Universidade do Minho
Escola de Engenharia

Nuno Miguel Ferreira Soares

Effect of chitosan solutions and water, applied directly on frozen fish as an edible coating, on shelf-life extension

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PhD Thesis in Chemical and Biological Engineering

Supervisor of the thesis
Professor Doutor António Augusto Martins de Oliveira Soares Vicente

June 2016

Statement of integrity

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 27 June 2016

Full name: Diana Miguel Ferreira Soares

Signature:

Agradecimentos

A lista de agradecimentos de quem desenvolveu o seu projeto de doutoramento enquanto mantinha uma atividade profissional na indústria só pode ser alargada. De facto, nada seria possível sem a ajuda de muitos e até de alguns que não terão consciência do facto. Optei por dividir os agradecimentos em dois grupos de pessoas distintas: as pessoas a quem devo tudo e as pessoas a quem devo o facto de o projecto ter sido possível.

No primeiro grupo estão desde logo os meus Pais, Irmão e as minhas Filipas aos quais tudo é pouco para descrever quanto lhes devo. Logo depois as minhas Tias ou, como o Rui diz, a minha mãe 2, 3 e 4. O Rui é também uma pessoa que só pode estar presente, com ele não partilho sangue mas tenho partilhado a vida, e não é menos família por isso. Para a Sofia agradeço por ser a prova viva de que as novas gerações têm sempre o potencial para suplantar as anteriores e que, com esforço e dedicação, serão capazes de chegar onde as atuais só sonham. Um agradecimento a toda a restante família, relembrando também aqueles que já não podem ler este texto, pois um pouco de todos está no que sou… e o nosso trabalho é sobretudo aquilo que somos!

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Effect of chitosan solutions and water, applied directly on frozen fish as an edible coating, on shelf-life extension.

Abstract

Even though this fact might not be fully realized by the business owners and consumers, the wide use of water glazing in frozen fish industry (as a layer of ice over the frozen fish products) works just like an edible coating that protects the product during storage. Glazing aims at acting as a physical barrier to cold storage deterioration especially when the product is subjected to inadequate cold storage. Nevertheless, in addition to having a high procedural cost due to the necessary equipment, glazing has long been shrouded in controversy over the amount of water applied to the product. In extreme cases, up to 40 % glaze can be observed for some seafood products, seeking clearly more than just guaranteeing product quality. A paradigm change would be the use of alternatives to simple water that could improve the protection during storage and add functionalities to the protective coating. On top of that, the industry should be able to define the optimum amount of protective coating necessary to fulfill its original purpose.

With this purpose the work was divided in five steps. Firstly it was necessary to identify the ability of a chitosan solution used directly on frozen salmon (in similar conditions as the ones used in the industry to glaze frozen fish) to achieve better results than simple water when, after thawing, samples were tested for freshness parameters (TVC, TVB-N, K-value, pH, TBA). At this first stage it was studied how different chitosan concentrations and coatings uptake could affect these parameters. Samples were stored at -5 ^{o}C with the purpose of accelerating any negative effect promoted by cold storage conditions.

Then, in a second step, the chitosan concentration that better performed on step 1 together with a three-times higher concentration (since none of the used in step 1 showed clear antimicrobial activity) were used and samples were stored during a six-months period at -22 ^{o}C. The parameters to assess the deterioration were basically the

same of the first step, but colour changes were also measured since this is, particularly in salmon, a very important factor in the quality perceived by consumers.

After identifying that chitosan coatings perform better in coating loss, colour change and demonstrate antimicrobial activity, in step 3 it was meant to study the effect that the coating application conditions have on its thickness. With this step it was possible not only to identify what could be the thickness of the coating corresponding to the samples that performed better in step 2 but also to predict which conditions to use when a defined thickness is desired. This step validated that the same thickness of water glazing can be obtained by chitosan coatings with energy savings (salmon and coating temperature can be higher). The heat transfer phenomenon that is in the origin of the coating formation was also studied and salmon temperature profiles during dipping time were presented and analyzed. The introduction of the concept of Safe Dipping Time (SDT) was proposed, challenging the industry to reflect on the possible effects of temperature rise in frozen fish during dipping.

Realizing that in the "real world" storage conditions are not always perfect and (especially during transport/retail) frozen products can face temperature fluctuations, the ability of chitosan coating to outperform water glazing under those conditions was evaluated (step 4). Product was stored during 70 days in a freezing chamber continuously fluctuating between -15 ^{o}C and -5 ^{o}C and freshness parameters (pH, Total Volatile Basic Nitrogen (TVB-N), Total Viable Count (TVC)), as well as coating loss and colour parameters were evaluated.

Finally, step 5 was meant to verify if the use of chitosan coatings would be noticeable by consumers due to alterations of the salmon's organoleptic characteristics, when stored at -18 ^{o}C during a period of six months. A trained panel was used to evaluate chitosan coatings and water glazing against control samples after two, four and six months of storage.

Overall, chitosan demonstrated to be an alternative to the use of simple water for the coating/glazing of frozen fish since: a) it presented a slower rate of coating loss, b) it reduced the microbial contamination of the product, c) it preserved the colour of the product and, d) at the moment of consumption, no differences were perceived by consumers between samples treated with water and chitosan solution.

Efeito de soluções de quitosano e água, aplicadas directamente em peixe congelado como revestimento edível, na extensão do tempo de conservação.

Resumo

Mesmo que esse facto possa não ser reconhecido pelos empresários e consumidores, a ampla utilização da vidragem na indústria de pescado congelado (como uma camada de gelo que envolve o pescado congelado) funciona como um revestimento edível que protege o produto durante o armazenamento. A vidragem visa atuar como uma barreira física para proteção da qualidade do pescado congelado durante o armazenamento a frio, especialmente quando o produto é submetido a temperaturas inadequadas. No entanto, além de ter um custo elevado, devido ao processo e equipamento necessário, o uso de vidragem tem sido envolvido em controvérsia devido à quantidade de água aplicada. Em casos extremos, é observado que esta pode representar 40% do peso do produto final, procurando vantagens para além de melhorias na qualidade do produto. Para mudar este paradigma seria necessário procurar alternativas para o revestimento de água que permitissem melhorar a proteção e adicionar funcionalidades ao revestimento. Para além disso, a indústria deveria ser capaz de definir a quantidade de revestimento necessária para cumprir o seu propósito original.

Para este efeito, o trabalho foi dividido em cinco etapas. Primeiro foi necessário identificar a capacidade de uma solução de quitosano, aplicada diretamente no salmão congelado (em condições similares às utilizadas na indústria), em alcançar melhores resultados do que a água, quando, após descongelação, são avaliados parâmetros de frescura (TVC, ABVT, valor-K, pH, TBA) e do desempenho do revestimento. Nesta primeira fase, foi estudado como diferentes concentrações de quitosano e quantidade de revestimento aplicado podem afetar os resultados. As amostras foram armazenadas a -5 ^{o}C para acelerar efeitos negativos que possam surgir pela conservação em frio.

Em seguida, numa segunda etapa, utilizou-se a concentração de quitosano que registou melhor desempenho na etapa 1 e outra 3 vezes mais elevada (uma vez que nenhuma das utilizadas na etapa 1 mostrou uma clara actividade antimicrobiana) durante 6 meses e em condições de temperatura de conservação semelhantes às utilizadas na indústria (-22 ^{o}C). Os parâmetros de avaliação da deterioração foram basicamente os mesmos da primeira etapa,

mas foi também avaliada a mudança de cor uma vez que, particularmente no salmão, é um factor muito importante para a qualidade percebida pelos consumidores.

Depois de identificar que os revestimentos de quitosano tiveram um melhor desempenho na perda de revestimento, na alteração de cor e que demonstram atividade antimicrobiana, na etapa 3 foi estudado o efeito que as condições de aplicação de revestimento têm na espessura de revestimento. Com este passo foi possível não só identificar qual poderia ser a espessura de revestimento correspondente às amostras com melhor desempenho na etapa 2, mas também, prever as condições a usar para obter uma determinada espessura. Este passo validou que a mesma espessura de vidragem poderá ser obtida por revestimentos de quitosano com poupança de energia (utilizando temperatura de salmão e de revestimento mais elevadas). O fenómeno de transferência de calor que está na origem da formação de revestimento foi também estudado e definidos os perfis de temperatura no interior do salmão durante o tempo de imersão. A introdução do conceito de Tempo Seguro de Imersão foi proposta com o objetivo de desafiar a indústria a refletir sobre o efeito do aumento de temperatura no peixe congelado enquanto este está imerso para aplicação da vidragem.

Reconhecendo que no mundo real nem sempre as condições de conservação dos produtos congelados são as ideais e que (em particular durante o transporte e distribuição) o produto sofre oscilações de temperaturas, foi avaliado se a vidragem com quitosano teria um melhor desempenho que a água simples nessas condições (etapa 4). O produto foi armazenado durante 70 dias numa arca congeladora que oscilava continuamente entre -15 ^{o}C e -5 ^{o}C procedendo-se à avaliação da perda de cor e vidragem bem como dos parâmetros TVC, ABVT e pH.

Finalmente, na etapa 5, verificou-se se o uso de revestimentos de quitosano seria percetível pelo consumidor devido a alterações nas características organolépticas do salmão, quando armazenado à temperatura de -18 ^{o}C durante um período de seis meses. Um painel treinado foi usado para avaliar revestimentos de quitosano e a de água simples quando comparados com amostras controlo, após dois, quatro e seis meses.

Em geral, o quitosano demonstrou ser uma alternativa ao uso de água no revestimento de peixe congelado, uma vez que: a) apresenta uma menor taxa de perda de revestimentos, b) reduz a contaminação microbiana do produto, c) preserva a cor do produto e d) no momento do consumo, não é percebido de forma diferente pelos consumidores.

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List of Symbols and Abbreviations

a_W - Water activity

ADP - Adenosine diphosphate

AHA - American Heart Association

AMP - Adenosine monophosphate

ANOVA - Analysis of variance

ATP - Adenosine triphosphate

CIE - Commission Internationale de l'Eclairage

CSIRO - Commonwealth Scientific and Industrial Research Organization

DD - Degree of deacetylation

DHA - Docosahexaenoic acid

DSC - Differential scanning calorimetry

EC- European Commission

EFSA - European Food Safety Authority

EPA - Eicosapentaenoic acid

EU - European union

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

GRAS - Generally regarded as safe

HPLC - High performance liquid chromatography

Hx - Hypoxanthine

HxR - Inosine

IPCV-ESTG - Instituto Politécnico de Viana de Castelo – Escola Superior de Tecnologia e Gestão

IMP - Inosine monophosphate

ISO - International Organization for Standardization

LC-PUFA - Long-chain polyunsaturated fatty acids

MIC - Minimum growth inhibitory concentration

MDA - Malondialdehyde

OECD - Organisation for Economic Co-operation and Development

PCA - Principal Component Analysis

QDA - Quantitative Descriptive Analysis

QIM - Quality index method

SACN/COT - Scientific Advisory Committee on Nutrition (Committee on Toxicity)

SDT - Safe dipping time

SSO - Specific Spoilage Organisms

TBARS - Thiobarbituric acid reactive substances

TBA - Thiobarbituric acid

TFRU - Tasmanian Food Research Unit

TPA - Texture profile analysis

TVB-N - Total volatile basic nitrogen

TVC - Total viable count

US - United States

USA - United States of America

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My “why” and thesis outline

During my first eight years in the seafood industry as plant and quality manager so many times the subject of conversations with managers, clients, regulatory authorities, colleagues, workers, and suppliers was about glazing, the amount of glazing and its use or abuse! In fact, the use of glazing is so much widespread through the industry that I am quite sure my experience is not unique.

It is difficult to clearly indicate when glazing started to be a technique used to protect frozen fish. In the first (1861) U.S. patent describing a process to artificially freezing and preserving fish, Enoch Piper claims the invention of a new and improved method of preserving fish that includes a 24 h freezing process and suggests glazing the fish, by a dip in cold water, forming a coat of about one-eighth of an inch in thickness. Later, in 1926, the Appendix VIII to the report of the U. S. Commissioner of Fisheries mentions that fish must be frozen in metal pans and then warmed slightly by spraying or immersing in cold water to loosen them. The report states that glazing tanks are commonly made from wooden or concrete, provided with a movable wooden platform suspended by ropes to a windlass, by which it was moved up and down the tank. Despite so many years have gone by, much of the focus of the seafood industry have been more on the equipment and efficiency of the glazing application (*i.e.* safer equipment and increase the amount of glaze uptake without reducing productivity) than in: a) developing a coating that is more than a sacrificing agent for water sublimation or to mechanical damage and b) defining the amount of glaze necessary to protect the product during the defined shelf-life.

This is why I was motivated for this work. I believe that the seafood industry paradigm of using just water to glaze frozen seafood and in a discretionary amount must be changed. The use of excessive glazing, that in truth no one really knows what it means, since there are no limits imposed and studies to support such limits, is a long controversy that is been fought more by imposing labeling rules then by scientific studies. This thesis, the outline of which is presented just below, aims to start changing this paradigm, but is humbly presented as only a small (first) step in a long journey.

The thesis is organized in four sections: Section I - Literature review, Section II - Experimental work, Section III - Conclusions and future work, and section IV – Appendix.

Section I is constituted by chapters 1 to 4 and presents an overview on the importance of seafood and its industry and the most relevant subjects to the development of the experimental work. Section II has 5 chapters (chapters 5 to 9) throughout which the outcomes of work done during the PhD are presented according to the articles that were produced.

Chapter 1 identifies the importance of seafood and its industry in the modern society. It unveils briefly the chemical composition due to its importance in seafood degradation and benefits. The use of the traditional glazing or edible coatings, as alternative, is presented in Chapter 2. Chapter 3 approaches seafood common preservation processes and methods for freshness evaluation. In Chapter 4 the relevant transfer phenomenon associates with glazing and glazed frozen fish are presented.

Section II is initiated with Chapter 5, where chitosan coating with different concentrations were tested in comparison with the control and traditional water glazing. In Chapter 6 two chitosan coating concentration were tested in similar conditions to industrial preservation chambers for six-months. Chapter 7 presents a work where the conditions of the application of the glazing and the thickness of the glazing obtained are studied; a theoretical approach is presented to the energy transfer that occurs at the moment of glazing application. Chapter 8 assesses how chitosan glazing compares with water glazing when the product is subject to severe temperature fluctuations during storage. Finally, Chapter 9 focuses on verifying if the use of chitosan glazing instead of water glazing could affect the consumer organoleptic perception.

Section III is dedicated to the main conclusions of the work, future work and how we hope it can impact the industry.

Section IV is the Appendix.

