Universidade do Minho
Escola de Engenharia

Marina Soraia Gomes de Oliveira

Edible coatings on frozen fish: Effect of applying a chitosan-based coating on the quality of frozen salmon

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UMinho | 2013

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biológica
Ramo Tecnologia Química e Alimentar

Trabalho realizado sob a orientação do
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outubro de 2013

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Cartão de Cidadão: 13795213

Título

Revestimentos edíveis em pescado congelado: Efeito da aplicação de um revestimento à base de quitosano na qualidade de salmão congelado

Edible coatings on frozen fish: Effect of applying a chitosan-based coating on the quality of frozen salmon

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Ano de conclusão

2013

Mestrado Integrado em Engenharia Biológica

Universidade do Minho, 31/10/2013

Assinatura: _____

Dedicatória

"Não sei se estou perto ou longe demais, sei apenas que sigo em frente, vivendo dias iguais de forma diferente. Levo comigo cada recordação, cada vivência, cada lição. E mesmo que tudo não ande da forma que eu gostaria, saber que já não sou a mesma de ontem me faz perceber que valeu a pena. Há um tempo em que é preciso abandonar (...) e esquecer os nossos caminhos que nos levam sempre aos mesmos lugares... É o tempo da travessia... e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos."

Fernando Teixeira de Andrade

À minha família

Agradecimentos

À família e amigos pelo amor incondicional, em especial aos insubstituíveis: pais, irmãos, cunhado, padrinho, Margarida, Francisco, Ana, Teresa, Raquel, Filipa e G.J.E..

Ao Engenheiro Nuno Soares pela presença assídua, pelo apoio e companheirismo constantes e pela irrepreensível e generosa parceria.

Ao Professor Vicente por todo o amparo, pela simpatia e otimismo sempre presentes.

Ao Departamento de Engenharia Biológica, nas pessoas que nele trabalham, em especial ao pessoal do Laboratório de Instrumentação e Processo e à Plataforma de Microscopia, na pessoa da Doutora Ana Nicolau, pela paciência e cooperação.

À Vanibru pela disponibilização das instalações e equipamentos e pela hospitalidade dos seus trabalhadores.

À empresa Castro Pinto & Costa (LabMaia) pela disponibilização do equipamento de HPLC e pelo fantástico acolhimento e boa disposição permanente, em especial à Engenheira Inês, Filipa e Elizabete, ao João e à Vânia.

Ao IPMA, na pessoa da Doutora Helena Lourenço, pela partilha de conhecimento na aplicação da Norma Portuguesa 3356:2009.

À empresa Konica Minolta/Aquateknica, na pessoa do Físico David Roldán, pelo oportuno esclarecimento na avaliação da cor.

À Tânia, ao Tiago e à Cristina pela atenciosa disponibilidade e gentil companhia.

À Engenheira Susana pelo desafiante convite e pela oportunidade tão bem-vinda de aprendizagem na superfície comercial MercAtlas.

A todos aqueles que direta ou indiretamente colaboraram comigo para que a realização deste trabalho fosse possível, o meu mais sincero OBRIGADA.

Resumo

O aumento do consumo de peixe, devido às suas características nutricionais, obrigou a uma dinamização da indústria do pescado, no que respeita ao melhoramento dos processos de conservação do mesmo. A congelação e a vidragem são técnicas comumente usadas na redução da incidência dos processos de deterioração no pescado. Com o objetivo de encontrar uma alternativa que complementasse a congelação e substituísse o vidrado de água, o presente trabalho visou avaliar o efeito da aplicação de revestimentos edíveis de 0.5% e 1.5% de quitosano na qualidade do pescado congelado. Ambos os revestimentos – o vidrado de água e os revestimentos de quitosano – foram aplicados diretamente em salmão do Atlântico (*Salmo salar*) congelado e armazenado durante 6 meses a -22 °C. Comparando ambos os revestimentos entre si e com amostras controlo não revestidas, diversos parâmetros como perda de revestimento/vidrado, perda de peso, perda por gotejamento, TVC, TBA, TVB-N, K-value, pH e coordenadas de cor L*a*b* foram periodicamente avaliados. Encontraram-se resultados favoráveis para as amostras de salmão revestidas com 0.5% de quitosano no controlo da perda de revestimento e para as amostras revestidas com 1.5% de quitosano na manutenção da cor do salmão e no controlo da contaminação microbiana de amostras congeladas e descongeladas. Neste trabalho vários parâmetros, como a perda de revestimento/vidrado, perda de peso, perda por gotejamento, TVC, TBA, TVB-N e K-value, revelaram-se bastante estáveis devido à proteção providenciada por uma correta temperatura de armazenamento e por um controlo apropriado da sua manutenção.

Palavras-chave: salmão, qualidade, congelação, vidragem, quitosano.

Abstract

The increase of fish consumption due to its nutritional characteristics, led to a stimulation of fishing industry, as regards the improvement of the processes for its conservation. Freezing and glazing are techniques commonly used in reducing the incidence of fish deterioration processes. In order to find an alternative to complement freezing and replace water glaze, the present work aimed at evaluating the effect of edible coatings of 0.5% and 1.5% chitosan on the quality of frozen fish. Both coatings - water glazing and chitosan coatings - were applied directly on Atlantic salmon (*Salmo salar*) frozen and stored for 6 months at -22 °C. Comparing both coatings with each other and with control uncoated samples, several parameters such as coating/glazing loss, weight loss, drip loss, TVC, TBA, TVB-N, K-value, pH and color coordinates L*a*b* were periodically evaluated. Favorable results were found for salmon samples coated with 0.5% chitosan in the control of coating loss and for the samples coated with 1.5% chitosan in maintaining the color of the salmon and controlling microbial contamination of samples frozen and thawed. In this work several parameters, such as coating loss, weight loss, drip loss, TVC, TBA, TVB-N, and K-value maintained quite stable due to the protection provided by a correct freezing temperature and a suitable control of its maintenance.

Keywords: salmon, quality, freezing, glazing, chitosan.

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List of Nomenclature

Abbreviations

Abs - Absorbance

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

APC - Aerobic Plate Counts

Art. - Article

ATP - Adenosine triphosphate

BS EN ISO - British, European and International Standard Organization

CFU - Colony forming units

Co. Ltd. - Company Limited

DL - Decree - Law

DSC - Differential Scanning Calorimetry

EDTA - Ethylenediaminetetraacetic acid

ESR - Electron Spin Resonance

FAO - Food and Agriculture Organization

FTIR - Fourier Transform Infrared Radiation

HPLC - High Performance Liquid Chromatography

HQL - High-quality life

Hx - Hypoxanthine

HxR - Inosine

ICMSF - International Commission on Microbiological Specifications for Foods

IIR - International Institute of Refrigeration

IMP - Inosine monophosphate

IV - Infra-red

JND - Just Noticeable Difference

K-value - ATP breakdown products

MA - Malonaldehyde

MDA - malondialdehyde

mg MDA/Kg sample - milligrams of malondialdehyde per 1000 g of sample

mg N/100 g sample – milligrams of nitrogen per 100 g of sample

MRD - Maximum Recovery Diluent

N - Nitrogen

NMR - Nuclear Magnetic Resonance

NP - Portuguese Standard

OSI - Oxidative Stability Instrument

p-AnV - p-anisidine value

PCA - Plate Count Agar

PPP - product-processing-packaging

PSL - Practical Storage Life

PV - Peroxide Value

RGB - Red Green Blue

s seconds

S.A. - Anonymous Society

SD - Standard Deviation

ssp. - specie

TBA - Thiobarbituric Acid

TBA-MDA - Thiobarbituric acid-malondialdehyde complex

TBARS - Thiobarbituric Acid Relative Substance

TCA - Trichloroacetic acid

TMA - Trimethylamine

TTT - Time Temperature Tolerance

TVB-N - Total Volatile Basic Nitrogen

TVC - Total viable counts

UV - Ultraviolet

UV/Vis - Ultraviolet-Visible

Symbols

a^* - Red and green direction

a_w - water activity

b^* - Yellow and blue direction

C - Concentration of malondialdehyde (μmol)

d - Dilution factor corresponding to the first dilution

ΔE^*ab - Color difference

Δa^* - Difference in the value a* found between the sample color and the color of the standard

Δb^* - Difference in the value b* found between the sample color and the color of the standard

ΔL^* - Difference in the value L* found between the sample color and the color of the standard

F_c - Volume correction factor (moisture of sample)

H - Moisture content of the sample (%)

L^* - Lightness

m - Mass of the taking the test (g)

M - Molar mass

m_s - Mass of the sample (g)

N - Number of microorganisms

n=3 - Triplicate samples

n_1 - Number of dishes retained in the first dilution

n_2 - Number of dishes retained in the second dilution

p - Significance

v - Volume of the extract (mL)

V_0 - Volume of hydrochloric acid added in the blank test (mL)

V_1 - Volume of hydrochloric acid added in the diffusion control test (mL)

V_2 - Volume of hydrochloric acid added in the extract test (mL)

V_3 - Volume of filtrate added in the periphery of the Conway cell (mL)

W_1 - Weight of the salmon sample before the coating application (g)

W_2 - Weight of the salmon sample after the coating application (g)

W_3 - Weight before the glaze is apply in the samples (g)

W_4 - Weight after the glaze is apply in the samples (g)

W_5 - Weight of the coated samples after the storage period (g)

W_6 - Weight of the glazed samples after the storage period (g)

W_7 - Weight of the uncoated samples (g)

W_8 - Weight of the uncoated samples after the storage period (g)

W_9 - Weight of frozen samples without coating/glazing and before being placed in the refrigerator (g)

W_{10} - Weight of thawed samples (g)

[...] - Concentration (μmol/mL)

$\sum C$ - Sum of colonies counted

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Introduction

Nowadays, the changes in dietary patterns and the benefits credited to a healthy diet revitalized intensively all sectors responsible for food production, leading to the search for alternative processing, preservation and chemical alteration of these products (Fai *et al.*, 2008). Marine foods attract great attention from consumers as a source of important nutritional components for a healthy diet (Simopoulos, 1997 cited in Rodriguez-Turienzo *et al.*, 2011). Consequently, the consumption of fish containing valuable nutrients has recently increased (Kilincceker *et al.*, 2009). In these contexts, the aim of this thesis and primary objective of this work focuses on assessing the effect of applying an edible chitosan-based coating on the quality of frozen fish, in order to study the possibility of using it as an alternative to water glaze, commonly applied in the fish industry. This coating was applied on frozen Atlantic salmon (*Salmo salar*) directly, and its effect on the shelf life of the fish was assessed, during frozen storage at -22 °C for 6 months.

With this in mind, this thesis was organized in two parts - Part I - State of The art and Part II - Experimental work. Part I is composed of two chapters, Chapter 1 and Chapter 2 and Part II is grouped in three chapters, Chapter 3, Chapter 4 and Chapter 5.

Chapter 1 provides an overview of the importance of fish in a healthy diet and the increased of his demand. This chapter also reflects on the changes in the fish industry in consequence of the increase of its consumption, while time providing an overview of this industry as a major employer. This chapter also mentions the traditional methods of fish conservation, such as freezing and glazing and other emerging methods, such as the edible coatings, in particular those that are based on chitosan. The concept of quality and its relationship with freshness, in addition to the different physical, chemical and biological processes that allows assessing it, are presented in Chapter 2.

Chapter 3 introduces methods, such as sample preparation, the preparation of the coating solutions, the transportation of samples and the determination of the values of TVC, TBA, TVB-N, K-value, pH, and color parameters, regularly used in the evaluation and control of fish quality during frozen storage. In Chapter 4, our results are discussed and in Chapter 5 the key findings of this thesis are summarized, as well as suggestions for improvement and future prospects.

Part I – State of Art

Chapter 1. Fish

The demand for food that promotes health and well-being has increased in recent years. The populations of many industrialized countries are becoming older, richer, more educated and more health conscious. Fish has a particular prominence in this respect, following mounting evidence confirming the health benefits of eating fish. More stringent demands to assurance food safety are another high-profile issue that has emerged in recent years, in order to earn and maintain consumer confidence in fish (FAO, 2012).

Consumers are increasingly requesting product attributes that depend on the production process. They now demand guarantees that their food has been produced, handled and commercialized in a way that is not dangerous to their health, respects the environment and addresses various other ethical and social concerns. At the same time, they also want convenience and palatability.

Besides trying to address consumer's requests, producers and major distributors are increasingly concerned about the sustainability, risk of depletion of marine stocks and the transparency in traceability systems – in order to trace the source, the quality, and the environmental and social impacts of food production and distribution (FAO, 2012).

Fuelled by changes in consumer taste and advances in technology, packaging, logistics and transport, the food industry produces appealing and healthy fish products. These diversified products include higher-value products, semi-processed and processed products, and products that are ready to eat or require little preparation before serving. This is accomplished by the insertion of improvements in storage and processing capacity, together with major innovations in refrigeration, ice-making, and food-packaging and fish-processing equipment (FAO, 2012).

1.1. Fish Industry

The growth of the supply and consumption of fish

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010, of which about 128 million tonnes was utilized as food for people. From the fish meant for direct human consumption, in 2010, 46.9% was live, fresh or chilled fish, followed by frozen fish with 29.3%. Prepared or preserved fish and cured fish represented 14.0% and 9.8% respectively from the total fish for human consumption (FAO, 2012).

The world food fish supply has grown in the last five decades, with an average growth rate of 3.2 percent per year in the period 1961–2009, outpacing the increase of 1.7 percent per year in the world's population. Per capita, the world food fish supply increased from an average of 9.9 kg (live weight equivalent) in the 1960s to 18.4 kg in 2009. There are large variations across countries and regions of the world in the amount of total fish supply for human consumption, reflecting different eating habits and traditions, availability of fish and other foods, prices, socio-economic levels, and seasons. Differences are also evident within countries, with consumption usually higher in coastal areas (FAO, 2012).

Fish industry as an employer

Fisheries and aquaculture also provided livelihoods and income for an estimated 54.8 million people engaged in the primary sector of fish production in 2010, of which an estimated 7 million were occasional fishers and fish farmers. In addition to the primary production sector, fisheries and aquaculture provide numerous jobs in additional activities such as processing, packaging, marketing and distribution, manufacturing of fish-processing equipment, net and gear making, ice production and supply, boat construction and maintenance, research and administration. All of this employment, together with dependants, is estimated to support the livelihoods of 660 - 820 million people, or about 10 – 12% of the world's population (FAO, 2012).

1.2. Fish - Chemical composition and structure

Due to its nutritional characteristics, fish is an important source of high grade protein and the knowledge of its composition is essential if the fullest use is to be made of it (Murray & Burt, 2001). In matter of fact, chemical composition of fish is very important not only for the consumer, but also for the processor, who needs to know the nature of the raw material before he can apply correctly the techniques of chilling, freezing, smoking or canning; the nutritionist, who wants to know what contribution fish can make to the diet and to health; and the cook, who must know the fish in order to prepare it for the table (Murray & Burt, 2001).

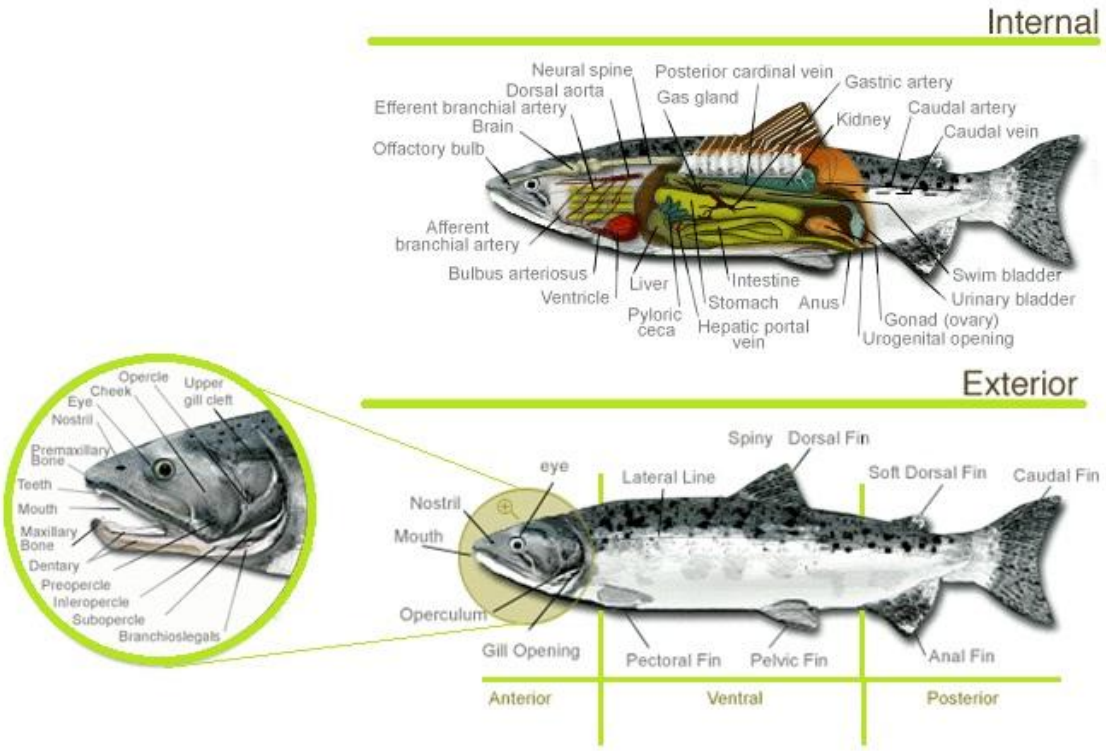
However, chemical composition varies widely from fish to fish of the same species and also within an individual fish, making accuracy impossible. Thus, measurement of constituents of fish products is necessary to meet specifications or to comply with regulations (Murray & Burt, 2001).

The scheme in Figure 1.1 shows the part internal and the exterior surface of salmon. The blocks of muscle are separated by thin sheets, which are known as connective tissue; these are curved within the fillet and run from the backbone to the skin. In fresh fish the muscle blocks are firmly attached to the connective tissue and the surface of a cut fillet is smooth and continuous. There are also tiny blood vessels running through the muscle. The connective tissue accounts for only a small percentage of the total weight of the muscle, making the fish less tough to eat than meat (Murray & Burt, 2001).

Fish muscle (Figure 1.2) is of two kinds, light muscle and dark muscle. In white fish, such as cod, there is a small strip of dark or red muscle just under the skin on both sides of the body, running beneath the lateral line. In fatty fish, such as salmon, the strips of dark muscle are much larger in proportion and contain higher concentrations of fat and certain vitamins. Since it is not usually practicable to separate the dark fatty muscle from the light muscle when preparing fish for cooking, as is made with the fat from meat, usually the values given in the tables for composition of flesh are for the total muscle, taking light and dark together (Murray & Burt, 2001).

Components of fish muscle

The principal components of fish muscle, the edible part of the fish, are water, proteins and fat. Other minor components are carbohydrates, minerals, vitamins and extractives (sugars, free amino acids and nitrogenous bases) (Murray & Burt, 2001).



Source: adapted from http://www.greatcanadianrivers.com/salmon/species-biology-internal.html, consulted in 09/05/2013

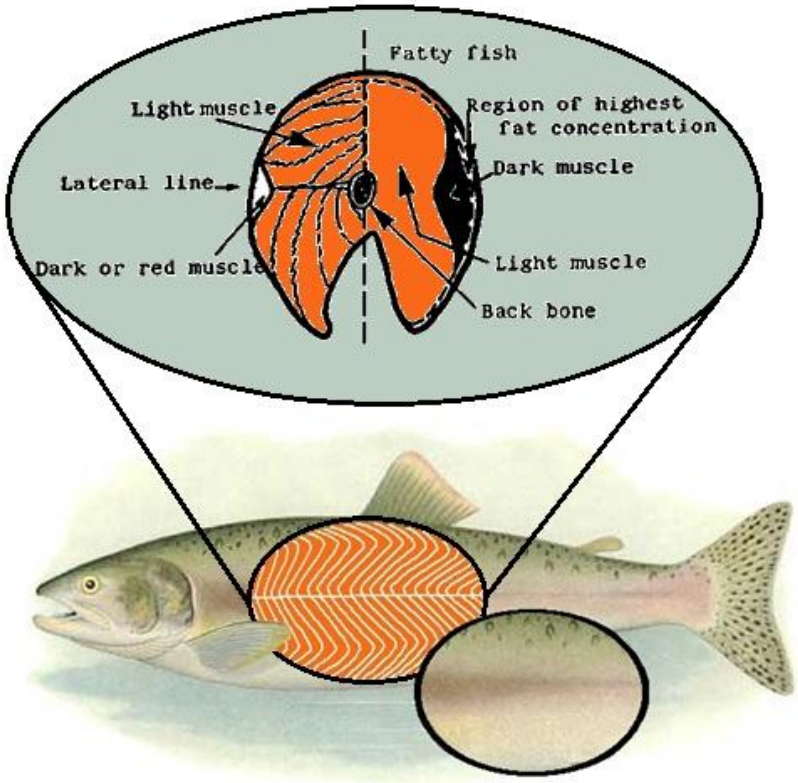
Figure 1.1. Representative scheme of internal and external anatomy of salmon.

According to FAO (2012), fish and fishery products represent a valuable source of nutrients of fundamental importance for diversified and healthy diets. Not only, fish is low in saturated fats, carbohydrates and cholesterol, but also provides high-value protein, a wide range of essential micronutrients, including various vitamins (D, A and B), minerals (including calcium, iodine, zinc, iron and selenium) and polyunsaturated omega-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid). Therefore, the consumption of fish, even in small amounts can have a significant positive nutritional impact by providing essential amino acids, fats and micronutrients that are scarce in vegetable-based diets. There is also evidence of beneficial effects of fish consumption in relation to coronary heart disease, stroke, age-related macular degeneration, mental health, and in terms of growth and development, in particular for women and children during gestation and infancy for optimal brain development (FAO, 2012).

Water

The main constituent of fish flesh is water, which represents 30 to 90 per cent of the fillet weight. Water, in fresh fish muscle, is tightly bound to proteins in the structure in such a way that it

cannot readily be expelled even under high pressure. After prolonged chilled or frozen storage, however, proteins are less able to retain all the water, and some of it, containing dissolved substances, is lost as drip. Frozen fish that are stored at too high a temperature, for example, will produce a large amount of drip and consequently quality will suffer (Murray & Burt, 2001).



Source: adapted from http://www.earthlife.net/fish/muscles.html and http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm, consulted in 09/05/2013

Figure 1.2. Diagram of fish muscle.

Proteins

The amount of protein in fish muscle is usually somewhere between 15% and 20%, but values lower than 15% or as high as 28% are occasionally met with in some species. All proteins, including those from fish, are chains of chemical units linked together to make one long molecule. These units, of which there are about twenty types, are called amino acids, and certain of them are essential in the human diet for the maintenance of good health. Two essential amino acids called lysine and methionine are generally found in high concentrations in fish proteins. Fish protein provides a good combination of amino acids that is highly suited to man's nutritional requirements (Murray & Burt, 2001).

Fat

The fat content can vary greatly among the various species of fish; even in the same specie it can be considered a seasonal variation in fat content of fatty fish. The term fat is used for simplicity, although the less familiar term "lipid" is more correct, since it includes fats, oils and waxes, as well as more complex, naturally-occurring compounds of fatty acids. As the fat content rises, so the water content falls, and *vice versa*; the sum of water and fat in a fatty fish is fairly constant at about 80%. Although protein content falls very slightly when the fat content falls it nevertheless remains fairly constant somewhere between 15% and 18%. The fat is not always uniformly distributed throughout the flesh of a fatty fish. For example in Pacific salmon there may be nearly twice as much fat in muscle from around the head as there is in the tail muscle (Murray & Burt, 2001).

1.3. Fish Conservation

Fish is a very versatile food commodity, appearing in a great variety of ways and product forms, such as live, fresh, chilled, frozen, heat-treated, fermented, dried, smoked, salted, pickled, boiled, fried, freeze-dried, minced, filleted, powdered or canned, or as a combination of two or more of these forms (FAO, 2012). While all of these methods of preserving fish at long-term are used, the most important ones are those based on the action of low temperatures, because they are better able to preserve the nutritional and sensory characteristics of the products (Gonçalves *et al.*, 2009).

