| Bicyclogermacrene | 1555 | 0.3 | 1-Octen-3-ol | 921 | 0.2 |
| α-Muurolene | 1558 | tr | 1-Methyl-3-(1-methylethyl)-benzene | 982 | 6.8 |
| β-Bisabolene | 1565 | 2.1 | *p*-Cymen-7-ol | 1181 | 0.1 |
| Selina-3,7(11)-diene | 1577 | 0.2 | Thymyl methyl ether | 1241 | 0.1 |
| β-Cadinene | 1583 | 0.2 | Carvacryl methyl ether | 1253 | 0.4 |
| *cis*-α-Bisabolene | 1602 | 0.1 | Thymol | 1316 | 12.6 |
| γ-Cadinene | 1709 | 0.1 | Methyleugenol | 1452 | tr |
| Copaene | 1713 | tr | Hexadecanoic acid | 2005 | tr |
| | | | 2,3,5,6-Tetramethylphenol | 2067 | 0.1 |
| | | | **Total identified** | | **92.3** |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
***** Normalized peak areas without correction factors.

Total phenolic content revealed statistically higher values in hot water extract, followed by essential oil, ethanolic extract, and cold water extract (Figure 2.2.1). The phenol values obtained for hot water and ethanolic extracts were lower than those reported by Chun and co-authors [177] for oregano. Such differences might be attributed to distinct plant origins, seasonal sampling periods (not specified), part of plant used (dried powder of the whole plant), and extraction methods (longer in hot water and several ethanol concentrations were used).

**Table 2.2.2.** Major constituents of *Origanum vulgare* essential oils reported in different studies and corresponding origin, sample seasonal period and extraction method employed.

| Origin | Sampling period | Extraction method details | Compounds and compounds concentration in the essential oil | Reference |
|---|---|---|---|---|
| Greece | n.d. | 4 h hydrodistillation (Clevenger apparatus)[3] | carvacrol (8.6-80.8 %); thymol (1.8-63.7 %); *p*-cymene (5.2-13.0 %); γ-terpinene (5.8-7.5 %) | [167] |
| Vilnius district (Lithuania) | 1995, 1997-99 | Hydrodistillation[1,2] | β-ocimene (10.8-21.6%); germacrene D (7.5-29.4 %); β-caryophyllene (2.3-16.6 %); sabinene (2.1-15.0 %) | [168] |
| Kumaon Himalaya (India) | August 2008 | 2 h steam distillation[1,2] | thymol (0-82.0 %); carvacrol (0-27.4 %); germacrene D (trace-13.3 %); germacrene D-4-ol (0-9.5 %); β-caryophyllene (0.4-8.8 %) | [169] |
| Poland | n.d. | hydrodistillation (Deryng)[1] | carvacrol (3.6-9.1 $g.kg^{-1}$); thymol (2.14-8.44 $g.kg^{-1}$); γ-terpinene (1.5-4.9 $g.kg^{-1}$) | [170] |
| Calabria (southern Italy) | June-July | 2 h hydrodistillation (Clevenger apparatus)[1,2] | thymol (7.9-55.5 %); carvacrol (0.3-56.6 %); γ-terpinene (12.6-32.6 %); *p*-cymene (3.6-9.7 %); methyl carvacryl ether (1.9-4.7 %) | [171] |
| Unknown origin (Portuguese market) | n.d. | 4 h hydrodistillation (Clevenger apparatus)[1,2] | thymol (32.6 %); γ-terpinene (25.9 %); *p*-cymene (10.7 %); β-caryophyllene (4.5 %) | [172] |
| Iti montain (central Greece) | n.d. | 2 h hydrodistillation[1,2] | thymol (45.2 %); carvacrol (33.1 %); *p*-cymene (7.4 %); γ-cymene (5.5 %) | [178] |
| Kozani (Greece) | July 2002 | 3 h steam distillation (Clevenger apparatus)[1,2] | carvacrol (88.7 %); *p*-cymene (3.4 %); γ-terpinene (3.2 %); β-caryophyllene (1.1 %) | [57] |
| Vojvodina province (Serbia and Montenegro) | July 2000 | hydrodistillation[1,2] | carvacrol (61.3 %); thymol (13.9 %); γ-terpinene (3.1 %) | [73] |
| Crete (Greece) | n.d. | 2 h using a microstream distillation-extraction apparatus[1] | thymol (63.3 %); γ-terpinene (12.7 %); *p*-cymene (9.9 %); carvacrol (7.8 %) | [179] |
| Argentina | November 2007 and 2008 | 1 h hydrodistillation (Clevenger apparatus) [1,2] | thymol (20.5-26.1 %); *trans*-sabinene hydrate (27.8-32.5 %); γ-terpinene (5.4-15.5 %); α-terpinene (4.2-4.6 %); terpinen-4-ol (3.5-5.0 %) | [180] |
| Taygetos Mountain (Peloponnese, Greece) | n.d. | 2 h hydrodistillation (Clevenger apparatus)[1,2] | carvacrol (74.6 %); *p*-cymene (9.7 %); γ-terpinene (5.9 %) | [181] |
| Dalmatia (Croatia) | October 1998 | 3 h hydrodistillation[1] | thymol (40.4 %); carvacrol (24.8 %); *p*-cymene (16.8 %) | [182] |
| Market in Pisa (Italy) | January 2004 | 2 h hydrodistillation (Clevenger apparatus)[1] | carvacrol (54.7 %); thymol (22.1 %); γ-terpinene (6.0 %); *p*-cymene (5.5 %) | [183] |
| Oltu valley, Erzorum (Turkey) | n.d. | 3 h hydrodistillation (Clevenger apparatus)[1] | caryophyllene (14.4 %); spathulenol (11.6 %); germacrene-D (8.1 %); α-terpineol (7.5 %); caryophyllene oxide (5.8 %) | [176] |
| Island of Euboea (Greece) | n.d. | 2 h hydrodistillation (Clevenger apparatus)[1,2] | carvacrol (79.6 %); *p*-cymene (8.8 %); thymol (2.5 %); γ-terpinene (2.1 %) | [184] |

*Abbreviations*: n.d. – not described.
Compounds detection method is represented by a superscript number in the 3rd column: [1] for gas chromatography/mass spectrometer; [2] for gas chromatography/flame ionization detector; and [3] for Fourier transform Raman spectroscopy.

Since phenols are able to prevent oxidation [163], the greater antioxidant activity of hot water extract could be attributed to the higher content of phenols in this extract, compared with the remaining extracts. Although high total phenolic content was detected in hot water extract and essential oil, the latter showed the weakest antioxidant activity with the reducing power and DPPH methods, but

not with the FRAP assay. Dambolena and co-authors [180] reported that other compounds beside phenols may also be responsible for the antioxidant activity.

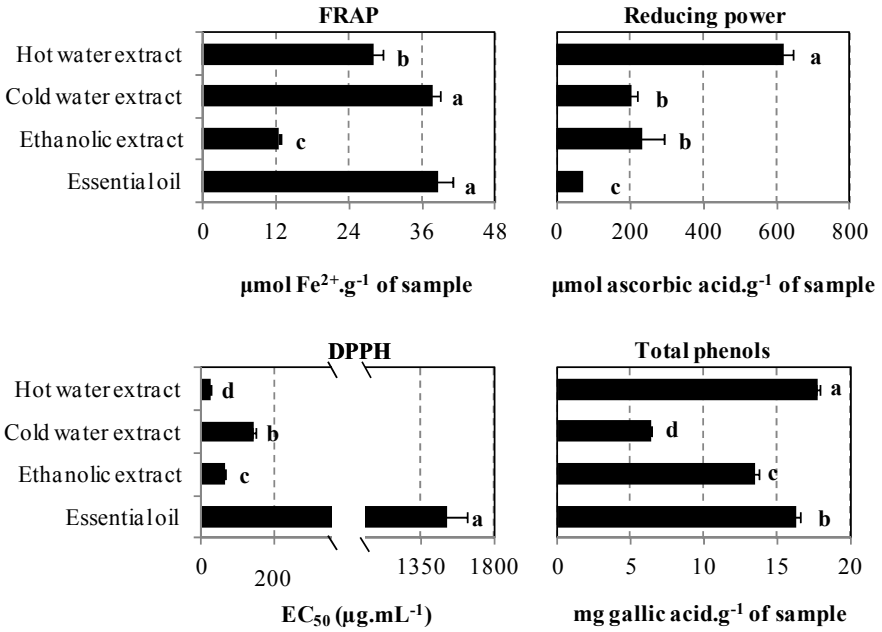


**Figure 2.2.1.** Antioxidant activity (FRAP, reducing power and DPPH) and phenol content of *Origanum vulgare* extracts and essential oil. The bars represent the average, and the error bars the standard deviation. In each graphic, different letters denote significant differences ($p < 0.05$). *Abbreviations*: FRAP – ferric reducing antioxidant power; DPPH – α,α-diphenyl-β-picrylhydrazyl; $EC_{50}$ – sample providing 50 % inhibition.

### 3.3. *Antibacterial activity of extracts and essential oil*

Almost all bacteria tested showed resistance to hot and cold water extracts, except *S. putrefaciens* and *B. thermosphacta* (Table 2.2.3). In contrast, the ethanolic extract was able to inhibit the growth of most bacteria, except *P. putida*, being *S. putrefaciens* and *B. thermosphacta* the most resistant and *E. coli* and *L. monocytogenes* the most sensitive bacteria (Table 2.2.3). A microdilution method (liquid medium) was used to quantify the bacterial reduction caused by the minimum inhibitory concentration (MIC) of extracts, and bacterial concentration was determined by counts of viable cells. In liquid medium, the MIC of ethanolic extract was still able to decrease *S. typhimurium, L. innocua*, and *L. monocytogenes* concentrations (Table 2.2.3).

*O. vulgare* essential oil was very effective in inhibiting the growth of all bacterial strains tested with MIC values below 5 $mg.mL^{-1}$ (Table 2.2.3). Taking into account the inhibition zone using undiluted essential oil, *P. putida* was the most resistant strain, despite revealing the lowest MIC value. The values obtained with *E. coli*, *S. typhimurium,* and *L. monocytogenes* are in accordance with those obtained by Sivropoulou and co-authors [184] and Faleiro and

co-authors [172], while other studies reported higher antibacterial activity [57; 73]. The results for the MIC of *O. vulgare* essential oil showed a strong reduction of most bacteria tested in liquid medium, particularly with both *Listeria* strains (Table 2.2.3). The antibacterial activity observed with the essential oil could be related to the presence of carvacrol and thymol, since these compounds are able to inhibit the growth of *E. coli* and *L. monocytogenes* [57; 172; 175]. Oregano essential oil affects the murein composition, influencing the number of muropeptides of *L. monocytogenes* and *E. coli* cell walls [185-186], and a similar inhibition mechanism is expected to occur with the remaining bacterial strains.

**Table 2.2.3.** Antibacterial activity of *Origanum vulgare* extracts and essential oil against foodborne spoilage and pathogenic bacteria.

| | ***Salmonella typhimurium*** | ***Escherichia coli*** | ***Listeria innocua*** | ***Listeria monocytogenes*** | ***Shewanella putrefaciens*** | ***Brochothrix thermosphacta*** | ***Pseudomonas putida*** |
|---|---|---|---|---|---|---|---|
| **Disc diffusion method** | | | | | | | |
| *Inhibition radius* (mm) | | | | | | | |
| Hot water extract (41.7 mg.mL$^{-1}$) | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Cold water extract (23.8 mg.mL$^{-1}$) | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Ethanolic extract (27.7 mg.mL$^{-1}$) | 2 | 1 | 2 | 2 | N.D. | N.D. | N.D. |
| Essential oil (896.6 mg.mL$^{-1}$) | 32 | 29 | 17 | 23 | 32 | 18 | 9 |
| *MIC* (mg.mL$^{-1}$) | | | | | | | |
| Hot water extract | N.D. | N.D. | N.D. | N.D. | 417 | 417 | N.D. |
| Cold water extract | N.D. | N.D. | N.D. | N.D. | 238 | N.D. | N.D. |
| Ethanolic extract | 13.9 | 6.9 | 13.9 | 6.9 | 277 | 277 | N.D. |
| Essential oil | 2.2 | 1.1 | 2.2 | 2.2 | 4.5 | 4.5 | 0.4 |
| **Effect of MIC on liquid medium** | | | | | | | |
| *Logarithmic reductions* | | | | | | | |
| Ethanolic extract | 4.7 | 0 | 5.2 | 4.5 | - | - | - |
| Essential oil | 7.2 | 6.9 | 8.0 | 8.0 | - | 4.5 | 1.2 |
| *Absorbance changes* | | | | | | | |
| Ethanolic extract | + 0.028 | - 0.083 | - 0.084 | - 0.307 | - | - | - |
| Essential oil | - 0.162 | - 0.317 | - 0.732 | - 0.550 | + 0.047 | - 0.096 | - 0.141 |

*Abbreviations*: MIC – minimum inhibitory concentration; N.D. – inhibition not detected.
Radius of paper disc is not included.
The values of absorbance change with "+" and "-" indicate an increase and decrease in the absorbance values, respectively.

Antibiotics are active at concentrations of 10 μg.mL$^{-1}$, and plant extracts active at concentrations of 100 μg.mL$^{-1}$ have a good potency level [187]. The extracts tested here are active at higher concentration levels, as the results for the disc diffusion method showed, but results obtained with the liquid medium assay suggest that active concentration could be lower if this method was used for the determination of the MIC.

The absorbance of the bacterial suspensions with the MIC of extracts was measured before and after the incubation period. Results (Table 2.2.3) revealed a reduction for all bacteria, which is in accordance with the bacterial counts. However, the results did not follow the same trend in ethanolic extract, and therefore, future studies using microdilution methods for MIC evaluation should focus on bacterial counts instead of absorbance or visual inspection.

## 4. Conclusions

This study suggests that the hot water extract of *O. vulgare* of Portuguese origin has strong antioxidant capacity. Additionally, the *O. vulgare* ethanolic extract and essential oil revealed antibacterial properties, though being stronger with the essential oil. In this context, *O. vulgare* extracts and essential oil have strong potential to be used as alternatives to synthetic chemicals in industries whereas oxidation and microbial contamination are problems. Nonetheless, safety and toxicity issues of these extracts and essential oil still need to be evaluated beforehand.

# CHAPTER 2.3.

# Chemical composition and antibacterial and antioxidant properties of commercial essential oils

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**Abstract**

The aim of this work was to determine the effectiveness of 17 essential oils to inhibit the growth of seven foodborne spoilage and pathogenic bacterial strains (*Brochothrix thermosphacta*, *Escherichia coli*, *Listeria innocua*, *Listeria monocytogenes*, *Pseudomonas putida*, *Salmonella typhimurium* and *Shewanella putrefaciens*). Additionally, the antioxidant activity (by free radical scavenging activity and ferric reducing power) and the chemical composition of these essential oils were evaluated. All essential oils inhibited the growth of at least four bacteria tested, and lower values of minimum inhibitory concentration (< 3.0 $mg.mL^{-1}$) were needed to inhibit *P. putida*. The highest reductions (8.0 log $CFU.mL^{-1}$) were achieved with coriander, Spanish oregano and rosemary essential oils for *L. innocua*, as well as with thyme essential oil for both *Listeria* strains. The results showed that for the evaluation of antibacterial activity of plant essential oils, bacterial counts should be performed instead of absorbance readings when using microdilution methods. Regarding the antioxidant activity, clove and Spanish oregano essential oils showed the strongest antioxidant properties. Essential oils showed a great variety of compounds in their chemical compositions, some of those with known antibacterial and antioxidant properties. In conclusion, all tested essential oils have very strong potential applicability as antibacterial and antioxidant agents for food and pharmaceutical industries.

**Keywords**

Antibacterial activity; antioxidant activity; essential oil; chemical composition

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## 1. Introduction

Essential oils are complex mixtures of volatile compounds with strong odour that are synthesized in several plant organs, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes [50; 63]. These volatile compounds have diverse ecological functions, acting as defensive substances against microorganisms and herbivores, but can also be important to attract insects for the dispersion of pollens and seeds [50].

Essential oils have therapeutic uses in human medicine due to its anticancer, antinociceptive, antiphlogistic, antiviral, antibacterial and antioxidant properties [51]. Additionally, the use of essential oils is becoming popular to increase the shelf life of food products, since consumers are more conscious about the health problems caused by several synthetic preservatives [83; 188]. Synthetic preservatives that are added in food items, as antimicrobials and antioxidants, are considered to be without potential adverse effects and are classified as generally recognized as safe. However, there have been problems concerning the safety of some chemicals, including the possibility of allergies from benzoic acid and sulphites, the formation of carcinogenic nitrosamines from nitrites, and the possible rodent carcinogenicity of butylated hydroxyanisole and butylated hydroxytoluene [5].

Studies with essential oils as food additives revealed to be advantageous, as observed by the increase in food shelf life [166]. Still, the amount of essential oils used was determinant for the acceptance, as strong aromas of essential oils might be imparted to food products [166]. Several factors influence the chemical composition of plant essential oils, including the species, part of the plant, season of harvesting, geographical origin, and also the extraction method, and consequently their bioactive properties [50; 67-71]. The antibacterial and antioxidant properties of many essential oils and constituents have been studied so far [52-55; 149; 189]. However, most results published so far are dispersed and employed different techniques, making comparison rather difficult. Additionally, several plant essential oils properties have not yet been studied. Therefore, the present study aimed to screen antibacterial properties of 17 commercially available essential oils against seven foodborne spoilage and pathogenic bacteria. In addition, the antioxidant activity and chemical composition of these essential oils were evaluated.

## 2. Material and methods

### 2.1. *Chemicals*

Phosphate buffer, trichloroacetic acid, ferric chloride and ascorbic acid were purchased from Fluka (Buchs, Germany); α,α-diphenyl-β-picrylhydrazyl (DPPH), potassium hexacyanoferrate (III) and dimethylsulfoxide (DMSO) from Sigma-Aldrich (Steinheim, Germany); tryptic soy agar and plate count agar from Merck (Darmstadt, Germany); butylated hydroxytoluene (BHT) from BDH (Poole, England); brain heart infusion broth from Oxoid (Basingstoke, Hampshire, England); ethanol had a purity grade of 99 % and the water used was Milli Q purified and distilled.

### 2.2. *Essential oils*

Essential oils of 17 plants were acquired from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany): 1) basil oil from *Ocimum basilicum* (W211907; lot 09802EH-038; leaves steam distillation; origin: The Island of Comoro); 2) carrot oil from *Daucus carota* (W224405; lot 06803KC-097; seeds steam distillation; origin: France); 3) celery oil from *Apium graveolens* (W227102; lot 05603BS-456; seeds steam distillation; origin: Mexico); 4) citronella oil from *Cymbopogon nardus* (W230804; lot 16202BS-208; leaves and stems steam distillation; origin: China); 5) clove oil from *Eugenia* spp. (C8392; lot 116K1861; buds distillation; origin: Indonesia); 6) coriander oil from *Coriandrum sativum* (W233404; lot 03513JH-208; ripe seeds steam distillation; origin: Austria); 7) garlic oil (biological source not specified; W250317; lot 04712EE-148; bulbs synthetic organic material; origin: Mexico); 8) grapefruit oil from *Citrus paradisi* (W253006; lot 05014CE-027; fresh peel of the fruit cold pressed; origin: Florida, USA); 9) lemon oil from *Citrus limon* (W262508; lot 03509JH-258; fresh peel of the fruit cold pressed; origin: California, USA); 10) marjoram oil from *Thymus mastichina* (W523208; lot 06606PI-326; flowering herbs steam distillation; origin: Spain); 11) onion oil from *Allium cepa* (W281719; lot 15119LB-268; bulbs steam distillation; origin: The Netherlands); 12) Spanish oregano oil from *Thymus capitatus* (W282812; lot 21417CL-214; dried flowering herb steam distillation; origin: Spain); 13) parsley oil from *Petroselinum sativum* (W283606; lot 10207JU-265; leaves and stems steam distillation; origin: USA); 14) rosemary oil from *Rosmarinus officinalis* (W299200; lot 03609JH-457; flowering tops distillation; origin: Spain); 15) sage oil from *Salvia officinalis* (W300306; lot 16508MA-124; flowers and leaves steam distillation; origin: Mexico); 16) tarragon oil from

*Artemisia dracunculus* (W241202; lot 10821MA-468; leaves and flowering tops steam distillation; origin: Spain); and 17) thyme oil from *Thymus vulgaris* (W306509; lot 05223CE-497; flowering plant distillation; origin: Spain).

### 2.3. *GC-MS analysis*

The essential oils were analyzed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the split mode (1:50) at 250 ºC and a fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 µm film thickness; HP-5MS; 5 % diphenyl 95 % dimethyl polydimethylsiloxane, Agilent Technologies) were used. The oven temperature was programmed at 45 ºC for 1 min, raised to 250 ºC at 5 ºC.min$^{-1}$ and maintained at 250 ºC for 5 min. Helium was used as carrier gas at 30 cm.s$^{-1}$ and the injection volume was 1 µL. The temperatures of transfer line, ion source, and quadrupole analyzer were maintained at 280 ºC, 230 ºC, and 150 ºC, respectively. A turbo molecular pump ($10^{-5}$ Torr) was used. In the full-scan mode, electron ionization mass spectra in the range 40-400 Da were recorded at 70 eV electron energy. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The identity of each compound was assigned by comparison of their retention indexes, relative to a standard mixture of n-alkanes [141], as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA). For semi-quantification purposes the normalized peak area abundances without correction factors were used.

### 2.4. *Antibacterial activity*

#### 2.4.1. *Strains and growth conditions*

The antibacterial activity tests included foodborne spoilage and pathogenic bacteria acquired from the American Type Culture Collection (ATCC) and the Spanish Type Culture Collection (CECT): *Brochothrix thermosphacta* (CECT 847), *Escherichia coli* (ATCC 25922), *Listeria innocua* (CECT 910), *Listeria monocytogenes* (CECT 5873), *Pseudomonas putida* (CECT 7005), *Salmonella typhimurium* (ATCC 14028) and *Shewanella putrefaciens* (CECT 5346). These strains kept at -70 ºC in a cryopreservative solution (Microbank, Pro-lab Diagnostics, Richmond Hill, ON, Canada) were inoculated in tryptic soy agar and incubated overnight at 30 ºC, except *L. monocytogenes* that was inoculated

in plate count agar at the same temperature. Subsequently, one colony from each culture was inoculated in brain heart infusion broth and incubated at 30 °C for 18-24 h with shaking (75 rpm), in order to obtain freshly cultured microbial suspensions ($10^8$-$10^9$ cells.mL$^{-1}$) for tests.

#### 2.4.2. *Disc diffusion method*

The antibacterial activity of essential oils was determined with the disc diffusion method [148]. Briefly, bacterial suspensions were adjusted to $1 \times 10^7$ CFU mL$^{-1}$ and spread in tryptic soy agar or plate count agar using sterile cotton swabs. Subsequently, filter paper discs (6 mm Ø; Whatman #1) were placed on the surface of Petri dishes and impregnated with 20 µL of essential oil at different concentrations (diluted in DMSO). Negative controls were prepared only with DMSO. After staying at 4 ºC (2 h), all Petri dishes were incubated at 30 ºC (24 h), except *L. monocytogenes* that was incubated during 48 h. All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of the inhibition zones to the nearest millimetre, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of bacteria.

#### 2.4.3. *Effect of MIC on liquid medium*

The MIC of essential oils determined with the disc diffusion method was also tested for antibacterial activity using liquid media. Bacterial suspensions were adjusted to $1 \times 10^7$ CFU.mL$^{-1}$ in brain heart infusion broth, and 180 µL were added per well of 96-well plates. Subsequently, 20 µL of essential oil (diluted in DMSO, in order to obtain the MIC) were added, or the same volume of DMSO without essential oil as a positive control. Plates were incubated at 4 ºC (2 h) and at 30 ºC (24 h). The absorbance at 595 nm was measured using a micro plate reader (BIO-RAD model 680, Hercules, CA, USA) before and after the incubation period for estimation of cell number, and bacterial concentration was determined by viable counts on tryptic soy agar or plate count agar. All determinations were performed in triplicate.

### 2.5. *Antioxidant activity tests*

#### 2.5.1. *Free radical scavenging effect*

The DPPH free radical scavenging effect was assessed by the method of Kondo and co-authors [145]. Briefly, 0.1 mL of each essential oil at different concentrations (from 0.07 to 100 % diluted in ethanol) was added to 2 mL DPPH (0.21 mM in 95 % ethanol). The mixture was shaken, left for 60 min at room temperature in the dark, and the absorbance was measured at 517 nm in a

spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). The percentage of DPPH inhibition was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ is the absorbance of the control reaction (blank with 0.1 mL ethanol and DPPH) and $Abs_{sample}$ is the absorbance of the sample reaction (0.1 mL essential oil diluted in ethanol and DPPH). The sample concentration (in 1 mL reaction mixture) providing 50 % inhibition ($EC_{50}$) was estimated by plotting the percentages of inhibition against essential oil concentrations. All determinations were performed in triplicate. As positive control, the $EC_{50}$ was estimated for the synthetic antioxidant reagent BHT.

To standardize DPPH results, the antioxidant activity index (AAI), proposed by Scherer & Godoy [146], was calculated as follows:

$$\text{AAI} = \frac{\text{DPPH concentration in reaction mixture } (\mu g.mL^{-1})}{EC_{50}\ (\mu g.mL^{-1})}$$

Samples were classified as showing poor antioxidant activity (AAI < 0.5), moderate (0.5 < AAI < 1.0), strong (1.0 < AAI < 2.0) and very strong (AAI > 2.0) [146].

### 2.5.2. *Ferric reducing power*

The capacity of essential oils to reduce iron (III) to iron (II) was determined according to the method of Oyaizu [144]. Briefly, the sample (1 mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexacyanoferrate III (1 % w/v). After 30 min of incubation at 50 ºC in the dark, 2.5 mL trichloroacetic acid (10 % w/v) were added and the mixture kept at room temperature for 10 min. Afterwards, 2.5 mL of this mixture was added to 2.5 mL water and 0.5 mL ferric chloride (0.1 % w/v), vigorously mixed, and the absorbance was measured at 700 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Essential oils were diluted in ethanol. All determinations were performed in triplicate. Negative (ethanol) and positive (ascorbic acid in the 10-40 $\mu g.mL^{-1}$ range) control reactions were performed, in order to plot the absorbance of ascorbic acid against its concentration. The results were expressed as μmol of ascorbic acid per g of sample.

### 2.6. *Statistical analysis*

Differences between essential oils were tested with analysis of variance. In order to satisfy analysis of variance assumptions data were transformed, followed by multiple comparisons tests (Tukey HSD) to identify differences

between groups. If transformed data could not meet assumptions, non-parametric analysis of variance (Kruskall-Wallis) was performed, followed by non-parametric multiple comparisons test (Dunn). The results obtained from absorbance measures and counts of bacteria viable cells were tested for correlation. All statistical analyses were tested at 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

### 3.1. *Essential oils chemical composition*

The number of identified compounds in each essential oil studied ranged between 7 and 36, comprising 59.0 to 99.5 % of the total essential oil volatile composition (Tables 2.3.1 and 2.3.2).

Grapefruit, lemon, coriander and garlic essential oils were particularly rich in monoterpene hydrocarbons (Table 2.3.1). The main component of grapefruit and lemon essential oils was α-limonene, coriander essential oil contains mainly $\Delta^3$-carene and γ-terpinene, and garlic essential oil has 1(7),5,8-*o*-menthatriene and di-2-propenyldisulfide.

Sage, rosemary, marjoram, thyme and Spanish oregano essential oils were mainly composed of oxygenated monoterpenes (> 40 %; Table 2.3.2). Sage and rosemary essential oils showed the most similar chemical composition, being particularly rich in (-)-camphor, eucalyptol and (-)-bornylacetate, whereas the major components in marjoram essential oil were eucalyptol and *endo*-5,5,6-trimethyl-2-norbornanone, *m*-thymol in thyme essential oil, and carvacrol and *m*-thymol in Spanish oregano essential oil.

The essential oils of citronella, celery and carrot showed higher content of sesquiterpene hydrocarbons (> 35 %; Table 2.3.2). Citronella essential oil is mainly composed of $\Delta^2$-carene and β-citronellal, in celery essential oil the major components were β-selinene, 2-propenylphenoxyacetate, α-limonene and 2-methyl-benzoxazole, whereas carrot essential oil contained mostly δ-cadinene.

Finally, tarragon, basil, parsley, onion and clove essential oils were rich in other compounds, particularly in methylchavicol (tarragon and basil essential oils), 4-methoxy-6-(2-propenyl)-1,3-benzodioxole, apiol and 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene in parsley essential oil, dipropyltrisulfide in onion essential oil and *p*-eugenol, aceteugenol and *trans*-caryophyllene in clove essential oil (Table 2.3.1).

**Table 2.3.1.** Chemical composition of volatiles in grapefruit, lemon, coriander, garlic, tarragon, basil, parsley, onion, and clove essential oils.

| Compounds | RI | Grapefruit | Lemon | Coriander | Garlic | Tarragon | Basil | Parsley | Onion | Clove |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Monoterpene hydrocarbons*** | | ***87.4*** | ***78.4*** | ***73.2*** | ***20.7*** | ***1.6*** | ***0.9*** | ***1.2*** | ***0.2*** | ***-*** |
| α-Pinene | 870 | 0.1 | 0.6 | 1.1 | - | 0.1 | - | 0.4 | - | - |
| β-Pinene | 920 | - | 7.0 | 0.2 | - | - | tr | 0.5 | - | - |
| Cymene | 980 | - | 0.1 | 1.3 | - | - | - | tr | - | - |
| β-Limonene | 986 | - | - | 1.2 | - | 1.3 | tr | 0.3 | - | - |
| α-Limonene | 995 | 84.7 | 60.1 | - | - | - | - | - | - | - |
| α-Fellandrene | 1025 | - | 9.0 | 2.2 | - | - | - | tr | - | - |
| $\Delta^3$-Carene | 1092 | - | - | 60.5 | - | - | - | - | - | - |
| 1,3,8-*p*-Menthatriene | 1231 | 1.3 | - | - | - | - | - | - | - | - |
| 3-Carene | 1276 | - | - | 5.7 | - | - | - | - | - | - |
| 1(7),5,8-*o*-Menthatriene | 1328 | - | - | - | 20.7 | - | - | - | - | - |
| ***Oxygenated monoterpenes*** | | ***1.7*** | ***-*** | ***6.5*** | ***5.1*** | ***0.4*** | ***0.9*** | ***0.1*** | ***0.2*** | ***-*** |
| *trans*-Limone oxide | 1128 | 0.5 | - | - | 3.8 | - | - | - | - | - |
| (+)-Camphor | 1135 | - | - | 6.5 | - | - | - | - | - | - |
| *endo*-5,5,6-Trimethyl-2-norbornanone | 1198 | - | - | - | 1.2 | 0.4 | tr | - | - | - |
| (*S*)-2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-one | 1261 | 1.2 | - | - | - | - | - | - | - | - |
| ***Sesquiterpene hydrocarbons*** | | ***1.3*** | ***1.0*** | ***18.2*** | ***0.7*** | ***-*** | ***7.9*** | ***0.2*** | ***6.6*** | ***14.8*** |
| γ-Terpinene | 1426 | - | - | 18.2 | - | - | tr | - | - | - |
| β-Elemene | 1434 | - | - | - | - | - | 0.5 | - | 5.6 | - |
| *trans*-Caryophyllene | 1465 | 0.5 | 1.0 | - | - | - | - | tr | 0.5 | 10.8 |
| 2,6-Dimethyl-6-(4-methyl-3-pentenyl)-bicyclo[3.1.1]hept-2-ene | 1483 | - | - | - | - | - | 4.1 | - | - | - |
| α-Humulene | 1501 | - | - | - | - | - | - | - | - | 2.0 |
| γ-Cadinene | 1565 | - | - | - | - | - | 1.1 | - | - | - |
| γ-Muurolene | 1698 | - | - | - | - | - | 1.9 | - | - | - |
| ***Oxygenated sesquiterpenes*** | | ***2.1*** | ***-*** | ***-*** | ***0.7*** | ***-*** | ***2.7*** | ***-*** | ***1.0*** | ***0.1*** |
| Methyleugenol | 1449 | - | - | - | - | - | 2.7 | - | - | 0.1 |
| (-)-Cedreanol | 1694 | - | - | - | - | - | - | - | 1.0 | - |
| Nootkatone | 1845 | 1.3 | - | - | - | - | - | - | - | - |
| ***Esters*** | | ***-*** | ***-*** | ***0.3*** | ***0.2*** | ***0.8*** | ***1.5*** | ***-*** | ***3.2*** | ***16.8*** |
| Aceteugenol | 1586 | - | - | - | - | - | - | - | - | 16.8 |
| Ethyl palmitate | 2001 | - | - | - | - | - | - | - | 1.4 | - |
| ***Others*** | | ***3.2*** | ***3.7*** | ***-*** | ***31.6*** | ***96.2*** | ***83.1*** | ***97.9*** | ***56.2*** | ***67.6*** |
| di-2-Propenyldisulfide | 1056 | - | - | - | 10.6 | - | - | - | - | - |
| Dipropyldisulfide | 1093 | - | - | - | - | - | - | - | 5.9 | - |
| *trans*-Propenylpropyldisulfide | 1101 | - | - | - | - | - | - | - | 1.7 | - |
| *o*-(4-Nitrophenyl)-hydroxylamine | 1147 | - | - | - | - | - | - | - | 5.5 | - |
| Methylchavicol | 1213 | - | - | - | - | 92.4 | 77.9 | - | - | - |
| Dipropyltrisulfide | 1363 | - | - | - | - | - | - | - | 28.4 | - |
| *cis*-2,3-Diphenyl-1-pentafluorophenylaziridine | 1371 | - | - | - | - | - | - | - | 3.1 | - |
| *cis*-Propenylpropyltrisulfide | 1376 | - | - | - | - | - | - | - | 3.8 | - |
| *p*-Eugenol | 1414 | - | - | - | - | - | - | - | - | 67.6 |
| Dimethyl tetrasulphide | 1419 | - | - | - | 4.3 | - | - | - | - | - |
| 1,2,3,5-Tetramethyl-benzene | 1424 | - | 1.3 | - | - | - | - | - | - | - |
| 1-(1,5-Dimethylhexyl)-4-methylbenzene | 1482 | - | 2.3 | - | - | - | - | - | - | - |
| 4-Methoxy-6-(2-propenyl)-1,3-benzodioxole | 1585 | - | - | - | - | - | - | 45.1 | - | - |
| di-2-Propenyltetrasulfide | 1592 | - | - | - | 9.2 | - | - | - | - | - |
| Elemicin | 1612 | - | - | - | - | - | - | 5.9 | - | - |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
The compounds listed show a minimum concentration of 1 % in at least one essential oil analyzed.
Values are presented as percentage of normalized peak areas without correction factors.

**Table 2.3.1. (cont.)**

| Compounds | RI | Grapefruit | Lemon | Coriander | Garlic | Tarragon | Basil | Parsley | Onion | Clove |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Others (cont.)*** | | | | | | | | | | |
| 3-Methoxycinnamaldehyde | 1620 | - | - | - | - | 3.8 | 3.7 | - | - | - |
| 3,3'-Thiobis-1-propene | 1644 | - | - | - | 2.1 | - | - | - | - | - |
| 1,2,3,4-Tetramethoxy-5-(2-propenyl)-benzene | 1655 | - | - | - | - | - | - | 16.9 | - | - |
| Apiol | 1740 | - | - | - | - | - | - | 29.9 | - | - |
| N-Difluorophosphinodimethylhdroxylamine | 1814 | - | - | - | - | - | - | - | 3.6 | - |
| Sulfur | 2030 | - | - | - | 3.7 | - | - | - | 1.4 | - |
| 7-Methoxy-8-(2-formyl-2-methylpropyl)coumarin | 2196 | 1.8 | - | - | - | - | - | - | - | - |
| **Total identified** | | **95.8** | **83.1** | **98.1** | **59.0** | **99.0** | **97.0** | **99.5** | **67.3** | **98.7** |
| **Number of compounds identified** | | **17** | **9** | **10** | **15** | **7** | **25** | **7** | **35** | **9** |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
The compounds listed show a minimum concentration of 1 % in at least one essential oil analyzed.
Values are presented as percentage of normalized peak areas without correction factors.

Previous studies found similar chemical compositions for basil, Spanish oregano, clove, thyme, grapefruit, and lemon essential oils, but in different proportions [52; 73; 190-192], while other studies revealed different main compounds, like *e.g.* Hussain and co-authors [193] that identified linalool as the main compound of basil essential oil. For several essential oils, including those of citronella, coriander, rosemary, and sage, distinct chemical compositions were obtained in comparison with the results of previous studies [179; 194-195]. The chemical composition of plant essential oils may vary according to species, part of the plant, season of harvesting and geographical origin [67-70]. In addition, the extraction method can also influence the type and amount of molecules extracted [50; 71].

To our knowledge, the pattern of chemical content in several essential oils analyzed in this work has not been reported so far (*e.g.* celery, onion, parsley and garlic essential oils). Several compounds found in these essential oils, such as thymol, carvacrol, (-)-camphor, (-)-bornyl acetate, *trans*-caryophyllene, γ-terpinene and methylchavicol, have known antioxidant properties [55-56]. Additionally, antibacterial properties have also been described for thymol, carvacrol, eugenol, camphor, α-pinene and citronellal [57-60].

**Table 2.3.2.** Chemical composition of volatiles in sage, rosemary, marjoram, thyme, Spanish oregano, citronella, celery, and carrot essential oils.

| Compounds | RI | Sage | Rosemary | Marjoram | Thyme | Spanish oregano | Citronella | Celery | Carrot |
|---|---|---|---|---|---|---|---|---|---|
| ***Monoterpene hydrocarbons*** | | ***16.3*** | ***15.2*** | ***12.9*** | ***7.1*** | ***3.4*** | ***27.6*** | ***14.1*** | ***1.1*** |
| α-Pinene | 870 | 1.3 | 4.9 | 1.1 | - | 0.1 | - | - | 0.4 |
| Camphene | 887 | 1.1 | 2.3 | 0.4 | 0.1 | - | - | - | tr |
| β-Pinene | 920 | 1.1 | 1.8 | 2.0 | 0.1 | tr | - | 0.1 | 0.1 |
| Cymene | 980 | 0.3 | 1.0 | - | 4.0 | 1.8 | - | - | tr |
| α-Limonene | 995 | - | - | - | - | - | - | 13.8 | - |
| α-Fellandrene | 1025 | 0.2 | 0.2 | 0.1 | 1.5 | 0.4 | - | - | tr |
| *S*-3-Carene | 1080 | - | 0.8 | 3.1 | - | 0.4 | 0.1 | - | tr |
| *m*-Cymene | 1178 | 0.4 | 0.2 | 2.4 | 0.6 | 0.4 | - | - | 0.1 |
| Mentha-1,4,8-triene | 1196 | 1.5 | 3.8 | - | - | tr | tr | - | - |
| $\Delta^2$-Carene | 1283 | 2.6 | - | 2.8 | - | - | 22.5 | - | - |
| 1,3,8-*p*-Menthatriene | 1321 | 2.5 | - | - | - | - | - | - | - |
| α-Terpinolene | 1390 | 4.4 | - | - | - | - | - | - | - |
| *cis*-2,6-Dimethyl-2,6-octadiene | 1392 | - | - | - | - | - | 4.5 | - | - |
| ***Oxygenated monoterpenes*** | | ***43.5*** | ***59.7*** | ***74.9*** | ***82.6*** | ***89.5*** | ***22.5*** | ***0.3*** | ***0.1*** |
| Eucalyptol | 996 | 12.2 | 18.2 | 54.0 | 0.1 | 0.1 | - | - | - |
| (*E*)-2,3-Epoxycarane | 1083 | 0.5 | 0.2 | - | 1.6 | - | - | - | - |
| (+)-Camphor | 1135 | - | - | 2.7 | - | - | - | - | - |
| (-)-Isopulegol | 1139 | - | - | - | - | - | 1.0 | - | - |
| (-)-Camphor | 1141 | 28.7 | 35.5 | - | - | - | - | - | - |
| β-Citronellal | 1155 | - | - | - | - | - | 11.9 | - | - |
| *endo*-Borneol | 1164 | 1.9 | 5.5 | 4.6 | - | - | - | - | - |
| *endo*-5,5,6-Trimethyl-2-norbornanone | 1198 | 0.3 | 0.4 | 13.6 | tr | - | - | - | - |
| β-Citronellol | 1248 | - | - | - | - | - | 9.0 | - | - |
| *m*-Thymol | 1333 | - | - | - | 75.4 | 10.9 | - | - | - |
| Carvacrol | 1345 | - | - | - | 5.4 | 78.4 | - | - | - |
| ***Sesquiterpene hydrocarbons*** | | ***6.5*** | ***7.7*** | ***6.5*** | ***3.2*** | ***3.0*** | ***38.3*** | ***52.7*** | ***83.3*** |
| γ-Terpinene | 1426 | - | - | - | - | - | 7.5 | - | 3.9 |
| β-Elemene | 1434 | - | - | - | - | - | 6.2 | 0.1 | 0.3 |
| *trans*-Caryophyllene | 1465 | 2.0 | 7.2 | 5.8 | 2.9 | 2.7 | - | 1.2 | 8.3 |
| 2,6-Dimethyl-6-(4-methyl-3-pentenyl)-bicyclo[3.1.1]hept-2-ene | 1483 | - | - | - | - | - | - | - | 3.0 |
| *trans*- β-Farnesene | 1504 | - | - | - | - | - | - | - | 4.7 |
| β-Selinene | 1536 | 0.1 | - | - | - | - | 0.2 | 42.9 | 2.2 |
| α-Selinene | 1545 | - | - | - | - | - | 0.4 | 7.4 | 1.7 |
| α-Muurolene | 1551 | - | - | - | - | - | 1.3 | - | - |
| β-Bisabolene | 1561 | 4.0 | - | - | - | - | - | - | 4.4 |
| (+)-δ-Cadinene | 1575 | - | - | - | - | - | 6.0 | - | 0.7 |
| *cis*-α-Bisabolene | 1594 | - | - | - | - | - | - | - | 1.0 |
| Eremophilene | 1604 | - | - | - | - | - | 9.8 | - | - |
| δ-Cadinene | 1659 | - | - | - | - | - | - | - | 48.6 |
| γ-Selinene | 1684 | - | - | - | - | - | 1.2 | - | - |
| (+)-β-Selinene | 1702 | - | - | - | - | - | 1.0 | - | - |
| (-)-α-Amorphene | 1705 | - | - | - | - | - | 2.8 | - | - |
| ***Oxygenated sesquiterpenes*** | | ***-*** | ***-*** | ***-*** | ***-*** | ***0.1*** | ***1.7*** | ***-*** | ***0.3*** |
| (-)-Cedreanol | 1694 | - | - | - | - | - | 1.7 | - | - |
| ***Esters*** | | ***12.6*** | ***13.4*** | ***0.3*** | ***0.1*** | ***0.1*** | ***1.6*** | ***16.0*** | ***0.6*** |
| (-)-Bornylacetate | 1312 | 12.6 | 13.4 | 0.3 | - | - | - | - | 0.4 |
| *m*-(Trimethylsiloxy)-cinnamic acid methyl ester | 1397 | - | - | - | - | - | 1.5 | - | - |
| 2-Propenylphenoxyacetate | 1773 | - | - | - | - | - | - | 16.0 | - |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
The compounds listed show a minimum concentration of 1 % in at least one essential oil analyzed.
Values are presented as percentage of normalized peak areas without correction factors.

**Table 2.3.2. (cont.)**

| Compounds | RI | Sage | Rosemary | Marjoram | Thyme | Spanish oregano | Citronella | Celery | Carrot |
|---|---|---|---|---|---|---|---|---|---|
| ***Others*** | | ***0.1*** | ***0.4*** | ***-*** | ***1.6*** | ***2.6*** | ***0.1*** | ***13.9*** | ***3.9*** |
| Pentylbenzene | 1153 | - | - | - | - | - | - | 1.5 | - |
| Valerophenone | 1393 | - | - | - | - | - | - | 1.0 | - |
| (3*E*,5*E*,8*E*)-3,7,11-Trimethyl-1,3,5,8,10-dodecapentaene | 1638 | - | - | - | 1.3 | 2.3 | - | - | 3.6 |
| 2-Methyl-benzoxazole | 1706 | - | - | - | - | - | - | 11.0 | - |
| **Total identified** | | **79.0** | **96.5** | **94.6** | **94.5** | **98.8** | **91.7** | **97.0** | **89.3** |
| **Number of compounds identified** | | **24** | **16** | **13** | **19** | **17** | **32** | **17** | **36** |

*Abbreviations*: tr – traces (< 0.05 %); RI – retention index.
The compounds listed show a minimum concentration of 1 % in at least one essential oil analyzed.
Values are presented as percentage of normalized peak areas without correction factors.

### 3.2. *Antibacterial activity*

The antibacterial activity of the essential oils was evaluated using the disc diffusion method (Table 2.3.3). Only *E. coli* and *S. typhimurium* were resistant to carrot, grapefruit, lemon, onion, and parsley essential oils, as well as *P. putida* against carrot and parsley essential oils. The resistance of these Gram-negative bacteria could be due to the complexity of their double layer cell membrane in comparison with the single membrane of Gram-positive bacteria [164]. In contrast, the remaining bacteria (*B. thermosphacta*, *L. innocua*, *L. monocytogenes* and *S. putrefaciens*) were susceptible to all essential oils tested. Twelve essential oils, namely basil, celery, citronella, clove, coriander, garlic, marjoram, Spanish oregano, rosemary, sage, tarragon and thyme, inhibited the growth of all bacteria tested. Thymol, carvacrol, eugenol, camphor, α-pinene and citronellal, which make part of the composition of several of these essential oils, may be in the base of the antibacterial activity observed. It has been reported that some essential oil components interfere with the lipids of cell membranes, cause leakage of intracellular materials, and ultimately the cell lysis [196]. However, for some essential oils studied here none of these compounds with known antibacterial activity were present in their compositions, and the antibacterial activity may be due to other compounds for which the antibacterial activity has not yet been tested, to the best of our knowledge.

Overall, lower MIC of essential oils were needed to inhibit *P. putida* (< 3.0 mg.mL$^{-1}$), whereas higher MIC was required to inhibit *S. putrefaciens* (6 values higher than 10 mg.mL$^{-1}$) and both *Listeria* strains (5-7 values higher than 10 mg.mL$^{-1}$) (Figure 2.3.1). The highest MIC was obtained with the

rosemary essential oil (90.8 mg.mL$^{-1}$) against *B. thermosphacta* and *S. typhimurium*. In contrast, lower concentrations were needed to inhibit all bacteria when garlic, citronella and Spanish oregano essential oils were tested. So far, few studies addressed the antibacterial activity of these essential oils with the bacteria strains tested. The antibacterial activity was similar (Spanish oregano against *L. innocua*) [190], lower (garlic against *L. monocytogenes*) [197], or higher (coriander against *E. coli* and *S. typhimurium*) [195] than in previous studies. The differences might be related with distinct composition of the essential oils tested.

**Table 2.3.3.** Mean radius of inhibition zones (mm) of 20 µL undiluted essential oils tested against seven bacterial strains.

| Essential oils | *B. thermosphacta* | *E. coli* | *L. innocua* | *L. monocytogenes* | *P. putida* | *S. typhimurium* | *S. putrefaciens* |
|---|---|---|---|---|---|---|---|
| **Basil** | 9 | 5 | 4 | 9 | 4 | 5 | 17 |
| **Carrot** | 12 | N.D. | 8 | 5 | N.D. | N.D. | 3 |
| **Celery** | 21 | 2 | 8 | 6 | 2 | 2 | 10 |
| **Citronella** | 13 | 1 | 12 | 15 | 1 | 1 | 14 |
| **Clove** | 11 | 8 | 6 | 7 | 11 | 9 | 19 |
| **Coriander** | 14 | 25 | 20 | 16 | 5 | 7 | 32 |
| **Garlic** | 7 | 3 | 15 | 11 | 6 | 3 | 32 |
| **Grapefruit** | 18 | N.D. | 5 | 7 | 4 | N.D. | 7 |
| **Lemon** | 17 | N.D. | 5 | 3 | 2 | N.D. | 4 |
| **Marjoram** | 22 | 6 | 6 | 6 | 3 | 8 | 24 |
| **Onion** | 7 | N.D. | 5 | 4 | 3 | N.D. | 32 |
| **Spanish oregano** | 34 | 27 | 34 | 20 | 24 | 27 | 32 |
| **Parsley** | 3 | N.D. | 1 | 3 | N.D. | N.D. | 2 |
| **Rosemary** | 37 | 6 | 4 | 8 | 4 | 3 | 27 |
| **Sage** | 17 | 5 | 8 | 10 | 4 | 4 | 14 |
| **Tarragon** | 10 | 3 | 2 | 2 | 3 | 3 | 10 |
| **Thyme** | 32 | 47 | 44 | 45 | 40 | 57 | 32 |

*Abbreviations*: N.D. – inhibition zone radius not detected.
Disc radius is not included.

The effect of MIC on bacteria viable cells was also tested in liquid medium. *S. putrefaciens* was sensitive to the solvent used to dilute the essential oils, particularly in liquid medium whereas DMSO is dispersed (in solid medium, the effect of DMSO on this strain was subtracted from the effect of essential oils diluted in the solvent). Consequently, it was impossible to determine the MIC effect of essential oils in liquid medium for this strain. Overall, essential oils showed a pronounced reduction on viable bacteria in liquid medium (Table 2.3.4). The greatest reductions (8.0 log CFU.mL$^{-1}$) were observed with *L. innocua* (coriander, Spanish oregano, rosemary and thyme) and *L. monocytogenes* (thyme). In contrast, no reduction was observed for *B. thermosphacta* (garlic and onion), *E. coli* (celery, rosemary and sage), both

*Listeria* strains (parsley) and *S. typhimurium* (basil, celery, citronella, garlic and tarragon).

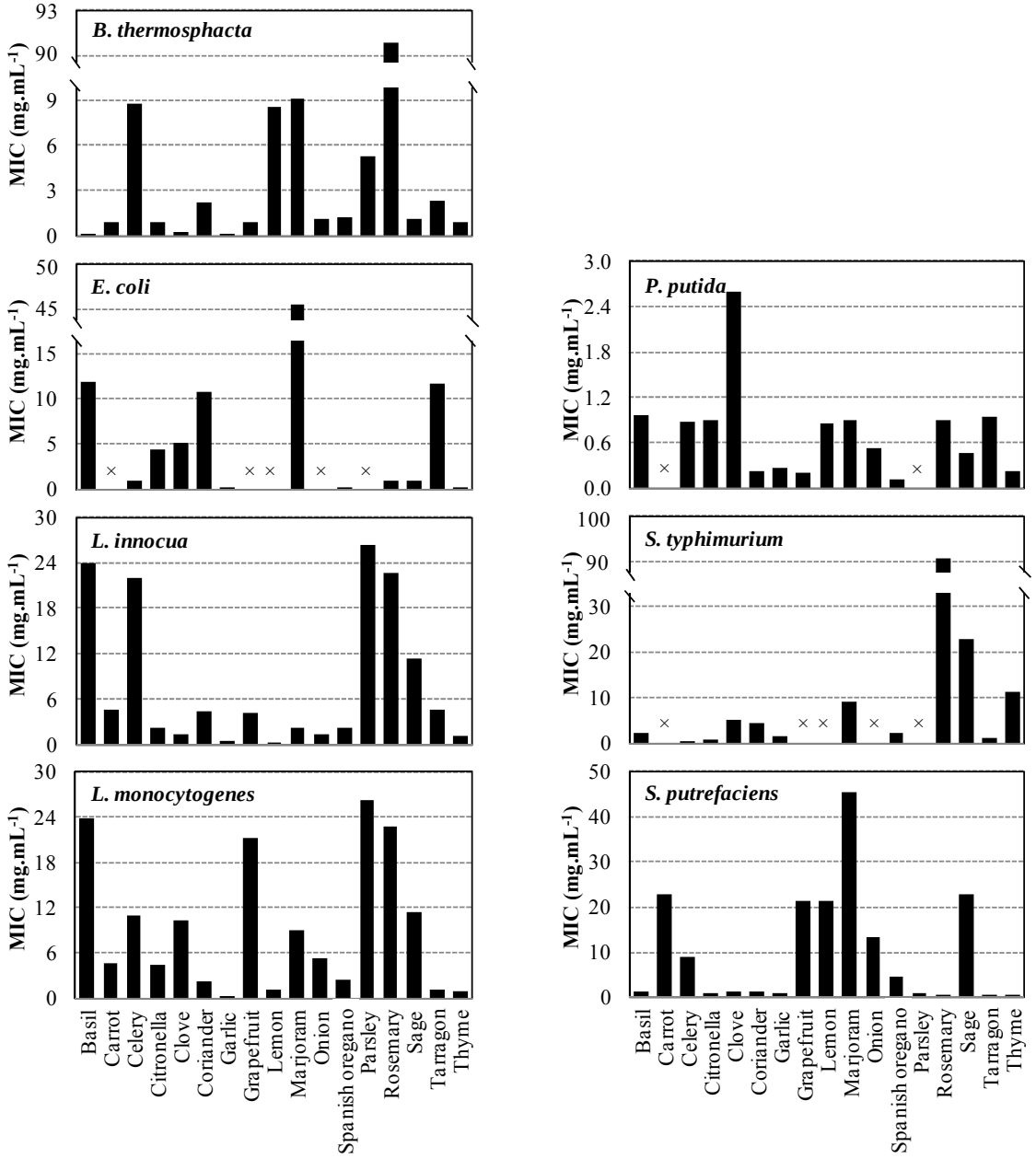


**Figure 2.3.1.** Minimum inhibitory concentration (MIC) of 17 essential oils against foodborne spoilage and pathogenic bacteria obtained with the disc diffusion method. The symbol × means that the essential oil did not inhibit the growth of the bacterial strain.

Very often, MIC of plant essential oils is determined with microdilution methods, and results are based on absorbance or visual inspection [133; 193]. However, the results obtained in the present study revealed that absorbance did not correlate with cells viability for most bacteria tested (Figure 2.3.2). Both

variables were only significantly correlated for *L. innocua* (r = -0.51; $p = 0.04$), and *S. typhimurium* (r = -0.61; $p = 0.04$). Therefore, future studies evaluating the antibacterial activity of plant essential oils should perform bacterial counts for MIC evaluation instead of absorbance when using microdilution methods.

**Table 2.3.4.** Effect of minimum inhibitory concentration (MIC) obtained with the disc diffusion method in liquid medium.

| Essential oils | *B. thermosphacta* | *E. coli* | *L. innocua* | *L. monocytogenes* | *P. putida* | *S. typhimurium* |
|---|---|---|---|---|---|---|
| **Basil** | 1.4 | 4.6 | 2.2 | 2.8 | 1.9 | 0.0 |
| **Carrot** | 2.4 | - | 5.7 | 6.4 | - | - |
| **Celery** | 3.3 | 0.0 | 3.5 | 3.6 | 1.7 | 0.0 |
| **Citronella** | 3.8 | 6.9 | 7.7 | 7.3 | 2.1 | 0.0 |
| **Clove** | 1.8 | 6.9 | 3.4 | 7.3 | 6.0 | 7.2 |
| **Coriander** | 4.5 | 6.6 | 8.0 | 4.3 | 0.8 | 5.3 |
| **Garlic** | 0.0 | 0.9 | 2.0 | 2.0 | 0.5 | 0.0 |
| **Grapefruit** | 3.7 | - | 2.1 | 3.9 | 2.0 | - |
| **Lemon** | 4.2 | - | 2.9 | 2.3 | 1.4 | - |
| **Marjoram** | 4.5 | 6.9 | 2.8 | 5.8 | 1.3 | 2.6 |
| **Onion** | 0.0 | - | 1.9 | 2.4 | 0.5 | - |
| **Spanish oregano** | 4.5 | 2.7 | 8.0 | 7.3 | 0.3 | 7.2 |
| **Parsley** | 1.8 | - | 0.0 | 0.0 | - | - |
| **Rosemary** | 4.5 | 0.0 | 8.0 | 7.3 | 1.1 | 1.7 |
| **Sage** | 2.5 | 0.0 | 6.1 | 6.1 | 0.9 | 7.2 |
| **Tarragon** | 2.4 | 6.9 | 6.9 | 1.9 | 2.1 | 0.0 |
| **Thyme** | 4.5 | 2.9 | 8.0 | 8.0 | 2.5 | 7.2 |

The values are presented as the mean bacterial CFU (colony forming units) logarithmic reductions per mL.
Values not shown correspond to essentials oils not able to inhibit the growth of the bacterial strain with the disc diffusion method.