Outputs from this thesis

This thesis is based on the following publications:

Articles in journals with peer review:

Soares, N. M., Mendes, T. S., & Vicente, A. A. (2013). Effect of chitosan-based solutions applied as edible coatings and water glazing on frozen salmon preservation - A pilot-scale study. *Journal of Food Engineering*, *119*, 316–323. http://doi.org/10.1016/j.jfoodeng.2013.05.018

Soares, N. M. F., Oliveira, M. S. G., & Vicente, A. A. (2015). Effects of glazing and chitosan-based coating application on frozen salmon preservation during six-month storage in industrial freezing chambers. *LWT - Food Science and Technology*, *61*, 524–531. http://doi.org/10.1016/j.lwt.2014.12.009

Soares, N. M., Fernandes, T. A., & Vicente, A. A. (2016). Effect of variables on the thickness of an edible coating applied on frozen fish - Establishment of the concept of safe dipping time. *Journal of Food Engineering*, *171*, 111–118. http://doi.org/10.1016/j.jfoodeng.2015.10.016

Articles in journals without peer review:

Soares, N. F. (2016). Updating Use of Glazing in the Frozen Seafood Industry. *Food Quality & Safety*, 33–36. Retrieved from http://www.foodqualityandsafety.com/article/updating-use-of-glazing-in-the-frozen-seafood-industry/

Poster:

Soares, N. M., Oliveira, M. S., Fernandes, T. A., Vicente, A. A (2014). Chitosan glazing thickness effect on Colour and TVC in frozen salmon – introducing the “safe dipping time” concept. II Simposio Científico Internacional para la Innovación en la Industria Marina y Alimentaria. ANFACO-CECOPESCA. Vigo-Espanha

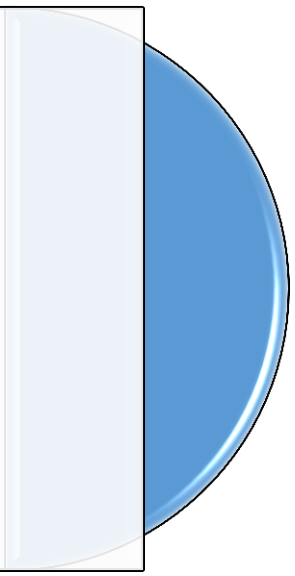
Articles submitted:

Soares, N., Silva, P., Barbosa, C., Pinheiro, R. Vicente, A. A. (2016). Comparing the effects of glazing and chitosan-based coating applied on frozen salmon on its organoleptic and physicochemical characteristics over six-months storage. Submitted to the Journal of Food Engineering (16/03/2016)

Silva, P., Soares, N., Vicente, A. A. (2016). Comparing the physicochemical and microbiological effects of glazing and chitosan- based coatings applied on frozen salmon over a 70-day storage period under thermal stress. Submitted to the Journal of Food Engineering (27/04/2016).

SECTION I

Literature Review



Chapter 1. Characterization of seafood and seafood industry

This chapter presents the seafood main constituents and a balance of the product major benefits and risks. The industry development, production and future are also introduced.

The chapter is based on Chapter 1 of the book:

Soares, N. F., Vicente, A. A., Martins, C. M. A. (2016). *Food safety in the seafood industry - A Practical guide for ISO 22000 and FSSC 22000 implementation* (1st ed.). Wiley-Blackwell.

1.1. Seafood

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 (FAO, 2012).

Seafood is unanimously considered as a great source of important nutritional components for a healthy diet (Rodriguez-Turienzo *et al.*, 2011). As stated by James (2013), the benefits of seafood consumption far outweigh its possible risks. Fish consumption provides energy, protein and important nutrients like long-chain ω-3 polyunsaturated fatty acids, which have positive impacts in the health of consumers.

Fish deterioration is most commonly a result of enzymatic autolysis, microbial growth, and oxidation and can be delayed using methods like freezing, salting, smoking, fermentation, drying and canning. About 30 % of landed fish is lost because of microbial activity. The increase of world's population and the need to store and transport fish are factors that enhance the importance of this issue and make fish preservation imperative in order to maintain its nutritional properties, flavour, colour, texture and extend its shelf-life (Ghaly *et al.*, 2010).

1.1.1 Chemical composition

The chemical composition of seafood is decisive to understand and predict seafood deterioration. The chemical constituents of fish flesh can be divided into two groups, the major and minor components (Murray & Burt, 2001):

- Major: water, protein and fat
- Minor: carbohydrates, minerals and vitamins

The specific amount of each component is dependent on extrinsic factors of the fish (*e.g.* the environment/season) and intrinsic factors (*e.g.* species, age, sex or spawning/migration period) (Huss, 1988, 1995).

Proteins

It is estimated in 15 % to 20 % the amount of protein present in fish muscle. Despite that, values as high as 28 % can be found in some species of fish (Murray & Burt, 2001) although there is, according to EFSA (2005), a tendency for protein content decreases with age-related increases in the lipid content. Proteins, which are constituted by chains of amino acids, are essential for a balanced human diet and good health. Fish is an important source of easily digestible protein and with high biological value (Bohl, 1999).

Water

As in all living organisms, water is the main constituent of fish, reaching typically 80 % of a lean fish fillet weight and about 70 % of a fatty fish flesh weight (Murray & Burt, 2001). The water content is reduced as fat percentage increases as several authors have reported (EFSA, 2005; Feeley *et al.,* 1972; Huss, 1988; Love, 1980, 1988; Osman *et al.,* 2001; Pirestani *et al.*, 2009).

The high level of water content, especially the one that is free and not bounded to any component, such as proteins or carbohydrates, is the main factor for seafood being perishable. In fact, this available water is a condition for the growth of microorganisms (Dauthy, 1995). It is common to define the available water in terms of water activity (a_w) that can oscillate between 0 and 1 (Aberoumand, 2010; Jay *et al.* , 2005; Neumeyer *et al.* 1997).

Table 1-1 shows the minimum values of a_w necessary for different microorganisms to grow. Fish is particularly vulnerable to degradation by microorganisms since its a_w is reported to be close to one (Martin *et al.,* 2000).

Lipids

The amount of lipids (also commonly referred to as *fat*) can be very different depending on the species. The designation of lean, intermediate and fatty fish is not consensual among the industry and the limits of each category often overlap. Fish with

a fat content of 5-20 % are commonly considered as fatty fish in opposition to the 1-2 % content of lean fish. In the range of 1-8 % the fish in designated as intermediate (EFSA, 2005). The lipid content is also characterized to be dependent on season, feeding and even on the location of the fish tissue (Murray & Burt, 2001).

Table 1-1 - Approximate minimum a_w values for growth of microorganisms important in foods.

Microorganism	Minimum a_w (FDA, 2011)	Minimum a_w (Jay *et al.*, 2005)
Bacillus cereus	0.92	----
Clostridium botulinum (type A)	0.94	0.94
C. botulinum (type E)	0.97	0.97
C. perfringens	0.93	----
Escherichia coli	0.95	0.96
Listeria monocytogenes	0.92	----
Salmonella spp.	0.94	----
Shigella spp.	0.96	----
Staphylococcus aureus	0.83	0.86
(toxin production)	0.85	----
Vibrio cholera	0.97	----
V. parahaemolyticus	0.94	0.94
V. vulnificus	0.96	----

One of the major characteristics of fish is that its lipids are composed of long-chain polyunsaturated fatty acids (LC-PUFA) containing many fatty acids with five or six double bonds (H. H. Huss, 1988; Stansby & Hall, 1967). Two particularly LC-PUFA are of great importance in human nutrition since they cannot be synthesized by the organism: eicosapentaenoic and docosahexaenoic acids. The LC-PUFA (also referred to ω-3 due to the first double bound being located after the third carbon atom from the methyl end of the chain) are associated with important functions like brain development in children (particularly in the last trimester of pregnancy) and disease

prevention, such as sudden cardiac death, coronary heart disease, atherosclerosis, and lowering blood pressure or anti-thrombotic actions (FAO, 2013). It is also important to clarify that despite the list of benefits being long, the consumer must be aware that the concentration of constituents differs among distinct products and the benefits obtained may not be linearly dependent on the amount consumed (Mozaffarian & Rimm, 2006).

Carbohydrates

The amount of carbohydrates in fish is small. In fish muscle it is common to be less that 1 %. Values from 2 % to 5 % can be found in the dark muscle of fatty fish or in some molluscs (Aitken *et al.,* 2001; Murray & Burt, 2001). One of the most relevant differences in the chemical composition between crustacean shellfish and molluscan shellfish is the higher content of carbohydrate in the second (Jay *et al.*, 2005).

Minerals and Vitamins

Regarding the mineral content of fish, iodine and selenium (very important in the development of brain of children) are of special relevance since they are almost exclusively found in foods from the aquatic environment (Toppe, 2012). The ability of selenium to bind with mercury and this way reducing its toxicity is also regarded as another major benefit of fish consumption (Ralston *et al.*, 2008).

Although the content of fish in vitamins varies significantly between different fish species and even between some parts of the same fish, in general it is a greater source of fat-soluble vitamins A, D and E than mammals muscle (Huss, 1988). From all the vitamins present, vitamin D is mostly significant since it is uncommon in other foods and presents many benefits for human health (*e.g.* bone health and reduction of type 2 diabetes). The storage and production conditions are very important for the final vitamins content at the moment of consumption since vitamins are sensitive to factors like light, heat, and temperature and storage time.

1.1.2 Benefits and risks

None of the benefits presented in section 1.1.1 comes without some risks associated with the consumption of fish. Recently, several works were published that assess the balance between risks and benefits associated with different amounts of seafood consumption (Cohen et al., 2005; Dovydaitis, 2008; FAO & WHO, 2011; James, 2013; Mozaffarian & Rimm, 2006; Sidhu, 2003; Torpy, 2006). In parallel and in accordance with these researches several national food safety agencies have established recommendations for the maximum number of fish servings per week (Table 1-2).

Most studies assess the positive contribution to human health from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and the risks of methyl mercury and/or dioxins. Overall, the benefits overcome the risks although it is necessary to consider the amount of servings, some particular categories of end consumers and also some types of fish, particularly the ones where mercury can be found in higher concentrations.

1.2. Development of Fish Industry

It is clear from recent reports from FAO (FAO, 2012, 2014; OECD & Food and Agriculture Organization of the United Nations, 2014) that seafood industry has evolved significantly over the last decades. From the factors that contributed for this tendency we can distinguish between extrinsic to the industry, such as population growth and a greater concern of consumers with a healthy diet, and intrinsic to the industry, like expansion of production and processing of fish, development of new packaging solutions and increasing efficiency of distribution channels.

In order to reach an increasing number of consumers, widely distributed throughout the globe, increasing importance has been given to new packaging solutions that preserve this perishable product from deterioration. Nevertheless, some methods of processing and preserving fish are not still available worldwide, and some developing countries are still limited by the absence of hygienic landing centres, electricity,

drinking water, roads, ice, cold rooms and appropriate refrigerated transport. Despite that, frozen fish production for human consumption has increased from 13 % to 24 % share between 1992 and 2012 (FAO, 2014).

Table 1-2 - Number of fish servings per week recommended by different food safety agencies.

Food Safety Agency	Country	Servings/week
SACN/COT	UK	General population: 4 Women in reproductive age, pregnant women and children under 16: 2 (1 serving/1 oily fish) (SACN, 2004)
EFSA	EU	General population: 1-2 up to 3-4 during pregnancy Pregnant women: avoid some species with high mercury levels (EFSA, 2014)
FDA/US EPA and AHA	USA	2-3 (FDA & EPA, 2014)
Health Canada	Canada	2 (Health Canada 2007)
FSANZ AND AHF	Australia and New Zealand	2-3 of most types of fish Exception of some types of fish with high levels of mercury especially for pregnant women (FSANZ, 2011)
MHLW	Japan	Limit consumption of some species, especially for pregnant women and children (MHLW, 2005)
WHO/FAO	-	1-2 (WHO & UNEP, 2008)

1.3. Fish production

According to the publication State of the World Fisheries and Aquaculture (SOFIA), from the Food and Agriculture Organization of the United Nations (FAO), the total amount of world fisheries has been steadily increasing over the past few decades, as shown in Figure 1-1. (FAO, 2014).

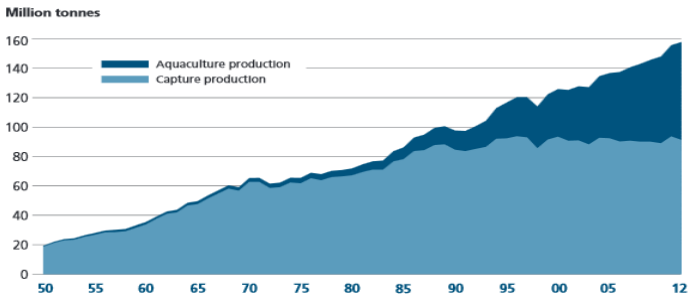


Figure 1-1 - World capture and aquaculture production (adapted from FAO, 2014).

When the world capture and aquaculture production is analyzed in retrospective (Figure 1-1) two main features are recognizable:

1. The increase in the total seafood production
2. Since the 90 ‘s the increase is almost only due to aquaculture production

In fact, in the last five decades the world fish production increased at an average annual rate of 3.2 %, outpacing population growth worldwide at 1.6 %. Between 2000 and 2012 aquaculture more than doubles its production from value of 32.4 million tons in 2000 to 66.6 million tons in 2012, with an average annual rate of 6.2 %.

The increase in aquaculture production, which in fact could already suppress the capture of fish destined to human consumption (estimated for 2015 but no official data available at the moment), brings a new reality to the fisheries sector. The more controlled process of aquaculture production and increasing focus of organizations in products with more aggregated value to the consumer may create opportunities for edible coatings to provide solutions that contribute to the increase of products safety and differentiation.

1.4. Unveiling the future

It is expected that, after the global financial crises, the long-term slow (but steady) economic recovery will continue to show a positive trend for fish trade in the future. Despite the instability in the last couple of years' stocks, preliminary estimates for 2013 indicate a further increase of fish trade and fishery products (FAO, 2014).

Aquaculture is at a historical turning point since is estimated to surpass capture as the main source of fish for human consumption in 2015 (OECD & FAO, 2013). The estimated reduction in its growth from the 5.6 % *p.a.* in the last decade to 2.5 % *p.a.* until 2023 (OECD & Food and Agriculture Organization of the United Nations, 2014) reflect the challenges this activity will face in the near future (Figure 1-2).