Fish processing is evolving, from simple gutting, heading or slicing to more advanced value-addition, such as breading, cooking and individual quick-freezing, depending on the commodity and market value (FAO, 2012).

1.3.1. Freezing

Freezing represents the main method of processing fish for human consumption and it is the most used method in the control and/or reduction of biochemical changes that occur in fish during storage. It accounted for 55.2% of total processed fish for human consumption and 25.3% of total fish production in 2010. The proportion of frozen fish grew from 33.2% of total production for human consumption in 1970 to reach a record high of 52.1% in 2010. Processors of traditional

products, in particular of canned products, have been losing market shares to suppliers of fresh and frozen products as a result of long-term shifts in consumer preferences (Fan *et al.*, 2009; FAO, 2012; Kilincceker *et al.*, 2009; Rodriguez-Turienzo *et al.*, 2011; Sathivel *et al.*, 2007). However, there are reports of progressive loss of intrinsic and sensory quality of frozen fish during storage (Vanhaecke *et al.*, 2010). In fact, if on one hand, the use of temperatures below -12 °C inhibit microbial growth and slows down enzymatic activity (Jiang & Lee, 2004 cited in Rodriguez-Turienzo *et al.*, 2011), on the other hand, freezing is not able to completely inhibit microbial and chemical reactions, such as lipid oxidation, protein denaturation and dehydration surface (sublimation and recrystallization of ice crystals) leading to deterioration of fish quality during prolonged storage, resulting in off-flavors, rancidity, dehydration, weight loss, loss of juiciness, drip loss and toughening, as well as microbial spoilage and autolysis (Fan *et al.*, 2009; Gonçalves *et al.*, 2009; Rodriguez-Turienzo *et al.*, 2011 Sathivel *et al.*, 2007).

Although, despite the occurrence of a certain deterioration of the quality of frozen foods during storage, freezing increases the shelf life of the products if carried out correctly. Thus, the extent of loss quality depends on many factors including the rate of freezing and thawing, storage temperature, temperature fluctuations, abuse of freeze-thaw during storage, transport, exposure and consumption. It should be noted that freezing does not improve product quality; the final quality depends of the quality of the product in the moment of freezing and the conditions during freezing, storage and distribution (Gonçalves *et al.*, 2009).

Effect of freezing rate on ice crystal structure

Hayes *et al.* (1984) cited in Evans (2008) define the freezing rate in relation to the velocity of movement of the ice-water freezing front. The rates of freezing determine the type, size and distribution of ice formation.

Evans (2008) points out that freezing removes water form the food matrix by forming ice crystals. Although ice crystals remain in the food, the remaining water that is in contact with the food matrix becomes concentrated with solutes and it's a_w becomes low. Foods with a lower water activity are more stable, since most microorganisms cease functioning below the water activity of about 0.7. However, the formation of ice crystals can downgrade the quality of the food by three mechanisms: mechanical damage to the food structure, cross-linking of proteins and limited re-absorption of water on thawing (drip loss).

According to Fellows (2000), different tissues have different resistances to freezing damage. Products with a more flexible fibrous structure which separates during freezing instead of breaking, are not seriously damage their texture, while products that have a more rigid cell structure may be damaged by ice crystals. The extent of damage depends on the size of the crystals and hence on the rate of heat transfer. During slow freezing, ice crystals grow in intercellular spaces and deform and rupture adjacent cell walls. Cells become dehydrated and permanently damaged by the growing crystals. On thawing, cells do not regain their original shape and turgidity. The food is softened and cellular material leaks out from ruptured cells, increasing the drip loss. In fast freezing, smaller ice crystals form within both cells and intercellular spaces. There is little physical damage to cells, and minimal dehydration of the cells. The texture of the food is thus retained to a greater extent. However, very high freezing rates may cause stresses within some foods that result in splitting or cracking of the tissues. Zhu *et al.* (2004) confirms this by saying that freezing process was generally much more important than thawing for drip loss, once slower freezing produces larger extra-cellular ice crystals, resulting in more tissue damage and thawing loss (Fennema, 1973 cited in Zhu *et al.*, 2004).

Another process which causes the same result is migratory recrystallisation, largely caused by fluctuations in the storage temperature. This process causes the melting of ice crystals and movement of moisture to regions of lower vapor pressure, which leads areas of the food nearest to the source of heat to become dehydrated (freezer burn). When the temperature falls again, the existing ice crystals increase their size. There is therefore a gradual reduction in the numbers of small crystals and an increase in the size of larger crystals, resulting in loss of quality similar to that observed in slow freezing. This is minimized by packaging in moisture-proof materials (Fellows, 2000). For this reason, as soon as seafood is removed from a freezer, they should be glazed or wrapped (unless they have been packaged before freezing) and immediately transferred to a low temperature store to rapidly refreeze and to preserve taste, smell and texture as well as to minimize thaw drip loss (Gonçalves *et al.*, 2009; Jose & Sherief, 1993 cited in Jacobsen & Fossan, 2001).

1.3.2. Glazing

The application of a thin layer of ice on the surface of the frozen products by spraying or dipping into a water bath, it is common practice in frozen fish industry, in a process termed glazing (Gonçalves *et al.*, 2009; Vanhaecke *et al.*, 2010). This technique aims at minimizing the impact of

undesirable changes on the quality of frozen products during storage (Gonçalves *et al.*, 2009; Vanhaecke *et al.*, 2010). This water glaze excludes air from the surface of the product, thus reducing the rate of oxidation and also serves as a protective barrier to temperature fluctuations, allowing the glaze evaporate instead tissue water, when an increase of temperature occurs (Fossan & Jacobsen, 2001 cited in Gonçalves *et al.*, 2009). It is intended that the entire surface of the product be completely and uniformly glazed, typically with a percentage of glazing applied between 4% and 10%, although it may vary between 2% and 20% (Vanhaecke *et al.*, 2010). This percentage depends on the immersion time, the temperature of fish and water and the size and shape of the fish (Fossan & Jacobsen, 2001; Johnston *et al.*, 1994; Pedersen & Jacobsen, 1997 cited in Gonçalves *et al.*, 2009). Thus, for a glazing less than 6%, a deficiency can occur in product protection and for a glazing greater than 12%, commercial disputes can be generated because excess water would entail additional costs for consumers (Vanhaecke *et al.*, 2010).

According to DL nº 37/2004, in an attempt to protect the consumers interests was adopted an official method for sampling and determination of drained net weight, which gave rise to NP 4355:2002. It has also become mandatory to show in the foodstuffs labels, information about your drained net weight and its price, which allowed consumers to know the amount of water that is being sold with the product.

1.3.3. Edible coatings/films

New technologies are being use to ensure the conformity of frozen fish during storage trying to satisfy the growing demand for this product. In some experiments, natural products were used to ensure the quality of the fish and the extension of its validity (Sathivel *et al.*, 2007; Souza *et al.*, 2010).

Edible films and coatings have become one of the most promising alternatives to protect the products against mechanical damage, physical, chemical and microbiological activities. These are thin layers of edible material that, when applied in food, assist in their preservation, distribution and marketing (Falguera *et al.*, 2011; Pinheiro *et al.*, 2010).

The application of edible coating in foods has been evaluated by several authors as: Ribeiro *et al.* (2007) on the surface of strawberries; Cerqueira *et al.* (2009b) in tropical fruits such as *acerola* (*Malpighia emarginata*), *cajá* (*Spondias lutea*), mango (*Mangifera indica*), *pitanga* (*Eugenia uniflora*) and *seriguela* (*Spondias purpurea*); Cerqueira *et al.* (2009a) on the surface of cheese;

Martins *et al.* (2010) in *Ricotta* cheese and Souza *et al.* (2010) in mango "Tommy Atkins" (cited in Pinheiro *et al.*, 2010). Other examples can be found regarding the development of coatings to protect food products like garlic (Botrel *et al.*, 2007), India cherries (*Ziziphus mauritina cv. Cuimi*) (Qiuping & Wenshui, 2007) and red *pitaya* (*Hylocereus undatus*) (Chien *et al.*, 2007) (cited in Fai *et al.*, 2008).

These coatings/films have particular properties that make them particularly useful for application in perishable products. Some of those properties are structural resistance to water and microorganisms, functional attributes (antibiotics, antifungal, antibacterial, etc.), mechanical properties (tension and flexibility), optical properties (brightness and opacity), the barrier effect against gases flow and high acceptability (Falguera *et al.*, 2011).

The main difference between films and coatings is how they are applied in food. While the coatings are applied by immersion of the product in a solution, the films are first shaped as solid sheets, like a package, and then applied to the product (Falguera *et al.*, 2011; Kilincceker *et al.*, 2009).

Coatings/films can be produced by a variety of biodegradable polymers such as polysaccharides, proteins, lipids, resins, with or without the addition of plasticizers and surfactants. The functionality and behavior of edible films and coatings depend mainly on their mechanical and transport properties, which in turn vary with their composition, formation process, and the method of application in the product (Pinheiro *et al.*, 2010; Rodriguez-Turienzo *et al.*, 2011).

These coatings/films create a modified atmosphere that restricts the transfer of gases such as O_2, CO_2 and aromatic compounds, influencing several parameters in fresh and minimally processed food, such as color, texture, sensory quality, antioxidant properties, production of ethylene volatile compounds and microbial growth as a result of anaerobic processes (Falguera *et al.*, 2011; Pinheiro *et al.*, 2010; Rodriguez-Turienzo *et al.*, 2011). Developed to reduce, inhibit or prevent the growth of microorganisms on food surfaces where microbial contamination is more significant, their application innovated the concept of active packaging (Falguera *et al.*, 2011; Pereira *et al.*, 2010).

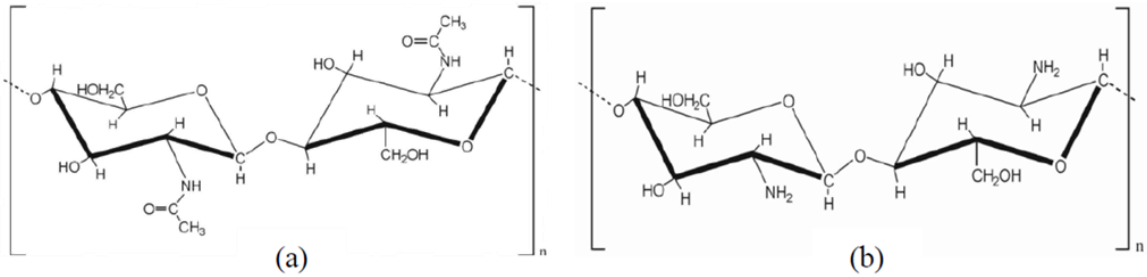
1.3.3.1. Chitosan

Chitosan (Figure 1.3 (b)) is a natural amino-cationic hetero-polymer composed of ß-1.4 D-glucosamine units, linked to N-acetylglucosamine residues, which can be obtained by chitin

deacetylation. Chitin (Figure 1.3 (a)) is an abundant natural polymer, a linear polysaccharide alkali-acid insoluble, which has ß-1,4 N-acetylglucosamine as monomeric unit. It can be found in the exoskeleton of crustaceans, insects and fungal cell walls (Fai *et al.*, 2008).

The use of chitin in many industrial processes generates a large amount of solid waste. The valorization of these residues through their transformation into other products, such as chitosan, is a way to reduce wastage (Pinheiro *et al.*, 2010).

Chitosan attracts much attention in the food industry due to its viscoelastic properties and its particular properties such as non-toxicity, bioactivity, biodegradability, biocompatibility, reactivity, selective permeability, polyelectrolytic action, the ability to form gels and films, the adsorption capacity, the ability antibacterial, antifungal, antimicrobial and antioxidant. Thus, the chitosan-based materials can be used for producing edible films and coatings resistant, durable and flexible, with mechanical properties comparable to commercial polymers (Fan *et al.*, 2009; Pinheiro *et al.*, 2010; Sathivel *et al.*, 2007).



Source: removed from Fai *et al.* (2008)

Figure 1.3. Chemical structure of chitin (a) and chitosan (b).

Chapter 2. Fish Quality - Freshness

Monitoring and control quality of frozen fish is one of the fundamental purposes of the seafood industry. Many parameters are involved in the definition of quality (Figure 2.1), including safety, nutritional and sensory properties, price, convenience and constancy, color packaging, availability and freshness (Ólafsdóttir *et al.*, 1997; Souza *et al.*, 2010). In fact, the maintenance of quality parameters, by developing effective techniques on their conservation, it is essential to make food more appealing to the end consumers (Gonçalves *et al.*, 2009; Pinheiro *et al.*, 2010). The change of one of these parameters affects largely the product acceptability by the consumers and consequently also the commercial value (Rodriguez-Turienzo *et al.*, 2011).

Freshness is one of the most important parameters for the quality of the final product. In fact, quality is a function of freshness, although this is not *a priori* a factor sufficient to guarantee it. Freshness can be translated by some sensory, (bio)chemical, physical and microbiological parameters determined by different analyzes, which extend from the time of harvesting to product deterioration. These tests claim to detect variations in flavor, texture, color, odor, and other parameters that affect the freshness and alter perception and consumer satisfaction (Kilincceker *et al.*, 2009; Ólafsdóttir *et al.*, 1997).

2.1. Microbiology

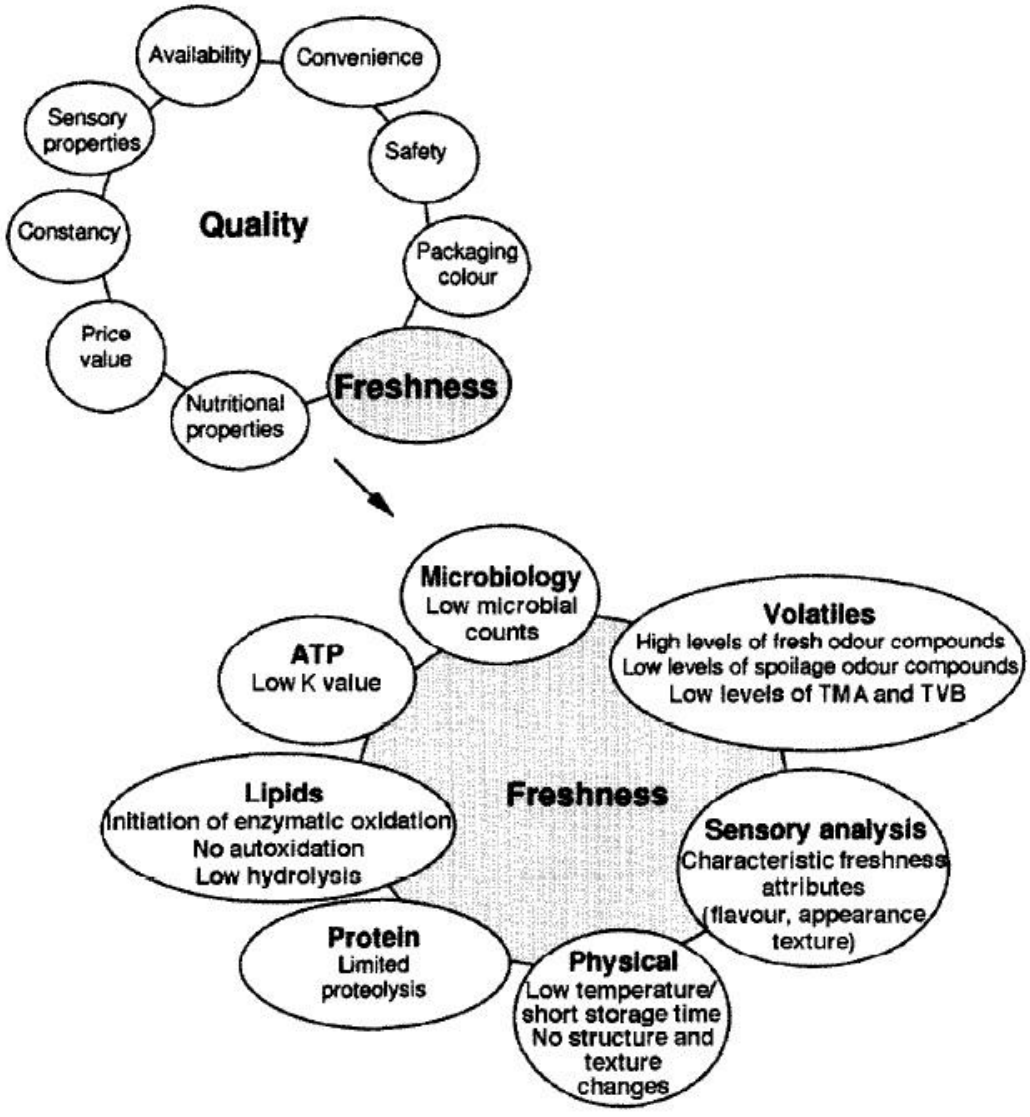
2.1.1. Total Viable Counts (TVC)

The microbial activity is the main factor limiting the shelf life of fresh fish, and the main cause of its deterioration. The total viable count (TVC) is used as an acceptability index in standards, guidelines and specifications, since this method can provide a useful measure of the freshness of fish (Ólafsdóttir *et al.*, 1997). According to ICMSF (1986), an aerobic plate count (APC) is recommended for all products as a good indicator from storage length and conditions of products prior to stabilizing processes such as freezing. Thus APC is indicative of general quality and, to a lesser extent, of handling and storage procedures.

Newly caught fish contain a diverse microflora, usually around 10^{2}-10^{6} CFU/g on whole fish and cut fillets. Usually, in fish products, TVC around 10^{7}-10^{8} CFU/g, lies at the point of sensory rejection. Nevertheless, standards, guidelines and specifications often use much lower TVC as

indices of acceptability. Microbial criteria based on low TVC, such as 10^6 CFU/g, are problematic to use, because a correlation between TVC and the remaining shelf life is assumed, although generally not known (Ólafsdóttir *et al.*, 1997). In matter of fact, an increase in APC to levels in excess of 10^6 per gram is usually indicative of long storage at chilling temperatures or temperature abuse prior to freezing (ICMSF, 1986).

In order to properly evaluate fish freshness, microbial methods are developed together with mathematical models, which express the effects of storage conditions - such as temperature and atmosphere - on the correlation between microbial numbers and remaining shelf life. The most promising results were obtained with relatively slow detection methods such as plate counts and other growth techniques involving an incubation period (Ólafsdóttir *et al.*, 1997).

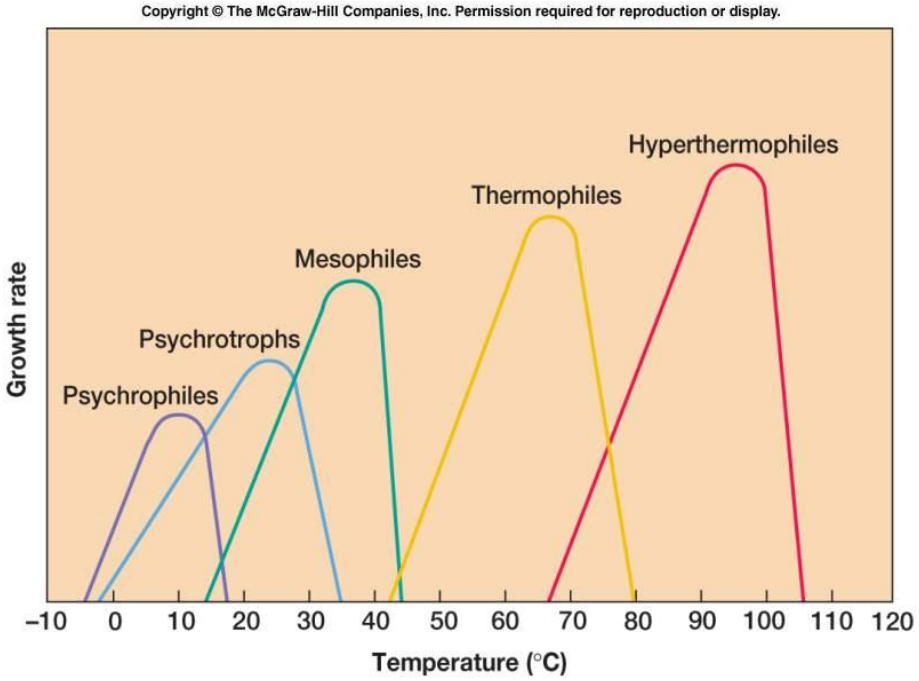


Source: removed from Ólafsdóttir *et al.* (1997)

Figure 2.1. Relationship between quality and freshness.

Psychrophilic and psychrotrophic microorganisms

Microorganisms can be classified according to their growth rate at different temperatures. According to this classification, microorganisms that are expected to grow at freezing temperatures will be psychrophiles and psychrotrophs (Figure 2.2).



Source: removed from http://classroom.sdmesa.edu/eschmid/Lecture4-Microbio.htm, consulted in 14/06/2013

Figure 2.2. Temperature ranges for different microbial life forms.

Much of life on Earth has evolved to colonize low-temperature environments. In fact, the cold biosphere (temperatures permanently below 5 °C) represents by far the largest fraction of the global biosphere (Casanueva *et al.*, 2010; Cavicchioli, 2006; Feller & Gerday 2003; Margesin & Miteva, 2011; Siddiqui & Cavicchioli, 2006 cited in Siddiqui *et al.*, 2013). Actually, the lowest temperature limit for life seems to be around -20 °C, which is the value reported for bacteria living in permafrost soil and in sea ice. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or the ice, and to brine channels. Aerobic and anaerobic bacteria are found at these temperatures (D'Amico *et al.*, 2006).

Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and Polar Regions. Some of these organisms, depending on their optimal growth temperature, are also known by the terms psychrotolerant or psychrotroph, but the general term used to designate all microorganisms growing well at

temperatures around the freezing point of water is psychrophiles. The ability of psychrophiles to survive and proliferate at low temperatures implies that they have overcome key barriers inherent to permanently cold environments, such as reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; protein cold-denaturation; inappropriate protein folding; and intracellular ice formation (D'Amico *et al.*, 2006).

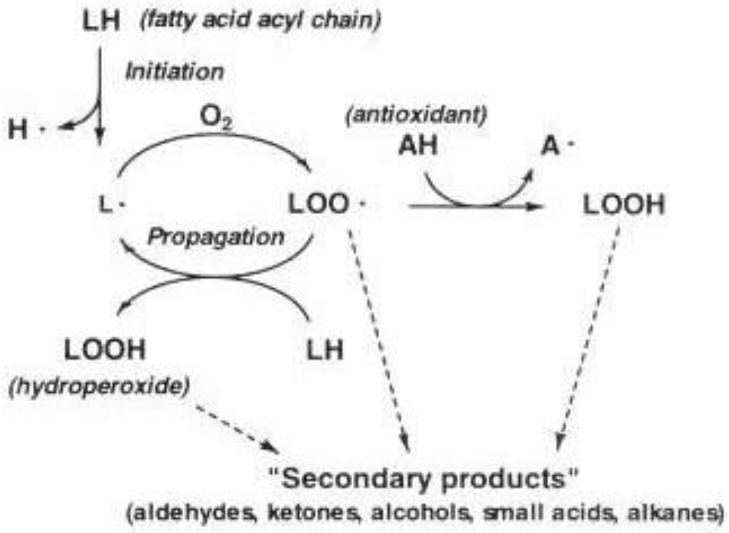
2.2. Lipids

2.2.1. 2-Thiobarbituric acid (TBA)

For measuring lipid oxidation in foods, different analytical methods are used. It is necessary to select a proper and adequate method for a particular application. Five groups divide the available methods to monitor lipid oxidation in foods, based on what they measure: the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products. A number of physical and chemical tests, including instrumental analyses, have been employed in laboratories and the industry for measurement of various lipid oxidation parameters. These include the weight-gain and headspace oxygen uptake method for oxygen absorption; iodometric titration, ferric ion complexes, and Fourier transform infrared (FTIR) method for peroxide value; chromatographic analysis for changes in reactants; spectrometry for conjugated dienes and trienes, 2-thiobarbituric acid (TBA) value, p-anisidine value (p-AnV), and carbonyl value; Rancimat and Oxidative Stability Instrument (OSI) method for oil stability index; and electron spin resonance (ESR) spectrometric assay for free-radical type and concentration; differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR). In addition, sensory tests provide subjective or objective evaluation of oxidative deterioration, depending on certain details (Shahidi & Zhong, 2005).