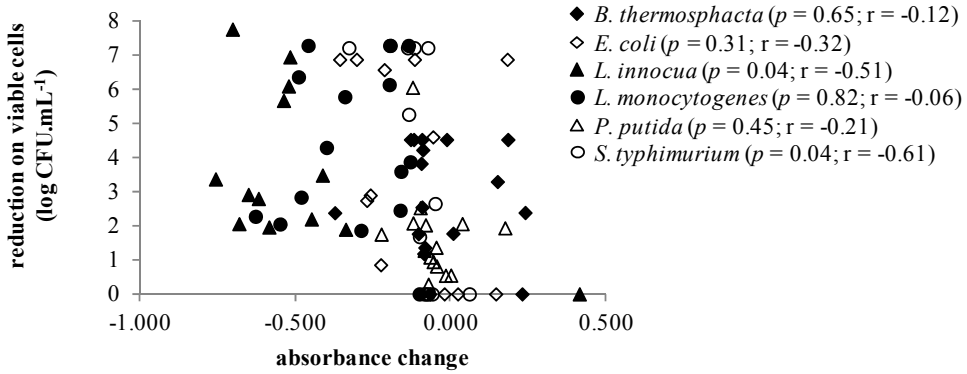


**Figure 2.3.2.** Relation between the reduction on viable cells and the absorbance (at 595 nm) of bacteria exposed to each essential oil minimum inhibitory concentration (MIC). *Abbreviations*: $p$ – p-value obtained for the correlation; r – correlation coefficient.

### 3.3. *Antioxidant activity*

The essential oils of celery, citronella, clove, Spanish oregano, parsley, tarragon, and thyme were able to inhibit 50 % of the radical scavenging activity of DPPH (Table 2.3.5). The remaining 10 essential oils revealed almost no antioxidant activity. The lowest $EC_{50}$ values were obtained with clove ($35.7 \pm 1.2$ µg.mL$^{-1}$) and Spanish oregano ($46.8 \pm 0.4$ µg.mL$^{-1}$), thus being

classified as very strong and strong antioxidants, respectively, accordingly to Scherer & Godoy [146] (Table 2.3.5). The antioxidant activity of clove and Spanish oregano essential oils was similar to that found for the synthetic antioxidant BHT, which was within values obtained in other studies [161-162]. In contrast, the remaining essential oils revealed poor antioxidant activity.

**Table 2.3.5.** Antioxidant activity of essential oils and BHT (butylated hydroxytoluene) measured by the DPPH (α,α-diphenyl-β-picrylhydrazyl) method.

| Essential oils / Compounds | DPPH | |
|---|---|---|
| | $EC_{50}$ (mg.mL$^{-1}$) | Antioxidant activity index (AAI) |
| **Celery** | 10.04 ± 0.39 $^{a}$ | < 0.1 |
| **Citronella** | 1.18 ± 0.02 $^{d}$ | < 0.1 |
| **Clove** | 0.04 ± 0.00 $^{f}$ | 2.2 ± 0.1 |
| **Spanish oregano** | 0.05 ± 0.00 $^{f}$ | 1.7 ± 0.0 |
| **Parsley** | 7.23 ± 0.16 $^{c}$ | < 0.1 |
| **Tarragon** | 8.81 ± 0.10 $^{b}$ | < 0.1 |
| **Thyme** | 0.25 ± 0.01 $^{e}$ | 0.3 ± 0.0 |
| **BHT** | 0.02 ± 0.00 $^{f}$ | 4.0 ± 0.5 |

*Abbreviations*: $EC_{50}$ – sample providing 50 % inhibition.
Values are presented as mean ± standard deviation.
Different letters (*a-f*) denote significant differences ($p < 0.05$) between essential oils/compounds.

The antioxidant activity measured with the ferric reducing power assay (Figure 2.3.3) revealed similar results to those obtained with the DPPH technique. The highest antioxidant activities were obtained for clove and Spanish oregano essential oils, followed by thyme and citronella, whereas the remaining essential oils had values below 5 μmol of ascorbic acid per g of essential oil.

Clove essential oil has been reported in previous studies as one of the strongest antioxidants, even higher than some synthetic antioxidants like BHT or butylated hydroxyanisole [198-200]. The strong activity of clove essential oil can be due to the presence of eugenol, the main constituent of this essential oil, which is known to have antioxidant activity [55; 200]. Spanish oregano essential oil is also known to have antioxidant activity, due in part to the presence of carvacrol, though higher antioxidant activity was detected than in the study of Bounatirou and co-authors [190]. Such differences might be related with different essential oil chemical compositions, such as the higher amounts of thymol found in Spanish oregano essential oil in these study (*ca.* 11 %) face to the 0.5 % maximum content described in the study of Bounatirou and co-authors [190]. The presence of huge amounts of *m*-thymol in thyme might be in the base of the strong antioxidant activity found, which is within the values obtained by other authors [73]. In citronella essential oil, the antioxidant activity

might be explained by the presence of β-citronellol and β-citronellal, with known antioxidant activity [55]. It would be expectable that the essential oils of coriander, sage, rosemary, tarragon, basil, and marjoram showed higher antioxidant activities. The compounds γ-terpinene, (-)-camphor, (-)-bornylacetate, eucalyptol, and methylchavicol, with known antioxidant activities [55-56; 201], were detected in their chemical compositions, but probably their amounts were not enough to reveal antioxidant activity.

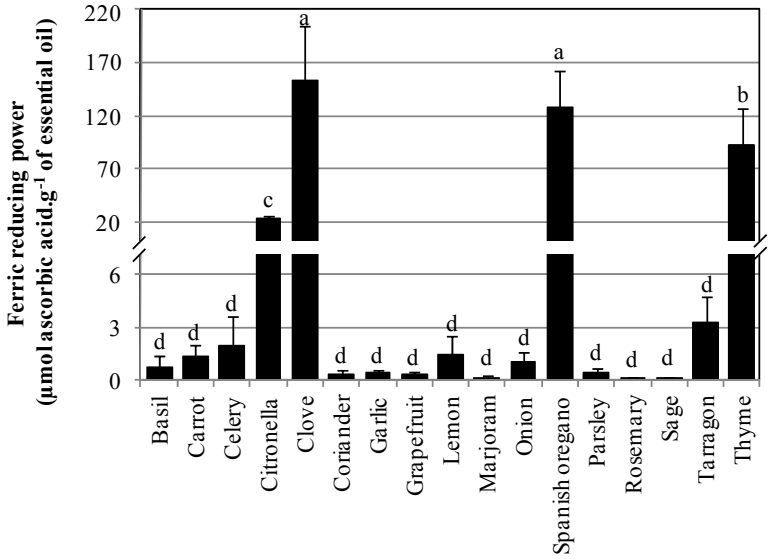


**Figure 2.3.3.** Antioxidant activity of essential oils measured with the ferric reducing power method. Bars represent the average and the error bars indicate the standard deviation. Different letters denote significant differences ($p < 0.05$) between essential oils.

## 4. Conclusions

This study showed that essential oils of basil, celery, citronella, clove, coriander, garlic, marjoram, Spanish oregano, rosemary, sage, tarragon and thyme were effective to inhibit the growth of all foodborne spoilage and pathogenic bacteria tested. In particular, lower amounts of garlic, citronella and Spanish oregano essential oils were needed to strongly inhibit the growth of all bacteria. Additionally, clove, Spanish oregano and thyme essential oils revealed the strongest antioxidant activity. Antibacterial and antioxidant activities of these essential oils could be due, in part, to the presence of several compounds, like carvacrol, thymol, citronellal and eugenol, in their chemical compositions. The results confirm the potential applicability of natural substances from plant origin as antibacterials and antioxidants. Still, for the application of essential

oils in food items, it must be assure a homogenous distribution of the essential oil, using for example carriers, like coatings and films.

# CHAPTER 2.4.

# Quality changes of sea bass (*Dicentrarchus labrax*) fillets preserved with Spanish oregano and lemon essential oils during refrigerated storage

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**Abstract**

In the present study, the effect of two essential oils (1.25 % v/w Spanish oregano, O; 1.25 % v/w Spanish oregano and 1.25 % v/w lemon, OL) and a synthetic preservative (0.25 % w/v potassium sorbate, PS) on the shelf life extension of fresh sea bass fillets stored under refrigeration (4 ºC) was investigated. Microbiological (psychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae), chemical (nucleotide, peroxide value, and malondialdehyde), physical (colour, texture, smell intensity, and pH), and sensory (odour, appearance, and texture) parameters were studied.

The dominant bacteria in the microflora of sea bass fillets were *Pseudomonas* spp. and hydrogen sulphide producing bacteria. Both were reduced with essential oils and PS in day 0. The treatments with essential oils increased the microbiological shelf life of sea bass fillets from 7 to 11 days. Although malondialdehyde concentration was kept within good quality limits in all treatments, lipid oxidation was reduced in treatments with essential oils. During storage, changes in the colour parameters chroma and hue were reduced in the PS treatment, but the variations in whiteness were smaller in treatment with both essential oils (OL). Results of sensory analysis indicate that PS extended the shelf life of sea bass fillets from 11 to 14 days. On the other hand, the odour of essential oils was pointed out as a negative attribute and crucial for its acceptability. Nucleotide degradation and pH were not affected by the addition of essential oils or PS.

In conclusion, the essential oils showed potential for preservation of fish products with increased microbiological shelf life. These essential oils could also delay rancidity in fish species and conditions with higher propensity for rancidity.

**Keywords**

Sea bass; Spanish oregano; *Thymus capitatus*; *Citrus limon*; essential oils; quality changes; shelf life extension; microbiology; sensory analysis

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## 1. Introduction

Consumers are increasingly demanding for high quality, fresh-like appearance, long shelf life, convenient, and minimally processed fish products. However, fish products are highly perishable due to autolytic reactions from endogenous enzymes, chemical and microbiological deterioration [2]. The search for new technologies that will maintain the quality of the fish has been the result of both economic drive to prevent waste and the consumers demand for mildly preserved products.

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species belonging to the family Moronidae, widely distributed in the Mediterranean Sea and Atlantic Ocean, and intensively farmed in several Mediterranean countries, *e.g.* Greece, Spain, and Italy [6]. This species is of high commercial value, great consumption worldwide, and is highly appreciated because of its excellent organoleptic properties and reasonable price [6]. In the retail stores, sea bass is most commonly sold as the whole fish, and ice storage has been used to prolong its shelf life. Whole fish presents a shelf life of 12-13 days in chilled conditions [7], and different attempts were done to extend sea bass shelf life, including the use of gamma radiation [12], and soluble gas stabilization with $CO_2$ [14-15].

Several studies have recommended the use of potassium sorbate to preserve fish. Potassium sorbate can extend the freshness period, reduce the production of total volatile base nitrogen [202], and inhibit the microbial growth [203], by dipping samples in potassium sorbate solutions with concentrations of 2-5 % (w/v).

Essential oils gained in recent years the interest of researchers as potential natural antioxidant and antimicrobial agents in food preservation, as consumers are increasingly aware about the risks associated with the ingestion of some synthetic preservatives. The effect of essential oils has been studied in various seafood products, including octopus [131], shrimps [204], and rainbow trout [79] and has revealed an extension of the shelf life of these seafood items either in aerobic, modified atmospheres, or vacuum package conditions.

The Spanish oregano essential oil is extracted from *Thymus capitatus*, a characteristics herb from the Mediterranean area and is rich in thymol and carvacrol, while the lemon essential oil of *Citrus limon* is mainly composed by

limonene [205]. These compounds are known for their antimicrobial [57; 206] and antioxidant [55] activities.

The aim of this study was to investigate the effect of Spanish oregano and lemon essential oils, as natural preservatives, on the quality and shelf life extension of vacuum packed sea bass fillets stored under refrigerated (4 ºC) conditions, by evaluating microbiological, chemical, physical, and sensory parameters. For comparison purposes, the synthetic preservative potassium sorbate was also tested.

## 2. Material and methods

### 2.1. *Chemicals*

Spanish oregano essential oil from *Thymus capitatus* (W282812; lot 21417CL-214; dried flowering herb steam distillation; origin: Spain), lemon essential oil from *Citrus limon* (W262508; lot 03509JH-258; fresh peel of the fruit cold pressed; origin: California, USA), potassium hydroxide, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), hypoxanthine (Hx), hydrogen peroxide, and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany); potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 2-thiobarbituric acid (TBA), ethylenediaminetetracetic acid (EDTA), propyl gallate, methanol, acetonitrile, hydrochloric acid, ammonium thiocyanate, iron (II) chloride, iron (III) chloride, chloroform, and plate count agar from Merck (Darmstadt, Germany); perchloric acid and trichloroacetic acid (TCA) from Panreac Química S.A.U. (Barcelona, Spain); inosine (HxR) from BDH Chemicals Ltd (Poole, England); maximum recovery diluent, violet red bile glucose agar, Pseudomonas agar base, and supplement SR 103 from Oxoid (Basingstoke, Hampshire, England); Lyngby iron agar from Scharlau Chemie (Sentmenat, Spain); potassium sorbate from F. Duarte (Indústria e Comércio Alimentar S.A., Rio de Mouro, Portugal); ethanol had a purity grade of 96 % and distilled and Milli Q purified water was used.

### 2.2. *Preparation of samples and treatments*

Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens with commercial quality were acquired from a local market and transported to the laboratory in foamed polystyrene boxes with ice. The average weight of each fish was 356 ± 39 g and the total length was 32 ± 1 cm. Fish samples (*ca*. 18 kg) were filleted and the skin was removed.

The fillets were randomly selected for each treatment and sampling day in order to reduce the effect of specimen variability. Four lots of samples were prepared: the first lot comprises the control samples, without any preservative (C); treatment O with 1.25 % v/w of Spanish oregano essential oil applied in both sides of fillets; treatment OL with 1.25 % v/w of Spanish oregano essential oil combined with 1.25 % v/w of lemon essential oil; and treatment PS with 0.25 % w/w of potassium sorbate. The lemon essential oil was not tested alone because the Spanish oregano essential oil revealed better *in vitro* antioxidant and antibacterial properties [205].

Fillets were vacuum packed at 40 mbar in polyamide/polyethylene barrier pouches (25 × 40 cm; 85 μm in thickness; oxygen permeability of 54.3 $cm^3.m^{-2}.day^{-1}$ at 75 % relative humidity and 23 ºC, carbon dioxide permeability of 176 $cm^3.m^{-2}.day^{-1}$ at 75 % relative humidity and 23 ºC, water vapour permeability of 5.2 $g.m^{-2}.day^{-1}$ at 100 % relative humidity and 38 ºC) with a vacuum packager (model A300/52, Multivac, Wolfertschwenden, Germany).

Samples were stored under refrigeration (4.0 ± 0.5 ºC) for a period of 14 days. During storage, two packages (*ca.* of 180 g of fillets per package) from each treatment (C, O, OL, and PS) were taken at predetermined intervals: 0, 4, 7, 11, and 14 days. Microbiological, physical, and sensory analyses were performed in sampling day, and part was homogenized and stored at -80 ºC until chemical analysis.

### 2.3. *Microbiological analysis*

Sea bass muscle (5 g) was aseptically collected to a Stomacher bag with filter. A primary ten-fold dilution was made with maximum recovery diluent and homogenized for one min at medium speed (230 rpm) using a Stomacher homogenizer (Laboratory blender STOMACHER 400, Seward Laboratory Systems Inc., Florida, USA). Appropriate series of decimal dilutions were prepared, and homogenates were spread on agar for psychrotrophic bacteria, *Pseudomonas* spp., and hydrogen sulphide producing bacteria enumeration, or poured into molten agar with a double layer to ensure anaerobic conditions for Enterobacteriaceae enumeration.

Psychrotrophic bacteria were enumerated on plate count agar, *Pseudomonas* spp. on Pseudomonas agar base supplemented with SR 103, and hydrogen sulphide producing bacteria (including *Shewanella putrefaciens*) on Lyngby iron agar, after incubation at 20 ºC for 4 days. Enterobacteriaceae were

enumerated on violet red bile glucose agar after incubation at 30 ºC for 24 h. For hydrogen sulphide producing bacteria only black colonies and those with a black centre were counted, whereas for Enterobacteriaceae the large colonies with purple haloes were counted and a representative number was tested for oxidation and fermentation reactions. Petri dishes containing 30-300 colony forming units (CFU) were selected for counting, and the results were expressed into logarithms of the CFU number per g of muscle. Determinations were performed in duplicate.

## 2.4. *Chemical analysis*

### 2.4.1. *Nucleotide*

Nucleotide and its breakdown products were extracted according to the method of Ryder [207]. During extract preparation, samples were kept in an ice bath. Minced sea bass muscle (5 g) was homogenized with perchloric acid (25 mL; 0.6 M) using an Ultra-turrax homogenizer (6,500 rpm, 1 min; Ultraturrax T25, Janke & Kunkel IKA®- Labortechnik). The homogenate was centrifuged (20,000 ×*g*, 10 min, 0 ºC; centrifuge 3K30, Sigma, Osterode, Germany), and 10 mL of the supernatant were neutralized with potassium hydroxide to a pH of 6.90. The neutralized supernatant stood for 30 min at 2 ºC to precipitate potassium perchlorate, which was then removed by filtration through sintered glass. The filtrate solution was made up to 20 mL with water, filtered (0.22 μm pore size), and kept at -80 ºC until injection.

An aliquot (20 μL) was injected into a high performance liquid chromatograph HP Agilent 1050 series (Agilent, USA) for nucleotide analysis. The chromatograph was equipped with a LiChrosorb RP-18 reverse-phase column (250 × 4.6 mm; 10 μm; VDS Optilab) operating isocratically with a mobile phase pumped at 1.6 $mL.min^{-1}$, and the detection wavelengths were set at 254 nm. The mobile phase was composed of potassium dihydrogen phosphate (0.04 M) and dipotassium hydrogen phosphate (0.06 M) with a pH of 6.90.

Nucleotides and its breakdown products were identified and quantified by comparison with standards. Standard curves for ATP and its degradation products (ADP, AMP, IMP, HxR, and Hx) were constructed in the 0.02-0.80 mM range. The peak areas were obtained with the software Agilent ChemStation for LC (Agilent, USA). All determinations were performed in triplicate. The $K_I$-index, which is a simplified form of the freshness indicator K-index, was estimated according to the following equation [33]:

$$K_I = \frac{HxR + Hx}{IMP + HxR + Hx}$$

**2.4.2. *Peroxide value***

The lipid fraction was extracted with methanol, chloroform, and water (40:20:16, v:v:v) following the method of Bligh & Dyer [208]. The peroxide value was determined using the ferric thiocyanate method accordingly to Shantha & Decker [209]. The procedure was conducted in subdued light. Briefly, the lipid fraction (200 µL), ethanol (10 mL; 95 %), ammonium thiocyanate (200 µL; 0.3 $g.mL^{-1}$), and iron chloride solution (200 µL; 4 $mg.mL^{-1}$ of iron II chloride in hydrochloric acid 37 %) were mixed. After 5 min, the absorbance was measured at 500 nm in a spectrophotometer (Spectrophotometer UNICAM UV-Vis, Helλos, Cambridge, UK).

For the calibration curve, a solution of iron (III) chloride was prepared by adding iron (III) chloride (1.45 g), hydrochloric acid (50 mL; 10 N), and hydrogen peroxide (2 mL; 30 %), and after boiling for 5 min, water was added to obtain a final volume of 500 mL. Different concentrations of this solution were prepared in ethanol (95 %), in the range 5-200 µg of Fe (III) per mL, and subjected to the same procedure as for the lipid fraction. The peroxide value was expressed as milliequivalents (meq) of peroxides per kg of muscle. All determinations were performed in triplicate.

**2.4.3. *Malondialdehyde***

Malondialdehyde (MDA) was quantified according to the method described by Seljeskog and co-authors [210] with modifications in the sample deproteinisation as described by Mendes and co-authors [211]. Briefly, minced sea bass (5 g) was homogenized with TCA solution (10 mL; 75 $g.L^{-1}$ TCA, 1 $g.L^{-1}$ EDTA, 1 $g.L^{-1}$ propyl gallate) using a Ultra-turrax homogenizer (5,000 rpm, 1 min). Then, the homogenate was filtered (Whatman #1) and the filtrate was centrifuged (5,000 rpm, 10 min). Sample supernatant (0.5 mL) was mixed with TBA (1.5 mL; 40 mM), heated (97 ºC, 60 min), and cooled (-20 ºC, 20 min). Methanol (3 mL) was added, and the resulting solution was filtered (0.22 µm pore size) and kept at -80 ºC until injection for MDA quantification.

Samples (10 µL) were injected into a high performance liquid chromatograph (Agilent 1100 series, Agilent, USA). Separation of MDA-TBA adduct was done using a reversed-phase column (4.6 × 150 mm; 5 µm; Phenomenex Gemini ODS C18 110Å, Phenomenex, Torrance, CA, USA), operating isocratically with a mobile phase (potassium dihydrogen phosphate 50 mM, methanol, and

acetonitrile in the proportion of 72:17:11; v:v:v) pumped at 1.0 mL.min$^{-1}$, and the spectrofluorimetric detector wavelengths were set at 525 nm (excitation) and 560 nm (emission).

MDA-TBA adduct was identified and quantified by comparison with TEP which was used as the MDA standard. A standard curve was made from TEP diluted in the TCA solution at concentrations in the range of 0.6-10.0 μM, without hydrolysis prior to the TBA reaction. The peak areas were obtained with the software Agilent ChemStation for LC (Agilent, USA). Results were expressed as mg of MDA per kg of muscle. All determinations were performed in triplicate.

## 2.5. *Physical analysis*

### 2.5.1. *Colour*

Colour measurements were assessed with a colorimeter (CR-410, Konica Minolta Camera, Co, Japan) in minced sea bass, to avoid colour heterogeneity of fillets. The colorimeter was calibrated against a white standard plate (CIE $L^*a^*b^*$ system: $L^* = 97.79$; $a^* = -0.02$; $b^* = 1.84$). Lightness ($L^*$), red-green value ($a^*$), and yellow-blue value ($b^*$) were measured. All determinations were performed in duplicate. Chroma (C*), hue (h*), and whiteness (W) were estimated according to Schubring [212] as follows:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$h^* = arctg\frac{b^*}{a^*}$$

$$W = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2}$$

### 2.5.2. *Texture*

Texture measurements were performed using a compression methodology that simulates the finger test as described by Botta [213]. The measurements were performed using an Instron texture analyser (Instron 4301, Bucks, United Kingdom) and a software for data acquisition and analysis (Series IX-3.0, Instron, Bucks, United Kingdom). A cylindrical probe with 12.5 mm diameter was used, and placed in contact with the surface of the fillet. Then, the probe was pressed downwards at a constant speed of 8 mm.s$^{-1}$ into the sample until a 5 N force was reached. At this time, the distance that the probe depresses the fillet was measured (deformation distance). Then, the fillet was allowed to rebound for 1 second. The distance the fillet rebounded was measured (rebound distance). Determinations were performed in five different locations of each

fillet. During fillet compression, the rate of load change (module of deformability) was also calculated using a least squares fit, being expressed in $N.mm^{-1}$. Deformation distances were standardized to remove the effect of fillets thickness as follows:

$$\text{Deformation}\,(\%) = \frac{\text{deformation distance}}{\text{thickness}} \times 100$$

Rebound was estimated as follows:

$$\text{Rebound}(\%) = \frac{\text{deformation distance} - \text{rebounded distance}}{\text{deformation distance}} \times 100$$

**2.5.3. *Smell intensity***

Instrumental smell intensity (number of molecules) was determined with a Portable Odour Level Indicator (Cosmos XP - 329 III R, New Cosmos Electric Co. Ltd, Osaka, Japan) that used a platinum heat coil covered with a high-sensitivity metal oxide ($SNO_2$/ZnO) semiconductor as a sensor kept at high temperature. Gas barrier bags were carefully perforated in one end by insertion of a pointed Teflon tube and a second hole was made in the opposite side of the bag in order to allow a stream of air through the product and into the Cosmos unit. Results were expressed as Cosmos units of smell intensity. Determinations were performed in duplicate.

**2.5.4. *pH***

The pH was measured directly on minced sea bass fillets using a surface calibrated pH electrode (SenTix 21, WTW, Weilheim, Germany) connected to a pH meter (microprocessor pH meter 539 WTW, Weilheim, Germany). Determinations were performed in duplicate.

**2.6. *Sensory analysis***

Sensory evaluation was conducted in individual booths under controlled conditions of light and temperature. The attributes of raw sea bass fillets were evaluated by a panel consisting of six experienced judges. A description of attributes and terminology used were discussed beforehand with the panel members. All treatments (C, O, OL, and PS) were presented individually on each sampling day. Panellists were asked to score odour (fresh odour), appearance (characteristic appearance), and texture (firmness) of samples. A 1-5 category intensity scale of attributes was used, where a score of 1 indicated "absence", and a score of 5 indicated "very intense". Panellists were also asked

for rejection/acceptability of samples. The results are the average of attributes scores of all judges.

**2.7. *Statistical analysis***

Differences between treatments were tested at a 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA). The effects of treatments and storage time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences between treatments.

## 3. Results and discussion

**3.1. *Microbiological analysis***

In live and healthy fish, microorganisms are mostly found on the outer surface (skin and slime), and in some inner surfaces like gills and gastro-intestinal tract, while the remaining inner tissues are sterile [43-44]. After fish death, the presence of bacteria in the muscle results from contamination during processing and handling, where a maximum of 5 log CFU.g$^{-1}$ in total counts is considered acceptable in a good quality product [47].

In the beginning of storage, the treatment C showed a low value of psychrotrophic bacteria counts (3.3 log CFU.g$^{-1}$; Figure 2.4.1). The treatment PS reduced bacteria load to 2.6 log CFU.g$^{-1}$, whereas in treatments with essential oils the load was below detection limit (1 log CFU.g$^{-1}$). For *Pseudomonas* spp. the effect of treatments was similar to psychrotrophic bacteria, thought with lower values in the treatment C (Figure 2.4.1). For hydrogen sulphide producing bacteria, treatments PS, O, and OL reduced the load below detection limit in day 0. Enterobacteriaceae was undetectable in all treatments in the beginning of storage (Figure 2.4.1).

These results are in agreement with previous studies where the application of thyme essential oil reduced bacterial flora of sea bass and swordfish [17; 77]. The bacterial reduction might be due to the action of components of essential oils with known antimicrobial properties (such as thymol, carvacrol, and limonene) [57; 206]. It is known that some essential oil components, such as eugenol, γ-terpinene, and α-terpineol, interfere with the lipids of cell membranes, cause leakage of intracellular materials, and ultimately the cell lysis [196]. Drosinos and co-authors [203] reported that the application of potassium sorbate reduced total viable counts in 2 log CFU.g$^{-1}$and extended microbiological shelf life of gilthead sea bream packed in modified atmosphere.

During storage, psychrotrophic bacterial load increased reaching the upper acceptability limit for fresh marine species [47] in day 7 in treatments C and PS (Figure 2.4.1). Both treatments with essential oils showed lower psychrotrophic bacterial loads, increasing the microbiological shelf life of sea bass fillets until day 11. The addition of lemon essential oil did not change the bacterial load of fillets compared with treatment only with Spanish oregano essential oil. In a previous study, thymol, lemon extract, and grapefruit seed extract acted in a synergic way to improve the microbiological stability of fish hamburgers, being the concentration of compounds determinant [214].

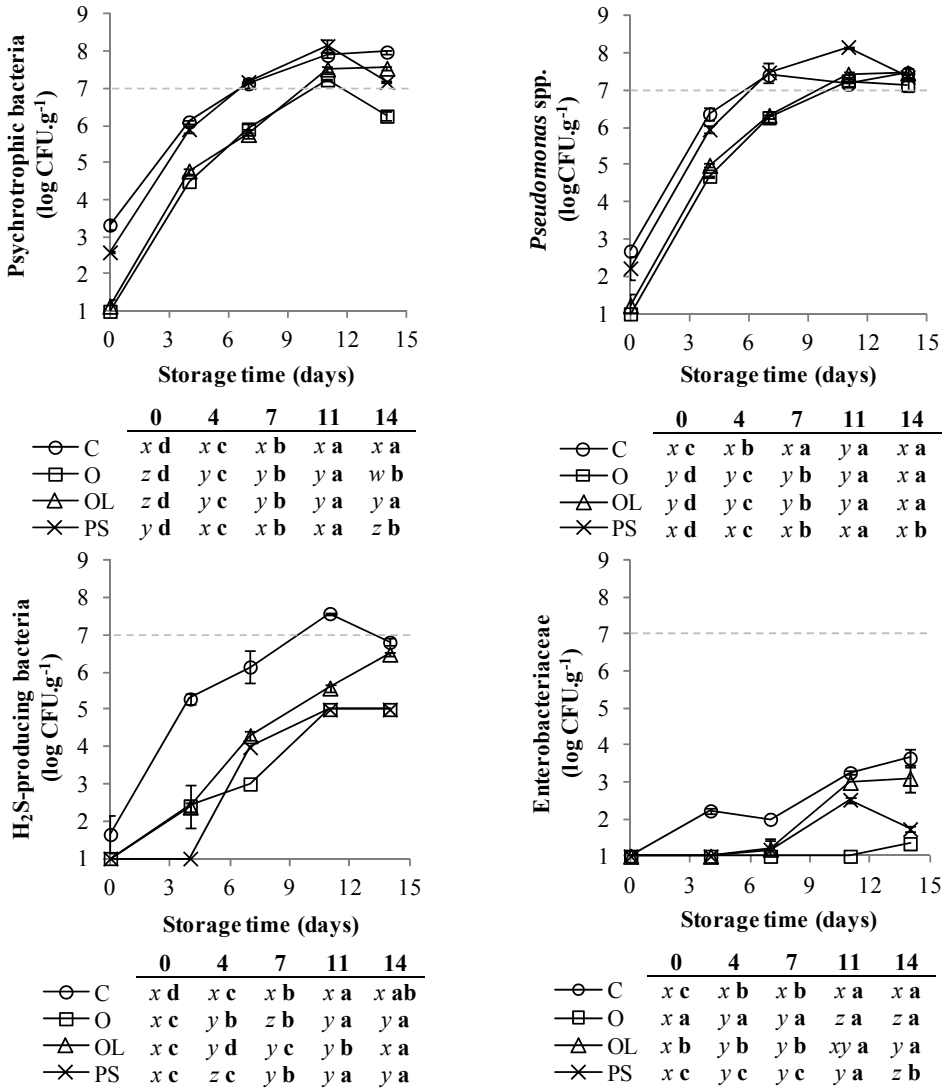


**Figure 2.4.1.** Changes in psychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae during refrigerated storage of sea bass control fillets (C), and preserved with Spanish oregano essential oil (O), Spanish oregano and lemon essential oils (OL), and potassium sorbate (PS). Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**d**). The grey dashed line indicates the upper acceptability limit for fresh marine species. *Abbreviations*: CFU – colony forming units.

For *Pseudomonas* spp., a similar evolution was observed as the one described for psychrotrophic bacteria (Figure 2.4.1). Concerning hydrogen sulphide producing bacteria, a load of 7 log CFU.g$^{-1}$ was reached at day 11 in treatment C. During almost all the storage period, the treatments O, OL, and PS showed values of at least 2 log CFU.g$^{-1}$ lower than the treatment C. Concerning Enterobacteriaceae, treatment C reached 3.7 log CFU.g$^{-1}$ by the end of storage, whereas the remaining treatments (O, OL, and PS) showed values close to the detection limit until day 7, and treatment O even delayed Enterobacteriaceae growth until the end of the storage period.

In previous studies, different fish species, packaging conditions, and essential oil concentrations were reported to be determinant in the shelf life extension of fish muscle. In sea bass treated with thyme essential oil, the microbiological shelf life was not extended in aerobic packaging conditions, but seven additional days were observed using modified atmosphere packaging [17], while in swordfish, three additional days were obtained independently of the packaging conditions used (aerobic or modified atmosphere) [77].

## 3.2. *Chemical analysis*

### 3.2.1. *Nucleotide degradation*

ATP breakdown products formed in fish after death result from a chain reaction in which metabolites typically rise and then fall as the next metabolite in the degradative chain begins to rise [27]. The initial stages in nucleotide degradation are thought to be mainly due to autolytic reactions, while the degradation of HxR to Hx is due to the presence of both autolytic and bacterial activities [27]. IMP has been recognized as having flavour-enhancing properties, while Hx contributes to the off-flavours typical of spoiled fish [27].

Low levels of ATP, ADP, and AMP were found in the beginning of storage (concentration levels below 0.4 μmol.g$^{-1}$; data not shown), which is in accordance with the baseline levels obtained for several fish species [29]. Taking into account that ATP, ADP, and AMP concentrations rapidly decrease and are negligible within 25 h after death [33], the simplified freshness indicator $K_I$ was used, that follows the degradation rate of IMP and its breakdown products.

Initially, the treatment C showed a $K_I$-index of 50.6 % (Table 2.4.1) and IMP concentration was 3.7 μmol.g$^{-1}$ (data not shown). The treatments OL and PS revealed higher $K_I$-index values (53.4 and 59.7 %, respectively) at day 0. During storage, $K_I$-index values increased reaching 97.9 %, as IMP depleted and Hx concentration increased to 3.6 μmol.g$^{-1}$ in treatment C (Table 2.4.1). For

the other treatments (O, OL, and PS) statistical differences were observed in the evolution of nucleotide degradation between treatments, but without a clear trend. During storage, $K_I$-index evolution of treatments with essential oils was comparable, and by the end of storage $K_I$-index was slightly lower in these treatments. This difference in the $K_I$-index might be due to the lower degradation of HxR to Hx, since bacterial activity was affected by essential oils. In previous studies, treatments with potassium sorbate delayed nucleotide degradation differently depending on the fish species, being more pronounced in black promfret *Parastromateus niger* than in pearlspot *Etroplus suratensis* [202].

**Table 2.4.1.** Changes in $K_I$-value, texture, smell intensity, and pH during refrigerated storage of sea bass control fillets (C), and preserved with Spanish oregano essential oil (O), Spanish oregano and lemon essential oils (OL), and potassium sorbate (PS).

| | Day 0 | Day 4 | Day 7 | Day 11 | Day 14 |
|---|---|---|---|---|---|
| ***$K_I$-value*** (%) | | | | | |
| **C** | $50.6 \pm 0.7^{z\,e}$ | $69.3 \pm 0.1^{z\,d}$ | $80.5 \pm 0.4^{y\,c}$ | $94.9 \pm 0.1^{x\,b}$ | $97.9 \pm 0.1^{x\,a}$ |
| **O** | $51.2 \pm 0.4^{z\,e}$ | $72.9 \pm 0.3^{y\,d}$ | $81.2 \pm 0.1^{y\,c}$ | $92.6 \pm 0.1^{y\,b}$ | $96.4 \pm 0.3^{y\,a}$ |
| **OL** | $59.7 \pm 0.9^{x\,e}$ | $73.0 \pm 0.1^{y\,d}$ | $80.8 \pm 0.5^{y\,c}$ | $92.2 \pm 0.1^{y\,b}$ | $96.1 \pm 0.2^{y\,a}$ |
| **PS** | $53.4 \pm 0.5^{y\,e}$ | $74.1 \pm 0.1^{x\,d}$ | $85.1 \pm 0.7^{x\,c}$ | $95.0 \pm 0.2^{x\,b}$ | $97.8 \pm 0.3^{x\,a}$ |
| ***Deformation*** (%) | | | | | |
| **C** | $45.5 \pm 8.7^{x\,a}$ | nd | $35.3 \pm 5.3^{x\,ab}$ | $29.8 \pm 6.4^{x\,b}$ | $30.7 \pm 6.1^{x\,b}$ |
| **O** | $37.9 \pm 6.3^{x\,a}$ | $29.2 \pm 6.5^{x\,a}$ | $32.2 \pm 6.6^{x\,a}$ | $28.0 \pm 3.1^{x\,a}$ | $34.8 \pm 9.6^{x\,a}$ |
| **OL** | $35.4 \pm 7.7^{x\,a}$ | $28.0 \pm 4.5^{x\,a}$ | $33.7 \pm 9.7^{x\,a}$ | $34.1 \pm 9.2^{x\,a}$ | $39.4 \pm 4.8^{x\,a}$ |
| **PS** | $36.9 \pm 5.2^{x\,a}$ | nd | $37.8 \pm 7.4^{x\,a}$ | $28.0 \pm 5.8^{x\,a}$ | $37.1 \pm 3.5^{x\,a}$ |
| ***Module of deformability*** ($N.mm^{-1}$) | | | | | |
| **C** | $0.72 \pm 0.07^{x\,b}$ | nd | $0.97 \pm 0.16^{x\,ab}$ | $0.91 \pm 0.17^{x\,b}$ | $1.36 \pm 0.02^{x\,a}$ |
| **O** | $0.76 \pm 0.08^{x\,a}$ | $0.84 \pm 0.18^{x\,a}$ | $0.85 \pm 0.10^{x\,a}$ | $0.99 \pm 0.08^{x\,a}$ | $0.98 \pm 0.17^{xy\,a}$ |
| **OL** | $0.91 \pm 0.14^{x\,a}$ | $1.12 \pm 0.09^{x\,a}$ | $1.08 \pm 0.14^{x\,a}$ | $1.13 \pm 0.04^{x\,a}$ | $1.24 \pm 0.23^{x\,a}$ |
| **PS** | $0.71 \pm 0.09^{x\,a}$ | nd | $0.78 \pm 0.08^{x\,a}$ | $0.84 \pm 0.10^{x\,a}$ | $0.71 \pm 0.05^{y\,a}$ |
| ***Rebound*** (%) | | | | | |
| **C** | $75.1 \pm 8.7^{x\,a}$ | nd | $48.6 \pm 10.2^{y\,b}$ | $65.1 \pm 7.7^{x\,ab}$ | $71.9 \pm 9.9^{x\,a}$ |
| **O** | $71.1 \pm 8.7^{x\,a}$ | $76.1 \pm 9.9^{x\,a}$ | $76.1 \pm 9.0^{x\,a}$ | $71.2 \pm 7.8^{x\,a}$ | $71.2 \pm 7.5^{x\,a}$ |
| **OL** | $60.8 \pm 10.2^{x\,a}$ | $71.9 \pm 9.3^{x\,a}$ | $75.1 \pm 8.3^{x\,a}$ | $76.8 \pm 1.4^{x\,a}$ | $71.2 \pm 7.4^{x\,a}$ |
| **PS** | $72.1 \pm 7.4^{x\,a}$ | nd | $63.7 \pm 8.7^{yx\,a}$ | $68.0 \pm 4.3^{x\,a}$ | $64.3 \pm 4.3^{x\,a}$ |
| ***Smell intensity*** (Cosmos units) | | | | | |
| **C** | $565 \pm 97^{x\,a}$ | $565 \pm 136^{x\,a}$ | $438 \pm 8^{x\,a}$ | $569 \pm 14^{x\,a}$ | $582 \pm 105^{x\,a}$ |
| **PS** | $545 \pm 40^{x\,a}$ | $511 \pm 7^{x\,a}$ | $583 \pm 159^{x\,a}$ | $409 \pm 12^{x\,a}$ | $602 \pm 128^{x\,a}$ |
| ***pH*** | | | | | |
| **C** | $6.4 \pm 0.0^{x\,a}$ | $6.4 \pm 0.0^{x\,a}$ | $6.4 \pm 0.1^{y\,a}$ | $6.4 \pm 0.0^{x\,a}$ | $6.3 \pm 0.0^{y\,a}$ |
| **O** | $6.4 \pm 0.0^{x\,ab}$ | $6.3 \pm 0.0^{x\,b}$ | $6.4 \pm 0.0^{y\,ab}$ | $6.4 \pm 0.0^{x\,ab}$ | $6.5 \pm 0.0^{x\,a}$ |
| **OL** | $6.3 \pm 0.1^{x\,b}$ | $6.3 \pm 0.0^{x\,ab}$ | $6.4 \pm 0.0^{y\,ab}$ | $6.5 \pm 0.0^{x\,a}$ | $6.4 \pm 0.0^{xy\,ab}$ |
| **PS** | $6.4 \pm 0.0^{x\,b}$ | $6.4 \pm 0.0^{x\,b}$ | $6.6 \pm 0.1^{x\,a}$ | $6.5 \pm 0.0^{x\,ab}$ | $6.5 \pm 0.0^{x\,ab}$ |

*Abbreviations*: $K_I$-value – freshness indicator index; nd – not determined.
Values are presented as average ± standard deviation.
Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*y*) or between sampling days (**a**-**e**).

### 3.2.2. *Lipid oxidation*

Fish is highly susceptible to lipid oxidation because its muscle is characterized by a high content of polyunsaturated fatty acids [26]. As a consequence of oxidation, lipid hydroperoxides are formed and decomposed to alcohols, aldehydes, ketones, and hydrocarbons [26].

Lipid oxidation measured by the peroxide value showed considerably low oxidation levels (< 0.1 meq of peroxides per kg of muscle) in all treatments during storage (data not shown), being within recommended values (< 10-20 meq.kg$^{-1}$ of fat) [215]. The low levels observed are possible due to rapidly broken down of hydroperoxides to secondary products [26].

Lipid oxidation secondary products measured by the MDA determination statistically increased with storage time in all treatments (Figure 2.4.2). In treatment PS, the accumulation of MDA increased at a higher rate than in control samples, while in the treatments O and OL a reduction in lipid oxidation was observed (Figure 2.4.2). In general, no significant differences were observed between treatments with essential oils during storage, except in day 14, where treatment O showed higher MDA values. The lower oxidation levels observed in treatments with essential oils might be due to the presence of carvacrol and thymol in Spanish oregano essential oil, and to limonene in lemon essential oil, all with known antioxidant activities [55].

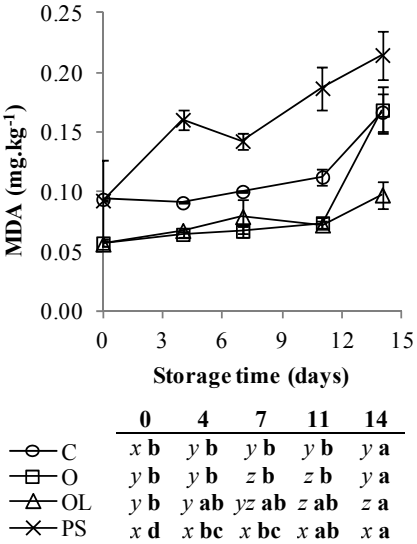


**Figure 2.4.2.** Changes in malondialdehyde (MDA) during refrigerated storage of sea bass control fillets (C), and preserved with Spanish oregano essential oil (O), Spanish oregano and lemon essential oils (OL), and potassium sorbate (PS). Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**d**).

Although MDA concentration increased during storage, rancid odours were not detected by panellists in sensory analysis and values were within the guidelines for MDA limit values (< 0.72 mg MDA.kg$^{-1}$) in seafood [216]. The positive effects of essential oils in lipid oxidation are not as noticeable, possible due to the low fat content in sea bass and short storage period.

In previous studies, the application of thyme essential oil also reduced lipid oxidation during storage, particularly in aerobic conditions compared to modified atmosphere (*e.g.* swordfish *Xiphias gladius* and sea bass) [17; 77]. The differences found for thyme essential oils might be related with thyme species and the amount of antioxidant components in each essential oil, fish species under study, and packaging treatments.

### 3.3. *Physical analysis*

#### 3.3.1. *Colour*

Colour is an important quality attribute of food as influences appearance and presentation, and might determine the acceptability by consumers and purchasing decisions [217]. The colour of fish muscle is related with carotenoids and hemepigments [22], with the muscle physical structure, and the amount of unbound water influencing light scattering [16].

In the beginning of storage, control samples showed whiteness values of 72.7, whereas hue was 63.4 and chroma 8.1 (Figure 2.4.3). In day 0, whiteness and chroma significantly decreased in treatment O, while hue and chroma statistically increased in treatment OL, compared with control samples. In treatment PS, chroma significantly increased, and whiteness and hue statistically decreased. The changes in colour parameters observed in treatments with essential oils might be related with their colour, which is slightly yellow.

During storage, control fillets became darker, reddish, and yellowish, the hue values varied around 60 and chroma increased (Figure 2.4.3). In treatments O, OL, and PS some fluctuations were observed in the colour parameters during storage. By the end of the storage period, treatment OL did not get so dark and both treatments with essential oils showed higher hue values. On the other hand, in treatment PS smaller variations were observed in hue and chroma during all storage period.

#### 3.3.2. *Texture*

The texture of fish is a quality parameter of great importance for consumers [218]. Several mechanisms are involved in texture changes that take place in fish muscle during storage. Autolytic and microbiological processes that occur after fish death lead to the softening of muscle tissues and to the lost of elasticity [218-219].

Texture was evaluated in terms of deformation and rebound (finger test), which are related with firmness and elasticity of fillets, respectively (Table 2.4.1). In the beginning of storage, treatments O, OL, and PS did not cause significant

changes in fillets deformation. In general, the module of deformability increased in treatments C, O, and OL with storage time, though only significantly in treatment C. This indicates that more force was needed to compress the fillet with storage time. In treatment PS, the values of module of deformability did not change during storage.

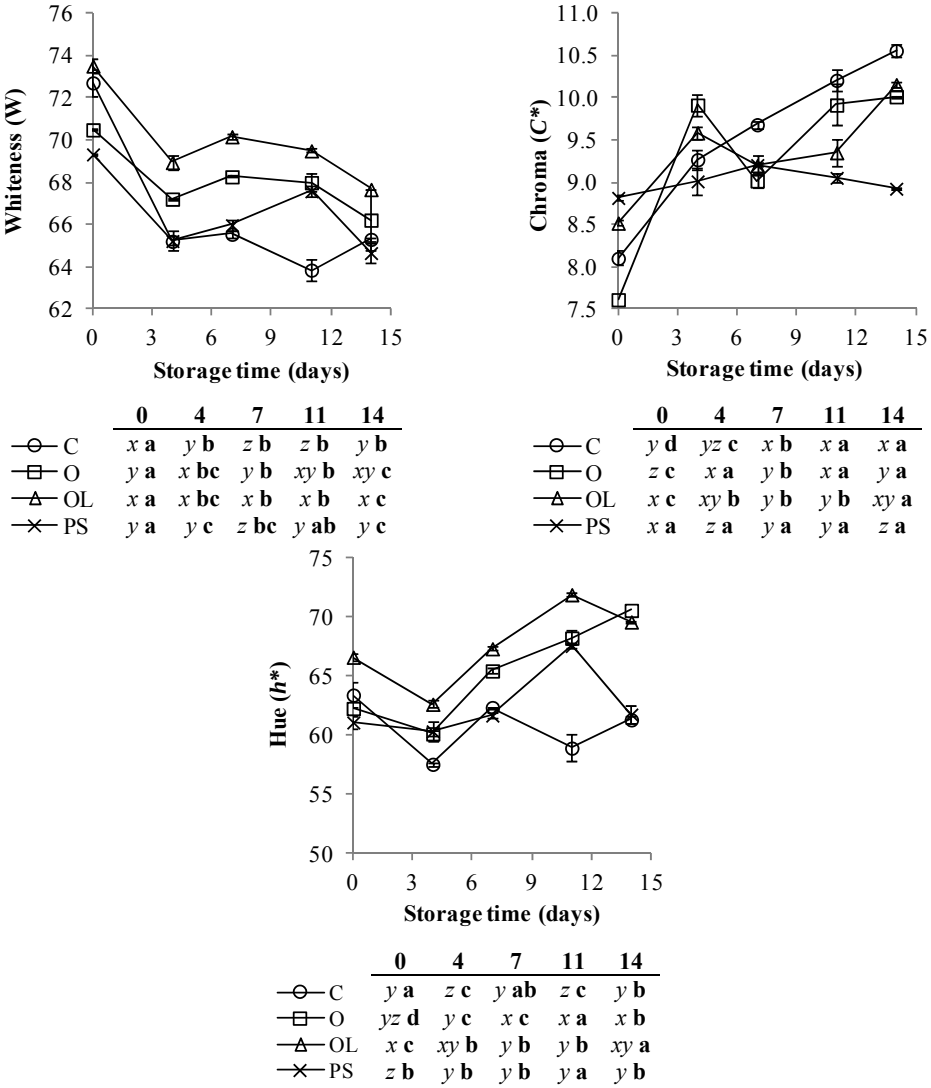


**Figure 2.4.3.** Changes in colour parameters (whiteness, chroma, and hue) during refrigerated storage of sea bass control fillets (C), and preserved with Spanish oregano essential oil (O), Spanish oregano and lemon essential oils (OL), and potassium sorbate (PS). Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**d**).

These results did not follow the same tendency detected by panellists in sensory analysis, where a decrease in fillets firmness was registered with storage time. In previous studies, instrumental measurements of hardness in meagre fillets

were not significantly different between storage days, and panellists also detected differences in fillets texture [220].

The variations observed in deformation values are within those reported for raw cod fillets [213]. These variations in texture are due to the fact that several measurements were performed to comprise different parts of fillets. On the other hand, manipulation of fillets might have also contributed to these variations. In fact, in a second compress test performed in the same location (data not shown), fillets deformation statistically increased in average from 35 to 49 % (paired samples t-student test, $p$-value < 0.05).

In day 0, rebound of fillets was 75 % in control samples, which means that in a fillet of 10 mm thickness, only *ca*. 1 mm did not recover the original shape taking into account a deformation of 45.5 %. With the exception of day 7 that showed the lowest rebound value in control samples, storage time and treatments did not affect the rebound of fillets (Table 2.4.1).

### 3.3.3. *Smell intensity*

Initially, smell intensity values were 565 Cosmos units in control samples (Table 2.4.1). Similar smell intensities were observed in the PS samples. In contrast, in treatments with essential oils, measurements exceeded 1200 Cosmos units in the beginning of storage, *i.e.* outside the apparatus reading range, and for this reason it was not possible to determine accurately and precisely the smell intensity of treatments O and OL during all experience. The strong smell intensity observed in treatments O and OL is not related with the typical off-odours formed during fish degradation, but were the direct result of essential oils addition. Also, in sensory analysis, the fresh odour characteristic of raw fish was not detected, because the volatiles of essential oils masked the original odour of fillets.

During storage, smell intensities in control and PS samples were not significantly different between treatments, neither between storage days (Table 2.4.1).

It would be expectable that smell intensity increased during storage due to off-odours release. In sensory analysis, fishy and putrid odours were detected from day 11 in control samples and at day 14 in PS samples. In a study with several fish species (*Bidyanus bidyanus*, *Salmo trutta*, *Tilapia niceotica* × *Tilapia aurea*, and *Lates calcarifer*) smell intensity remained almost constant during the first 15 days of refrigerated storage [221]. In other seafood products, such as edible crab and octopus, smell intensity increased with storage time,

reflecting the release of off-odours detected in sensory analysis [222-223]. In this sense, the results obtained in this study showed that this instrumental analysis was not adequate as spoilage indicator for sea bass fillets.

### 3.3.4. *pH*

*Post-mortem* glycolysis of fish muscle results in the accumulation of lactate and protons, which in turn lowers muscle pH [26], reduces the net surface charge of muscle proteins, and causes their partial denaturation [24]. The decrease in pH can lead to loss of water holding capacity [24]. Additionally, pH strongly influences the microbiology of fish muscle, especially pH sensitive spoilage bacteria [43].

In control samples, pH values did not change significantly during storage (pH of 6.3-6.4; Table 2.4.1). In general, treatments O, OL, and PS did not cause changes in fillets pH. This is in accordance with previous studies where pH values were not much affected by the application of thyme essential oil in swordfish and sea bass fillets using aerobic and modified atmosphere conditions [17; 77]. Other authors reported changes in pH values in fish products treated with potassium sorbate [203].

### 3.4. *Sensory analysis*

Initially, raw fillets of control samples were translucent, showed light colour, fresh characteristic odour, and the muscle was firm (Figure 2.4.4). In treatment PS, no major changes were registered, compared with control samples. However, the characteristic odour to fresh fish was lost in treatments with essential oils. Panellists always identified the intrinsic odour of essential oils used in treatments O and OL, though a more intense score was attributed to treatment O (average score 3.7) than to treatment OL (average score 2.9). The variations in colour obtained by instrumental analysis were not detected in sensory analysis in any treatment.

During storage, the sensory quality decreased in all treatments (Figure 2.4.4). Variations in scores of fresh odour and firmness were more evident than in the appearance of fillets. In control samples, fresh odour and firmness decreased with storage time. From day 11, off-odours typical of spoiled fish were detected in control samples, being rejected by panellists. During storage, the sensory changes of treatment PS were similar to those observed in control samples, but fillets of treatment PS were rejected later (day 14). Drosinos and co-authors [203] pointed out that potassium sorbate has a positive effect in odour of raw gilthead

sea bream. It is also able to extend the sensory shelf life of cooked pearlspot and black pomfret in 7 and 8 days, respectively [202].

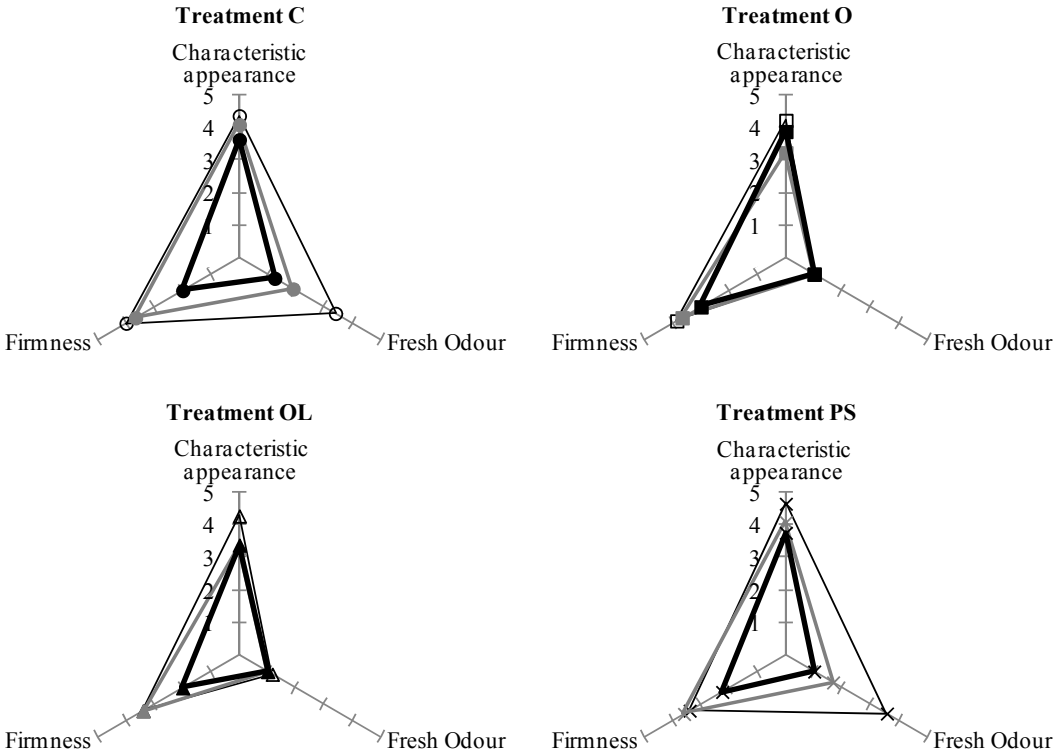


**Figure 2.4.4.** Sensory appreciation of raw sea bass control fillets (C), and preserved with Spanish oregano essential oil (O), Spanish oregano and lemon essential oils (OL), and potassium sorbate (PS). The markers represent days 1 (black thin line), 7 (grey solid line), and 14 (black solid line) of refrigerated storage. Category scale (1-5): 1, absence; 5, very intense. Standard deviation was lower than 1.5.

Odour intensity of the essential oils led to the rejection of fillets at an earlier stage. During storage, off-odours typical of spoiled fish were not detected in these treatments. Apart from odour evaluation, fillets firmness did not change in a greater extend in treatment O, contrarily to the remaining lots. In previous studies, the odours intensity of essential oils was an important factor in the acceptability and preference of panellists, being preferred the lowest concentrations [80; 131]. Lower concentrations (0.1-0.2 % v/w) of thyme essential oils (species not specified) were reported to increase the sensory shelf life of sea bass fillets in 3-4 days [17] and swordfish in 2-5 days [77] using different package conditions (aerobic and modified atmosphere).

## 4. Conclusions

Spanish oregano and lemon essential oils (*T. capitatus* and *C. limon*) increased the shelf life of vacuum packed sea bass fillets stored under refrigerated

conditions for four additional days, taking into account microbiological criteria. The synthetic preservative (potassium sorbate) did not increase the microbiological shelf life of fillets. The essential oils reduced lipid oxidation, while in the treatment with the synthetic preservative lipid oxidation increased. In contrast, in sensory analysis, the synthetic preservative increased fillets shelf life, while odour intensity was determinant for the acceptability of samples treated with essential oils by panellists. Essential oils showed great potential for preservation of fish products with increased microbiological shelf life. To overcome the odour intensity problem, essential oils might be used in lower concentrations combined with other preservation methods, like its incorporation in packaging films.

# CHAPTER 3. Development of methods to preserve sea bass fillets: films with natural substances

# CHAPTER 3.1.

# Characterization of fish protein films incorporated with essential oils of clove, garlic and Spanish oregano: physical, antioxidant and antibacterial properties

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**Abstract**

The increasing demand for high quality foods using environmental-friendly packaging systems and natural preservatives leads research to develop solutions like edible and biodegradable films and coatings with bioactive properties. In this context, films prepared with Cape hake by-products proteins and three essential oils (garlic, clove, and Spanish oregano) were characterized in terms of physical, mechanical, antioxidant, and antibacterial properties. Control films, without essential oils, were homogeneous, transparent, slight yellow, and mechanically resistant. The incorporation of garlic, clove, and Spanish oregano essential oils in films significantly decreased thickness, water solubility, breaking force and elongation. In contrast, the addition of essential oils increased the free radical scavenging activity of films. Modifications in other properties of films were specific for each essential oil. Clove films showed lower water vapour permeability than control films, and the highest antibacterial activity (against *Shewanella putrefaciens*). Garlic films were the most yellowish and showed the highest antioxidant activity. Spanish oregano films were similar to the control ones, particularly in colour, transparency, and reducing power. In conclusion, proteins recovered from Cape hake by-products can be used for the preparation of biodegradable films with potential to be used in new food packaging systems. Still, the potential application of each film depends on the product, as different products might have different problems to be improved.