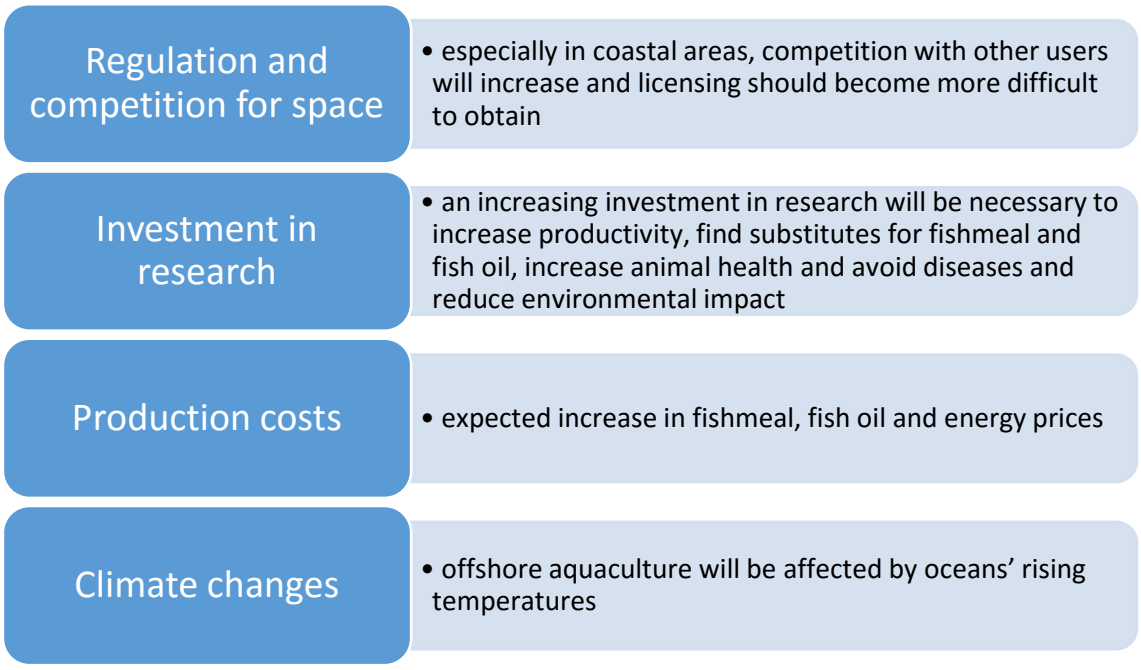


Figure 1-2 - Restrictions to the aquaculture growth.

There are two main factors that can reduce demand for fish products. First, although population rate is growing (1.2 % in 2010–2015) that growth will tend to become slower and is estimated to be 1.0 % during 2020–2025 (United Nations, Department of

Economic and Social Affairs, 2013). The second major factor will be the pressure on price (Figure 1-3) since not only world population is growing but also *per capita* consumption is estimated to increase (from the 19.2 kg in 2011–2013 to 20.9 kg in 2023). Despite the greater awareness of the general population towards the importance and great benefits of fish protein on human diet, the higher price of fish will lead to its substitution by other cheaper sources.

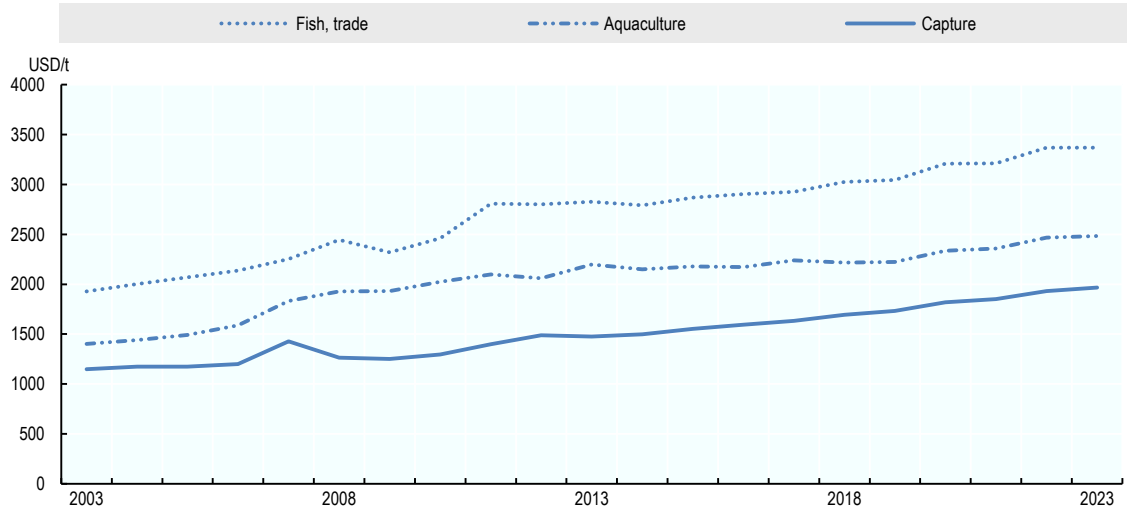


Figure 1-3 - World fish price development in nominal terms between 2003 and 2023 (Data from: http://stats.oecd.org/viewhtml.aspx?QueryId=58643&vh=0000&vf=0&l&il=&lang=en#).

The world total fisheries production is projected to reach 181 million tons in 2022. In 2013, exports reached a new record of over US$ 136 billion, more than 5 % than the previous year. Despite the decline in the average growth rate projected for the 2013–2022 period, fish products will still be one of the most highly traded goods in the word, with exports reaching 36 % of total fishery production in 2022 (FAO, 2014).

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Chapter 2. Glazing and edible coatings

This chapter introduces the process of glazing frozen fish, its present (limited) use and paradigms. Edible coatings are also present as a natural alternative to enhance food products characteristics and shelf-life. A particular emphasis is given on the use of chitosan as an edible coating solution.

2.1. Glazing

Traditional frozen storage of fish may lead to a progressive loss of intrinsic and sensory characteristics (Vanhaecke *et al.*, 2010). According to the Code of Practice for Fish and Fishery Products, glazing is the application of a protective layer of ice formed at the surface of a frozen product by spraying it with, or dipping it into, clean seawater, potable water or potable water with approved additives, as appropriate (Codex Alimentarius Comission, 2003). Glazing is a widely used technique to protect fish from this loss, and can be defined as the application of a layer of ice on frozen products' surface by spraying or brushing water or by dipping in a water bath (Žoldoš *et al.*, 2011). Figure 2-1 presents a magnified photograph from an actual chitosan coating applied on frozen salmon and Figure 2-2 is an illustration of the coating formation (dark blue surface growing in the arrows direction) around the product surface (grey).

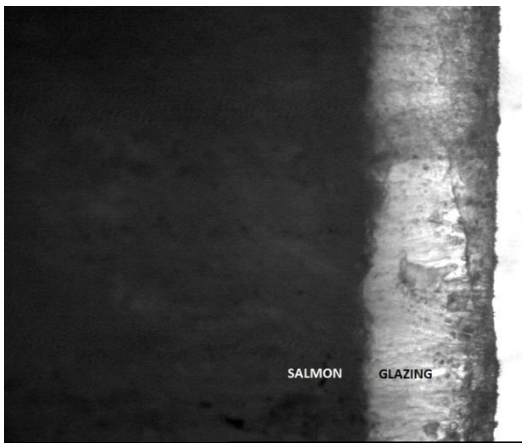

Figure 2-1 - Chitosan coating in frozen salmon Ice glazing by dipping a frozen product in cold water.

When frozen fish are to be stored, depending on packaging, they are exposed more or less to the cold air of the freezing chamber. Without glazing, the oxygen of the air will react with the fats (turning them rancid) and drying and dehydration of the product will not be prevented (which may lead to freezer burn). In addition, glazing is a physical barrier that protects the product from damage during production, packaging, transport, and retail.

Glazing is essential to minimize some of the factors that may deteriorate fish quality before reaching the consumer like the rate of freezing and thawing, temperature fluctuations, high storage temperature, incorrect transportation (high temperature and surface damage). However, glazing may also jeopardize fish quality because of partial thawing of the fish and slow refreezing in cold storage (Žoldoš et al., 2011). The major benefits and disadvantages of using glaze are presented in Figure 2-23. Glazing process must be controlled and guarantee a uniform thickness around the fish surface.

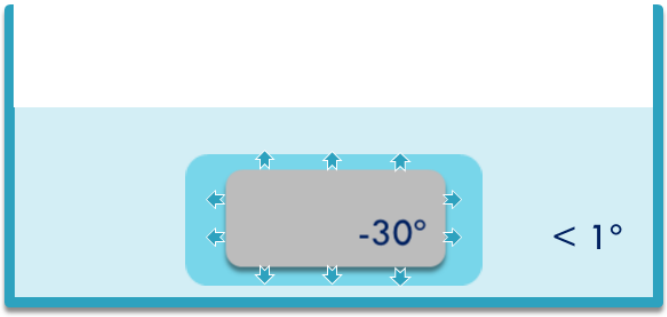

Figure 2-2 - Glazing by dipping a frozen product in cold water. A coat of frozen water is created all around the product surface.

The most common method of glazing is dipping, where frozen seafood products are immersed in a tank filled with cold water for a period of time, creating an ice coat that completely surrounds the product. Glazing carried out by spraying uses proper equipment to spray glazing solution over the product. Despite dipping is a relatively simple and cheaper method, with more production capacity than spraying, it is more difficult to control the amount and uniformity of glaze added. The factors that influence the amount of glazing applied are glazing time, water temperature, fish temperature and size and shape of the product (Johnston *et al.*, 1994). The determination of the amount of glazing applied can be very important for the evaluation of its protective function and for economic reasons and is presented as the percentage of water in the glazed product. From this number *per se* it cannot be directly inferred the protection ability of the glazing. In fact, a 10 % glazing will

correspond to different amount of glazing (in weight) and different glazing thickness depending on the weight or shape of the product, for instance.

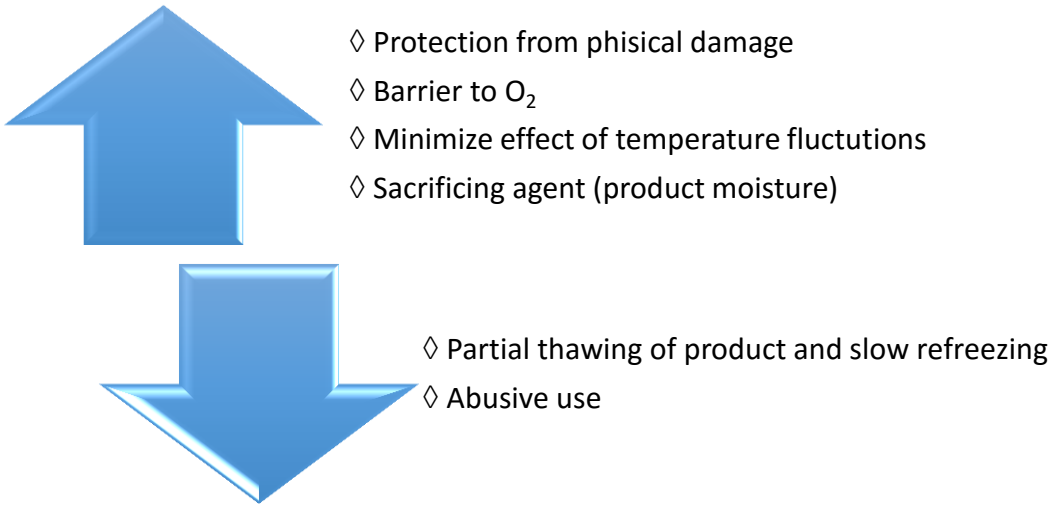

Figure 2-3 – Main advantages and disadvantages of glazing.

It is common to read expressions like *over glazing* or *excessive glazing*. Unless there is an agreement between client and manufacturer, these expressions only reflect a subjective judgment. In fact, in the cases where the product weight is intentionally mislabeled with the purpose of obtaining financial profit, then what we are really talking about is a fraud, once as there is no threshold defined for the amount of glazing that is necessary to protect the product, it cannot be considered objectively excessive. In order to start objectively addressing the problem of correct glazing in opposition to excessive glazing it is necessary to start thinking in terms of thickness of the coating. Even if glazing is thought as a sacrificing agent that protects the product water from sublimating, clearly the thickness of the coating would be the best way to predict how much time glazing will be able to perform this role. In opposition to the percentage of glazing, thickness value is independent of the kind of product (or its size and shape) and will clearly indicate the capacity of glazing to protect any product according to a set of storage conditions (*e.g.* storage temperature, temperature fluctuations and storage period).

The amount of glazing in frozen seafood varies greatly. It can be found from 4 % until 40 %, in some cases. At the lower level of glazing it is more usual to have heavier products and on the higher end the lighter ones. Although the economic motivation of using high amounts of glazing (representing gains for the producers if the weight of glaze is paid by clients or consumers) is being fought by some countries with labelling legislation (imposing the use of drained weight) consumers will still be eluded by the size of the product, and probably pay more if the product price changes according to its grade.

As presented before the amount of glazing that is formed is dependent on the product and solution temperatures, the immersion time, and the product itself (and its shape). If understanding these variables is almost empirical (and of common sense) more challenging and important for the fish industry is to answer questions such as those presented in Figure 2-4.

2.2. Edible coatings

Even when damage is not clear or noticeable, products can be exposed to conditions that promote its deterioration during storage and transportation time. In fact, the logistics of food products are increasingly longer and complex and edible coatings can work “silently” in providing protection during the time the product takes to reach the consumer. Despite freezing preservation efficiency, some undesirable changes such as:

- lipid oxidation
- surface dehydration
- protein denaturation
- recrystallization
- vitamin loss

might occur during frozen storage, thus negatively affecting the nutritional and sensory quality of frozen fish, influencing the acceptability of the product. Even when the product is glazed, temperature fluctuations often occur specially during handling and transport of frozen fish which causes losses in the glazing, reducing its protective

effect. Thus, it is of great importance to develop coatings that combine the mentioned positive features of glazing with increased resistance to temperature fluctuations and a longer protection period. Edible coatings have already been used in fruits, vegetables, and meat (Lin & Zhao, 2007; Rojas-grau *et al.*, 2009; Zhou *et al.*, 2010)

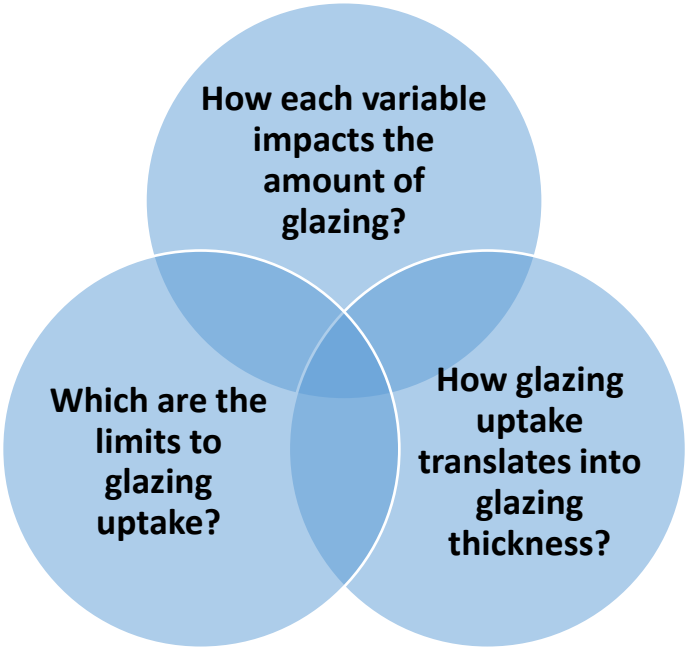

Figure 2-4- Questions to address glazing thickness.