Due to its lipid composition, fish is highly susceptible to oxidation, which translates into changes in odor, flavor, texture, color and nutritional value. Oxidation becomes an important factor of deterioration, particularly at temperatures below 0 °C (Ólafsdóttir *et al.*, 1997), and results from the instability of primary oxidation products, such as hydroperoxides, which decompose into secondary oxidation products such as aldehydes, ketones, alcohols, hydrocarbons, volatile organic

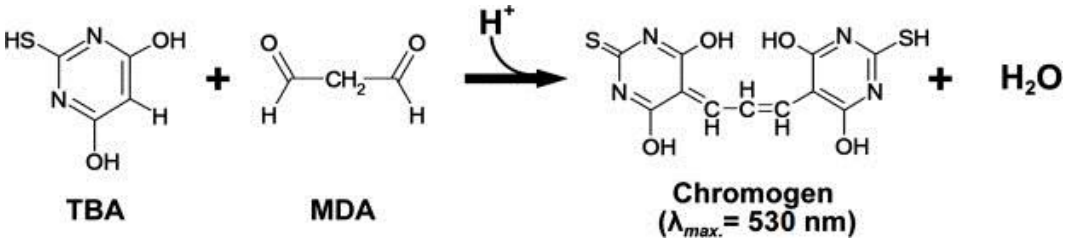
acids and epoxy compounds, among others (Figure 2.3) (Mendes *et al.*, 2009; Shahidi & Zhong, 2005).



Source: removed from Huss, 1995 available in http://www.fao.org/docrep/v7180e/V7180e06.htm#5.4%20Lipid%20oxidation%20and%20hydrolysis, consulted in 14/06/2013

Figure 2.3. Autoxidation of polyunsaturated lipid.

Malondialdehyde (MDA), one of the most important products of oxidation, is often used as marker of oxidative damage in biological samples and in food. A simple method for the determination of MDA is the spectrophotometric detection of thiobarbituric acid-malondialdehyde complex (TBA-MDA) obtained after reaction with 2-thiobarbituric acid (TBA) at low pH and high temperature (Figure 2.4) (Mendes *et al.*, 2009).



Source: adapted from Bastos *et al.* (2012)

Figure 2.4. TBA test reaction between 2-thiobarbituric acid and malonaldehyde, forming a colored compound, measured in a spectrophotometer at 530 nm.

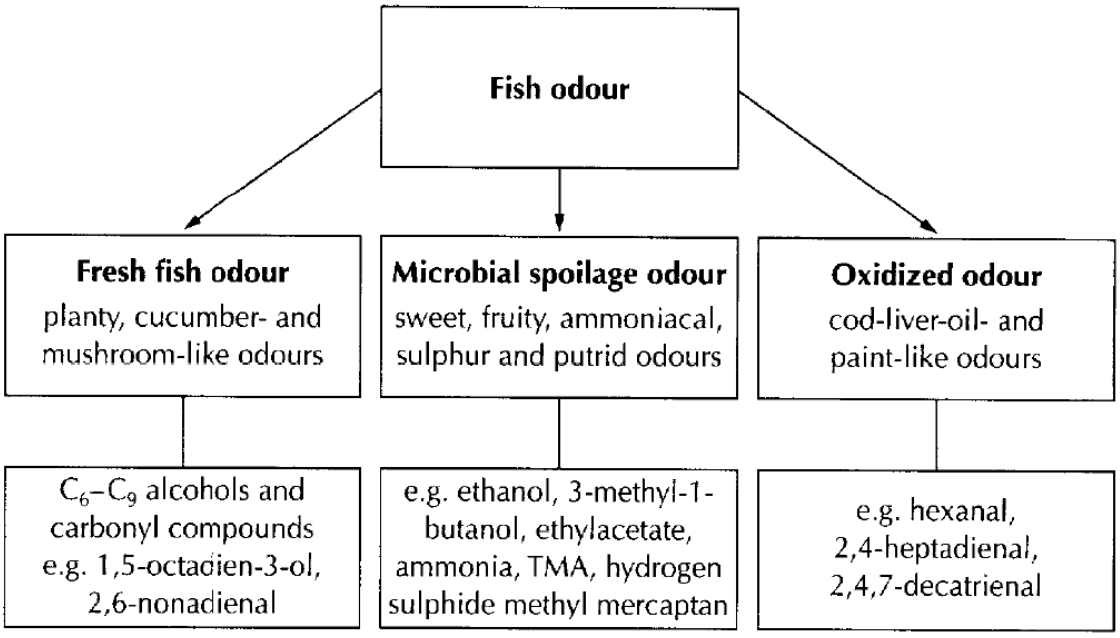
Although the TBA test is frequently used to assess the oxidative state of a variety of food systems, it has limitations, such as lack of specificity and sensitivity, since many other substances may react with the TBA reagent and contribute to absorption, causing an overestimation of the

intensity of color complex. These interferences may come from additional absorption of other alkanals, 2-alkenals, 2.4-alkdienals, ketones, ketosteroids, acids, esters, proteins, sucrose, urea, pyridines, and pyrimidines, also referred to as TBARS (Shahidi & Zhong, 2005).

2.3. Volatiles

2.3.1. Total Volatile Basic Nitrogen (TVB-N)

Odor is one of the most important parameters used to evaluate fish freshness, which can be monitored by measurement of characteristic volatile compounds. Several of these compounds can be used to monitor the freshness or spoilage stage of fish and to assess its quality, including the value of total volatile nitrogen (TVB-N). These volatile compounds can be divided into three groups based on their origin, as illustrated in Figure 2.5 (Ólafsdóttir *et al.*, 1997). Extractive compounds, particularly the volatiles, whose concentration in fish varies directly with time of storage, have long been studied since they may provide indicators of the quality of fish. When fish is stored after capture, the amount of some of the extractives present will change with time; thus measurement of the amount can often indicate the storage time and hence indirectly the quality (Murray & Burt, 2001).



Source: removed from Ólafsdóttir *et al.* (1997)

Figure 2.5. Categorization of fish odours and the volatile compounds that contribute to the characteristic odour of fresh, spoiled and oxidized fish.

The TVB-N limit established for the various fish categories is:

- 25 mg nitrogen/100 g
  - *Sebastes ssp.*
  - *Helicoelenus dactylopterus*
  - *Sebastichtys capensis*
- 30 mg nitrogen/100 g
  - species belonging to the family *pleuronecitidae* (with the exception of halibut) - *Hippoglossus sp.*
- 35 mg nitrogen/100 g
  - *Salmo Salar*
  - species belonging to the family *Merlucciidae*
  - species belonging to the family *Gadidae*

Routine methods used to control the threshold TVB-N are the following:

- microdiffusion method described by Conway and Byrne (1933)
- direct distillation method described by Antonacopoulos (1968)
- distillation of an extract deproteinized with trichloroacetic acid [*Codex Alimentarius* Committee on Fish and fishery products (1968)] (Directive 95/149/EC).

2.4. Adenosine-5'-triphosphate (ATP)

2.4.1. K-value

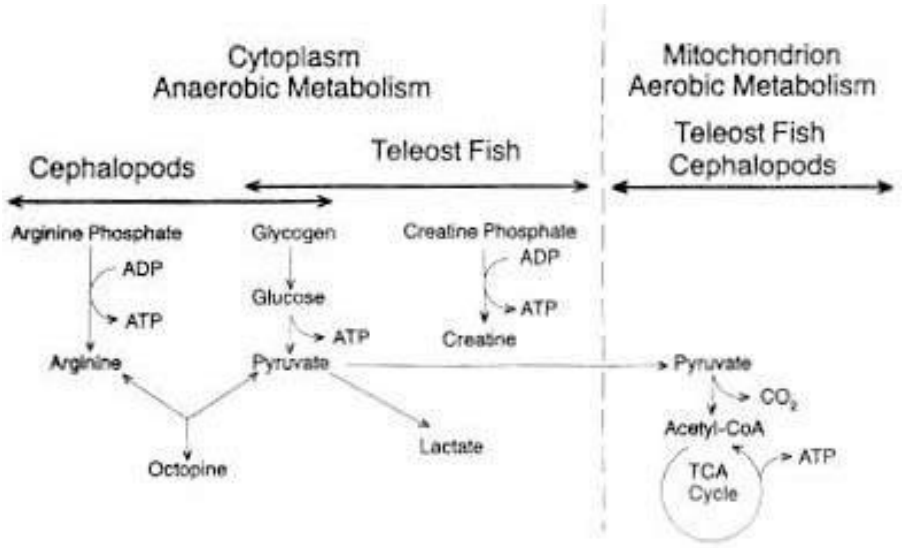
Oxidation starts immediately after catch, but becomes particularly important for shelf life only at temperatures below 0 °C, when oxidation rather than microbial activity becomes the major spoilage factor. The initiation of lipid oxidation arises from various early *post mortem* changes in fish tissues (Ólafsdóttir *et al.*, 1997).

Rigor mortis is one of the most prominent changes in muscle occurring soon after death. When fish are killed while relaxed, creatine phosphate is degraded prior to the breakdown of adenosine triphosphate (ATP) (Figure 2.6). When the creatine phosphate and ATP reach about the same concentration as ATP, ATP content begins to decrease and *rigor mortis* starts. *Rigor mortis* occurs when crossbridge cycling between myosin and actin in myofibrils ceases, and permanent actin and myosin linkages are formed. However, *rigor mortis* is resolved after some time. Possible

causes of *post mortem* tenderization include a weakening of Z-discs of myofibrils, a degradation of connective tissue or a weakening of myosin-actin junctions (Wang *et al.*, 1998). In *post mortem* fish muscle degradation of adenosine triphosphate (ATP) proceeds according to the sequence (Ólafsdóttir *et al.*, 1997; Özogul *et al.*, 1999; Souza *et al.*, 2010):

ATP (adenosine thriphosphate) → **ADP** (adenosine diphosphate) → **AMP** (adenosine monophosphate) → **IMP** (inosine monophosphate) → **HxR** (inosine) → **Hx** (hypoxanthine)

Following death, ATP is rapidly degraded to IMP by endogenous enzymes (autolysis). The further degradation of IMP to inosine and hypoxanthine is much slower, and is catalyzed mainly by endogenous IMP phosphohydrolase and inosine ribohydrolase, with a contribution from bacterial enzymes as storage time increases. Therefore, the degradation of ATP is parallel to loss of freshness of the fish. Thus, a chemical index of fish freshness is appealing because it is quantifiable, objective and lends itself to automation. ATP alone cannot be used because it is so rapidly converted to IMP and the concentrations of its intermediate degradation products rise and fall, making them unreliable indexes of freshness. As a result, attention has focused on inosine and hypoxanthine, the terminal catabolites of ATP. Inosine accumulates in some species of fish whereas hypoxanthine accumulates in others as terminal catabolites (Ólafsdóttir *et al.*, 1997). The K-value is used as an index for the estimation of fish freshness and it is defined as the ratio of the sum of inosine and hypoxanthine concentrations to the total concentration of ATP metabolites (Lin, 1993; Ólafsdóttir *et al.*, 1997; Souza *et al.*, 2010). So, a fresh fish will have a low K-value. A shortcoming of the K-value as a freshness index is its dependence on a variety of variables. It varies between species owing to differences in rates of ATP degradation. It also varies with *post mortem* time and temperature storage conditions, handling conditions and method of kill. Thus, a profile of K-value versus time must be established for each species and its specific handling and storage conditions before K-value measurements can be used to evaluate freshness. Following acid extraction and neutralization, metabolites are separated by ion-exchange chromatography or HPLC and quantified by their absorbance. Although other methods have been used, HPLC method is the most reliable (Ólafsdóttir *et al.*, 1997).



Source: removed from Huss, 1995 available in http://www.fao.org/docrep/v7180e/V7180e06.htm#5.4%20Lipid%20oxidation%20and%20hydrolysis, consulted in 14/06/2013)

Figure 2.6. Aerobic and anaerobic breakdown of glycogen in fish muscle.

2.5. pH

High water-holding capacity, neutral pH values, enzymes contained in the tissues, and lower connective tissue content have acceleratory effects on the process of spoiling (Kilincceker *et al.*, 2009). According to Huss (1995), the knowledge about the pH of fish meat may give valuable information about its condition. At the moment of fish death, the normal respiration process cannot occur, the oxygen supply is interrupted, the blood circulation fails and the production of energy is limited. So, in the beginning, because of *rigor mortis*, the maximum level of lactic acid is present in the structure, decreasing the pH. Although, the pH of the fish fillet increases, in the *post mortem* period, in consequence of the decomposition of nitrogenous compounds, by the proteolytic bacteria and autolytic enzymes. This increase in pH has a negative effect on the quality of the product during storage; especially, the sensorial characteristics such as odor, color, and texture (Shenderyuk & Bykowski, 1989 cited in Kilincceker *et al.*, 2009).

Fan *et al.* (2009) confirms this trend claiming the pH value decreases initially and then increases, indicating similar observations by Alasalvar *et al.* (2001) and Manju *et al.* (2007). The initial pH decrease may be attributed to the dissolution of CO_2 in the fish sample, while the increase of pH was postulated to be due to an increase in volatile bases produced, e.g. ammonia and trimethylamine (TMA), by either endogenous or microbial enzymes (Manat *et al.*,2005; Ruiz-Capillas & Moral, 2001 cited in Fan *et al.*,2009).

According to Sigurgisladottir *et al.* (2000) cited in Rodriguez-Turienzo *et al.* (2011), freezing cause changes in pH values of fish muscle; this is probably due to the increase in concentration of substances in the water that remains unfrozen in frozen foods and modifies the acid–base equilibrium; there is a tendency towards higher acidity (Jiang & Lee, 2004 cited in Rodriguez-Turienzo *et al.*, 2011).

2.6. Sensory Analysis

2.6.1. Color

Color is all around us. Although, an infinite number of colors surround us, nothing really has color. Color is a perception and subjective interpretation, generated in the eye-brain system in response to given stimuli. Color communicates and sells, driving the sale of virtually every consumer product in the world. It evokes a wide range of emotions that draw the buyer to the product (Hewlett-Packard Development Company, 2008; Konica Minolta, 2003; X-Rite, 2004).

Color, as one aspect of appearance, is one of the major attributes that affect the consumer perception of quality. It has to be within an expected range for food acceptance, and the degree of acceptability is judged within that range. Nearly every food product has an acceptable range of color, depending on a wide range of factors, like ethnic origin of the consumer and of the food, age and sex of the consumer, physical surroundings at the time of viewing, health consciousness of the consumer, physical well-being, among others (Francis, 1995; HunterLab, 2008). This makes color an important marketing communication tool and a crucial part of the selling process, interfering in buyer decision (Garber *et al.*, 2000). So, measurement is the key to total production control, allowing the establishment of precise measurement standards that can be repeated in the process production of identical items within quality tolerances (X-Rite, 2004). To use color effectively, it must be kept under control, answering the customer's specifications. The best way to control color is by measuring it, because if you can measure color, you can control it.

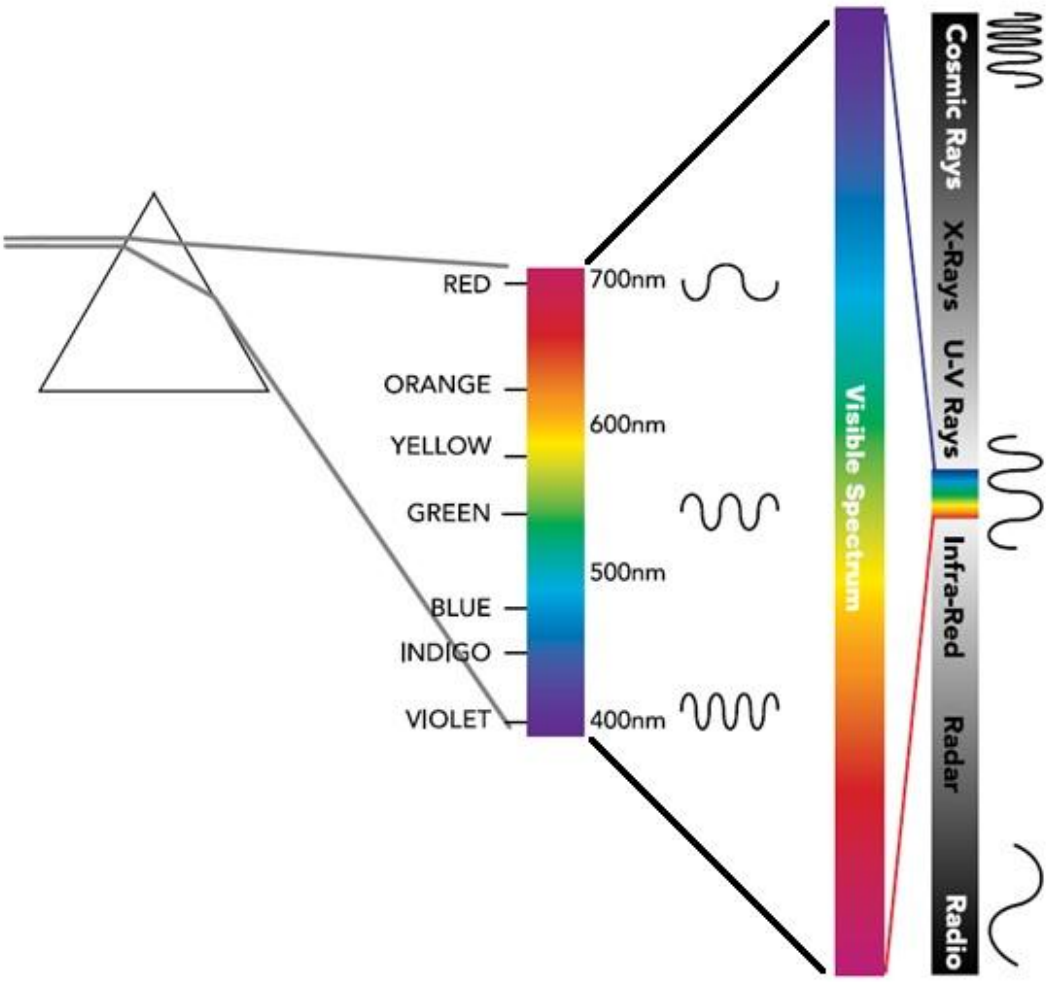
Light, object and viewer are three essential elements to perceive color, since it results from an interaction between them. In matter of fact, in total darkness and if we close our eyes, we cannot see anything, not even colors. And if there is only an object, the color simply does not exist. All three elements must be present for the color as we know it exists. For the viewer to perceive the light as a distinct color, it must be modified by the object (Konica Minolta, 2003; X-Rite, 2004).

Light

The light is just one of several electromagnetic waves that exist in space and which together constitute the electromagnetic spectrum (Figure 2.7). The region of visible light is only a small part of the spectrum located between about 400-700 nm. The light reflected from an object, which we recognize as being his color, is the mixture of light at various wavelengths, within the visible region. The human eye can see light in the visible region of the electromagnetic spectrum, however, "light" is not the same as "color". The light is radiation that stimulating the retina of the eye, making it vision possible. The stimulation of the eye is transmitted to the brain, and it is here that the concept of "color" first occurs as the brain's response to information received from the eye. These stimuli are perceived by the brain as a particular color. Exactly which color is perceived depends on the composition of wavelengths in the light waves. However, our vision system responds to each individual wavelength. For instance, in passing a beam of white light through a prism, the light is dispersed and different colors are seen as respond of the eyes to each individual wavelength. So, different wavelengths cause us to see different colors. On the other hand, we rarely see all wavelengths at once (pure white light), or just one wavelength at once. Color appears when light is modified into a new composition of many wavelengths by interaction with an object. This is how all objects get their color - by modifying light, which is send to our eyes as a unique composition of wavelengths (HunterLab, 2008; Konica Minolta, 2003; X-Rite, 2004).

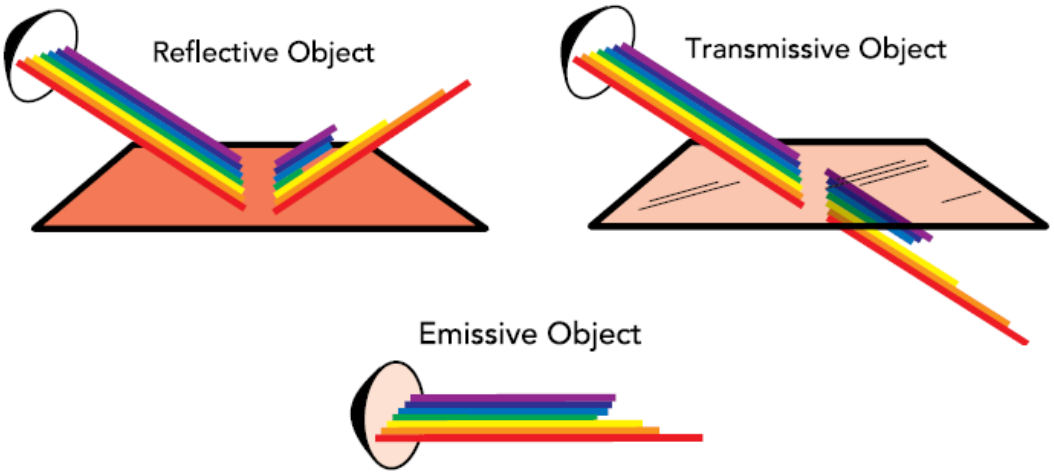
Object

Every object absorbs and reflects light spectrum into portions and different amounts. When light waves strike an object, the object's surface absorbs some of the spectrum's energy, while other parts of the spectrum are reflected back from the object. The modified light that is reflected from the object has an entirely new composition of wavelengths. Light can be modified by striking a reflective object such as paper; or by passing through a transmissive object such as film. Reflected, transmitted, or emitted light is, in the purest of terms, "the color of the object" (Figure 2.8). There are as many different colors as there are different object surfaces, because each object affects light in its own unique way. The color of the object varies with the viewing conditions, viewing angle and angle measurement (Konica Minolta, 2003; X-Rite, 2004).



Source: adapted from X-Rite (2004)

Figure 2.7. Electromagnetic and visible spectra.



Source: removed from X-Rite (2004)

Figure 2.8. Different objects surfaces modifying the light.

Viewer

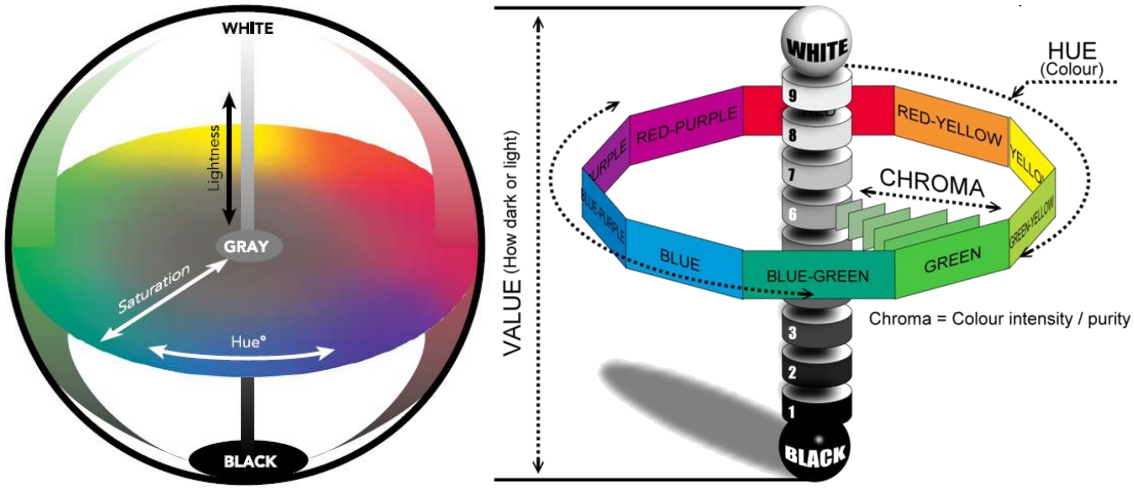
Without the viewer, there would be no sensory response that would recognize or register the wavelengths as a unique "color." If an object is not seen, he doesn't have color. Technically, color is there in the form of wavelengths. So, the concept of color only happens in our minds, after our visual sensory system has responded to those wavelengths. The basis for human vision is the network of light sensors in our eyes. These sensors respond to different wavelengths by sending unique patterns of electrical signals to the brain. In the brain, these signals are processed into the sensation of sight - of light and of color. Our memory system recognizes distinct colors; this system breaks the visible spectrum into its most dominant regions of red, green and blue, and then concentrates on these colors to calculate color information (Figure 2.9) (Konica Minolta, 2003; X-Rite, 2004).