**Keywords**

Hake muscle proteins; biodegradable films; active packaging; antibacterial properties; antioxidant properties; physical properties

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## 1. Introduction

In recent years, edible and biodegradable films and coatings prepared with proteins, polysaccharides, and lipids have received increasing attention. Some examples of their commercial applications are: collagen casings for sausages, confectioner's glaze made from shellac, corn zein and gelatin-based coatings for pharmaceuticals, and waxes on various fruits [86]. Factors contributing to the interest in films and coatings development include: consumers demand for high quality foods and natural preservatives; food processors' needs for new storage techniques; environmental concerns over disposal of non-renewable food packaging materials; and opportunities for creating new market outlets for under-utilized film-forming ingredients [86].

Edible and biodegradable films must meet a number of specific functional requirements, including colour, appearance, barrier properties, mechanical, and rheological characteristics, which are dependent on the type of material used and type of application [88]. Films primarily composed of proteins usually have suitable mechanical and optical properties, but show poor water vapour barrier properties because of their hydrophilic nature [88]. Active compounds like essential oils can be added to films to improve their functional properties, such as water vapour permeability, as well as antimicrobial and antioxidant properties [107-108; 111].

In the seafood processing industry, a substantial amount of by-products are generated that can be used to recover proteins to prepare films and restructured seafood products. Protein films have been successfully prepared using fish proteins, including myofibrillar and sarcoplasmic proteins [100; 224]. Essential oils of aromatic plants like clove, garlic, and Spanish oregano show strong antimicrobial and antioxidant properties [190; 198; 225]. Therefore, incorporation of essential oils in films can improve functionalities of films. In this context, the aim of the current work was to study the physical, mechanical, antioxidant, and antibacterial properties of films prepared with fish proteins recovered from Cape hake by-products and essential oils from aromatic plants (clove, garlic and Spanish oregano).

## 2. Material and methods

**2.1. *Chemicals*** Essential oils of clove from *Eugenia* spp. (C8392; lot 116K1861; buds distillation; origin: Indonesia), garlic (biological source not specified;

W250317; lot 04712EE-148; bulbs synthetic organic material; origin: Mexico), and Spanish oregano from *Thymus capitatus* (W282812; lot 21417CL-214; dried flowering herb steam distillation; origin: Spain), glycerol, α,α-diphenyl-β-picrylhydrazyl (DPPH), sodium bromide, trizma hydrochloride (Tris-HCl), sodium azide, and potassium hexacyanoferrate III were obtained from Sigma-Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany); phosphate buffer, ferric chloride, trichloroacetic acid, and ascorbic acid were purchased from Fluka (Buchs, Germany); tryptic soy agar and plate count agar from Merck (Darmstadt, Germany); brain heart infusion broth and maximum recovery diluent from Oxoid (Basingstoke, Hampshire, England); ethylene diamine tetracetic acid (EDTA calibration sample) from LECO (LECO corporation, St. Joseph, USA); ethanol had a purity grade of 99 % and the water used was Milli-Q purified and distilled.

**2.2. *Film preparation***

Fish proteins were recovered from frozen by-products resulting from the portioning (fish 'sawdust' and cut offs) of Cape hake (*Merluccius capensis*) by alkaline solubilisation following a methodology previously described [102]. Recovered proteins (90 % w/w protein content) were freeze-dried, packed under vacuum conditions, and stored at -30 ºC until utilisation.

Hake protein powder (30 g) was added to water (2 L) and homogenized (5,000 rpm, 1 min) using a Polytron homogenizer. The pH was adjusted to 11 with 1 M sodium hydroxide and mechanically stirred, followed by centrifugation (10,000 ×*g*, 15 min, 5 ºC) to remove insoluble material. The protein concentration of the soluble fraction was determined (see methodology below), glycerol was added at 59 % (w/w) of protein, and the mixture was gently stirred (30 min). Afterwards, the essential oils of clove, garlic, and Spanish oregano were added to protein film forming solutions, emulsified in a Polytron homogenizer (13,500 rpm, 2 min) and the emulsion was degassed under vacuum (20 min) and casted on plates to obtain films with 4 mg of protein and 1 μL of essential oil per $cm^2$ when present. Control films had the same amount of protein per surface area. The plates were placed on levelled surfaces to obtain films with homogeneous thickness, dried in a ventilated drying chamber (30 ºC, 50 % relative humidity, 20 h), peeled off, and stored at room temperature at 57 % relative humidity in desiccators with saturated solutions of sodium bromide. Four types of films were prepared (treatments):

a) without essential oils; b) with clove essential oil; c) with garlic essential oil; and d) with Spanish oregano essential oil.

The protein content of the soluble fraction was determined using a FP-528 LECO nitrogen analyser (LECO, St. Joseph, USA), calibrated with EDTA (carbon – 41.07 ± 0.17, hydrogen – 5.55 ± 0.02, nitrogen – 9.57 ± 0.03), according to the Dumas method [226]. All determinations were performed in triplicate.

**2.3. *Colour*** Films colour parameters ($L^*$, $a^*$ and $b^*$) were measured (n = 6 for each film) using a colorimeter (CR-410, Konica Minolta Camera, Co, Ozaka, Japan) with a measure cell opening of 50 mm. For measurements, films were placed on a white standard plate. Chroma ($C^*$), hue ($h^*$) and whiteness (W) were estimated using the following equations, accordingly to Atarés and co-authors [227]:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$h^* = arctg\frac{b^*}{a^*}$$

$$W = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2}$$

Colour was expressed as the difference of colour ($\Delta E^*$) in the different parameters, accordingly to the equation [96]:

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

where $\Delta L^*$, $\Delta a^*$ and $\Delta b^*$ correspond to the variation between the colour parameter of film and that of the white standard plate used as background.

**2.4. *Transparency*** The absorbance of films (600 nm; spectrophotometer UNICAM Uv/Vis UV2; ATI-UNICAM, Cambridge, United Kingdom) and film thickness (mm; in the same location of absorbance readings) were measured (n = 5 for each film) in order to address film transparency using the following equation:

$$\text{Transparency} = \frac{A_{600}}{x}$$

where $A_{600}$ is the absorbance of films and $x$ is the film thickness (mm) [228]. Films less transparent show higher transparency values.

**2.5. *Opacity*** Opacity was measured accordingly to the Hunterlab method [229] using the same equipment of colour measurement. The opacity (%) of films was calculated

with reflectance measurements of each film (n = 6) with standard black and white backing plates, accordingly to the following equation:

$$\text{Opacity} = \frac{Y_{black\ backing}}{Y_{white\ backing}} \times 100$$

where $Y$ is the CIE tristimulus value of the film with the black ($Y_{black\ backing}$) or white ($Y_{white\ backing}$) backing plates.

**2.6. *Thickness***

Film thickness was measured in nine different locations of each film, using a Digimatic tube micrometer model BMD-25D (MITUTOYO MFG Co. Ltd., Kawasaki, Japan).

**2.7. *Water vapour permeability***

Water vapour permeability (WVP) was measured in duplicate for each film using the method described by Gontard and co-authors [230]. The films were sealed in cells containing silica gel (0 % relative humidity) and then stored at 30 ºC in desiccators with water. The cells were weighed at regular intervals for a period of 30 h, and the WVP estimated as follows:

$$\text{WVP} = \frac{w \times x}{A \times t \times (P_2 - P_1)}$$

where $w$ is the weight gain (g), $x$ is the film thickness (m), $A$ is the exposed area of film ($m^2$), $t$ is the time of weight gain (s), and $P_2$-$P_1$ is the vapour pressure differential across the film (Pa).

**2.8. *Film and protein solubility***

Film solubility in water (n = 5) was determined according to the method of Hoque and co-authors [231]. Films (3 × 2 $cm^2$) were weighted and immersed in water (10 mL) with sodium azide (0.1 % w/v). The mixture was continuously shaken (24 h; room temperature) and centrifuged (3,000 ×$g$; 10 min; 25 ºC). Unsolubilised films were dried (24 h; 105 ºC) and film solubility was determined by subtracting the weight of unsolubilised dry matter from the initial weight of dry matter and expressed as a percentage of the total weight.

The protein concentration in the water (n = 5) was determined using the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad, Hercules, California, USA). Protein solubility was expressed as percentage of total solubilised film protein at 20 ºC for 24 h.

**2.9. *Mechanical properties***

Prior to measuring the mechanical properties, films were conditioned in desiccators with saturated solutions of sodium bromide (57 % relative humidity)

at room temperature during 72 h. The puncture and tensile tests were performed using an Instron texture analyser (Instron 4301, Bucks, United Kingdom) and data obtained with the Instron Corporation software (version 5.02, 1985-1990).

The force and deformation at the films breaking point were determined using a puncture test. Each film (10 × 10 $cm^2$) was fixed between two cells with a circular hole (16 mm Ø) in the centre of the cells, where a 3 mm diameter plunger moving at 6 $cm.min^{-1}$ perforated the films. The puncture deformation (PF) was calculated considering that the stress was homogeneously distributed along the film at the breaking point [232], with the following equation:

$$PF = \frac{\Delta l}{l_0} = \frac{\sqrt{D^2 + l_0^2} - l_0}{l_0}$$

where $D$ is the stretch distance of films at the breaking point, $l$ is the final length of the film and $l_0$ is the initial length of the film (equal to the radius of the cell hole). Five films were used per treatment, with nine measurements recorded per film.

Tensile strength (*i.e.* force at breaking point per initial cross-sectional area) and elongation percentage were measured using film strips (10 × 2 $cm^2$) fixed in the extremities of a self-aligning grip. The initial grip separation was 5 cm and crosshead speed was 6 $cm.min^{-1}$. Tensile tests were carried out in 16 film strips per treatment.

**2.10. *Antioxidant activity*** The antioxidant activity of films was evaluated using film powders. Films were ground with a pestle in a mortar, using liquid nitrogen.

**2.10.1. *Free radical scavenging*** The scavenging effect of DPPH free radical was determined in triplicate according to the method of Weng and co-authors [233]. Film powders (20 mg) in 2 mL Tris-HCl buffer (0.1 M, pH 7.4) were added to 2 mL of 0.2 mM DPPH in ethanol (95 % v/v). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The mixture was centrifuged (4,500 ×*g*, 10 min) and the absorbance of supernatant was recorded at 517 nm in a spectrophotometer (UNICAM UV/Vis UV2; ATI-UNICAM, Cambridge, United Kingdom). A negative control was prepared using all reagents, except the film powder. DPPH radical-scavenging activity was calculated with the following formula:

$$\text{Percentage of inhibition } (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{sample}$ and $Abs_{control}$ correspond to the absorbance of sample and control, respectively.

**2.10.2. *Reducing power***

The reducing power of films was determined in triplicate according to the modified method of Oyaizu [144] as described by Weng and co-authors [233]. Film powders (20 mg) with 2 mL water, 2 mL phosphate buffer (0.2 M, pH 6.6) and 2 mL potassium hexacyanoferrate III (1 % w/v) were incubated at 50 ºC for 20 min. Afterwards, 2 mL trichloroacetic acid (10 % w/v) were added and the mixture was centrifuged (1,500 ×*g,* 10 min, room temperature). A 2 mL aliquot of the supernatant was mixed with 2 mL water and 0.4 mL ferric chloride (0.1 % w/v). The absorbance was recorded after 10 min at 700 nm in a spectrophotometer (UNICAM UV/VIS UV2; ATI-UNICAM, Cambridge, United Kingdom). A negative control was prepared using all reagents except the film powder, whereas a positive control was also prepared (10-70 $\mu g.mL^{-1}$ range) in order to plot the absorbance of ascorbic acid against concentration. The results were expressed as mg of ascorbic acid per g of film.

**2.11. *Antibacterial activity***

The antibacterial activity tests included foodborne spoilage and pathogenic bacteria acquired from the American Type Culture Collection (ATCC) or the Spanish Type Culture Collection (CECT): *Brochothrix thermosphacta* (CECT 847), *Escherichia coli* (ATCC 25922), *Listeria innocua* (CECT 910), *Listeria monocytogenes* (CECT 5873), *Pseudomonas putida* (CECT 7005), *Salmonella typhimurium* (ATCC 14028), and *Shewanella putrefaciens* (CECT 5346). These strains, kept at -70 ºC in a cryopreservative solution (Microbank, Pro-lab Diagnostics, Richmond Hill, ON, Canada), were inoculated in tryptic soy agar and incubated overnight at 30 ºC, except *L. monocytogenes* that was inoculated in plate count agar. Subsequently, one colony from each culture was inoculated in brain hearth infusion broth and incubated at 30 °C for 18-24 h with shaking (75 rpm), in order to obtain freshly cultured microbial suspensions ($10^8$-$10^9$ $CFU.mL^{-1}$) for tests.

Antibacterial activity of the films was evaluated by the agar diffusion method. Freshly bacterial suspensions were adjusted to $1 \times 10^7$ $CFU.mL^{-1}$ in brain heart infusion broth and spread on the surface of tryptic soy agar or plate count agar using a sterile cotton swab. Subsequently, edible film discs (1.5 cm Ø) were placed on the agar surface. After staying at 4 ºC for 2 h, Petri dishes were incubated at 30 ºC for 24 h, except *L. monocytogenes* that was incubated during

48 h. Antibacterial activity was evaluated by measuring the total diameter of the inhibition zone, including the film disc, to the nearest millimetre.

The antibacterial properties of films were also evaluated with the macrodilution method [234]. Films ($5 \times 5$ cm$^2$) were immersed in 10 mL of the bacterial suspensions ($1 \times 10^7$ CFU.mL$^{-1}$) in brain heart infusion broth. After staying 4 h at 30 ºC with shaking (75 rpm) ten-fold dilutions were performed in maximum recovery diluent and spread in tryptic soy agar or plate count agar for the determination of the bacterial concentration.

In both methods, bacterial suspensions without films were used as positive controls, whereas films without bacterial strains were used as negative controls. All determinations were performed in triplicate.

**2.12. *Statistical analysis***

Differences between films were tested with analysis of variance, followed by multiple comparisons tests (Tukey HSD). If data could not meet analysis of variance assumptions, non-parametric analysis of variance (Kruskall-Wallis) was performed, followed by non-parametric multiple comparisons test (Dunn). All statistical analyses were tested at 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

**3.1. *Physical properties***

Colour parameters of films are shown in Table 3.1.1. Fish protein films showed high $L^*$ values and were slightly yellowish. Similar results were previously reported for fish muscle proteins-based films [93; 235-236]. The addition of essential oils affected some colour parameters depending on the essential oil used, *i.e.* darker, yellowish, higher chroma, lower hue and less whiteness films (garlic and clove), slightly less green (Spanish oregano) and slightly more green (garlic). In previous studies, the addition of essential oils also affected film colour parameters, being influenced by the amount and type of essential oil used [227; 237].

Fish protein films without essential oil and with Spanish oregano were more transparent than clove and garlic films (Table 3.1.2). Similar transparency values of fish protein films were reported in previous studies [100], whereas lower transparency has also been referred [93], which could be attributed to different protein content in films. The change in transparency of films with different essential oils might be related with the type and amount of essential oil, as well as film thickness.

Opacity did not reveal statistical differences among films, with values ranging between 15.9 and 16.4 (Table 3.1.2). Previous studies reported lower opacity values in fish protein films subjected to different thermal treatments [96].

**Table 3.1.1.** Colour parameters of fish protein films (n = 6).

| | Fish proteins films | | | |
|---|---|---|---|---|
| | **No essential oil** | **Garlic** | **Clove** | **Spanish oregano** |
| **Lightness** (*L**) | $93.6 \pm 0.6^{a}$ | $91.9 \pm 0.3^{b}$ | $91.9 \pm 1.0^{b}$ | $93.4 \pm 0.3^{a}$ |
| **Red-green value** (*a**) | $-1.7 \pm 0.1^{b}$ | $-1.9 \pm 0.1^{c}$ | $-1.7 \pm 0.1^{b}$ | $-1.4 \pm 0.0^{a}$ |
| **Yellow-blue value** (*b**) | $5.9 \pm 0.6^{c}$ | $12.0 \pm 1.0^{a}$ | $8.9 \pm 2.6^{b}$ | $5.8 \pm 0.2^{c}$ |
| **Chroma** (*C**) | $6.1 \pm 0.6^{c}$ | $12.2 \pm 1.0^{a}$ | $9.1 \pm 2.6^{b}$ | $5.9 \pm 0.2^{c}$ |
| **Hue** (*h**) | $106.6 \pm 0.9^{a}$ | $99.2 \pm 0.6^{b}$ | $101.2 \pm 2.4^{b}$ | $104.0 \pm 0.5^{a}$ |
| **Whiteness** (W) | $91.1 \pm 0.8^{a}$ | $85.4 \pm 0.9^{b}$ | $87.8 \pm 2.6^{b}$ | $91.1 \pm 0.4^{a}$ |
| **ΔE*** | $4.7 \pm 0.6^{b}$ | $11.0 \pm 1.0^{a}$ | $8.0 \pm 2.8^{a}$ | $4.6 \pm 0.2^{b}$ |

*Abbreviations*: ΔE* – difference of colour between colour parameters of film and that of the white standard plate used as background.
In each line, different letters denote significant differences ($p < 0.05$) within films.
Values are presented as average ± standard deviation.

**Table 3.1.2.** Physical and mechanical properties of fish protein films tested.

| | | Fish protein films | | | |
|---|---|---|---|---|---|
| | **n** | **No essential oil** | **Garlic** | **Clove** | **Spanish oregano** |
| **Transparency** | 5 | $1.8 \pm 0.1^{c}$ | $22.9 \pm 2.3^{a}$ | $7.0 \pm 3.3^{b}$ | $2.3 \pm 0.2^{c}$ |
| **Opacity** | 6 | $15.9 \pm 0.9^{a}$ | $16.2 \pm 0.6^{a}$ | $16.4 \pm 0.9^{a}$ | $16.2 \pm 1.0^{a}$ |
| **Thickness** (μm) | 9 | $28.3 \pm 4.0^{a}$ | $12.8 \pm 7.0^{c}$ | $12.3 \pm 4.7^{c}$ | $17.2 \pm 6.0^{b}$ |
| **Water vapour permeability** ($\times 10^{-11}$ g.m$^{-1}$.s$^{-1}$.Pa$^{-1}$) | 2 | $5.9 \pm 0.1^{ab}$ | $4.3 \pm 1.0^{bc}$ | $3.8 \pm 0.9^{c}$ | $7.8 \pm 0.8^{a}$ |
| **Puncture tests** | 9 | | | | |
| Force (N) | | $6.1 \pm 2.0^{a}$ | $3.7 \pm 0.4^{b}$ | $3.5 \pm 1.1^{b}$ | $6.3 \pm 1.1^{a}$ |
| Deformation (mm) | | $7.0 \pm 1.1^{b}$ | $8.9 \pm 1.3^{b}$ | $15.2 \pm 2.0^{a}$ | $13.2 \pm 1.3^{a}$ |
| Puncture deformation (%) | | $33.5 \pm 9.7^{b}$ | $50.4 \pm 12.1^{b}$ | $115.0 \pm 22.4^{a}$ | $93.0 \pm 14.2^{a}$ |
| **Tensile tests** | 16 | | | | |
| Force (N) | | $3.5 \pm 0.9^{a}$ | $2.1 \pm 0.9^{b}$ | $1.8 \pm 0.6^{b}$ | $2.4 \pm 1.5^{b}$ |
| Elongation (%) | | $147.9 \pm 31.8^{a}$ | $53.3 \pm 21.1^{b}$ | $55.7 \pm 31.7^{b}$ | $83.2 \pm 50.3^{b}$ |
| Tensile strength (MPa) | | $6.1 \pm 1.7^{a}$ | $6.6 \pm 2.7^{a}$ | $7.3 \pm 2.3^{a}$ | $6.4 \pm 4.0^{a}$ |

In each line, different letters denote significant differences ($p < 0.05$) between films.
Values are presented as average ± standard deviation.

Fish protein films had an average thickness of 28 μm (Table 3.1.2), which was similar to values registered in previous studies with different proportions of protein and polyvinyl alcohol per surface area [236]. Still, higher thickness values were obtained in previous studies [235], and these differences might be related with different protein content in films [238]. The incorporation of essential oils in films significantly decreased thickness to 12-17 μm, being garlic and clove films those with lower thickness values. Pires and co-authors [239] also found a decrease in films thickness in the presence of essential oil at the concentrations used in the current study. Previous studies reported that essential oils of oregano, thyme, clove, and cinnamon increased thickness of alginate and chitosan-based films [240-241], while oregano, lemongrass, and cinnamon essential

oils did not affect thickness of alginate-apple puree films [242]. The differences among studies might be related with the interaction between essential oils, casting surface and film forming solutions.

The results of water vapour permeability of films varied between 3.8 and $7.8 \times 10^{-11}$ $g.m^{-1}.s^{-1}.Pa^{-1}$ (Table 3.1.2). Water vapour permeability of control films was similar to values obtained in previous studies with fish muscle protein-based films [236], whereas, few studies revealed one order of magnitude lower values [97]. Protein concentration may be responsible for such differences [238]. The incorporation of clove essential oil significantly reduced the permeability of films to water vapour, while no statistical differences were detected with films containing garlic or Spanish oregano essential oils. In previous studies, the addition of edible oils (*e.g* peanut and corn oils) to fish protein films also decreased water vapour permeability [243], while the incorporation of garlic essential oil in alginate-based films increased the water vapour permeability [244]. The distinct composition of essential oils could be responsible for the differences observed, as the water vapour transfer process depends on the hydrophilic-hydrophobic ratio of film components. Still, it cannot be assumed that the water vapour permeability of edible films is reduced simply by adding a hydrophobic component, but the impact of lipid addition on the microstructure of the emulsified film is also a determining factor [237].

Films and protein solubility results are shown in Figure 3.1.1. Fish protein films immersed in water become hydrated and thicker, without visually loosing their integrity. Control films showed 34 % soluble fraction, which was lower than several other values reported earlier [98; 235]. The protein solubility of control films was within the values obtained in other studies [100]. The incorporation of essential oils in films reduced films solubility, principally for Spanish oregano and clove films (10 %), which was similar to the findings of Hosseini and co-authors [241] in chitosan-based films with clove and cinnamon, but not with thyme essential oil. In contrast, protein solubility was significantly higher in films incorporated with clove, whereas statistically lower values were obtained with films with Spanish oregano.

### 3.2. *Mechanical properties*

The results from tensile and puncture tests of fish proteins films are summarized in Table 3.1.2.

In tensile tests, the force needed to break fish protein films without essential oils, as well as its elongation percentage, were statistically higher than in films

with essential oils. The elongation at the breaking point of films without essential oils was higher than those reported in previous studies (*ca.* 17-125 %) [92-93; 238]. In contrast, its tensile strength was similar to values obtained by Prodpran & Benjakul [238] and by Sabato and co-authors [245], whereas higher values were obtained by Artharn and co-authors [92]. The differences found in the force and elongation of films tested in the current study might be related with their thickness, as the results of tensile strength (force at breaking point per initial cross-sectional area) were not statistically different. Similarly, Atarés and co-authors [227] concluded that the presence, type, and content of essential oils does not affect tensile strength of sodium caseinate-based films.

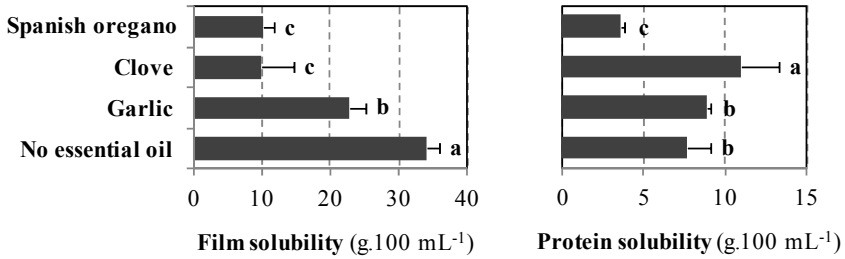


**Figure 3.1.1.** Films solubility and protein solubility of fish protein films tested. In each graphic, different letters denote significant differences ($p < 0.05$) between films. Bars represent average values, whereas error bars indicate the standard deviation (n = 5).

The force needed to puncture films without essential oil was statistically higher than in films incorporated with garlic and clove, whereas no differences were detected with Spanish oregano films. Additionally, films with clove and Spanish oregano significantly increased films puncture deformation compared to films without essential oil and garlic films. In the case of tilapia films, the puncture force was lower (lower than 4 N) for the same levels of plasticizer [96], and the differences could be attributed to the thermal treatments performed in tilapia films forming solutions. In a previous study, puncture force decreased with the incorporation of essential oil with the same concentration used in the current study [239].

### 3.3. *Antioxidant activity*

The results of DPPH radical-scavenging activity and reducing power of fish protein films are shown in Figures 3.1.2 and 3.1.3, respectively. Fish protein films showed some antioxidant activity that can be due to the presence of free sulfhydryl groups and other aminoacids such as tryptophan, methionine, and tyrosine on hake fish proteins as mentioned by Taguchi and co-authors [246] and

Faraji and co-authors [247]. The incorporation of essential oils significantly increased the DPPH radical-scavenging activity of fish protein films. The highest antioxidant activity was obtained with clove and garlic films (72 % of inhibition). The differences found in the antioxidant activity of films with different essential oils, are due to different compounds with antioxidant activity in essential oils composition. The DPPH radical-scavenging activity of films with Spanish oregano and clove was lower than that of the free essential oil, whereas garlic films revealed higher activity than the essential oil itself. The decrease in the activity could be due to interactions between the components of films and essential oils that could no longer be available to interact in the antioxidant activity reactions, and to the loss of volatile compounds of essential oils during the films drying process. The increase in the antioxidant activity observed for the garlic films might be due to the presence of di-2-propenyldisulfide and di-2-propenyltetrasulfide in this essential oil, both with disulfide bonds that might be cleaved in the film forming solutions and are able to act as scavenging hydroxyl radicals increasing the antioxidant activity in the DPPH method, and not in the ferric reducing power method.

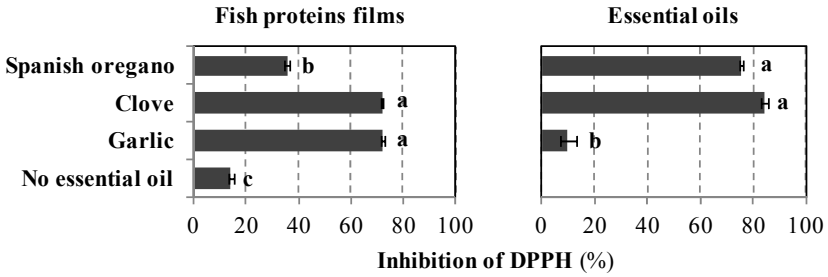


**Figure 3.1.2.** Antioxidant activity of essential oils and fish protein films tested, measured with the free radical scavenging method. In each graphic, different letters denote significant differences ($p < 0.05$) within films or essential oils. Bars represent average values, whereas error bars indicate the standard deviation (n = 3). *Abbreviations*: DPPH – α,α-diphenyl-β-picrylhydrazyl.

The reducing power of films without essential oil was lower than that of surimi films of Alaska pollack [233]. The incorporation of clove and garlic essential oils significantly increased films antioxidant activity, but not for Spanish oregano. Once again, the reducing power of films with Spanish oregano and clove was lower than that of the free essential oil, whereas garlic films revealed higher activity than the essential oil itself.

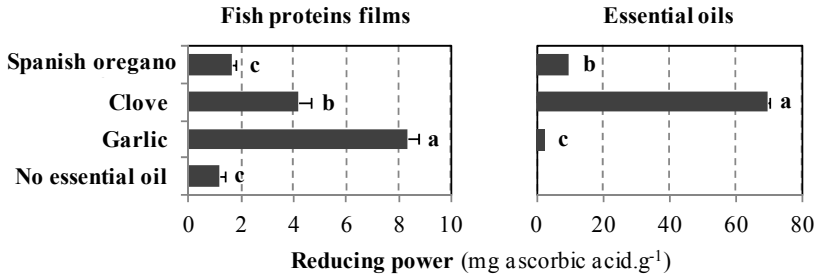


**Figure 3.1.3.** Antioxidant activity of essential oils and fish protein films tested, measured with the reducing power method. In each graphic, different letters denote significant differences ($p < 0.05$) within films or essential oils. Bars represent average values, whereas error bars indicate the standard deviation (n = 3).

### 3.4. *Antibacterial properties*

The antibacterial activity of films is shown in Table 3.1.3. The agar diffusion method revealed only inhibition with both *Listeria* strains. Inhibition of *L. monocytogenes* only occurred with garlic and Spanish oregano films, whereas *L. innocua* was only inhibited by films without essential oil and films with Spanish oregano.

**Table 3.1.3.** Antibacterial activity of fish protein films tested.

| | Fish protein films | | | |
|---|---|---|---|---|
| | **No essential oil** | **Garlic** | **Clove** | **Spanish oregano** |
| **Agar diffusion method** (cm) | | | | |
| *Brochothrix thermosphacta* | N.D. | N.D. | N.D. | N.D. |
| *Escherichia coli* | N.D. | N.D. | N.D. | N.D. |
| *Listeria innocua* | 1.6 ± 0.0 | N.D. | N.D. | 1.6 ± 0.0 |
| *Listeria monocytogenes* | N.D. | 3.0 ± 1.7 | N.D. | 1.7 ± 0.2 |
| *Pseudomonas putida* | N.D. | N.D. | N.D. | N.D. |
| *Salmonella typhimurium* | N.D. | N.D. | N.D. | N.D. |
| *Shewanella putrefaciens* | N.D. | N.D. | N.D. | N.D. |
| **Macrodilution method** (%) | | | | |
| *Brochothrix thermosphacta* | N.D. | 60.2 ± 5.3 | N.D. | 41.5 ± 32.3 |
| *Escherichia coli* | N.D. | N.D. | N.D. | N.D. |
| *Listeria innocua* | N.D. | 86.9 ± 1.8 | 20.5 ± 13.1 | 66.5 ± 8.5 |
| *Listeria monocytogenes* | N.D. | 68.7 ± 22.0 | 41.4 ± 27.6 | N.D. |
| *Pseudomonas putida* | 87.2 ± 6.7 | N.D. | N.D. | N.D. |
| *Salmonella typhimurium* | N.D. | N.D. | N.D. | N.D. |
| *Shewanella putrefaciens* | 54.0 ± 31.7 | 77.2 ± 30.1 | 92.2 ± 3.0 | N.D. |

*Abbreviations*: N.D. – inhibition not detected.
Results obtained with the agar diffusion method represent the inhibition diameter, including the disc film (1.5 cm Ø), whereas the macrodilution method indicates the percentage reduction of bacterial viable cells obtained with 5 × 5 $cm^2$ films in 10 mL of bacterial suspensions ($1 \times 10^7$ $CFU.mL^{-1}$).
Values are presented as average ± standard deviation (n = 3).

In contrast, the macrodilution method used to quantify the bacterial reduction caused by films (5 × 5 $cm^2$) revealed inhibitions with all films tested. None of the films was able to inhibit *E. coli* and *S. typhimurium*. In contrast, *B. thermosphacta* and *L. monocytogenes* were inhibited by garlic and Spanish oregano films, *L. innocua* inhibition occurred with all films with essential oils, *P. putida* inhibition only occurred with films without essential oil, and *S. putrefaciens* was inhibited by films without essential oil and by garlic and

clove films. The addition of essential oils to films did not reduce the growth of Gram-negative bacteria like *E. coli*, *P. putida*, and *S. typhimurium* when comparing with films without essential oil. The resistance of these bacterial strains could be due to the complexity of their double layer cell membrane in comparison with the simpler cell membrane of Gram-positive bacteria [164].

Several studies tested the antibacterial activity of edible films incorporated with different natural substances [248], but fewer focused on films incorporated with essential oils. Garlic films showed similar results to those obtained by Pranoto and co-authors [244], but different than those reported in the study of Seydim & Sarikus [108], where *L. monocytogenes* was not inhibited by garlic films. Inhibition of *L. monocytogenes* by films with clove and *Thymus* essential oils was also obtained by Hosseini and co-authors [241]. The differences among studies could be due to the diversity of active agents in the essential oils, interactions between essential oil compounds and films constituents (alginate, whey protein, and chitosan), and also due to differences in the amount of essential oil per area of film (not always specified).

## 4. Conclusions

Hake proteins recovered from by-products of seafood processing industries can be used for the preparation of biodegradable films. The incorporation of essential oils in these films reduced film thickness as well as the solubility in water, affected film mechanical properties, and improved antioxidant activity. Clove films showed the lowest water vapour permeability and the highest antibacterial activity (against *S. putrefaciens*); garlic films presented the highest antioxidant activity; and Spanish oregano films were more similar to control films. The results of this study indicate that hake protein films, prepared with by-products, incorporated with clove, garlic, and Spanish oregano essential oils, show interesting properties (water vapour barrier and antibacterial and antioxidant activities) to be good candidates to be used in biodegradable food packaging systems.

# CHAPTER 3.2.

# Effect of fish protein films incorporated with essential oils in microbiological shelf life of sea bass (*Dicentrarchus labrax*) fillets during refrigerated storage

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**Abstract**

This study aimed to evaluate the effectiveness of fish protein films incorporated with essential oils in microbiological shelf life of sea bass fillets during refrigerated storage. Fresh sea bass fillets were microbiologically contaminated ($10^2$ CFU.$g^{-1}$) and fish protein films were used to preserve them. The essential oils of citronella, garlic, and thyme were incorporated in films and then applied in contaminated fillets to study their effects in bacterial load.

Films without essential oils increased the microbiological shelf life of sea bass fillets from 4 to 6 days, when compared with fillets without films. The growth of both *Pseudomonas* spp. and Enterobacteriaceae was slower in treatments with these films. However, the addition of essential oils to films did not increase the microbiological safety, neither the shelf life of fillets. Other formulations with different concentrations of essential oils might be more advantageous. In conclusion, the films prepared with the essential oils of citronella, garlic, and thyme did not show potential to be used for preservation of sea bass fillets, taking into account microbiological criteria.

**Keywords**

Protein films; challenge tests; microbiology; shelf life; sea bass; essential oils; citronella; garlic; thyme

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## 1. Introduction

The use of essential oils is becoming popular to increase the shelf life of food products, since consumers are more conscious about the health problems caused by several preservatives [83; 188]. Studies with essential oils as food additives revealed to be advantageous, as shelf life was increased [166]. However, the application of essential oils in foods might be limited because strong odours of essential oils might be imparted to food products, and this is determinant for acceptance by consumers.

In recent years, edible and biodegradable films and coatings prepared with proteins, polysaccharides, and lipids have received attention by researchers. Films primarily composed of proteins usually have suitable mechanical and optical properties, but showed poor water vapour barrier properties because of their hydrophilic nature [88]. Active compounds like essential oils can be added to films to improve their functional properties, such as water vapour permeability, as well as antimicrobial and antioxidant properties [107-108; 111].

A few studies tested films incorporated with essential oils to preserve seafood, including cod [83] and Asian sea bass [75]. Results of these studies showed a slower growth of fish spoilage bacteria when essential oils were incorporated in films.

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species widely distributed in the Mediterranean Sea and Atlantic Ocean and intensively farmed in several Mediterranean countries, *e.g.* Greece, Spain, and Italy [6]. This species is highly appreciated because of its excellent organoleptic properties and reasonable price [6]. In the retail stores, sea bass is most commonly sold as the whole fish, and ice storage has been used to prolong its shelf life. Although slowly, it seems that the consumers are also interested to purchase fillets.

In this context, the aim of the current work was to study the effectiveness of films prepared with fish proteins recovered from Cape hake by-products and essential oils from aromatic plants in microbiological shelf life of sea bass (*Dicentrarchus labrax*) fillets during refrigerated storage. The essential oils of citronella, garlic, and thyme were chosen to be incorporated in films due to their antibacterial properties [205].

## 2. Material and methods

### 2.1. *Chemicals*

Essential oils of citronella from *Cymbopogon nardus* (W230804; lot 16202BS-208; leaves and stems steam distillation; origin: China), garlic (biological source not specified; W250317; lot 04712EE-148; bulbs synthetic organic material; origin: Mexico), and thyme from *Thymus vulgaris* (W306509; lot 05223CE-497; flowering plant distillation; origin: Spain), and sodium bromide, sodium hydroxide, and glycerol were acquired from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany); ethylene diamine tetracetic acid (EDTA calibration sample) from LECO (LECO corporation, St. Joseph, USA); plate count agar from Merck (Darmstadt, Germany), maximum recovery diluent, violet red bile glucose agar, Pseudomonas agar base, and supplement SR 103 from Oxoid (Basingstoke, Hampshire, England); Lyngby iron agar from Scharlau Chemie (Sentmenat, Spain); and the water used was distilled and Milli-Q purified.

### 2.2. *Films preparation*

Fish proteins were recovered from frozen by-products resulting from the portioning (fish 'sawdust' and cut offs) of Cape hake (*Merluccius capensis*) by alkaline solubilisation following a methodology previously described [102]. Recovered proteins (90 % w/w protein content) were freeze-dried, packed under vacuum conditions, and stored at -30 ºC until utilisation.

Hake protein powder (30 g) was added to water (2 L) and homogenized (5,000 rpm, 1 min) using a Polytron homogenizer (Polytron PT-MR 3000, Kinematica, Littau, Switzerland). The pH was adjusted to 11 with 1 M sodium hydroxide and mechanically stirred, followed by centrifugation (10,000 ×*g*, 15 min, 5 ºC) to remove insoluble material. The protein concentration of the soluble fraction was determined, glycerol was added at 59 % (w/w) of protein, and the mixture was gently stirred (30 min). Afterwards, the essential oils of citronella, garlic, and thyme were added to protein film forming solutions, emulsified in a Polytron homogenizer (13,500 rpm, 2 min) and the emulsion was degassed under vacuum (20 min) and casted on plates to obtain films with 4 mg of protein and 1 µL of essential oil, when present, per $cm^2$. Control films had the same amount of protein per surface area. The plates were placed on levelled surfaces to obtain films with homogeneous thickness, dried in a ventilated drying chamber (30 ºC, 50 % relative humidity, 20 h), peeled off, and stored at room temperature at 57 % relative humidity in desiccators with

saturated solutions of sodium bromide. Four types of films were prepared: without essential oils; with citronella essential oil; with garlic essential oil; and with thyme essential oil.

The protein content of the soluble fraction was determined using a FP-528 LECO nitrogen analyser (LECO, St. Joseph, USA), calibrated with EDTA sample calibration (carbon – 41.07 ± 0.17, hydrogen – 5.55 ± 0.02, nitrogen – 9.57 ± 0.03), according to the Dumas method [226]. All determinations were performed in triplicate.

### 2.3. *Contaminated fish juice preparation*

Minced sea bass muscle (*ca*. 300 g) was packed in styrofoam trays, wrapped in cling film, and kept at 10 ºC for 7 days to promote bacterial growth. Then, minced muscle was frozen (-20 ºC), and part was unfrozen to determine the level of bacterial contamination, following the methodology described later in the material and methods section of this chapter (2.5. Microbiological analysis).

For the preparation of contaminated fish juice, minced muscle was unfrozen, and then it was homogenized and diluted in maximum recovery diluent in a Stomacher bag with filter for one min at medium speed (230 rpm) using a Stomacher homogenizer (Laboratory blender STOMACHER 400, Seward Laboratory Systems Inc., Florida, USA). Then, the necessary dilutions were performed, in maximum recovery diluent, to obtain a final concentration of $3 \times 10^3$ colony forming units (CFU) per mL.

### 2.4. *Preparation of samples and treatments*

Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens, with commercial quality, were acquired from a local market and transported to the laboratory. The average weight of each fish was 362 ± 47 g and the total length was 33 ± 1 cm. Fish samples (n = 16) were filleted by hand and skin removed.

Fillet portions were distributed in several bags with approximately 600 g in each, and mixed with 20 mL of contaminated fish juice (contamination was $10^2$ CFU per g of fillets). After the contamination step, fillet portions were placed between two films, packed in styrofoam trays, wrapped in cling film, and store under refrigerated conditions (4.0 ± 0.5 ºC) for a period of 8 days. During storage, three fillet portions (*ca*. 15 g each) from each treatment were taken for microbiological analysis at predetermined intervals: 0, 2, 4, 6, and 8 days.

A total of 5 treatments was studied: fillets without films and essential oils; fillets with films but without essential oils; fillets with films incorporated with

citronella essential oil; fillets with films incorporated with garlic essential oil; and fillets with films incorporated with thyme essential oil.

### 2.5. *Microbiological analysis*

Sea bass muscle (10 g) was aseptically collected to a Stomacher bag with filter. A primary ten-fold dilution was made with maximum recovery diluent and homogenized for one min at medium speed (230 rpm) using a Stomacher homogenizer. Appropriate series of decimal dilutions were then prepared. Homogenates were spread on agar for *Pseudomonas* spp. and hydrogen sulphide producing bacteria enumeration, or poured into molten agar for psychrotrophic bacteria and Enterobacteriaceae (with a double layer to ensure anaerobic conditions) enumeration.

*Pseudomonas* spp. were enumerated on Pseudomonas agar base supplemented with SR 103, hydrogen sulphide producing bacteria (including *Shewanella putrefaciens*) on Lyngby iron agar, and psychrotrophic bacteria were enumerated on plate count agar after incubation at 20 ºC for 4 days. Enterobacteriaceae were enumerated on violet red bile glucose agar after incubation at 30 ºC for 1 day. For the hydrogen sulphide producing bacteria only the black colonies or those with a black centre were counted, whereas for Enterobacteriaceae the large colonies with purple haloes were counted and a representative number were tested for oxidation and fermentation reactions. Petri dishes containing 30-300 CFU were selected for counting, and the results were expressed into logarithms of the CFU number per g of muscle. All determinations were performed in duplicate.

### 2.6. *Statistical analysis*

The effects of treatments and storage time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences between treatments. All statistical analyses were tested at a 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA.

## 3. Results and discussion

The characterization of fish proteins films (without essential oils) used in the current study was reported in Chapter 3.1 of this thesis taking into account physical, mechanical, antioxidant, and antibacterial properties. The same properties of films incorporated with garlic essential oils were also reported in

Chapter 3.1., while those with citronella and thyme essential oils were reported by Pires and co-authors [249].

The contaminated fish juice had 9.0 log CFU.g$^{-1}$ of psychrotrophic bacteria, 8.1 log CFU.g$^{-1}$ of *Pseudomonas* spp., 7.0 log CFU.g$^{-1}$ of hydrogen sulphide producing bacteria, and 7.8 log CFU.g$^{-1}$ of Enterobacteriaceae.

Initially, contaminated sea bass fillets had 4.7 log CFU.g$^{-1}$ of psychrotrophic bacteria, and the initial microflora consisted mainly in *Pseudomonas* spp. and hydrogen sulphide producing bacteria (Figure 3.2.1). Initial levels of Enterobacteriaceae were near the detection limit (1 log CFU.g$^{-1}$). During storage, bacterial load of contaminated fillets increased and achieved 7 log CFU.g$^{-1}$ in day 4.

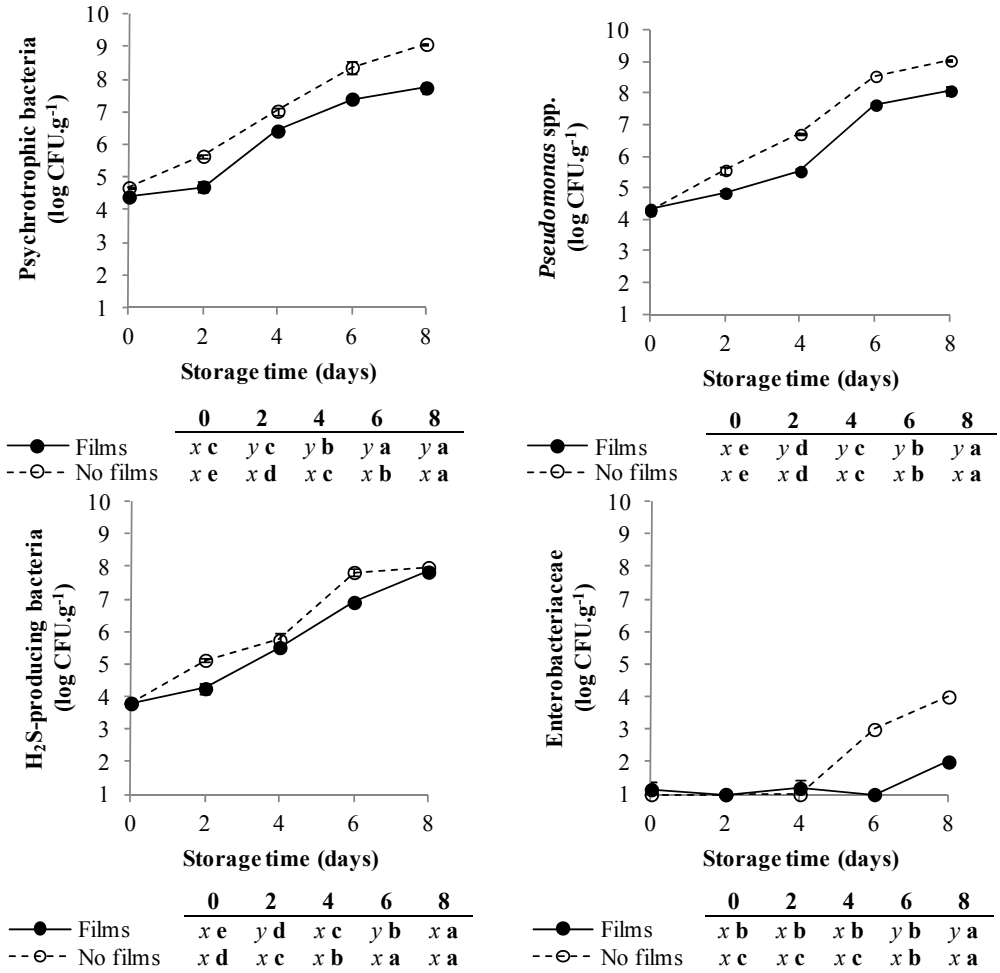


**Figure 3.2.1.** Changes in bacterial microflora during refrigerated storage of sea bass fillets without films and preserved with fish protein films. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*y*) or between sampling days (**a**-**e**). *Abbreviations*: CFU – colony forming units.

The use of fish protein films to preserve fillets did not cause significant changes in initial bacteria counts (Figure 3.2.1). During storage, psychrotrophic bacteria load increased and reached 7 log CFU.g$^{-1}$ in day 6, increasing the microbiological shelf life of sea bass fillets, compared with the treatment without films. Concerning *Pseudomonas* spp. the trend was similar to the one described for psychrotrophic bacteria. However, the utilization of films was not so effective against hydrogen sulphide producing bacteria. Only in days 4 and 6, hydrogen sulphide producing bacteria counts were significantly lower in treatments with films than in the treatments without films. Taking into account Enterobacteriaceae, the treatment with films reduced the growth in 2 log CFU.g$^{-1}$ in days 6 and 8. The contact of films with fillets surface might cause anaerobic conditions that are responsible for a slower bacterial growth.

In general, the incorporation of essential oils in fish protein films did not cause significant changes in bacterial load in day 0 (Table 3.2.1). Only the films with garlic caused changes in initial bacterial counts, increasing psychrotrophic bacteria counts to $4.8 \pm 0.1$ log CFU.g$^{-1}$. During storage, *Pseudomonas* spp. increased faster in treatments with essential oils of citronella, garlic, and thyme. In day 6, all treatments with essential oils reached 7 log CFU.g$^{-1}$ and did not extend the shelf life of fillets, compared with the treatments with films without essential oils.

The essential oils of citronella, garlic, and thyme showed antibacterial activity against spoilage bacteria common in fish products, which is due to the presence of some compounds in their compositions with known antibacterial activities [205]. *In vitro* tests of fish protein films incorporated with essential oils revealed also antibacterial activity against *Shewanella putrefaciens* (garlic and thyme films) and *Pseudomonas putida* (citronella films).

In previous studies, films incorporated with essential oils increased microbiological shelf life of cod and Asian sea bass [75; 83]. In the study with the Asian sea bass, gelatine films were incorporated with lemongrass essential oil at similar concentrations, and in the study with cod more than 10 μL of clove essential oil were used per cm$^2$ of film [83]. The differences in films formulation and in essential oils concentration and composition might explain the distinct effectiveness to inhibit the growth of bacteria.

**Table 3.2.1.** Changes in bacterial microflora during refrigerated storage of sea bass fillets preserved with fish protein films.

| | Day 0 | Day 2 | Day 4 | Day 6 | Day 8 |
|---|---|---|---|---|---|
| **Psychrotrophic bacteria** (log CFU.g$^{-1}$) | | | | | |
| *Films without essential oils* | 4.4 ± 0.1$^{y\,c}$ | 4.7 ± 0.2$^{y\,c}$ | 6.4 ± 0.1$^{x\,b}$ | 7.4 ± 0.0$^{z\,a}$ | 7.7 ± 0.2$^{y\,a}$ |
| *Films with essential oils* | | | | | |
| Citronella | 4.3 ± 0.1$^{y\,e}$ | 5.2 ± 0.0$^{x\,d}$ | 6.4 ± 0.0$^{x\,c}$ | 6.9 ± 0.2$^{w\,b}$ | 8.1 ± 0.1$^{y\,a}$ |
| Garlic | 4.8 ± 0.1$^{x\,d}$ | 5.4 ± 0.1$^{x\,c}$ | 6.4 ± 0.0$^{x\,b}$ | 8.7 ± 0.0$^{x\,a}$ | 8.8 ± 0.1$^{x\,a}$ |
| Thyme | 4.6 ± 0.0$^{xy\,e}$ | 5.1 ± 0.0$^{x\,d}$ | 6.3 ± 0.1$^{x\,c}$ | 8.0 ± 0.0$^{y\,b}$ | 8.6 ± 0.1$^{x\,a}$ |
| ***Pseudomonas* spp.** (log CFU.g$^{-1}$) | | | | | |
| *Films without essential oils* | 4.3 ± 0.0$^{x\,c}$ | 4.8 ± 0.1$^{z\,c}$ | 5.5 ± 0.1$^{y\,b}$ | 7.6 ± 0.1$^{y\,a}$ | 8.1 ± 0.2$^{x\,a}$ |
| *Films with essential oils* | | | | | |
| Citronella | 3.9 ± 0.1$^{x\,e}$ | 5.3 ± 0.2$^{yz\,d}$ | 6.8 ± 0.2$^{x\,c}$ | 7.5 ± 0.0$^{y\,b}$ | 8.2 ± 0.1$^{x\,a}$ |
| Garlic | 4.4 ± 0.1$^{x\,d}$ | 5.4 ± 0.1$^{xy\,c}$ | 6.6 ± 0.0$^{x\,b}$ | 8.5 ± 0.0$^{x\,a}$ | 8.3 ± 0.2$^{x\,a}$ |
| Thyme | 4.3 ± 0.4$^{x\,c}$ | 5.9 ± 0.0$^{x\,b}$ | 6.3 ± 0.0$^{x\,b}$ | 8.0 ± 0.2$^{xy\,a}$ | 8.2 ± 0.0$^{x\,a}$ |
| **$H_2S$-producing bacteria** (log CFU.g$^{-1}$) | | | | | |
| *Films without essential oils* | 3.8 ± 0.1$^{x\,d}$ | 4.3 ± 0.2$^{x\,d}$ | 5.5 ± 0.0$^{xy\,c}$ | 6.9 ± 0.0$^{y\,b}$ | 7.8 ± 0.0$^{y\,a}$ |
| *Films with essential oils* | | | | | |
| Citronella | 3.7 ± 0.1$^{x\,e}$ | 4.4 ± 0.0$^{x\,d}$ | 5.8 ± 0.3$^{x\,c}$ | 6.6 ± 0.3$^{y\,b}$ | 7.8 ± 0.1$^{x\,a}$ |
| Garlic | 4.0 ± 0.0$^{x\,d}$ | 4.6 ± 0.1$^{x\,c}$ | 5.5 ± 0.0$^{xy\,b}$ | 7.9 ± 0.0$^{x\,a}$ | 7.6 ± 0.1$^{y\,a}$ |
| Thyme | 3.8 ± 0.0$^{x\,d}$ | 4.6 ± 0.0$^{x\,c}$ | 5.0 ± 0.4$^{y\,c}$ | 6.9 ± 0.0$^{y\,b}$ | 7.8 ± 0.1$^{y\,a}$ |
| **Enterobacteriaceae** (log CFU.g$^{-1}$) | | | | | |
| *Films without essential oils* | 1.2 ± 0.2$^{x\,a}$ | 1.0 ± 0.0$^{x\,a}$ | 1.2 ± 0.3$^{x\,a}$ | 1.0 ± 0.0$^{y\,a}$ | 2.0 ± 0.0$^{y\,a}$ |
| *Films with essential oils* | | | | | |
| Citronella | 1.0 ± 0.0$^{x\,b}$ | 1.0 ± 0.0$^{x\,b}$ | 1.2 ± 0.3$^{x\,b}$ | 1.3 ± 0.4$^{y\,b}$ | 3.0 ± 0.0$^{y\,a}$ |
| Garlic | 1.0 ± 0.0$^{x\,c}$ | 1.0 ± 0.0$^{x\,c}$ | 1.5 ± 0.7$^{x\,c}$ | 3.0 ± 0.0$^{x\,b}$ | 4.5 ± 0.7$^{x\,a}$ |
| Thyme | 1.2 ± 0.3$^{x\,b}$ | 1.2 ± 0.2$^{x\,b}$ | 1.0 ± 0.0$^{x\,b}$ | 2.0 ± 0.0$^{xy\,ab}$ | 3.0 ± 0.0$^{y\,a}$ |

*Abbreviations*: CFU – colony forming units.
Values are presented as average ± standard.
Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*w*) or between sampling days (**a**-**e**).

## 4. Conclusions

Films without essential oils increased the microbiological shelf life of sea bass fillets from 4 to 6 days, reducing the growth of *Pseudomonas* spp. and Enterobacteriaceae. However, the incorporation of essential oils in films did not cause important changes in bacterial counts. Films prepared with the essential oils of citronella, garlic, and thyme did not show potential to be used for preservation of sea bass fillets, taking into account microbiological criteria. Other formulations with different concentrations of essential oils or combining different essential oils might be more advantageous.

# CHAPTER 4. Development of methods to preserve sea bass fillets: high pressure processing

# CHAPTER 4.1.

# Changes of enzymes activity and protein profiles caused by high pressure processing in sea bass (*Dicentrarchus labrax*) fillets

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**Abstract**

High pressure processing (HPP) is a technology of growing interest for food preservation, due to its ability to control the activity of degradative enzymes. The effect of three variables (pressure levels of 100, 250, and 400 MPa; pressure holding times of 0, 5, 15, and 30 min; and pressurization rates of 8 and 14 MPa.$s^{-1}$) on the activity of the enzymes acid phosphatase, cathepsins (B and D), lipase, and calpains was studied using sea bass fillets as a case study model. Additionally, the effect of HPP on sarcoplasmic proteins was studied by SDS-PAGE and isoelectric focusing electrophoreses. The increase in pressure level and holding time decreased the protein concentration in sarcoplasmic extracts, and also the activity of calpains. As compared to non-treated samples (0.1 MPa), acid phosphatase activity was lower at 400 MPa, and for cathepsin D lower activities were observed at 100 and 400 MPa. The increase in pressurization rate increased the activity of cathepsin D, lipase, and calpains, although it was not always significant. In contrast, cathepsin B and lipase activities were less affected by HPP treatments. Electrophoresis separation of sarcoplasmic proteins showed that the intensity of many protein bands changed mainly due to pressure level and holding time. The results of this study suggest that HPP causes lysosomes disruption and also denaturation, aggregation, and fragmentation of sarcoplasmic proteins, and this evidence might be related to the decrease in enzymes activity especially at 400 MPa. In conclusion, HPP can be a potential tool to control the activity of degradative enzymes, which might prevent the softening of sea bass muscle due to autolytic reactions.

**Keywords**

High pressure processing; sea bass; enzymes; water soluble proteins; cathepsins; calpains; acid phosphatase; lipases; SDS-PAGE; IEF

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## 1. Introduction

The increase of consumer's preference toward fresh and minimally processed food products, rather than processed and frozen ones, demands for research on new processing and preservation methods, especially for products with short shelf life. High pressure processing (HPP) is of growing interest for the processing and preservation of food. This technology has the potential to better retain food's nutritional and organoleptic characteristics, when compared to traditional thermal processing [114]. It is also a potential tool for the creation of new product textures by the food industry because of its ability to induce modifications on food functional properties [117]. HPP treatments have been used to inactivate microorganisms to extend food shelf life [114]. HPP is known to affect non-covalent chemical bonds of molecules, thus inducing modifications in water, proteins, polysaccharides, and lipids [3].

Freshness of seafood is rapidly lost in the initial stages of *post mortem* degradation due to autolytic degradation by endogenous enzymes, leading to an initial loss in prime quality [28]. *Post mortem* changes, pre-processing, and processing methods can lead to tissue damage and disruption of cellular organelles, like mitochondria and lysosomes, releasing several enzymes into the cellular fluid [250]. The impact of autolytic enzymes on textural quality will limit the shelf life and cause an early downgrade of food products [251]. Therefore, enzymatic activity of fish has been used as an indicator of quality changes [250], and calpains and cathepsins (B and D) have been reported as the most important enzymes involved in the softening of fish muscle tissues [28; 39-40].