2.2.1 Definition and Function

Edible coatings can be defined as a solution prepared from edible materials that can be applied directly on the food to create a primary package. Several materials can be used in their composition (see section 2.2.2) and they can be used individually or in combination with edible films or even non-edible films. Despite both edible coating and film are used as a primary packaging prepared from edible ingredients, the first is typically applied directly to food and the second is formed and then used to wrap food.

Edible coatings (and films) have become a promising alternative to protect food products against mechanical damage, as a gas and moisture barrier, to enhance sensory perceptions, and for protection of microbiological contamination (Falguera *et al.*, 2011; Pascall & Lin, 2012; Pinheiro *et al.*, 2010). The incorporation of additives

could add to edible coating other functional effects (*e.g.* antioxidants, fungicides, flavours, pigments). Figure 2-5 shows some of the main functions of edible coatings.

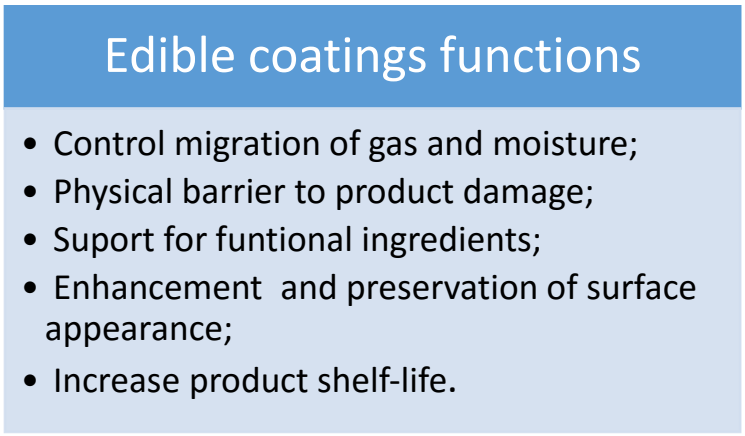

Figure 2-5 – Main functions of edible coatings.

The application of edible coatings is commonly done by the immersion of the food product in the coating solution with the objective of obtaining a uniform thin layer of the material covering the entire food product.

Another positive impact that the use of edible coatings can have in the food industry is that they may be obtained from food wastes or by-products. In the fish industry crustaceans' shells may be used to obtain chitosan, fish wastes may be used to obtain proteins (Ferraro *et al.*, 2010; Gómez-Guillén *et al.*, 2009), corn zein can be obtained from the corn industry (Lai & Padua, 1997), whey protein from the dairy industry (Regalado *et al.*, 2006), etc. All these materials may be used to produce films and coatings.

2.2.2 Materials

The literature shows different approaches on how to classify edible coatings according to their components. One general classification was proposed by Srinivasa & Tharanathan (2007) who divided biobased polymers as "Directly extracted from biomass", "Synthesized from bio-derived monomers", and "Produced by

microorganisms". Another way is grouping according with the nature of their components (*e.g.* hydrocolloids, lipids, and composites).

Figure 2-6 presents examples of hydrocolloids (polysaccharides and proteins) and lipids used in edible coatings and the main properties they introduce in the coating.

As a general rule, proteins are utilized to provide mechanical stability, polysaccharides are applied to control oxygen and other gases transmission and fats are used to reduce water transfer (Pavlath & Orts, 2009).

Polysaccharides

Monosaccharides unites joined together by glycosidic bounds constitute the basic structure of polysaccharides and can occur in plants (*e.g.* guar gum and pectin), microorganisms (*e.g.* dextran and xantan gum) and animals (*e.g.* chitosan) (Nelson & Cox, 2011; Sinha & Kumria, 2001). In nature they have two main roles:

- Structural component (*e.g.* cellulose and chitin)
- Energy storage (*e.g.* starch and glycogen)

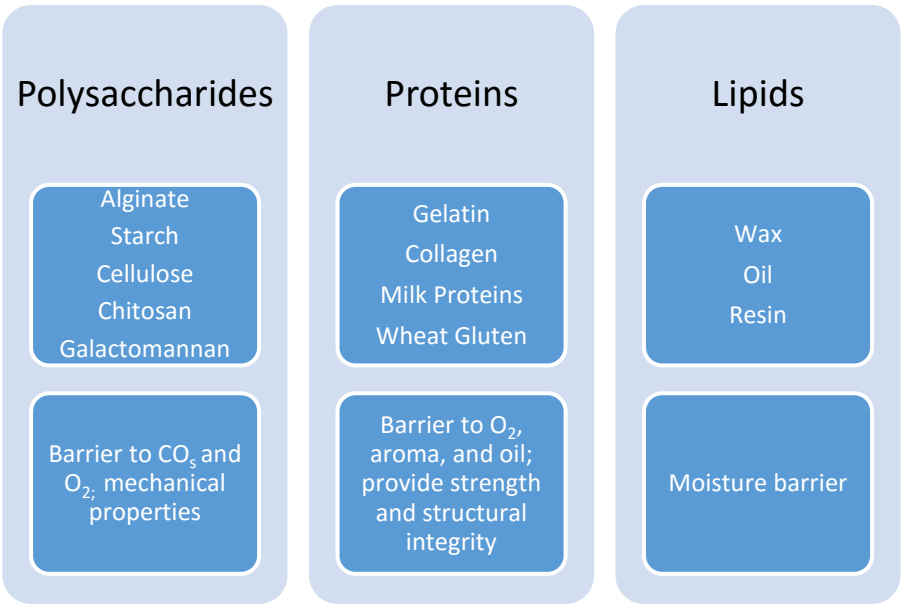

Figure 2-6 - Examples of polysaccharides, proteins, and lipids and the properties they add to the coating.

Their biological roles are dependent on several characteristics (Nelson & Cox, 2011):

- Frequent monosaccharides units
- Length of the chains
- Types of bonds
- Degree of branching

Besides the properties presented in Figure 2-6 the fact that polysaccharides are abundant in nature and at low cost make them one of the most used materials to produce edible coatings. The main limitation commonly attributed to the use of edible coatings based on polysaccharides is related to their poor barrier to water vapor and sensitivity to moisture due to their natural hydrophilicity (Azeredo, 2012). From all the polysaccharides a special focus will be given to chitosan since it was the forming material used during the experimental work.

a) Chitin

Chitin (Figure 2-7) is present in the exoskeleton of insects, crabs, and lobsters and is also a structural constituent of fungi cell walls. It is the second most abundant biopolymer in nature (after cellulose) and is a linear copolymer of N-acetylglucosamine and N-glucosamine with a β-1,4 linkage (Dash *et al.*, 2011).

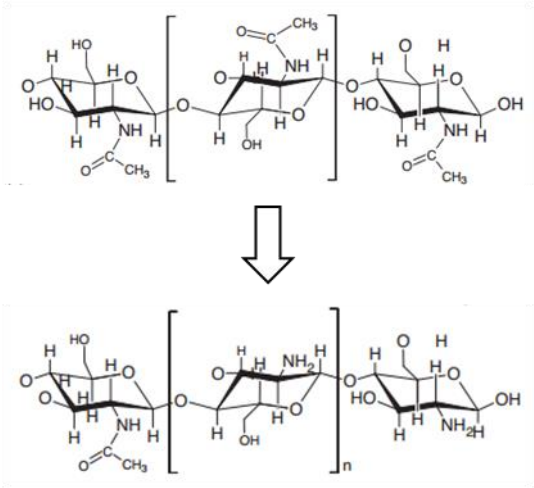

Figure 2-7 Chemical structure of chitin (above) and chitosan (adapted from Shiekh *et al.*, 2013).

The extraction of chitin is commonly a chemical method based on three steps: demineralization, deproteinization, and decolourization. When the objective is to produce chitosan a final step of deacetylation is introduced (Figure 2-7) (Shiekh *et al.*, 2013).

b) Chitosan

Chitosan can also be found as a component of the structure of certain fungi but mainly it is obtained from chitin by two different methods (Castro & Paulín, 2012):

- Thermochemical deacetylation
- Enzymatic hydrolysis

Chitosan structural backbone consists of D-glucosamine units linked (ß-1,4) to N-acetyl-D-glucosamine. The ratio of glucosamine to acetylglucosamine influences chitosan properties and is known as the degree of deacetylation (DD). The DD commonly ranges between 40 % to 98 % and is the key characteristic to define its physiological activities and functional properties (*e.g.* moisture absorption, charge distribution, intrinsic viscosity, and solubility in aqueous solutions). Depending on the preparation method the molecular weight of chitosan may fluctuate between 5 X 10^4 and 2 X 10^6 and is another attribute that defines its characteristics and applicability (Pedro *et al.*, 2009; Raafat & Sahl, 2009). Given the properties of the chitosan solution (an aqueous solution) the same factors presented in section 2.1 for glazing with water are expected to directly influence the amount of chitosan coating uptake and thickness.

Chitosan attracts much attention in the food industry because it is non-toxic, bioactive (anti-microbial, anti-oxidant), biodegradable, biocompatible and has also a very interesting reactivity, selective permeability, polyelectrolytic action, adsorption capacity and ability to form gels and films, a consequence of its visco-elastic properties once in solution (Fan *et al.*, 2009; Pinheiro *et al.*, 2010; Sathivel *et al.*, 2007).

In Table 2-1 is presented the main mechanisms and properties that influence chitosan anti-fungal, anti-microbial, anti-viral and anti-bacterial activity. For anti-fungal and anti-bacterial is also presented the minimum growth inhibitory concentration (MIC) of chitosan against fungi and bacteria, respectively.

The use of chitosan industrially in food, particularly in frozen fish, will be conditioned to economic, regulatory and consumer considerations.

1- Economically the first aspect to take into consideration is how the use of chitosan in glazing can increase product price. Using a reference price of 20-25 €/kg of chitosan and considering the range of 0.5-1.5 % chitosan concentration used in this work, the increase in product price would be about 0.012-0.038 €/kg for a 10 % glazing uptake. The second aspect would be if the same equipments can be used. The only limitation that clearly can be foreseen is in the case of high chitosan concentration (>1 %). In this case the chitosan solution becomes closer to a gel that will make it difficult to use in conventional glazing machines. On the other side, as this work showed, the use of chitosan will make possible to achieve the same thickness of glazing uptake with less energy spent in cooling and even open the possibly of doing it with less equipments. In any case, each organization must make its own trade off, adjusted to its own reality.
2- Despite of chitosan not being added to the product, its presence in glazing will include it in the category of food aditive and therefore it must be included in the ingredients list. Besides taking into consideration how the consumer will react to this new ingredient (adressed in the next point), it must be also guaranteed that its use would be approved by local authoroties. Chitosan was approved to food use in Canada (Baldwin, 2007) and has been used and aproved by the Food and Drug Administration (FDA) for medical uses (*e.g.* drug encapsulation and bandages). In 2001, an Iceland-based company that manufactures and supplies chitosan and chitin derivatives announced the GRAS[1] (Generally Recognized As Safe) self-affirmed status in the US market of

[1] The GRAS designation is considered mandatory by FDA for any substance or chemical to be added to foods and beverages.

one of their products (Raafat & Sahl, 2009). A Scientific Opinion by the European Food Safety Authority (EFSA), published in 2011, has also proclaimed the scientific confirmation between the consumption, by adults, of 3 g of chitosan daily and the occurrence of normal blood LDL-cholesterol concentrations (EFSA, 2005). From these examples it is possible to antecipate that after facing the usual bureaucracy for these processes, chitosan will be aproved by authoroties for food use.

3- The market of edible films and coatings has a great potential as companies must continue searching for alternatives (perceived as healthier) to satisfy the demand for extended shelf-life and maintenance of the organoleptic original characteristics of their products. A growing tendency of consumers to appreciate clean labels, with no additives, is clear. Equally, consumers value the use of natural alternatives in opposition to synthetic chemicals or modifying genetically food products. The natural background of chitosan, the fact that it can be extracted from waste of the fish industry, and the advantages that brings to the product safety may be enough to overcome the more negative perception of consumers.

Proteins

Proteins' composition and structure is of high complexity due to the multiple interactions and different position, type, and energy of bindings. Made up of one or more long chains of amino acids residues (from a pool of 20 different amino acids) they are involved in key aspects of plants and animals (*e.g.* transport, defence and regulation). Some of the functional properties associated with proteins are solubility, elasticity, and cohesion-adhesion (Li-Chan, 2004).

As mentioned before, some of the properties presented by proteins coatings are their excellent oxygen, oil and aroma barrier and their capacity of providing structural integrity. The main limitation is their weak moisture barrier.

Property	Mechanisms	Characteristics	Organism	MIC (ppm)
Anti-fungal activity		Molecular weight; Concentration; Types of functional groups. (Ing *et al*, 2012)	*Botrytis cinerea* *Fusarium oxysporum* *Drechstera sorokiana* *Micronectriella nivalis* *Piricularia oryzae* *Trichophyton equinum* (Rabea *et al*, 2003)	10 100 10 10 5000 2500
Anti-bacterial activity	Altering cell walls permeability. (Chung *et al*, 2004)	Degree of polymerization; Molecular weight. (Qi *et al,*. 2004)	*Agrobacterium tumefaciens* *Bacillus cereus* *Corinebacterium michiganence* *Erwinia* sp. *Erwinia carotovora* subsp. *Escherichia coli* *Klebsiella pneumoniae* *Micrococcus luteus* *Pseudomonas fluorescens* *Staphylococcus aureus* (Rabea *et al*, 2003)	100 1000 10 500 200 20 700 20 500 20
Anti-viral activity	Decrease viability of cells; Neutralize infection of phage; Block replication of viral phage. (Chirkov, 2002)	Concentration; Polymerization degree; Positive charge. (Rabea *et al.*, 2003)		
Anti-microbial activity	Amino groups interaction with cell membranes; Changing barrier properties; Reducing cell viability. (Castro & Paulín, 2012; Raafat & Sahl, 2009)	Sequence of molecular processes. (Raafat & Sahl, 2009)		

Table 2-1 - Mechanisms of action and main characteristics that influence some of the chitosan's properties. MIC for several organisms that are affected by chitosan activity.