Source: removed from X-Rite (2004)

Figure 2.9. RGB system – Color's Additive Primaries.

Dimensions of color

When one says 'measure the color' what it means is 'locate the color' in terms of coordinates in a three-dimensional color solid (Figure 2.10) (Francis, 1995). Color has three attributes - hue (basic color), saturation (vividness or dullness) and lightness (brightness or darkness) - which can be arranged together to create a three-dimensional solid. The wavelength determines the color's hue; wave purity determines saturation; and wave amplitude (height) determines lightness. Hue, saturation, and lightness demonstrate that visible color is three-dimensional. These attributes provide three coordinates that can be used to "map" visible color in a color space. There are many different types of color spaces and they can be used to describe the range of visible or reproducible colors of a viewer or device. This three-dimensional format is also a very convenient way to compare the relationship between two or more colors (Konica Minolta, 2003; X-Rite, 2004).

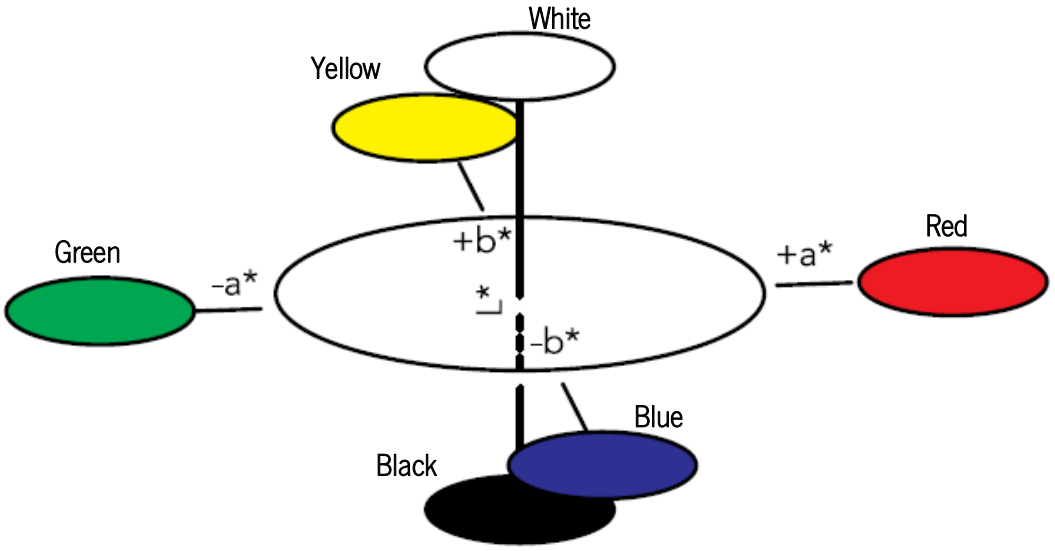


Source: adapted from X-Rite (2004) and http://www.paintbasket.com/members/index.php?topic=121.0, consulted in 29/08/2013

Figure 2.10. Munsell Color System (HSL – the three dimensions of color).

Color space (CIE L*a*b*)

The color space CIE *L*a*b** is uniform color space, which uses a repeatable system of color communication standards. These standards' most important function was to provide a universal framework for color matching. The *L*a*b** color model uses rectangular coordinates based on the perpendicular yellow-blue and green-red axes. One of the advantages of spectral data is its ability to predict the effects of different light sources on an object's appearance. This color space has been defined by the CIE in 1976 to reduce one of the biggest problems of space *Yxy* original: that equal distances on the chromaticity diagram *x,y* do not correspond to an equal perception of color differences. In this improved space, equal distances in the coordinates of the diagram correspond to equally perceived color differences. Thus, *L** indicates lightness and *a** and *b** are the chromaticity coordinates. In the *a*,b** chromaticity diagram, *a** and *b** indicate color directions: *+a** is the red direction, *-a** is the green direction, *+b** is the yellow direction and *-b** is the blue direction (Figure 2.11). The center is achromatic. With the increase of the values of *a** and *b** and the point moves away from the center, the color saturation increases (Konica Minolta, 2003; X-Rite, 2004).



Source: removed from X-Rite (2004)

Figure 2.11. Color space (CIE *L*a*b**) – Mapping Color's dimensions.

The colorimeter

Unlike the human eye, a colorimeter can measure a color accurately and easily. As previously noted, unlike subjective expressions commonly used by people to describe the colors verbally, the colorimeters express colors numerically according to international standards. In addition, the personal perception of a particular color may vary depending on the background or the light source used. The colorimeters correspond to the functions of the human eye, but as they always make measurements using the same light source and the same lighting method, the measurement conditions are always the same, day, night, indoor or outdoor environments (X-Rite®, 2004). In the color space *L*a*b** color difference can be expressed by a single value, *ΔE*ab*, which indicates the size of the color difference, but doesn't shows how the colors are different. This is, *ΔE*ab* indicates the degree of color difference, but not the direction. *ΔE*ab* is defined by the following Equation 1:

$$\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \qquad \text{Equation 1}$$

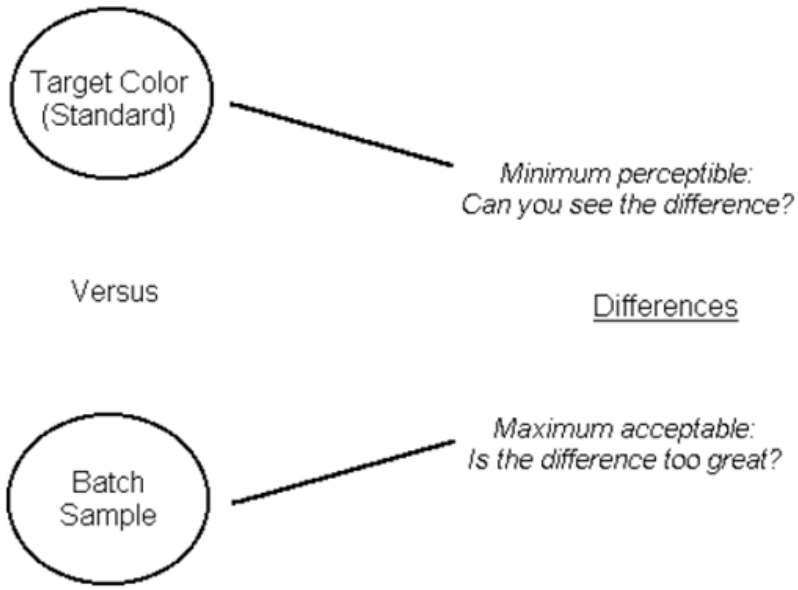
Where *ΔL**, *Δa**, *Δb** indicate the difference in values *L**, *a** and *b** found between the sample color and the color of the standard (Konica Minolta, 2003; X-Rite, 2004).

CIE L*a* b* Tolerance Method

Generally, the color of a product may be judge to be "acceptable" or "unsatisfactory". Such judgments can be visually or instrumentally based on a perceived difference between an ideal product standard and a sample. When this difference is quantified, tolerances can be established. Tolerances are limits within a product are considered acceptable. Any product falling outside the tolerances is unacceptable. There are two levels of visual color differences that are used to establish color tolerances (Figure 2.12):

- Minimum perceptible difference, which defines a just-noticeable difference between standard and sample;
- Maximum acceptable difference, which is the largest acceptable difference between standard and sample.

Manufactures are generally concerned about the maximum acceptable color difference rather than minimum perceptible difference, and color tolerances are usually based on it. Any difference than that would cause the sample to be rejected (HunterLab, 2008).

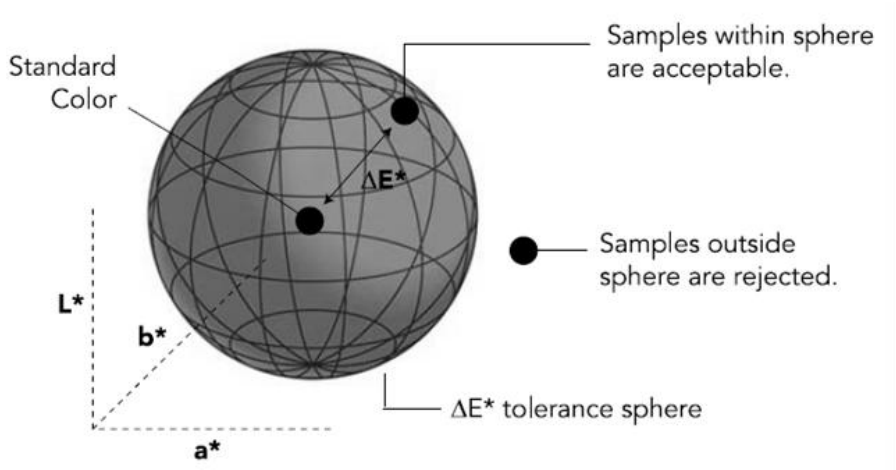


Source: removed from HunterLab (2008)

Figure 2.12. Illustrative scheme of perceptible versus acceptable differences.

Using CIE *L*a*b**, the standard color - or original specification - is pinpointed by its measurement data in the *L*a*b** color space. Then, a theoretical "tolerance sphere" is plotted around the color. This sphere represents the acceptable amount of difference between the standard

and other measured samples (the color output). Data that falls within the tolerance sphere represents an acceptable color. Measurements that fall outside the tolerance sphere are unacceptable (X-Rite, 2004).



Source: removed from X-Rite (1998)

Figure 2.13. Tolerance sphere for acceptable color difference.

The size of the tolerance sphere (Figure 2.13) is determined by customer's specifications for acceptable color difference, which is expressed in delta (Δ) units such as ΔE (delta error) (X-Rite, 2004). According to X-Rite (2004), typical customer tolerance in the graphic arts industry usually lies between 2 and 6 ΔE. Differences between colors in an image that are within 4Δ units of each other often are not visible to most viewers. According to Ahmad (2006), a minor difference in color perceived by the human eye is in the range of ΔE^*ab 0.3 to 0.5. However, the acceptable thresholds are much higher, standing at intervals of 1.1 to 2.1.

2.7. Physical properties

2.7.1. Temperature and storage time

According to Evans (2008), the shelf-life of a frozen food is a complex concept that depends on the characteristics of the food product and the environmental conditions to which the food is exposed after being subjected to the freezing process. The International Institute of Refrigeration (IIR, 1986) has recommended two definitions: practical storage life (PSL) and high-quality life (HQL). The PSL (or acceptability time) is "the period of proper frozen storage after freezing of an initial high-quality product, through which the frozen food retains its quality

characteristics and is suitable for consumption or for use in further process". HQL parameter (or Just Noticeable Difference (JND)) is defined as "the storage period through which the initial quality was maintained from the time of freezing up to the point where 70% of the trained taste panel members are capable of detecting a noticeable difference between the frozen food stored at different temperatures and the corresponding controls stored at -40 °C in a triangular sensory test" (Evans, 2008). However, these concepts diverge from the concept of storage life that is acceptable to consumers, which may be acceptable for three to six times longer than the PSL or HQL. These methods therefore measure the period that food remains essentially the same as when it was frozen. Fluctuating temperatures and the type of packaging used are the main causes of loss of storage life. Since temperature fluctuation has a cumulative effect on food quality, the proportion of PSL or HQL lost can be found by integrating losses over time. Time temperature tolerance (TTT) and product processing packaging (PPP) concepts are used to monitor and control the effects of temperature fluctuations on frozen food quality during production, distribution and storage (Fellows, 2000).

Knowledge of the various changes that occurs in fish, immediately after harvest or catch, must be known, especially the changes in properties that take place over time. This information can be gained by performing controlled storage experiments that extend from the time of harvest until spoilage.

Freshness, loss of freshness and spoilage can also be monitored using different techniques. However, only a few of these techniques are routinely applied in the fish industry, because they are time-consuming, require expensive laboratory equipment and trained personnel (Ólafsdóttir *et al.*, 1997). In matter of fact, freezing and frozen storage can give a shelf-life of more than one year if properly carried out (Chevalier *et al.*, 2001; Johnston *et al.*, 1994; Regenstein & Regenstein, 1991 cited in Gonçalves *et al.*, 2009).

According to Reid *et al.* (2003), shelf-life estimation for frozen foods can be a long process because of the long duration of shelf-life at the lower temperatures of storage. The purpose of low-temperature storage is to achieve extended shelf-lives. A reliable procedure that allows an effective estimation of the low-temperature storage life of a product, utilizing data collected on the product in question, requires around 60 days, while effectively estimating the storage temperatures required to achieve target shelf-lives of 1 year, 18 months, 2 years or even longer.

There is a need for procedures that will allow for the estimation of extended shelf-lives, especially at low temperatures, without requiring too long an evaluation period. Many procedures

exist for accelerated shelf-life testing at refrigeration temperatures, but fewer procedures have been developed for the prediction of frozen storage temperatures. Since any extrapolation based on such a narrow temperature range is fraught with uncertainty, the challenge becomes one of identifying alternative information sources that can assist with the estimation of shelf-life, or accepting that the initial evaluation of the temperature-dependent stability of a product will necessarily occupy significant time (Reid *et al.*, 2003). Ólafsdóttir *et al.* (1997) confirms that although, there are many different physical measurements that provide information on parameters related to fish freshness, none of these methods is able to determine unambiguously whether a fish is fresh or not.

In general, the lower the temperature of frozen storage, the lower the rate of microbiological and biochemical changes. The article 5 of DL nº 37/2004 of February 26 describes that frozen products should be kept at a temperature of -18 °C, or below, in all its points. Transportation and sale admit the following maximum tolerances for temperature of frozen and deep-frozen products:

- In the carriage: 3 °C;
- In sales displays: 6 °C.

However, freezing and frozen storage do not inactivate enzymes and have a variable effect on microorganisms (Fellows, 2000). Relatively high storage temperatures (between -4 °C and -10 °C) have a greater lethal effect on microorganisms than do lower temperatures (between -15°C and -30 °C). Different types of microorganism also vary in their resistance to low temperatures; vegetative cells of yeasts, moulds and gram negative bacteria (for example coliforms and *Salmonella* species) are most easily destroyed; Gram-positive bacteria (for example *Staphylococcus aureus* and *Enterococci*) and mould spores are more resistant, and bacterial spores (especially *Bacillus* species and *Clostridium* species such as *Clostridium botulinum*) are virtually unaffected by low temperatures. At normal frozen storage temperatures (-18 °C), there is a slow loss of quality owing to both chemical changes and, in some foods, enzymatic activity. These changes are accelerated by the high concentration of solutes surrounding the ice crystals, the reduction in water activity (to 0.82 at -20 °C in aqueous foods) and by changes in pH and redox potential. If enzymes are not inactivated, the disruption of cell membranes by ice crystals allows them to react to a greater extent with concentrated solutes. The main changes to frozen foods during storage are degradation of pigments, loss of vitamins, residual enzyme activity, and oxidation of lipids (Fellows, 2000).

Part II – Experimental Work

Chapter 3. Materials and Methods

3.1. Fish preparation

Frozen and packaged Atlantic salmon fillet (*Salmo salar*) was obtained from a local company (Vanibru – Comércio de Produtos Alimentares, Braga, Portugal). After unpacking, the salmon fillets were cut into slices (samples) with the dimensions 10 cm × 5 cm × 2-3 cm (Figure 3.1) and an average weight of (113.4±7.4) g, using a vertical bone sawing machine (FK 32, BIZERBA, Germany). This process was carried out in a refrigerated (~ 5 – 8 °C) room to minimize temperature uptake. For each treatment, salmon samples (*n=3*) were individually packed in zip-lock polyethylene freezer bags and stored in an industrial freezing chamber maintained at (-21.4±1.6) °C, for 6 months.

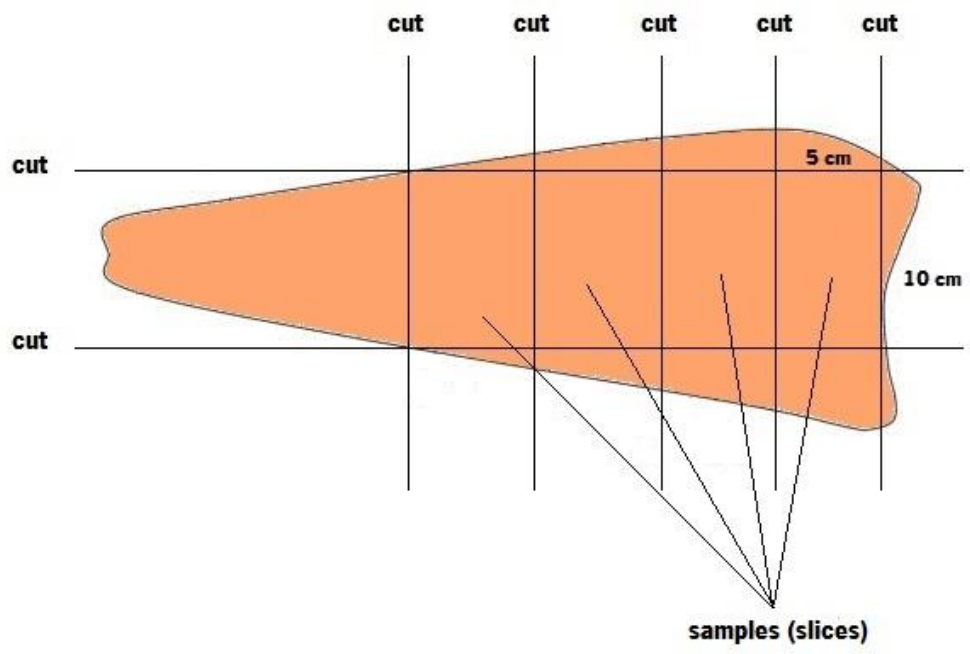


Figure 3.1. Illustration of the salmon fillet, exemplifying the scheme of cuts used.

3.2. Preparation of the coating solutions

Chitosan from Golden-shell Biochemical Co. Ltd. (China) with a 91% degree of deacetylation was used. The coating solutions were prepared by dissolving the chitosan (0.5% and

1.5%) in a 1% lactic acid solution with agitation, using a magnetic stirrer, at a temperature of 45 °C, until complete dissolution.

3.3. Preparation of the samples

3.3.1. Preparation of the samples coated with chitosan

The frozen salmon samples (-21.4±1.6) °C were weighted (W_1), dipped in chitosan coating solutions (5.18±0.49) °C for 0.5% chitosan solution and (8.10±0.57) °C for 1.5% chitosan solution, the solution temperature was monitored by the probe (HANNA Instruments, HI765PW, Romania) of the infrared Pronto Plus thermometer (HANNA Instruments, HI99556-10, Romania)). The samples were dipped 35 seconds in 0.5% chitosan solution and 10 seconds in 1.5% chitosan solution, drained for 2 minutes and weighted again (W_2). These dives were performed in a pilot-scale glazing tank (Figure 3.2) with the help of a stainless steel mesh, who collected the samples from inside the tank, in order to minimize the interference with the amount of coating applied. Following the Equation 2, the coating uptake was calculated, where W_1 and W_2 indicate the weight of the salmon sample before and after the coating application, respectively. An average of (9.6±0.1)% and (10.0±0.2)% of coating uptake (wt%) was obtained for the chitosan solutions of 0.5% and 1.5%, respectively.

$$Coating\ uptake\ (\%) = \frac{W_2 - W_1}{W_2} \times 100 \qquad \text{Equation 2}$$

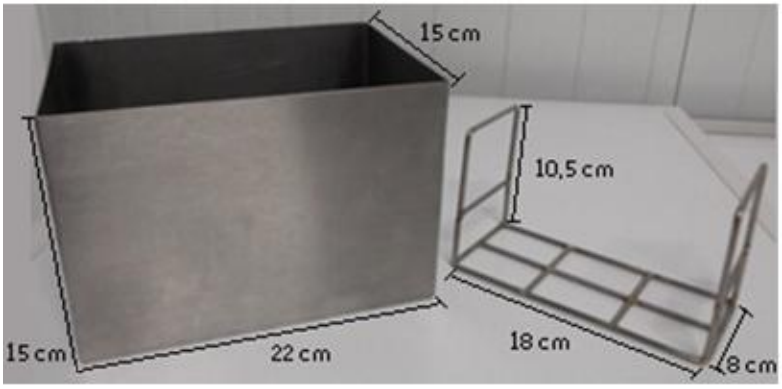


Figure 3.2. Pilot-scale glazing tank (left) and dipping instrument – mesh (right) constructed in A151 316 stainless steel.

3.3.2. Preparation of the samples glazed with water

A similar process was followed for the salmon samples glazed. These samples were weighted (W_3), dipped in water (0.28±0.08) °C for 40 s, drained for 1 minute and weighted again (W_4). The glazing uptake obtained was calculated by Equation 3, where W_3 and W_4 indicate the weight before and after the glaze is apply in the samples, respectively. An average of glazing uptake of (8.4±0.3)% was obtained.

$$Glazing\ uptake\ (\%) = \frac{W_4 - W_3}{W_4} \times 100 \qquad \text{Equation 3}$$

3.3.3. Preparation of the control samples

The samples that have integrated the control group were left untreated. These non-coated samples were used for comparison with the remaining groups of samples.

3.4. Storage and transport of the samples

All salmon samples were individually packed in zip-lock polyethylene freezer bags, inside corrugated boxes, and stored in an industrial freezing chamber maintained at (-21.4±1.6) °C, for 6 months. This temperature was monitored using a data logger (DS7922 1Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., U.S.A.).

Salmon samples were then transported to the laboratory in polystyrene boxes with an appropriate quantity of ice accumulators. Once in the laboratory, the salmon samples were kept in a common freezer at ~20 °C until further use. Fish samples were taken from each package, and microbiological and physicochemical analyses were performed at regular intervals. All analysis was done in triplicate.

3.5. Samples Analysis

3.5.1. Coating Loss

After the storage period, the coated samples were weighed again (W_5) and the coating loss was determined using Equation 4.

$$Coating\ loss\ (\%) = \frac{W_5 - W_2}{W_2 - W_1} \times 100 \qquad \text{Equation 4}$$

3.5.2. Glazing Loss

After the storage period, the glazed samples were weighed again (W_6) and the glazing loss was determined with Equation 5.

$$Glazing\ loss\ (\%) = \frac{W_6 - W_4}{W_4 - W_3} \times 100 \qquad \text{Equation 5}$$

3.5.3. Weight Loss

The control samples left untreated don't have any coating. In this case, the uncoated samples were initially weighed (W_7) and after the storage period were weighed again (W_8) and the weight loss determined with Equation 6:

$$weight\ loss\ (\%) = \frac{W_8 - W_7}{W_8} \times 100 \qquad \text{Equation 6}$$

3.5.4. Drip Loss

To calculate Drip Loss, all the frozen samples were removed from the freezer, kept for 22 hours in the refrigerator at 5 °C, removed from the zip-lock polyethylene bag and placed on a rack for 2 minutes to release drip, and the thawed samples were weighed. The Drip Loss was determined by Equation 7, were W_9 indicates the weight of frozen samples without coating/glazing

and before being placed in the refrigerator; W_{10} indicate the weight of thawed samples (Sathivel *et al.*, 2007).

$$Drip\ Loss\ (\%) = \frac{W_9 - W_{10}}{W_9} \times 100 \qquad \text{Equation 7}$$

3.5.5. Determination of TVC

Total Aerobic Plate counts were estimated by the procedure based on the BS EN ISO 4833:2003 Standard Protocol. A 25 g sub-sample of product was required for testing. Since the package should be cleaned and sanitized before opening, the external surface of rigid or semi-rigid packages of fish was cleaned with detergent and water ensuring no contamination of the package contents occurs. The opening of the package was carried out using sterile scalpels, scissors or forceps. All operations during and after opening were carried out under aseptic conditions without interruption.

The testing sample was obtained removing randomly -selected individual sub-samples, each of not more than 1 g mass to produce an appropriate mass of sample for testing. The test sample was added to a stomacher bag containing 9 volumes of maximum recovery diluent (MRD) and stomached for 1 minute. Using a fresh sterile pipette 1mL of the initial inoculum was transferred into 9 mL of MRD and the procedure was repeated for as many decimal dilutions as required (Figure 3.3). The dilutions were mixed using a vortex mixer for 5 to 10 seconds. From each dilution, 1 mL of the initial inoculum was aseptically inoculated into a labeled Petri dish.