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species widely distributed in the Mediterranean Sea and Atlantic Ocean and intensively farmed in several Mediterranean countries, *e.g.* Greece, Spain, and Italy [6]. This species is highly appreciated because of its excellent organoleptical properties and reasonable price [6].

The effect of pressure level in the activity of degradative enzymes has been studied in fish muscle, and results showed that the activity of degradative enzymes such as calpains and cathepsin D is reduced [39; 251]. However, other pressure variables like pressure holding time and pressurization rate have not been a focus of attention. It is important to further investigate the effect of HPP in fresh fish to better understand the resultant effects on the quality and protein characteristics.

This study aims to understand the effect of HPP on the activity of several degradative enzymes of fresh sea bass fillets. In particularly, pressure level (0.1-400 MPa), pressure holding time (0-30 min), and pressurization rate (8 and 14 MPa.$s^{-1}$) were tested to evaluate their effect on enzymatic activity of acid phosphatase, cathepsins (B and D), lipases, and calpains. Additionally, sarcoplasmic proteins of HPP sea bass were separated according to sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and isoelectric focusing (IEF) electrophoreses, to identify changes on protein profiles.

## 2. Material and methods

### 2.1. *Chemicals*

The chemicals sodium dodecyl sulphate (SDS), trichloroacetic acid, and acetic acid were obtained from Fluka (Buchs, Germany); bromophenol blue was from Merck (Darmstadt, Germany); potassium hydroxide was from Panreac Quimica S.A.U. (Barcelona, Spain); Coomassie Brilliant Blue R-250 was from Bio-Rad (Philadelphia, USA); Coomassie Phastgel blue R was from Amersham Pharmacia Biotech (Uppsala, Sweden); *p*-nitrophenylphosphate, L-methionine-7-amido-4-methylcoumarin triflouroacetic salt, Z-arginine-arginine-7-amido-4-methylcoumarin hydrochloride, hemoglobin from bovine blood, olive oil, trizma hydrochloride (Tris-HCl), dithiothreitol (DTT), 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris), ethylene diamine tetracetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), glycine, potassium chloride, glycerol, magnesium chloride, sodium acetate, calcium chloride, thymolphtalein, potassium phosphate monobasic, sodium hydroxide, monochloroacetic acid, β-mercaptoethanol, citric acid, trisodium citrate, and bovine serum albumin were from Sigma-Aldrich (Steinheim, Germany); ethanol had a purity grade of 99 %; and the water used was Milli-Q purified and distilled.

### 2.2. *Preparation of samples*

Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens, with commercial quality, were acquired from a local market. The average weight of each fish was 465 ± 57 g and the total length was 35 ± 1 cm. Fish samples (n = 16) were filleted, and skin was removed. Fillets were divided in portions of *ca*. 15 g, and eight portions randomly selected were used for each treatment to reduce the effect of specimen variability. Fillet portions were vacuum packed in low-oxygen permeable barrier bags (Colamin XX 100e, Obermühle, Pössneck,

Germany) with a vacuum packager (Packman, Albipack, Águeda, Portugal) before pressurization treatments.

### 2.3. *High pressure treatments*

HPP treatments were carried out in a hydrostatic press (high pressure system U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature (6 ºC), using a mixture of propylene glycol and water (1:1) as pressurizing fluid. Samples (8 fillet portions per treatment) were processed at pressure levels of 100, 250, and 400 MPa for 5, 15, and 30 min using a pressurization rate of *ca*. 8 MPa.s$^{-1}$. A higher pressure rate increment, *ca.* 14 MPa.s$^{-1}$, was also tested to process samples at the same pressure levels (100, 250, and 400 MPa) for 5 min. Control treatments of 0 min pressure holding time were also performed subjecting the fillets to pressurization and immediate depressurization. Nineteen treatments were studied (Table 4.1.1) including non-treated samples (0.1 MPa). After HPP treatments, samples were immediately frozen in liquid nitrogen and stored at -20 ºC until further analysis.

**Table 4.1.1.** Treatment conditions tested.

| | Pressurization rate | Pressure level | Pressure holding time |
|---|---|---|---|
| **Non-treated** samples not subjected to high pressure processing | | | |
| **Treated** samples subjected to high pressure processing | 8 MPa.s$^{-1}$ | 100 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | | 250 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | | 400 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | 14 MPa.s$^{-1}$ | 100 MPa | 0 min |
| | | | 5 min |
| | | 250 MPa | 0 min |
| | | | 5 min |
| | | 400 MPa | 0 min |
| | | | 5 min |

The time indicated in each treatment does not include the duration of pressurization and depressurization.

## 2.4. *Enzymatic activity*

### 2.4.1. *Preparation of extracts*

The extraction of enzymes was performed following the methodology previously described by Lakshmanan and co-authors [251], with minor modifications. Briefly, minced sea bass (20 g) was homogenized with cold

water (40 mL; 4 ºC) using an Ultra Turrax homogenizer (2 min, 8,000 rpm; Ultraturrax T25, Janke & Kunkel IKA®- Labortechnik). The homogenate was kept on ice for 30 min with occasional stirring and centrifuged (14,600 ×*g*, 4 ºC, 20 min; centrifuge 3K30, Sigma, Osterode, Germany). The supernatant was filtered (Macherey-Nagel MN 640 W), frozen in liquid nitrogen, and stored at -20 ºC until further analyses (enzymatic analysis and protein concentration).

#### 2.4.2. *Acid phosphatase*

Acid phosphatase activity was assayed with *p*-nitrophenylphosphate as a substrate following the methodology of Ohmori and co-authors [252]. Extracts (1 mL) were mixed with substrate solution (0.9 mL, 4 mM *p*-nitrophenylphosphate in 0.1 mM sodium acetate buffer and 1 mM EDTA, pH 5.5) and incubated at 37 ºC during 15 min. The reaction was stopped by the addition of potassium hydroxide (4 mL, 0.1 M) and the *p*-nitrophenol released was measured spectrophotometrically (Perkin Elmer Instruments Lambda 35 UV/VIS spectrometer) at 400 nm. Acid phosphatase activity was expressed as absorbance units (AU) change per min per g of muscle. Four replicates were performed per treatment.

### 2.4.3. *Cathepsins*

#### 2.4.3.1. *Cathepsin B*

The activity of cathepsin B was assayed by the Barrett & Kirschke [253] method. Enzyme extract (0.1 mL) was mixed with substrate solution (0.1 mL, 0.0625 mM Z-arginine-arginine-7-amido-4-methylcoumarin hydrochloride in 100 mM Bis-Tris, 20 mM EDTA, 4 mM DTT, pH 6.5) and incubated at 37 ºC during 5 min. The reaction was stopped by the addition of SDS (1 mL, 3 % w/v in 50 mM Bis-Tris, pH 7.0) and the fluorescence of 7-amino-4-methylcoumarin liberated was measured (excitation = 360 nm; emission = 460 nm) using a spectrofluorometer (FluoroMax 3 Spectrofluorometer, Horiba Jovin Yvon, New Jersey, USA). Cathepsin B activity was expressed as fluorescence units (FU) change per min per g of muscle. Four replicates were performed per treatment.

#### 2.4.3.2. *Cathepsin D*

The cathepsin D assay used was based on the procedure described by Anson [254], with small modifications. Enzyme extract (0.5 mL) was mixed with substrate solution (1.5 mL of 2 % w/v denatured hemoglobin from bovine blood in 0.2 M citrate buffer, pH 3.7) and incubated at 37 ºC during 3 h. The reaction was stopped by the addition of trichloroacetic acid (1.5 mL, 10 % w/v). After being vigorously stirred, the precipitate was removed by centrifugation

(18,000 ×*g*, 15 min) and soluble peptides were measured spectrophotometrically at 280 nm. Cathepsin D activity was expressed as AU change per h per g of muscle. Four replicates were performed per treatment.

**2.4.4. *Lipase*** The activity of lipase was assayed with olive oil as substrate following the titrimetric enzymatic assay described by Sigma-Aldrich [255]. Enzyme extract (1 mL) was mixed with substrate solution (1.5 mL of olive oil, 1.25 mL of water, and 0.5 mL of 200 mM Tris-HCl buffer pH 7.7) and incubated at 37 ºC during 24 h. The reaction was stopped by adding ethanol (2 mL, 95 % v/v) and the liberated free fatty acid was titrated against sodium hydroxide (25 mM) using thymolphtalein as indicator. Lipase activity was expressed in units per g of muscle, where one unit corresponds to the amount of enzyme that hydrolyzes one microequivalent of fatty acids from triglycerides in one h. Four replicates were performed per treatment.

**2.4.5. *Calpains*** Activity of calpains was measured using the method of Sasaki and co-authors [256]. Enzyme extract (0.1 mL) was mixed with substrate solution (0.1 mL, 0.125 mM L-methionine-7-amido-4-methylcoumarin trifluoroacetic salt in 100 mM Bis-Tris, 5 mM calcium chloride, pH 6.5) and incubated at 37 ºC during 2 min. The reaction was stopped by adding 30 mM monochloroacetic acid, 21 mM acetic acid, and 9 mM sodium acetate (3 mL, pH 4.3). The fluorescence of 7-amino-4-methylcoumarin liberated was measured (excitation = 360 nm; emission = 460 nm) using a spectrofluorometer (FluoroMax 4 Spectrofluorometer, Horiba Jobin Yvon, New Jersey, USA). Calpains activity was expressed as FU change per min per g of muscle. Four replicates were performed per treatment.

### 2.5. *Electrophoreses*

**2.5.1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*** Muscle samples were added to cold buffer (75 mM potassium chloride, 10 mM potassium phosphate monobasic, 2 mM magnesium chloride, 2 mM EGTA, pH 7.0; 4 ºC) and homogenized using an Ultra Turrax homogenizer. The homogenate was centrifuged (10,000 ×*g*, 4 ºC, 10 min) and the supernatant containing the sarcoplasmic proteins was frozen until further use. Sarcoplasmic protein solutions (with the same adjusted protein concentration) were diluted 1:1 in loading buffer (62.5 mM Tris-HCl, 20 % v/v glycerol, 2 % w/v SDS, 5 %

v/v β-mercaptoethanol, and 0.01 % w/v bromophenol blue at pH 6.8) and heated (100 ºC, 3 min) prior to gel running.

For SDS-PAGE, gels with different resolving power were used (10, 12, and 15 % polyacrylamide) with stacking gels of 4 %. The amount of sample loaded per lane was 100 μg of protein in gels with 10 and 12 % polyacrylamide, and 300 μg of protein in gels with 15 % polyacrylamide. Molecular weight standards (Precision Plus Protein™ Standards All Blue, Bio-Rad, Philadelphia, USA) were used in every gel. Electrophoresis was performed in a vertical unit (Hoefer SE 600 series, Amersham Pharmacia Biotech, Uppsala, Sweden) at 10 ºC (LAUDA Ecoline Staredition RE 104, Lauda-Konigshofen, Germany) with running buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1 % SDS, pH 9), and the running conditions limited to 25 mA (EPS 601, Amersham Pharmacia Biotech, Uppsala, Sweden). Protein bands were fixed and stained with Coomassie Brilliant Blue R-250. Determinations were performed in triplicate.

### 2.5.2. *Isoelectric focusing (IEF) with CleanGel*

For IEF of sarcoplasmic extracts, the homogenization of muscle was performed with cold water (4 ºC) to avoid band disturbances due to the presence of salts. The homogenates were centrifuged (40,000 ×*g*, 4 ºC, 20 min) and the supernatants used as sarcoplasmic proteins.

Sarcoplasmic protein solutions (1 mg.mL$^{-1}$) were analysed by IEF using a CleanGel (Amersham Pharmacia Biotech, Uppsala, Sweden) according to Silva and co-authors [257] with modification on gel rehydration. For a full-size CleanGel, 21 mL of a solution containing ampholine preblended (0.26 mL, pH 3.5-9.5; Pharmacia Biotech, Uppsala, Sweden), and ampholine (1.31 mL, pH 3.5-5.0; Pharmacia Biotech, Uppsala, Sweden) was used to rehydrate. Electrode wicks (Serva 42972, Heidelberg, Germany) were soaked with anode fluid (Serva 42984, Heidelberg, Germany) and cathode fluid (Serva 42986, Heidelberg, Germany). The electrophoresis running conditions were: prefocusing (30 min) – 500 V, 8 mA, 8 W; sample entrance (20 min) – 500 V, 8 mA, 8 W; and isoelectric focusing (5,000 Vh) – 2,000 V, 14 mA, 14 W (MultiDrive XL, Amersham Pharmacia Biotech, Uppsala, Sweden) at 10 ºC (Multitemp II thermostatic circulator model 2219, Amersham, Sweden). After prefocusing, the sample application pieces (Amersham Pharmacia Biotech, Uppsala, Sweden) were placed 2 cm in front of the cathodic wick, and 30 μL of samples and 7.5 μL of isoelectric point (pI) marker solutions (isoelectric focusing calibration kit pH 3-10 and pH 5-10.5, Amersham Pharmacia Biotech,

Uppsala, Sweden) were applied. After IEF run, proteins were fixed and stained with Coomassie Phastgel blue R. Determinations were performed in triplicate.

**2.5.3. *Identification and quantification of protein bands***

Gels were scanned using a densitometer (model GS-800, Bio-Rad, Philadelphia, USA) and the images were analysed with PDI Discovery Series software (module Quantity One version 2.4; Bio-Rad, Philadelphia, USA). Molecular weights, pI values, and changes in integrated optical density (IOD) of protein bands were calculated.

**2.6. *Protein concentration in extracts***

Concentration of proteins in extracts, for enzymatic activity and electrophoreses analyses, was determined with the Bradford method [258] using bovine serum albumin as standard. Four replicates were performed per treatment.

**2.7. *Statistical analysis***

Differences between treatments were tested at a 0.05 level of probability with the software STATISTICA™ 6.1 (Statsoft, Inc., Tulsa, OK, USA). The Dunnett test was used to identify differences between HPP and non-treated samples. The effects of pressure level and pressure holding time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences between treatments. To study the effect of pressurization rate, a t-test for independent samples was used.

## 3. Results and discussion

**3.1. *Protein concentration***

Protein concentration in water extracts was *ca*. 90 $mg.mL^{-1}$ in non-treated samples. The solubility of proteins of sea bass fillets treated with HPP was found to be dependent on pressure level and holding time (Figure 4.1.1). In fact, apart from the treatment at 100 MPa during 0 min, HPP induced a significant reduction in protein solubility with pressure level and holding time. Water soluble proteins reduced 4.5 times in samples treated at 400 MPa (5-30 min) when compared to non-treated samples. In contrast, pressurization rate did not influence sarcoplasmic protein solubility for the short-time treatments (0 and 5 min; Table 4.1.2).

The decrease of protein solubility with the increase of pressure level is in accordance with the results obtained with bovine muscle by Marcos and co-authors [259]. The protein solubility represents a measure of protein denaturation, and the decrease observed with the raise of pressure level could be due to the formation of protein aggregates that can no longer be solubilized in

water [259], which were pronounced with the increase in pressure holding time. These results also indicate that the increase in pressure level and holding time might decrease the amount of enzymes available, as soluble proteins in these extracts include mainly enzymes [260].

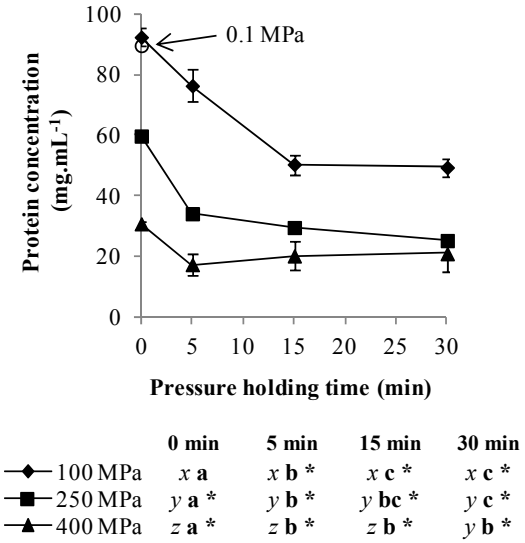



**Figure 4.1.1.** Protein concentration in sarcoplasmic extracts of sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x-z*) or between pressure holding times (**a-c**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa).

**Table 4.1.2.** Protein concentration in sarcoplasmic extracts and enzymatic activities of sea bass fillets treated with high pressure at 8 and 14 MPa.s$^{-1}$ pressurization rates.

| | Pressure level (MPa) | 0 min | | 5 min | |
|---|---|---|---|---|---|
| | | 8 MPa.s$^{-1}$ | 14 MPa.s$^{-1}$ | 8 MPa.s$^{-1}$ | 14 MPa.s$^{-1}$ |
| **Protein concentration in sarcoplasmic extracts** (mg.mL$^{-1}$) | 100 | 92.4 ± 3.1$^{a}$ | 91.0 ± 5.9$^{a}$ | 76.4 ± 5.2$^{A}$ | 76.7 ± 2.3$^{A}$ |
| | 250 | 59.8 ± 1.4$^{a}$ | 71.4 ± 14.0$^{a}$ | 34.2 ± 1.0$^{A}$ | 33.6 ± 7.5$^{A}$ |
| | 400 | 30.9 ± 0.8$^{a}$ | 31.6 ± 0.8$^{a}$ | 17.5 ± 3.7$^{A}$ | 19.8 ± 0.2$^{A}$ |
| **Acid phosphatase activity** (AU.min$^{-1}$.g$^{-1}$) | 100 | 5.4 ± 0.4$^{a}$ | 5.3 ± 0.7$^{a}$ | 5.6 ± 0.3$^{A}$ | 6.0 ± 0.3$^{A}$ |
| | 250 | 4.6 ± 0.1$^{a}$ | 5.9 ± 0.1$^{a}$ | 5.3 ± 0.6$^{A}$ | 5.1 ± 0.5$^{A}$ |
| | 400 | 4.5 ± 0.4$^{a}$ | 4.4 ± 0.4$^{a}$ | 4.4 ± 1.0$^{A}$ | 4.3 ± 0.3$^{A}$ |
| **Cathepsin B activity** (×10$^{3}$ FU.min$^{-1}$.g$^{-1}$) | 100 | 3.7 ± 0.2$^{a}$ | 3.6 ± 1.4$^{a}$ | 4.4 ± 0.4$^{A}$ | 4.9 ± 1.5$^{A}$ |
| | 250 | 2.8 ± 0.2$^{a}$ | 3.8 ± 1.7$^{a}$ | 5.3 ± 1.5$^{B}$ | 7.1 ± 1.3$^{A}$ |
| | 400 | 6.3 ± 1.7$^{a}$ | 7.7 ± 1.1$^{a}$ | 6.9 ± 1.2$^{A}$ | 5.5 ± 0.6$^{B}$ |
| **Cathepsin D activity** (AU.h$^{-1}$.g$^{-1}$) | 100 | 0.15 ± 0.04$^{b}$ | 0.22 ± 0.02$^{a}$ | 0.20 ± 0.05$^{A}$ | 0.27 ± 0.05$^{A}$ |
| | 250 | 0.25 ± 0.05$^{a}$ | 0.25 ± 0.04$^{a}$ | 0.21 ± 0.01$^{B}$ | 0.28 ± 0.06$^{A}$ |
| | 400 | 0.31 ± 0.02$^{b}$ | 0.42 ± 0.07$^{a}$ | 0.10 ± 0.04$^{A}$ | 0.15 ± 0.09$^{A}$ |
| **Lipase activity** (×10$^{-2}$ units.g$^{-1}$) | 100 | 3.64 ± 0.86$^{a}$ | 4.47 ± 0.52$^{a}$ | 1.11 ± 0.48$^{B}$ | 3.92 ± 1.20$^{A}$ |
| | 250 | 3.33 ± 0.77$^{a}$ | 4.36 ± 0.55$^{a}$ | 5.20 ± 2.22$^{A}$ | 5.10 ± 1.65$^{A}$ |
| | 400 | 4.89 ± 2.10$^{a}$ | 5.82 ± 0.76$^{a}$ | 3.95 ± 1.73$^{A}$ | 6.01 ± 1.61$^{A}$ |
| **Calpains activity** (×10$^{4}$ FU.min$^{-1}$.g$^{-1}$) | 100 | 13.7 ± 1.9$^{a}$ | 14.9 ± 1.5$^{a}$ | 12.2 ± 1.0$^{B}$ | 15.3 ± 1.5$^{A}$ |
| | 250 | 11.0 ± 0.6$^{b}$ | 13.5 ± 1.3$^{a}$ | 5.3 ± 0.5$^{A}$ | 4.7 ± 0.8$^{A}$ |
| | 400 | 5.3 ± 1.1$^{b}$ | 7.2 ± 1.4$^{a}$ | 1.4 ± 0.1$^{B}$ | 1.6 ± 0.1$^{A}$ |

*Abbreviations*: AU – absorbance units; FU – fluorescence units.
Values are presented as average ± standard deviation.
Different letters denote significant differences ($p < 0.05$) between pressurization rates at the same pressure level for 0 min (a-b) or 5 min (A-B).

## 3.2. *Enzymatic activity*

### 3.2.1. *Acid phosphatase*

Phosphatase activities in fish muscles, particularly ATP-, ADP-, and IMP-degrading enzymatic activities, have been reported to be related to the freshness index, K-value [261].

The activity of acid phosphatase in non-treated samples (5.6 AU.min$^{-1}$.g$^{-1}$) was not significantly different from that found in 100 MPa (0-30 min) and 250 MPa (5-15 min) samples, and these values were significantly higher than in the remaining treatments (Figure 4.1.2). In general, pressure holding time and pressurization rate did not affect the activity of acid phosphatase (Figure 4.1.2; Table 4.1.2), but still the activity of acid phosphatase decreased to a minimum of 3.7 AU.min$^{-1}$.g$^{-1}$ in samples treated at 400 MPa (15 min). HPP treatments that cause inactivation of acid phosphatase might be suitable for the preservation of fish muscle, as a decrease in its activity indicates less degradation of IMP, and thus pleasant flavors might be kept for longer.

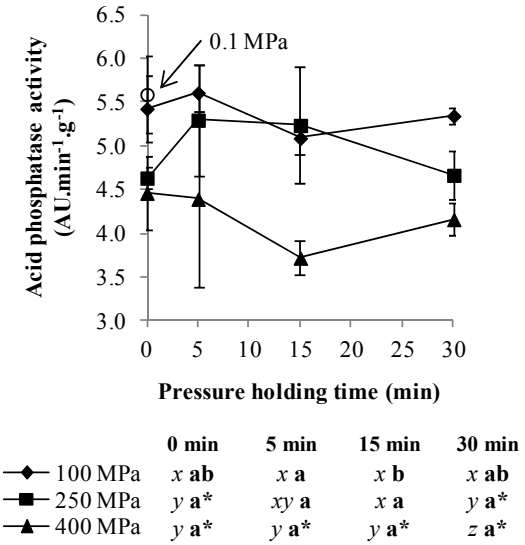



**Figure 4.1.2.** Acid phosphatase activity in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*z*) or between pressure holding times (**a-b**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa). *Abbreviations*: AU – absorbance units.

Ohmori and co-authors [252] obtained similar results in bovine liver pressurized during 10 min at levels up to 500 MPa. At lower pressure levels (up to 300 MPa), it was observed a gradually increase in acid phosphatase activity in the cytosolic fraction and a decrease in the lysosomal fraction, but the total activity remained almost the same [252]. This effect was explained by the enzyme being released from lysosomes due to their disruption, as approximately

40-60 % of this enzyme is bound to lysosomes membranes [252]. The decrease in the phosphatase activity with the increase in pressure level, can be a result of enzyme inactivation caused by HPP treatments. HPP affects proteins differently in minced and whole fish muscle, as in HPP minced sea bass values increased *ca*. 2.5 times in samples treated at 500 MPa (5 min) compared to non-treated samples [39].

### 3.2.2. *Cathepsins*

#### 3.2.2.1. *Cathepsin B*

Cathepsins are a group of proteases responsible for the softening of the muscle tissue [28]. This group of enzymes is found in lysosomes and can be released into the cytoplasm and intracellular spaces as a consequence of lysosomal disruption, mainly during *post mortem* storage [39]. In particular, cathepsin B is a cysteine carboxypeptidase that is regulated *in vivo* by a inhibitor named cystatin [39].

The effect of HPP on the activity of cathepsin B is shown in Figure 4.1.3 and Table 4.1.2. No significant differences were determined between non-treated samples ($5.2 \times 10^3$ FU.min$^{-1}$.g$^{-1}$) and samples treated at 100 MPa (0-30 min). In what concerns pressurization rate, the activity of this enzyme at 250 MPa (5 min) increased with the increase in pressurization rate, while at 400 MPa (5 min) the opposite was observed (Table 4.1.2).

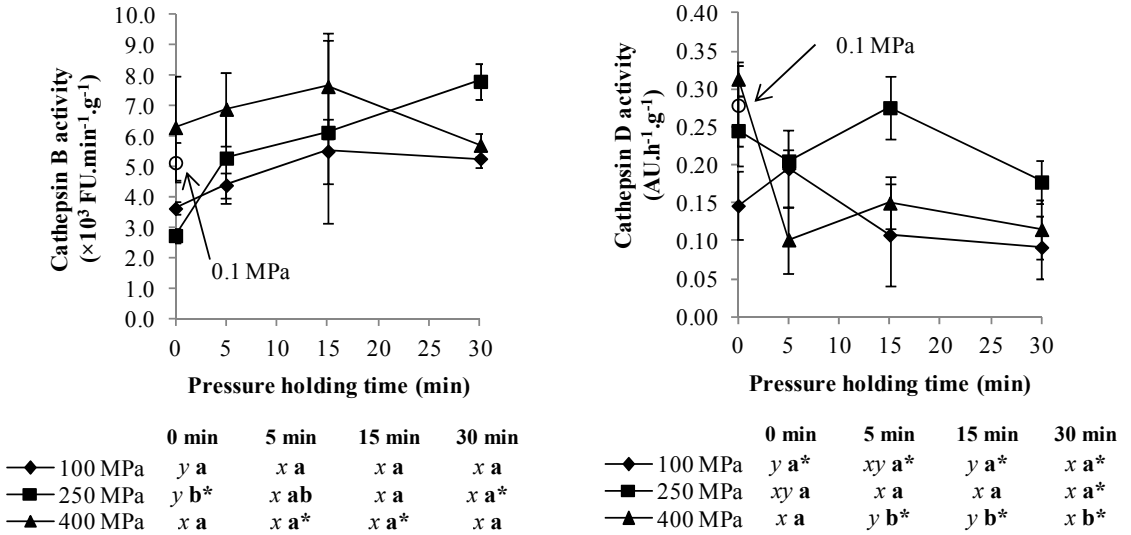



**Figure 4.1.3.** Cathepsin B and D activities in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x-y*) or between pressure holding times (**a-b**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa). *Abbreviations*: FU – fluorescence units.

In general, pressure level did not affect significantly the activity of cathepsin B. In previous studies, the activity of cathepsin B increased almost two times with the increase in pressure level from 0.1 to 300 MPa (5 min) [262].

In the current study, the highest values were observed in treatments at 250 MPa (30 min) and 400 MPa (5-15 min). At 250 MPa, the increase in pressure holding time from 0 to 30 min, increased cathepsin B activity in 2.8 times. The cathepsin B activity increase might be due to disruption of lysosomes and consequent release of enzymes that became available to interact with substrate, and this can promote the softening of sea bass muscle tissues.

#### 3.2.2.2. *Cathepsin D*

Cathepsin D is considered to be the most important enzyme in *post mortem* degradation due to the absence of a specific inhibitor in the fish muscle [39]. This enzyme is an aspartic acid protease that occurs in the lysosomal system [263].

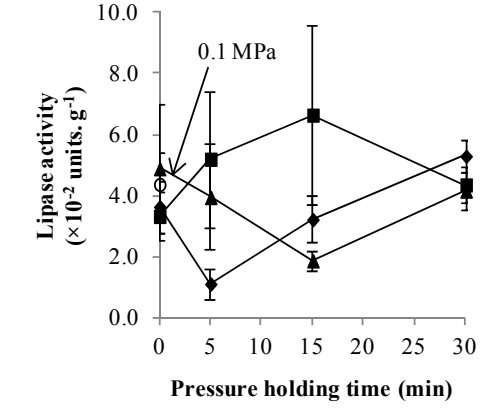
The activity of cathepsin D in non-treated samples was 0.28 $AU.h^{-1}.g^{-1}$, and the variations caused by HPP treatments did not exceed the activity values observed in non-treated samples (Figure 4.1.3). With the exception of the 0 min treatments for which the increase in pressure level increased cathepsin D activity, the treatments at 100 and 400 MPa showed lower activity values. Pressure holding time only affected the activity of cathepsin D in the 400 MPa treatments, decreasing the activity with the increase in pressure holding time. In relation to pressurization rate, higher values of cathepsin D activity were, in general, observed in samples treated with the fastest pressurization rate treatment, but not always significant (Table 4.1.2).

In what concerns the effect of pressure level, the activity of cathepsin D followed a similar trend in previous studies with minced and whole muscle of sea bass, but the activity in non-treated samples was lower than in treatments at higher pressure levels [39; 262]. At lower pressure levels (100 MPa), the variations in cathepsin D activity seems to be due principally to inactivation of this enzyme. With the increase in pressure level to 250 MPa, the rupture of lysosomes and consequent release of enzymes might caused an increase of cathepsin D activity. It is not set aside the hypothesis that HPP continues to inactivate the enzyme, but proportionally the release of enzymes from lysosomes might be more important at this pressure level. At 400 MPa, inactivation predominates again the effects of HPP on the activity of this enzyme, and these treatments (pressure holding time of at least 5 min) might be suitable for the preservation of fish muscle.

### 3.2.3. *Lipase*

Lipases are triacylglycerol acylhydrolases that degrade phospholipids and catalyse the hydrolysis of fatty acid ester bonds in triacylglycerols to free fatty acids and glycerol [42; 264]. Accumulation of free fatty acids has been associated with quality deterioration due to changes on textural properties by stimulation of protein denaturation and production of off-flavours by promotion of lipid oxidation [42].

Lipase activity (Figure 4.1.4) in non-treated samples was $4.4 \times 10^{-2}$ units.g$^{-1}$, and it was not significantly affected in most of the HPP treatments. In what concerns pressurization rate, higher values of lipase activity were only observed in samples treated at 100 MPa during 5 min with the fastest pressurization rate treatment (Table 4.1.2).




| | 0 min | 5 min | 15 min | 30 min |
|---|---|---|---|---|
| 100 MPa | *x* **ab** | *x* **b*** | *xy* **ab** | *x* **a** |
| 250 MPa | *x* **a** | *x* **a** | *x* **a** | *x* **a** |
| 400 MPa | *x* **a** | *x* **a** | *y* **a** | *x* **a** |

**Figure 4.1.4.** Lipase activity in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*y*) or between pressure holding times (**a**-**b**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa).

Published data show that the degradation of lipids is enhanced by HPP treatments in salmon and tuna [265], but lipid degradation inhibition has also been reported in tuna [266]. Differences between species, as well on product processing conditions (*e.g.* fresh, smoked, fillet, *carpaccio*, and minced fish) might explain these differences. Nayak and co-authors [267] suggested that differences in lipase activity can also be attributed to feeding habits, food composition, and physiological status of fish.

Nevertheless, the increase in lipids degradation with HPP treatments was not associated with a reduction in texture quality due to changes in structural proteins of fish muscle [265]. Particularly, the reactions related to lipid

degradation are of less importance in fish with low fat content, where storage conditions are the determinant factors in the evolution of lipid degradation [265].

### 3.2.4. *Calpains*

Calpains are one of the main proteinase groups that hydrolyze myofibrillar proteins, affecting the texture of *post mortem* fish muscle [40]. Calpains are intracellular cysteine proteases, free in the cells cytoplasm, active at neutral pH, dependent upon calcium, and with their activity regulated by the endogenous inhibitor calpastatin [268].

The overall activity of calpains was high ($11.4 \times 10^4$ FU.min$^{-1}$.g$^{-1}$ in non-treated samples). In general, samples treated at 100 MPa showed calpain activities similar to non-treated samples (Figure 4.1.5). The increase in pressure level and holding time inactivated this enzyme to levels of $1.5 \times 10^4$ FU.min$^{-1}$.g$^{-1}$ in samples treated at 250 (15-30 min) and 400 MPa (5-30 min). The increase in pressurization rate caused a significant increase in calpains activity in samples treated at 100 MPa (5 min), 250 MPa (0 min), and 400 MPa (0-5 min) (Table 4.1.2).

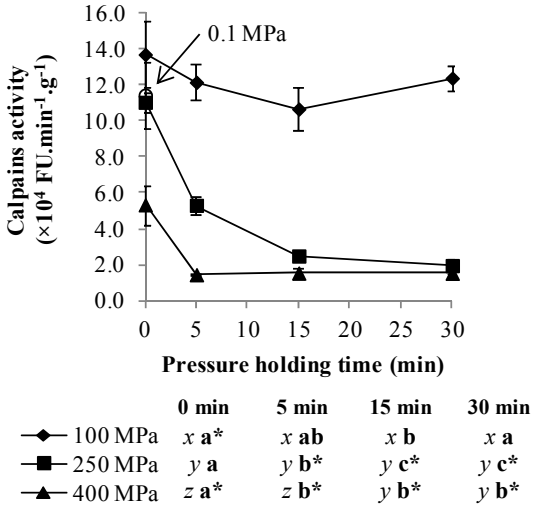



**Figure 4.1.5.** Calpains activity in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*z*) or between pressure holding times (**a**-**c**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa). *Abbreviations*: FU – fluorescence units.

The decrease in calpains activity at higher pressure levels and longer holding times was probably caused by the dissociation of both subunits of calpains [269]. A similar decrease of calpains activity with pressure level was observed in previous studies with minced sea bass [269], which may delay *post mortem*

degradation due to the higher hardness of HPP samples during refrigerated storage [16].

### 3.3. *Electrophoreses*

#### 3.3.1. *SDS-PAGE*

Molecular weights of major sarcoplasmic proteins in sea bass fillets were 84.3, 52.6-54.0, 41.0, 20.9-27.2, 14.1-15.6, 10.8, 3.9, and 1.6-2.2 kDa (Figure 4.1.6). The protein bands with 48.3-41.0 kDa, those with 27.2-24.7 kDa, and those with 80.6 and 27.2 kDa showed molecular weights similar to those of cathepsin D, cathepsin B, and calpains, respectively [268; 270]. Still, different proteins might be included in the same protein bands.

Changes in sarcoplasmic proteins profile from HPP sea bass fillets were followed by SDS-PAGE after protein adjustment to the same concentration (Figure 4.1.6 and Table 4.1.3). Electrophoretic profiles show that a total of 34 protein bands in the range 1.6-158.8 kDa presented significant differences among HPP treatments. Also, protein profiles of HPP control samples (0 min of pressure holding time at 100, 250, and 400 MPa) presented 16 protein bands with higher intensities than the same bands in 0.1 MPa samples (158.8, 146.9, 76.0, 51.6-54.0, 38.3-41.0, 22.2-24.7, 14.1-19.2, and 10.8-12.2 kDa). A similar pattern was found in HPP squid treated at 300 MPa [271].

Concerning pressure level, 400 MPa treatments caused marked changes in the profile of sarcoplasmic proteins, as compared to 100 MPa (Figure 4.1.6). The intensity of protein bands with 20.9, 19.2, 18.8, 12.2, and 7.4 kDa increased with pressure level in all pressure holding times, while the opposite was observed with the protein band with 91.8 kDa, which was undetectable at 250 and 400 MPa. On the other hand, the protein band with 13.6 kDa appeared only at 400 MPa (5-30 min).

In what concerns pressure holding time, the decrease in intensity of protein bands with higher molecular weights was accompanied by an increase in intensities of protein bands with lower molecular weights. At 400 MPa, the intensity of 14 protein bands (158.8, 146.9, 84.3, 76.0, 54.0, 52.6, 48.3, 41.0, 24.7, 18.8, 15.6, 14.1, 13.6, and 10.8 kDa) gradually decreased with longer processing times. The same patterns were observed in the protein bands with 52.6 and 24.7 kDa at 250 MPa. In contrast, the intensity of the protein band with 20.9 kDa increased with pressure holding time at 250 MPa, and those with 5.3, 3.3, and 2.8 kDa increased at 400 MPa. These variations with pressure

holding time do not seem to happen in squid, as it was not highlighted in the study of Gou and co-authors [271] where squid was treated at 300 MPa (0-20 min).

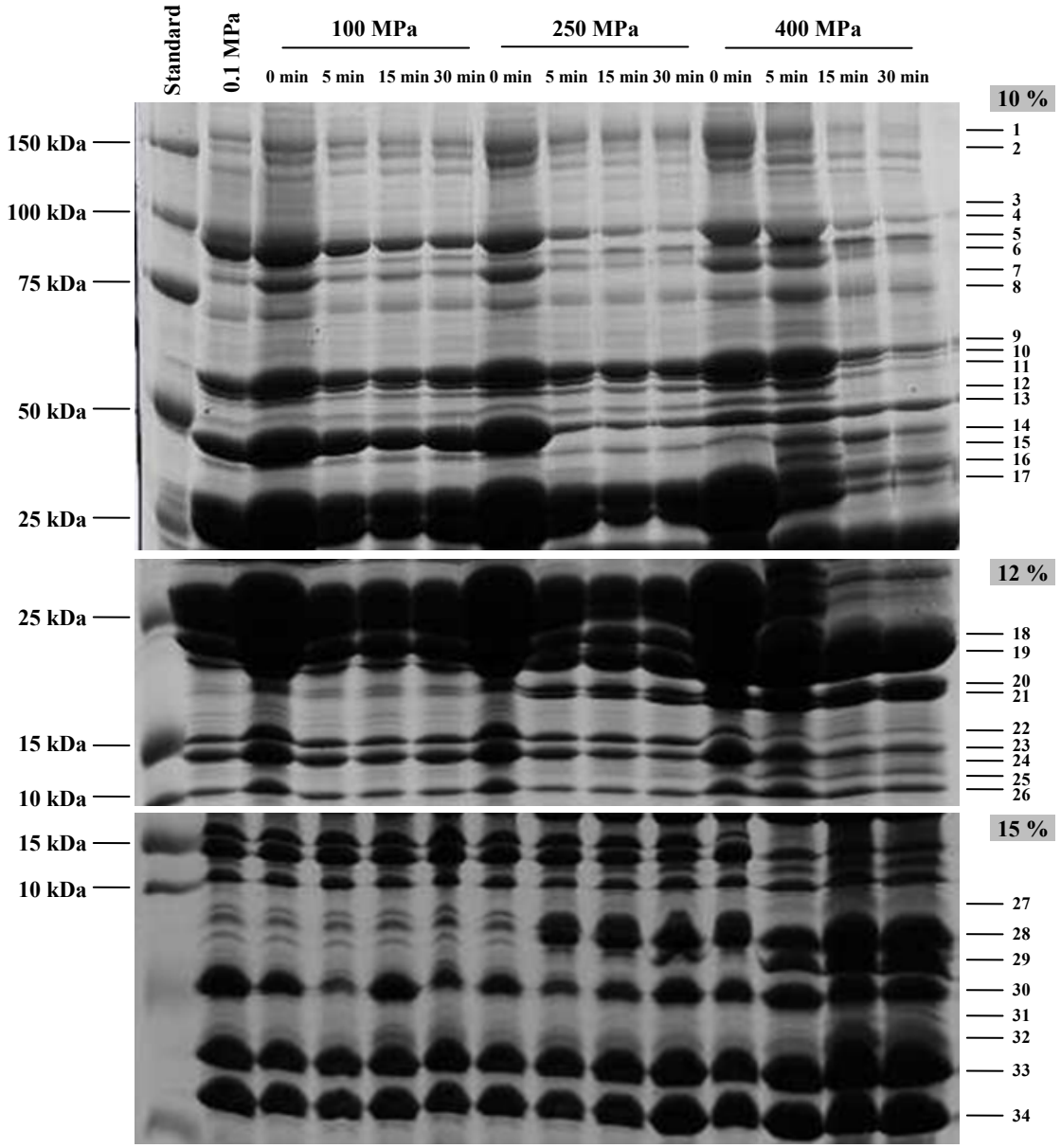



**Figure 4.1.6.** SDS-PAGE protein profiles of sarcoplasmic proteins extracted from sea bass fillets treated with high pressure. Proteins separation was achieved in gels containing 10, 12, and 15 % of polyacrilamide (identified in the right side of the figure). Molecular weight of standard proteins is also indicated on the left side of the figure. Protein bands were numbered (numbers aligned with protein bands) and the molecular weight is indicated in Table 4.1.3 .

In terms of pressurization rate (data not shown), the intensity of most protein bands decreased with the increase in pressurization rate at 250 MPa, while the opposite was observed at 400 MPa. However, no clear pattern was observed at

100 MPa, as the fastest rate decreased the intensity of several protein bands in samples treated during 0 min, but not during 5 min.

**Table 4.1.3.** Statistical differences in the integrated optical densities (IOD) of protein bands obtained by SDS-PAGE of sarcoplasmic proteins extracted from sea bass fillets treated with high pressure (electropherograms shown in Figure 4.1.6).

| Band number | Molecular weight (kDa) | 100 MPa | | | | 250 MPa | | | | 400 MPa | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 min | 5 min | 15 min | 30 min | 0 min | 5 min | 15 min | 30 min | 0 min | 5 min | 15 min | 30 min |
| 1 | 158.8 ± 10.2 | *y* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *xy* **a** * | *x* **a** * | *x* **b** | *x* **b** |
| 2 | 146.9 ± 10.8 | *x* **a** * | *x* **b** | *x* **b** | *x* **b** | *x* a * | *x* **b** | *x* **b** | *x* **b** | *x* **a** * | *x* **ab** | *x* **b** | N.D.* |
| 3 | 91.8 ± 9.9 | a * | b * | b * | b * | N.D.* | N.D.* | N.D.* | N.D.* | N.D.* | N.D.* | N.D.* | N.D.* |
| 4 | 84.3 ± 7.7 | *x* **a** | *x* **b** * | *x* **b** | *x* **b** | *y* **a** | *y* **b** | *y* **b** | *y* **b** | *z* **a** * | *x* **a** | *y* **b** | *y* **b** |
| 5 | 80.6 ± 7.2 | *x* **a** | *y* **a** | *y* **a** | *x* **a** | *x* **a** | *y* **a** | *y* **a** * | *x* **a** | *x* **b** * | *x* **a** * | *x* **b** * | *x* **b** * |
| 6 | 76.0 ± 6.7 | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *y* **b** | *x* **b** | *x* **b** * | *y* **a** * | *x* **b** * | *x* **c** | *x* **c** * |
| 7 | 70.5 ± 4.5 | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **b** | *x* **a** * | *x* **b** | *x* **b** |
| 8 | 67.8 ± 4.8 | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **ab** | *x* **a** * | *x* **b** | *x* **b** |
| 9 | 54.0 ± 3.4 | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *x* **a** * | *x* **b** * | *y* **b** * |
| 10 | 52.6 ± 3.1 | *y* **a** * | *y* **b** | *x* **b** | *x* **b** | *y* **a** * | *y* **b** | *xy* **bc** | *y* **c** * | *x* **a** * | *x* **b** * | *y* **c** * | *y* **c** * |
| 11 | 51.6 ± 2.9 | *x* **a** * | *y* **b** | *x* **b** | *xy* **b** | *x* **a** * | *y***b** | *x* **b** * | *x* **ab** * | *x* **b** * | *x* **a** * | *x* **c** | *y* **c** |
| 12 | 48.3 ± 4.0 | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** * | *y* **a** | *x* **a** | *x* **a** | *x* **a** * | *x* **a** * | *x* **b** | *y* **b** * |
| 13 | 41.0 ± 2.6 | *x* **a** * | *x* **bc** | *x* **c** | *x* **b** * | *x* **a** * | *z* **b** * | *y* **b** * | *y* **b** * | *y* **a** * | *y* **a** * | *y* **b** * | *y* **b** * |
| 14 | 38.3 ± 2.5 | *x* **a** * | *y* **b** | *y* **b** | *y* **b** | *x* **a** * | *y* **a** | *y* **a** | *y* **a** | *x* **c** * | *x* **a** * | *x* **b** * | *x* **c** * |
| 15 | 33.1 ± 2.3 | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **b** | *x* **a** * | *x* **b** | *x* **b** |
| 16 | 27.2 ± 1.6 | *x* **a** * | *x* **a** | *x* **a** | *x* **a** | *x* **a** * | *x* **ab** | *x* **b** | *x* **ab** | *x* **a** | *y* **b** * | *y* **b** * | *y* **b** * |
| 17 | 24.7 ± 1.0 | *x* **a** * | *x* **b** | *x* **b** | *x* **b** | *x* **a** * | *x* **b** | *x* **bc** * | *y* **c** * | *x* **a** * | *x* **b** | *y* **c** * | *z* **c** * |
| 18 | 22.2 ± 0.2 | *x* **a** * | *y* **b** | *xy* **b** | *x* **b** | *x* **a** * | *y* **b** | *y* **b** | *y* **b** | *x* **b** * | *x* **a** * | *x* **c** | *xy* **c** |
| 19 | 20.9 ± 0.2 | *y* **a** | *z* **a** | *y* **a** | *y* **a** | *y* **b** * | *y* **ab** * | *x* **a** * | *x* **a** * | *x* **a** * | *x* **a** * | *x* **a** * | *x* **a** * |
| 20 | 19.2 ± 0.3 | *y* **a** * | *z* **a** | *z* **a** | *z* **a** | *y* **a** * | *y* **a** * | *y* **a** * | *y* **a** * | *x* **a** * | *x* **a** * | *x* **a** * | *x* **a** * |
| 21 | 18.8 ± 0.3 | *y* **a** * | *z* **b** | *y* **b** | *y* **b** | *y* **ab** * | *y* **ab** * | *xy* **b** * | *x* **a** * | *x* **a** * | *x* **a** * | *x* **b** * | *x* **b** * |
| 22 | 15.6 ± 0.3 | *x* **a** * | *x* **b** | *x* **b** | *x* **b** | *x* **a** * | *x* **b** | *x* **b** | *x* **b** | *y* **a** * | *x* **ab** | *y* **bc** * | *y* **c** * |
| 23 | 14.1 ± 0.5 | *xy* **a** * | *y* **b** | *x* **b** | *x* **b** | *y* **a** * | *y* **b** | *x* **b** | *y* **b** * | *x* **a** * | *x* **b** * | *x* **c** * | *y* **c** * |
| 24 | 13.6 ± 0.5 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | **a** * | **b** * | **c** * |
| 25 | 12.2 ± 0.4 | *z* **a** * | *y* **b** | *y* **ab** | *y* **ab** | *y* **a** * | *y* **b** | *y* **b** | *y* **b** * | *x* **b** * | *x* **a** * | *x* **bc** * | *x* **c** * |
| 26 | 10.8 ± 0.4 | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *y* **b** | *x* **b** | *x* **b** | *x* **a** * | *x* **a** * | *x* **b** * | *x* **b** |
| 27 | 9.5 ± 0.4 | *x* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **a** | *xy* **a** | *y* **a** | *y* **a** | *x* **c** | *x* **bc** | *x* **a** * | *x* **b** * |
| 28 | 7.4 ± 0.3 | *y* **a** | *y* **a** | *z* **a** | *z* **a** * | *y* **b** | *x* **a** * | *y* **a** * | *y* **a** * | *x* **b** * | *x* **c** * | *x* **a** * | *x* **ab** * |
| 29 | 5.3 ± 0.2 | *x* **a** | *y* **a** | *y* **a** | *z* **a** | *x* **b** | *y* **b** | N.D. | *y* **a** * | N.D. | *x* **b*** | *x* **a*** | *x* **a*** |
| 30 | 3.9 ± 0.1 | *x* **b** * | *y* **c** * | *x* **a** | *y* **b** * | *x* **b** * | *y* **c** * | *y* **bc** * | *x* **a** * | *x* **b** | *x* **a** | *x* **a** | *x* **a** * |
| 31 | 3.3 ± 0.0 | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **b** | *x* **b** | *x* **a** * | *x* **a** * |
| 32 | 2.8 ± 0.1 | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **b** | *x* **b** | *x* **a** * | *x* **a** * |
| 33 | 2.2 ± 0.1 | *x* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **a** | *xy* **a** | *y* **a** | *xy* **a** | *x* **c** | *x* **bc** | *x* **a** * | *x* **ab** * |
| 34 | 1.6 ± 0.2 | *x* **a** | *y* **a** * | *y* **a** | *y* **a** | *x* **b** | *y* **b** | *y* **b** | *x* **a** | *x* **b** | *x* **ab** * | *x* **a** * | *x* **a** * |

*Abbreviations*: N.D. – band not detected.
Only bands with IOD of one or higher in at least one treatment are shown.
Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*z*) or between pressure holding times (**a-c**), and the symbol * denotes significant differences with non-treated samples (0.1 MPa).

The results obtained in the current study revealed a higher effect of pressure level in the protein profiles of sarcoplasmic proteins compared to other food matrixes like turkey [272] and bovine [259]. The muscle of terrestrial animals and fish tenderizes at different rates, and fish tenderization diminishes firmness and acceptability by consumers [22]. The results suggest that fish proteins may be more susceptible to HPP treatments, as compared to terrestrial animals. Chéret and co-authors [262] also studied the effect of pressure level in sea bass fillets,

but protein profiles of sarcoplasmic extracts revealed less differences, which might be due to the smaller amount of proteins loaded for the electrophoresis.

The decrease in the intensity of protein bands might be due to protein degradation or fragmentation, and to insolubilization of sarcoplasmic proteins as a result of the formation of proteins aggregates [259]. On the other hand, the increase in the intensity of some protein bands could be explained by an increase in the solubility of myofibrillar proteins as a result of modifications in the protein structure with the HPP treatment [259].

### 3.3.2. *IEF*

The effect of HPP in the protein profiles of native sarcoplasmic proteins was followed by IEF (Figure 4.1.7). The pI values of major sarcoplasmic proteins in sea bass fillets were *ca*. 8.04-8.63, 5.98, and 4.36-4.50. Comparing the profiles of HPP samples obtained by IEF and SDS-PAGE, the results of IEF showed less changes, as only 12 protein bands were detected in the IEF profiles. Once again, the differences in protein bands intensities were mainly due to variations in pressure level and holding time, and not as much with pressurization rate.

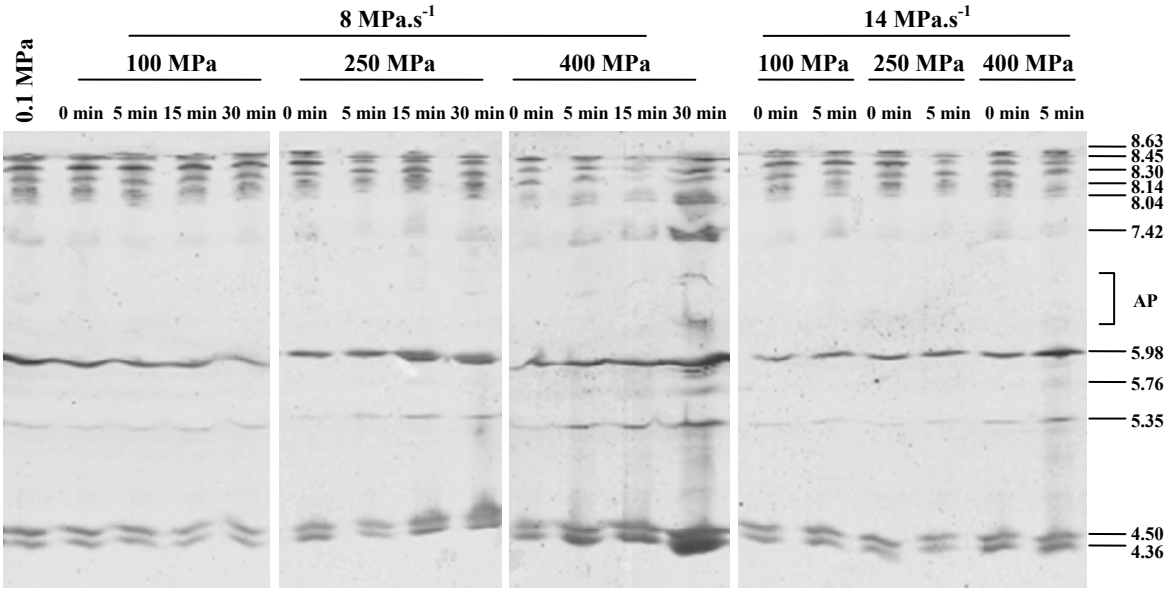



**Figure 4.1.7.** IEF protein profiles of sarcoplasmic proteins extracted from sea bass fillets treated with high pressure. Isoelectric point of proteins bands is indicated on the right side of the figure. *Abbreviations*: AP – sample application point.

The increase in pressure level and holding time lead to the intensification of the protein bands with pI in the range from 4.36 to 7.42. Although this increased has been observed for most of protein bands, samples treated at 400 MPa (15 min) showed lower intensities in protein bands with pI of 8.30-8.63 than

400 MPa (5 and 30 min). Additionally, the protein band with pI value of 5.76 appeared only in the treatments at 400 MPa (5-30 min). In relation to pressurization rate, most differences in IOD values were not evident by human visual inspection of electropherograms. The exceptions were for protein bands with pI of 8.45, 8.30, and 8.04, where higher intensities were observed in the fastest pressurization rate treatments at 400 MPa.

HPP treatments effects in IEF profiles were studied in minced salmon and saithe samples [273], but sarcoplasmic proteins of these species behaved differently when compared to the current study. In fact, three protein bands with pI near 5.85 were not detected in samples treated at 350 MPa (15 min). Still, the IEF profile was differently affected with the HPP treatment according to fish species.

## 4. Conclusions

In conclusion, HPP induced significant changes in the activities of several enzymes of sea bass muscle with pressure level and holding time, but only minor changes were detected with pressurization rate. The increase in pressure level and holding time decreased the protein concentration in sarcoplasmic extracts, and also the activity of calpains. The highest reductions were observed at 400 MPa, for acid phosphatase, cathepsin D, and calpains activities. HPP also caused noticeable modifications in sarcoplasmic proteins profiles (SDS-PAGE and IEF) accompanying pressure level and holding time, which might be due to denaturation, fragmentation, and aggregation of proteins. This study brought new evidence about the effect of HPP in sarcoplasmic protein profiles and in the activity of degradative enzymes, which might be useful to control the softening of fish muscle tissues due to autolytic reactions. Nevertheless, further studies are still needed to understand the effect of HPP treatments in the quality attributes of sea bass fillets during refrigerated storage.

# CHAPTER 4.2.

# Effect of high pressure processing in the quality of sea bass (*Dicentrarchus labrax*) fillets: pressurization rate, pressure level and holding time

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**Abstract**

High pressure processing (HPP) is a technology of growing interest for food preservation, namely due to its ability to reduce microorganisms and to improve food functional properties. The effect of three variables (pressure levels of 100, 250, and 400 MPa; pressure holding times of 0, 5, 15, and 30 min; and pressurization rates of 8 and 14 MPa.s$^{-1}$) in microbiological and physical (pH, colour, and water holding capacity - WHC) analyses was studied in sea bass fillets. Additionally, the effect of HPP on myofibrillar proteins was studied by SDS-PAGE and isoelectric focusing electrophoreses.

Fillets treated at 100 MPa were not significantly different from non-treated samples. Nonetheless, pressurization rate induced changes in all parameters evaluated, though in a lesser extent than pressure level and holding time. The magnitude of changes increased with the pressure level (250 and 400 MPa) and holding time (0-30 min), being 400 MPa during 30 min the treatment responsible for the major changes. Generally, microbiological load and WHC decreased, fillets become whitish and similar to cooked fish, pH increased, and myofibrillar proteins with molecular weights below 30 kDa increased, whereas those with lower isoelectric point values decreased. The microbiological safety of sea bass fillets was improved with HPP treatments, suggesting a potential extension in shelf life. In conclusion, HPP can be a potential tool to process sea bass fillets and to delay the degradation of fish muscle during storage.

**Keywords**

High pressure processing; sea bass; myofibrillar proteins; microbiology; SDS-PAGE; IEF

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## 1. Introduction

The increase in consumer's preference for fresh and minimally processed food products, rather than processed and frozen ones, demands for research on new processing and preservation methods, especially for products with short shelf life. Freshness of fish and fish products is lost rapidly during *post mortem* degradation due to autolytic degradation by the action of endogenous enzymes, leading to prime quality loss [28]. The degradation of proteins due to enzymatic action creates ideal conditions for the growth of microorganisms that contaminate the muscle after fish death, and is responsible for spoilage [16; 21].

High pressure processing (HPP) is a technology of growing interest for the processing and preservation of food. This technology has the potential to better retain food's nutritional and organoleptical characteristics, when compared with traditional thermal processing [114]. HPP can also be used by the food industry to create new product textures, since it induces modifications on food functional properties [117]. HPP treatments have also the ability to inactivate spoilage and pathogenic microorganisms thus extending food shelf life [114], and non-covalent chemical bonds of molecules are affected by HPP inducing modifications in water, proteins, polysaccharides, and lipids [39].