Lipids

The main roles of lipids in nature are as structural material and energy storage. The use of lipids in coatings introduces special complexity due to the presence of a waxy taste and texture, potential rancidity, and insolubility in water (Quezada Gallo, 2000). On the other hand, they provide an excellent moisture barrier. The use of lipids in combination with proteins or polysaccharides is a very good example of a way of enhancing their good properties while minimizing the limitations.

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Chapter 3. Seafood preservation and freshness evaluation

This chapter presents the mainframe for understanding the difference between quality and freshness and describe the main instrumental methods for freshness evaluation (microbiological, physical and chemical). Freshness evaluation by sensory analysis is introduced and the main characteristics of descriminative tests, descriptive tests, and scoring method are described.

Raw seafood is a highly perishable product. Organizations should focus all their efforts in maintaining the freshness characteristics of seafood, since that is the only way to maximize its value. The economic value of fish products is strongly correlated with the consumer perception of its freshness, that can ultimately be translated by some sensory, (bio)chemical, physical and microbiological parameters (Ólafsdóttir et al., 1997). Although lack of freshness is clearly considered by the consumer as lack of quality, a fresh product may not be consider as a quality product since quality is a perception that consumers have on a series of attributes of the product, according to their own scale of appreciation. According to the standard ISO 8402:1994 (ISO/IEC, 2005) quality is "the totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs". These needs can change due to personal taste, education/area in the world where the consumer lives, or even the level of information available to the consumer. In fish products the quality features most commonly considered are associated with nutrition, safety, consistency, and organoleptic features. (FAO, 2015). Figure 3-1 points out that freshness is only one feature of the seafood “quality” and presents some of the most common parameters of its evaluation.

The principal methods for the assessment of fresh fish quality can be split into two categories: sensory and instrumental.

Sensory methods are particularly significant in the food industry since they attempt to interpret and translate the perception of human senses about the product tested. The major strength of these methods that is being closer to the expected experience of the consumers is also their main weakness since they can be affected by the subjectivity of individuals’ preferences or backgrounds. For that reason, sensory evaluation should be done by a properly trained panel. In the last decades some advances have been made in developing instruments that can substitute humans in the evaluation of food sensory characteristics like texture (texturometer), odor (“the artificial nose”), and colour (colourimeter). Technology promises in the next years to go even further and significant progresses are being made using optical scanning in the “reading” of food products instantly in order to identify food characteristics (e.g. nutritional content or how long has the product been in cold storage).

The instrumental methods include chemical, physical and microbiological methods and have two main limitations. First, they are usually time consuming (and expensive), and second an individual result often fails to guarantee that the product will satisfy the consumer expectations.

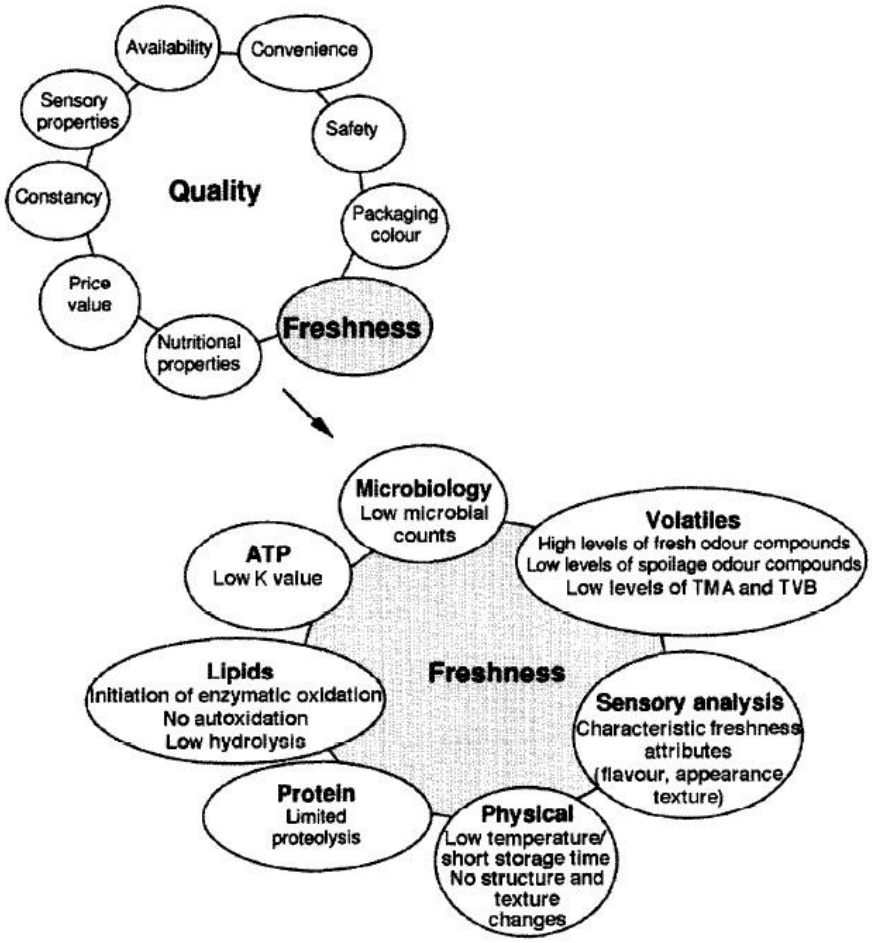

Figure 3-1 - Quality and freshness Parameters (adapted from Ólafsdóttir et al., 1997).

3.1. Non-sensory methods

3.1.1. Microbiological methods

Pathogenic bacteria are the main agents responsible for cases of infections/food poisoning. Food infections are characterized by ingestion of viable bacteria, which multiply inside the human body. On the other hand, food poisoning is caused by the

ingestion of food previously contaminated with toxins produced by pathogenic bacteria, which are present in high numbers in food (Huss *et al.* 2004).

According to Huss *et al.* (2004), pathogenic bacteria associated with diseases caused by the ingestion of fish can be divided into three major groups:

- Indigenous pathogenic bacteria of the aquatic environment – *Clostridium botulinum* (non-proteolytic type B, E and F), *Vibrio* (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) and *Plesiomonas shigelloides*;
- Indigenous pathogenic bacteria of the general environment – *Listeria monocytogenes*, *Clostridium botulinum* (proteolytic type A and B), *Clostridium perfringens*, *Bacillus spp.*;
- Indigenous pathogenic bacteria of humans and animals – *Salmonella spp.*, *Shigella spp.*, *Escherichia coli*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Staphylococcus aureus*.

Although several factors can influence the growth rate of microorganisms, temperature is undoubtedly one of the most relevant. The minimal growth temperatures of pathogenic bacteria associated with diseases caused by contaminated fish ingestion are represented in Table 3-1.

The use of Total Viable Counts (TVC) to quantify the amount of microorganisms in food products is a widely used methodology that gives a general idea of the level of contamination of the product and has the advantage of the results being easily compared with published references. Other options like measuring psychrophiles or Specific Spoilage Organisms (SSO) would be more product/conditions specific but the results would not be so straightforwardly benchmarked with industry experience.

Total Viable Counts

Since microbial activity (including that from pathogenic bacteria) is the main factor for the deterioration of the product, limiting its shelf-life, the estimation of the number of viable microorganisms in the food products has been for a long time used as a measure of its freshness. Nevertheless, this value can vary widely according to the

product origin and characteristics. Values in the range of 10^2 – 10^6 are usual in fish but higher values 10^7 – 10^8 are closer to the point of consumer sensory rejection (Ólafsdóttir et al., 1997). Individual result**s** of TVC may not be enough to take conclusions regarding the handling of the fish before sampling, temperature conditions and packaging efficiency but systematic and comparative results can provide an overall degree of bacterial contamination and hygiene utilized.

Table 3-1 - Growth temperatures of pathogenic bacteria.

Microorganism	**Temperature (°C/°F)** **(FDA, 2011)**	**Temperature (°C/°F)** **(Jay *et al.*, 2005)**	**Temperature (°C/°F)** (H. H. Huss et al., 2004)
Clostridium botulinum (proteolytic, types A and B)	10/50	----	10/50
Clostridium botulinum (non-proteolytic, types B, E and F)	3.3/37.4	-----	3.3/37.4
Clostridium perfringens	10/50	-----	----
Vibrio cholerae	10/50	-----	10/50
V. parahaemolyticus	5/41	5/41	5/41
V. vulnificus	8/46.4	----	8/46.4
Vibrio spp.	----	- 5/23	----
Plesiomonas shigelloides	----	----	8/46.4
Listeria monocytogenes	- 0.4/31.28	1/33.8	0–2/32–35.6
Salmonella spp.	5.2/41.36	7/44.6	5/41
Shigella spp.	6.1/42.98	----	6
Escherichia coli	6.5/43.7	----	7/44.6
Staphylococcus aureus (toxin production)	7/44.6 10/50	6.7/44.06 ----	7/44.6 10/50
Yersinia enterocolitica	- 1.3/29.66	- 2/28.4	- 1.3/29.66
Bacillus cereus	4/39.2	7/44.6	----

3.1.2. Chemical methods

The main advantages that chemical methods have are their objectivity, being easily comparable and being capable of distinguishing between marginal quality differences. Chemical methods rely on the measurement of metabolites that in some cases are the result of the microbiological activity (spoilage). Since it is a quantitative method, its assessment throughout the food chain can give a good perception of where are the main steps/sources of contamination (Huss, 1995; FAO, 2015). One of the most widely used methods is the Total Volatile Basic Nitrogen (TVB-N).

TVB-N

Lack of freshness in fish is commonly associated with the presence of a non-characteristic odor consequence of the production of volatile compounds. TVB-N is broadly used to evaluate fish deterioration and even, in some cases like the European Union, considered as an acceptability index for certain fish species (Table 3-2).

The method presents some limitations. In fact, TVB-N includes the measurement of trimethylamine (produced by spoilage bacteria), dimethylamine (produced by autolytic enzymes during frozen storage), ammonia (produced by the deamination of amino-acids and nucleotide catabolites) and other volatile basic nitrogenous compounds and does not distinguish if the source of spoilage is autolytic or from bacterial activity. Another disadvantage is that it is only giving useful information in later stages of spoilage (Huss, 1995)

K value

Endogenous enzymes of fish start immediately after catch to degrade Adenosine Triphosphate (ATP) and *rigor mortis* is established. The sequence of ATP degradation until Hypoxanthine (Hx) is presented in Figure 3-2.

The equation 3-1 was developed in order to quantify fish freshness throughout this cycle where [ATP], [ADP], [AMP], [IMP], [Ino] and [Hx] represent the relative

concentrations of these compounds in fish muscle measured during storage time (Saito *et al.*, 1959).

Table 3-2 - Fish species which have TVB-N limit established (Commission of the European Communities, 1995).

Fish category	TVB-N limit (mg nitrogen/100 g fish)
Sebastes spp. *Helicolenus dactylopterus* *Sebastichthys capensis*	25
Pleuronectidae (except *Hippoglossus* sp.)	30
Salmo salar Merluccidae Gadidae	35

The output of this equation is the ratio of the sum of inosine and hypoxanthine concentrations to the total concentration of ATP metabolites and has been used as a reliable indicator of freshness in fish. As K value increases, fish freshness reduces. The costs associated with this method, normally requiring an acid extraction and neutralization followed by an ion-exchange chromatography or high-performance liquid chromatography (HPLC) and then quantified by absorbance, compromises its generalized use in the industry (Ólafsdóttir et al., 1997).

$$\mathrm{K}\,\% = \frac{[\mathrm{Ino}]+[\mathrm{Hx}]}{[\mathrm{ATP}]+[\mathrm{ADP}]+[\mathrm{AMP}]+[\mathrm{IMP}]+[\mathrm{Ino}]+[\mathrm{Hx}]} \times 100 \qquad \text{Equation 3-1}$$

Thiobarbituric acid (TBA)

As presented in section 1.1.1., it is common that fish is divided according to the fat percentage of body weight. Especially fatty fish are highly susceptible to lipid oxidation (lipids include fats, oils, waxes, and other compounds of fatty acids) resulting in changes in the odor, flavor, texture, colour and nutritional value of the product. When fish is maintained at temperatures below 0 °C lipid oxidation becomes the major factor

of deterioration (rather than microbial activity) and particularly important in defining shelf-life (Ólafsdóttir et al., 1997).

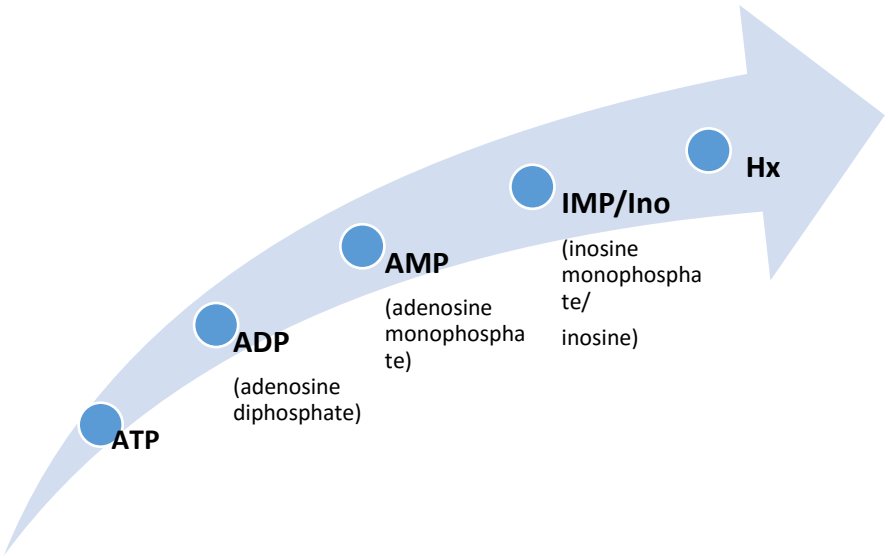

Figure 3-2 - ATP degradation after *post mortem.*

The primary oxidation products, lipid hydroperoxides, are highly unstable and therefore not a good indicator of the extension on lipid oxidation. In fact, hydroperoxides are difficult to relate with sensory quality since they are inodorous and flavorless and, since they break down to secondary oxidation products, a small value could reflect an early stages but also a late stage of oxidation (Kanner & Rosenthal, 1992). In later stages of oxidation hydroperoxides decompose into secondary oxidation products such as aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, among others. The reaction of molondialdehyde (MDA) with 2-thiobarbituric acid (TBA) can be used to determine the extension of lipid oxidation since the resulting TBA-MDA complex can be detected in a spectrophotometer (Figure 3-3).