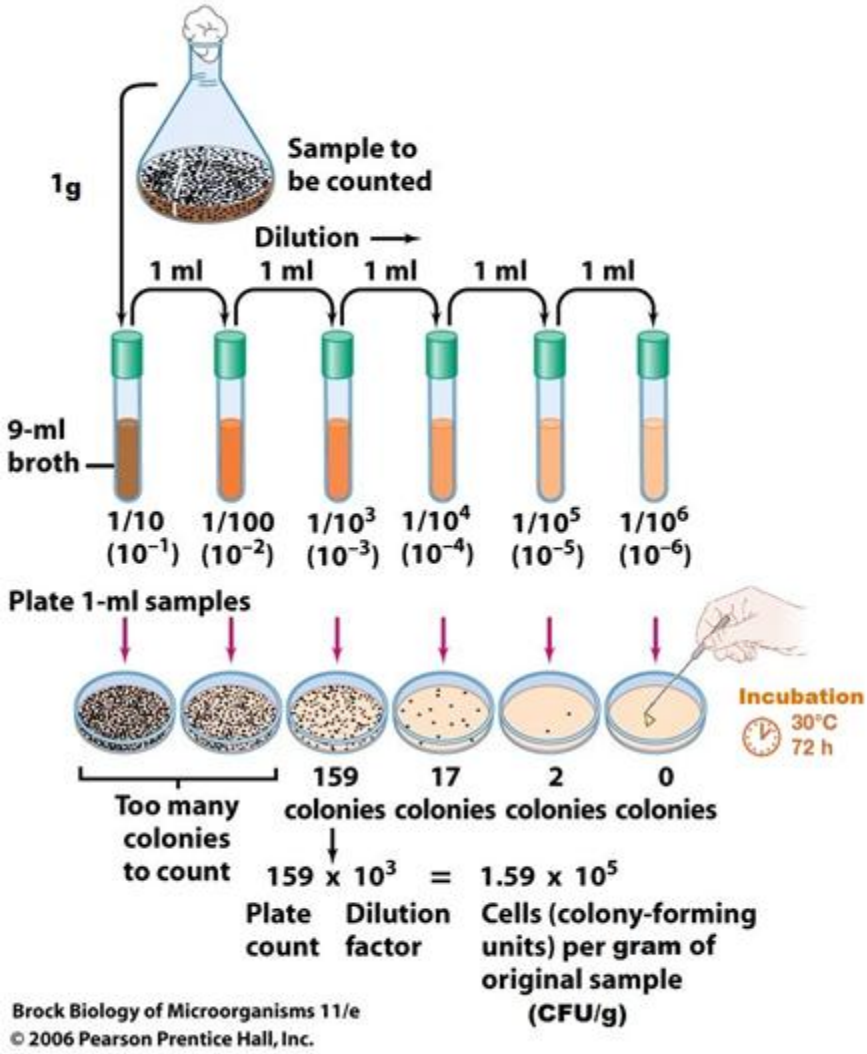
In parallel, a boiling bath melted a 500 mL pre-sterilized bottle of Plate Count Agar (PCA) in 60-90 minutes. Bottles were warmed slightly before placing in very hot water (to avoid cracking), taking care not to overheat the agar. After melting, the agar was left on the bench for 20 minutes to ensure that the bottle will not crack and then it was placed in a water bath of temperature (46±1) °C (a single bottle took roughly 1 hour to cool to 46 °C).

Subsequently, 15 mL of tempered (46±1) °C PCA agar was added to each Petri dish by mixing and swirling six times clockwise, six times left to right, six times anticlockwise and six times up and down (the time elapsing between the preparation of the initial suspension and contact with the agar did not exceed 45 minutes). After solidifying, were inverted and placed into an incubator at (30±1) °C for (72±3) hours.

The colonies from each Petri dish containing more than 15 and fewer than 300 colonies were counted and the number of microorganisms (M) present in the test sample was calculated using Equation 8, where $\sum c$ is the sum of colonies counted, n_1 is the number of dishes retained in the first dilution, n_2 is the number of dishes retained in the second dilution and d is the dilution factor corresponding to the first dilution. The results were reported as the number of microorganisms per gram of sample.

The Total Viable Counts (TVC) were estimated for samples frozen (-20 °C) and thawed samples (5.9 °C).

$$N = \frac{\sum c}{(n_1 - n_2)d}$$ Equation 8



Source: adapted from http://sciencelane.com/?p=689, consulted in 13/08/2013

Figure 3.3. Example of serial dilution from an initial sample.

3.5.6. Determination of TBA

TBA (2-thiobarbituric acid) was determined by spectrophotometric detection according to the standard procedure described in NP 3356:2009.

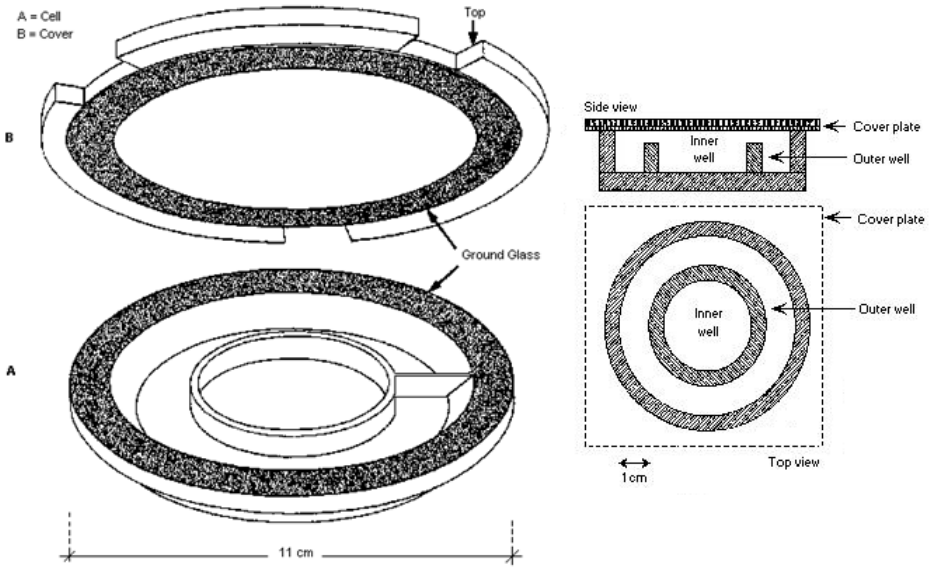
A portion of 15 g of sample was accurately weighed on an analytical balance (Mettler AE200) and homogenized in a blender (BECKEN coffee grinder, Worten, Portugal) at least twice. A solution of trichloroacetic acid was prepared by adding 75 g of trichloroacetic acid, 1 g of EDTA (ethylenediaminetetraacetic acid Titriplex II), 20 mL of an alcoholic solution of propyl gallate 5% into a 1000 mL volumetric flask, making up the volume with distilled water and homogenizing. The milled sample was placed into a 50 mL falcon tube and a 30 mL of solution of trichloroacetic acid 7.5% was added with a pipette. This solution waited about 2 minutes for the extraction was complete. Then, the extract was filtered with filter paper Whatmann n° 1 for beaker, obtaining a clear extract. Next, it was accurately pipetted to a test tube 5 mL of extract and 5 mL of 0.02 M thiobarbituric acid (TBA). The test tube was capped and placed in a boiling water bath (~100°C) (VWR, VMS-C7 Advanced magnetic hotplate stirrer supplied with a glass coated PT1000 probe) for 40 minutes. After this time, the tubes were removed from the bath and cooled under cold running water, opened carefully and stirred to avoid the formation of air bubbles. The content of each tube was transferred to a quartz cell of 10 mm and the absorbance was measured with a spectrophotometer (Jasco V-560 UV/Vis spectrophotometer, Japan) at 530 nm, using distilled water in the reference cell. A reagent blank was run at the same time. This reagent blank was produced under the same conditions, but replacing the volume of extract for an equal volume of distilled water. The TBA index is given in the amount of malondialdehyde, expressed in mg per 1000 g of sample (mg MDA/Kg sample), according to Equation 9, where C is the concentration of malondialdehyde, expressed in micromoles; v is the volume, in milliliters of the extract; H is the moisture content of the sample, expressed as a percentage; m is the mass, in grams, of the taking the test (Appendix A1).

$$TBA\ value = \frac{72 \times C}{m \times v} \times \left(30 + \frac{m \times H}{100}\right) \qquad \text{Equation 9}$$

3.5.7. Determination of TVB-N

The value of TVB-N was determined by the method of Conway as set out in NP 2930:2009. A 50 g sample (m_s) was homogenized with 100 mL of 5% trichloroacetic acid and, after 2 minutes, the mixture was filtered through gauze. Then, it was transferred 1 mL of boric acid (H_3BO_3) to the center of the Conway cell (Figure 3.4). On the periphery of the Conway cell was added 1 mL of filtrate (V_3), 0.5 mL distilled water and 1 mL of potassium carbonate (K_2CO_3) saturated solution. The Conway cell was carefully closed without stirring the solutions and placed in an incubator at 40 °C for 90 minutes. After that period, the boric acid was titrated with 0.02 mol/L hydrochloric acid, until a pink coloration was acquired. A blank and a diffusion control were performed by replacing the volume of extract by an equal volume of distilled water and 0.1% ammonium sulfate, ($(NH_4)_2SO_4$) respectively (Appendix A2). The TVB-N value was determined according to the Equation 10, where V_0, V_1 and V_2 represent the volumes of hydrochloric acid (mL) added in the blank test, in the diffusion control test, and in the extract test, respectively, and F_c is a volume correction factor (moisture of sample). The results were expressed in mg of nitrogen per 100 g of sample (mg N/100 g sample).

$$TVB - N\ value = \frac{21 \times (V_2 - V_0)}{(V_1 - V_0) \times V_3 \times m_s} \times (100 + F_c) \qquad \text{Equation 10}$$



Source: adapted from http://www.ufrgs.br/imunovet/molecular_immunology/invitrocellfree.html and http://www.inchem.org/documents/antidote/antidote/ant02.htm, consulted in 14/08/2013

Figure 3.4. Representation of the Conway cell.

3.5.8. Determination of K-value

The K-value was determined according to the method of Ryder (1985) as described by Souza *et al.* (2010). A 5 g of sample was homogenized (BECKEN coffee grinder, Worten, Portugal) with 25 mL of chilled 0.6 mol/L perchloric acid ($HClO_4$) at 0 °C for 1 minute. The homogenate was centrifuged (EBA 20, Hettich zentrifugen, Germany) at 3000×g (6000 rpm) for 10 minutes. Using a pH meter, 10 mL of the supernatant was adjusted to pH 6.5-6.8 with 1 mol/L potassium hydroxide (KOH) (Metrohm 620 pH meter, Swiss made). After standing at flaked ice for 30 min, the potassium perchlorate that precipitated was removed by filtration using a Whatman n° 1 filter paper. The filtrate was diluted to 20 mL with Milli-Q purified distilled water, passed through a 0.20 µm Fioroni membrane, and stored at -80 °C until the subsequent analyses.

Twenty microliter aliquots of all samples were analyzed using a HPLC (Hitachi High-Technologies Corporation chromatograph (VWR, Tokyo, Japan)) equipped with a Organizer (Elite Lachrom), Pump (Elite Lachrom L-2130), UV-Vis detector (Elite Lachrom L-2420) at 254 nm, Autosampler (Elite Lachrom L-2200) and Column oven (Elite Lachrom L-2300) with a Purospher®Star RP-18e (endcapped particles, 5 µm particle size, LichroCART® 250-4 HPLC Cartridge, ART. 1.50252.0001, Sorbent Lot Hx947476, Merck, Germany) column (Figure 3.5). Separation of the nucleotide products was achieved using a mobile phase of 0.04 mol/L potassium dihydrogen orthophosphate (KH_2PO_4) and 0.06 mol/L dipotassium hydrogen orthophosphate (K_2HPO_4) dissolved in 1:1 ratio in Milli-Q purified distilled water, at a flow rate of 1 mL/min. The peaks obtained from fish muscle extracts were identified and quantified through standard solution curves (Appendix A3). ATP breakdown products comprising ATP, ADP, AMP, IMP, HxR, and Hx were measured, and the K value was calculated using Equation 11 described by Saito *et al.* (1959):

$$K - value\ (\%) = \frac{[Hx]+[HxR]}{([ATP]+[ADP]+[AMP]+[IMP]+[Hx]+[HxR])} \times 100 \qquad \text{Equation 11}$$

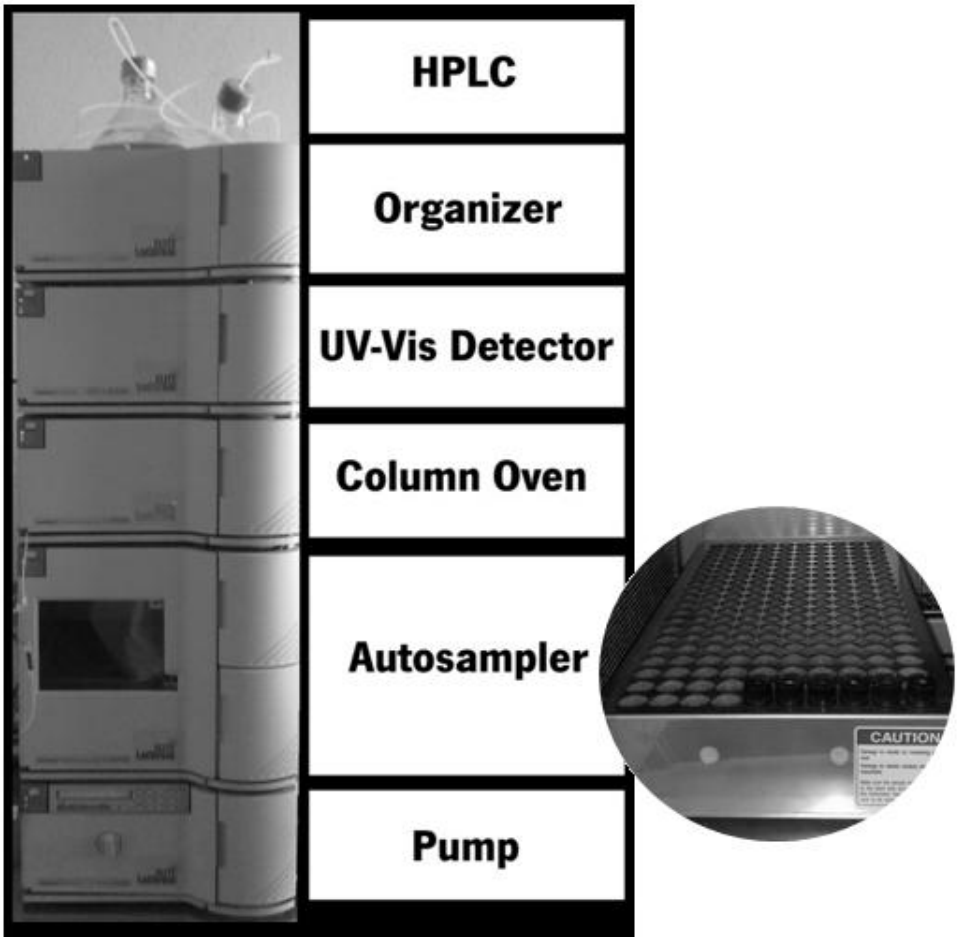
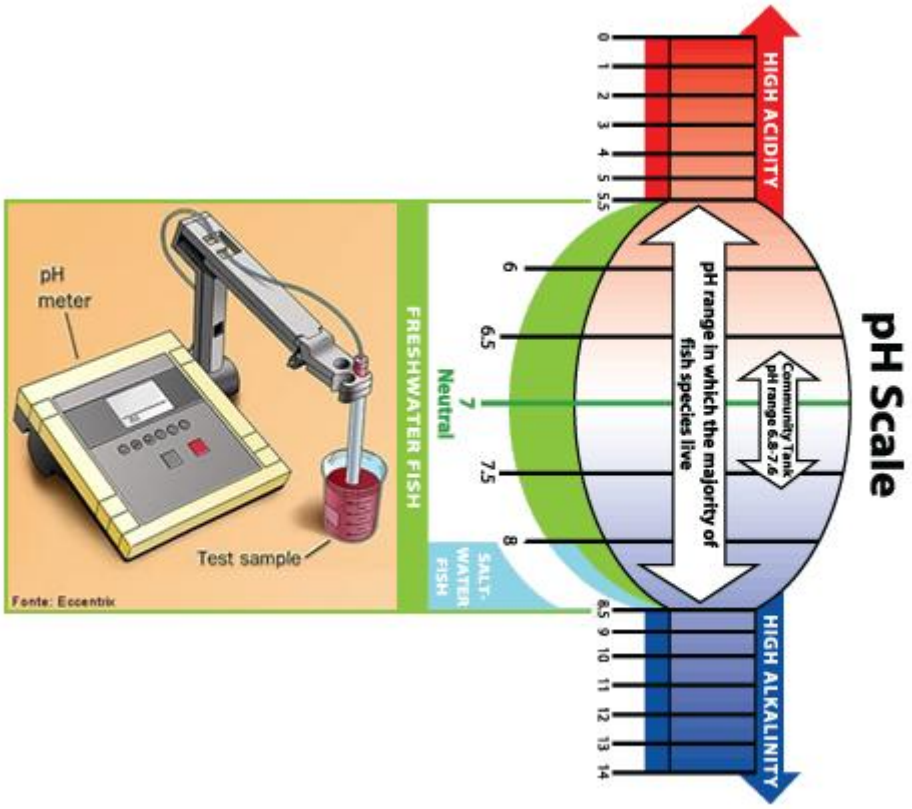


Figure 3.5. Organizational scheme of the HPLC equipment used.

3.5.9. Determination of pH-value

After removing the coating/glazing with a knife in order to prevent changes in the samples, a 5 g portion of each sample was homogenized with 50 mL of Milli-Q purified distilled water in a mixer (BECKEN coffee grinder, Worten, Portugal) for 30 s and the pH value of the suspension was measured using a pH meter (Metrohm 620 pH meter, Swiss made) (Figure 3.6) (Fan *et al.*, 2009; Sathivel *et al.*, 2007; Souza *et al.*, 2010).



Source: adapted from http://www.quimica.seed.pr.gov.br/modules/galeria/detalhe.php?foto=1488&evento=4 and http://www.pet-tabs.us/freshwater_quality/category/ph, consulted in 14/05/2013

Figure 3.6. Illustration of the pH meter and pH range typical for freshwater fish.

3.5.10. Determination of color parameters

The instrumental measurement of color salmon was performed using a colorimeter (CHROMA METER CR-400/410, AQUATEKNICA, SA, Konica Minolta, Japan). The results were expressed using the CIE $L^*a^*b^*$ system. The parameters measured were the luminosity L^* (L^*=0 for black and L^*=100 for white) and color coordinates a^* (-a^* for green and +a^* for red) and b^* (-b^* for blue and +b^* for yellow). The salmon samples presented, to the "naked eye", a similar color for the same groups, i.e. for the control group (uncoated), the glazed group, the group coated with 0.5% chitosan and the group coated with 1.5% chitosan, the samples appeared identical colors. The values obtained for the first trial served as a reference standard. These values were discounted to the values of the following tests to calculate the ΔE^*ab. The samples, with approximately 1 cm of thickness, were evaluated at six different points, 3 points on the right side and three points on the left side to obtain an average value that minimizes the color difference within the same sample. The equipment was calibrated using the white calibration plate (Figure 3.7). Salmon samples were

stored in a controlled temperature chamber at (-15.8±1.7) °C and they were taken at different time points for evaluation (Appendix A4). This temperature was monitored using a data logger (DS7922 1-Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., U.S.A.).

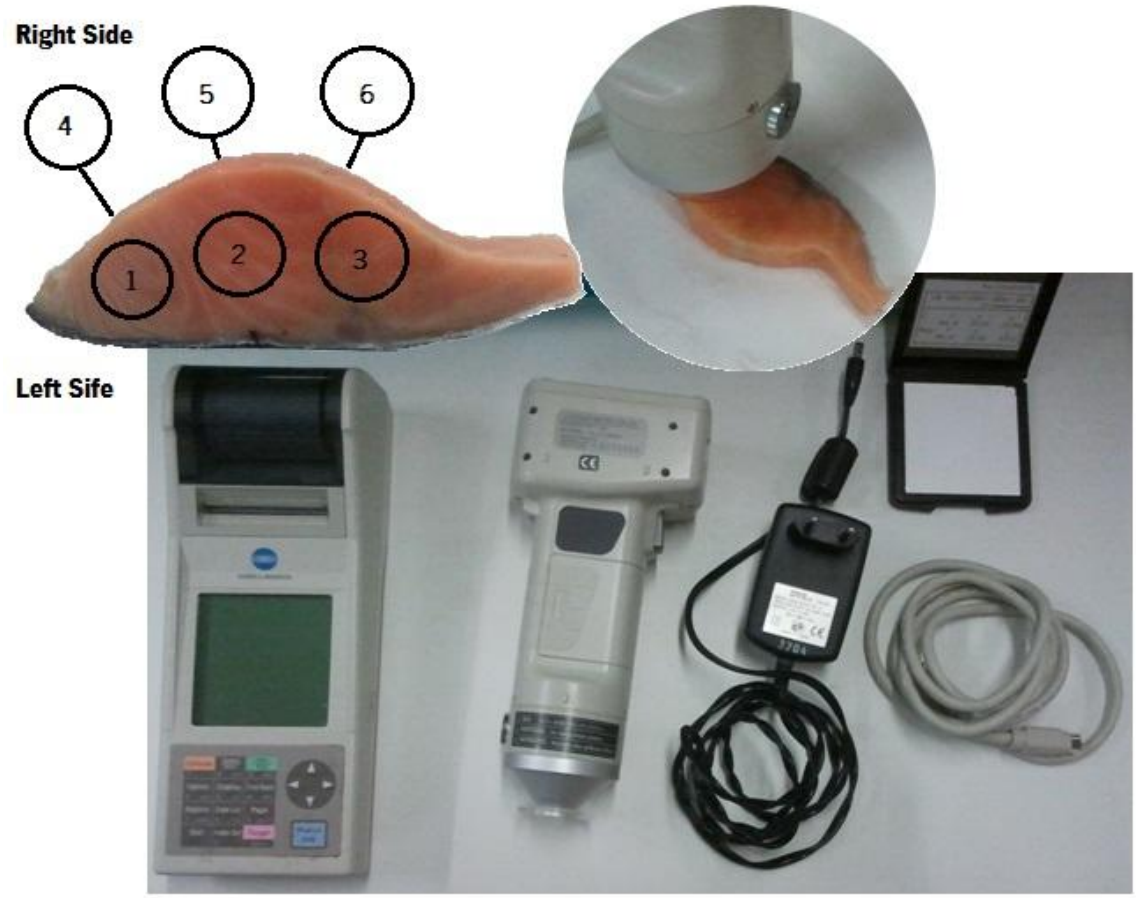


Figure 3.7. Illustration of the methodology and equipment used in the measurement of the color of salmon.

3.5.11. Statistical analysis

All experiments were performed in triplicate. The mean values of those 3 independent determinations were calculated for each treatment at every moment. The statistical significance of differences among treatment means was evaluated by analysis of variance (ANOVA) followed by the Tukey test with significance at $p<0.05$. Data were evaluated statistically using the software STATISTICA version 7.0 (StatSoft Inc. 2004).

Chapter 4. Results and Discussion

4.1. Coating Loss

The effect of chitosan coatings, water glazing and storage time on the coating/glazing loss of salmon samples during storage at -22 °C is shown in Figure 4.1.

The application of both coatings, chitosan coating and water glazing, presented no statistically significant effect in the initial coating/glazing loss values. Although stable in the first moments, after 13 and 39 days of storage, where the glazed samples presented a coating loss of (2.64±0.73)% and (2.47±0.93)% and the samples coated with 0.5% chitosan showed a coating loss of (2.09±0.96)% and (1.80±1.17)%, the samples coated with 0.5% chitosan showed a tendency to have smaller values than the glazed samples. This tendency was evident in the two subsequent periods, after 68 and 125 days of storage, when this difference becomes significantly different. In fact, the glazed samples presented a coating loss of (4.72±1.22)% against (1.91±0.83)% for the 0.5% chitosan coated samples, after 68 days of storage. After 125 days of storage, the glazed samples showed a coating loss of (3.43±1.17)% against (1.29±0.48)% for the samples coated with 0.5% chitosan. Such decrease was also observed in the last moment, after 182 days of storage, although not significantly different was found, since the size of the deviation standard. However, it is possible to detect an effect more pronounced and effective of the 0.5% chitosan coating in the control of water transfer of the samples when compared with the samples glazed with water.