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species widely distributed in the Mediterranean Sea and Atlantic Ocean and intensively farmed in several Mediterranean countries, *e.g.* Greece, Spain, and Italy [6]. This species is highly appreciated because of its excellent organoleptic properties and reasonable price [6]. Published data shows that the effect of HPP on fresh fish quality varies with species. In sea bass, pressure level has been only studied in fillets subjected to short-time treatments (5-10 min) [16; 274]. However, longer pressure holding times and different pressurization rates have not yet been addressed, and both variables might influence the quality of fresh fish.

This study aims to understand the effect of HPP treatments on quality parameters of fresh sea bass fillets. In particularly, pressure level (0.1-400 MPa), pressure holding time (0-30 min), and pressurization rate (8 and 14 $MPa.s^{-1}$) were tested to evaluate their influence on microbiological and physical analyses, namely pH, colour, and water holding capacity. Additionally, myofibrillar proteins of HPP sea bass were separated according to sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and isoelectric focusing (IEF) electrophoreses, to identify changes on protein profiles.

## 2. Material and methods

### 2.1. *Chemicals*

The chemicals trizma hydrochloride (Tris-HCl), dithiothreitol (DTT), urea, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), glycine, potassium chloride, glycerol, magnesium chloride, β-mercaptoethanol, sodium phosphate dibasic, sodium dihydrogen phosphate, and potassium phosphate monobasic were obtained from Sigma-Aldrich (Steinheim, Germany); sodium dodecyl sulphate (SDS) from Fluka (Buchs, Germany); plate count agar and bromophenol blue from Merck (Darmstadt, Germany); Coomassie Brilliant Blue R250 from Bio-Rad (Philadelphia, USA); Phastgel blue R from Amersham Pharmacia Biotech (Uppsala, Sweden); maximum recovery diluent from Oxoid (Hampshire, England); ethylene diamine tetracetic acid (EDTA calibration sample) from LECO (LECO corporation, St. Joseph, USA); the water used was Milli-Q purified and distilled.

### 2.2. *Preparation of samples*

Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens, with commercial quality, were acquired from a local market. The average weight of each fish was 490 ± 75 g and the total length was 35 ± 2 cm. Fish samples (n = 14) were filleted and the skin was removed. Fillets were divided in portions of *ca*. 15 g, and eight portions randomly selected were used for each treatment to reduce the effect of specimen variability. Fillet portions were vacuum packed in low-oxygen permeable barrier bags (Colamin XX 100e, Obermühle, Pössneck, Germany) using a vacuum packager (Packman, Albipack, Águeda, Portugal) before pressurization treatments.

### 2.3. *High pressure treatments*

HPP treatments were carried out in a hydrostatic press (High pressure system U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature, using a mixture of propylene glycol and water (1:1) as pressurizing fluid. Samples (8 fillet portions per treatment) were processed at pressure levels of 100, 250, and 400 MPa during 5, 15, and 30 min using a pressurization rate of *ca*. 8 MPa.s$^{-1}$ at 6 ºC. A higher pressure rate increment, *ca.* 14 MPa.s$^{-1}$, was also tested to process samples at the same pressure levels (100, 250, and 400 MPa) for 5 min. Control treatments of 0 min pressure holding time were performed subjecting the fillets to pressurization and immediate depressurization. Nineteen treatments were

studied (Table 4.2.1) including non-treated samples (0.1 MPa). After HPP treatments, samples were immediately frozen in liquid nitrogen and stored at -20 ºC until further analyses, except those for microbiological analysis.

**Table 4.2.1.** Treatment conditions tested.

| | Pressurization rate | Pressure level | Pressure holding time |
|---|---|---|---|
| **Non-treated** samples not subjected to high pressure processing | | | |
| **Treated** samples subjected to high pressure processing | 8 $MPa.s^{-1}$ | 100 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | | 250 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | | 400 MPa | 0 min |
| | | | 5 min |
| | | | 15 min |
| | | | 30 min |
| | 14 $MPa.s^{-1}$ | 100 MPa | 0 min |
| | | | 5 min |
| | | 250 MPa | 0 min |
| | | | 5 min |
| | | 400 MPa | 0 min |
| | | | 5 min |

The time indicated in each treatment does not include the duration of pressurization and depressurization.

**2.4. *Microbiological analysis*** Sea bass muscle (10 g) was aseptically collected to a Stomacher bag with filter. A primary ten-fold dilution was made with maximum recovery diluent and homogenized for one minute at 230 rpm using a Stomacher homogenizer (Laboratory blender STOMACHER 400, Seward Laboratory Systems Inc., Florida, USA). Appropriate series of decimal dilutions were then prepared in maximum recovery diluent, and homogenates were poured into molten plate count agar for enumeration. Total counts were performed after incubation at 30 ºC for 3 days. Petri dishes containing 30-300 colony forming units (CFU) were selected for counting, and the results were expressed in logarithms of the CFU number per g of muscle. Determinations were performed in duplicate.

## 2.5. *Physical properties*

**2.5.1. *Colour*** Colour measurements were assessed with a colorimeter (CR-410, Konica Minolta Camera, Co, Japan) with minced sea bass, to avoid colour heterogeneity of fillets. The colorimeter was initially calibrated against a specific white standard plate (CIE $L^*a^*b^*$ system: $L^* = 97.79$; $a^* = -0.02$; $b^* = 1.84$). Lightness ($L^*$), red-green value ($a^*$), and yellow-blue value ($b^*$) were measured, and chroma ($C^*$) and whiteness (W) estimated according to

Hernández and co-authors [220] and Ramirez-Suarez & Morrissey [266], as follows:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$W = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2}$$

Four measurements were recorded per treatment.

**2.5.2. *pH***

The pH was measured directly on minced sea bass fillets using a surface calibrated pH electrode (SenTix 21, WTW, Weilheim, Germany) connected to a pH meter (microprocessor pH meter 539 WTW, Weilheim, Germany). Four measurements were recorded per treatment.

**2.5.3. *Water holding capacity***

The water holding capacity (WHC) was measured with the modified method of Sánchez-González and co-authors [275]. Briefly, a piece of fillet (2 g; *ca.* 1.5 $cm^3$; $W_s$) wrapped in two filter papers (also weighted, $W_i$; Whatman #1) was centrifuged (3,000 ×*g*, 10 min, 20 ºC; centrifuge 3K30, Sigma, Osterode, Germany), and after centrifugation, the sample was removed and the filter papers weighted ($W_f$). WHC was expressed as g of water in sample after centrifugation per 100 g of water initially present in sample:

$$WHC = \frac{W_s \times \frac{H}{100} - (W_f - W_i)}{W_s \times \frac{H}{100}} \times 100$$

where H is moisture (%). Four determinations were performed per treatment.

## 2.6. *Electrophoreses*

**2.6.1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)***

Muscle samples were added to cold buffer (75 mM potassium chloride, 10 mM potassium phosphate monobasic, 2 mM magnesium chloride, 2 mM EGTA, pH 7.0; 4 ºC) and homogenized using an Ultra turrax homogenizer (Ultraturrax T25, Janke & Kunkel IKA®- Labortechnik). The homogenate was centrifuged (10,000 ×*g*, 10 min, 4 ºC) and the supernatant (sarcoplasmic proteins) discarded. Fresh buffer was added to the pellet and the process (homogenization and centrifugation) repeated three times to obtain a pellet that was used as myofibrillar proteins. Myofibrillar protein solutions (with the same adjusted protein concentration) were diluted 1:1 in loading buffer (62.5 mM Tris-HCl, 20 % v/v glycerol, 2 % w/v SDS, 5 % v/v β-mercaptoethanol, and 0.01 % w/v bromophenol blue at pH 6.8) and heated (100 ºC, 3 min) prior to gel running.

For SDS-PAGE, gels with different resolving power were used (10 and 12 % of polyacrylamide) with stacking gels of 4 %. The amount of sample loaded per lane was 200 µg of protein. Molecular weight standards (Precision Plus Protein™ Standards All Blue, Bio-Rad, Philadelphia, USA) were used in every gel. Electrophoresis was performed in a vertical unit (Hoefer SE 600 series, Amersham Pharmacia Biotech, Uppsala, Sweden) at 10 ºC (LAUDA Ecoline Staredition RE 104, Lauda-Konigshofen, Germany) with running buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1 % SDS, pH 9), and the running conditions limited to 25 mA (EPS 601, Amersham Pharmacia Biotech, Uppsala, Sweden). Protein bands were fixed and stained with Coomassie Brilliant Blue R-250. Determinations were performed in triplicate.

**2.6.2. *Isoelectric focusing (IEF) with CleanGel***

To prepare myofibrillar extracts for IEF, muscle samples were homogenized with cold water (4 ºC) to avoid band disturbances due to the presence of salts. The homogenate was centrifuged (40,000 ×*g*, 4 ºC, 20 min) and the supernatant (sarcoplasmic proteins) discarded. Then, the pellet was homogenized with extraction solution (8 M urea and 0.1 M DTT in 20 mM sodium phosphate buffer at pH 6.5) and centrifuged (20,000 ×*g*, 20 ºC, 15 min). The protein concentration of the supernatant (myofibrillar proteins) was adjusted to 10 mg.mL$^{-1}$ with the same extraction solution.

Myofibrillar protein solutions were analysed by IEF using a CleanGel (Amersham Pharmacia Biotech, Uppsala, Sweden) according to Silva and co-authors [257] with minor modifications. For a full-size CleanGel, 21 mL of a solution containing urea (8 M), ampholine preblended (0.26 mL, pH 3.5-9.5; Pharmacia Biotech, Uppsala, Sweden), and ampholine (1.31 mL, pH 3.5-5.0; Pharmacia Biotech, Uppsala, Sweden) was used to rehydrate. Electrode wicks (Serva 42972, Heidelberg, Germany) were soaked with anode fluid (Serva 42984, Heidelberg, Germany) and cathode fluid (Serva 42986, Heidelberg, Germany). The electrophoresis running conditions were: prefocusing (30 min) – 500 V, 8 mA, 8 W; sample entrance (20 min) – 500 V, 8 mA, 8 W; and isoelectric focusing (5,000 Vh) – 2,000 V, 14 mA, 14 W (MultiDrive XL, Amersham Pharmacia Biotech, Uppsala, Sweden) at 15 ºC (Multitemp II thermostatic circulator model 2219, Amersham, Sweden). After prefocusing, the sample application pieces (Amersham Pharmacia Biotech, Uppsala, Sweden) were placed 2 cm in front of the cathodic wick, and 20 µL of samples and 7.5 µL of isoelectric point (pI) marker solutions (isoelectric focusing

calibration kit pH 3-10 and pH 5-10.5, Amersham Pharmacia Biotech, Uppsala, Sweden) were applied. After IEF run, proteins were fixed and stained with Coomassie Phastgel blue R. Determinations were performed in triplicate.

#### 2.6.3. *Identification and quantification of proteins bands*

Gels were scanned using a densitometer (model GS-800, Bio-Rad, Philadelphia, USA) and the images analysed with PDI Discovery Series software (module Quantity One version 2.4; Bio-Rad, Philadelphia, USA). Molecular weights, pI values, and changes in integrated optical density (IOD) of protein bands were calculated.

#### 2.6.4. *Protein concentration in myofibrillar extracts*

The protein content of myofibrillar protein solutions used for SDS-PAGE was determined using a FP-528 LECO nitrogen analyser (LECO, St. Joseph, USA), calibrated with EDTA calibration sample (carbon – 41.07 ± 0.17, hydrogen – 5.55 ± 0.02, nitrogen – 9.57 ± 0.03), according to the Dumas method [226]. The protein concentration of myofibrillar protein solutions used for IEF was determined through absorbance measurements at 280 nm [276]. Three replicates were performed per treatment.

### 2.7. *Statistical analysis*

Differences between treatments were tested at a 0.05 level of probability with the software STATISTICA™ 6.1 (Statsoft, Inc., Tulsa, OK, USA). The Dunnett test was used to identify differences between HPP and non-treated samples. The effects of pressure level and pressure holding time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences between treatments. To study the effect of pressurization rate, a t-test for independent samples was used.

## 3. Results and discussion

### 3.1. *Microbiological analysis*

In live fish, microorganisms are found on the outer surface (skin and slime), and in some inner surfaces like gills and gastro-intestinal tract, while the remaining inner tissues of a healthy fish are sterile [43-44]. After fish death, the presence of bacteria in the muscle results from contamination during processing and handling, where a maximum of 5 log CFU.g$^{-1}$ of total counts is considered the upper limit for seafood having good quality [47]. High pressures cause collapse of intracellular vacuoles, and damage to cell walls and cytoplasmic membranes, and bacteria in the log phase of growth are, in general, more barosensitive than cells in the stationary or dormant phases [3].

The results of total viable counts are shown in Figure 4.2.1. Non-treated samples showed a level of bacterial load below 5 log CFU.g$^{-1}$, and most of the treatments at 100 MPa did not cause significant changes in those numbers. Bacteria counts decreased with pressure holding time at 250 and 400 MPa, and this decrease was more accentuated at 400 MPa. In fact, samples treated at 400 MPa during 30 min showed a 2 log units reduction compared with non-treated samples.

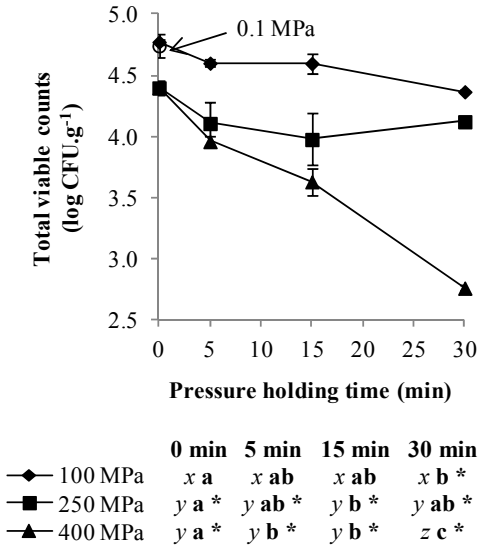



**Figure 4.2.1.** Mesophilic aerobic bacterial variation in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x-z*) or pressure holding times (**a-c**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa). *Abbreviations*: CFU – colony forming units.

In what concerns the pressurization rate (Table 4.2.2), statistical differences were found in mesophilic bacterial counts in 0 min treatments for all pressure levels. Still, those differences were lower than 0.5 log units.

Previous studies reported similar results for sea bass fillets treated at 100 MPa, though higher reductions were attained at higher pressure levels (300-500 MPa; 5 min) [16]. Recently, fresh salmon showed microbial reductions of 3 log units when treated at 150 MPa during 15 min [124], though at a higher pressure level and holding time (200 MPa, 20 min) total counts did not decrease in cold smoked salmon [277]**.** Product characteristics, experimental procedures, and the presence of a different microflora might explain the different results between studies.

**Table 4.2.2.** Variations in quality parameters of sea bass fillets treated with high pressure at 8 and 14 MPa.s$^{-1}$ pressurization rates.

| | Pressure level (MPa) | 0 min | | 5 min | |
|---|---|---|---|---|---|
| | | 8 MPa.s$^{-1}$ | 14 MPa.s$^{-1}$ | 8 MPa.s$^{-1}$ | 14 MPa.s$^{-1}$ |
| **Mesophilic aerobic bacteria** (log CFU.g$^{-1}$) | 100 | 4.77 ± 0.03$^{b}$ | 5.01 ± 0.00$^{a}$ | 4.60 ± 0.03$^{A}$ | 4.77 ± 0.06$^{A}$ |
| | 250 | 4.36 ± 0.01$^{b}$ | 4.77 ± 0.03$^{a}$ | 3.99 ± 0.01$^{A}$ | 4.29 ± 0.03$^{A}$ |
| | 400 | 4.40 ± 0.03$^{a}$ | 4.18 ± 0.00$^{b}$ | 3.96 ± 0.04$^{A}$ | 3.87 ± 0.00$^{A}$ |
| **Colour parameters** | | | | | |
| **Red-green value** (*a**) | 100 | 7.8 ± 0.8$^{a}$ | 5.2 ± 1.8$^{b}$ | 7.2 ± 0.7$^{A}$ | 5.5 ± 1.7$^{A}$ |
| | 250 | 6.4 ± 0.3$^{a}$ | 4.4 ± 1.1$^{b}$ | 5.0 ± 0.2$^{A}$ | 5.1 ± 0.8$^{A}$ |
| | 400 | 6.9 ± 0.2$^{a}$ | 5.9 ± 1.2$^{a}$ | 2.6 ± 0.5$^{A}$ | 2.4 ± 0.2$^{A}$ |
| **Yellow-blue value** (*b**) | 100 | 12.2 ± 0.3$^{a}$ | 10.3 ± 1.6$^{a}$ | 12.1 ± 1.1$^{A}$ | 9.9 ± 1.4$^{B}$ |
| | 250 | 11.6 ± 0.4$^{a}$ | 9.3 ± 1.0$^{b}$ | 6.9 ± 1.6$^{A}$ | 7.5 ± 1.1$^{A}$ |
| | 400 | 8.0 ± 0.3$^{a}$ | 8.1 ± 0.8$^{a}$ | 5.6 ± 0.2$^{B}$ | 6.2 ± 0.2$^{A}$ |
| **Chroma** (C*) | 100 | 14.5 ± 0.7$^{a}$ | 11.6 ± 2.3$^{b}$ | 14.1 ± 1.3$^{A}$ | 11.3 ± 2.1$^{A}$ |
| | 250 | 13.3 ± 0.5$^{a}$ | 10.3 ± 1.4$^{b}$ | 8.6 ± 1.3$^{A}$ | 9.0 ± 1.3$^{A}$ |
| | 400 | 10.6 ± 0.2$^{a}$ | 10.0 ± 1.3$^{a}$ | 6.2 ± 0.3$^{B}$ | 6.7 ± 0.2$^{A}$ |
| **Whiteness** (W) | 100 | 63.0 ± 1.9$^{b}$ | 69.0 ± 2.2$^{a}$ | 63.6 ± 1.8$^{B}$ | 68.6 ± 1.6$^{A}$ |
| | 250 | 65.9 ± 0.4$^{b}$ | 70.9 ± 1.2$^{a}$ | 72.8 ± 0.8$^{A}$ | 72.6 ± 0.5$^{A}$ |
| | 400 | 68.5 ± 1.6$^{a}$ | 72.0 ± 2.9$^{a}$ | 77.5 ± 0.5$^{A}$ | 77.0 ± 0.5$^{A}$ |
| **pH** | 100 | 6.25 ± 0.04$^{a}$ | 6.17 ± 0.03$^{b}$ | 6.24 ± 0.04$^{A}$ | 6.12 ± 0.10$^{A}$ |
| | 250 | 6.06 ± 0.06$^{b}$ | 6.22 ± 0.06$^{a}$ | 6.54 ± 0.05$^{A}$ | 6.21 ± 0.06$^{B}$ |
| | 400 | 6.15 ± 0.05$^{a}$ | 6.19 ± 0.03$^{a}$ | 6.54 ± 0.04$^{A}$ | 6.35 ± 0.03$^{B}$ |
| **Water holding capacity** (%) | 100 | 57.9 ± 3.7$^{a}$ | 60.7 ± 1.9$^{a}$ | 56.6 ± 3.2$^{A}$ | 61.3 ± 3.1$^{A}$ |
| | 250 | 56.5 ± 5.5$^{b}$ | 65.6 ± 4.1$^{a}$ | 42.3 ± 2.6$^{A}$ | 46.7 ± 3.1$^{A}$ |
| | 400 | 50.2 ± 6.2$^{b}$ | 59.9 ± 4.0$^{a}$ | 42.6 ± 4.0$^{A}$ | 46.6 ± 2.3$^{A}$ |

*Abbreviations*: CFU – colony forming units.
Values are presented as average ± standard deviation.
Different letters denote significant differences ($p < 0.05$) between pressurization rates at the same pressure level for 0 min (a-b) or 5 min (A-B).

### 3.2. *Physical properties*

#### 3.2.1. *Colour*

Colour is an important quality attribute of food as influences appearance and presentation, and might determine the acceptability by consumers and purchasing decision [217]. The colour of fish muscle is related not only with carotenoids and hemepigments, namely myoglobin and haemoglobin [22], but also with the muscle physical structure and the amount of unbound water that influences light scattering [16].

The evolution of colour parameters according to pressure level and holding time is shown in Figure 4.2.2. Lightness (*L**) and whiteness (W) in non-treated samples were not significantly different to those observed in 100, 250, and 400 MPa control samples. The increase in pressure holding time did not significantly affect any colour parameter of samples treated at 100 MPa. However, samples lost translucency and became whiter with higher pressure levels (250 and 400 MPa) and with a further increase in pressure holding time, revealing a cooked appearance.

The effect of HPP treatments in the red-green and yellow-blue values was not as pronounced as in lightness and whiteness. In fact, the increase in pressure

holding time significantly lowered the red-green value for 400 MPa treatments, while the yellow-blue value significantly decreased with pressure holding time at 250 MPa, and both values decreased with pressure level. Chroma values of samples treated at 250 and 400 MPa (5-30 min) were significantly lower than in non-treated samples or samples treated at 100 MPa.

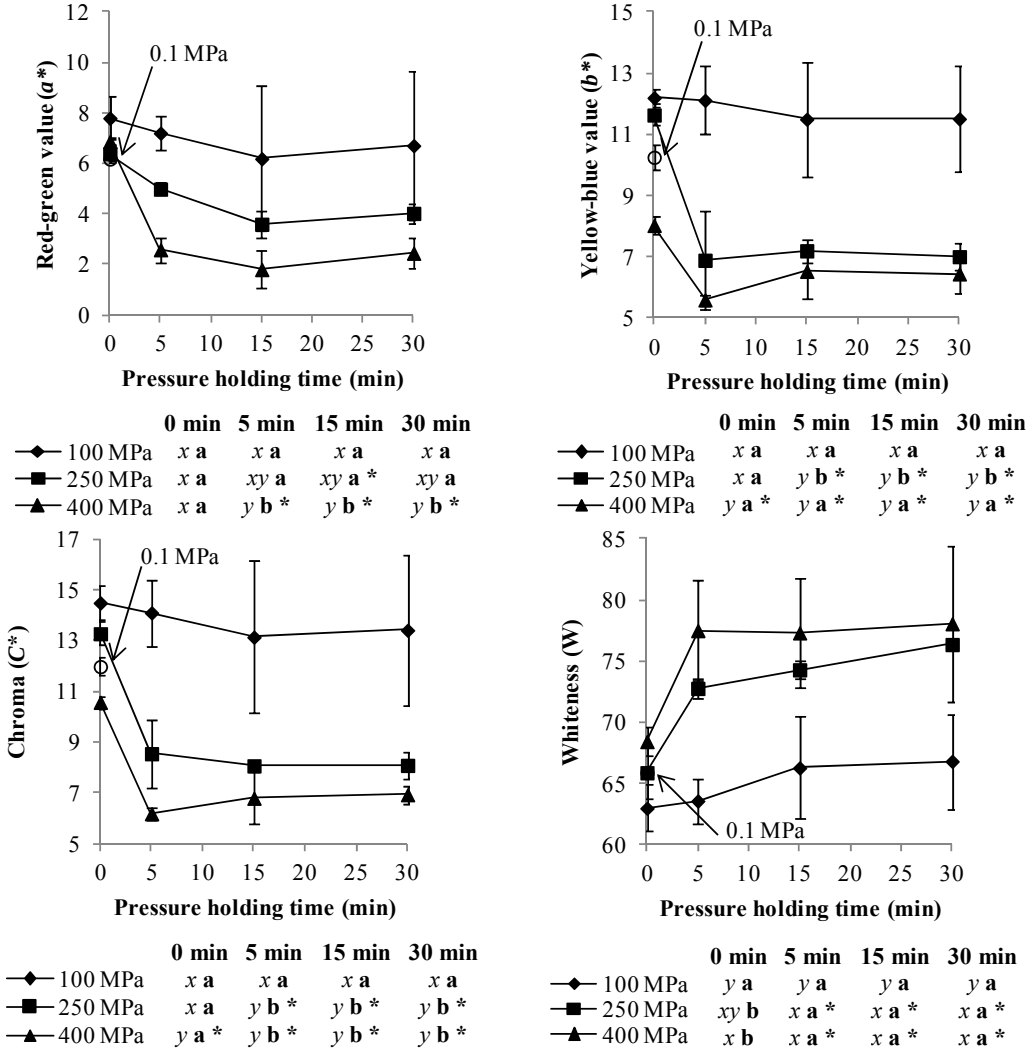



**Figure 4.2.2.** Colour parameters of sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*y*) or pressure holding times (**a-b**), and the symbol ***** denotes significant differences with non-treated samples (0.1 MPa).

Pressurization rate also significantly influenced the colour parameters of sea bass fillets (Table 4.2.2). Generally, at 0 min, whiteness values increased with pressurization rate, while chroma and red-green value decreased, though the effect of pressurization rate was not so evident with higher pressure holding times (5 min) and at 400 MPa.

A similar trend in *L** values with pressure level was described in previous studies with sea bass [16; 274], salmon [123], and sea bream [127]. However, different patterns were reported for the remaining colour parameters with pressure level [16; 274], which varied with the temperature used to pressurized samples [274]. Colour changes in sea bass muscle caused by HPP are mainly due to modifications in the protein matrix [16]. Similar colour changes occur in cooked fish muscle due to denaturation of myofibrillar and sarcoplasmic proteins [124; 129]. Still, the lowered fillet redness suggests that HPP treatments might cause pigments degradation.

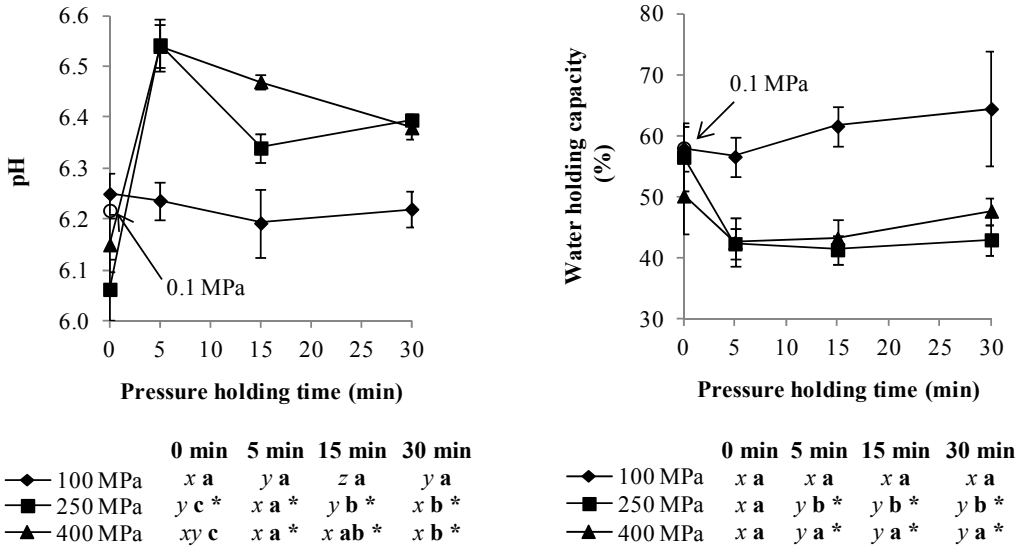
### 3.2.2. *pH*

*Post mortem* glycolysis of fish muscle results in the accumulation of lactate and $H^+$, which in turn lowers muscle pH [26], reduces the net surface charge on muscle proteins, and causes their partial denaturation [24]. The decrease in pH can lead to some WHC loss [24]. Additionally, pH strongly influences the microbiology of fish muscle, specially pH sensitive spoilage bacteria [43].

The pH of non-treated samples (pH = 6.2) was not significantly different to that found in control samples of 100 and 400 MPa treatments (Figure 4.2.3). At 100 MPa, pressure holding time did not reveal statistical variations in pH. However, at higher pressure levels (250 and 400 MPa) pH values significantly increased as pressure holding time rose to 5 min (pH = 6.4), though decreased with longer pressure holding times (15 and 30 min). Pressurization rate caused changes in pH values, although no pattern was observed (Table 4.2.2).

The variation in pH was attributed to conformational changes in muscle proteins associated with their denaturation, due to a more or less exposure of acidic and basic aminoacids groups. A similar mechanism has been recognized with cooked fish muscle, where a pH increase is related with the available acidic groups [278].

Previous studies with cod and tuna muscle revealed that pH increased with pressure level, and for tuna pH increased also with pressure holding time in short-time treatments (2-6 min) [266; 278]. In contrast, sea bream treated at 250 MPa during 5 min showed lower pH compared with non-treated samples [127]. Higher pH values in fish muscle have been associated with shorter shelf life, but it is not determinant [24].




| | 0 min | 5 min | 15 min | 30 min |
|---|---|---|---|---|
| 100 MPa | *x* **a** | *y* **a** | *z* **a** | *y* **a** |
| 250 MPa | *y* **c** * | *x* **a** * | *y* **b** * | *x* **b** * |
| 400 MPa | *xy* **c** | *x* **a** * | *x* **ab** * | *x* **b** * |

| | 0 min | 5 min | 15 min | 30 min |
|---|---|---|---|---|
| 100 MPa | *x* **a** | *x* **a** | *x* **a** | *x* **a** |
| 250 MPa | *x* **a** | *y* **b** * | *y* **b** * | *y* **b** * |
| 400 MPa | *x* **a** | *y* **a** * | *y* **a** * | *y* **a** * |

**Figure 4.2.3.** pH and water holding capacity variations in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*y*) or pressure holding times (**a-c**), and the symbol * denotes significant differences with non-treated samples (0.1 MPa).

### 3.2.3. *WHC*

Several quality parameters of fish products such as tenderness, juiciness, or succulence are influenced by the ability of muscle proteins to hold water molecules [260]. Free water accounts about 90 % of water in fish tissues, being held mainly in intracellular locations, where its presence is influenced by changes in protein structure, distribution of fluids between intra- and extracellular locations, pH, and ionic strength [279-280]. HPP treatments may degrade proteins and change the intracellular architecture of fibrils, which influences the ability of muscle cells to retain water [128].

The effect of HPP treatments in WHC of sea bass fillets is shown in Figure 4.2.3. The WHC in non-treated samples (58 %) was not significantly different from those in 100 (all pressure holding times), 250 (0 min), and 400 MPa (0 min). At 250 MPa, pressure holding time significantly decreased WHC achieving values of *ca*. 41 %. In general, WHC decreased about 20 % with the increase in pressure level from 100 MPa to higher values. The pressurization rate also affected WHC of sea bass fillets (Table 4.2.2), though only significantly increased in fillets subjected to 250 and 400 MPa during 0 min. Though no significant differences were observed in 5 min fast pressurization rate, absolute values were higher, particularly at 100 MPa.

Chéret and co-authors [16] reported higher WHC of sea bass (80 % in non-treated samples) and that the pressure level did not affect this parameter in such a accentuated way. The differences in the results might be related with the

methodologies used. These authors evaluated WHC as the ability of proteins to bind water after muscle homogenization, instead of using a portion of intact muscle.

Tironi and co-authors [281] showed that after cooking, the WHC of sea bass treated with HPP was similar to control samples. These results indicate that the differences caused by HPP in the WHC of fillets might disappear with the cooking process.

### 3.3. *Electrophoreses*

#### 3.3.1. *SDS-PAGE*

The myofibrillar proteins in sea bass fillets presented a similar protein profile to previously described results of muscle proteins [270; 282]. Proteins bands were tentatively identified as: myosin heavy chains (band 1), M protein (band 2), C protein (band 3), actinin (band 4), desmin (band 7), actin (band 9), tropomyosin (band 16), myosin light chains (bands 19 e 20), and parvalbumin (band 24) (Figure 4.2.4). The molecular weights of major proteins were 137.2-205.6, 75.2-107.0, 33.6, 22.1, 18.1-18.6, 12.1, 5.5-8.3, and 3.9 kDa.

To identify changes caused by HPP treatments in myofibrillar proteins profile of sea bass fillets, SDS-PAGE was performed (Figure 4.2.4 and Table 4.2.3). A total of 15 protein bands with molecular weights of 205.6, 71.5-147.4, 53.0-55.9, and 33.6 kDa did not show significant differences between HPP treatments when evaluating the effects of pressure level and holding time. Twenty eight protein bands showed significant differences, being the most evident changes observed in the IOD of protein bands with molecular weights below 30 kDa (Table 4.2.3). In addition, HPP treatments led the appearance of protein bands with 29.9, 22.7, 21.4, 20.8, and 10.9 kDa, principally in treatments at higher pressure levels and longer holding times.

Pressure level caused changes in the intensity of several protein bands. In general, samples treated at 100 MPa kept a protein profile closer to that of non-treated samples, as well as the control of 250 MPa treatments. The protein bands with 23.5, 22.7, 19.5, 15.9, 15.3, and 14.2 kDa significantly increased in intensity with pressure level in treatments of 5 to 30 min of pressure holding time, while those with 22.1, 18.1, 8.3, and 6.9 kDa decreased with pressure level for all pressure holding times tested.

In relation to pressure holding time, the IOD of protein bands with 5.0 and 9.4 kDa statistically decreased at 100 MPa. At 250 MPa, the IOD of proteins

bands with 48.3, 22.7, 22.1, 18.6, 18.1, 6.9, and 5.0 kDa significantly decreased, while the opposite was observed for those with 23.5, 21.4, 19.5, 14.2, and 12.1 kDa. At 400 MPa, a significant increase in the IOD of 16 protein bands (158.8, 61.2, 59.4, 48.3, 27.9, 27.8, 23.5, 21.4, 20.8, 15.9, 14.2, 10.9, 9.4, 5.0, 4.7, and 4.4 kDa) was detected.

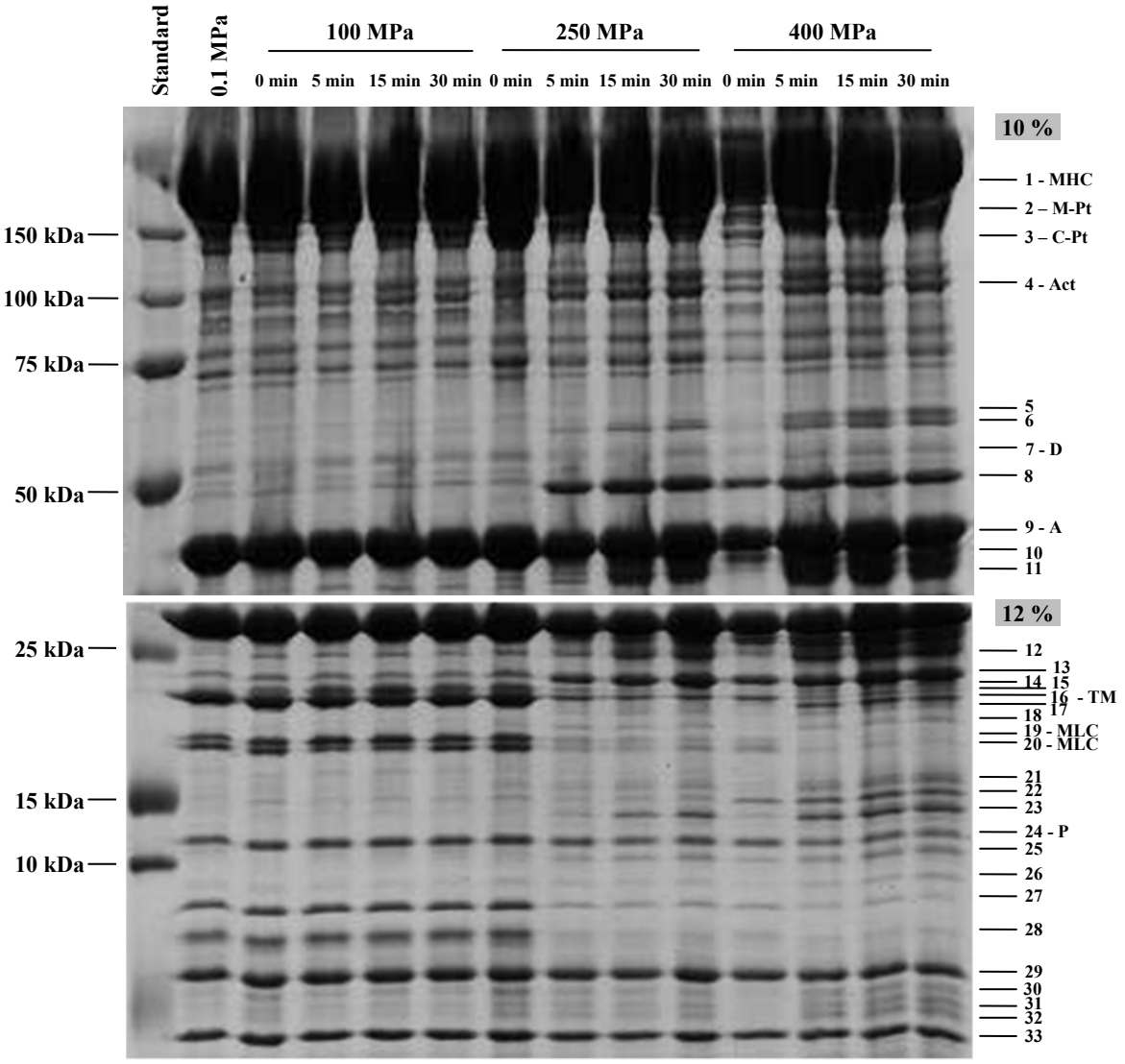



**Figure 4.2.4.** SDS-PAGE protein profiles of myofibrillar proteins extracted from sea bass fillets treated with high pressure. Proteins separation was achieved in gels containing 10 and 12 % of polyacrilamide (identified in the right side of the figure). Molecular weight of standard proteins are indicated on the left side of the figure. *Abbreviations*: MHC – myosin heavy chains; M-Pt – M protein; C-Pt – C protein; Act – actinin; D – desmin; A – actin; TM – tropomyosin; MLC – myosin light chains; P – parvalbumin.

In terms of pressurization rate (data not shown), the higher rate was responsible for a slight increase in protein bands intensity in almost all treatments. The only

exception was for the 100 MPa treatment during 5 min, with about half of the protein bands decreasing with pressurization rate.

Chéret and co-authors [262] reported similar effects: protein profile of sea bass fillets was not substantially changed at 100 MPa (5 min); at 300 MPa (5 min) the intensity of protein bands with 200, 150, 37, 32, and 20 kDa decreased. This effect could be due to denaturation or structure modification of myofibrillar proteins by HPP treatments, which could modify their extractability [262]. Additionally, Sequeira-Munoz and co-authors [283] attributed the increase in the intensity of lower molecular weights protein bands to the fragmentation of myofibrillar proteins, in treatments of carp fillets at 250 MPa during 30 min.

**Table 4.2.3.** Statistical differences in the integrated optical density (IOD) of protein bands obtained by SDS-PAGE of myofibrillar proteins extracted from sea bass fillets treated with high pressure (electropherograms shown in Figure 4.2.4).

| Band number | Molecular weight (kDa) | 100 MPa | | | | 250 MPa | | | | 400 MPa | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 min | 5 min | 15 min | 30 min | 0 min | 5 min | 15 min | 30 min | 0 min | 5 min | 15 min | 30 min |
| 2 | 158.8 ± 22.8 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **b** | *x* **a** | *x* **a** | *x* **a** |
| 5 | 61.2 ± 3.6 | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **b** | *x* **b** | *x* **ab*** | *x* **a*** |
| 6 | 59.4 ± 3.2 | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *xy* **a** | *x* **b** | *x* **ab** | *x* **ab** | *x* **a*** |
| 8 | 48.3 ± 3.4 | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **b** | *x* **ab** | *x* **ab*** | *xy* **a*** | *x* **b** | *x* **ab** | *x* **ab*** | *x* **a*** |
| 10 | 29.9 ± 3.0 | N.D. | N.D. | N.D. | N.D. | N.D. | *x* **a** | *x* **a** | *x* **a*** | *x* **a** | *x* **a*** | *x* **a*** | *x* **a*** |
| 11 | 27.9 ± 2.6 | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *xy* **a** | *x* **b** | *x* **ab** | *x* **ab*** | *x* **a*** |
| 12 | 27.8 ± 2.2 | *x* **a** | *x* **a** | *x* **a** | *y* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **a*** | *x* **b** | *x* **b*** | *x* **b*** | *x* **a*** |
| 13 | 23.5 ± 1.6 | *x* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **c** | *x* **bc*** | *x* **b*** | *x* **a*** | *x* **b*** | *x* **a*** | *x* **a*** | *x* **a*** |
| 14 | 22.7 ± 1.3 | *x* **c*** | *y* **b*** | *y* **a*** | *y* **b*** | *x* **a*** | *x* **b*** | *x* **b*** | N.D. | N.D. | *x* **b*** | *x* **a*** | *x* **ab*** |
| 15 | 22.1 ± 1.2 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **b*** | *y* **b*** | *y* **b*** | *y* **a*** | *y* **a*** | *y* **a*** | *y* **a*** |
| 16 | 21.4 ± 1.2 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | *y* * | **c** | **b*** | **ab*** | *x* **a*** |
| 17 | 20.8 ± 1.2 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | **a*** | **a*** | **a*** |
| 18 | 19.5 ± 0.9 | *x* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **b** | *xy* **b*** | *xy* **ab*** | *x* **a*** | N.D. | *x* **b*** | *x* **ab*** | *x* **a*** |
| 19 | 18.6 ± 0.7 | *x* **a*** | *x* **a*** | *x* **a*** | *x* **a*** | *x* **a*** | *y* **b*** | *y* **b*** | *y* **b*** | *y* **a*** | *y* **a*** | *y* **a*** | *y* **a*** |
| 20 | 18.1 ± 0.6 | *x* **ab** | *x* **c** | *x* **bc** | *x* **a** | *x* **a** | *xy* **b*** | *y* **b*** | *y* **b*** | *y* **a*** | *y* **a*** | *y* **a*** | *y* **a*** |
| 21 | 15.9 ± 0.4 | *x* **a** | *y* **a** | *z* **a** | *z* **a** | *x* **a** | *y* **a** | *y* **a*** | *y* **a*** | *x* **c** | *x* **b*** | *x* **a*** | *x* **a*** |
| 22 | 15.3 ± 0.2 | *y* **a** | *y* **a** | *y* **a** | *y* **a** | *xy* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **c*** | *x* **b*** | *x* **a*** | *x* **ab*** |
| 23 | 14.2 ± 0.4 | *x* **a** | *y* **a** | *y* **a** | *z* **a** | *x* **c** | *x* **b*** | *y* **b*** | *y* **a*** | *x* **c** | *x* **b*** | *x* **a*** | *x* **a*** |
| 24 | 12.1 ± 0.3 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **b*** | *xy* **b** | *xy* **ab** | *x* **a** | *y* **a** | *y* **a** | *y* **a** | *x* **a** |
| 25 | 10.9 ± 0.3 | N.D. | N.D. | N.D. | N.D. | N.D. | *x* **ab** | *x* **a*** | *y* **ab*** | **b** | *x* **ab*** | *x* **a*** | *x* **a*** |
| 26 | 9.4 ± 0.2 | *x* **a*** | *x* **b*** | *x* **b** | *y* **b** | *y* **a** | *x* **a*** | *x* **a*** | *y* **a** | *y* **b*** | *x* **b*** | *x* **b*** | *x* **a*** |
| 27 | 8.3 ± 0.2 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a*** | *y* **c*** | *y* **c*** | *y* **b*** | *y* **a*** | *y* **a*** | *y* **a*** | *z* **a*** |
| 28 | 6.9 ± 0.1 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *y* **b*** | *y* **b*** | *y* **b*** | *y* **a*** | *y* **a*** | *y* **a*** | *y* **a*** |
| 29 | 5.5 ± 0.2 | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a*** | *x* **b** | *y* **b** | *x* **a** | *y* **a** | *x* **a** | *xy* **a** | *y* **a** |
| 30 | 5.0 ± 0.2 | *x* **a*** | *x* **b** | *y* **b** | *y* **b** | *x* **a*** | *x* **b** | *y* **b** | *y* **b** | *y* **b** | *x* **b** | *x* **a*** | *x* **a*** |
| 31 | 4.7 ± 0.3 | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **b** | *x* **b** | *x* **a*** | *x* **a*** |
| 32 | 4.4 ± 0.4 | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **a** | *x* **a** | *y* **a** | *y* **a** | *x* **c** | *x* **c** | *x* **b*** | *x* **a*** |
| 33 | 3.9 ± 0.4 | *x* **a*** | *x* **a** | *x* **a** | *x* **a** | *x* **a*** | *x* **b** | *y* **b** | *x* **a** | *y* **a*** | *x* **a** | *xy* **a** | *x* **a** |

*Abbreviations*: N.D. – band not detected.
Only the bands with significant differences in the IOD are presented.
Different letters denote significant differences ($p < 0.05$) between pressure levels (*x*-*z*) or pressure holding times (**a-c**), and the symbol * denotes significant differences with non-treated samples (0.1 MPa).

The general pattern observed in the protein profiles follows a comparable tendency as the one found in WHC results. The major changes occurred in

protein bands at 250 and 400 MPa for pressure holding times of 5-30 min might be linked to the lost of WHC in muscle. Additionally, the changes observed in proteins might also be related with modifications in the texture of fillets. Chéret and co-authors [16] noticed that HPP decreased hardness, gumminess, and chewiness at 100-300 MPa, but at 400 and 500 MPa hardness is not affected, while gumminess and chewiness increases.

### 3.3.2. *IEF*

The effect of HPP in protein profiles of myofibrillar proteins was also assessed with IEF (Figure 4.2.5). The pI values of major myofibrillar proteins in sea bass fillets were *ca*. 9.35-9.83, 5.28-5.59, and 4.35. IEF profiles showed much less changes than SDS-PAGE ones, as only 12 bands were revealed in IEF profiles. The presence of urea in protein solutions shifts pI values [284], but taking into account the pI values of C protein, desmin, myosin heavy chain, myosin light chains, and parvalbumins [282] it can be assumed that the intense bands in the anodic section of the gel include these proteins.

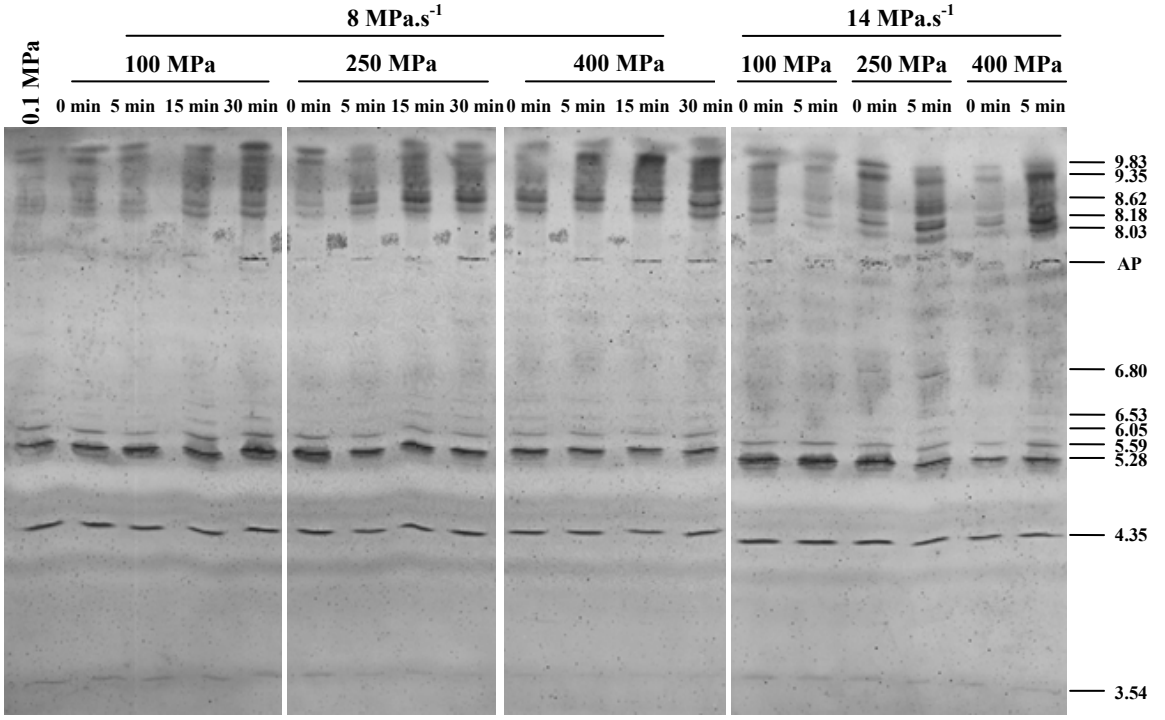



**Figure 4.2.5.** IEF protein profiles of myofibrillar proteins extracted from sea bass fillets treated with high pressure. Isoelectric point of proteins bands are indicated on the right side of the figure. *Abbreviations*: AP – sample application point.

The intensity of five protein bands at 100 MPa (pI of 9.83, 8.62, 5.59, 5.28, and 3.54), two bands at 250 MPa (pI of 8.62 and 8.18), and two bands at 400 MPa

(pI of 9.83 and 9.35) increased with pressure holding time. Pressure holding time also decreased the IOD of two protein bands at 250 and 400 MPa (pI of 3.54 and 8.18, respectively). For each pressure holding time, at least seven protein bands were gradually affected with pressure level. In general, protein bands with lower pI values (5.59, 5.28, 4.35, and 3.54) decreased in intensity at higher pressure levels, while the band with pI value of 6.80 increased. This trend is similar to the one observed for the proteins bands with 8.3 and 6.9 kDa in SDS-PAGE, which indicates that the proteins bands with lower pI values compose the parvalbumin pool.

Concerning pressurization rate, the IOD of protein bands with pI values of 9.83, 6.05, 5.59, 4.35, and 3.54 decreased with the increase in pressurization rate in 0 min treatments. In samples treated at 100 MPa during 5 min, proteins with higher pI values decreased in intensity with the increase in pressurization rate, while those with lower pI values increased their intensity. At 250 and 400 MPa, during 5 min, protein bands with higher pI values increased in intensity with the increase in pressurization rate.

The distinct proportion of proteins with altered pI values might explain the differences found in fillets pH. Nevertheless, the IEF was performed under denaturing conditions, and pH was measured with proteins in their native structure.

## 4. Conclusions

HPP considerably changed the quality of sea bass muscle, though varying with pressure variables (pressure level, pressure holding time, and pressurization rate). The treatments at 100 MPa did not cause significant changes when compared with non-treated samples, and the changes caused by the pressurization rate were of minor importance when compared with other pressure variables. The increase in pressure level and holding time enabled improvements in microbiological quality of fillets thus supporting that shelf life can be increased. Fillets WHC and colour were negatively affected. Changes in the protein profiles (SDS-PAGE and IEF analyses) might explain the effect of HPP on fish muscle physical properties. Depending on the principal goal for the application of HPP (*e.g.* microbiological safety or a new texture), different HPP conditions should be chosen. Future studies are still needed to understand the effect of HPP treatments in the quality attributes of fish fillets during storage,

that will enable to assess the contribution of HPP to extend their shelf life and potential use by the seafood industry.

# CHAPTER 4.3.

# Effects of high pressure processing on the quality and shelf life of sea bass (*Dicentrarchus labrax*) fillets during refrigerated storage

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**Abstract**

The aim of this study was to evaluate the effect of high pressure processing (HPP) on the quality of fresh sea bass fillets using two pressure levels (250 and 400 MPa). Vacuum packed fillets were used as control samples and all fillets were stored under refrigerated conditions for 18 days. The microbiological, chemical, physical, and sensory parameters were followed.

Both HPP treatments increased the microbiological shelf life of sea bass fillets. In day 14, control samples reached the upper acceptability limit (7 log CFU.g$^{-1}$), while fillets treated at 250 and 400 MPa had 3.2 and 1.4 log CFU.g$^{-1}$, respectively. In general, hydrogen sulphide producing bacteria and Enterobacteriaceae loads were below detection limit in HPP treatments. Results from nucleotides analysis indicate that HPP treatments reduced the conversion of inosine 5'-monophosphate to inosine. HPP also influenced fillets sensory characteristics. The most evident changes in fillets were the increase in whiteness, the loss of translucency, and a firmer consistency. The effect was more pronounced in the treatment at 400 MPa. Lipid oxidation increased in HPP treatments, being more accentuated in the treatment at 400 MPa. Instrumental smell intensity increased in both HPP treatments, though the sensory panel did not detect any rancid or other unpleasant odours. No effect was observed in the amount of volatile bases or in pH values. In conclusion, HPP treatments showed potential application for new fish products development with increased microbiological safety and shelf life, longer freshness, and with unique characteristics (*e.g.* firmer and whitish).

**Keywords**

High pressure processing; sea bass; quality changes; shelf life; microbiology; sensory analysis

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## 1. Introduction

The increase in consumer's preference for fresh and minimally processed food products, rather than processed and frozen ones, demands for research on new processing and preservation methods, especially for highly perishable products such as fish. Fish freshness is rapidly lost during *post-mortem* phase due to autolytic degradation by the action of endogenous enzymes, leading to prime quality loss [28]. This creates ideal conditions for the growth of microorganisms responsible for spoilage [16; 21].

High pressure processing (HPP) is a technology of growing interest for the processing and preservation of food. This technology has the potential to better retain food's nutritional and organoleptical characteristics, compared with traditional thermal processing [114]. HPP can also be used by the food industry to create new product textures, since it induces modifications on food functional properties [117]. HPP treatments have also the ability to inactivate spoilage and pathogenic microorganisms thus extending food shelf life [114].

Several studies with different HPP conditions have been conducted in seafood, including red mullet [129], squid [271], gilthead sea bream [127], and salmon [124]. Reported results are not always in agreement due to differences in conditions: HPP (*e.g.* pressure level and holding time), species under study, packaging, and storage. In general, microbiological shelf life increased in HPP treatments, while the effect of HPP in other quality parameters varied between studies.

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species widely distributed in the Mediterranean Sea and Atlantic Ocean and intensively farmed in several Mediterranean countries, *e.g.* Greece, Spain, and Italy, being highly appreciated due to its excellent organoleptic properties and reasonable price [6]. In the retail stores, sea bass is most commonly sold as whole fish and stored in ice, presenting a shelf life of 12-13 days [7]. Still, accordingly to FAO, fish losses caused by spoilage account for around 10 % of the total production from capture fisheries and aquaculture [4]. In this sense, different attempts have been done to extend sea bass shelf life [12; 14-15].

The aim of this work was to study the effect of HPP treatments on the quality and shelf life of fresh sea bass fillets vacuum packed and stored under refrigerated conditions. In particular, two pressure levels (250 and 400 MPa) were tested and compared with non-treated samples to evaluate their influence on microbiological, chemical, physical, and sensory parameters.

## 2. Material and methods

### 2.1. *Preparation of samples and treatments*

Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens were acquired from a local aquaculture farm (maximum *post mortem* time: 24 h). The average weight of each fish was 434 ± 78 g and the total length was 33 ± 2 cm. Fish samples (n = 27) were filleted and the skin was removed. All fillets (including those of control treatment) were vacuum packed individually in low-oxygen permeable barrier bags (Colamin XX 100e, Obermühle, Pössneck, Germany) with a vacuum packager (Packman, Albipack, Águeda, Portugal) before pressurization treatments.

HPP treatments were carried out in a hydrostatic press (High pressure system U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature (6 ºC), using a mixture of propylene glycol and water (1:1) as pressurizing fluid. Samples were processed at pressure levels of 250 and 400 MPa during 5 min using a pressurization rate of *ca*. 8 MPa.s$^{-1}$. Control samples (0.1 MPa) were also included in the study. After treatments, samples were stored under refrigeration (1.9 ± 0.3 ºC) during 18 days. During storage, three packages from each treatment (0.1, 250, and 400 MPa) were taken for microbiological, chemical, physical, and sensory analyses at predetermined intervals: 1, 4, 7, 11, 14, and 18 days of storage.

### 2.2. *Chemicals*

Potassium hydroxide, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), hypoxanthine (Hx), and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany); potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 2-thiobarbituric acid (TBA), ethylenediaminetetracetic acid (EDTA), propyl gallate, methanol, acetonitrile, and plate count agar from Merck (Darmstadt, Germany); perchloric acid and trichloroacetic acid (TCA) from Panreac Química S.A.U. (Barcelona, Spain); inosine (HxR) from BDH Chemicals Ltd (Poole, England); maximum recovery diluent, violet red bile glucose agar, Pseudomonas agar base, and supplement SR 103 from Oxoid (Basingstoke, Hampshire, England); Lyngby iron agar from Scharlau Chemie (Sentmenat, Spain); and the water used was distilled and Milli Q purified.

### 2.3. *Microbiological analysis*

Sea bass muscle (10 g) was aseptically collected to a Stomacher bag with filter. A primary ten-fold dilution was made with maximum recovery diluent and homogenized for one min at medium speed (230 rpm) using a Stomacher homogenizer (Laboratory blender STOMACHER 400, Seward Laboratory Systems Inc., Florida, USA). Appropriate series of decimal dilutions were then prepared. Homogenates were spread on agar for *Pseudomonas* spp. and hydrogen sulphide producing bacteria enumeration, or poured into molten agar for psychrotrophic bacteria and Enterobacteriaceae (with double layer to ensure anaerobic conditions) enumeration.