TBA value is an excellent way to evaluate lipid oxidation and is expressed as µmol or mg of malonaldehyde used per kg of fat or fish used. Nevertheless it has some limitations particularly due to the fact that TBA can react with other substances (alkanals, 2-alkenals, 2.4-alkdienals, ketones, ketosteroids, acids, esters, proteins, sucrose, urea, pyridines, and pyrimidines) also referred to as TBARS (Thiobarbituric

acid reactive substances) leading to an overestimation of the TBA value (Shahidi & Zhong, 2005).

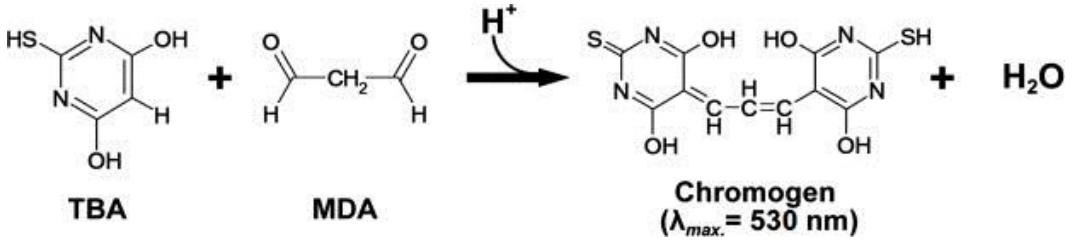

Figure 3-3 – Reaction between TBA and MDA producing a coloured complex (adapted from Bastos *et al.*, 2012).

3.1.3. Physical methods

Some of the most often limitations associated with physical methods are of being insufficiently reliable and dependent on the species. On the other hand these methods are commonly faster than microbial and chemical analysis.

pH

Despite there is no pH value defined as optimal for fish muscle, its acidity can give precious information about the muscle condition. Several factors can determine the pH value. Soon after death, as the as *rigor mortis* is established, pH tends to decrease due to the presence of lactic acid. In a second phase, microbial growth can be responsible for the pH increase since spoilage bacteria activity can provoke the buildup of ammonia components (Rodriguez-Turienzo *et al.*, 2011). Freezing is associated to the increase of acidity in fish muscle as a result from the higher concentration of substances in the unfrozen substance which alters the product acid-base equilibrium (Jiang & Lee, 2004; Sigurgisladóttir *et al.*, 2000)

Texture

Texture is a very important characteristic of fish muscle and one that is directly tied with the consumer quality perception either by touching or when eating the product. The texture of fish products can change due to the product degradation or as consequence of frozen storage. In fact, the basis for the development of Texture Profile Analysis (TPA) was to develop a method that could objectively quantify the assessment of a trained panel of judges about products texture (Huss, H. 1995). The development of an instrumental approach to TPA was decisive to guarantee its wider use by:

- Reducing the analysis time and cost;
- Allowing repeatable analyses (using different laboratories, operators and food products;

TPA tests simulate how samples behave when chewed and become known also as the "two bite test" due to the double compression used to determine the textural properties of foods. The texture analyzer recollects information of the samples behavior and typically plots a graph (Figure 3-4).

In the same test it is possible to measure several parameters (Rosenthal, 2010) :

1. Hardness – Resistance to scratching (value from the first compression peak)
2. Cohesiveness – ability to resist the second compression relative to the first compression (Area 2/Area 1)
3. Springiness – height recovery between compressions (Distance 2/Distance 1)

Chewiness can be calculated by multiplying the 3 parameters presented above.

Colour

Colour is one of the food products' attributes that has a decisive role in the consumer perception of quality. In fact, consumers have an expected range of colour for food acceptance but the degree of acceptability can depend on their personnel experience, age, sex and even education. For that reason organizations that produce food products know the importance of controlling colour to be successful in satisfying consumers'

expectations. To control any characteristic of a product it is necessary to know how to measure it and therefore be able to "locate" its coordinates in an established three-dimensional space (Francis, 1995).

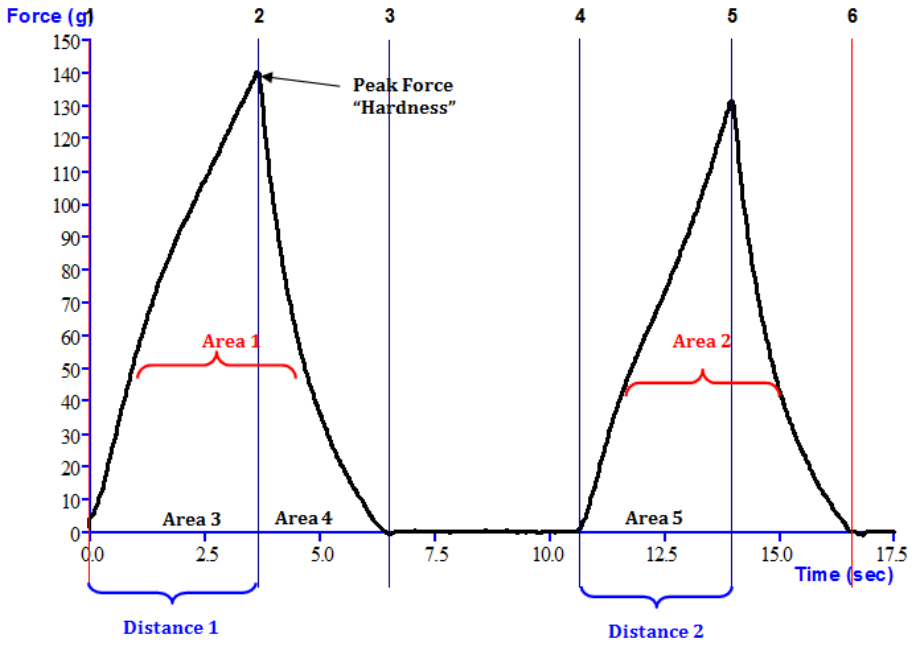

Figure 3-4 - Double compression test (adapted from Texture Technologies Corporation, 2015).

The *Commission Internationale de l'Eclairage* (CIE) created in 1976 a uniform colour scale, CIELAB ($L^*a^*b^*$), where colours are located in space (Figure 3-5) where movement in the $+a$ direction depicts a shift towards red (away from green) and a $+b$ movement represents a shift towards yellow (away from blue). The center L^* axis shows $L = 0$ (black or total absorption) at the bottom. At the center of this plane is neutral or gray (X-Rite, 2007).

The use of an instrument like a colourimeter to measure colour brings three major benefits when compared with the human eye:

- Allows expressing colour as a numerical value (avoiding subjective expressions)
- Reduces perception error using the same light source and light method (maintaining measurements conditions)

- Increases sensibility identifying differences in colour undetected to the human eye

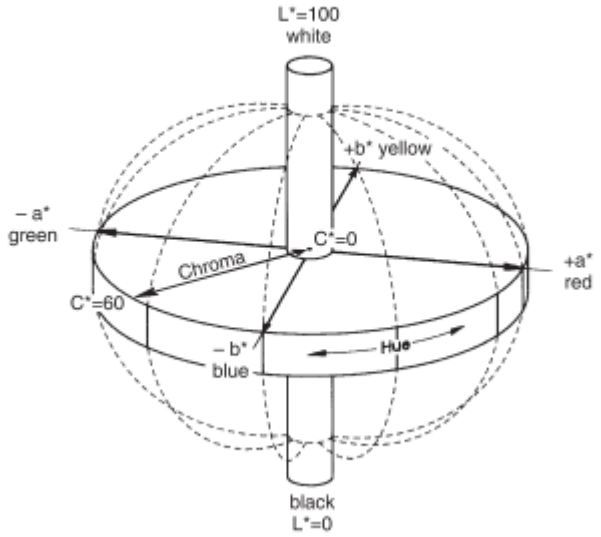

Figure 3-5 - CIELAB three dimensional space (adapted from X-Rite, 2007).

Most of the times more important than the measurement of the colour to determine product quality is the measuring of colour difference. In the CIE *L*a*b** colour system, the colour difference (Δ*E*ab*) can be calculated by equation 3-2 and expressed through a single value. The main limitation of this number is that although it quantifies the difference, it does not indicate how colours are different (X-Rite, 2007).

$$\Delta E^*ab = \sqrt{(\Delta L)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation 3-2}$$

In which the parameters Δ*L**, Δ*a**, Δ*b** regard the difference in the *L**, *a** and *b** values between two different colours (Minolta, 2007).

Defining Δ*E*ab* values tolerances can be very important in order to avoid consumer rejection of the product. Companies may want to define the minimum Δ*E*ab* that is perceptible for consumers, guaranteeing that until that moment no difference from

the standard will be notable, or to define a maximum Δ*E*ab* after which the product will most probably be rejected by consumers, or even both. There is no clear consensus in literature about what is the minimum Δ*E*ab* perceptible to the human but some numbers are relatively common. A colour difference below 2 is most of the times considered undetectable or only by trained eyes and values above 3.5 are detectable by the majority of people (Cruse, 2015; Pritchard, 2010).

3.2. Sensory Analysis

Product specification and quality standards in seafood industry are most commonly based on the assessment of the fish sensory characteristics and their correlation with non-sensory methods like chemical, microbial and physical assessment (Green, 2010). According to Huss (1995), the scientific approach to sensory analysis can be divided in three steps as presented in Figure 3-6.

To assess fish freshness there are two main kinds of sensory testing methods: a) objective and b) subjective.

Subjective assessment is more used in product development and market research as assessors are invited to express natural feelings of liking, pleasure and validation aiming to understand how consumer think about the product. In objective tests it is avoided that the personal bias and feelings influence results since its purpose is either to describe a specific aspect of quality - descriptive - or to distinguish between two or more products - discriminative (Torry Research Station, 2001).

The level of training necessary for the panel to assess the product will be conditioned by the experience of the judges and the method used. Huss (1995) advocated that a laboratory panel must consist of 8 to 10 members and should regularly be trained. Some of the advantages of using a panel of judges for sensory assessment are: a) faster perception of what ordinary consumers may experience (compared with non-sensory methods) and b) being acceptable for writing into specifications for quality. On the other hand, it may have as disadvantages: a) judges can become fatigued, b) time-

consuming and costly process of training, and c) problematic and controversial interpretation of the results (CAMO Software AS, 2015).

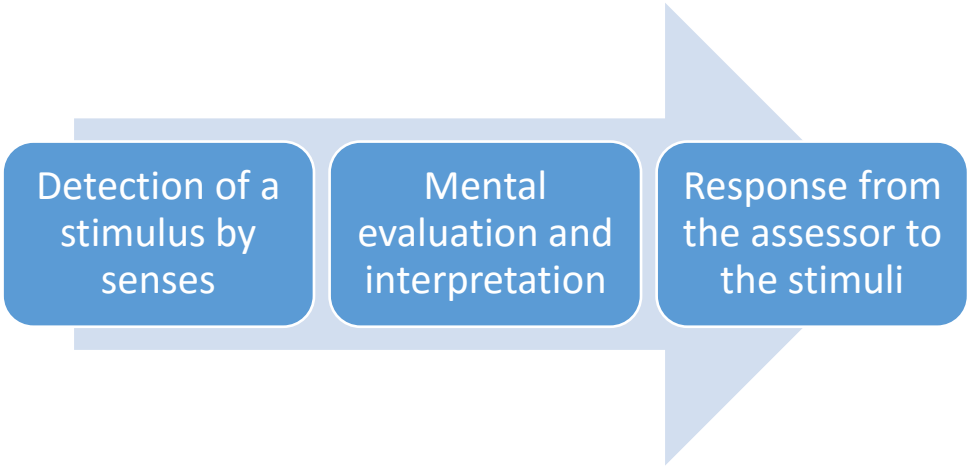

Figure 3-6 - Scientific approach to the human assessment of sensory analysis.

3.2.1. Discriminative tests

ISO 4120:2004 describes what is known as the triangle test. The test aims to determine whether a perceptible sensory difference or similarity exists between samples of two products. This test is applicable even when the nature of the difference is unknown but only if the products are fairly homogeneous (ISO, 2004). Judges are given three coded samples, and are asked to determine which one differs from the other two. Another discriminative test that is usually less time consuming to prepare and trains judges is the ranking test. This method aims at ranking several samples according to the degree with which they exhibit some specified characteristic (*e.g.* sweetness) (Huss, 1995).

3.2.2. Descriptive tests

Descriptive tests used in the sensory evaluation of fish include methods such as structured scaling and profiling (Lawless & Heymann, 2010). While in scaling a several

degrees of intensity scale (with objective terms to describe the attribute) is provided to the judges, profiling allows for a complete description of the product being assessed, and it is a way to describe it. Quantitative Descriptive Analysis (QDA) allows obtaining a detailed description of organoleptic characteristics present in the product assessed in a quantitative way. Judges are given a wide selection of reference samples and use the samples in order to define a terminology that accurately describes the product in question (Lawless & Heymann, 2010).

Statistic analysis with multivariate analysis allows correlating single attributes to a change in the sensory properties of a product. The results can be seen in "spiders web" representation (Figure 3-7) (Huss, 1995).

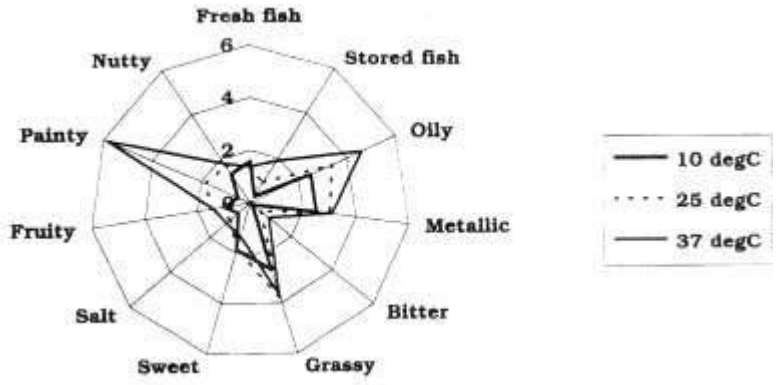

Figure 3-7 - Flavor profiles of several components of fish oil (adapted from Huss, H. 1995).

3.2.3. Scoring methods

Scoring methods rate the degree of freshness of individual physical characteristics of fish (*e.g.* skin, eyes, gills, flesh) of raw seafood, independently evaluated, to determine an overall result. *Torry Scale* has been considered the first modern method and was developed by Torry Research Station in Scotland. More recently (late 1970s and early 1980s) the Tasmanian Food Research Unit developed the Quality Index Method (QIM) that is having growing acceptance in the industry. For each fish species a set of physical characteristics to assess is defined and for each one a description of the level of

deterioration is provided together with a demerit point. This way, what judges are evaluating is the degree or rate of deterioration or change. At the end of the assessment, the sum of the demerits can then be related to corresponding days of storage and remaining shelf-life (Green, 2010).