Samples coated with 1.5% chitosan were also tested on two occasions, 68 days and 182 days of storage. Though these samples did not show significant differences compared to the other coatings, they appear to lose less coating than the glazed samples. These samples presented a coating loss of (3.19±0.89)% against (4.72±1.22)% for the glazed samples, after 68 days of storage and a coating loss of (2.49±1.61)% compared with the glazing loss of (3.36±0.53)%, after 182 days of storage. The same tendency did not occur for samples coated with 0.5% chitosan, where samples coated with 1.5% chitosan seem to suffer a greater coating loss. The samples coated with 1.5% chitosan lost (3.19±0.89)% against the coating loss of (1.91±0.83)% for the samples coated with 0.5% chitosan, after 68 days of storage. After 182 days of storage, a coating loss of (2.49±1.61)% for the samples coated with 1.5% chitosan still was higher than (2.37±1.37)% to samples coated with 0.5% chitosan.

In matter of fact and according to Gonçalves *et al.* (2009), the weight loss by dehydration during freezing and storage can be reduced by both methods: covering the surface with packaging material or surrounding the product with a thin layer of ice. If, on one hand, Jacobsen & Fossan (2001) argue that if the product is subject to inadequate cold storage, the glaze will evaporate instead of the tissue water itself, on the other hand, Kilincceker *et al.* (2009) claims that a barrier is formed against the water oozing out, with the process of coating, conserving the majority of the water inside the product. However, Kester & Fennema (1986), cited by Sathivel *et al.* (2007) and Rodriguez-Turienzo *et al.* (2011), reported that chitosan coatings may function as moisture-sacrificing agents instead of moisture barriers, thus moisture loss from the product could be delayed until the moisture contained within the chitosan coating had been evaporated. That is, while coatings loose their water by sublimation during storage, they prevent losses of food moisture.

A study performed by Soares *et al.* (2013) reports that coating loss from frozen salmon stored at -5 °C increases during storage. The different storage temperatures may explain the different results, since at -5 °C ice is closer to its melting point and more liquid water is available than at -18 °C. The apparent stability of the coating loss values for the different coatings indicate that an adequate freezing temperature (< -18 °C) can be effective in reducing coating loss during storage and by so increase fish protection.

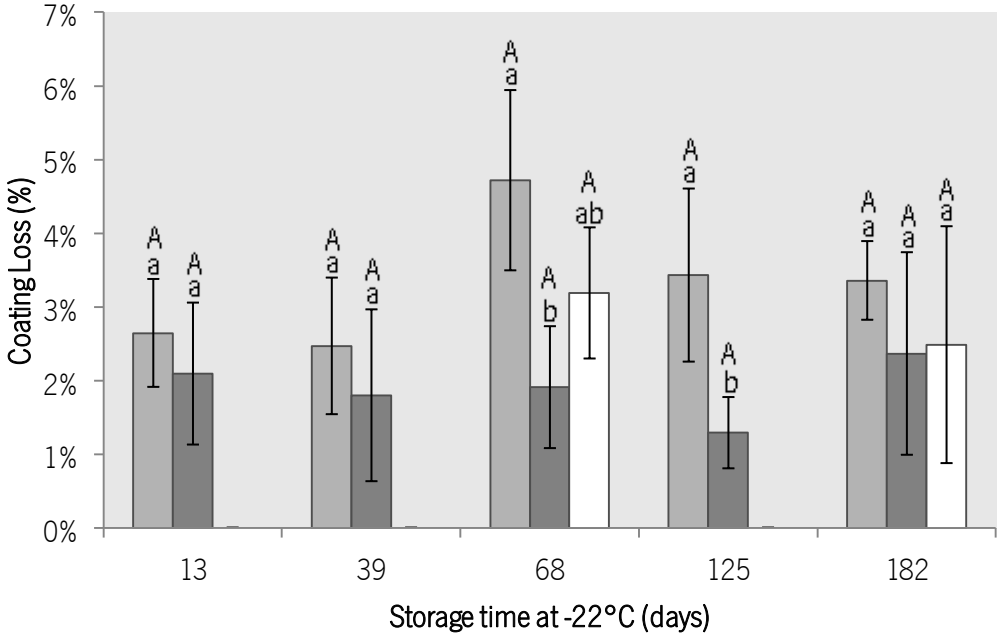


Figure 4.1. Coating Loss (%) for salmon samples glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same

day and different capital letters in bars with the same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.2. Weight Loss

The weight loss of salmon samples during storage at -22 °C is shown in Figure 4.2. These values showed no significant differences throughout storage. However, despite being very small, these values show an increasing tendency along the entire storage, starting at (0.08±0.04)% and ending at (0.16±0.07)%.

Johnston *et al.* (1994) states that weight loss due to dehydration in a freezer depends on the type of freezer, freezing time, type of product, air velocity and freezer operating conditions. These reduced values might be explained by a well controlled storage temperature, since the temperature profile from the industrial chamber used showed an amplitude of temperature values less than 2 °C (Appendix A4 – Figure A.11) and due to the fact that all samples, including the control samples without coating, are stored in polyethylene bags, inside corrugated boxes, which also act as protection.

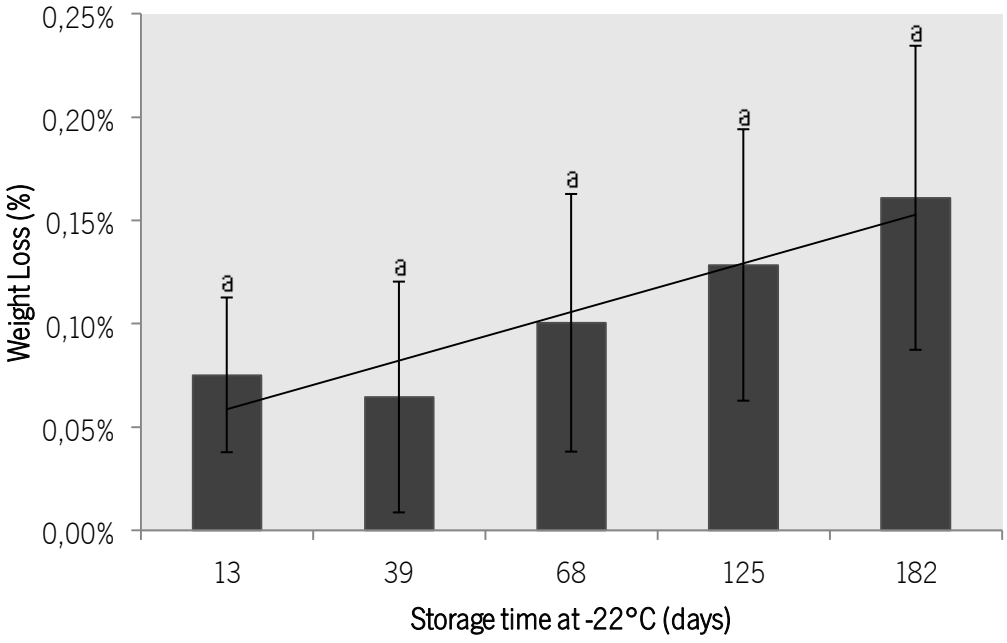


Figure 4.2. Weight Loss (%) of uncoated salmon samples from the control group (■ QS) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different letters indicate a statistically significant difference (Tukey test, $p<0.05$).

4.3. Drip Loss

The drip loss of salmon samples during storage at -22 °C is shown in Figure 4.3. The different coatings did not appear to interfere in a significant way to the drip loss, since the values do not show significant statistical differences The initial drip loss value for the control sample, without coating, was (1.7±0.5)%. The values for these control samples increased over the storage from (0.5±0.2)%, passing through (1.7±0.1)% and (2.3±1.5)%, to (4.8±1.0)% at the end, after 40, 69, 126 and 183 days of storage, respectively. Except for the 126 days of storage, all coatings also increased the amount of drip loss. The glazed samples started with a drip loss of (1.7±0.3)%, after 40 days of storage and (2.3±0.3)% after 69 days of storage, ending with (5.0±0.6)% after 183 days of storage. The samples coating with 0.5% of chitosan passed by (0.9±0.4)%, (3.1±0.3)%, (4.3±0.5)%, after 40, 69, 183 days of storage, respectively. Also the samples coating with 1.5% of chitosan increased their values from (2.2±1.1)% to (3.6±0.2)%, after 69 and 183 days of storage.

A study performed by Sathivel *et al.* (2007) showed drip losses of (0.5±0.3)% for uncoated salmon samples, (2.9 ± 1.2)% for glazed samples and (6.1±0.9)% for chitosan coated samples, after 8 months of frozen storage at -35 °C. Mackie (1993) cited in Sathivel *et al.* (2007) reported that myosin aggregation in frozen fish fillets during storage leads to muscle toughening and drip loss during thawing. Drip loss is also dependent on thawing temperature and rate of thawing.

As a whole, drip loss followed a growing trend during storage, increasing significantly to almost twice, in the last moment, for all samples. According to Fellows (2000), temperature fluctuation has a cumulative effect on food quality. During thawing, in samples subjected to slow freezing or recrystallisation, cells do not regain their original shape and turgidity because the growing ice crystals deform and rupture adjacent cell walls, increasing the release of cell constituents (water-soluble nutrients) to form drip losses. Very high freezing rates may also cause stresses within some foods that result in splitting or cracking of the tissues. Furthermore, drip losses form substrates for enzyme activity and microbial growth.

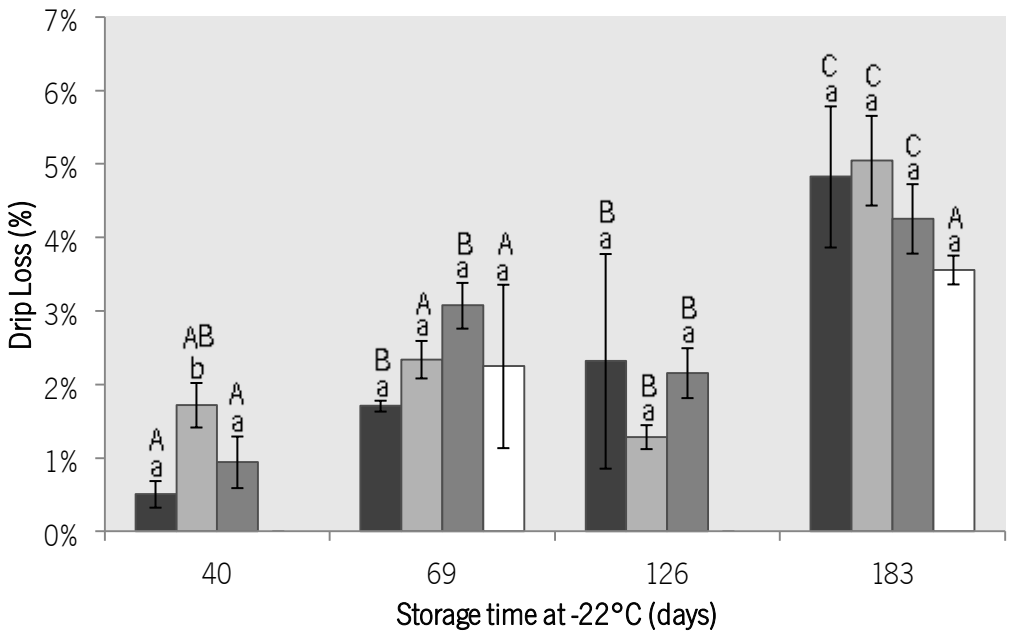


Figure 4.3. Drip Loss (%) of salmon samples from the control group (■ QS), glazed with water (■ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.4. TVC

The total viable counts (TVC) values for frozen salmon samples (-20 °C) during 6 months of storage at -22 °C are presented in Table 4.1. Based upon these data it is possible to notice that uncoated samples showed values constantly greater than glazed samples, except for the last moment when the value of the glaze sample ((3.65E+04±5.15E+04) CFU/g) is greater than the value of the control sample ((2.97E+03±2.65E+03) CFU/g). Both of these values are greatly affected by the standard deviations due to the reduced number of concordant repetitions. In turn, the samples coated with chitosan showed favorable values of TVC when compared with the uncoated samples or the glazed samples. While samples coated with 1.5% chitosan constantly show values below 10, the same did not happen with samples coated with 0.5% chitosan. In fact, the samples coated with 0.5% chitosan presented a TVC value of 1.50E+02, 2.10E+02, 2.60E+02 CFU/g, after 40, 118 and 181 days of storage, for one of the samples belonging to the same triplicate. Both coatings - water and chitosan - acted in the reduction and maintenance of the microbial composition of the frozen samples. However, the samples coated with chitosan showed the most promising results in microbial protection of frozen fishery products, especially the samples

coated with 1.5% chitosan. This ability of chitosan coatings to reduce, inhibit or prevent growth of microorganisms on food surfaces has been referenced by several authors, including Falguera *et al.* (2011) and Pereira *et al.* (2010).

Freezing also seems to have been effective, since all TVC values, including for uncoated samples, are below the acceptable threshold around 10E+07—10E+08 CFU/g, which lies at the point of sensory rejection (Ólafsdóttir *et al.*, 1997) and never exceeded the microbiological limit of 5E+05 CFU/g recommended by ICMSF (1986) for frozen fish of good quality.

Table 4.1. Total viable counts (TVC) values for frozen salmon samples (-20 °C) from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (☐ Q15) after 6 months of storage at -22 °C; standard deviation corresponds to three replications

TVC (-20 °C)	Storage Time (days)	Sample 1 (CFU/g)	Sample 2 (CFU/g)	Sample 3 (CFU/g)	Mean (CFU/g)	SD
QS	0	6.00E+03	5.80E+02	1.60E+03	2.73E+03	2.88E+03
	13	3.60E+02	1.50E+02	5.80E+02	3.63E+02	2.15E+02
	40	1.00E+03	1.70E+03	5.40E+02	1.08E+03	5.84E+02
	62	<10	<10	<10	<10	-
	118	5.10E+02	2.70E+02	6.40E+02	4.73E+02	1.88E+02
	181	1.10E+03	1.80E+03	6.00E+03	2.97E+03	2.65E+03
Q0	13	7.90E+02	7.20E+02	5.60E+02	6.90E+02	1.18E+02
	40	5.80E+02	1.20E+03	3.50E+02	7.10E+02	4.40E+02
	62	3.50E+02	3.30E+02	1.20E+02	2.67E+02	1.27E+02
	118	4.80E+02	2.10E+02	7.80E+02	4.90E+02	2.85E+02
	181	9.60E+04	7.00E+03	6.60E+03	3.65E+04	5.15E+04
Q5	13	<10	<10	<10	<10	-
	40	<10	1.50E+02	<10	-	-
	62	<10	<10	<10	<10	-
	118	2.10E+02	<10	<10	-	-
	181	2.60E+02	<10	<10	-	-
Q15	62	<10	<10	<10	<10	-
	181	<10	<10	<10	<10	-

The total viable counts (TVC) values for the unfrozen salmon samples (5.9 °C) after 24 h, during 6 months of storage at -22 °C are presented in Table 4.2. After analyzing these data, the same trend observed for frozen salmon could be confirmed. Again, all values are below the threshold of rejection and uncoated samples have higher TVC values than glazed and coated samples. The coated samples show TVC values lower than glazed samples. However, all coatings, especially chitosan coatings, are effective in protecting the thawed samples at refrigeration

temperatures. Coatings with 1.5% chitosan again showed consistent protection of the samples, this time thawed, maintaining the TVC below 10 in all samples, throughout the entire storage period. This way, 1.5% chitosan coatings demonstrated to be effective in protecting thawed samples at refrigerated temperatures, simulating the thawing conditions of fish at consumers' homes.

Table 4.2. Total viable counts (TVC) values for refrigerated salmon samples (5.9 °C) from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (☐ Q15) after 6 months of storage at -22 °C; standard deviation corresponds to three replications

TVC (5.9 °C)	Storage Time (days)	Sample 1 (CFU/g)	Sample 2 (CCFU/g)	Sample 3 (CFU/g)	Mean (CFU/g)	SD
QS	0	7.30E+03	2.00E+03	3.30E+03	4.20E+03	2.76E+03
	13	2.80E+03	2.20E+02	1.50E+03	1.51E+03	1.29E+03
	40	4.50E+03	9.50E+03	7.10E+03	7.03E+03	2.50E+03
	62	1.50E+02	1.40E+02	1.00E+02	1.30E+02	2.65E+01
	118	2.80E+05	3.00E+02	1.20E+03	9.38E+04	1.61E+05
	181	1.10E+05	3.70E+03	1.00E+04	4.12E+04	5.96E+04
Q0	13	1.10E+03	1.10E+03	1.40E+03	1.20E+03	1.73E+02
	40	1.60E+03	1.40E+03	8.70E+02	1.29E+03	3.77E+02
	62	7.10E+02	3.70E+02	1.40E+03	8.27E+02	5.25E+02
	118	1.20E+03	3.20E+03	3.70E+03	2.70E+03	1.32E+03
	181	8.60E+04	7.60E+03	8.50E+03	3.40E+04	4.50E+04
Q5	13	<10	1.00E+02	2.50E+02	-	-
	40	<10	2.40E+02	1.50E+02	-	-
	62	<10	<10	<10	<10	-
	118	3.20E+02	3.00E+02	<10	-	-
	181	1.40E+03	1.50E+02	8.40E+02	7.97E+02	6.26E+02
Q15	62	<10	<10	<10	<10	-
	181	<10	<10	<10	<10	-

4.5. TBA

Thiobarbituric acid (TBA) values for frozen salmon samples during storage are shown in Figure 4.4. The initial TBA value for the control uncoated sample was (0.2234±0.0305) mg MDA/kg sample. Although TBA values are very similar, for 39 days of storage, the samples have higher TBA values as (0.3179±0.0462), (0.2541±0.0219), (0.3948±0.0550) mg MDA/kg sample, for the uncoated, glazed and 0.5% chitosan coated samples, respectively. This may have been due

to TBA test limitations, such as lack of sensitivity and specificity of the spectrophotometric method, since many other substances may react with the TBA reagent (Shahidi & Zhong, 2005).

A study conducted by Sathivel *et al.* (2007), using the same method, began with similar TBA value for the uncoated sample with (0.25 ±0.08) mg MDA/kg fish. However, after 8 months of storage at -35 °C, the TBA value of the control sample increased to (7.4±1.4) mg MDA/kg fish, while the glazed sample presented a TBA value of (1.8±0.5) mg MDA/kg fish and the sample coated with 1% chitosan displays a TBA value of (1.3±0.6) mg MDA/kg fish. This study concluded that distilled water and 1% chitosan coatings were effective in reducing lipid oxidation in salmon fillets, when compared with uncoated samples. The same conclusion can not be drawn by analyzing the TBA values of the present work, since all samples appear to have a certain stability, except for 39 days of storage. In fact, there was no visible influence of different coatings in the control of lipid oxidation, which is confirmed by the absence of statistically significant differences. The freezing process seems to have been the major factor influencing lipid oxidation and the storage time may not have been long enough to show a clear difference.

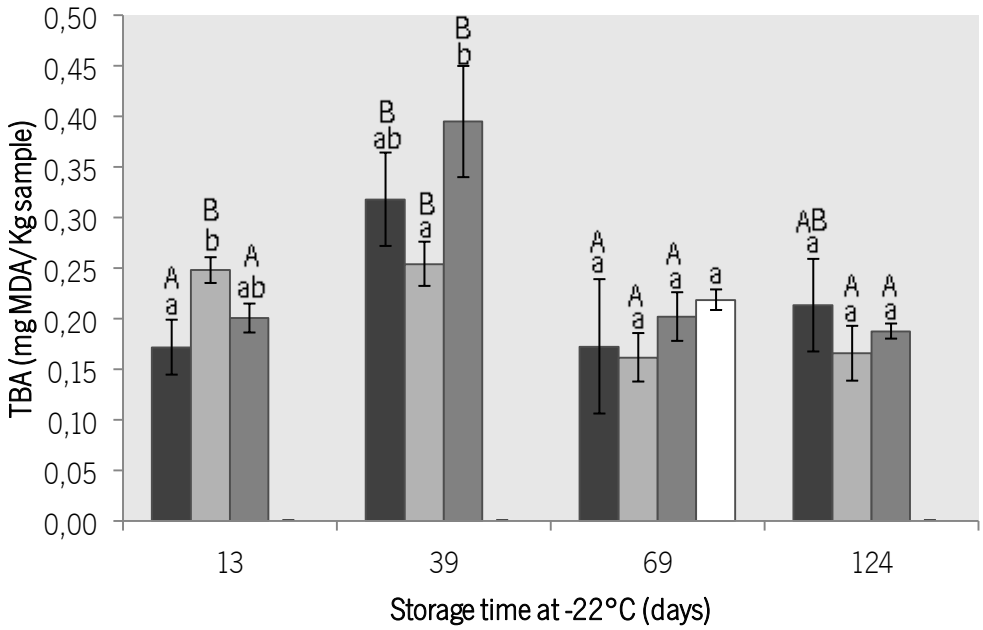


Figure 4.4. Thiobarbituric acid (TBA) values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 5 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.6. TVB-N

The TVB-N values for frozen salmon samples during storage are presented in Figure 4.5. The initial TVB-N value for the uncoated sample was (6.96±1.01) mg nitrogen/100 g sample. In the first moments, after 13 and 69 days, the TVB-N values suffered no great changes, decreasing slightly. The uncoated samples decreased from (5.82±0.87) to (4.81±0.01), the glazed samples from (4.43±0.08) to (4.30±0.01) and the samples coated with 0.5% chitosan fell from (5.63±0.63) to (5.14±0.82) nitrogen/100 g sample. However, all the samples increased at the last moment, after 188 days of storage for (6.02±1.09), (8.45±1.69) and (6.66±1.12) nitrogen/100 g sample, for the uncoated, glazed and 0.5% chitosan coated samples, respectively. The same is true for the samples coated with 1.5% chitosan, who increased from (4.29±0.02) to (7.27±1.37) nitrogen/100 g sample, after 69 and 188 days of storage, respectively.

In general, the TVB-N values are quite similar for all the coatings, contributing to the lack of statistically significant differences. Such lack of significant differences did not allow the detection of any contribution by the different coatings. These low values, far below the 35 mg nitrogen/100 g fish established as limit of acceptability of salmon by Directive 95/149/EC, indicate a good state of fish preservation. The increase of TVB-N values, in the last moment, might have been due to the activity of spoilage bacteria and endogenous enzymes, which are slowed down at low temperatures. Thus, if the experiments were maintained, it would be expected that degradation becomes clearer. It is also important to note that the storage temperature was a relevant factor, being able to inhibit changes in volatile compounds and consequently increasing the TVB-N, after 6 months of frozen storage.