*Pseudomonas* spp. were enumerated on Pseudomonas agar base supplemented with SR 103, hydrogen sulphide producing bacteria (including *Shewanella putrefaciens*) on Lyngby iron agar, and psychrotrophic bacteria were enumerated on plate count agar after incubation at 20 ºC for 4 days. Enterobacteriaceae were enumerated on violet red bile glucose agar after incubation at 30 ºC for 1 day. For the hydrogen sulphide producing bacteria only the black colonies or those with a black centre were counted, whereas for Enterobacteriaceae the large colonies with purple haloes were counted and a representative number were tested for oxidation and fermentation reactions. Petri dishes containing 30-300 colony forming units (CFU) were selected for counting, and the results were expressed in logarithms of the CFU number per g of muscle (log $CFU.g^{-1}$). Determinations were performed in duplicate.

### 2.4. *Chemical analysis*

#### 2.4.1. *Nucleotide degradation*

Nucleotide and its breakdown products were extracted according to the method of Ryder [207]. During extract preparation, samples were kept in ice bath. Minced sea bass muscle (5 g) was homogenized with perchloric acid (25 mL; 0.6 M) using an Ultra turrax homogenizer (1 min; 6,500 rpm; Ultraturrax T25, Janke & Kunkel IKA® - Labortechnik). The homogenate was centrifuged (20,000 ×*g*, 10 min, 0 ºC; centrifuge 3K30, Sigma, Osterode, Germany), and 10 mL of the supernatant were neutralized with potassium hydroxide to a pH of 6.90. The neutralized supernatant stood for 30 min at 2 ºC to precipitate most of the potassium perchlorate, which was then removed by filtration through sintered glass. The filtrate solution was made up to 20 mL with water, filtered (0.22 µm pore size), and kept at -80 ºC until injection.

An aliquot (20 µL) was injected into an high performance liquid chromatograph (HP Agilent 1100 series; Agilent, USA) equipped with a LiChrosorb RP-18

reverse-phase column (250 × 4.6 mm; 10 µm; VDS Optilab) operating isocratically with a mobile phase pumped at 1.6 mL.min$^{-1}$, and the detection wavelength set at 254 nm. The mobile phase was composed of potassium dihydrogen phosphate (0.04 M) and dipotassium hydrogen phosphate (0.06 M), with a pH of 6.90.

Nucleotide and its breakdown products were identified and quantified by comparison with standards. Standard curves for ATP and each compound involved in its degradation pathway, ADP, AMP, IMP, HxR, and Hx, were constructed in the 0.02-0.8 mM range. The peak areas were obtained with the software Agilent ChemStation for LC (Agilent, USA). All determinations were performed in triplicate. The $K_I$-index, which is a simplified form of the freshness indicator K-index, was estimated according to the following equation [33]:

$$K_I\ (\%) = \frac{HxR + Hx}{IMP + HxR + Hx} \times 100$$

**2.4.2. *Malondialdehyde***

Malondialdehyde (MDA) was quantified according to the method described by Seljeskog and co-authors [210] with modifications in the sample deproteinisation as described by Mendes and co-authors [211]. Briefly, minced sea bass muscle (5 g) was homogenized with TCA solution (10 mL; 75 g.L$^{-1}$ TCA, 1 g.L$^{-1}$ EDTA, 1 g.L$^{-1}$ propyl gallate) using an Ultra turrax homogenizer (1 min; 6,500 rpm). Then, the homogenate was filtered (Whatman #1) and the filtrate was centrifuged (5,000 rpm, 10 min). Sample supernatant (0.5 mL) was mixed with TBA (1.5 mL; 40 mM), heated (97 ºC, 60 min), and cooled (-20 ºC, 20 min). Methanol (3 mL) was added, the resulting solution was filtered (0.22 µm pore size), and kept at -80 ºC until injection.

An aliquot (10 µL) was injected into a high performance liquid chromatograph (Agilent 1100 series, Agilent, USA). Separation of MDA-TBA adduct was done using a reversed-phase column (4.6 × 150 mm; 5 µm; Phenomenex Gemini ODS C18 110Å, Phenomenex, Torrance, CA, USA), operating isocratically with a mobile phase pumped at 1.0 mL.min$^{-1}$, and the spectrofluorimetric detector wavelengths were set at 525 nm (excitation) and 560 nm (emission). The mobile phase was composed of potassium dihydrogen phosphate (50 mM), methanol, and acetonitrile in the proportion of 72:17:11 (v:v:v).

MDA-TBA adduct was identified and quantified by comparison with TEP which was used as MDA standard. A standard curve was made for TEP diluted

in TCA solution at concentrations ranging 0.6-10.0 μM without hydrolysis prior to the TBA reaction. The peak areas were obtained with the software Agilent ChemStation for LC (Agilent, USA). All determinations were performed in triplicate.

**2.4.3. *Total volatile basic nitrogen and trimethylamine nitrogen***

The total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) contents were determined with the microdiffusion method using Conway diffusion cells [285]. Briefly, minced sea bass muscle (5 g) was homogenized with TCA (10 mL; 5 % w/v) using an Ultra turrax homogenizer (2 min; 6,500 rpm), and the homogenate was filtered (Whatman #1). After alkalinisation with saturated potassium carbonate, the volatile base components were absorbed by boric acid, which was then titrated with hydrochloric acid (0.01 N). For TMA-N determination, formaldehyde was added to the samples. Results were expressed as mg N per 100 g of muscle. All determinations were performed in triplicate.

**2.5. *Physical analysis***

**2.5.1. *Colour***

Colour measurements were assessed with a colorimeter (CR-410, Konica Minolta Camera, Co, Japan) in minced sea bass muscle, to avoid colour heterogeneity of fillets. The colorimeter was calibrated against a white standard plate (CIE $L^*a^*b^*$ system: $L^* = 97.79$; $a^* = -0.02$; $b^* = 1.84$). Lightness ($L^*$), red-green value ($a^*$), and yellow-blue value ($b^*$) were measured. All determinations were performed in triplicate. Chroma ($C^*$), hue ($h^*$), and whiteness (W) were estimated according to Schubring [212], as follows:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$h^* = arctg\frac{b^*}{a^*}$$

$$W = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2}$$

**2.5.2. *Smell intensity***

Instrumental smell intensity (number of molecules) was determined with a Portable Odour Level Indicator (Cosmos XP - 329 III R, New Cosmos Electric Co. Ltd, Osaka, Japan) that uses platinum heat coil covered with a high-sensitivity metal oxide ($SNO_2$/ZnO) semiconductor as a sensor, kept at high temperature during use. Packages were carefully perforated in one end by insertion of a pointed Teflon tube and a second hole made in the opposite side

of the bag in order to allow a stream of air through the product into the Cosmos unit. Results were expressed as Cosmos units of smell intensity. Determinations were performed in each package (three packages for each treatment and sampling day). All determinations were performed in triplicate.

**2.5.3. *pH***

The pH was measured directly on minced sea bass muscle using a surface calibrated pH electrode (SenTix 21, WTW, Weilheim, Germany) connected to a pH meter (microprocessor pH meter 539 WTW, Weilheim, Germany). All determinations were performed in duplicate.

**2.6. *Sensory analysis***

Sensory evaluation was conducted in a specific room equipped with individual booths under controlled conditions of light and temperature. The attributes of raw sea bass fillets were evaluated by a panel consisting of at least six experienced judges. A description of attributes and terminology used were discussed with the panel members. HPP treated (250 and 400 MPa) and control fillets were presented individually on each sampling day. Panellists were asked to score odour, appearance, and texture (firmness) of samples. A 0-4 category intensity scale of attributes was used, where 0 indicate "absence", 2 indicate "moderate", and 4 indicate "very intense". The results were reported as the average of scores.

**2.7. *Statistical analysis***

The effects of treatments and storage time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences. In sensory analysis, the results of colour (rosy) in the control treatment were evaluated using one-way analysis of variance, followed by Tukey HSD to identify the differences between storage days. All statistical analyses were tested at a 0.05 level of probability with the software STATISTICA$^{TM}$ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

## 3. Results and discussion

**3.1. *Microbiological analysis***

In live and healthy fish, microorganisms are found on the outer surface (skin and slime), and in some inner surfaces like gills and gastro-intestinal tract, while the remaining tissues are sterile [43-44]. After death, the presence of bacteria in the fish muscle results from contamination during processing and handling, where a maximum of 5 log CFU.g$^{-1}$ is considered acceptable in a good quality product [47].

The initial bacteria load (psychrotrophic bacteria) was 2.3 log CFU.g$^{-1}$ and values reached 7 log CFU.g$^{-1}$, the upper acceptability limit for fresh marine species [47], after 14 days of storage in control samples (Figure 4.3.1). HPP treatments at 250 and 400 MPa resulted in a considerable reduction in bacterial load, in the beginning of storage (Figure 4.3.1). In samples treated at 250 and 400 MPa, counts increased to 3.7 and 2.6 log CFU.g$^{-1}$, respectively, in the end of experience.

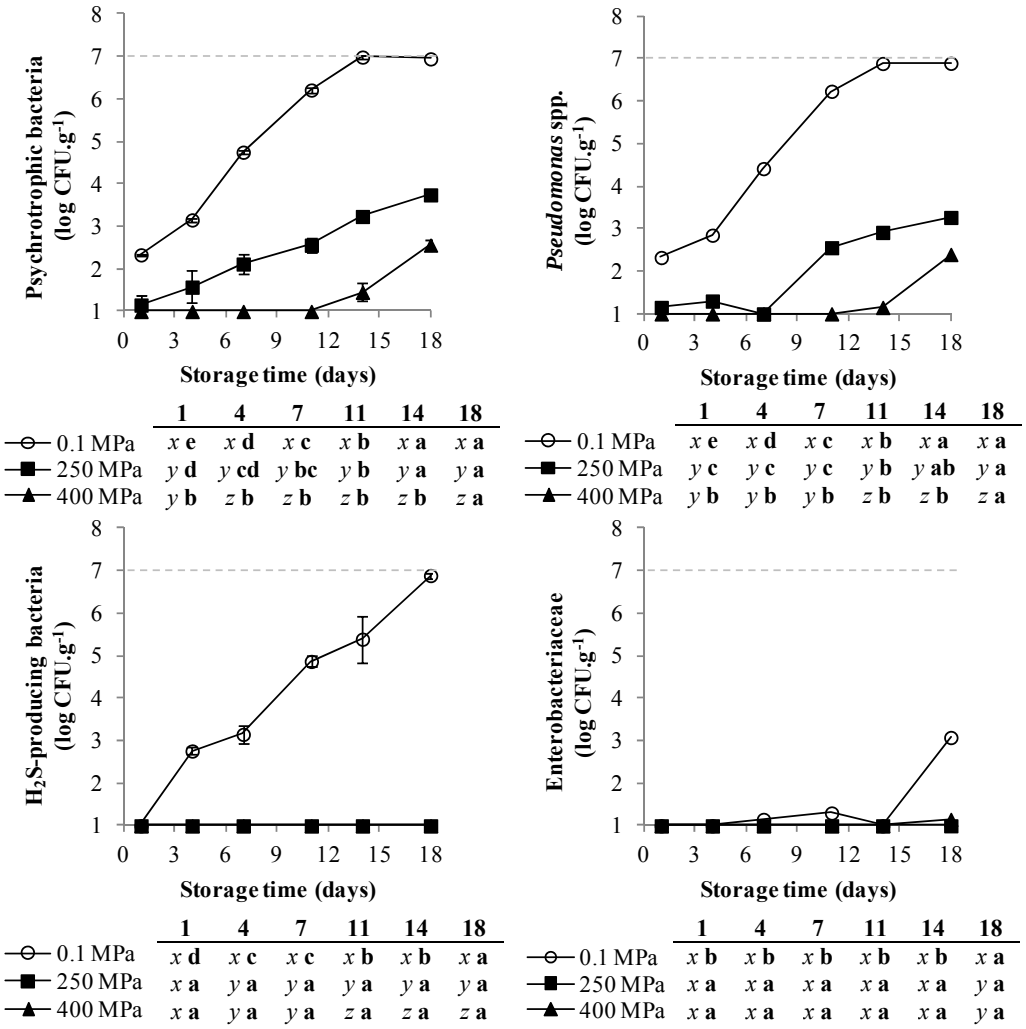



**Figure 4.3.1.** Changes in psychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa. Control samples are indicated as 0.1 MPa. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**e**). The grey dashed line indicates the upper acceptability limit for fresh marine species. *Abbreviations*: CFU – colony forming units.

Despite the evident reduction in bacterial load induced by HPP, the initial contamination of fish muscle seems also to be of great importance to extend

shelf life. In a previous study, a 3 log reduction in total aerobic counts was obtained at 400 MPa, but 6 log CFU.g$^{-1}$ were reached after only 7 days of storage [16]. In a study carried out by Yagiz and co-authors [124], fresh salmon treated with 300 MPa (15 min pressure holding time) showed similar results as those obtained in 400 MPa treatments tested in the present study.

The initial microflora of fillets consisted mainly of *Pseudomonas* spp. (Figure 4.3.1). This group of bacteria followed a similar trend to the one described for psychrotrophic bacteria during storage for all treatments. In what concerns hydrogen sulphide producing bacteria, counts in control samples were below the detection limit in the beginning of storage, and during storage increased up to 6.9 log CFU.g$^{-1}$ in the day 18 (Figure 4.3.1). In contrast, this bacterial group was kept below detection limit in HPP treatments during all storage time. Enterobacteriaceae load was low in all treatments during the first 14 days of storage, only reaching 3.1 log CFU.g$^{-1}$ at day 18 in control samples (Figure 4.3.1).

In previous studies, it was observed that HPP treatments of 50 and 100 MPa were more effective to reduce *Pseudomonas* spp. than hydrogen sulphide producing bacteria [123]. These authors also observed that treatments at 200 MPa (pressure holding times of 10-60 min) were able to reduce counts of *Pseudomonas* spp. and hydrogen sulphide producing bacteria in *ca*. 2 log units.

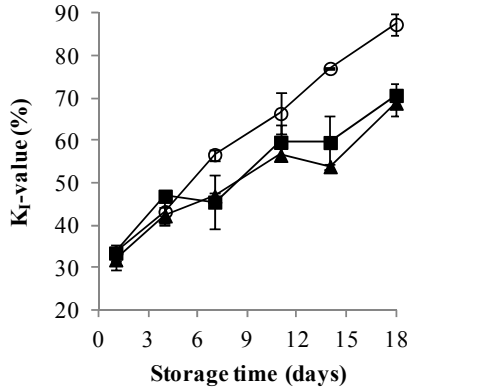
In the current study and because maximum microbiological limits were never reached in fillets treated with HPP, it was not possible to determine the microbiological shelf life, but it was extended at least 4 days. Data published previously also indicates that HPP can extend the shelf life of fresh fish, including sea bass [16], gilthead sea bream [127-128], red mullet [129], and salmon [124], principally in the treatments at higher pressure levels.

## 3.2. *Chemical analysis*

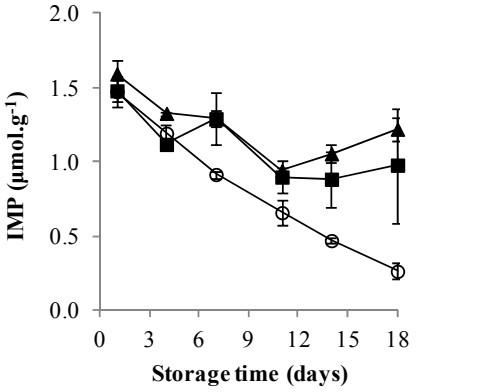
### 3.2.1. *Nucleotide degradation*

ATP breakdown products formed in fish after death result from a chain reaction in which metabolites typically rise and then fall as the next metabolite in the degradative chain begins to rise [27]. The initial stages in nucleotide degradation are thought to be mainly due to enzymatic reactions, while the degradation of HxR to Hx is also due to bacterial action [27]. IMP has been recognized as having flavour-enhancing properties, while Hx contributes to the off-flavours typical of spoiled fish [27].

Low levels of ATP, ADP, and AMP were found in the beginning of storage (concentration levels were below 0.15 μmol.g$^{-1}$; Figure 4.3.2), which is in accordance with baseline levels obtained for several fish species [29]. Taking into account that ATP, ADP, and AMP concentrations rapidly decrease and are negligible within 25 h after death [33], the simplified freshness indicator $K_I$ was used with IMP and its breakdown products.




| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *x* **f** | *x* **e** | *x* **d** | *x* **c** | *x* **b** | *x* **a** |
| 250 MPa | *x* **d** | *x* **c** | *y* **c** | *xy* **b** | *y* **b** | *y* **a** |
| 400 MPa | *x* **e** | *x* **d** | *y* **cd** | *y* **b** | *y* **bc** | *y* **a** |




| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *x* **a** | *x* **ab** | *y* **bc** | *x* **cd** | *y* **de** | *y* **e** |
| 250 MPa | *x* **a** | *x* **bc** | *x* **ab** | *x* **c** | *x* **c** | *x* **bc** |
| 400 MPa | *x* **a** | *x* **ab** | *x* **ab** | *x* **c** | *x* **bc** | *x* **bc** |

**Figure 4.3.2.** Changes in $K_I$-value and IMP concentration during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa. Control samples are indicated as 0.1 MPa. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*y*) or between sampling days (**a**-**f**). *Abbreviations*: $K_I$-value – freshness indicator index; IMP – inosine 5'-monophosphate.

Initially, fillets from all treatments showed a $K_I$-index of *ca*. 33 % and IMP concentrations around 1.5 μmol.g$^{-1}$ (Figure 4.3.2). $K_I$-index values increased with storage time, and by the end of storage reached 87 %, while IMP decreased to 0.3 μmol.g$^{-1}$ in control samples. HPP treatments reduced nucleotide degradation as observed by the lower $K_I$-index values, principally at the end of storage (Figure 4.3.2). From day 7, differences were clear in treatments at 400 MPa, while at 250 MPa differences were only evident after day 14. In fact, no significant differences were found in IMP concentration between days 11, 14, and 18 in 250 and 400 MPa treatments, while in control samples IMP concentration continued to decrease with storage time. The lower degradation of IMP to HxR, observed in HPP treatments at 250 and 400 MPa, indicates that pleasant flavours of fresh fish might be kept for longer periods compared with control samples.

In a previous study, the slowdown in nucleotide degradation was not observed in salmon treated with HPP, possibly as a consequence of employing weaker pressure levels (135-200 MPa) and lower holding time (30 seconds) conditions [125].

Acid phosphatase together with 5′ nucleotidase and nucleoside phosphorylase are the enzymes responsible for the conversion of IMP to HxR and HxR to Hx in fish muscle [27]. The results of the current study indicates that HPP treatments might reduce the activity of some enzymes involved in the nucleotide degradation process, and this effect might be more pronounced at higher pressure levels (400 MPa). This evidence is in accordance with previous studies that we carry out on the effect of HPP treatments in the activity of acid phosphatase.

### 3.2.2. *Lipid oxidation*

Fish is highly susceptible to lipid oxidation because its muscle is characterized by a high content of polyunsaturated fatty acids [26]. As a consequence, lipid hydroperoxides are formed and then decomposed to alcohols, aldehydes, ketones, and hydrocarbons [26].

MDA levels were monitored during storage, as a measure of secondary oxidation products formed in fillets. In the beginning of storage, MDA concentration was 0.2 $mg.kg^{-1}$ in control samples. After HPP treatments, lipid oxidation statistically increased in both treatments (Figure 4.3.3). During storage, MDA increased in all treatments, being more pronounced at 400 MPa where 1.7 $mg.kg^{-1}$ were reached by the end of storage.

According to guidelines for MDA concentration in seafood, fish muscle with values above 0.72 $mg.kg^{-1}$ will probably develop rancid flavours [286]. This limit was over passed at days 7, 11, and 18 in samples treated at 400 MPa, 250 MPa, and in control samples, respectively. However, panellists of sensory analysis did not detect rancid odours in fillets from any treatment during storage.

Similar variations in MDA concentration occurred in a previous study with cod treated at higher pressure levels [278]. However, Amanatidou and co-authors [123] did not report an increase in MDA concentration in salmon treated at a lower pressure level (150 MPa, pressure holding time of 10 min). Lipid oxidation was reported to increase at a lower rate with storage time in HPP treatments at 220-330 MPa, compared with non-treated samples [124; 127; 129]. Differences observed between studies might be related with different fish species, HPP

conditions (*e.g.* pressure holding time), package characteristics, and storage conditions.

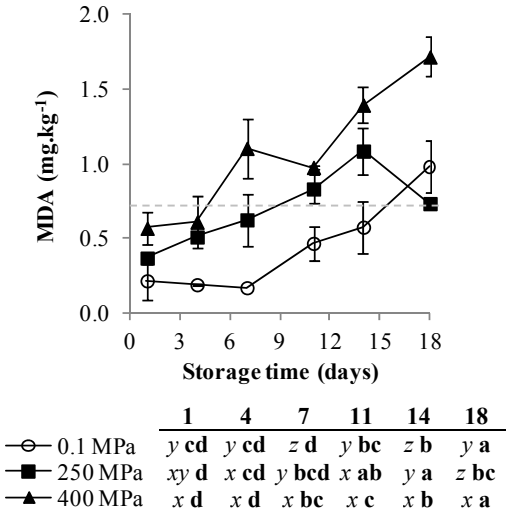



**Figure 4.3.3.** Changes in malondialdehyde (MDA) during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa. Control samples are indicated as 0.1 MPa. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**d**). The grey dashed line represents the limit from which rancid flavours might develop.

In previous studies, the differences in the effect of HPP in lipid oxidation was explained by possible differences in the fat characteristics, as well as in the content of unsaturated fats in different fish species that may be responsible for the non-uniform sensitivity of fats from different fish sources to pressure [127]. The accelerated oxidation in pressurized fish muscle may be due to denaturation of haem protein by pressure which releases metal ions promoting lipid auto-oxidation [287].

### 3.2.3. *Total volatile basic nitrogen and trimethylamine nitrogen*

Total volatile bases comprise amines with one atom of nitrogen per molecule, and is one of the most widely used measurements of spoilage evaluation of fishery products [34]. Total volatile bases include trimethylamine, produced by spoilage bacteria; dimethylamine, produced by autolytic enzymes; ammonia, produced by the deamination of aminoacids and nucleotide catabolites; and other volatile basic nitrogenous compounds associated with seafood spoilage [24; 34].

The initial TVB-N concentration in control samples was 17.0 mg N.100 $g^{-1}$ (Table 4.3.1). The HPP treatments did not significantly change TVB-N values, and during storage values did not increase significantly. Still, lower values were observed in HPP samples in days 14 and 18. TVB-N values did not reach the

maximum allowable value established by the European Commission [36] in any treatment. In previous studies, HPP treatments of 250 MPa increased TVB-N values of red mullet and decreased those of gilthead sea bream [127; 129].

**Table 4.3.1.** Changes in total volatile basic nitrogen (TVB-N) and pH values during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa.

| | Day 1 | Day 4 | Day 7 | Day 11 | Day 14 | Day 18 |
|---|---|---|---|---|---|---|
| **TVB-N** (mg N.100 $g^{-1}$) | | | | | | |
| 0.1 MPa | 17.0 ± 1.4$^{a}$ | 18.4 ± 1.5$^{a}$ | 18.0 ± 1.3$^{a}$ | 17.9 ± 1.0$^{a}$ | 18.7 ± 0.5$^{a}$ | 20.6 ± 1.3$^{a}$ |
| 250 MPa | 19.8 ± 1.9$^{a}$ | 18.9 ± 2.2$^{a}$ | 17.2 ± 1.3$^{a}$ | 16.4 ± 0.5$^{a}$ | 16.9 ± 0.6$^{a}$ | 19.3 ± 1.7$^{a}$ |
| 400 MPa | 18.7 ± 2.0$^{a}$ | 18.4 ± 0.8$^{a}$ | 18.1 ± 0.5$^{a}$ | 17.8 ± 1.0$^{a}$ | 17.0 ± 0.6$^{a}$ | 19.0 ± 0.6$^{a}$ |
| **pH value** | | | | | | |
| 0.1 MPa | 6.3 ± 0.0$^{abc}$ | 6.2 ± 0.1$^{c}$ | 6.3 ± 0.0$^{bc}$ | 6.4 ± 0.0$^{ab}$ | 6.4 ± 0.1$^{abc}$ | 6.5 ± 0.0$^{a}$ |
| 250 MPa | 6.4 ± 0.1$^{a}$ | 6.1 ± 0.1$^{b}$ | 6.4 ± 0.0$^{a}$ | 6.5 ± 0.0$^{a}$ | 6.4 ± 0.0$^{a}$ | 6.5 ± 0.0$^{a}$ |
| 400 MPa | 6.5 ± 0.0$^{a}$ | 6.2 ± 0.0$^{b}$ | 6.4 ± 0.0$^{a}$ | 6.6 ± 0.0$^{a}$ | 6.5 ± 0.1$^{a}$ | 6.6 ± 0.1$^{a}$ |

Control samples are indicated as 0.1 MPa.
Values are presented as average ± standard deviation.
Different letters denote significant differences ($p < 0.05$) between sampling days (**a-c**).
Significant differences between treatments were not observed.

Trimethylamine oxide is naturally present in the living tissue of many marine fish species as part of the non protein nitrogen fraction, and is an important compound for maintenance of several physiological functions in fish [37]. Trimethylamine oxide reduction by bacteria forms trimethylamine, which is often associated with the typical fishy odour of spoiled seafood [24].

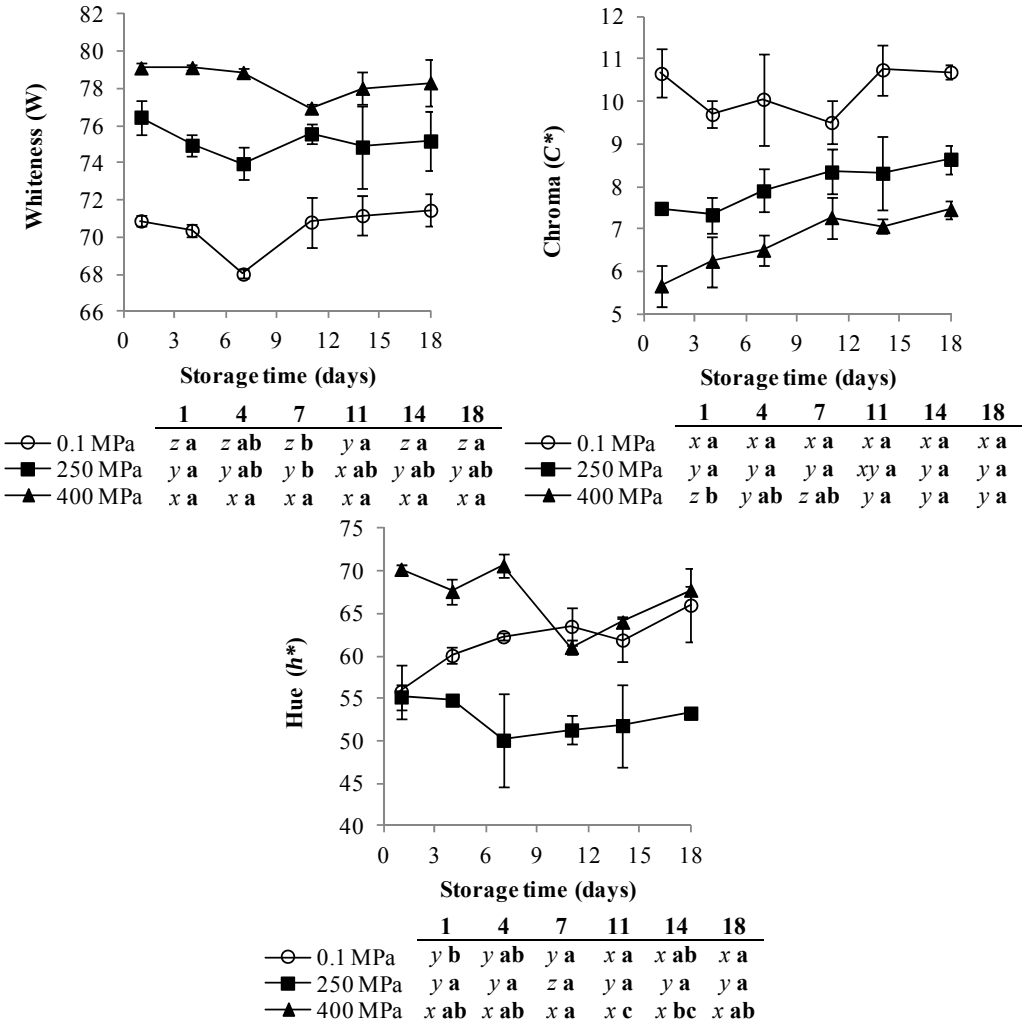
At the beginning of storage period, TMA-N values of control and HPP samples were lower than 0.5 mg N.100 $g^{-1}$ (data not shown). The HPP treatments did not significantly change TMA-N values, and during storage values did not change, being in all cases below the several limits of acceptability (5-12 mg N.100 $g^{-1}$) proposed in previous studies [288-289]. In studies with red mullet and gilthead sea bream, HPP treatments of 250 MPa (pressure holding time of 5 min at 7 ºC) did not cause significant changes in TMA-N values, but other combinations of pressure level, temperature, and pressure holding time affected TMA-N values differently [127; 129].

### 3.3. *Physical analysis*

#### 3.3.1. *Colour*

Colour is an important quality attribute of food as influences appearance and presentation, and might determine the acceptability by consumers and purchasing decision [217]. The colour of fish muscle is related with carotenoids and hemepigments [22], with the muscle physical structure and the amount of unbound water that influences light scattering [16].

Control fillets showed whiteness, chroma, and hue values of 70.9, 10.0, and 55.9, respectively (Figure 4.3.4). Both HPP treatments caused an increase in whiteness and a decrease in chroma, thus samples became whiter and revealed a cooked appearance especially at 400 MPa. During storage, some fluctuations around the initial values were observed in colour parameters in all treatments (Figure 4.3.4). By the end of storage, fillets did not get darker in any treatment, hue increased in control samples, and chroma increased in the 400 MPa treatment. Comparing both HPP treatments, whiteness and hue were lower in the 250 MPa treatment and chroma was higher. Still, by the end of the experience no significant differences were found in chroma between both treatments.




| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *z* **a** | *z* **ab** | *z* **b** | *y* **a** | *z* **a** | *z* **a** |
| 250 MPa | *y* **a** | *y* **ab** | *y* **b** | *x* **ab** | *y* **ab** | *y* **ab** |
| 400 MPa | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** |

| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** |
| 250 MPa | *y* **a** | *y* **a** | *y* **a** | *xy* **a** | *y* **a** | *y* **a** |
| 400 MPa | *z* **b** | *y* **ab** | *z* **ab** | *y* **a** | *y* **a** | *y* **a** |

| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *y* **b** | *y* **ab** | *y* **a** | *x* **a** | *x* **ab** | *x* **a** |
| 250 MPa | *y* **a** | *y* **a** | *z* **a** | *y* **a** | *y* **a** | *y* **a** |
| 400 MPa | *x* **ab** | *x* **ab** | *x* **a** | *x* **c** | *x* **bc** | *x* **ab** |

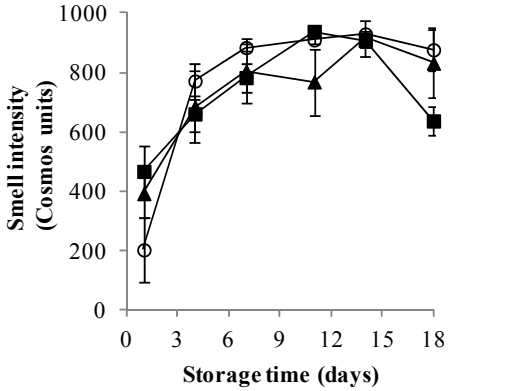
**Figure 4.3.4.** Changes in colour parameters (whiteness, chroma, and hue) during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa. Control samples are indicated as 0.1 MPa. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*z*) or between sampling days (**a**-**b**).

Colour changes in sea bass muscle caused by HPP are mainly due to modifications in the protein matrix [16]. Similar colour changes occur in cooked fish muscle due to denaturation of myofibrillar and sarcoplasmic proteins [124; 129].

In previous studies, no major changes were observed in colour parameters with storage time in HPP treatments in fresh salmon [123-124]. However, the effect of HPP in colour parameters evolution was reported to depend on the pressure level applied in red mullet [129] and on the temperature used during HPP treatment in gilthead sea bream [127].

### 3.3.2. *Smell intensity*

Initially smell intensity values were *ca*. 200 Cosmos units in control fillets (Figure 4.3.5). The application of HPP treatments caused an increase in the intensity, especially at 250 MPa which attained *ca*. 470 Cosmos units (Figure 4.3.5). With the increase in storage time, smell intensity increased to levels of 800-900 in day 7, and the differences between treatments disappeared. Then, smell intensity remained constant throughout storage, except in day 18 that the 250 MPa treatment showed lower values than other treatments.




| | 1 | 4 | 7 | 11 | 14 | 18 |
|---|---|---|---|---|---|---|
| 0.1 MPa | *y* **b** | *x* **a** | *x* **a** | *x* **a** | *x* **a** | *x* **a** |
| 250 MPa | *x* **c** | *x* **bc** | *x* **ab** | *x* **a** | *x* **a** | *y* **bc** |
| 400 MPa | *xy* **c** | *x* **b** | *x* **ab** | *x* **ab** | *x* **a** | *xy* **ab** |

**Figure 4.3.5.** Changes in smell intensity during refrigerated storage of sea bass fillets treated with high pressure processing at 250 and 400 MPa. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between treatments (*x*-*y*) or between sampling days (**a**-**c**).

In a previous study, a different pattern was observed: the smell intensity of several fresh fish species (*Bidyanus bidyanus*, *Salmo trutta*, *Tilapia niceotica* × *Tilapia aurea*, and *Lates calcarifer*) remained almost constant during the first 15 days of refrigerated storage, and the increase observed latter reflected a decrease in organoleptic properties [221].

The increase in MDA concentration with pressure level and with storage time could explain part of the variations observed in smell intensity. However, the smell intensity evolution obtained by instrumental analysis did not follow the same tendency as sensory analysis. This evidence happened possibly because the odour disappear with package opening during sensory analysis and ultimately because panellists did not notice the small variations that could remain in the headspace of fillets.

### 3.3.3. *pH*

*Post mortem* glycolysis of fish muscle results in the accumulation of lactate and $H^+$, which in turn lowers muscle pH [26], reduces the net surface charge on muscle proteins, and causes their partial denaturation [24]. The decrease in pH can lead to some water holding capacity loss [24]. Additionally, pH strongly influences the microbiology of fish muscle, specially pH sensitive spoilage bacteria [43].

In the beginning of the experiment, pH values were 6.3 in control samples (Table 4.3.1). HPP treatment caused a slight increase in initial pH values, although not statistically (Table 4.3.1). In day 4, pH values dropped in HPP treatments, but during storage pH values increased to values of *ca*. 6.5-6.6, without revealing differences between treatments.

In previous studies, HPP treatments with longer pressure holding times (20 min) did not affect the initial pH values of samples treated at 200 or 400 MPa, but after 7 days of storage pH values were lower in the 200 MPa treatment than in other treatments [278]. In contrast, Erkan and co-authors [129] observed a reduction in pH values in the beginning of storage time and an increase by the end in samples treated at 330 MPa. In gilthead sea bream, temperature used during HPP treatment influenced the evolution of pH during storage [127].

## 3.4. *Sensory analysis*

Initially, raw control fillets were rosy, translucent, and bright, with fresh odour characteristic of raw fish, and the muscle was firm recovering the shape after the finger test. HPP promoted changes in fillets that influenced sensory characteristics (Figure 4.3.6). The characteristic colour was lost in HPP fillets as denoted by the white colour revealed, quite similar to cooked fish, which is in accordance with the colour results obtained by instrumental analysis (Figures 4.3.4 and 4.3.6). Additionally, HPP treatments caused a considerable loss of translucency and the brightness looked like glassy/vitreous. In samples treated at 400 MPa, the brightness was intensified, and fillets become firmer.

Fresh odour decreased in HPP treatments, thought not statistically. Overall, the sensory acceptance of the HPP products was high.

Panellists decreased the score given to colour, translucency, brightness, fresh odour, and firmness with storage time in control fillets (Figure 4.3.6). Other attributes such as yellowish and brownish were scored as "absence" or "weak intensity" during storage. Unpleasant odours like fermented, sour, and putrid were not detected by most panellists. In the end of storage, control samples revealed changes in all attributes evaluated.

During storage of fillets treated with HPP, panellists decreased the score given to the attribute white in samples treated at 400 MPa, although this evidence is not in accordance with the instrumental measurements. In fillets treated at 250 MPa, the initial characteristics were kept for a longer period (Figure 4.3.6). In the end of storage, the fillets treated at 250 MPa showed some changes in the appearance, and those treated at 400 MPa lost firmness and the gapping increased.

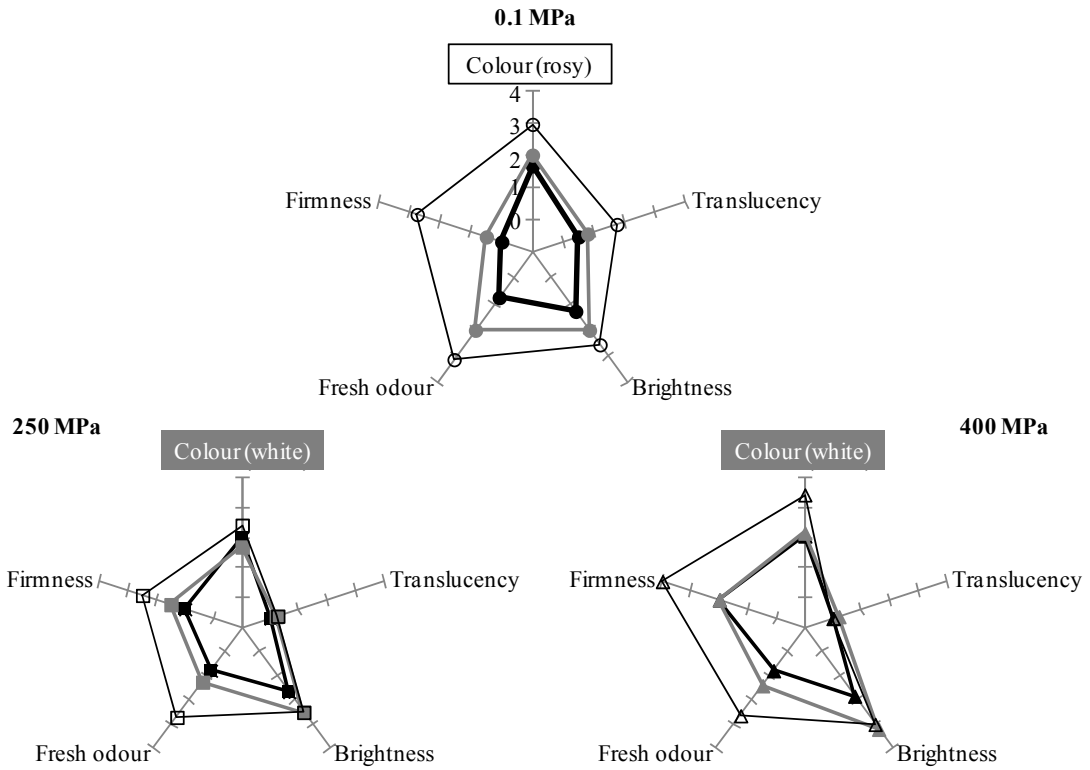



**Figure 4.3.6.** Sensory appreciation of raw sea bass treated with high pressure processing at 250 and 400 MPa. Control samples are indicated as 0.1 MPa. The unfilled markers represent the day 1, and the filled markers correspond to day 11 (grey) and 18 (black) of refrigerated storage. Category scale (0-4): 1, absence; 2, moderate; 4, very intense. Standard deviation was lower than 1.5.

In previous studies with gilthead sea bream and red mullet, sensory scores for appearance seems not to have been affected by HPP treatments in the first day of storage, and samples treated with HPP were better scored than control samples during storage [127; 129]. However, these authors also reported differences in instrumental colour results, in a similar way to the current study. The difference in the results can be explained by the use of a different sensory scale, where a score of 9-10 indicated "very good" quality and a score of 0-3.9 denote "spoiled".

## 4. Conclusions

Taking in to account microbiological criteria, HPP increases the shelf life of sea bass fillets for at least 4 extra days under refrigerated conditions. Both HPP treatments induced a reduction in the conversion of IMP to HxR and a slower decrease of freshness. HPP promoted changes in fillets that influenced sensory characteristics, conferring unique characteristics such as whiteness increase, translucency loss, and firmer consistency. Lipid oxidation and smell intensity (instrumental measurement) also increased in HPP treatments, but no rancid or unpleasant odours were detected in sensory analysis. In general, the effects of HPP treatments were more pronounced in the treatment at 400 MPa. No effect of HPP treatments was observed in the amount of volatiles bases, neither in pH values. Both HPP treatments showed potential for preservation of new fish products with increased microbiological safety and shelf life, longer freshness, and with unique characteristics (*e.g.* firmer and whitish).

# CHAPTER 5. General discussion and Conclusions

## INDEX

# General discussion

## 1. Development of methods to preserve sea bass fillets: natural substances

In Chapter 2 of this thesis, 19 essential oils and six extracts (hot water, cold water, and ethanolic) of 19 aromatic plants (European pennyroyal *Mentha pulegium*, oregano *Origanum vulgare*, basil *Ocimum basilicum*, carrot *Daucus carota*, celery *Apium graveolens*, citronella *Cymbopogon nardus*, clove *Eugenia* spp., coriander *Coriandrum sativum*, garlic *Allium* spp., grapefruit *Citrus paradisi*, lemon *Citrus limon*, marjoram *Thymus mastichina*, onion *Allium cepa*, Spanish oregano *Thymus capitatus*, parsley *Petroselinum sativum*, rosemary *Rosmarinus officinalis*, sage *Salvia officinalis*, tarragon *Artemisia dracunculus*, and thyme *Thymus vulgaris*) were studied in terms of antioxidant and antibacterial properties. The main aim was to screen a large number of plant extracts and essential oils for bioactive properties, comparing all of them using the same methodologies.

Afterwards, two essential oils (Spanish oregano and lemon) were selected to preserve fresh sea bass fillets under refrigerated conditions. The effect of essential oils on the quality and shelf life of fillets was evaluated taking into account microbiological, chemical, physical, and sensory criteria.

### 1.1. *Characterization of extracts and essential oils*

#### 1.1.1. *Antioxidant activity*

The results of antioxidant activity of extracts and essential oils of all 19 plants studied are summarized in Table 5.1, considering only α,α-diphenyl-β-picrylhydrazyl (DPPH) and reducing power methods (see Chapters 2.1-2.3 for further details).

Regarding the results of DPPH method, the hot water extracts of European pennyroyal and oregano, and the essential oil of clove were considered as very strong antioxidants, and the ethanolic extract of oregano and the Spanish oregano essential oil were classified as strong antioxidants. Concerning the results of reducing power method, the highest antioxidant activities were observed in all oregano extracts (hot water, cold water, and ethanolic; > 200 μmol ascorbic acid.$g^{-1}$), followed by extracts of European pennyroyal and the essential oils of clove, Spanish oregano, thyme, oregano, and citronella (20-200 μmol ascorbic acid.$g^{-1}$), whereas the remaining essential oils had lower antioxidant activities (< 4 μmol ascorbic acid.$g^{-1}$).

**Table 5.1.** Antioxidant activity of extracts and essential oils measured by DPPH and reducing power methods.

| Extracts and essential oils | DPPH | | Reducing power (μmol ascorbic acid.g$^{-1}$) |
|---|---|---|---|
| | $EC_{50}$ (mg.mL$^{-1}$) | Antioxidant activity index (AAI) | |
| **Hot water extracts** | | | |
| **European pennyroyal** *Mentha pulegium* | 0.02 ± 0.00 | 4.83 ± 0.12 (very strong) | 154.2 ± 16.8 |
| **Oregano** *Origanum vulgare* | 0.03 ± 0.00 | 3.16 ± 0.29 (very strong) | 621.7 ± 24.0 |
| **Cold water extracts** | | | |
| **European pennyroyal** *Mentha pulegium* | 0.17 ± 0.00 | 0.45 ± 0.01 (poor) | 116.6 ± 9.4 |
| **Oregano** *Origanum vulgare* | 0.14 ± 0.00 | 0.55 ± 0.02 (moderate) | 203.3 ± 18.0 |
| **Ethanolic extracts** | | | |
| **European pennyroyal** *Mentha pulegium* | 0.10 ± 0.00 | 0.80 ± 0.01 (moderate) | 137.4 ± 11.7 |
| **Oregano** *Origanum vulgare* | 0.06 ± 0.00 | 1.23 ± 0.02 (strong) | 232.7 ± 58.7 |
| **Essential oils** | | | |
| **European pennyroyal** *Mentha pulegium* | 6.20 ± 0.19 | <0.10 (poor) | 2.2 ± 0.7 |
| **Oregano** *Origanum vulgare* | 1.51 ± 0.12 | <0.10 (poor) | 74.5 ± 0.5 |
| **Basil** *Ocimum basilicum* | - | - | 0.8 ± 0.7 |
| **Carrot** *Daucus carota* | - | - | 1.3 ± 0.7 |
| **Celery** *Apium graveolens* | 10.04 ± 0.39 | <0.10 (poor) | 2.0 ± 1.7 |
| **Citronella** *Cymbopogon nardus* | 1.18 ± 0.02 | <0.10 (poor) | 23.5 ± 2.7 |
| **Clove** *Eugenia* spp. | 0.04 ± 0.00 | 2.21 ± 0.07 (very strong) | 153.4 ± 51.1 |
| **Coriander** *Coriandrum sativum* | - | - | 0.4 ± 0.2 |
| **Garlic** *Allium* spp. | - | - | 0.4 ± 0.2 |
| **Grapefruit** *Citrus paradisi* | - | - | 0.3 ± 0.1 |
| **Lemon** *Citrus limon* | - | - | 1.5 ± 1.1 |
| **Marjoram** *Thymus mastichina* | - | - | 0.2 ± 0.1 |
| **Onion** *Allium cepa* | - | - | 1.1 0.5 |
| **Spanish oregano** *Thymus capitatus* | 0.05 ± 0.00 | 1.69 ± 0.01 (strong) | 127.5 ± 35.3 |
| **Parsley** *Petroselinum sativum* | 7.23 ± 0.16 | <0.10 (poor) | 0.5 ± 0.2 |
| **Rosemary** *Rosmarinus officinalis* | - | - | 0.1 ± 0.1 |
| **Sage** *Salvia officinalis* | - | - | 0.1 ± 0.1 |
| **Tarragon** *Artemisia dracunculus* | 8.81 ± 0.10 | <0.10 (poor) | 3.3 ± 1.5 |
| **Thyme** *Thymus vulgaris* | 0.25 ± 0.01 | 0.32 ± 0.02 (poor) | 92.4 ± 34.0 |

*Abbreviations*: $EC_{50}$ – concentration providing 50 % of inhibition; DPPH – α,α-diphenyl-β-picrylhydrazyl.
Values are presented as average ± standard deviation.
Missing data correspond to essential oils that were not able to inhibit 50 % of DPPH.
Antioxidant categories (poor – AAI < 0.5; moderate – 0.5 < AAI < 1.0; strong – 1.0 < AAI < 2.0; very strong – AAI > 2.0) are presented in the AAI column in brackets.
Data from Chapters 2.1-2.3.

The antioxidant activities observed may be due in part to the presence of phenols, that in extracts had concentrations of 5-20 mg gallic acid.g$^{-1}$ (see Chapters 2.1-2.2 for further details). Phenols are organic compounds that contain a hydroxyl group directly bound to the aromatic ring, and the H-atom of the hydroxyl group can trap peroxyl radicals, preventing other compounds to be oxidized [163].

In essential oils, the antioxidant activity may be due to the presence of phenols such as thymol, carvacrol, and *p*-eugenol, but also due to other components like γ-terpinene, β-citronellol, and β-citronellal, all with known antioxidant activities [55]. The major constituents of essential oils are summarized in Table 5.2 (see Chapters 2.1-2.3 for further details).

The knowledge about bioactive properties of natural substances makes easier its choice for a future application to preserve food. Previous studies in the same field reported that intrinsic characteristics of plants, like origin, season, part of plant, and also the extraction procedure can contribute to a distinct composition of extracts and essential oils, and thus for their antioxidant activity, which explains differences between studies (discussed previously in Chapters 2.1, 2.2, and 2.3). In this sense, the results obtained in the current study for six extracts and 19 essential oils allowed to differentiate these natural substances in terms of antioxidant effectiveness.

**Table 5.2.** Major constituents of essential oils.

| Essential oils | Compounds and compounds concentration |
|---|---|
| **European pennyroyal** *Mentha pulegium* | menthone (35.9 %), pulegone (23.2 %), neo-menthol (9.2 %) |
| **Oregano** *Origanum vulgare* | carvacrol (14.5 %), thymol (12.6 %), γ-terpinene (11.6 %), β-fenchyl alcohol (12.8 %), δ-terpineol (7.5 %), 1-methyl-3-(1-methylethyl)-benzene (6.8 %) |
| **Basil** *Ocimum basilicum* | methylchavicol (77.9 %) |
| **Carrot** *Daucus carota* | δ-cadinene (48.6 %), *trans*-caryophyllene (8.3 %) |
| **Celery** *Apium graveolens* | β-selinene (42.9 %), 2-propenylphenoxyacetate (16.0 %), α-limonene (13.8 %), 2-methyl-benzoxazole (11.0 %), α-selinene (7.4 %) |
| **Citronella** *Cymbopogon nardus* | $\Delta^2$-carene (22.5 %), β-citronellal (11.9 %), eremophilene (9.8 %), β-citronellol (9.0 %), γ-terpinene (7.5 %), β-elemene (6.2 %), (+)-δ-cadinene (6.0 %) |
| **Clove** *Eugenia* spp. | *p*-eugenol (67.6 %), aceteugenol (16.8 %), *trans*-caryophyllene (10.8 %) |
| **Coriander** *Coriandrum sativum* | $\Delta^3$-carene (60.5 %), γ-terpinene (18.2 %), (+)-camphor (6.5 %), 3-carene (5.7 %) |
| **Garlic** *Allium* spp. | 1(7),5,8-*o*-menthatriene (20.7 %), di-2-propenyldisulfide (10.6 %), di-2-propenyltetrasulfide (9.2 %) |
| **Grapefruit** *Citrus paradisi* | α-limonene (84.7 %) |
| **Lemon** *Citrus limon* | α-limonene (60.1 %), α-fellandrene (9.0 %), β-pinene (7.0 %) |
| **Marjoram** *Thymus mastichina* | eucalyptol (54.0 %), *endo*-5,5,6-trimethyl-2-norbornanone (13.6 %), *trans*-caryophyllene (5.8 %) |
| **Onion** *Allium cepa* | dipropyltrisulfide (28.4 %), dipropyldisulfide (5.9 %), β-elemene (5.6 %), *o*-(4-nitrophenyl)-hydroxylamine (5.5 %) |
| **Spanish oregano** *Thymus capitatus* | carvacrol (78.4 %), *m*-thymol (10.9 %) |
| **Parsley** *Petroselinum sativum* | 4-methoxy-6-(2-propenyl)-1,3-benzodioxole (45.1 %), apiol (29.9 %), 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene (16.9 %), elemicin (5.9 %) |
| **Rosemary** *Rosmarinus officinalis* | (-)-camphor (35.5 %), eucalyptol (18.2 %), (-)-bornylacetate (13.4 %), *trans*-caryophyllene (7.2 %), *endo*-borneol (5.5 %) |
| **Sage** *Salvia officinalis* | (-)-camphor (28.7 %), (-)-bornylacetate (12.6 %), eucalyptol (12.2 %), |
| **Tarragon** *Artemisia dracunculus* | methylchavicol (92.4 %) |
| **Thyme** *Thymus vulgaris* | *m*-thymol (75.4 %), carvacrol (5.4 %) |

Only the compounds with a concentration of at least 5 % of the essential oil composition are listed.
Data from Chapters 2.1-2.3.

### 1.1.2. *Antibacterial activity*

The results of antibacterial activity of extracts and essential oils of all 19 plants studied are summarized in Table 5.3, considering only the minimum inhibitory concentration (MIC) obtained with the disc diffusion method and its effects in bacterial viable cells in suspensions (see Chapters 2.1-2.3 for further details).

Hot and cold water extracts of European pennyroyal and oregano did not inhibit the growth of most bacterial strains tested, and when inhibition occurred the MIC values were high (> 200 mg.mL$^{-1}$). The ethanolic extract of European pennyroyal inhibit the growth of all Gram-positive bacteria (*Brochothrix thermosphacta*, *Listeria innocua*, and *Listeria monocytogenes*), and that of oregano inhibit almost all bacteria tested, but it was not very effective to inhibit the growth of spoilage bacteria common in fish products.

**Table 5.3.** Antibacterial activity of extracts and essential oils.

| Extracts and essential oils | *Brochothrix thermosphacta* | *Escherichia coli* | *Listeria innocua* | *Listeria monocytogenes* | *Pseudomonas putida* | *Salmonella typhimurium* | *Shewanella putrefaciens* |
|---|---|---|---|---|---|---|---|
| **Hot water extracts** | | | | | | | |
| **European pennyroyal** *Mentha pulegium* | - | - | - | - | - | - | **323** |
| **Oregano** *Origanum vulgare* | **417** | - | - | - | - | - | **417** |
| **Cold water extracts** | | | | | | | |
| **European pennyroyal** *Mentha pulegium* | - | - | - | - | - | - | - |
| **Oregano** *Origanum vulgare* | - | - | - | - | - | - | **238** |
| **Ethanolic extracts** | | | | | | | |
| **European pennyroyal** *Mentha pulegium* | **30.5** (1.8) | - | **15.3** (0.0) | **15.3** (0.0) | - | - | - |
| **Oregano** *Origanum vulgare* | **277** | **6.9** (0.0) | **13.9** (5.2) | **6.9** (4.5) | - | **13.9** (4.7) | **277** |
| **Essential oils** | | | | | | | |
| **European pennyroyal** *Mentha pulegium* | **4.6** (0.8) | **2.3** (3.2) | **9.3** (1.8) | **2.3** (2.6) | **1.2** (1.3) | **9.3** (3.8) | **2.3** |
| **Oregano** *Origanum vulgare* | **4.5** (4.5) | **1.1** (6.9) | **2.2** (8.0) | **2.2** (8.0) | **0.4** (1.2) | **2.2** (7.2) | **4.5** |
| **Basil** *Ocimum basilicum* | **0.1** (1.4) | **12.0** (4.6) | **23.9** (2.2) | **23.9** (2.8) | **1.0** (1.9) | **2.4** (0.0) | **1.2** |
| **Carrot** *Daucus carota* | **0.9** (2.4) | - | **4.6** (5.7) | **4.6** (6.4) | - | - | **22.8** |
| **Celery** *Apium graveolens* | **8.8** (3.3) | **0.9** (0.0) | **22.0** (3.5) | **11.0** (3.6) | **0.9** (1.7) | **0.1** (0.0) | **8.8** |
| **Citronella** *Cymbopogon nardus* | **0.9** (3.8) | **4.4** (6.9) | **2.2** (7.7) | **4.4** (7.3) | **0.9** (2.1) | **0.9** (0.0) | **0.9** |
| **Clove** *Eugenia* spp. | **0.3** (1.8) | **5.2** (6.9) | **1.3** (3.4) | **10.4** (7.3) | **2.6** (6.0) | **5.2** (7.2) | **1.3** |
| **Coriander** *Coriandrum sativum* | **2.2** (4.5) | **10.9** (6.6) | **4.3** (8.0) | **2.2** (4.3) | **0.2** (0.8) | **4.3** (5.3) | **1.1** |
| **Garlic** *Allium* spp. | **0.1** (0.0) | **0.3** (0.9) | **0.5** (2.0) | **0.3** (2.0) | **0.3** (0.5) | **1.3** (0.0) | **1.1** |
| **Grapefruit** *Citrus paradisi* | **0.9** (3.7) | - | **4.3** (2.1) | **21.3** (3.9) | **0.2** (2.0) | - | **21.3** |
| **Lemon** *Citrus limon* | **8.5** (4.2) | - | **0.2** (2.9) | **1.1** (2.3) | **0.9** (1.4) | - | **21.3** |
| **Marjoram** *Thymus mastichina* | **9.1** (4.5) | **45.5** (6.9) | **2.3** (2.8) | **9.1** (5.8) | **0.9** (1.3) | **9.1** (2.6) | **45.5** |
| **Onion** *Allium cepa* | **1.1** (0.0) | - | **1.3** (1.9) | **5.3** (2.4) | **0.5** (0.5) | - | **13.4** |
| **Spanish oregano** *Thymus capitatus* | **1.2** (4.5) | **0.1** (2.7) | **2.3** (8.0) | **2.3** (7.3) | **0.1** (0.3) | **2.3** (7.2) | **4.7** |
| **Parsley** *Petroselinum sativum* | **5.3** (1.8) | - | **26.3** (0.0) | **26.3** (0.0) | - | - | **1.1** |
| **Rosemary** *Rosmarinus officinalis* | **90.8** (4.5) | **0.9** (0.0) | **22.7** (8.0) | **22.7** (7.3) | **0.9** (1.1) | **90.8** (1.7) | **0.1** |
| **Sage** *Salvia officinalis* | **1.1** (2.5) | **0.9** (0.0) | **11.4** (6.1) | **11.4** (6.1) | **0.5** (0.9) | **22.8** (7.2) | **22.8** |
| **Tarragon** *Artemisia dracunculus* | **2.3** (2.4) | **11.7** (6.9) | **4.7** (6.9) | **1.2** (1.9) | **0.9** (2.1) | **1.2** (0.0) | **0.5** |
| **Thyme** *Thymus vulgaris* | **0.9** (4.5) | **0.1** (2.9) | **1.1** (8.0) | **0.9** (8.0) | **0.2** (2.5) | **11.5** (7.2) | **0.5** |

Values in bold correspond to the minimum inhibitory concentration (MIC; mg.mL$^{-1}$) obtained with the disc diffusion method, and the values in brackets represent the effect of essential oils MIC in liquid medium (logarithmic reductions of colony forming unit per mL).
Missing data, indicated by "-" correspond to extracts/essential oils that did not inhibit the growth of the corresponding bacterial strains.
Data from Chapters 2.1-2.3.