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Chapter 4. Relevant transfer phenomena

This chapter explains how heat transfer is a phenomenon that is on the basis of glazing application. The major differences between the processes of heat transfer (conduction, convection and radiation) are explored, while describing how glazing of frozen fish is a transient state heat transfer process.

Mass transfer is introduced and presented as a similar process to heat tranfer from the point of view of mathematical description. Moisture migraton and glazing sublimation are described as two of the mass transfer phenomena with a potentialy significant impact on fish organopleptic properties.

4.1. Heat transfer

Heat transfer is a process of energy transmission through a medium driven by a temperature gradient that stops when the temperatures are the same. The process of heat transfer from the high temperature region to the low temperature one requires different mechanisms where thermodynamic and fluid flow principles are involved. Heat transfer is a process that comprises the exchange and/or conservation of energy and for that reason follows the first and second law of Thermodynamics:

1st law: During the interaction between a system and the surroundings the amount of energy gained by the system is exactly the same lost by the neighborhood.

2nd law: In an isolated system, entropy either increases (in an irreversible process) or remains constant (if the process is reversible).

It is common to divide heat transfer in three distinct processes (Figure 4-1): conduction (A), convection (B), and radiation (C). If we think solo in terms of temperature gradient as the only drive for heat transfer conduction and radiation are the processes that rigorously obey to that heat transfer principle. In convection the heat transfer is also influenced be the fluid motion.

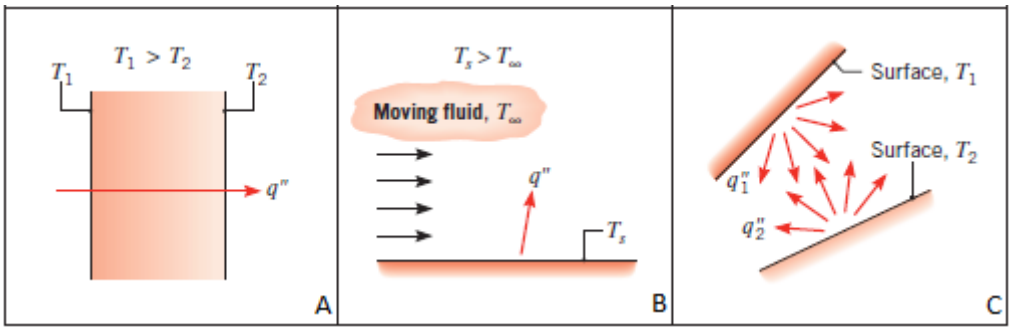

Figure 4-1 - The different processes of heat transfer (adapted from Bergman *et al.* 2011).

4.1.1. Conduction

Heat transfer by conduction can be viewed has the transfer of energy from the molecules with higher energy (higher temperatures) by interaction with neighbour low energy molecules (lower temperature). These interactions are more frequent in the cases where the molecules are closer (*e.g.* interactions in liquids when compared with gases).

In conduction the heat transfer per unit of area is proportional to the temperature gradient times the thermal conductivity of the medium as described by the Fourier 1st Law (Equation 4-1). The minus sign is the outcome of conduction through a homogeneous medium following the second law of thermodynamic and the temperature gradient being negative when *x* increases.

$$q''_x = -k\frac{dT}{dx} \qquad \text{Equation 4-1}$$

where: q''_x is the heat flux (W/m^2)
k is thermal conductivity (W/m K)
T is the temperature (K)
x is the distance in the direction of the heat flow (m)

Thermal conductivity

Despite being common to neglect the variation of thermal conductivity with temperature, listed values in literature are always presented to a specific temperature. The amount and the way (increase/decrease) thermal conductivity varies with temperature varies widely according to the material and its physical state. As general rule pure metals have the higher values and gases the lowest. Liquids come in between.

In gases, the continuous random motion of molecules rest on their kinetic energy (temperature). As molecules with higher energy collide with lower energy (in the lower

temperature side of the gradient) some of its energy is transferred. This way, the thermal conductivity will depend on the kinetic energy of the gas and therefore on its temperature. In liquids, the mechanism of thermal conductivity is similar and as general rule the values decrease with increasing temperature and molecular weight. Solid materials are known to conduct energy by the migration of free electrons and lattice vibration. Electrons are more effective in transport energy than vibrations and that is the reasons for metal solids have higher thermal conductivity (Kreith *et al.*, 2011). Values of thermal conductivity for fish and water are presented in Table 4-1.

Table 4-1 - Thermal conductivity values for different materials. Sources: 1- (Johnston *et al.*, 1994a); 2- (van der Sman, 2008); 3- (Sengers & Watson, 1986).

Material	Thermal Conductivity (W/m K)
Unfrozen white fish at 0 °C[(1)]	0.43 to 0.58
Frozen white fish at -1 °C[(1)]	1.30 to 1.73
Frozen white fish at -40 °C[(2)]	1.50 to 1.80
Water at 0 °C [(3)]	0.56

4.1.2. Convection

As represented in Figure 4-1(B) heat transfer by convection comprises two separate mechanisms: conduction and fluid (molecular) motion. This motion can be associated with the presence of a density (temperature) gradient ($T_S > T_\infty$) and in some cases of an external force. At the surface of the material the fluid velocity will be zero as result of viscous forces and all the thermal transfer will occur by the mechanism of conduction.

In some literature, convection is classified as forced when external means are the reason for the liquid flow and as natural when no mechanical equipment induces motion. Independent of the type on convection the equation that reflects this mechanism is known as *Newtons law of cooling* and is expressed as:

$$q" = h\ (T_S\ - T_\infty)$$ Equation 4-2

where: $q"$ is the convective heat flux (W/m^2)
h is the convection heat transfer coefficient (W/m^2 K)
Ts is the surface temperature (K)
T_∞ is the fluid temperature (K)

The convection heat transfer coefficient depends on the geometry of the surface, velocity of fluid and its properties and even the temperature difference (Bergman *et al.*, 2011; Kreith *et al.*, 2011). Therefore it is possible to have a wide range of values depending particularly on the fluid and if is natural or forced as presented in Table 4-2.

In the common industrial process of glazing application the heat transfer from water to the ice (see section 4.2) follows a forced convection mechanism due to the constant agitation of water.

Table 4-2 - Range of convection for gases and liquids (adapted from Cengel & Ghajar, 2015).

Type of convection	h (W/m^2 K)
Free convection of gases	2–25
Forced convection of gases	25-250
Free convection of liquids	10–1000
Forced convection of liquids	50-20,000

4.1.3. Radiation

Radiation, contrarily to conduction and convection, is a mechanism of thermal transfer that does not require a material medium since the energy is transported by

electromagnetic waves. The energy emitted by matter is due to changes in the electron configurations of molecules and depends on the absolute temperature and the nature of the surface. If we are in the presence of an ideal radiator the emissive power (*E*) per unit of area is calculated by multiplying the Stefan-Boltzmann constant (σ = 5.67 X 10^{-8} W/(m^2K^4)) by the absolute temperature of the surface (T_S). In real surfaces, the radiation emitted is lower that the ideal (blackbodies) according to the surface emissivity (ε) (equation 4-3).

$$E = \varepsilon \sigma T_S^4$$

Equation 4-3

4.1.4. Heat transfer during glazing

The process of glazing frozen fish by immersion in a bath of cold water leading to the formation o a layer of ice around the product surface is a process of heat transfer. The temperature gradient established when the frozen fish dips into the water bath impel the transmission of heat from the higher temperature (water) to the lower temperature (frozen fish). But there is another important factor that complicates analyzes of this processes. In the solid-liquid interface water change phase and a growing layer of ice will separate the frozen product from the liquid water.

Heat transfer will after glazing formation flow by convection from water to ice and then by conduction from ice to the frozen fish. Part of the heat transfer from the water to the frozen fish will be used to remove the latent heat of water until phase change and to remove heat from a by-the-second thicker layer of ice (Figure 4-2).

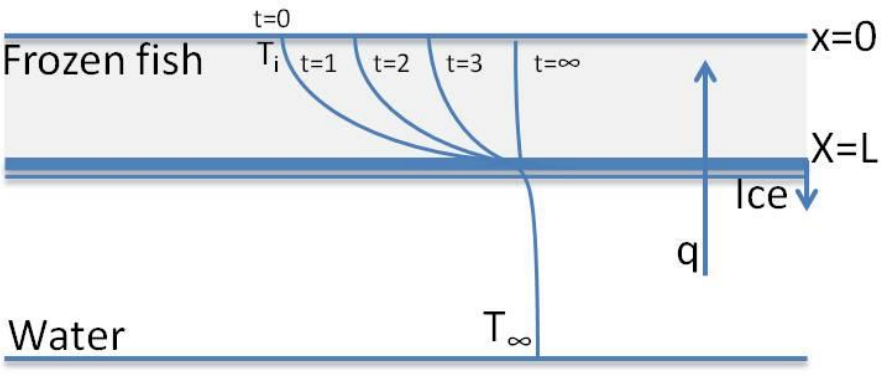

Figure 4-2 – Heat transfer profiles establish when frozen fish is dipped in cold water.

4.1.5. Steady state heat conduction

In some particular cases the process of heat transfer can occur under steady conditions and surface temperatures. In these cases the heat is continuously transferred through a medium from the higher (T_1) to the lower (T_2) temperature side. Another important aspect to appreciate is if there is more than one temperature gradient or if those other gradients can be considered negligible. In the simplest of cases heat transfer can be classified as one direction steady-state (Figure 4-3).

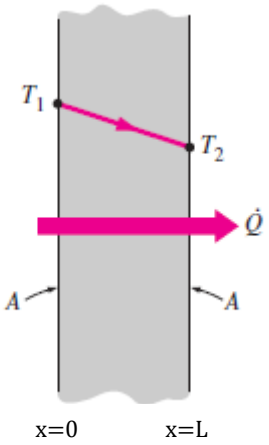

Figure 4-3 - Heat conduction in one direction steady-state condition (adapted from Cengel & Ghajar, 2015).

The Fourier law (Equation 4-1) for heat transfer can be adapted in this case, considering that $\frac{dT}{dx}$ is constant (temperature distribution is a straight line), and expresses as:

$$\dot{Q} = -kA\frac{T_2 - T_1}{L} \qquad \text{Equation 4-4}$$

Where $\dot{Q}$ is the heat flux, A the surface area, T_2 and T_1 the surface temperatures (constant) and L the surface thickness. The equation allows concluding that in a steady

state one direction phenomenon the rate of heat conduction is proportional to the thermal conductivity (k), surface area (A), temperature gradient ($T_2 - T_1$), and inversely to surface thickness (L) (Cengel & Ghajar, 2015).

4.1.6. Transient heat conduction

Most of the times the simple steady state approach to heat transfer is not applicable since changing conditions over time (besides changing conditions over space) must also be considered. One common example of a transient conduction situation is when a solid is subject to an abrupt environment temperature change as is the case of glazing (*i.e.* frozen fish dipped in water) as presented in Figure 4-2.

A simpler approach to this phenomenon is when the temperature gradient inside the solid is considered negligible and therefore temperature will be spatially constant at each moment. This will be clearly the case when the solid has no conduction resistance. Is common the use of the Biot number (*Bi*) to access if temperature gradient inside solids can be negligible (equation 4-5).

$$Bi = \frac{h}{k} \times \frac{V}{A_S} \qquad \text{Equation 4-5}$$

It is generally accepted that for Bi ≤ 0.1 a uniform temperature distribution throughout the solid can be assumed. Equation 4-5 presents two different fractions. In the first ($\frac{h}{k}$) the convection on the surface of the body (h) is compared with the conduction inside of it (k). The higher the conduction relatively to the convection the lower the *Bi* number obtained. The second fraction ($\frac{V}{A_S}$) assesses the relation between the volume of the solid (V) and its superficial area (A_S). Solids that present volumes relatively high to their surface area tend to have temperature profile when surface temperature changes. In short, only relatively small bodies of highly conductive materials can fulfill both requirements (Cengel, 2008).

In the case of frozen fish when dipped in agitated water and using Tables 4-1 and 4-2 values in Equation 4-5:

$$Bi = \frac{50}{1.8} \times \frac{V}{A_S} = 27{,}8 \times \frac{V}{A_S}$$

From this result we can conclude that only when the superficial area is at least[2] 278 times higher that the volume of the frozen fish the *Bi* would be less than 0.1.

When *Bi* > 0.1 then the error of assuming uniform temperature distribution inside the solid is high and the use of this simple approach will compromise the accuracy of results. Figure 4-4 illustrates the temperature profiles when a solid at initial temperature T_i is exposed to convection heat transfer from its surfaces to a fluid at temperature T_∞ and $T_i > T_\infty$. At the initial time (*t*=0) the temperature of the solid is constant (*T*i) but as soon as it is exposed to T_∞ the temperature near surfaces starts reducing (*t*=1) creating a temperature gradient inside the solid initiating heat conduction from the centre through the surfaces. In the next two periods (*t*=2 e *t*=3) the temperature continues falling until *t*=∞ where temperature is uniform and equal to T_∞.

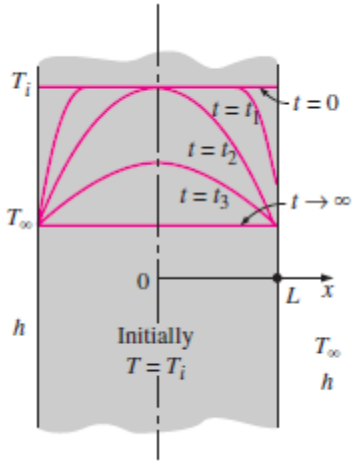

Figure 4-4 - Temperature profiles in transient state (Cengel & Ghajar, 2015).

These situations can be addressed by Fourier's 2nd law:

[2] It was chosen the higher value from *k* and the lowest from *h* in order to obtain the most conservative result

$$\frac{\partial T}{\partial t} = \alpha \frac{\partial^2 T}{\partial x^2}$$ Equation 4-6

Where T is temperature, t is time, α is thermal difussivity and x is distance.

Modifications in the boundary conditions or establishment of temperature gradients inside the solids are characteristic of transient conditions, whish lead to a multiple of solutions to equation, depending on the boundary conditions that better describe each particular situation. Solutions for a significant number of cases are available from Crank (1975).