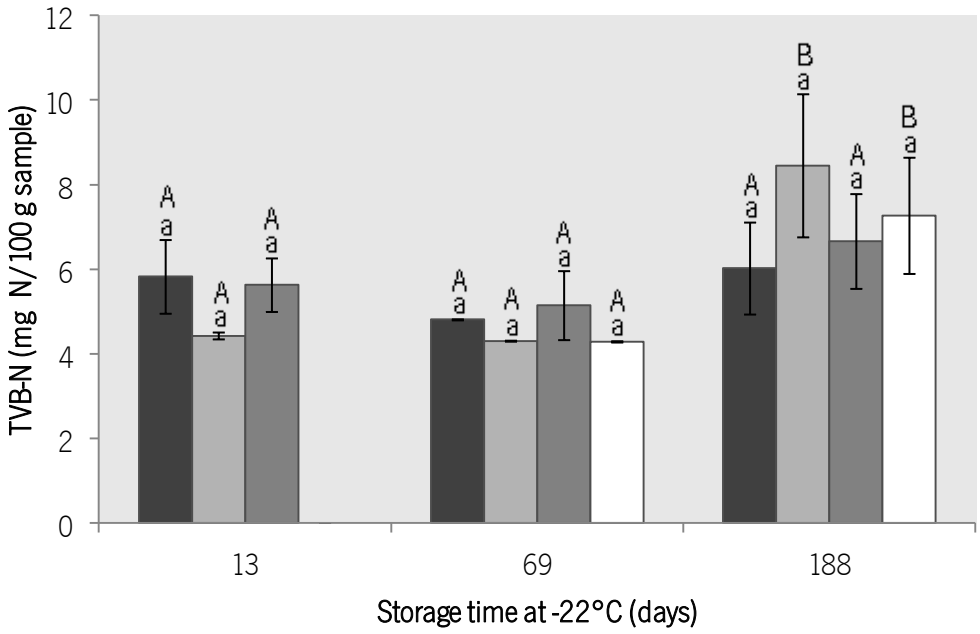


Figure 4.5. Total volatile basic nitrogen (TVB-N) values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with te same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.7. K-value

The K-values for frozen salmon samples, during 6 months of storage, are presented in Figure 4.6. From the data analysis it is not possible to draw any conclusions about the effect of coatings and glaze in ATP degradation of salmon samples, since the results are very similar and no statistically significant difference was detected. The initial K-value for the uncoated sample was (90.47±5.05)%. The K-values for the first moments, after 14 and 41 days of storage, were very high, with all above 80%. For the remaining time, after 69, 126 and 182 days of storage, the K-values are lower, never exceeding 67%. This can be explained by the occurrence of some error in the first moments that may have compromised these values, which appeared more reasonable over the latest times. Nevertheless, it was expected that the samples could exhibit high K-value results, since they came from a processed product with source in aquaculture.

According to Erikson *et al.* (1997), it seems reasonable to propose an upper K-value limit of 70 to 80% for good-quality Atlantic salmon (ice stored), and tentatively, lower than 40 to 50% for excellent quality. Disregarding the values of the two initial moments, it can be stated that the

remaining salmon samples indicate a fish of good quality, since they are below the maximum rejection limit of 80%.

Again the storage temperature emerges as an important factor in the stabilization of K-values. According to the study conducted by Soares *et al.* (2013), for 14 weeks of storage at -5 °C, the K-values showed an increasing trend, which did not happen in this experiment at -22 °C, where the K-values returned quite similar.

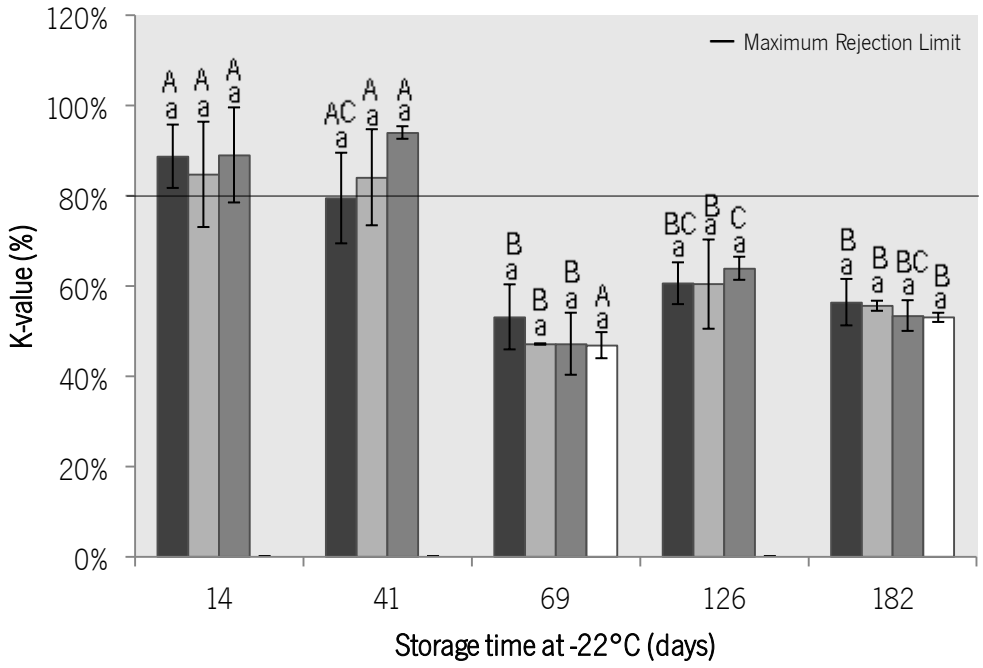


Figure 4.6. K-values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.8. pH-value

pH values during frozen storage are represented in Figure 4.7. The initial pH of the uncoated sample was found to be (6.43±0.05). After 14 days, the pH of all samples increased to (6.82±0.03), (6.85±0.09) and (6.85±0.05) for the uncoated, glazed and 0.5% chitosan coated samples, respectively. Throughout the remaining storage time, the pH values show a significant decreasing trend, which is supported by the results of the statistical analysis (capital letters). According to Rodriguez-Turienzo *et al.* (2011), freezing cause changes in pH values of fish muscle

towards higher acidity, probably due to the increase in concentration of substances in the water that remains unfrozen and modifies the acid–base equilibrium. The mean pH of all the samples were not higher than the limit of 6-6.5, recommended by Varlık *et al.* (1993) and Gülyavuz & Ünlüsayın (1999) cited in Kilincceker *et al.* (2009), with the exception for the first moment (13 days).

Similar results were reported in a study conducted by Sathivel *et al.* (2007) during 8 months at -35 °C. The uncoated samples showed a pH of (6.6±0.1), glazed samples a pH of (6.5±0.1) and samples coated with chitosan a pH of (6.4±0.1). In this case, the effect of pH on the coatings was unimportant, since did not vary significantly.

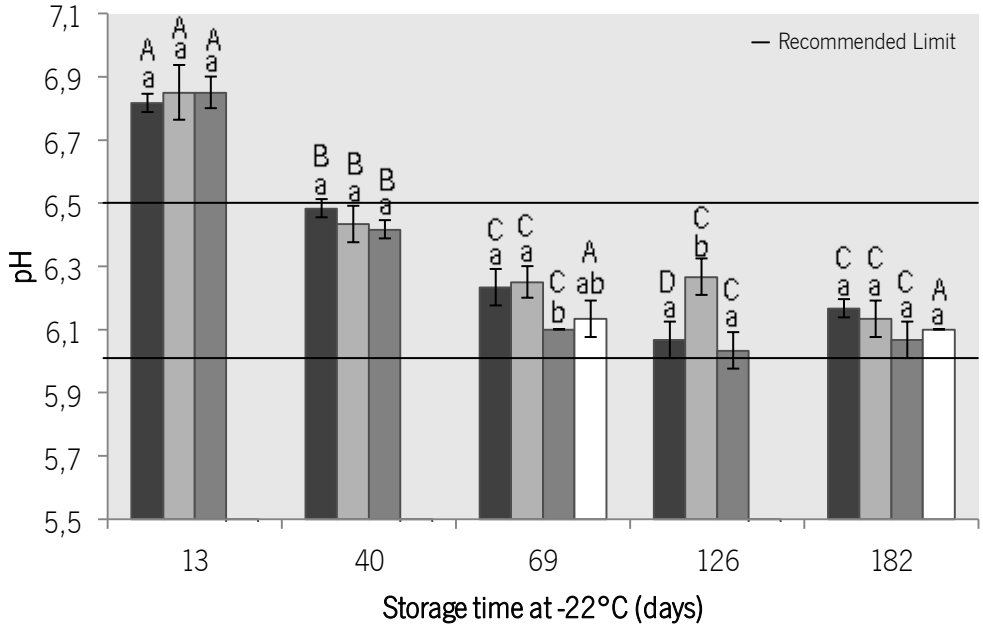


Figure 4.7. pH values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same color indicate a statistically significant difference (Tukey test, $p<0.05$).

4.9. Color parameters

As is commonly known salmon has natural color variations. For this reason, it was not possible to compare the color match between different coatings, since each sample group was obtained from different salmon fillets. These variations would greatly affect the color parameters during storage, which would invalidate any conclusions. Therefore, it was only evaluated the

variation of the color parameters for each group of samples - uncoated, glazed and coated samples - over time, separately.

The color parameters for the samples uncoated, glazed, coated with 0.5% chitosan and 1.5% chitosan are presented in Figure 4.8, Figure 4.9, Figure 4.10 and Figure 4.11, respectively. The temperature profile of the freezing chamber which contained the samples during storage is visible in Appendix A4 – Figure A.12.

The results for L*a*b* obtained during the experiment did not present significantly variations or any kind of tendency. Probably, a longer period of time may show more significant results.

Figure 4.12 shows the variation in perceived color differences of the salmon samples during storage (ΔE^*ab). By the graphical analysis is noticeable a large variability of the values, especially for the samples coated with 0.5% chitosan. This may have been due to a colorimeter reading error of the first sample coated with 0.5% chitosan, which serves as a standard for the remaining time. The samples coated with 1.5% chitosan were those that showed the most promising results. During the storage, these samples showed greater stability, presenting, for the majority of time, the lowest values of ΔE^*ab, even below the acceptable thresholds of 1.1 to 2.1 (Ahmad, 2006). For this reason, this coating may be the one who better protects fish color, since this was the coating who caused minor color differences when applied on frozen salmon samples, perhaps being imperceptible and better accept by consumers. According to Rodriguez-Turienzo *et al.* (2011), the type of plasticizer does not affect the color of frozen samples, since it is possible that the structure of the coating was different (perhaps more homogeneous) when it was applied on a frozen surface. In fact, when coating is applied after freezing, might better protect the carotenoids against oxidation. Moreover, carotenoids are bound to some myofibrillar proteins, thus the degree of protein denaturation also influences color changes (Ojagh *et al.*, 2011 cited in Rodriguez-Turienzo *et al.*, 2011).

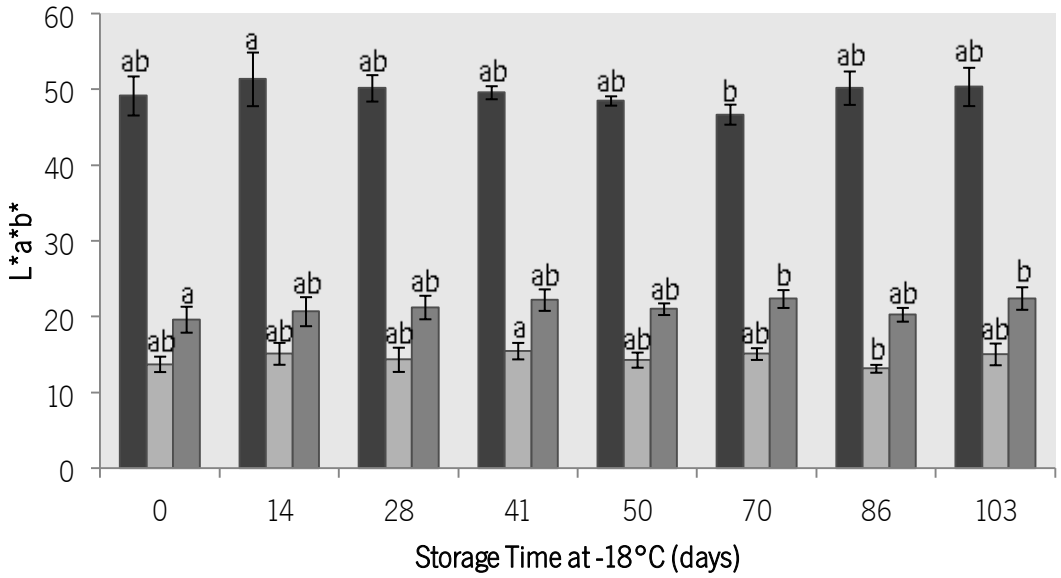


Figure 4.8. Color parameters for salmon samples from the control group for the coordinates L* (■ QSL*), a* (□ QSa*) and b* (■ QSb*) during 3.5 months of storage at -18 °C. Each bar represents the mean ± standard deviation of three replications. Different letters in the same color coordinate indicate a statistically significant difference (Tukey test, $p<0.05$).

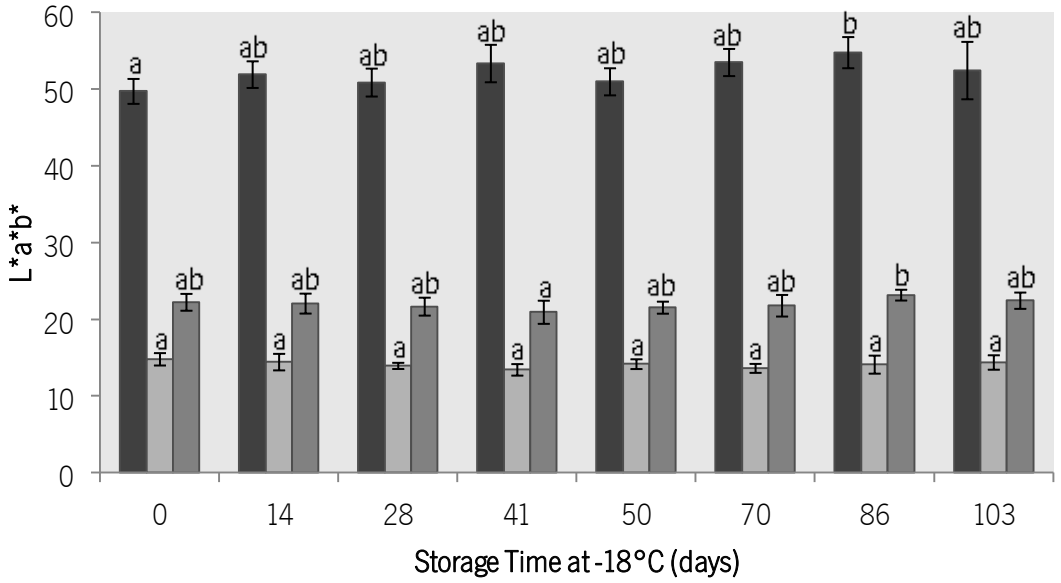


Figure 4.9. Color parameters for salmon samples from the group glazed with water for the coordinates L* (■ Q0L*), a* (□ Q0a*) and b* (■ Q0b*) during 3.5 months of storage at -18 °C. Each bar represents the mean ± standard deviation of three replications. Different letters in the same color coordinate indicate a statistically significant difference (Tukey test, $p<0.05$).

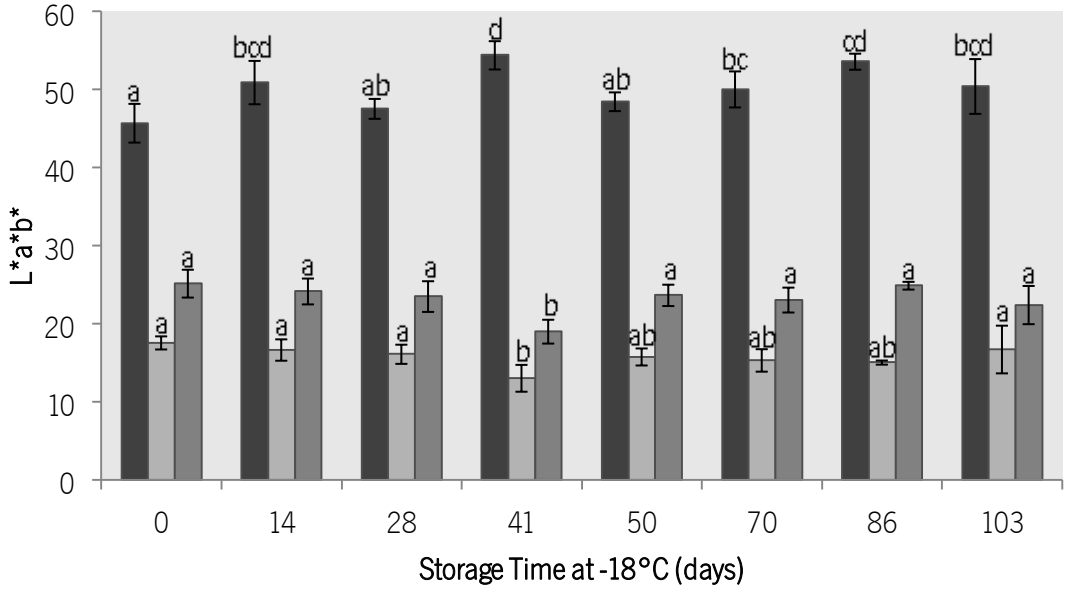


Figure 4.10. Color parameters for salmon samples from the group coated with 0.5% of chitosan for the coordinates L* (■ Q5L*), a* (□ Q5a*) and b* (■ Q5b*) during 3.5 months of storage at -18 °C. Each bar represents the mean ± standard deviation of three replications. Different letters in the same color coordinate indicate a statistically significant difference (Tukey test, p<0.05).

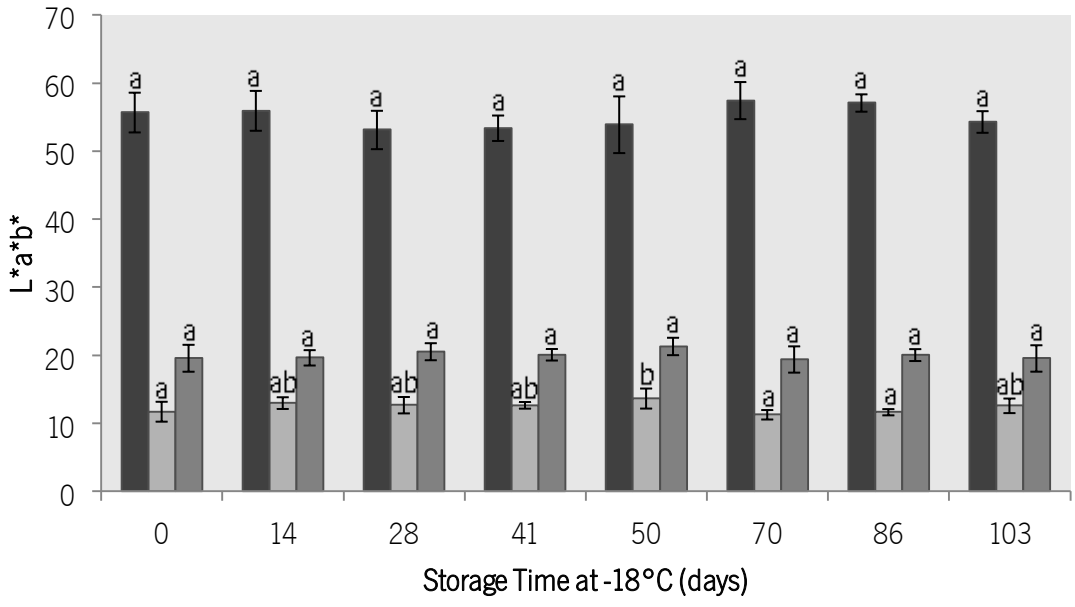


Figure 4.11. Color parameters for salmon samples from the group coated with 1.5% of chitosan for the coordinates L* (■ Q15L*), a* (□ Q15a*) and b* (■ Q15b*) during 3.5 months of storage at -18 °C. Each bar represents the mean ± standard deviation of three replications. Different letters in the same color coordinate indicate a statistically significant difference (Tukey test, p<0.05).

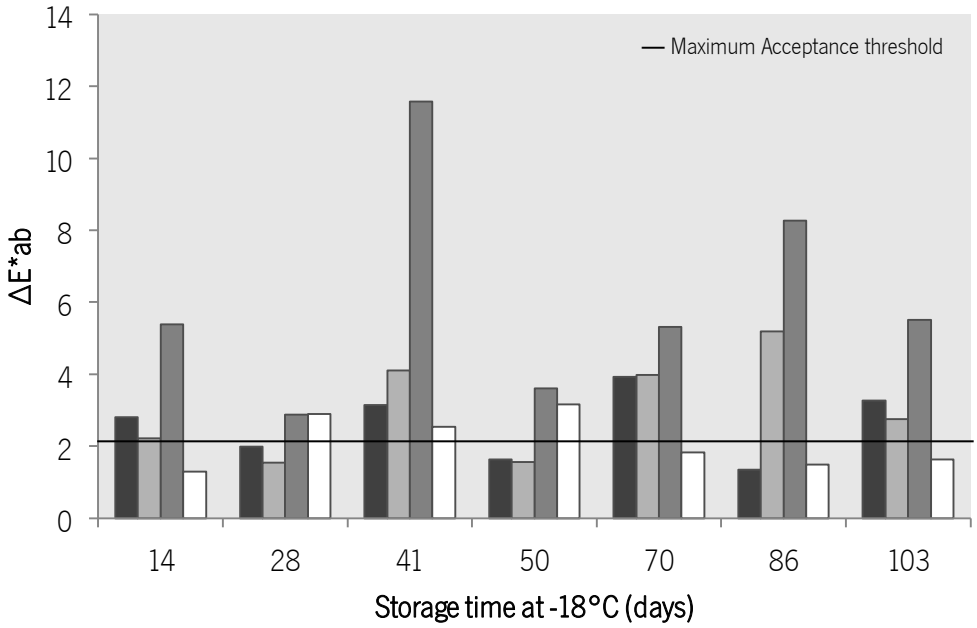


Figure 4.12. *ΔE*ab* values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and coated with 0.5% chitosan (■ Q5) and 1.5% chitosan (□ Q15) during 3,5 months of storage at -18 °C.

Chapter 5. Conclusions and future perspectives

Introducing **chitosan coatings** as an alternative to **water glazing**, usually carried out at industrial level represented a challenge. To prove their reliability a series of analysis were performed for evaluated the stability of the different parameters that affect **fish quality** during storage. Parameters such as coating loss, weight loss, drip loss, TVC, TBA, TVB-N, K-value, pH and L*a*b* coordinates give information about the **freshness** of the salmon samples, indicating the state of superficial dehydration, microbiological contamination, lipid oxidation, protein denaturation, and changes in odor and color.

Coating loss showed that water glazed samples loose more glazing when compared with the other samples. Both chitosan coatings present better results than the glaze with water. The apparent stability of the coating loss values for the different coatings indicates that an adequate freezing temperature can be effective in fish protection. However, the most promising results have been given by coating with 0.5% chitosan, which show the lowest values of coating loss during storage. Thus, this coating may be the one that best protects the samples against temperature fluctuations and other harmful factors during prolonged freezing.

Weight loss appears to have a growing trend during the storage; the values obtained were very low. This may help to conclude that one effective control of the storage prevents moisture loss by sublimation due to temperature fluctuations.

Regarding **drip loss**, the values show no significant influence of the different coatings on the test progress. As a whole, drip loss seems to increase at the last moment probably because of the accumulated damage caused by recrystallization and temperature fluctuations. However, given the slow progression of the increase in drip loss it is likely that the correct storage of the samples delayed their deterioration.

TVC results demonstrate that different coatings acted in the control of microbial flora. However, chitosan coatings, in particular the 1.5% chitosan coating, are those that return best results. Both chitosan coatings showed to be effective in protecting frozen and thawed samples (after 24 hours). The samples coated with 1.5% chitosan were even able to maintain the same level of protection in frozen and thawed samples. So, these coatings act as an additional barrier to overcome the contamination of salmon, thus improving the microbiological safety of salmon fillets

during storage. Also freezing appears to be useful in controlling microbial growth, since all samples, including the uncoated samples, remain below the acceptable limit of TVC during the entire storage.

The low **TBA** values allow concluding about product stability, where the different coatings do not appear to have a role; this may be due to the fact that freezing temperatures stabilize lipid oxidation. On the other hand, these low values may imply that a longer storage time would be needed in order to see some effects of coating usage.

TVB-N tests returned quite stable, indicating a fish in good condition. In fact, the low TVB-N values were far below the limit of acceptability established for salmon. Since the different coatings do not seem to affect this parameter, the stability suggests an efficiency of freezing in the maintenance of TVB-N values.

K-values show that the effect of glazing and coatings on the ATP degradation of the salmon samples is not relevant. Moreover, the statistic differences do not support any conclusion. However, in general, the samples indicate a fish of good quality, since the maximum rejection limit was not exceeded. Again the storage temperature emerges as an important factor in the stabilization of K-values.

pH-values show a decreasing trend. The effect of pH on the coatings was unimportant, since did not vary significantly. The mean pH of all the samples is not higher than the recommended limit.

Due to the **color** data stability no clear differences were found in the statistical analysis during storage. Regarding the ΔE^*ab values, the 1.5% chitosan coating was the one with best results maintaining, after 103 days, the color closer to the initial salmon and below values that can be considered acceptable for consumers.