The essential oils were more effective than extracts to inhibit the growth of bacterial strains tested, and 14 essential oils (European pennyroyal, oregano, basil, celery, citronella, clove, coriander, garlic, marjoram, Spanish oregano,

rosemary, sage, tarragon, and thyme) inhibit the growth of all bacteria strains. Lower concentrations (MIC < 5 $mg.mL^{-1}$) were needed to inhibit all bacteria when oregano, citronella, garlic, and Spanish oregano essential oils were used.

Taking into account the effect of MIC in bacteria viable cells in liquid medium, the greatest reductions, higher than 6 log colony forming units (CFU) per mL, were observed with 8 essential oils (oregano, carrot, citronella, clove, Spanish oregano, rosemary, sage, and thyme) against *L. monocytogenes*. Concerning the remaining bacterial strains, similar reductions were observed with the essential oils of oregano (*Escherichia coli*, *L. innocua*, and *Salmonella typhimurium*), clove (*E. coli*, *Pseudomonas putida*, and *S. typhimurium*), Spanish oregano (*L. innocua* and *S. typhimurium*), citronella, coriander, and tarragon (*E. coli* and *L. innocua*), thyme (*L. innocua* and *S. typhimurium*), marjoram (*E. coli*), rosemary (*L. innocua*), and sage (*S. typhimurium*).

The presence of compounds like pulegone, menthone, neo-menthol, thymol, carvacrol, eugenol, camphor, α-pinene, and citronellal in essential oils composition (Table 5.2) may explain the antibacterial activities observed [59; 149; 290]. However, for some essential oils studied here none of these compounds with known antibacterial activity were present in their compositions, and thus the antibacterial activity may be due to other compounds for which the antibacterial activity has not been yet study. It has been reported that some essential oil components interfere with cell wall and cytoplasmic membrane, disrupts the structure of their different layers of polysaccharides, fatty acids, and phospholipids, cause leakage of intracellular materials, and ultimately the cell lysis [50; 196].

In previous studies, the antibacterial properties of extracts and essential oils were evaluated using several methodologies, which makes comparisons difficult and not conclusive. Furthermore, several factors contribute for differences in chemical composition of natural substances and in its antibacterial properties. In the current study, the results of 25 natural substances (6 extracts and 19 essential oils), evaluated taking into account two different approaches, allowed to distinguish their effectiveness against seven bacterial strains.

### 1.2. *Effect of essential oils during refrigerated storage of sea bass fillets*

Based on the findings of antioxidant and antibacterial properties of plant extracts and essential oils (Chapters 2.1-2.3), the essential oil of Spanish oregano was selected to preserve fresh sea bass fillets under refrigerated conditions. Additionally, to improve sensory properties of sea bass fillets treated with Spanish oregano, the essential oil of lemon was added in a treatment combined with Spanish oregano essential oil. A treatment with a chemical preservative (potassium sorbate) was also studied. The quality of fillets was evaluated taking into account microbiological, chemical, physical, and sensory criteria (for further details see Chapter 2.4). The major quality changes in sea bass fillets obtained in this experimental assay are summarized in Table 5.4.

**Table 5.4.** Quality changes during refrigerated storage of sea bass fillets preserved with essential oils and potassium sorbate.

| | **Essential oils** | | |
|---|---|---|---|
| | **Spanish oregano (*Thymus capitatus*)** | **Spanish oregano and Lemon (*Thymus capitatus* and *Citrus limon*)** | **Potassium sorbate** |
| **Chemical** | | | |
| | - Lipid oxidation decreased in day 0<br>- Lipid oxidation did not increase until day 11 | - Lipid oxidation decreased in day 0<br>- Lipid oxidation did not increase until day 14 | - Lipid oxidation increased in day 0<br>- Lipid oxidation increased at a higher rate |
| **Microbiological** | | | |
| | - Bacterial load decreased 2.3 log CFU.g$^{-1}$ in day 0<br>- *Pseudomonas* spp. and $H_2S$-producing bacteria loads decreased in day 0<br>- Enterobacteriaceae load did not increase<br>- Higher shelf life (11 days) | - Bacterial load decreased 2.3 log CFU.g$^{-1}$ in day 0<br>- *Pseudomonas* spp. and $H_2S$-producing bacteria loads decreased in day 0<br>- Enterobacteriaceae load increased in day 11<br>- Higher shelf life (11 days) | - Bacterial load decreased 0.7 log CFU.g$^{-1}$ in day 0<br>- *Pseudomonas* spp. and $H_2S$-producing bacteria loads decreased in day 0<br>- Enterobacteriaceae load increased in day 11 |
| **Sensory** | | | |
| | - Fresh fish odour was lost<br>- Odour intensity of essential oil was high (3.7)<br>- No off-odours detected | - Fresh fish odour was lost<br>- Odour intensity of essential oil was moderate (2.9)<br>- No off-odours detected | - Off-odours were detected later (day 14) |

*Abbreviations*: CFU – colony forming units.
Descriptions reflect differences in relation to control treatment.
Data from Chapter 2.4.

#### 1.2.1. *Chemical analysis*

Lipid oxidation was evaluated by peroxide value and malondialdehyde (MDA) concentration (see Chapter 2.4 for further details). Since peroxide values were low (< 0.1 meq.kg$^{-1}$ of muscle), within recommended values (< 10-20 meq.kg$^{-1}$ of fat) [215], and did not vary during storage in all treatments, the following discussion reflects only MDA changes.

In Chapter 2.3 of this thesis, the results of antioxidant activity of Spanish oregano essential oil were reported, and based on those findings it would be expected that lipid oxidation in sea bass fillets was prevented by Spanish oregano essential oil.

The treatments with essential oils caused a decrease in lipid oxidation, which is explained by the strong antioxidant activity of Spanish oregano essential oil. During storage, Spanish oregano essential oil prevented lipid oxidation over 11 days of storage, and the treatment with Spanish oregano and lemon essential oils extended this effect for a longer period (14 days). The results of Chapter 2.3 showed a very low antioxidant activity of lemon essential oil. However, this essential oil contains α-limonene (60.1 %) and β-pinene (7.0 %) both with known antioxidant activity [55], which might explain the evolution in lipid oxidation in the treatment with Spanish oregano and lemon essential oils. In the treatment with potassium sorbate, lipid oxidation increased in day 0, and during storage a higher lipid oxidation rate was observed.

Although lipid oxidation increased during storage, MDA concentration did not increase to levels above recommended guidelines for seafood (0.72 mg MDA.kg$^{-1}$) [216], neither rancid odours were detected by panellists in sensory analysis. Packaging conditions (barrier pouches with low oxygen permeability and vacuum) might have been responsible for this type of evolution in lipid oxidation [291]. Additionally, the positive effects of essential oils in lipid oxidation are not as noticeable, possible due to the low fat content in sea bass and short storage period.

In conclusion, the combination of lemon and Spanish oregano essential oils was the best treatment to prevent lipid oxidation in sea bass fillets, and their application might be advantageous in products and storage conditions susceptible to lipid oxidation.

### 1.2.2. *Microbiological analysis*

A total of four microbiological analyses were performed, including psychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae (see Chapter 2.4 for further details).

In Chapter 2.3, the results of antibacterial activities of Spanish oregano and lemon essential oils were reported, and those results indicate that both essential oils inhibited the growth of all bacterial strains tested and even reduced them. Thus, it was expected a similar effect in sea bass fillets treated with essential oils. Additionally, results indicate that Spanish oregano essential oil was more

effective to inhibit the growth of *P. putida* and *S. putrefaciens* (spoilage bacteria common in fish products) than lemon essential oil, as lower MIC values were obtained with the former.

The treatments with essential oils (Spanish oregano *per se* and Spanish oregano combined with lemon) reduced psychrotrophic bacteria counts in 2.3 log CFU.$g^{-1}$, and *Pseudomonas* spp. and hydrogen sulphide producing bacteria loads decreased to levels below detection limit (1 log CFU.$g^{-1}$). No additional effect related with the presence of lemon essential oil was observed immediately after treatments.

In general, the evolution of psychrotrophic, *Pseudomonas* spp., and hydrogen sulphide producing bacteria was similar in both treatments with essential oils during storage, indicating that lemon essential oil did not increase the microbiological safety of fillets. In fact, the addition of lemon interfered with the antibacterial effect of Spanish oregano essential oil against Enterobacteriaceae, as shown by the higher counts in the treatment with both essential oils from day 11.

In treatments with essential oils, the upper acceptability limit for fresh marine species (7 log CFU.$g^{-1}$) [47] was achieved in day 11, increasing 4 days in the shelf life of sea bass fillets. The treatment with potassium sorbate also reduced bacteria load, but did not increase the microbiological shelf life of fillets.

In conclusion, the treatment with Spanish oregano essential oil showed the highest potential for microbiological preservation of sea bass fillets, as denoted by the increased microbiological safety and shelf life. Although similar results were obtained with the addition of lemon essential oil, its application is redundant as no improvements were observed, taking into account microbiological criteria.

### 1.2.3. *Sensory analysis*

In the treatment with potassium sorbate no major sensory changes were observed in sea bass fillets immediately after treatments, compared with control samples. The initial scores were kept for longer in the treatment with potassium sorbate and the typical off-odours of spoiled fish were noticed later. The sensory shelf life in this treatment increased from 11 (in control) to 14 days.

Essential oils application did not cause appearance variations (*e.g.* colour) in sea bass fillets, but panellists noticed that the fresh odour characteristic of raw fish was lost and fillets revealed an intense odour typical of the essential oils used. In the treatment with Spanish oregano essential oil, the odour intensity was

higher than in the treatment with Spanish oregano and lemon essential oils. During storage, no off-odours typical of spoiled fish were detected in treatments with essential oils. The odour intensity of essential oils was determinant for acceptance of fillets, and those that had softer odours were preferred by panellists.

The direct application of essential oils in sea bass fillets might be limited. To overcome the strong odours imparted to fillets, different approaches could be followed, like the application of essential oils in lower concentrations, but this could comprise its effectiveness. In alternative, essential oils could be incorporated in films. This strategy is described in the following section, in which three essential oils were incorporated in films prepared with fish proteins.

## 2. Development of methods to preserve sea bass fillets: films with natural substances

Based on the findings of antioxidant and antibacterial properties described in Chapters 2.1-2.3, the essential oils of clove, garlic, Spanish oregano, citronella, and thyme were selected to be incorporated in biodegradable fish protein films. The three former films were selected to be characterized taking into account physical, mechanical, antioxidant, and antibacterial properties (Chapter 3.1). Films with citronella, garlic, and thyme essential oils were used to preserve sea bass fillets under refrigerated conditions in a microbiological challenge test (Chapter 3.2). In all films, the perceived odours were much lower intense in comparison to free essential oils and film forming solutions.

### 2.1. *Characterization of fish protein films incorporated with essential oils*

#### 2.1.1. *Physical and mechanical properties*

Fish proteins films, without essential oils, were homogeneous, transparent, slight yellow, and mechanically resistant. However, the incorporation of essential oils (1 μL.cm$^2$) caused several changes in physical and mechanical properties of films (Table 5.5). In general, films with essential oils became thinner, less soluble in water, and less resistant to elongation. Other variations were dependent on the essential oil used.

The major variations in colour parameters were observed in films with clove and garlic essential oils, and both became darker and yellowish. These films also become less transparent, and less resistant in puncture tests. Additionally, films with clove essential oil became less permeable to water vapour, but the

protein solubility increased. In contrast, films with Spanish oregano were more similar to films without essential oils in terms of colour, transparency, and water vapour permeability. Still, some changes were observed in films with Spanish oregano essential oil, as films became less greenish, more deformable in a puncture test, and the protein solubility increased.

**Table 5.5.** Changes in physical and mechanical properties in fish protein films incorporated with essential oils.

| General changes in films with essential oils | Particular changes in films with essential oils |
|---|---|
| - Thickness decreased<br>- Elongation decreased (tensile test)<br>- Breaking force decreased (tensile test)<br>- Film solubility in water decreased | **Clove** (*Eugenia* spp.)<br>- Lightness, whiteness, and hue values decreased<br>- Yellow-blue and chroma values increased<br>- Transparency increased (less transparent)<br>- Water vapour permeability decreased<br>- Puncture deformation increased<br>- Puncture force decreased<br>- Protein solubility increased<br>**Garlic** (*Allium* spp.)<br>- Lightness, whiteness, hue, and red-green values decreased<br>- Yellow-blue and chroma values increased<br>- Transparency increased (less transparent)<br>- Puncture force decreased<br>**Spanish oregano** (*Thymus capitatus*)<br>- Red-green value increased<br>- Puncture deformation increased<br>- Protein solubility increased |

Descriptions reflect the differences in relation to films without essential oils.
Data from Chapter 3.1.

### 2.1.2. *Antioxidant activity*

The results of antioxidant activity of films incorporated with clove, garlic, and Spanish oregano essential oils are summarized in Table 5.6, taking into account DPPH and reducing power methods (see Chapter 3.1 for further details). Results obtained with the former method indicate that the antioxidant activity of films increased with the incorporation of essential oils. The reducing power of films also increased with the incorporation of clove and garlic essential oils, but not with Spanish oregano essential oil.

The antioxidant activity was lower in films with clove and Spanish oregano essential oils, than in the free essential oils. This decrease in the antioxidant activity could be due to interactions between components of films and essential oils that could no longer be available to interact as antioxidants, and also to the loss of volatile compounds of essential oils during films drying process.

In contrast, films with garlic essential oil showed the highest antioxidant activity, even higher than the essential oil alone. This result might be related with the presence of di-2-propenyldisulfide and di-2-propenyltetrasulfide in garlic essential oil composition (Table 5.2), both with disulfide bonds that might

be cleaved in the film forming solution and be able to act as scavenging hydroxyl radicals, as in others thiol antioxidants [292], increasing the antioxidant activity in the DPPH method.

**Table 5.6.** Antioxidant activity of films incorporated with essential oils measured by DPPH (α,α-diphenyl-β-picrylhydrazyl) and reducing power methods.

| Essential oils incorporated in films | DPPH (% of inhibition) | Reducing Power (μmol of ascorbic acid.g$^{-1}$ of films) |
|---|---|---|
| **Clove** (*Eugenia* spp.) | 71.9 ± 0.3 | 23.6 ± 3.0 |
| **Garlic** (*Allium* spp.) | 72.1 ± 0.8 | 47.5 ± 2.1 |
| **Spanish oregano** (*Thymus capitatus*) | 35.7 ± 1.0 | 9.6 ± 0.7 |

Values are presented as average ± standard deviation.
Data from Chapter 3.1.

### 2.1.3. *Antibacterial activity*

The results of antibacterial activity of films with essential oils are summarized in Table 5.7. Films with garlic essential oil showed antibacterial activity against a higher number of bacterial strains. Films with clove essential oil were more effective against the growth of *Shewanella putrefaciens* (spoilage bacteria common in fish products). None of the films with essential oils inhibited the growth of *P. putida* (spoilage bacteria common in fish products), neither *E. coli* nor *S. typhimurium*.

**Table 5.7.** Antibacterial activity of films incorporated with essential oils.

| Essential oils incorporated in films | *Brochothrix thermosphacta* | *Escherichia coli* | *Listeria innocua* | *Listeria monocytogenes* | *Pseudomonas putida* | *Salmonella typhimurium* | *Shewanella putrefaciens* |
|---|---|---|---|---|---|---|---|
| **Clove** (*Eugenia* spp.) | - | - | ✓ | ✓ | - | - | ✓ |
| **Garlic** (*Allium* spp.) | ✓ | - | ✓ | ✓ | - | - | ✓ |
| **Spanish oregano** (*Thymus capitatus*) | ✓ | - | ✓ | ✓ | - | - | - |

The symbol ✓ indicates antibacterial activity against the corresponding bacteria.
Missing data, indicated by "-" correspond to films that did not inhibit the growth of the corresponding bacterial strains.
Data from Chapter 3.1.

The incorporation of clove, garlic, and Spanish oregano essential oils in films increased the antibacterial activity of films. However, taking into account the results obtained for antibacterial activity of free essential oils (Chapter 2.3), a lower antibacterial activity was observed when essential oils were incorporated in films. Still, the film's effectiveness was dependent on the essential oil used and in the methodology employed for this evaluation (see Chapter 3.1 for further details).

In a previous study with similar films incorporated with citronella, coriander, tarragon, and thyme essential oils, those with thyme essential oil revealed antibacterial activity against a higher number of bacterial strains, and the highest against *S. putrefaciens* [249]. However, films with thyme essential oil did not reveal antibacterial activity against *P. putida*, while films with citronella essential oil did [249].

***2.2. Effect of fish protein films incorporated with essential oils during refrigerated storage of sea bass fillets***

The study described in Chapter 3.2 was developed according to the findings of Chapter 3.1 and with the results about films with citronella, clove, coriander, and thyme essential oils reported by Pires and co-authors [249]. Films incorporated with citronella, garlic, and thyme essential oils were selected to test its effectiveness to preserve sea bass fillets during refrigerated storage. In this experimental assay, a total of four microbiological analyses were performed including phychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae, in a microbiological challenge test (see Chapter 3.2 for further details).

Figure 5.1 shows the phychrotrophic bacteria evolution in sea bass fillets preserved without and with films prepared with fish proteins.

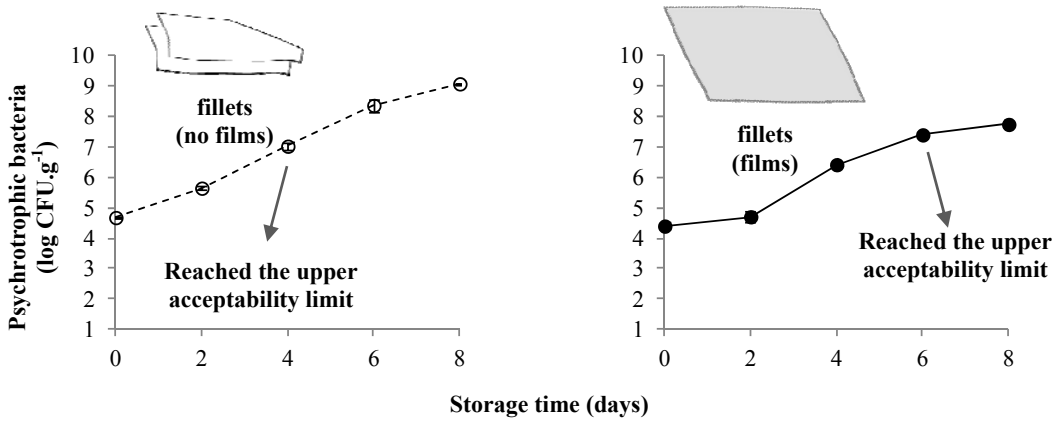



**Figure 5.1.** Changes in psychrotrophic bacteria counts during refrigerated storage of sea bass fillets without and with films. Vertical error bars represent the standard deviation. The upper acceptability limit for fresh marine species is 7 log CFU.g$^{-1}$. *Abbreviations*: CFU – colony forming units. Data from Chapter 3.2.

Films without essential oils inhibited the growth of both *Pseudomonas* spp. and Enterobacteriaceae, but not hydrogen sulphide producing bacteria, and increased the microbiological shelf life of sea bass fillets from 4 to 6 days (see Chapter 3.2 for further details). The contact of films with fillets surface might

have caused anaerobic conditions that were responsible for a slower bacterial growth [291].

Results of Chapter 3.1 and those of Pires and co-authors [249] showed that films with essential oils had antibacterial activity against *S. putrefaciens* (garlic and thyme essential oils) and *P. putida* (citronella essential oil). However, these films did not increase the microbiological safety or shelf life of contaminated sea bass fillets, compared with films without essential oils.

The results of this study indicate that these films are not suitable enough to preserve sea bass fillets, taking into account microbiological criteria. Other formulations with different concentrations of essential oils or combining different essential oils might be more advantageous.

## 3. Development of methods to preserve sea bass fillets: high pressure processing

In Chapter 4 of this thesis, HPP treatments were applied in sea bass fillets to study its effects on enzymes activity. Pressure level (100-400 MPa), pressure holding time (0-30 min), and pressurization rate (8-14 MPa.s$^{-1}$) were the variables studied in a total of 18 treatments. Several quality parameters (*e.g.* colour, water holding capacity, and mesophilic bacteria) were also evaluated in sea bass fillets treated with the same pressure conditions. Then, two pressure conditions (250 and 400 MPa, 5 min, 14 MPa.s$^{-1}$) were selected to process sea bass fillets and follow its quality during refrigerated storage. The effect of HPP treatments in the quality and shelf life of fillets was evaluated taking into account microbiological, chemical, physical, and sensory criteria.

### 3.1. *Effect of high pressure processing in enzymatic activity*

The effect of HPP treatments in the enzymatic activity of acid phosphatase, lipase, cathepsins (B and D), and calpains was evaluated immediately after HPP treatments (see Chapter 4.1 for further details).

In general, the treatments at 100 MPa (lower pressure level tested) did not cause changes in enzymes activity, neither the treatments of 0 min of pressure holding time. Pressure level and holding time were the variables responsible for most changes, and only minor changes were detected due to pressurization rate. HPP treatments affected differently the activity of the enzymes studied, which is discussed in the following sections.

**3.1.1. *Acid phosphatase***

Pressure levels of 100 (0-30 min) and 250 MPa (5-15 min) did not change the overall activity of acid phosphatase (Figure 5.2). HPP might have inactivated acid phosphatase in the cytosol, and at the same time released the enzyme from lysosomes membranes, since in previous works the activity increased in the cytosolic fraction, and decreased in the lysosomal fraction [252]. At higher pressure levels (400 MPa), the activity of this enzyme decreased, and thus HPP treatments might have inactivate acid phosphatase.

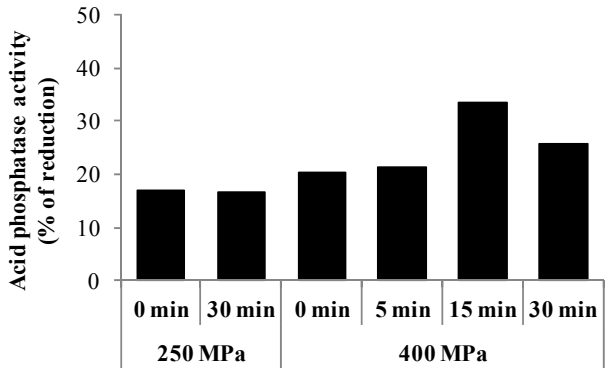



**Figure 5.2.** Changes in acid phosphatase activity in sea bass fillets treated with high pressure. Values represent the reduction in the activity of acid phosphate in relation to non-treated samples (0.1 MPa). Only the treatments with significant lower activities are shown. Data from Chapter 4.1.

It is known that acid phosphatase is an enzyme involved in nucleotide degradation of fish muscle, particularly in the degradation of IMP to HxR [27]. Thus, it is expectable that HPP treatments at 400 MPa cause a reduction in nucleotide degradation and an extension in the freshness period of sea bass fillets.

**3.1.2. *Lipase***

In sea bass fillets, lipase activity was not affected by most HPP treatments. Lipases are responsible for the accumulation of free fatty acids, which in turn are associated with quality deterioration, including changes in texture characteristics and production of off-flavours [42]. In this sense, the results obtained in the current study indicate that HPP might not influence the quality of sea bass that is related with lipase activity.

**3.1.3. *Proteolytic enzymes***

The activity of cathepsin B increased with pressure conditions of 250 (30 min) and 400 MPa (5-15 min). HPP treatments also increased the activity of cathepsin D with the increase in pressure level in treatments of 0 min of pressure holding time. On the other hand, the activity of cathepsin D decreased

in the treatments at 100 (0-30 min), 250 (30 min), and 400 MPa (5-30 min) and also with pressure holding time at 400 MPa.

Cathepsins B and D are lysosomal enzymes [28]. The disruption of lysosomes by HPP treatments causes the release of enzymes, promoting the contact with the substrate, which may explain the increase in the activity of these enzymes in several HPP treatments. In other treatments, the decrease in cathepsin D activity seems to be due principally to their inactivation.

Concerning calpains, HPP treatments decreased its activity with the increase in pressure level and holding time, and the lowest activity values were observed in treatments at 250 (15-30 min) and 400 MPa (5-30 min) (Figure 5.3). The effect of HPP treatments in calpains was different from that of cathepsins, since calpains are mainly in the cytosol [40]. Calpains inactivation with pressure level and holding time may be due to the dissociation of both subunits of calpains [269].

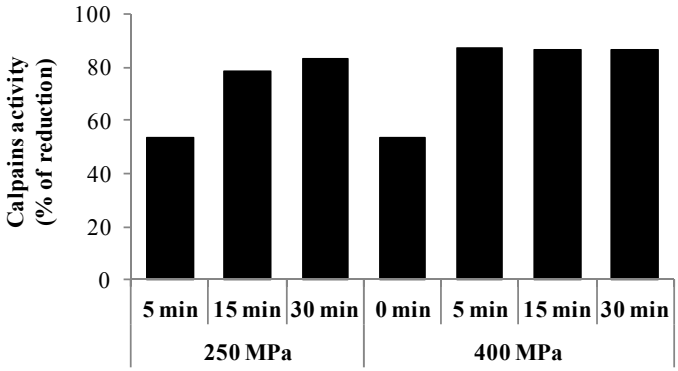



**Figure 5.3.** Changes in calpains activity in sea bass fillets treated with high pressure. Values represent the reduction in the activity of calpains in relation to non-treated samples (0.1 MPa). Only the treatments with significant lower activities are shown. Data from Chapter 4.1.

Calpains and cathepsins are proteolytic enzymes responsible for the softening of muscle tissues [28]. HPP treatments at 250 (30 min) and 400 MPa (5-30 min) caused the lowest activities of calpains and cathepsin D. However, for some of these treatments the activity of cathepsin B increased. Still, the decrease in the activity of calpains and cathepsin D was more important. Thus, results indicate that the application of treatments at 250 (30 min) and 400 MPa (5-30 min) in sea bass fillets could decrease the softening of muscle tissues during storage.

### 3.1.4. *Water soluble proteins*

The concentration of water soluble proteins decreased with the increase of pressure level and holding time, indicating a decrease in the concentration of enzymes, as water soluble proteins include mainly enzymes [260].

Electrophoretic profiles of water soluble proteins showed that a total of 34 protein bands with molecular weights of 1.6-158.8 kDa presented differences among HPP treatments. The intensity of several bands changed mainly due to variations in pressure level and holding time (this also happened in isoelectric focusing profiles of sarcoplasmic proteins; Chapter 4.1). The general pattern of water soluble protein profiles followed a comparable tendency to the one observed in the activity of acid phosphatase and calpains, and some changes might be linked. The decrease in the intensity of protein bands with higher molecular weights was accompanied with an increase in the intensity of protein bands with lower molecular weights. This result could be due to protein degradation or fragmentation, or to insolubilization of sarcoplasmic proteins as a result of the formation of proteins aggregates [259].

## 3.2. *Effect of high pressure processing in sea bass quality*

The effect of HPP treatments on the quality of sea bass fillets was evaluated immediately after treatments, taking into account microbiological and physical analyses (see Chapter 4.2 for further details). In a similar way of what was observed in Chapter 4.1, pressure level and holding time were responsible for the major changes on sea bass quality. Specific variations caused by HPP treatments were discussed in the following sections.

### 3.2.1. *Microbiological analysis*

Total viable counts of mesophilic bacteria were performed in sea bass fillets treated with HPP, and results are represented in Figure 5.4. Results showed that bacterial load decreased with the increase in pressure level, and also with pressure holding time, especially at 400 MPa. The greatest reduction (2 log CFU.g$^{-1}$) was observed in samples treated at 400 MPa for 30 min of pressure holding time. It is known that high pressures cause the collapse of intracellular vacuoles, and damage to cell walls and cytoplasmic membranes [3].

HPP treatments showed potential to increase the microbiological safety of sea bass fillets, being the most intense treatments (higher pressure levels/longer holding times) those that caused the greatest reductions in bacterial counts. These results indicate also that HPP might extend microbiological shelf life of sea bass fillets.

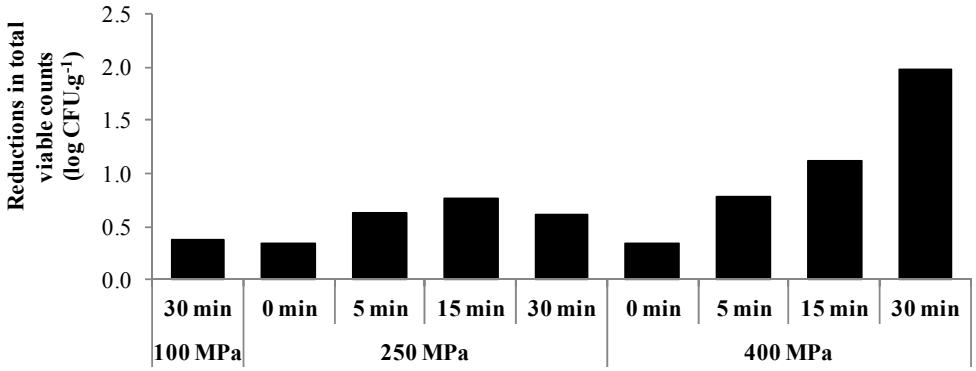



**Figure 5.4.** Variations in mesophilic bacteria counts in sea bass fillets treated with high pressure. Values represent the reduction in total viable counts in relation to non-treated samples (0.1 MPa). Only the treatments with significant lower values are shown. *Abbreviations*: CFU – colony forming units. Data from Chapter 4.2.

### 3.2.2. *Physical analysis*

The colour of sea bass fillets was deeply influenced by HPP treatments, especially the whiteness parameter (Figure 5.5). Fillets lost translucency and became whiter with higher pressure levels (250 and 400 MPa) and also with the increase in pressure holding time, revealing a cooked appearance. Similar colour changes occur in cooked fish muscle due to denaturation of myofibrillar and sarcoplasmic proteins [124; 129].

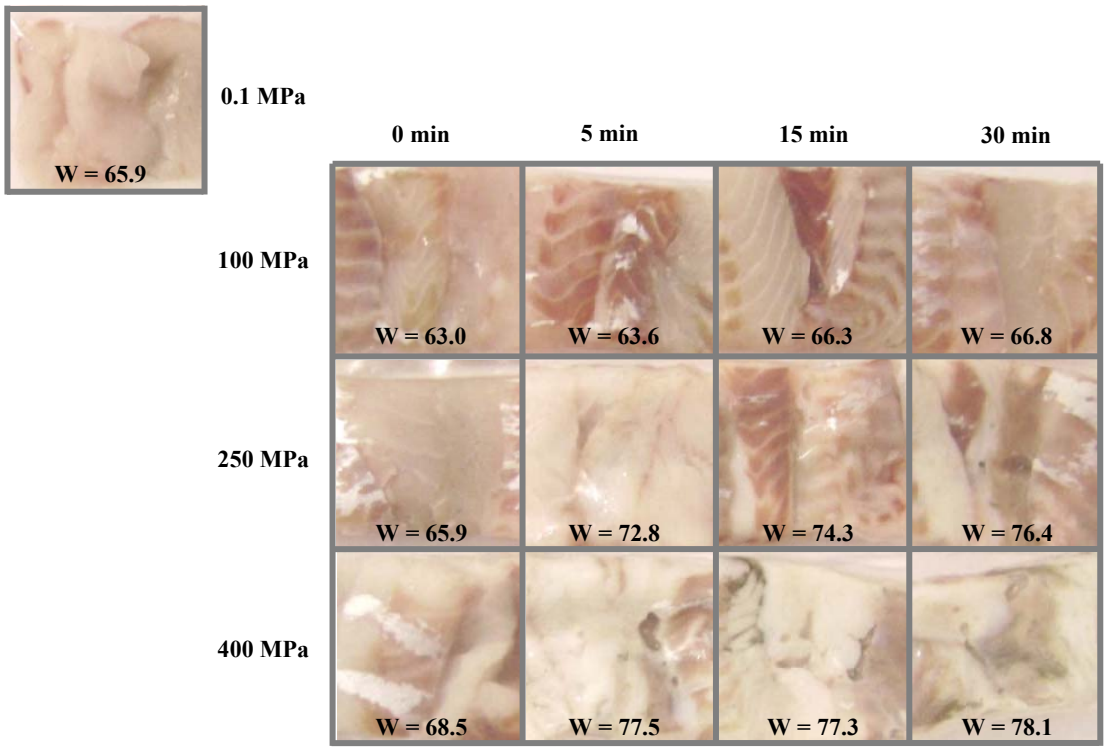



**Figure 5.5.** Changes in whiteness (W) and appearance of sea bass fillets treated with high pressure. Samples not treated with high pressure are indicated as 0.1 MPa. Data from Chapter 4.2.

The water holding capacity (WHC) of sea bass fillets was not too much affected with treatments at 100 (all pressure holding times), 250 (0 min) and 400 MPa (0 min). However, at 250 and 400 MPa treatments for 5-30 min of pressure holding time, the WHC decreased *ca*. 20 %. HPP treatments may degrade proteins and change the intracellular architecture of fibrils, which influences the ability of muscle cells to retain water [128]. The decrease of muscle proteins ability to hold water molecules might compromise several quality parameters, such as tenderness, juiciness, or succulence [260].

Profiles of myofibrillar proteins of sea bass fillets showed that a total of 28 protein bands presented differences among HPP treatments (see Chapter 4.2 for further details). In general, the treatments at 100 MPa caused fewer changes in the intensity of protein bands, and its magnitude increased with pressure level and holding time. The most evident changes were observed in protein bands with molecular weights below 30 kDa. The variations in the intensity of protein bands could be due to denaturation or structure modification of myofibrillar proteins [262] and to fragmentation of myofibrillar proteins [283] caused by HPP treatments. The general pattern in protein profiles followed a comparable tendency as the one observed in WHC results, and thus some changes in protein profiles might be linked to the loss of WHC in muscle. Furthermore, changes in protein profiles might also be related with modifications in the texture of fillets.

### 3.3. *Effects of high pressure processing during refrigerated storage of sea bass fillets*

Based on the findings of HPP treatments on enzymatic activity and quality changes in sea bass fillets (Chapters 4.1 and 4.2), two pressure levels of 250 and 400 MPa (5 min pressure holding time; 14 MPa.s$^{-1}$ pressurization rate) were selected to process and preserve fresh sea bass fillets under refrigerated conditions. The quality of fillets was evaluated taking into account microbiological, chemical, physical, and sensory criteria (for further details see Chapter 2.4). In Table 5.8 are summarized the major quality changes in sea bass fillets obtained in this experimental assay.

#### 3.3.1. *Chemical analysis*

Nucleotide degradation was followed during storage of sea bass fillets, considering the freshness indicator $K_I$-index and the inosine 5'-monophosphate (IMP) concentration (see Chapter 4.3 for further details). Lipid oxidation was also monitored during storage following MDA concentration, as a measure of the secondary oxidation products formed (see Chapter 4.3 for further details).

Considering nucleotide analysis, initial levels of $K_I$-index were *ca*. 33 % and IMP concentration was *ca*. 1.5 μmol.g$^{-1}$. By the end of storage, $K_I$-index in HPP treatments increased to *ca*. 70 % and IMP decreased to *ca*. 1.1 μmol.g$^{-1}$. In control treatments, nucleotide degradation evolved more rapidly ($K_I$-index was 87 % and IMP concentration was 0.3 μmol.g$^{-1}$ in day 18). Thus, HPP treatments caused a reduction in nucleotide degradation rate increasing the freshness period of sea bass fillets. These results indicate that HPP treatments may keep pleasant flavours for longer, as IMP has been recognized as having flavour-enhancing properties [27].

**Table 5.8.** Quality changes during refrigerated storage of sea bass fillets treated with high pressure processing.

| | High pressure processing conditions | |
|---|---|---|
| | **250 MPa** | **400 MPa** |
| **Chemical** | | |
| | - Nucleotide degradation rate decreased<br>- Freshness period increased<br>- Lipid oxidation rate increased | - Nucleotide degradation rate decreased<br>- Freshness period increased<br>- Lipid oxidation rate increased |
| **Microbiological** | | |
| | - Bacterial load decreased 2.3 log CFU.g$^{-1}$ in day 1<br>- *Pseudomonas* spp. load decreased in day 1<br>- $H_2S$-producing bacteria and Enterobacteriaceae counts were *ca*. 1 log CFU.g$^{-1}$ during all storage<br>- Higher shelf life (> 18 days) | - Bacterial load decreased 2.3 log CFU.g$^{-1}$ in day 1<br>- *Pseudomonas* spp. load decreased in day 1<br>- $H_2S$-producing bacteria and Enterobacteriaceae counts were *ca*. 1 log CFU.g$^{-1}$ during all storage<br>- Higher shelf life (> 18 days) |
| **Sensory** | | |
| | - Characteristic colour was lost<br>- Fillets become whiter<br>- Glassy/vitreous brightness | - Characteristic colour was lost<br>- Fillets become whiter, looked like cooked<br>- Glassy/vitreous brightness<br>- Fillets become firmer |

*Abbreviations*: CFU – colony forming units.
Descriptions reflect differences in relation to control treatment (0.1 MPa).
Data from Chapter 4.3.

Acid phosphatase is an enzyme responsible for the conversion of IMP to HxR in fish muscle [27]. The results obtained in nucleotide analysis may be explained by the inactivation of acid phosphatase in fillets treated with HPP, as it was observed in Chapter 4.1.

Concerning the results of lipid oxidation, HPP caused an increase in MDA concentration. This effect was more pronounced in the treatment at 400 MPa, and 1.7 mg MDA.kg$^{-1}$ were reached in the end of storage in this treatment. Although recommended values of MDA concentration for seafood (0.72 mg MDA.kg$^{-1}$) [216] were over passed in days 7 and 11 in fillets treated at 400 and 250 MPa, respectively, rancid odours were not detected in any treatment, during all storage.

Results of previous studies showed that the effect of pressure in lipids and lipid oxidation was different depending on the type of fat [127]. Also the content of unsaturated fats in fish species may be responsible for the non-uniform sensitivity of the fats from different fish sources to pressure [127]. The accelerated oxidation in pressurized fish muscle may be due to denaturation of haem proteins by pressure which releases metal ions promoting auto-oxidation of lipids [287].

### 3.3.2. *Microbiological analysis*

Four microbiological analyses were performed in sea bass fillets, including psychrotrophic bacteria, *Pseudomonas* spp., hydrogen sulphide producing bacteria, and Enterobacteriaceae (see Chapter 4.3 for further details).

HPP treatments reduced psychrotrophic bacteria counts in *ca*. 2.3 log CFU.g$^{-1}$. During storage, psychrotrophic bacteria counts increased to 3.7 log CFU.g$^{-1}$ in day 18 in samples treated at 250 MPa, while in the fillets treated at 400 MPa values were below the detection limit (1 log CFU.g$^{-1}$) until day 11 and increased to 2.6 log CFU.g$^{-1}$ in day 18. *Pseudomonas* spp. followed a similar trend as psychrotrophic bacteria. Hydrogen sulphide producing bacteria and Enterobacteriaceae counts were kept around the detection limit (1 log CFU.g$^{-1}$) in the HPP treatments during all storage.

Since bacterial counts in HPP treatments did not reach the upper acceptability limit for fresh marine species (7 log CFU.g$^{-1}$) [47], it was not possible to determine the microbiological shelf life of sea bass fillets treated at 250 and 400 MPa, but at least 4 extra days were achieved.

### 3.3.3. *Sensory analysis*

Panellists denoted that the characteristic colour of raw sea bass fillets was lost in HPP treatments, and a whiter colour was revealed. HPP also caused a loss of translucency, and the brightness on fillets surface looked like glassy/vitreous. The fillets from the 400 MPa treatment had an appearance more similar to cooked fish and were firmer, compared with the other treatments. Although several changes were observed in fillets due to HPP treatments, the overall sensory acceptance was high. Thus, HPP showed potential for process sea bass fillets and create a new product with special attributes.

With the increase in storage time, the score given to the attribute colour-white gradually decreased in HPP treatments, which is not in accordance with the results of colour instrumental measurements. Odour variations were not detected by panellists. In the 250 MPa treatments, the initial characteristics were

kept for longer. In the end of storage (day 18), one third of the panellist rejected HPP treatments based on changes in appearance, such as some lost of firmness and the gapping increase in fillets treated at 400 MPa. In contrast, 50 % of panellist rejected fillets due to changes not only in appearance, but also in fresh odour and firmness, in control samples in day 18.

## Conclusions

The results of this thesis allow to highlight the following conclusions:

The hot water extracts of European pennyroyal and oregano, and the essential oil of clove showed very strong antioxidant activities, measured by DPPH method. Considering the ferric reducing power method, the cold water and ethanolic extracts of European pennyroyal and oregano, and the essential oils of oregano, citronella, Spanish oregano, and thyme were also between the strongest antioxidant substances studied.

Taking into account the antibacterial activity results, the essential oils were more efficient than extracts (hot water, cold water, and ethanolic) to inhibit the growth of a larger number of bacterial strains, including both human pathogenic bacteria and spoilage bacteria common in fish products. Fourteen essential oils inhibited the growth of all bacterial strains tested, and the essential oils of oregano, citronella, garlic, and Spanish oregano presented the lowest MIC values ($< 5 mg.mL^{-1}$).

The application of Spanish oregano essential oils in sea bass fillets increased its microbiological shelf life from 7 to 11 days, under refrigerated conditions. The addition of lemon essential oil in the treatment with Spanish oregano essential oil had a negative effect in the antibacterial properties against Enterobacteriaceae. However, the treatment with both essential oils (Spanish oregano and lemon) decreased lipid oxidation. Furthermore, the addition of lemon essential oil resulted in lower odour intensity compared with the treatment with Spanish oregano essential oil *per se*. The odour intensity of essential oils was determinant for acceptance of sea bass fillets, and those that had softer odours were preferred by panellists.

Films incorporated with essential oils showed lower odours intensity when compared with the characteristic odour of free essential oils. Several physical and mechanical properties of films were changed with the incorporation of essential oils. In general, films became thinner, less soluble in water, and less resistant to elongation. Other variations were specific for each essential oil added to films. The antioxidant activity of films increased with the incorporation of essential oils, especially in those with garlic essential oil. These films also showed antibacterial activity against a higher number of bacterial strains.

The application of films (without essential oils) to contaminated sea bass fillets increased its microbiological shelf life from 4 to 6 days, under refrigerated conditions. These films inhibited the growth of both *Pseudomonas* spp. and Enterobacteriaceae. The incorporation of citronella, garlic, and thyme essential oils in films did not increase the microbiological safety or shelf life of sea bass fillets. Thus, these films did not show potential to be used for the preservation of sea bass fillets, taking into account microbiological criteria.

The study of natural substances to preserve sea bass fillets allowed concluding that essential oils can extend the shelf life of sea bass fillets, taking into account microbiological and chemical criteria. However, the strong odours of essential oils had a negative impact in sensory properties of sea bass fillets, which is determinant for its acceptance. The application of essential oils in films improved the sensory properties, but the concentration tested (1 $\mu L.cm^2$) did not show potential to increase the microbiological safety or shelf life of sea bass fillets.

HPP treatments changed the activity of several degradative enzymes and also the quality of sea bass muscle; being the pressure level and holding time the parameters responsible for the major changes. In general, treatments at lower pressure levels (100 MPa) did not affect immediately the activity of enzymes or the quality of sea bass fillets. The magnitude of changes increased with pressure level and holding time, and the major changes were observed in the treatment at 400 MPa during 30 min of pressure holding time.

In fillets treated at 250 (30 min) and 400 MPa (5-30 min) the lowest activities were observed for the proteolytic enzymes. In these conditions, lipase activity was not affected and acid phosphatase activity decreased further with the increase in pressure level and holding time. Water soluble protein profiles changes seem to be linked to differences in the activity of enzymes. In general, the increase in pressure level and holding time decreased bacterial load and water holding capacity; fillets became whitish and similar to cooked fish and the intensity of myofibrillar proteins with molecular weights below 30 kDa increased. Moreover, HPP treatments might cause protein denaturation, aggregates, degradation, or fragmentation.

HPP treatments (250 and 400 MPa, 5 min) increased the microbiological shelf life of sea bass fillets for at least 4 days. Bacterial counts did not reach the upper

limit considered acceptable for a good quality product, during 18 days of refrigerated storage. This technology also increased the freshness period of sea bass fillets. Although lipid oxidation rate increased in fillets treated with HPP, no rancid or unpleasant odours were noticed in sensory analysis during storage. HPP treatments showed potential for processing sea bass fillets and create a new product with unique characteristics, like the increase in whiteness, the loss of translucency, and a firmer consistency. In general, the effects of HPP treatments were more pronounced in the 400 MPa treatment.

The study of HPP treatments to preserve sea bass fillets allowed concluding that pressure level and holding time were the determinant variables for the general appearance of fillets. If the goal is a product with similar characteristics to fresh sea bass fillets, lower pressure levels and holding times should be used. Instead, if a new product with a different appearance and texture shows no problem of acceptance, higher pressure levels and holding times can be used, and an increase in microbiological safety and shelf life of HPP sea bass fillets will be attained, as well as an increase in the freshness period.

Comparing natural substances and HPP, both methods showed potential to preserve sea bass fillets. The combined use of natural substances and HPP might be more advantageous than each method *per se*. For example, to use essential oils, lower concentrations are suggested to improve sensory properties, but its effectiveness would be probably compromised. Combining both methods, HPP could ensure the microbiological safety and increase freshness for longer, while the essential oils, in its turn, contribute to control lipid oxidation reactions. These type of effects can be additive or synergistic, falling their application within the so called hurdle technologies, which consist in an intelligent application of several processes to improve the overall quality of food products [293]. In fact, few studies were performed combining natural substances and HPP, being the focus of most studies the inactivation of bacterial strains. Reported results in cold-smoked sardine, showed that the combination of rosemary and oregano extracts incorporated in gelatine films and HPP, yielded the best results both for preventing oxidation and inhibiting microbial growth [109]. However, fresh fish was not yet studied in these conditions, and may be one interesting approach for future research studies on preservation of fresh fish.

# REFERENCES


[1] Simopolpoulos, A.P., (1997). Seafood from producer to consumer, natural aspects of the fish, *in*: Lutten, J.B., Borrensen, T., Oehlenschläger, J. (Ed.), *Seafood from producer to consumer, integrated approach to quality*. Elsevier Amsterdam, The Netherlands, pp. 587-607.

[2] Ashie, I.N.A., Smith, J.P., Simpson, B.K., Haard, N.F., (1996). Spoilage and shelf-life extension of fresh fish and shellfish. Critical Reviews in Food Science and Nutrition 36(1), 87-121.

[3] Fellows, P.J., (2000). *Food processing technology: principles and practice* (2 ed). Woodhead Publishing in Food Science and Technology, CRC Press, England.

[4] FAO, (2005-2012). World inventory of fisheries. Reducing post-harvest losses. Issues Fact Sheets. Text by Lahsen Ababouch. FAO Fisheries and Aquaculture Department [online]. Rome, http://www.fao.org/fishery/topic/12369/en.

[5] Parke, D.V., Lewis, D.F.V., (1992). Safety aspects of food preservatives. Food Additives and Contaminants 9(5), 561-577.

[6] Ayala, M.D., Albors, O.L., Blanco, A., Alcázar, A.G., Abellán, E., Zarzosa, G.R., Gil, F., (2005). Structural and ultrastructural changes on muscle tissue of sea bass, *Dicentrarchus labrax* L., after cooking and freezing. Aquaculture 250(1-2), 215-231.

[7] Taliadourou, D., Papadopoulos, V., Domvridou, E., Savvaidis, I.N., Kontominas, M.G., (2003). Microbiological, chemical and sensory changes of whole and filleted Mediterranean aquacultured sea bass (*Dicentrarchus labrax*) stored in ice. Journal of the Science of Food and Agriculture 83(13), 1373-1379.

[8] FAO, (2010). Cultured Aquatic Species Information Programme - *Dicentrarchus labrax* (Linnaeus, 1758 ). FAO - Fisheries and Aquaculture Information and Statistics Service.

[9] Grigorakis, K., (2007). Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. Aquaculture 272(1-4), 55-75.

[10] Kyrana, V.R., Lougovois, V.P., (2002). Sensory, chemical and microbiological assessment of farm-raised European sea bass (*Dicentrarchus labrax*) stored in melting ice. International Journal of Food Science and Technology 37(3), 319-328.

[11] Kılınc, B., Caklı, S., Cadun, A., Dıncer, T., Tolasa, S., (2007). Comparison of effects of slurry ice and flake ice pretreatments on the quality of aquacultured sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) stored at 4°C. Food Chemistry 104(4), 1611-1617.

[12] Özden, Ö., Inugur, M., Erkan, N., (2007). Effect of different dose gamma radiation and refrigeration on the chemical and sensory properties and microbiological status of aqua cultured sea bass (*Dicentrarchus labrax*). Radiation Physics and Chemistry 76(7), 1169-1178.

[13] Torrieri, E., Cavella, S., Villani, F., Masi, P., (2006). Influence of modified atmosphere packaging on the chilled shelf life of gutted farmed bass (*Dicentrarchus labrax*). Journal of Food Engineering 77(4), 1078-1086.

[14] Mendes, R., Gonçalves, A., (2008). Effect of soluble $CO_2$ stabilization on the quality of fillets from farmed gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Journal of Aquatic Food Product Technology 17(4), 342-366.

[15] Mendes, R., Gonçalves, A., (2008). Effect of soluble $CO_2$ stabilisation and vacuum packaging in the shelf life of farmed sea bream and sea bass fillets. International Journal of Food Science and Technology 43(9), 1678-1687.

[16] Chéret, R., Chapleau, N., Delbarre-Ladrat, C., Verrez-Bagnis, V., Lamballerie, M.d., (2005). Effects of high pressure on texture and microstructure of sea bass (*Dicentrarchus labrax* L.) fillets. Journal of Food Science 70(8), e477-e483.

[17] Kostaki, M., Giatrakou, V., Savvaidis, I.N., Kontominas, M.G., (2009). Combined effect of MAP and thyme essential oil on the microbiological, chemical and sensory attributes of organically aquacultured sea bass (*Dicentrarchus labrax*) fillets. Food Microbiology 26(5), 475-482.

[18] Poli, B.M., Parisi, G., Zampacavallo, G., Mecatti, M., Lupi, P., Gualtieri, M., Franci, O., (2001). Quality outline of European sea bass (*Dicentrarchus labrax*) reared in Italy: shelf life, edible yield, nutritional and dietetic traits. Aquaculture 202(3-4), 303-315.

[19] Papadopoulos, V., Chouliara, I., Badeka, A., Savvaidis, I.N., Kontominas, M.G., (2003). Effect of gutting on microbiological, chemical, and sensory properties of aquacultured sea bass (*Dicentrarchus labrax*) stored in ice. Food Microbiology 20(4), 411-420.

[20] Alparslan, Y., Gürel, Ç., Metin, C., Hasanhocaoğlu, H., Baygar, T., (2012). Determination of sensory and quality changes at treated sea bass (*Dicentrarchus labrax*) during cold-storage. Journal of Food Processing & Technology 3(10).

[21] Oehlenschläger, J., Rehbein, H., (2009). Basic facts and figures, *in*: Rehbein, H., Oehlenschläger, J. (Eds.), *Fishery products: quality, safety and authenticity*. Blackwell Publishing Ltd, John Wiley & Sons, United Kingdom, pp. 1-18.

[22] Hui, Y.H., Cross, N., Kristinsson, H.G., Lim, M.H., Nip, W.K., Siow, L.F., Stanfield, P.S., (2006). Biochemistry in seafood processing, *in*: Hui, Y.H., Nip, W., Nollet, L.M.L., Paliyath, G., Simpson, B.K. (Eds.), *Food biochemistry and food processing*, 1 ed. Blackwell Publishing, Ames, Iowa, USA, pp. 351-378.

[23] Hyldig, G., (2007). Sensory profiling of fish, fish product, and shellfish, *in*: Nollet, L.M.L., Boylston, T., Chen, F., Coggins, P.C., Gloria, M.B., Hyldig, G., Kerth, C.R., McKee, L.H., Hui, Y.H. (Eds.), *Handbook of meat, poultry and seafood quality*, 1 ed. Blackwell Publishing, Ames, Iowa, USA, pp. 511-528.

[24] Huss, H.H., (1995). *Quality and quality changes in fresh fish*. Food and Agriculture Organization of the United Nations, Rome, Italy.

[25] Stroud, G.D., (2001). *Rigor in fish - the effect on quality*. Ministry of Technology, Torry Research Station.

[26] Erickson, M.C., (2002). Lipid oxidation of muscle foods, *in*: Akoh, C.C., Min, D.B. (Eds.), *Food lipids - chemistry, nutrition, and biotechnology*, 2 ed. Marcel Dekker, Inc., New York, USA, pp. 365-411.

[27] Gill, T., (2000). Nucleotide-degrading enzymes, *in*: Haard, N.F., Simpson, B.K. (Eds.), *Seafood enzymes - utilization and influence on postharvest seafood quality*. Marcel Dekker Inc., Basel, Switzerland, pp. 37-68.

[28] Nielsen, M.K., Nielsen, H.H., (2006). Seafood enzymes, *in*: Hui, Y.H., Nip, W., Nollet, L.M.L., Paliyath, G., Simpson, B.K. (Eds.), *Food biochemistry and food processing*. Blackwell Publishing, Ames, Iowa, USA, pp. 379-400.

[29] Mendes, R., Quinta, R., Nunes, M.L., (2001). Changes in baseline levels of nucleotides during ice storage of fish and crustaceans from the Portuguese coast. European Food Research and Technology 212(2), 141-146.

[30] Lakshmanan, P.T., Antony, P.D., Gopakumar, K., (1996). Nucleotide degradation and quality changes in mullet (*Liza corsula*) and pearlspot (*Etroplus suratensis*) in ice and at ambient temperatures. Food Control 7(6), 277-283.

[31] Özyurt, G., Kuley, E., Özkütük, S., Özogul, F., (2009). Sensory, microbiological and chemical assessment of the freshness of red mullet (*Mullus barbatus*) and goldband goatfish (*Upeneus moluccensis*) during storage in ice. Food Chemistry 114(2), 505-510.

[32] Saito, T., Arai, K., Matsuyoshi, M., (1959). A new method for estimating the freshness of fish. Bulletin of the Japanese Society of Scientific Fisheries 24, 749-750.

[33] Karube, I., Matsuoka, H., Suzuki, S., Watanabe, E., Toyama, K., (1984). Determination of fish freshness with an enzyme sensor system. Journal of Agricultural and Food Chemistry 32(2), 314-319.

[34] Howgate, P., (2009). Traditional methods, *in*: Rehbein, H., Oehlenschläger, J. (Eds.), *Fishery products: quality, safety and authenticity*. Blackwell Publishing Ltd, John Wiley & Sons, United Kingdom, pp. 19-41.

[35] Botta, J.R., (1995). *Evaluation of seafood freshness quality*. VCH Publishers, Inc., Canada.

[36] EC, (2005). Commission Regulation No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation No 854/2004 of the European Parliament and of the Council and Regulation No 882/2004 of the European Parliament and of the Council, derogating from Regulation No 852/2004 of the European Parliament and of the Council and amending Regulations No 853/2004 and No 854/2004 *L 338, 22 December 2005*, Official Journal, pp. 27-59.

[37] Sotelo, C.G., Rehbein, H., (2000). TMAO-degrading enzymes, *in*: Haard, N.F., Simpson, B.K. (Eds.), *Seafood enzymes - utilization and influence on postharvest seafood quality*. Marcel Dekker Inc., Basel, Switzerland, pp. 167-190.

[38] Careche, M., Barroso, M., (2009). Instrumental texture measurement, *in*: Rehbein, H., Oehlenschläger, J. (Eds.), *Fishery products: quality, safety and authenticity*. Blackwell Publishing Ltd, John Wiley & Sons, United Kingdom, pp. 214-239.

[39] Chéret, R., Delbarre-Ladrat, C., Lamballerie-Anton, M.d., Verrez-Bagnis, V., (2005). High-pressure effects on the proteolytic enzymes of sea bass (*Dicentrarchus labrax* L.) fillets. Journal of Agricultural and Food Chemistry 53(10), 3969-3973.

[40] Jiang, S.-T., (2000). Enzymes and their effects on seafood texture, *in*: Haard, N.F., Simpson, B.K. (Eds.), *Seafood enzymes - utilization and influence on postharvest seafood quality*. Marcel Dekker Inc., Basel, Switzerland, pp. 411-450.