One of the main **conclusions** of this work is the confirmation of the importance of a proper freezing and the relevance of storage control. Indeed, various parameters such as the coating loss, weight loss, drip loss, TVC, TBA, TVB-N, and K-value returned very stable due to the protection provided by a correct freezing temperature and a suitable control of its maintenance. Another important finding was the effective protection of the 1.5% chitosan coating in maintaining the color of salmon and in the control of microbial activity. In fact, the samples coated with 1.5% chitosan showed less perceptible color differences and provided a consistent protection for both - frozen and thawed samples - against microbiological contamination. Thus, this coating is a viable alternative to water glaze, not affecting the perception of quality of consumers.

In conclusion, edible coatings together with a correct freezing and storage control not only help in retarding the growth of microorganisms, but also help in the maintenance of chemical constituents, therefore reducing superficial dehydration, lipid oxidation, protein denaturation and changes in odor and color.

In the future, it is proposed to continue color analyses, for a longer period of time, as well as the use of sensory methods that complement the information given by the physical, chemical and micro(biological) parameters presented in this work. A study that enables to define more precisely the amount of coating applied in order to be able to realize the influence of coating thickness on samples' weight loss, can equally be very useful. It would also be appropriate to assess the migration of edible coating for frozen fish and the insertion of nanoencapsulated bioactive compounds in these same coatings.

An economic analysis to assess the viability of an effective industrially application of chitosan as a substitute for glazing would be relevant to a greater visibility of this study. Keeping this in mind, the costs should reveal the higher cost of chitosan solutions in relation to water, the need for a high concentration and its greater thickness which would require a large investment in adaptation of industrial equipment already existent. Also take into account, the need of lower uptakes and temperature maintenance and a smaller dive time, which may represent an energy and time savings.

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Appendixes

A1 – Standard Curve Determination for TBA method

Table A.1. Schematic representation of Standard Curve Determination for TBA method

Calibration	TBA (mL)	TCA (mL)*	TEP (mL)	MDA (µmol)	Abs**
Blank	5	5	-	-	-
P1	5	0	5	0,05	1
P2	5	1	4	0,04	0,8
P3	5	2	3	0,03	0,6
P4	5	3	2	0,02	0,4
P5	5	4	1	0,01	0,2

Source: adapted from Lemon (1975)

*Extraction solution (Working Standard Solution)
**Approximate Resulting Absorbance

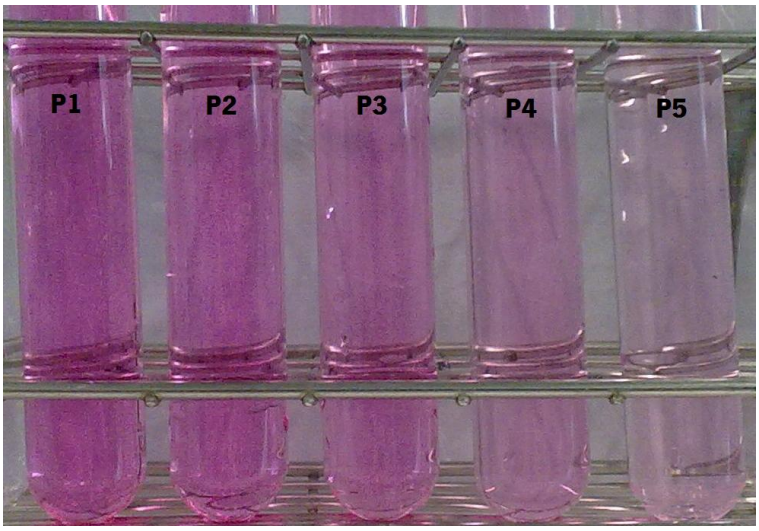


Figure A.1. Color grading obtained on the calibration curve.

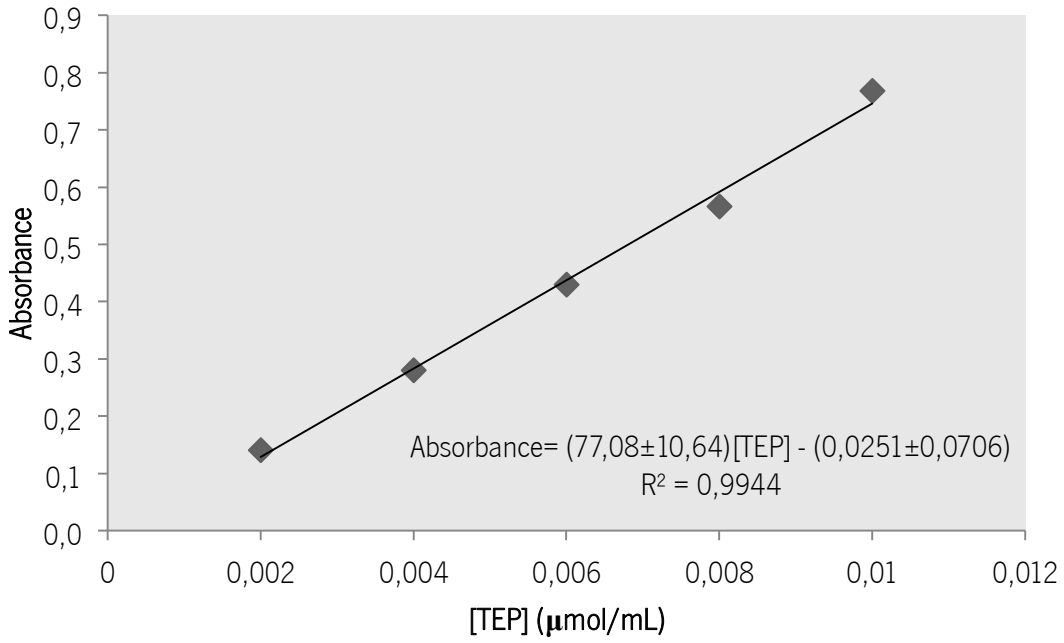
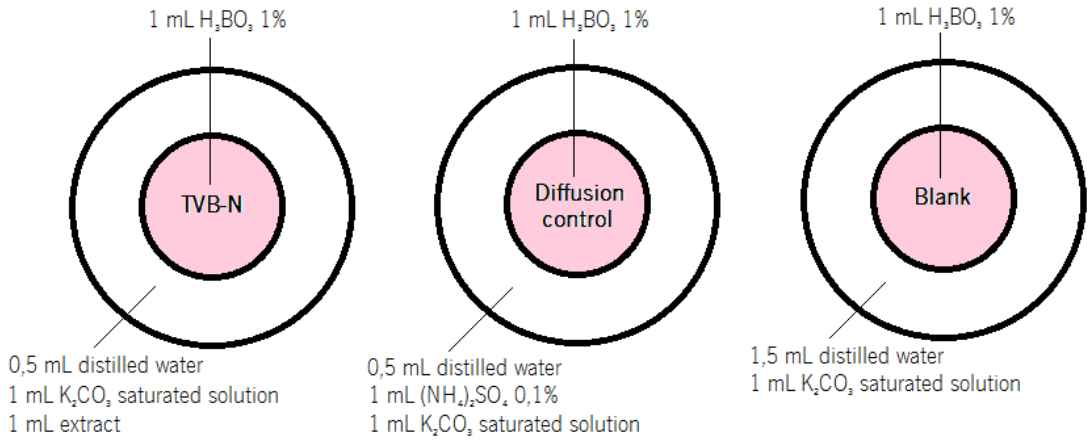


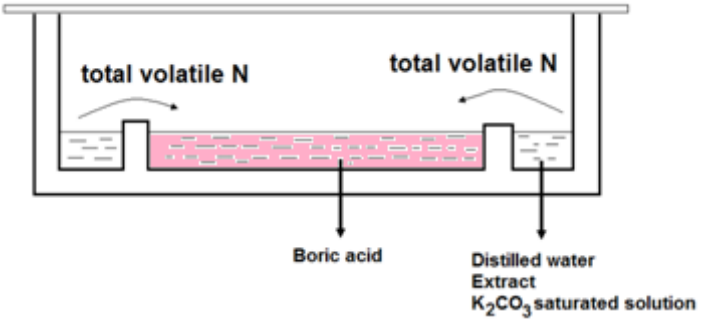
Figure A.2. Calibration curve for the TBA method.

A2 – Illustrations assistants to the implementation of standard NP2930:2009



Source: adapted from NP 2930:2009

Figure A.3. Scheme illustrates the preparation of the various tests on Conway cell.



Source: adapted from NP 2930:2009

Figure A.4. Representation of the alkalization by the action of potassium carbonate to release volatile bases and their reception in a boric acid solution followed by titration, in the interior of Conway cell.

A3 – Calibration curves for HPLC determinations

Table A.2. Scheme followed to obtain the calibration curves for K-value method

Standards	Concentration (µmol/mL)
"Mother solution"	2,00
1	1,00
2	0,75
3	0,60
4	0,30
5	0,15
6	0,05

The mother solution was made from the mixture of 6 compounds: IMP, ATP, ADP, AMP, Hx and HxR. The standards are obtained from dilutions of the mother solution.

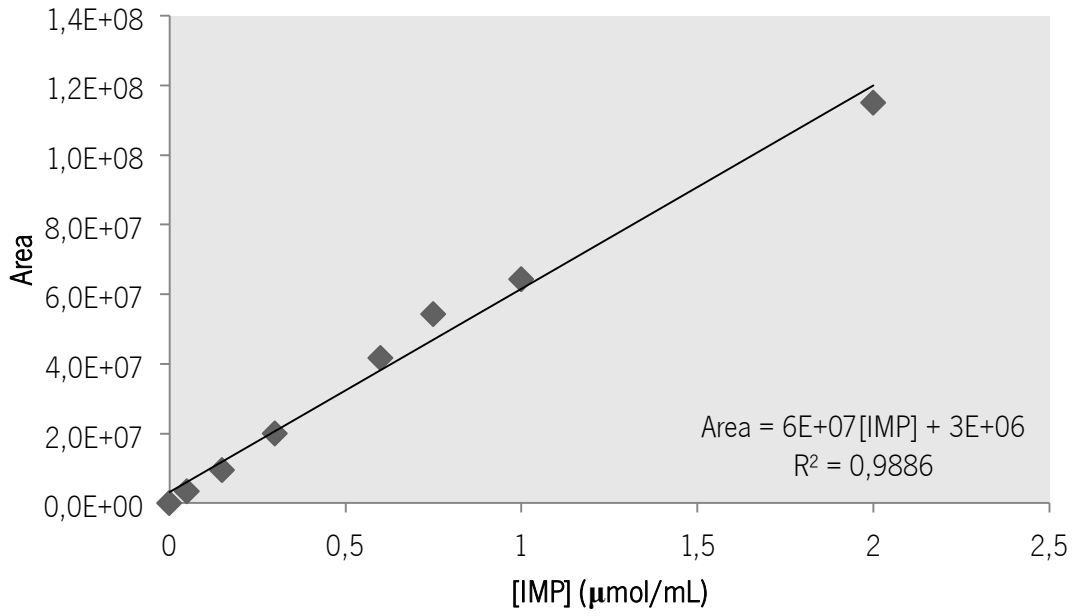


Figure A.5. Calibration curve of IMP.

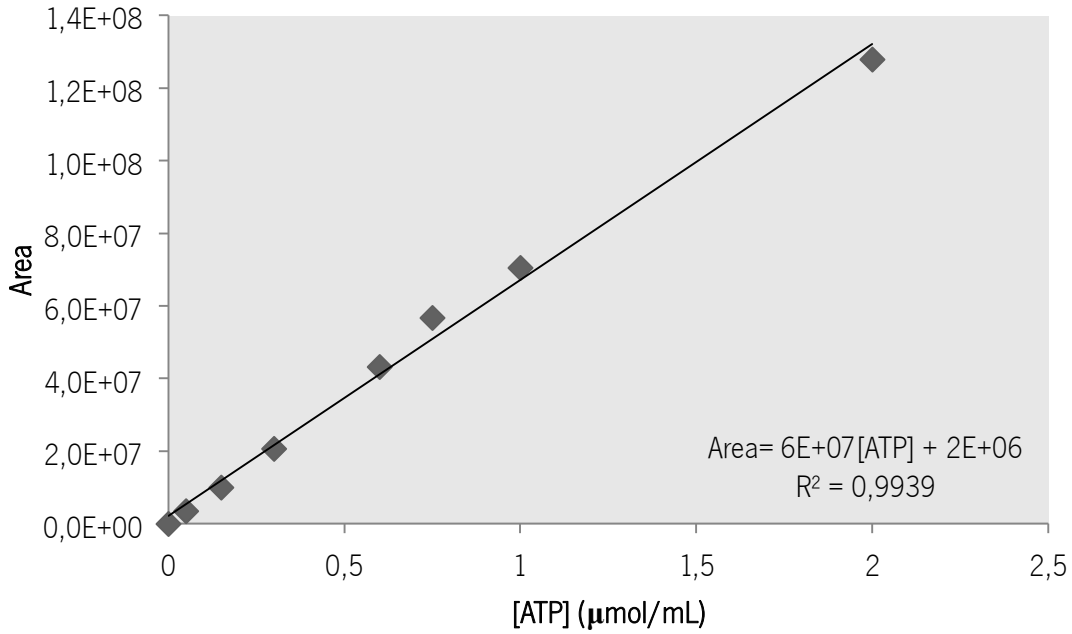


Figure A.6. Calibration curve of ATP.

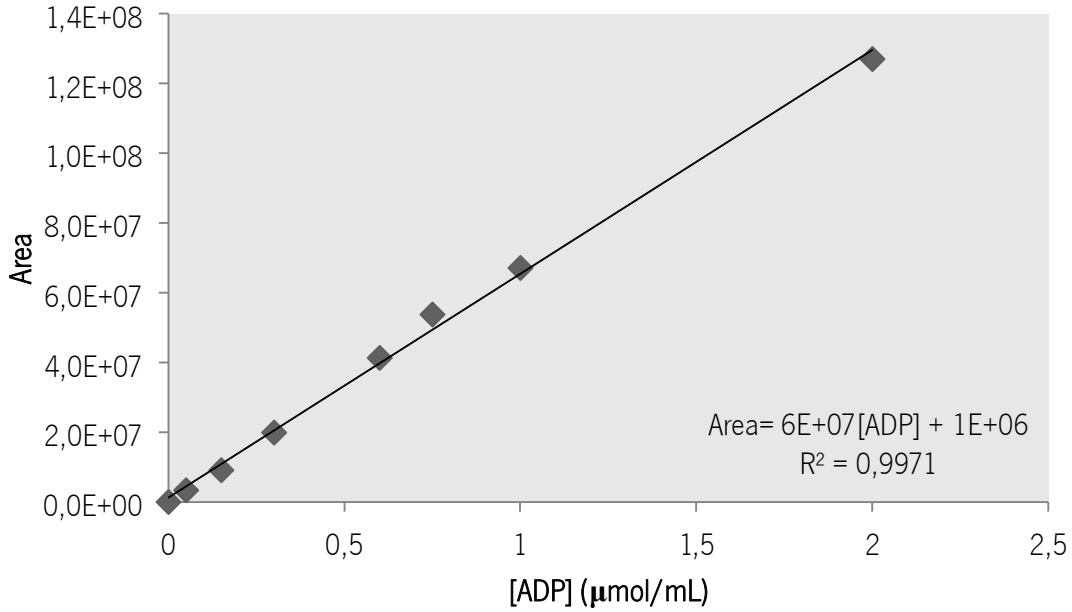


Figure A.7. Calibration curve of ADP.

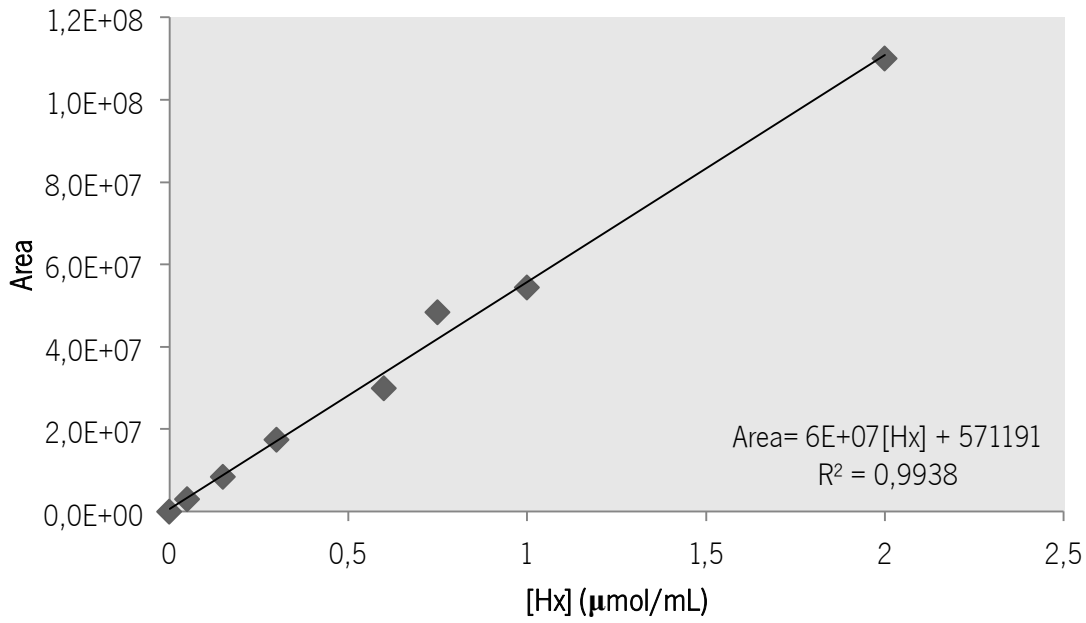


Figure A.8. Calibration curve of Hx.

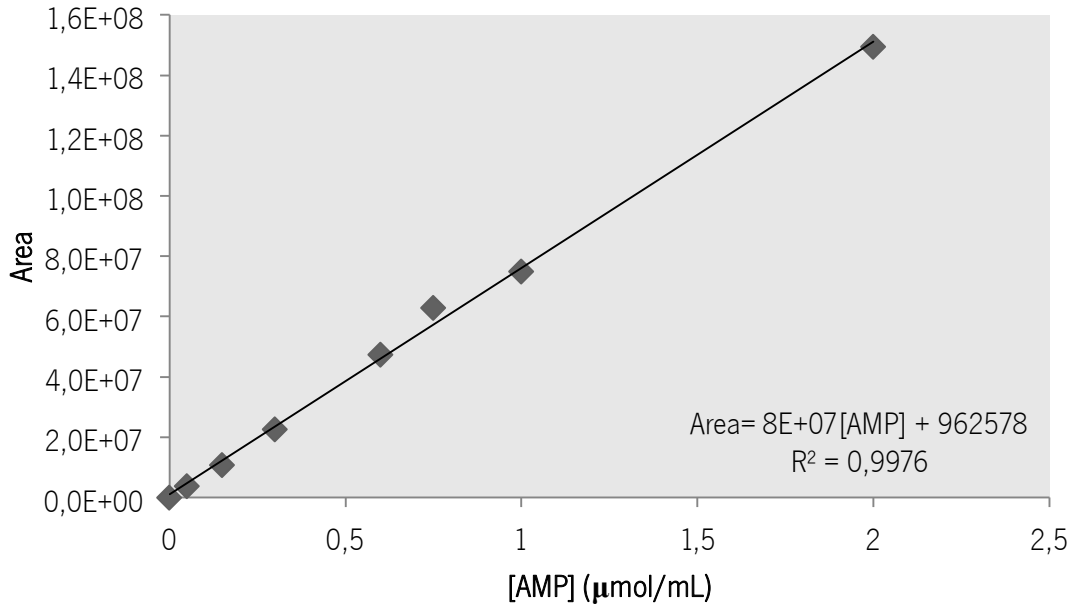


Figure A.9. Calibration curve of AMP.

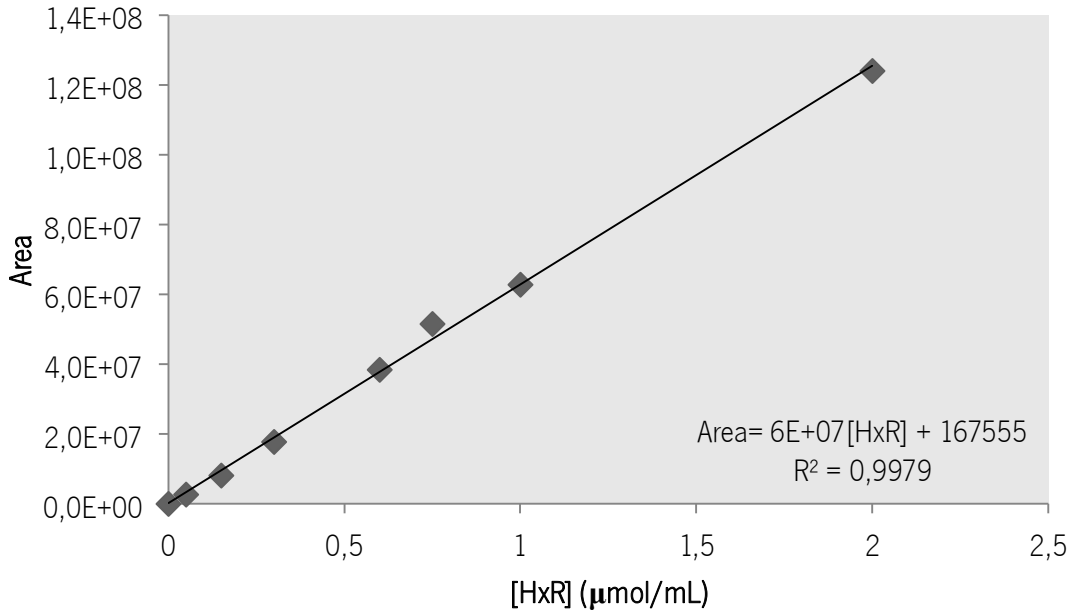


Figure A.10. Calibration curve of HxR.

A4 – Measurement of freezing chamber temperature during storage

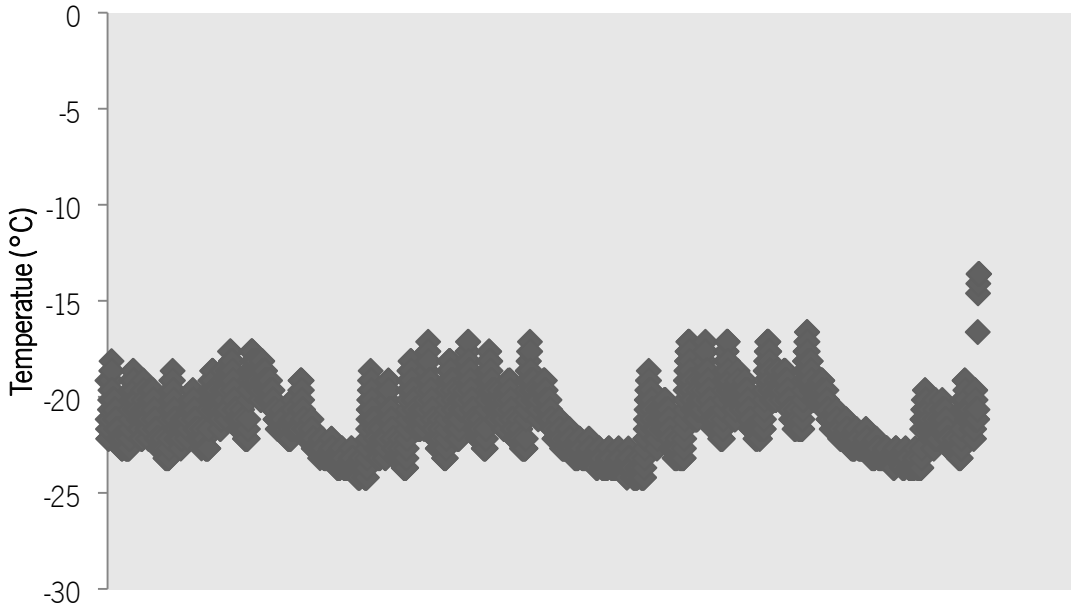


Figure A.11. Air temperature of industrial freezing chamber registered every 10 minutes by a data logger during frozen storage.

Figure A.12. Air temperature of freezing chamber registered every 10 minutes by a data logger during frozen storage.