[41] Love, R.M., (1997). Biochemical dynamics and the quality of fresh and frozen fish, *in*: Hall, G.M. (Ed.), *Fish processing technology*, 2 ed. Blackie Academic and Professional, Chapman & Hall, London, pp. 1-31.

[42] López-Amaya, C., Marangoni, A.G., (2000). Lipases, *in*: Haard, N.F., Simpson, B.K. (Eds.), *Seafood enzymes - utilization and influence on postharvest seafood quality*. Marcel Dekker Inc., Basel, Switzerland, pp. 121-146.

[43] Gram, L., Huss, H.H., (1996). Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33(1), 121-137.

[44] Jay, J.M., Loessner, M.J., Golden, D.A., (2005). *Modern food microbiology* (7 ed). Springer Science, Business Media, Inc., USA.

[45] Lyhs, U., (2009). Microbiological methods, *in*: Rehbein, H., Oehlenschläger, J. (Eds.), *Fishery products: quality, safety and authenticity*. Blackwell Publishing Ltd, John Wiley & Sons, United Kingdom, pp. 318-348.

[46] EC, (2005). Commission Regulation No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs *L 338, 22 December 2005*, Official Journal, pp. 1-26.

[47] ICMSF, (1986). *Microorganisms in foods. 2. Sampling for microbiological analysis: principles and specific applications* (2 ed). University of Toronto Press, Buffalo, NY.

[48] FAO, WHO, (2001). Guidelines for sensory evaluation of fish and shellfish in laboratories, *in*: FAO, WHO (Eds.), *Codex Alimentarius: Fish and Fishery Products*. Food and Agriculture Organization of the United Nations, World Health Organization, Rome, Italy, pp. 113-140.

[49] EC, (2008). Regulation (EC) No 1333/2008 of the European parliament and of the council of 16 December 2008 on food additives, *L 354, 16 December 2008*, Official Journal of the European Union, pp. 16-33.

[50] Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., (2008). Biological effects of essential oils - a review. Food and Chemical Toxicology 46(2), 446-475.

[51] Buchbauer, G., (2010). Biological activities of essential oils, *in*: Başer, K.H.C., Buchbauer, G. (Eds.), *Handbook of essential oils: science, technology, and applications*. CRC Press, Taylor & Francis Group, Boca Raton, pp. 235-280.

[52] Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G., Ruberto, G., (1998). Antimicrobial and antioxidant properties of some commercial essential oils. Flavour and Fragrance Journal 13(4), 235-244.

[53] Smith-Palmer, A., Stewart, J., Fyfe, L., (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Applied Microbiology 26(2), 118-122.

[54] Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., Bruni, R., (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chemistry 91(4), 621-632.

[55] Ruberto, G., Baratta, M.T., (2000). Antioxidant activity of selected essential oil components in two lipid model systems. Food Chemistry 69(2), 167-174.

[56] Wang, H.-F., Yih, K.-H., Huang, K.-F., (2010). Comparative study of the antioxidant activity of forty-five commonly used essential oils and their potential active components. Journal of Food and Drug Analysis 18(1), 24-33.

[57] Chorianopoulos, N., Kalpoutzakis, E., Aligiannis, N., Mitaku, S., Nychas, G.-J., Haroutounian, S.A., (2004). Essential oils of *Satureja*, *Origanum*, and *Thymus* species: chemical composition and antibacterial activities against foodborne pathogens. Journal of Agricultural and Food Chemistry 52(26), 8261-8267.

[58] Devi, K.P., Nisha, S.A., Sakthivel, R., Pandian, S.K., (2010). Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. Journal of Ethnopharmacology 130(1), 107-115.

[59] Inouye, S., Takizawa, T., Yamaguchi, H., (2001). Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. Journal of Antimicrobial Chemotherapy 47(5), 565-573.

[60] Kim, J., Marshall, M.R., Wei, C.-i., (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. Journal of Agricultural and Food Chemistry 43(11), 2839-2845.

[61] Regnier, T., Combrinck, S., Plooy, W.D., (2012). Essential oils and other plants extracts as food preservatives, *in*: Bhat, R., Alias, A.K., Paliyath, G. (Eds.), *Progress in food preservation*. John Wiley & Sons Ltd, West Sussex, United Kingdom, pp. 539-580.

[62] Burt, S., (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology 94(3), 223-253.

[63] Franz, C., Novak, J., (2010). Sources of essential oils, *in*: Başer, K.H.C., Buchbauer, G. (Eds.), *Handbook of essential oils: science, technology, and applications*. CRC Press, Taylor & Francis Group, Boca Raton, pp. 39-82.

[64] Harborne, A.J., (1998). *Phytochemical methods - a guide to modern techniques of plant analysis*. Chapman & Hall, London, United Kingdom.

[65] Raaman, N., (2006). *Phytochemical techniques*. New India Publishing Agency, New Delhi, India.

[66] Schmidt, E., (2010). Production of essential oils, *in*: Başer, K.H.C., Buchbauer, G. (Eds.), *Handbook of essential oils: science, technology, and applications*. CRC Press, Taylor & Francis Group, Boca Raton, pp. 83-120.

[67] Putievsky, E., Ravid, U., Dudai, N., (1986). The essential oil and yield components from various plant parts of *Salvia fruticosa*. Journal of Natural Products 49(6), 1015-1017.

[68] Müller-Riebau, F.J., Berger, B.M., Yegen, O., Cakir, C., (1997). Seasonal variations in the chemical compositions of essential oils of selected aromatic plants growing wild in Turkey. Journal of Agricultural and Food Chemistry 45(12), 4821-4825.

[69] Jordán, M.J., Martínez, R.M., Goodner, K.L., Baldwin, E.A., Sotomayor, J.A., (2006). Seasonal variation of *Thymus hyemalis* Lange and Spanish *Thymus vulgaris* L. essential oils composition. Industrial Crops and Products 24(3), 253-263.

[70] Viljoen, A.M., Subramoney, S., Vuuren, S.F.v., Başer, K.H.C., Demirci, B., (2005). The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. Journal of Ethnopharmacology 96(1-2), 271-277.

[71] Mejri, J., Abderrabba, M., Mejri, M., (2010). Chemical composition of the essential oil of *Ruta chalepensis* L: Influence of drying, hydro-distillation duration and plant parts. Industrial Crops and Products 32(3), 671-673.

[72] Miguel, M.G., (2010). Antioxidant activity of medicinal and aromatic plants. A review. Flavour and Fragrance Journal 25(5), 291-312.

[73] Bozin, B., Mimica-Dukic, N., Simin, N., Anackov, G., (2006). Characterization of the volatile composition of essential oils of some Lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. Journal of Agricultural and Food Chemistry 54(5), 1822-1828.

[74] Harpaz, S., Glatman, L., Drabkin, V., Gelman, A., (2003). Effects of herbal essential oils used to extend the shelf life of freshwater-reared Asian sea bass fish (*Lates calcarifer*). Journal of Food Protection 66(3), 410-417.

[75] Ahmad, M., Benjakul, S., Sumpavapol, P., Nirmal, N.P., (2012). Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil. International Journal of Food Microbiology 155(3), 171-178.

[76] Erkan, N., Tosun, S.Y., Ulusoy, S., Uretener, G., (2011). The use of thyme and laurel essential oil treatments to extend the shelf life of bluefish (*Pomatomus saltatrix*) during storage in ice. Journal Fur Verbraucherschutz Und Lebensmittelsicherheit - Journal of Consumer Protection and Food Safety 6(1), 39-48.

[77] Kykkidou, S., Giatrakou, V., Papavergou, A., Kontominas, M.G., Savvaidis, I.N., (2009). Effect of thyme essential oil and packaging treatments on fresh Mediterranean swordfish fillets during storage at 4 °C. Food Chemistry 115(1), 169-175.

[78] Giatrakou, V., Kykkidou, S., Papavergou, A., Kontominas, M.G., Savvaidis, I.N., (2008). Potential of oregano essential oil and MAP to extend the shelf life of fresh swordfish: a comparative study with ice storage. Journal of Food Science 73(4), M167-M173.

[79] Mexis, S.F., Chouliara, E., Kontominas, M.G., (2009). Combined effect of an oxygen absorber and oregano essential oil on shelf life extension of rainbow trout fillets stored at 4 °C. Food Microbiology 26(6), 598-605.

[80] Frangos, L., Pyrgotou, N., Giatrakou, V., Ntzimani, A., Savvaidis, I.N., (2010). Combined effects of salting, oregano oil and vacuum-packaging on the shelf-life of refrigerated trout fillets. Food Microbiology 27(1), 115-121.

[81] Ojagh, S.M., Rezaei, M., Razavi, S.H., Hosseini, S.M.H., (2010). Effect of chitosan coatings enriched with cinnamon oil on the quality of refrigerated rainbow trout. Food Chemistry 120(1), 193-198.

[82] Goulas, A.E., Kontominas, M.G., (2007). Combined effect of light salting, modified atmosphere packaging and oregano essential oil on the shelf-life of sea bream (*Sparus aurata*): Biochemical and sensory attributes. Food Chemistry 100(1), 287-296.

[83] Gómez-Estaca, J., Lacey, A.L.d., López-Caballero, M.E., Gómez-Guillén, M.C., Montero, P., (2010). Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. Food Microbiology 27(7), 889-896.

[84] Quitral, V., Donoso, M.L., Ortiz, J., Herrera, M.V., Araya, H., Aubourg, S.P., (2009). Chemical changes during the chilled storage of Chilean jack mackerel (*Trachurus murphyi*): Effect of a plant-extract icing system. LWT - Food Science and Technology 42(8), 1450-1454.

[85] Pavlath, A.E., Orts, W., (2009). Edible films and coatings: why, what, and how?, *in*: Embuscado, M.E., Huber, K.C. (Eds.), *Edible films and coatings for food applications*. Springer, New York, USA, pp. 1-23.

[86] Gennadios, A., Hanna, M.A., Kurth, L.B., (1997). Application of edible coatings on meats, poultry and seafoods: a review. Lebensmittel-Wissenschaft und-Technologie 30(4), 337-350.

[87] Quezada-Gallo, J.-A., (2009). Delivery of food additives and antimicrobials using edible films and coatings, *in*: Embuscado, M.E., Huber, K.C. (Eds.), *Edible films and coatings for food applications*. Springer, New York, USA, pp. 315-333.

[88] Guilbert, S., Gontard, N., Gorris, L.G.M., (1996). Prolongation of the shelf-life of perishable food products using biodegradable films and coatings. Lebensmittel-Wissenschaft und-Technologie 29(1-2), 10-17.

[89] Avena-Bustillos, R.d.J., McHugh, T.H., (2012). Role of edible film and coating additives, *in*: Baldwin, E.A., Hagenmaier, R., Bai, J. (Eds.), *Edible coatings and films to improve food quality*. CRC Press, Taylor & Francis Group, Boca Raton, Philadelphia, USA, pp. 157-184.

[90] Baldwin, E.A., Hagenmaier, R.D., (2012). Introduction, *in*: Baldwin, E.A., Hagenmaier, R., Bai, J. (Eds.), *Edible coatings and films to improve food quality*. CRC Press, Taylor & Francis Group, Boca Raton, Philadelphia, USA, pp. 1-12.

[91] Dangaran, K., Tomasula, P.M., Qi, P., (2009). Structure and function of protein-based edible films and coatings, *in*: Embuscado, M.E., Huber, K.C. (Eds.), *Edible films and coatings for food applications*. Springer, New York, USA, pp. 24-56.

[92] Artharn, A., Benjakul, S., Prodpran, T., (2008). The effect of myofibrillar/sarcoplasmic protein ratios on the properties of round scad muscle protein based film. European Food Research and Technology 227(1), 215-222.

[93] Chinabhark, K., Benjakul, S., Prodpran, T., (2007). Effect of pH on the properties of protein-based film from bigeye snapper (*Priacanthus tayenus*) surimi. Bioresource Technology 98(1), 221-225.

[94] Jongjareonrak, A., Benjakul, S., Visessanguan, W., Prodpran, T., Tanaka, M., (2006). Characterization of edible films from skin gelatin of brownstripe red snapper and bigeye snapper. Food Hydrocolloids 20(4), 492-501.

[95] Cuq, B., Gontard, N., Cuq, J.L., Guilbert, S., (1997). Selected functional properties of fish myofibrillar protein-based films as affected by hydrophilic plasticizers. Journal of Agricultural and Food Chemistry 45(3), 622-626.

[96] García, F.T., Sobral, P.J.d.A., (2005). Effect of the thermal treatment of the filmogenic solution on the mechanical properties, color and opacity of films based on muscle proteins of two varieties of Tilapia. LWT - Food Science and Technology 38(3), 289-296.

[97] Hamaguchi, P.Y., WuYin, W., Tanaka, M., (2007). Effect of pH on the formation of edible films made from the muscle proteins of Blue marlin (*Makaira mazara*). Food Chemistry 100(3), 914-920.

[98] Shiku, Y., Hamaguchi, P.Y., Benjakul, S., Visessanguan, W., Tanaka, M., (2004). Effect of surimi quality on properties of edible films based on Alaska pollack. Food Chemistry 86(4), 493-499.

[99] Gómez-Guillén, M.C., Ihl, M., Bifani, V., Silva, A., Montero, P., (2007). Edible films made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (*Ugni molinae* Turcz). Food Hydrocolloids 21(7), 1133-1143.

[100] Benjakul, S., Artharn, A., Prodpran, T., (2008). Properties of protein-based film from round scad (*Decapterus maruadsi*) muscle as influenced by fish quality. LWT - Food Science and Technology 41(5), 753-763.

[101] Pérez-Gago, M.B., (2012). Protein-based films and coatings, *in*: Baldwin, E.A., Hagenmaier, R., Bai, J. (Eds.), *Edible coatings and films to improve food quality*. CRC Press, Taylor & Francis Group, Boca Raton, Philadelphia, USA, pp. 13-77.

[102] Batista, I., Pires, C., Nelhas, R., Godinho, V., (2006). Acid and alkaline-aided protein recovery from Cape hake by-products, *in*: Luten, J.B., Jacobsen, C., Bekaert, K., Sæbø, A., Oehlenschläger, J. (Eds.), *Seafood research from fish to dish. Quality, safety and processing of wild and farmed fish*. Wageningen Academic Publishers, The Netherlands, pp. 427-438.

[103] Cardoso, C.M.L., (2011). Underutilized fish species and fish processing by-products upgrading strategies, *Instituto de Ciências Biomédicas Abel Salazar*. Universidade do Porto, Porto, Portugal, p. 330.

[104] Jaczynski, J., (2008). Protein and lipid recovery from food processing by-products using isoelectric solubilization/precipitation, *in*: Papadopoulos, K.N. (Ed.), *Food chemistry research developments*. Nova Science Publishers, Inc, New York, pp. 167-198.

[105] Han, J.H., (2005). New technologies in food packaging: overview, *in*: Han, J. (Ed.), *Innovations in food packaging*. Elsevier Academic Press, San-Diego, California, USA, pp. 3-11.

[106] Debeaufort, F., Quezada-Gallo, J.-A., Voilley, A., (1998). Edible films and coatings: tomorrow's packagings: a review. Critical Reviews in Food Science and Nutrition 38(4), 299 - 313.

[107] Oussalah, M., Caillet, S., Salmiéri, S., Saucier, L., Lacroix, M., (2004). Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of whole beef muscle. Journal of Agricultural and Food Chemistry 52(18), 5598-5605.

[108] Seydim, A.C., Sarikus, G., (2006). Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Research International 39(5), 639-644.

[109] Gómez-Estaca, J., Montero, P., Giménez, B., Gómez-Guillén, M.C., (2007). Effect of functional edible films and high pressure processing on microbial and oxidative spoilage in cold-smoked sardine (*Sardina pilchardus*). Food Chemistry 105(2), 511-520.

[110] Pelissari, F.M., Grossmann, M.V.E., Yamashita, F., Pineda, E.A.G., (2009). Antimicrobial, mechanical, and barrier properties of cassava starch−chitosan films incorporated with oregano essential oil. Journal of Agricultural and Food Chemistry 57(16), 7499-7504.

[111] García, M.A., Martino, M.N., Zaritzky, N.E., (2000). Lipid addition to improve barrier properties of edible starch-based films and coatings. Journal of Food Science 65(6), 941-944.

[112] Mertens, B., (1999). Hydrostatic pressure treatment of food: equipment and processing, *in*: Gould, G.W. (Ed.), *New methods of food preservation*. Aspen Publishers, Inc., pp. 135-158.

[113] Patterson, M.F., (2005). Microbiology of pressure-treated foods. Journal of Applied Microbiology 98(6), 1400-1409.

[114] Patterson, M.F., Linton, M., Doona, C.J., (2007). Introduction to high pressure processing of foods, *in*: Doona, C.J., Feeherry, F.E. (Eds.), *High pressure processing of foods*. Blackwell Publishing and the Institute of Food Technologists, Ames, USA, pp. 1-14.

[115] Singh, R.P., (2001). Part I: technical elements, *Technical elements of new and emerging non-thermal food technologies*. Food and Agriculture Organization, Rome, Italy.

[116] Patterson, M.F., Ledward, D.A., Rogers, N., (2006). High Pressure Processing, *in*: Brennan, J.G. (Ed.), *Food Processing Handbook*. Wiley, Weinheim, Germany, pp. 173-200.

[117] Chapleau, N.J., Lamballerie-Anton, M.I.d., (2003). Changes in myofibrillar proteins interactions and rheological properties induced by high-pressure processing. European Food Research and Technology 216(6), 470-476.

[118] Smelt, J.P.P.M., (1998). Recent advances in the microbiology of high pressure processing. Trends in Food Science & Technology 9(4), 152-158.

[119] Yaldagard, M., Mortazavi, S.A., Tabatabaie, F., (2008). Review: The principles of ultra high pressure technology and its application in food processing/preservation: A review of microbiological and quality aspects. African Journal of Biotechnology 7(16), 2739-2767.

[120] Messens, W., Camp, J.V., Huyghebaert, A., (1997). The use of high pressure to modify the functionality of food proteins. Trends in Food Science & Technology 8(4), 107-112.

[121] Hendrickx, M., Ludikhuyze, L., Broeck, I.V.d., Weemaes, C., (1998). Effects of high pressure on enzymes related to food quality. Trends in Food Science & Technology 9(5), 197-203.

[122] Matser, A.M., Stegeman, D., Kals, J., Bartels, P.V., (2000). Effects of high pressure on colour and texture of fish. High Pressure Research: An International Journal 19(1), 109-115.

[123] Amanatidou, A., Schlüter, O., Lemkau, K., Gorris, L.G.M., Smid, E.J., Knorr, D., (2000). Effect of combined application of high pressure treatment and modified atmospheres on the shelf life of fresh Atlantic salmon. Innovative Food Science & Emerging Technologies 1(2), 87-98.

[124] Yagiz, Y., Kristinsson, H.G., Balaban, M.O., Welt, B.A., Ralat, M., Marshall, M.R., (2009). Effect of high pressure processing and cooking treatment on the quality of Atlantic salmon. Food Chemistry 116(4), 828-835.

[125] Ortea, I., Rodríguez, A., Tabilo-Munizaga, G., Pérez-Won, M., Aubourg, S.P., (2010). Effect of hydrostatic high-pressure treatment on proteins, lipids and nucleotides in chilled farmed salmon (*Oncorhynchus kisutch*) muscle. European Food Research and Technology 230(6), 925-934.

[126] Briones, L.S., Reyes, J.E., Tabilo-Munizaga, G.E., Pérez-Won, M.O., (2010). Microbial shelf-life extension of chilled Coho salmon (*Oncorhynchus kisutch*) and abalone (*Haliotis rufescens*) by high hydrostatic pressure treatment. Food Control 21(11), 1530-1535.

[127] Erkan, N., Üretener, G., (2010). The effect of high hydrostatic pressure on the microbiological, chemical and sensory quality of fresh gilthead sea bream (*Sparus aurata*). European Food Research and Technology 230(4), 533-542.

[128] Campus, M., Addis, M.F., Cappuccinelli, R., Porcu, M.C., Pretti, L., Tedde, V., Secchi, N., Stara, G., Roggio, T., (2010). Stress relaxation behaviour and structural changes of muscle tissues from gilthead sea bream (*Sparus aurata* L.) following high pressure treatment. Journal of Food Engineering 96(2), 192-198.

[129] Erkan, N., Üretener, G., Alpas, H., (2010). Effect of high pressure (HP) on the quality and shelf life of red mullet (*Mullus surmelutus*). Innovative Food Science & Emerging Technologies 11(2), 259-264.

[130] Zare, Z., (2004). High pressure processing of fresh tuna fish and its effects on shelf life, *Department of Food Science and Agricultural Chemistry*. McGill University, Montreal, Quebec, Canada, p. 94.

[131] Atrea, I., Papavergou, A., Amvrosiadis, I., Savvaidis, I.N., (2009). Combined effect of vacuum-packaging and oregano essential oil on the shelf-life of Mediterranean octopus (*Octopus vulgaris*) from the Aegean Sea stored at 4 °C. Food Microbiology 26(2), 166-172.

[132] Mosqueda-Melgar, J., Raybaudi-Massilia, R.M., Martín-Belloso, O., (2008). Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices. Food Microbiology 25(3), 479-491.

[133] Cao, L., Si, J.Y., Liu, Y., Sun, H., Jin, W., Li, Z., Zhao, X.H., Pan, R.L., (2009). Essential oil composition, antimicrobial and antioxidant properties of *Mosla chinensis* Maxim. Food Chemistry 115(3), 801-805.

[134] Sökmen, A., Sökmen, M., Daferera, D., Polissiou, M., Candan, F., Ünlü, M., Akpulat, H.A., (2004). The *in vitro* antioxidant and antimicrobial activities of the essential oil and methanol extracts of *Achillea biebersteini* Afan. (Asteraceae). Phytotherapy Research 18(6), 451-456.

[135] Gruenwald, J., Brendler, T., Jaenicke, C., (2000). *PDR for herbal medicines.* Medical Economic Company, Inc., Montvale, NJ.

[136] Agnihotri, V.K., Agarwal, S.G., Dhar, P.L., Thappa, R.K., Baleshwar, Kapahi, B.K., Saxena, R.K., Qazi, G.N., (2005). Essential oil composition of *Mentha pulegium* L. growing wild in the north-western Himalayas India. Flavour and Fragrance Journal 20(6), 607-610.

[137] Aghel, N., Yamini, Y., Hadjiakhoondi, A., Pourmortazavi, S.M., (2004). Supercritical carbon dioxide extraction of *Mentha pulegium* L. essential oil. Talanta 62(2), 407-411.

[138] Petrakis, E.A., Kimbaris, A.C., Pappas, C.S., Tarantilis, P.A., Polissiou, M.G., (2009). Quantitative determination of pulegone in pennyroyal oil by FT-IR spectroscopy. Journal of Agricultural and Food Chemistry 57(21), 10044-10048.

[139] Reis-Vasco, E.M.C., Coelho, J.A.P., Palavra, A.M.F., (1999). Comparison of pennyroyal oils obtained by supercritical $CO_2$ extraction and hydrodistillation. Flavour and Fragrance Journal 14(3), 156-160.

[140] Mata, A.T., Proença, C., Ferreira, A.R., Serralheiro, M.L.M., Nogueira, J.M.F., Araújo, M.E.M., (2007). Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. Food Chemistry 103(3), 778-786.

[141] Adams, R.P., (2001). *Identification of essential oil components by gas chromatography/quadrupole mass spectrometry.* Allured Publishing Corporation, Carol Stream, IL.

[142] Benzie, I.F.F., Strain, J.J., (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 239(1), 70-76.

[143] Deighton, N., Brennan, R., Finn, C., Davies, H.V., (2000). Antioxidant properties of domesticated and wild *Rubus* species. Journal of the Science of Food and Agriculture 80(9), 1307-1313.

[144] Oyaizu, M., (1986). Studies on products of browning reaction: antioxidative activity of products of browning reaction. Japanese Journal of Nutrition 44, 307-315.

[145] Kondo, S., Tsuda, K., Muto, N., Ueda, J.-e., (2002). Antioxidative activity of apple skin or flesh extracts associated with fruit development on selected apple cultivars. Scientia Horticulturae 96(1-4), 177-185.

[146] Scherer, R., Godoy, H.T., (2009). Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. Food Chemistry 112(3), 654-658.

[147] Asami, D.K., Hong, Y.-J., Barrett, D.M., Mitchell, A.E., (2003). Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. Journal of Agricultural and Food Chemistry 51(5), 1237-1241.

[148] NCCLS, (2002). *Performance standards for antimicrobial susceptibility testing; Twelfth International Supplement; M100-S12*. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.

[149] Sivropoulou, A., Kokkini, S., Lanaras, T., Arsenakis, M., (1995). Antimicrobial activity of mint essential oils. Journal of Agricultural and Food Chemistry 43(9), 2384-2388.

[150] Mahboubi, M., Haghi, G., (2008). Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil. Journal of Ethnopharmacology 119(2), 325-327.

[151] Dorman, H.J.D., Kosar, M., Kahlos, K., Holm, Y., Hiltunen, R., (2003). Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. Journal of Agricultural and Food Chemistry 51(16), 4563-4569.

[152] Beghidja, N., Bouslimani, N., Benayache, F., Benayache, S., Chalchat, J., (2007). Composition of the oils from *Mentha pulegium* grown in different areas of the East of Algeria. Chemistry of Natural Compounds 43(4), 481-483.

[153] Díaz-Maroto, M.C., Castillo, N., Castro-Vázquez, L., González-Viñas, M.Á., Pérez-Coello, M.S., (2007). Volatile composition and olfactory profile of pennyroyal (*Mentha pulegium*) plants. Flavour and Fragrance Journal 22(2), 114-118.

[154] Hajlaoui, H., Trabelsi, N., Noumi, E., Snoussi, M., Fallah, H., Ksouri, R., Bakhrouf, A., (2009). Biological activities of the essential oils and methanol extract of tow cultivated mint species (*Mentha longifolia* and *Mentha pulegium*) used in the Tunisian folkloric medicine. World Journal of Microbiology and Biotechnology 25(12), 2227-2238.

[155] Kamkar, A., Javan, A.J., Asadi, F., Kamalinejad, M., (2010). The antioxidative effect of Iranian *Mentha pulegium* extracts and essential oil in sunflower oil. Food and Chemical Toxicology 48(7), 1796-1800.

[156] Marzouk, B., Fredj, M.B.H., Chraief, I., Mastouri, M., Boukef, K., Marzouk, Z., (2008). Chemical composition and antimicrobial activity of essential oils from Tunisian *Mentha pulegium* L. Journal of Food, Agriculture & Environment 6(1), 78-82.

[157] Müller-Riebau, F., Berger, B., Yegen, O., (1995). Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey. Journal of Agricultural and Food Chemistry 43(8), 2262-2266.

[158] Mkaddem, M., Boussaid, M., Fadhel, N.B., (2007). Variability of volatiles in Tunisian *Mentha pulegium* L. (Lamiaceae). Journal of Essential Oil Research 19(3), 211-214.

[159] Nickavar, B., Alinaghi, A., Kamalinejad, M., (2008). Evaluation of the antioxidant properties of five Mentha species. Iranian Journal of Pharmaceutical Research 7(3), 203-209.

[160] Guimarães, R., Barreira, J.C.M., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R., (2011). Effects of oral dosage form and storage period on the antioxidant properties of four species used in traditional herbal medicine. Phytotherapy Research 25(4), 484-492.

[161] Borneo, R., León, A.E., Aguirre, A., Ribotta, P., Cantero, J.J., (2009). Antioxidant capacity of medicinal plants from the Province of Córdoba (Argentina) and their *in vitro* testing in a model food system. Food Chemistry 112(3), 664-670.

[162] Gourine, N., Yousfi, M., Bombarda, I., Nadjemi, B., Stocker, P., Gaydou, E.M., (2010). Antioxidant activities and chemical composition of essential oil of *Pistacia atlantica* from Algeria. Industrial Crops and Products 31(2), 203-208.

[163] Nguyen, M.T., Kryachko, E.S., Vanquickenborne, L.G., (2003). General and theoretical aspects of phenols, *in*: Rappoport, Z. (Ed.), *The chemistry of phenols*. John Wiley & Sons Ltd, Chichester, West Sussex, England, pp. 1-198.

[164] Hogg, S., (2005). *Essential microbiology.* John Wiley & Sons, Ltd, Chichester, West Sussex, England.

[165] Duru, M.E., Öztürk, M., Ugur, A., Ceylan, Ö., (2004). The constituents of essential oil and *in vitro* antimicrobial activity of *Micromeria cilicica* from Turkey. Journal of Ethnopharmacology 94(1), 43-48.

[166] Chouliara, E., Karatapanis, A., Savvaidis, I.N., Kontominas, M.G., (2007). Combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, stored at 4 °C. Food Microbiology 24(6), 607-617.

[167] Daferera, D.J., Tarantilis, P.A., Polissiou, M.G., (2002). Characterization of essential oils from Lamiaceae species by Fourier transform Raman spectroscopy. Journal of Agricultural and Food Chemistry 50(20), 5503-5507.

[168] Mockute, D., Bernotiene, G., Judzentiene, A., (2001). The essential oil of *Origanum vulgare* L. ssp. *vulgare* growing wild in Vilnius district (Lithuania). Phytochemistry 57(1), 65-69.

[169] Bisht, D., Chanotiya, C.S., Rana, M., Semwal, M., (2009). Variability in essential oil and bioactive chiral monoterpenoid compositions of Indian oregano (*Origanum vulgare* L.) populations from northwestern Himalaya and their chemotaxonomy. Industrial Crops and Products 30(3), 422-426.

[170] Figiel, A., Szumny, A., Gutiérrez-Ortíz, A., Carbonell-Barrachina, Á.A., (2010). Composition of oregano essential oil (*Origanum vulgare*) as affected by drying method. Journal of Food Engineering 98(2), 240-247.

[171] Russo, M., Galletti, G.C., Bocchini, P., Carnacini, A., (1998). Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart): a preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. Inflorescences. Journal of Agricultural and Food Chemistry 46(9), 3741-3746.

[172] Faleiro, L., Miguel, G., Gomes, S., Costa, L., Venancio, F., Teixeira, A., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., (2005). Antibacterial and antioxidant activities of essential oils isolated from *Thymbra capitata* L. (Cav.) and *Origanum vulgare* L. Journal of Agricultural and Food Chemistry 53(21), 8162-8168.

[173] Safaei-Ghomi, J., Ebrahimabadi, A.H., Djafari-Bidgoli, Z., Batooli, H., (2009). GC/MS analysis and *in vitro* antioxidant activity of essential oil and methanol extracts of *Thymus caramanicus* Jalas and its main constituent carvacrol. Food Chemistry 115(4), 1524-1528.

[174] Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., Raneva, V.G., (1999). Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chemistry 64(1), 59-66.

[175] Aligiannis, N., Kalpoutzakis, E., Mitaku, S., Chinou, I.B., (2001). Composition and antimicrobial activity of the essential oils of two *Origanum* species. Journal of Agricultural and Food Chemistry 49(9), 4168-4170.

[176] Şahin, F., Güllüce, M., Daferera, D., Sökmen, A., Sökmen, M., Polissiou, M., Agar, G., Özer, H., (2004). Biological activities of the essential oils and methanol extract of *Origanum vulgare* ssp. *vulgare* in the eastern Anatolia region of Turkey. Food Control 15(7), 549-557.

[177] Chun, S.-S., Vattem, D.A., Lin, Y.-T., Shetty, K., (2005). Phenolic antioxidants from clonal oregano (*Origanum vulgare*) with antimicrobial activity against *Helicobacter pylori*. Process Biochemistry 40(2), 809-816.

[178] Adam, K., Sivropoulou, A., Kokkini, S., Lanaras, T., Arsenakis, M., (1998). Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. Journal of Agricultural and Food Chemistry 46(5), 1739-1745.

[179] Daferera, D.J., Ziogas, B.N., Polissiou, M.G., (2000). GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. Journal of Agricultural and Food Chemistry 48(6), 2576-2581.

[180] Dambolena, J.S., Zunino, M.P., Lucini, E.I., Olmedo, R., Banchio, E., Bima, P.J., Zygadlo, J.A., (2010). Total phenolic content, radical scavenging properties, and essential oil composition of *Origanum* species from different populations. Journal of Agricultural and Food Chemistry 58(2), 1115-1120.

[181] Karpouhtsis, I., Pardali, E., Feggou, E., Kokkini, S., Scouras, Z.G., Mavragani-Tsipidou, P., (1998). Insecticidal and genotoxic activities of oregano essential oils. Journal of Agricultural and Food Chemistry 46(3), 1111-1115.

[182] Milos, M., Mastelic, J., Jerkovic, I., (2000). Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp. *hirtum*). Food Chemistry 71(1), 79-83.

[183] Prieto, J.M., Iacopini, P., Cioni, P., Chericoni, S., (2007). *In vitro* activity of the essential oils of *Origanum vulgare*, *Satureja montana* and their main constituents in peroxynitrite-induced oxidative processes. Food Chemistry 104(3), 889-895.

[184] Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., Arsenakis, M., (1996). Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agricultural and Food Chemistry 44(5), 1202-1205.

[185] Caillet, S., Lacroix, M., (2006). Effect of gamma radiation and oregano essential oil on murein and ATP concentration of *Listeria monocytogenes*. Journal of Food Protection 69(12), 2961-2969.

[186] Caillet, S., Shareck, F., Lacroix, M., (2005). Effect of gamma radiation and oregano essential oil on murein and ATP concentration of *Escherichia coli* O157:H7. Journal of Food Protection 68(12), 2571-2579.

[187] Rios, J.L., Recio, M.C., Villar, A., (1988). Screening methods for natural products with antimicrobial activity: A review of the literature. Journal of Ethnopharmacology 23(2-3), 127-149.

[188] Holley, R.A., Patel, D., (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology 22(4), 273-292.

[189] Hersch-Martínez, P., Leaños-Miranda, B.E., Solórzano-Santos, F., (2005). Antibacterial effects of commercial essential oils over locally prevalent pathogenic strains in Mexico. Fitoterapia 76(5), 453-457.

[190] Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M.M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. Food Chemistry 105(1), 146-155.

[191] Guan, W., Li, S., Yan, R., Tang, S., Quan, C., (2007). Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. Food Chemistry 101(4), 1558-1564.

[192] Uysal, B., Sozmen, F., Aktas, O., Oksal, B.S., Kose, E.O., (2011). Essential oil composition and antibacterial activity of the grapefruit (*Citrus paradisi* L) peel essential oils obtained by solvent-free microwave extraction: comparison with hydrodistillation. International Journal of Food Science & Technology 46(7), 1455-1461.

[193] Hussain, A.I., Anwar, F., Sherazi, S.T.H., Przybylski, R., (2008). Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. Food Chemistry 108(3), 986-995.

[194] Kanko, C., Sawaliho, B.E.-H., Kone, S., Koukoua, G., N'Guessan, Y.T., (2004). Étude des propriétés physico-chimiques des huiles essentielles de *Lippia multiflora*, *Cymbopogon citratus*, *Cymbopogon nardus*, *Cymbopogon giganteus*. Comptes Rendus Chimie 7(10-11), 1039-1042.

[195] Matasyoh, J.C., Maiyo, Z.C., Ngure, R.M., Chepkorir, R., (2009). Chemical composition and antimicrobial activity of the essential oil of *Coriandrum sativum*. Food Chemistry 113(2), 526-529.

[196] Oyedemi, S.O., Okoh, A.I., Mabinya, L.V., Pirochenva, G., Afolayan, A.J., (2009). The proposed mechanism of bactericidal action of eugenol, α-terpineol and γ-terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*. African Journal of Biotechnology 8(7), 1280-1286.

[197] Ross, Z.M., O'Gara, E.A., Hill, D.J., Sleightholme, H.V., Maslin, D.J., (2001). Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. Appl. Environ. Microbiol. 67(1), 475-480.

[198] Misharina, T., Samusenko, A., (2008). Antioxidant properties of essential oils from lemon, grapefruit, coriander, clove, and their mixtures. Applied Biochemistry and Microbiology 44(4), 438-442.

[199] Jirovetz, L., Buchbauer, G., Stoilova, I., Stoyanova, A., Krastanov, A., Schmidt, E., (2006). Chemical composition and antioxidant properties of clove leaf essential oil. Journal of Agricultural and Food Chemistry 54(17), 6303-6307.

[200] Wei, A., Shibamoto, T., (2010). Antioxidant/lipoxygenase inhibitory activities and chemical compositions of selected essential oils. Journal of Agricultural and Food Chemistry 58(12), 7218-7225.

[201] Mitić-Ćulafić, D., Žegura, B., Nikolić, B., Vuković-Gačić, B., Knežević-Vukčević, J., Filipič, M., (2009). Protective effect of linalool, myrcene and eucalyptol against *t*-butyl hydroperoxide induced genotoxicity in bacteria and cultured human cells. Food and Chemical Toxicology 47(1), 260-266.

[202] Manju, S., Gopal, T.K.S., Jose, L., Ravishankar, C.N., Kumar, K.A., (2007). Nucleotide degradation of sodium acetate and potassium sorbate dip treated and vacuum packed Black Pomfret (*Parastromateus niger*) and Pearlspot (*Etroplus suratensis*) during chill storage. Food Chemistry 102(3), 699-706.

[203] Drosinos, E.H., Lambropoulou, K., Mitre, E., Nychas, G.J.E., (1997). Attributes of fresh gilt-head seabream (*Sparus aurata*) fillets treated with potassium sorbate, sodium gluconate and stored under a modified atmosphere at 0±1°C. Journal of Applied Microbiology 83(5), 569-575.

[204] Mastromatteo, M., Danza, A., Conte, A., Muratore, G., Nobile, M.A.D., (2010). Shelf life of ready to use peeled shrimps as affected by thymol essential oil and modified atmosphere packaging. International Journal of Food Microbiology 144(2), 250-256.

[205] Teixeira, B., Marques, A., Ramos, C., Neng, N.R., Nogueira, J.M.F., Saraiva, J.A., Nunes, M.L., (2013). Chemical composition and antibacterial and antioxidant properties of commercial essential oils. Industrial Crops and Products 43(0), 587-595.

[206] Vuuren, S.F.v., Viljoen, A.M., (2007). Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. Flavour and Fragrance Journal 22(6), 540-544.

[207] Ryder, J.M., (1985). Determination of adenosine triphosphate and its breakdown products in fish muscle by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 33(4), 678-680.

[208] Bligh, E.G., Dyer, W.J., (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

[209] Shantha, N.C., Decker, E.A., (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. Journal of AOAC International 77(2), 421-424.

[210] Seljeskog, E., Hervig, T., Mansoor, M.A., (2006). A novel HPLC method for the measurement of thiobarbituric acid reactive substances (TBARS). A comparison with a commercially available kit. Clinical Biochemistry 39(9), 947-954.

[211] Mendes, R., Cardoso, C., Pestana, C., (2009). Measurement of malondialdehyde in fish: A comparison study between HPLC methods and the traditional spectrophotometric test. Food Chemistry 112(4), 1038-1045.

[212] Schubring, R., (2009). Colour measurement, *in*: Rehbein, H., Oehlenschläger, J. (Eds.), *Fishery products: quality, safety and authenticity*. Blackwell Publishing Ltd, John Wiley & Sons, United Kingdom, pp. 127-172.

[213] Botta, J.R., (1991). Instrument for nondestructive texture measurement of raw Atlantic cod (*Gadus morhua*) fillets. Journal of Food Science 56(4), 962-964.

[214] Corbo, M.R., Speranza, B., Filippone, A., Granatiero, S., Conte, A., Sinigaglia, M., Nobile, M.A.D., (2008). Study on the synergic effect of natural compounds on the microbial quality decay of packed fish hamburger. International Journal of Food Microbiology 127(3), 261-267.

[215] Connell, J.J., (1975). *Control of fish quality*. Fishing News (Books) Ltd., Farnham, Surrey, UK.

[216] Ke, P.J., Nash, D.M., Ackman, R.G., (1976). Quality preservation in frozen mackerel. Canadian Institute of Food Science and Technology Journal - Journal de L'Institut Canadien de Science et Technologie Alimentaires 9(3), 135-138.

[217] Sahin, S., Sumnu, S.G., (2006). *Physical properties of food.* Springer Science and Business Media, LLC, New York.

[218] Hyldig, G., (2007). Texture of fish, fish products, and shellfish, *in*: Nollet, L.M.L., Boylston, T., Chen, F., Coggins, P.C., Gloria, M.B., Hyldig, G., Kerth, C.R., McKee, L.H., Hui, Y.H. (Eds.), *Handbook of meat, poultry and seafood quality*, 1 ed. Blackwell Publishing, Ames, Iowa, USA, pp. 549-561.

[219] Olafsdottir, G., Nesvadba, P., Natale, C.D., Careche, M., Oehlenschläger, J., Tryggvadóttir, S.a.V., Schubring, R., Kroeger, M., Heia, K., Esaiassen, M., Macagnano, A., Jørgensen, B.M., (2004). Multisensor for fish quality determination. Trends in Food Science & Technology 15(2), 86-93.

[220] Hernández, M.D., López, M.B., Álvarez, A., Ferrandini, E., García, B.G., Garrido, M.D., (2009). Sensory, physical, chemical and microbiological changes in aquacultured meagre (*Argyrosomus regius*) fillets during ice storage. Food Chemistry 114(1), 237-245.

[221] Gelman, A., Drabkin, V., Glatman, L., (2003). A rapid non-destructive method for fish quality control by determination of smell intensity. Journal of the Science of Food and Agriculture 83(6), 580-585.

[222] Anacleto, P., Teixeira, B., Marques, P., Pedro, S., Nunes, M.L., Marques, A., (2011). Shelf-life of cooked edible crab (*Cancer pagurus*) stored under refrigerated conditions. LWT - Food Science and Technology 44(6), 1376-1382.

[223] Mendes, R., Silva, H.A., Anacleto, P., Cardoso, C., (2011). Effect of $CO_2$ dissolution on the shelf life of ready-to-eat *Octopus vulgaris*. Innovative Food Science & Emerging Technologies 12(4), 551-561.

[224] Cuq, B., Gontard, N., Guilbert, S., (1997). Thermal properties of fish myofibrillar protein-based films as affected by moisture content. Polymer 38(10), 2399-2405.

[225] Benkeblia, N., (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). Lebensmittel-Wissenschaft und-Technologie 37(2), 263-268.

[226] Saint-Denis, T., Goupy, J., (2004). Optimization of a nitrogen analyser based on the Dumas method. Analytica Chimica Acta 515(1), 191-198.

[227] Atarés, L., Bonilla, J., Chiralt, A., (2010). Characterization of sodium caseinate-based edible films incorporated with cinnamon or ginger essential oils. Journal of Food Engineering 100(4), 678-687.

[228] Shiku, Y., Hamaguchi, P.Y., Tanaka, M., (2003). Effect of pH on the preparation of edible films based on fish myofibrillar proteins. Fisheries Science 69(5), 1026-1032.

[229] Anonymous, (2008). *HunterLab method. Applications note: Opacity*.

[230] Gontard, N., Guilbert, S., Cuq, J.-L., (1992). Edible wheat gluten films: influence of the main process variables on film properties using response surface methodology. Journal of Food Science 57(1), 190-195.

[231] Hoque, M.S., Benjakul, S., Prodpran, T., (2011). Properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin incorporated with cinnamon, clove and star anise extracts. Food Hydrocolloids 25(5), 1085-1097.

[232] Sobral, P.J.A., Menegalli, F.C., Hubinger, M.D., Roques, M.A., (2001). Mechanical, water vapor barrier and thermal properties of gelatin based edible films. Food Hydrocolloids 15(4-6), 423-432.

[233] Weng, W., Osako, K., Tanaka, M., (2009). Oxygen permeability and antioxidative properties of edible surimi films. Fisheries Science 75(1), 233-240.

[234] NCCLS, (1999). *Methods for determining bactericidal activity of antimicrobial agents. Approved guideline. NCCLS document M26-A.* National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.

[235] Artharn, A., Benjakul, S., Prodpran, T., Tanaka, M., (2007). Properties of a protein-based film from round scad (*Decapterus maruadsi*) as affected by muscle types and washing. Food Chemistry 103(3), 867-874.

[236] Limpan, N., Prodpran, T., Benjakul, S., Prasarpran, S., (2010). Properties of biodegradable blend films based on fish myofibrillar protein and polyvinyl alcohol as influenced by blend composition and pH level. Journal of Food Engineering 100(1), 85-92.

[237] Atarés, L., Jesús, C.D., Talens, P., Chiralt, A., (2010). Characterization of SPI-based edible films incorporated with cinnamon or ginger essential oils. Journal of Food Engineering 99(3), 384-391.

[238] Prodpran, T., Benjakul, S., (2005). Effect of acid and alkaline solubilization on the properties of surimi based film. Songklanakarin Journal of Science and Technology 27(3), 563-574.

[239] Pires, C., Ramos, C., Teixeira, G., Batista, I., Mendes, R., Nunes, L., Marques, A., (2011). Characterization of biodegradable films prepared with hake proteins and thyme oil. Journal of Food Engineering 105(3), 422-428.

[240] Benavides, S., Villalobos-Carvajal, R., Reyes, J.E., (2011). Physical, mechanical and antibacterial properties of alginate film: Effect of the crosslinking degree and oregano essential oil concentration. Journal of Food Engineering 110(2), 232-239.

[241] Hosseini, M.H., Razavi, S.H., Mousavi, M.A., (2009). Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. Journal of Food Processing and Preservation 33(6), 727-743.

[242] Rojas-Graü, M.A., Avena-Bustillos, R.J., Olsen, C., Friedman, M., Henika, P.R., Martín-Belloso, O., Pan, Z., McHugh, T.H., (2007). Effects of plant essential oils and oil compounds on

mechanical, barrier and antimicrobial properties of alginate-apple puree edible films. Journal of Food Engineering 81(3), 634-641.

[243] Tanaka, M., Ishizaki, S., Suzuki, T., Takai, R., (2001). Water vapor permeability of edible films prepared from fish water soluble proteins as affected by lipid type. Journal of the Tokyo University of Fisheries 87, 31-37.

[244] Pranoto, Y., Salokhe, V.M., Rakshit, S.K., (2005). Physical and antibacterial properties of alginate-based edible film incorporated with garlic oil. Food Research International 38(3), 267-272.

[245] Sabato, S.F., Nakamurakare, N., Sobral, P.J.A., (2007). Mechanical and thermal properties of irradiated films based on Tilapia (*Oreochromis niloticus*) proteins. Radiation Physics and Chemistry 76(11-12), 1862-1865.

[246] Taguchi, K., Iwami, K., Kawabata, M., Ibuki, F., (1988). Antioxidant effects of wheat gliadin and hen's egg white in powder model systems: protection against oxidative deterioration of safflower oil and sardine oil. Agricultural and Biological Chemistry 52(2), 539-545.

[247] Faraji, H., McClements, D.J., Decker, E.A., (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. Journal of Agricultural and Food Chemistry 52(14), 4558-4564.

[248] Sivarooban, T., Hettiarachchy, N.S., Johnson, M.G., (2008). Physical and antimicrobial properties of grape seed extract, nisin, and EDTA incorporated soy protein edible films. Food Research International 41(8), 781-785.

[249] Pires, C., Ramos, C., Teixeira, B., Batista, I., Nunes, M.L., Marques, A., (2013). Hake proteins edible films incorporated with essential oils: physical, mechanical, antioxidant and antibacterial properties. Food Hydrocolloids 30(1), 224-231.

[250] Gopakumar, K., (2000). Enzymes and enzyme products as quality indices, *in*: Haard, N.F., Simpson, B.K. (Eds.), *Seafood enzymes - utilization and influence on postharvest seafood quality*. Marcel Dekker Inc., Basel, Switzerland, pp. 337-363.

[251] Lakshmanan, R., Patterson, M.F., Piggott, J.R., (2005). Effects of high-pressure processing on proteolytic enzymes and proteins in cold-smoked salmon during refrigerated storage. Food Chemistry 90(4), 541-548.

[252] Ohmori, T., Shigehisa, T., Tagi, S., Hayashi, R., (1992). Biochemical effects of high hydrostatic pressure on the lysosome and proteases involved in it. Bioscience, Biotechnology and Biochemistry 56(8), 1285-1288.

[253] Barrett, A.J., Kirschke, H., (1981). Cathepsin B, cathepsin H and cathepsin L, *in*: Lorand, L. (Ed.), *Methods in enzymology. Proteolytic enzymes, Part C*. Academic Press, New York, pp. 535-561.

[254] Anson, (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. The Journal of General Physiology 22(1), 78-89.

[255] Sigma-Aldrich, (1999). Enzymatic assay of lipase (EC 3.1.1.3) (olive oil as substrate). Sigma Aldrich Chemie GmbH, http://www.sigmaaldrich.com.

[256] Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Murachi, T., (1984). Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. Journal of Biological Chemistry 259(20), 12489-12494.

[257] Silva, H., Mendes, R., Nunes, M., Empis, J., (2006). Protein changes after irradiation and ice storage of horse mackerel (*Trachurus trachurus*). European Food Research and Technology 224(1), 83-90.

[258] Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1-2), 248-254.

[259] Marcos, B., Kerry, J.P., Mullen, A.M., (2010). High pressure induced changes on sarcoplasmic protein fraction and quality indicators. Meat Science 85(1), 115-120.

[260] Tahergorabi, R., (2011). Seafood proteins, *in*: Phillips, G.O., Williams, P.A. (Eds.), *Handbook of food proteins*. Woodhead Publishing Limited, Sawston, Cambridge, UK, pp. 116-149.

[261] Gill, T.A., (1992). Chemical and biological indices in seafood quality, *in*: Huss, H.H., Jacobsen, M., Liston, J. (Eds.), *Quality assurance in the fish industry*. Elsevier, Amsterdam, pp. 337-387.

[262] Chéret, R., Hernández-Andrés, A., Delbarre-Ladrat, C., Lamballerie, M.d., Verrez-Bagnis, V., (2006). Proteins and proteolytic activity changes during refrigerated storage in sea bass (*Dicentrarchus labrax* L.) muscle after high-pressure treatment. European Food Research and Technology 222(5), 527-535.

[263] Mommsen, T.P., (2004). Salmon spawning migration and muscle protein metabolism: the August Krogh principle at work. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139(3), 383-400.

[264] Hasan, F., Shah, A.A., Hameed, A., (2009). Methods for detection and characterization of lipases: A comprehensive review. Biotechnology Advances 27(6), 782-798.

[265] Gómez-Estaca, J., López-Caballero, M.E., Gómez-Guillén, M.C., Lacey, A.L.d., Montero, P., (2009). High pressure technology as a tool to obtain high quality *carpaccio* and *carpaccio*-like products from fish. Innovative Food Science & Emerging Technologies 10(2), 148-154.

[266] Ramirez-Suarez, J.C., Morrissey, M.T., (2006). Effect of high pressure processing (HPP) on shelf life of albacore tuna (*Thunnus alalunga*) minced muscle. Innovative Food Science & Emerging Technologies 7(1-2), 19-27.

[267] Nayak, J., Nair, P.G.V., Ammu, K., Mathew, S., (2003). Lipase activity in different tissues of four species of fish: rohu (*Labeo rohita* Hamilton), oil sardine (*Sardinella longiceps* Linnaeus), mullet (*Liza subviridis* Valenciennes) and Indian mackerel (*Rastrelliger kanagurta* Cuvier). Journal of the Science of Food and Agriculture 83(11), 1139-1142.

[268] Delbarre-Ladrat, C., Chéret, R., Taylor, R., Verrez-Bagnis, V., (2006). Trends in *postmortem* aging in fish: understanding of proteolysis and disorganization of the myofibrillar structure. Critical Reviews in Food Science and Nutrition 46(5), 409-421.

[269] Chéret, R., Delbarre-Ladrat, C., Verrez-Bagnis, V., Lamballerie, M.d., (2007). Effect of high pressure on the calpain–calpastatin system in fish muscle. Journal of Food Science 72(6), C313-C316.

[270] Obinata, T., Maruyama, K., Sugita, H., Kohama, K., Ebashi, S., (1981). Dynamic aspects of structural proteins in vertebrate skeletal muscle. Muscle & Nerve 4(6), 456-488.

[271] Gou, J., Lee, H.-Y., Ahn, J., (2010). Effect of high pressure processing on the quality of squid (*Todarodes pacificus*) during refrigerated storage. Food Chemistry 119(2), 471-476.

[272] Chan, J.T.Y., Omana, D.A., Betti, M., (2011). Application of high pressure processing to improve the functional properties of pale, soft, and exudative (PSE)-like turkey meat. Innovative Food Science & Emerging Technologies 12(3), 216-225.

[273] Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa, H., Martínez, I., (2001). Species identification of formed fishery products and high pressure-treated fish by electrophoresis: a collaborative study. Food Chemistry 72(1), 105-112.

[274] Erkan, N., Üretener, G., Alpas, H., (2010). Effects of high pressure treatment on physicochemical characteristics of fresh sea bass (*Dicentrarchus labrax*). Journal für Verbraucherschutz und Lebensmittelsicherheit 5(1), 83-89.

[275] Sánchez-González, I., Carmona, P., Moreno, P., Borderías, J., Sánchez-Alonso, I., Rodríguez-Casado, A., Careche, M., (2008). Protein and water structural changes in fish surimi during gelation as revealed by isotopic H/D exchange and Raman spectroscopy. Food Chemistry 106(1), 56-64.

[276] Piñeiro, C., Barros-Velázquez, J., Pérez-Martín, R.I., Martínez, I., Jacobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie, I.M., Jessen, F., (1999). Development of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis reference method for the analysis and identification of fish species in raw and heat-processed samples: A collaborative study. Electrophoresis 20(7), 1425-1432.

[277] Lakshmanan, R., Dalgaard, P., (2004). Effects of high-pressure processing on *Listeria monocytogenes*, spoilage microflora and multiple compound quality indices in chilled cold-smoked salmon. Journal of Applied Microbiology 96(2), 398-408.

[278] Angsupanich, K., Ledward, D.A., (1998). High pressure treatment effects on cod (*Gadus morhua*) muscle. Food Chemistry 63(1), 39-50.

[279] Lakshmanan, R., Parkinson, J.A., Piggott, J.R., (2007). High-pressure processing and water-holding capacity of fresh and cold-smoked salmon (*Salmo salar*). LWT - Food Science and Technology 40(3), 544-551.

[280] Huff-Lonergan, E., Lonergan, S.M., (2005). Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. Meat Science 71(1), 194-204.

[281] Tironi, V., Lamballerie, M.d., Le-Bail, A., (2010). Quality changes during the frozen storage of sea bass (*Dicentrarchus labrax*) muscle after pressure shift freezing and pressure assisted thawing. Innovative Food Science & Emerging Technologies 11(4), 565-573.

[282] Terova, G., Addis, M.F., Preziosa, E., Pisanu, S., Pagnozzi, D., Biosa, G., Gornati, R., Bernardini, G., Roggio, T., Saroglia, M., (2011). Effects of postmortem storage temperature on sea

bass (*Dicentrarchus labrax*) muscle protein degradation: Analysis by 2-D DIGE and MS. Proteomics 11(14), 2901-2910.

[283] Sequeira-Munoz, A., Chevalier, D., LeBail, A., Ramaswamy, H.S., Simpson, B.K., (2006). Physicochemical changes induced in carp (*Cyprinus carpio*) fillets by high pressure processing at low temperature. Innovative Food Science & Emerging Technologies 7(1-2), 13-18.

[284] Gelsema, W.J., Ligny, C.L.d., Veen, N.G.v.d., (1979). Isoelectric points of proteins, determined by isoelectric focusing in the presence of urea and ethanol. Journal of Chromatography A 171, 171-181.

[285] Conway, E.J., Byrne, A., (1933). An absorption apparatus for the micro-determination of certain volatile substances: the micro-determination of ammonia. Biochemical Journal 27(2), 419-429.

[286] Ke, P.J., Nash, D.M., Ackman, R.G., (1976). Quality preservation in frozen mackerel. Canadian Institute Food Science and Technology Journal 9, 135-138.

[287] Chevalier, D., Bail, A.L., Ghoul, M., (2001). Effects of high pressure treatment (100-200 MPa) at low temperature on turbot (*Scophthalmus maximus*) muscle. Food Research International 34(5), 425-429.

[288] Ruiz-Capillas, C., Moral, A., (2005). Sensory and biochemical aspects of quality of whole bigeye tuna (*Thunnus obesus*) during bulk storage in controlled atmospheres. Food Chemistry 89(3), 347-354.

[289] Erkan, N., Özden, Ö., (2008). Quality assessment of whole and gutted sardines (*Sardina pilchardus*) stored in ice. International Journal of Food Science & Technology 43(9), 1549-1559.

[290] Sato, K., Krist, S., Buchbauer, G., (2006). Antimicrobial effect of trans-cinnamaldehyde, (-)-perillaldehyde, (-)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. Biological & Pharmaceutical Bulletin 29(11), 2292-2294.

[291] Brown, H., Williams, J., (2003). Packaged product quality and shelf life, *in*: Coles, R., McDowell, D., Kirwan, M.J. (Eds.), *Food Packaging Technology*. Blackwell Publishing Ltd, Boca Raton, USA, pp. 65-94.

[292] Mišík, V., Mak, I.T., Stafford, R.E., Weglicki, W.B., (1993). Reactions of captopril and epicaptopril with transition metal ions and hydroxyl radicals: An EPR spectroscopy study. Free Radical Biology and Medicine 15(6), 611-619.

[293] Leistner, L., (2000). Basic aspects of food preservation by hurdle technology. International Journal of Food Microbiology 55(1-3), 181-186.