For the boundary conditions of an infinite plane wall:

$$\frac{T - T_W}{T_0 - T_W} = \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \cos\left(\frac{(2n+1)\pi . x}{2L}\right) \exp\left(-\frac{\alpha . (2n+1)^2 \pi^2 t}{4L^2}\right)$$ Equation 4-7

$$\text{with: } \begin{cases} t = 0 & \forall, & T = T_0 \\ t > 0 & x = 0 & \frac{\partial T}{\partial x} = 0 \\ t > 0 & x = L & T = T_W \end{cases}$$

where T (K) is wall temperature at the coordinate x, T_W (K) is wall surface temperature, T_0 (K) is the initial wall temperature, L (m) is the wall semi-thickness and α ($m^2\ s^{-1}$) is:

$$\alpha = \frac{k}{\rho . C_p}$$ Equation 4-8

Where: k ($W\ m^{-1}\ K^{-1}$) is thermal conductivity, ρ ($kg\ m^{-3}$) is density and C_p ($J\ kg^{-1}\ K^{-1}$) is heat capacity.

The thermal diffusivity (α) can be described as the quotient of the conducted heat and the stored heat and is defined as the speed that heat diffuses through a material (Cengel & Ghajar, 2015).

This solution (Equation 4-7) can be used to predict the thermal gradient inside a frozen fish when being glazed (Figure 4-2).

4.2. Mass transfer

The similitudes between heat transfer and mass transfer start with the most basic of their principles. Like in heat transfer, mass transfer starts when a gradient is established (in this case from different concentrations) that promotes movement from the higher to the lower side of the gradient. For example, when ice sublimates we are in presence of a solid-to-gas mass transfer.

Mass transfer mechanisms include diffusion (equivalent to conduction in heat transfer) and convection and are known to be dependent on factors such as temperature, pressure, solute size, molecular weight and viscosity. Similar to heat transfer, the diffusion rate m_{dif} per unit area of section is proportional to the concentration gradient $\frac{\partial C}{\partial x}$ as expressed by the Fick's law of diffusion (equation 4-9)

$$m_{\mathrm{dif}} = -\mathrm{D}\frac{\partial \mathrm{C}}{\partial \mathrm{x}} \qquad \text{Equation 4-9}$$

where *D* is the diffusion coefficient.

When the mass transfer phenomenon is between a surface and a moving fluid, the transfer involves not only mass diffusion but also bulk fluid motion and is designated as mass convection and can be expressed as:

$$\mathrm{m}_{\mathrm{conv}} = \mathrm{h}_{\mathrm{conv}}\,(\mathrm{C}_{\mathrm{S}} - \mathrm{C}_{\infty}) \qquad \text{Equation 4-10}$$

where m_{conv} is the rate of mass convection per unit of area, h_{conv} is the mass transfer coefficient, and $(C_S - C_\infty)$ is the concentration difference (Cengel & Ghajar, 2015).

4.2.1. Moisture migration and freezer burn

Moisture migration is a phenomenon with severe consequences for frozen fish products organoleptic characteristics. From the other consequences of this phenomenon it should be taken in particular consideration: a) moisture loss by sublimation that, besides reducing the product weight with most probably economic consequences for the producers/retailers[3], it alters the product appearance and texture (*e.g.* spongy consistency of frozen fish filets and the emergence of a characteristic yellow/brown pigmentation on filet surface) commonly designated as freezer burn; and b) recrystallization that is a consequence from redistribution of free water inside the frozen fish, especially when frozen product is subjected to frequent and large temperature oscillations, leading to an increase in ice crystal size affecting product texture and appearance.

Temperature fluctuation is a common denominator of these two phenomena since it also largely contributes to moisture sublimation. The fluctuations modify the water-holding capacity of the air around the frozen product leading to absorption of water from the product or glaze. An indirect consequence of this phenomenon is easily observed by the accumulation of ice crystals inside in the inner-surface of packaging or in the evaporator coils (Garthwait, 1997). Regarding recrystallization, as temperatures raise small ice crystals melt. The effect of the temperature raise is not linear since the unfrozen fraction of water raises quickly near freezing point. The moisture "released" during the temperature raise tends to diffuse to larger crystals when product temperature drops again.

[3] For exposed meat carcasses during frozen storage it have been reported up to 1% per month weight lost (Pham & Mawson, 2010).

4.2.2. Glazing sublimation

If the molecules of water near the surface of the glazing have enough energy to suppress the surface binding energy, a mass transfer phenomenon will take place between glaze and the gas phase. The partial pressure of water molecules at the ice/gas interface is determined by the *Raoults law* (equation 4-11),

$$p_A = x_A p_{A,sat} \qquad \text{Equation 4-11}$$

where p_A is the partial pressure of A in the gas phase, x_A is the mole fraction of species A in the solid, and $p_{A,sat}$ is the saturation pressure of species A at the surface temperature (Bergman *et al.*, 2011). By this equation, it is clear that water sublimation from frozen fish is more difficult than from glazing due to the lower saturation pressure (*i.e.* x_A in fish will be smaller than in water glaze). A similar approach can be made when solutes are mixed in water glaze. In this case, besides reducing $x_A$it has to be taken in consideration the fact that water will be encapsulated inside the gel matrix originated by the presence of chitosan.

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SECTION II

Experimental work

Chapter 5. Effect of chitosan-based solutions applied as edible coatings and water glazing on frozen preservation – A pilot-scale study

This chapter is based in the article:

Soares, N. M., Mendes, T. S., & Vicente, A. A. (2013). Effect of chitosan-based solutions applied as edible coatings and water glazing on frozen salmon preservation - A pilot-scale study. *Journal of Food Engineering*, *119*, 316–323. http://doi.org/10.1016/j.jfoodeng.2013.05.018

5.1. Introduction

The aim of this research was to compare the effect of chitosan glazing on frozen salmon preservation with that of water glazing. It was particularly important to assess if chitosan glazing presented a higher resistance to sublimation during frozen storage, reduce the microbiological contamination and other parameters of fish freshness like pH, TVB-N, TBA, K Value. For this purpose, three chitosan solutions (0.25 %, 0.50 % and 0.75 % w/v) and water were applied in different amounts (6 %, 8 % and 11 % of coated fillet weight) directly on the surface of frozen salmon. In order to accelerate the deterioration processes, salmon was stored during 14 weeks at -5 °C.

Microbial and chemical indices were used to assess deterioration during storage and the coating sublimation was evaluated through weight loss measurements. The results obtained showed that chitosan coatings can protect frozen fish from deterioration. Microbial growth, assessed by total viable counts (TVC), and total volatile basic nitrogen (TVB-N) were maintained below the maximum limits recommended which are 5 x 10^5 CFU/g and 35 mg nitrogen/100 g fish, respectively. The use of 0.50 % and 0.75 % chitosan solutions generally demonstrated to be more efficient in preventing salmon weight loss.

5.2. Materials and methods

5.2.1 Fish Samples

Frozen and vacuum packaged skinless Atlantic salmon (*Salmo salar*) fillets from aquaculture were kindly provided by *Lerøy Seafood Group*. After unpacking, a vertical bone sawing machine (FK 32, BIZERBA, Germany) was used to cut the salmon fillets in pieces with the dimensions 10 cm × 5 cm × 2-3 cm and an average weight of 79.1 ± 5.2 g. This process was carried out in a refrigerated room to minimize temperature uptake and the salmon pieces were stored at -18 °C until further use.

5.2.2 Glazing solutions

The chitosan solutions used in this study were prepared using chitosan from Golden-shell Biochemical Co. Ltd. (China) with a 91 % degree of deacetylation. In a 5 L Erlenmeyer a 2 L solution of chitosan (0.25, 0.50 and 0.75 % w/v) were prepared dissolving 5, 10 and 15 g in a 1 % lactic acid solution (90 % w/w purity) and the volume was completed up to 2 L with distilled water. The solution was stirred with a magnetic stirrer in a heating plate (VWR; Model: VMS-C7 Advanced) at 70 °C, until complete dissolution of the chitosan. The heating was then turned off and the solution remained in agitation overnight. The solution was then transferred to a closed glass container and stored at 8 °C. Distilled water was used for water glazing.

5.2.3 Glazing application

The frozen fish pieces (-18 °C) were weighted, dipped in chitosan glazing solutions (5 °C) or in water (0 °C), for different dipping times (water: 10, 40 and 90 s; chitosan 0.25%: 5, 30, and 90s; chitosan 0.50%: 5, 30, and 60s; chitosan 0.75% 5 and 30 s), drained for 2 minutes and weighted again. This glazing process was carried out in a pilot-scale glazing tank; samples were collected from the tank with a stainless steel mesh, in order to minimize the interference with the amount of coating applied. Coating and glazing uptake were calculated according to Equation 5-1, where W_{salmon} and W_i represent the weight of the salmon portion before and after the coating/glazing application, respectively. Samples groups with an average coating uptake of 6.1 ± 0.6 %, 8.1 ± 0.7 %, and 10.5 ± 0.9 % were obtained. Salmon pieces belonging to the control group were left untreated.

$$Glazing\ uptake\ (\%) = \frac{W_i - W_{salmon}}{W_i} \times 100 \qquad \text{Equation 5-1}$$

All samples were individually packed in polyethylene freezer bags and stored at -5.0 ± 0.6 °C for 14 weeks. This temperature was monitored and registered every 20 min using a data logger (DS1923 temperature/humidity logger iButton®, Dallas Semiconductors, USA). During storage, samples were taken in triplicate and separately assessed.

5.2.4 Glazing loss

After the storage period, samples were weighted (W_f) and the glazing loss was determined by the following equation.

$$Glazing\ loss\ (\%) = \frac{W_f - W_i}{(W_i - W_{salmon})} \times 100 \qquad \text{Equation 5-2}$$

5.2.5 Microbiological analyses

The TVC were estimated according to the procedure described in the standard ISO 4833-1:2013. A 1 g portion of sample was added to 9 mL of diluent and 3 successive dilutions of this suspension were performed. From each dilution, including the initial suspension, 1 mL (V) was transferred to a Petri dish and about 15 mL of plate count agar at 44 °C to 47 °C was added (Figure 5-1).

The inocula were mixed with the medium by rotating the dishes and, after solidifying, were inverted and placed into an incubator at 30 °C for 72 h. The colonies from each Petri dish containing more than 15 and fewer than 300 colonies were counted and the number of microorganisms (N) present in the test sample was calculated using

equation 5-3, where $\sum C$ is the sum of the colonies from two successive dilutions and d is the number corresponding to the first dilution counted.

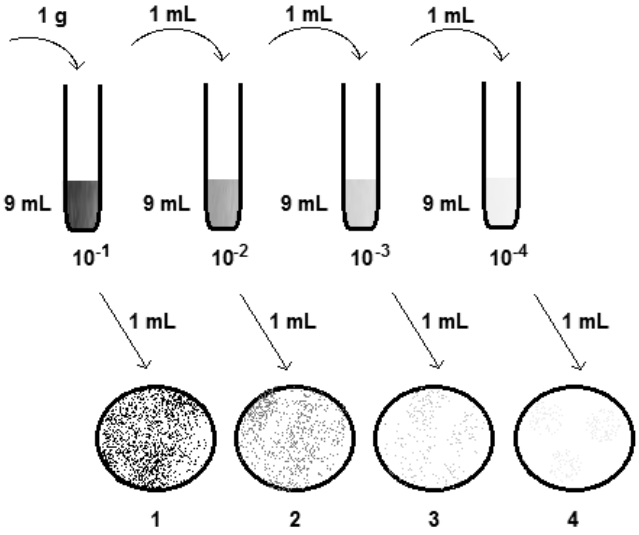

Figure 5-1 - Representation of successive dilutions from initial sample

The results were reported as the number of microorganisms per gram of sample.

$$N = \frac{\sum C}{V \times 1.1 \times d}$$ Equation 5-3

5.2.6 Chemical analyses

Determination of pH

After removing the 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 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, Switzerland).

Determination of 2-thiobarbituric acid (TBA)

The 2-thiobarbituric acid (TBA) value was evaluated colourimetrically using the method of Pokorny & Dieffenbacher (1989). 500 mg portion of each sample was weighted and added to 25 mL of 1-butanol. Using a pipette, 5 mL of the sample solution and 5 mL of TBA reagent were transferred to a dry test tube. The test tube was stoppered, thoroughly mixed using a vortex, and placed in a thermostated water bath at 95°C for 120 min. After cooling in running tap water, the optical density was measured at 530 nm in a 10 mm quartz cell, using distilled water in the reference cell, in a Jasco V-560 UV/Vis spectrophotometer (Japan). A reagent blank was run at the same time.

Determination of total volatile basic nitrogen (TVB-N)

The TVB-N value was determined according to the procedure described in the standard NP 2930:2009. A 50 g portion of fish sample (m) was homogenized with 100 mL of 5 % (w/v) trichloroacetic acid and, after standing for 2 min, the mixture was filtered through gauze. A 1 mL volume of boric acid was transferred to the centre of a Conway cell and 1 mL of filtrate (V_3), 0.5 mL of distilled water, and 1 mL of potassium carbonate (K_2CO_3) saturated solution were added to the periphery as represented in Figure 5-2. The Conway cell was closed carefully without mixing the solutions and placed into an incubator at 40 ^{o}C during 90 min. After that period, the boric acid solution was titrated with 0.02 mol/L hydrochloric acid until acquiring a pink colouration. A blank and a diffusion control were also performed, replacing the volume of extract by an equal volume of distilled water and 0.1% (w/v) ammonium sulphate respectively.

The amount of TVB-N was calculated using the Equation 5-4, 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.

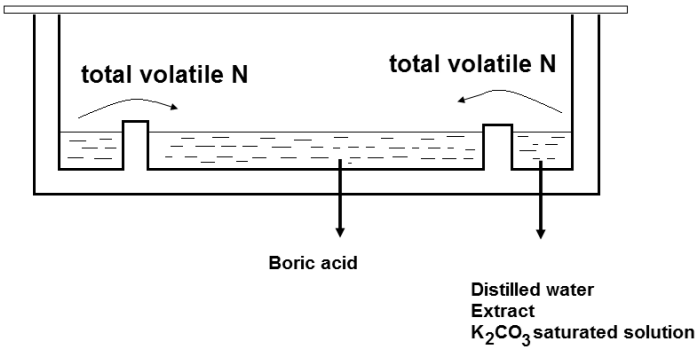

Figure 5-2 - Representation of a Conway cell (adapted from NP 2930)

$$\frac{21\times(V_2-V_0)}{(V_1-V_0)\times V_3\times m}(100+F_c)$$ Equation 5-4

Determination of K value

The K value was estimated according to the method of Ryder (1985) as described by Souza *et al.*, (2010). A 5 g sample was homogenized with 25 mL of chilled 0.6 mol/L perchloric acid at 0 °C for 1 min. The homogenate was centrifuged (EBA 20, Hettich zentrifugen, Germany) at 3000×g for 10 min, and 10 mL of the supernatant adjusted to pH 6.5-6.8 with 1 mol/L potassium hydroxide using a digital pH meter (HI 8711E, HANNA Instruments, Italy). After standing at 1 °C for 30 min, the potassium perchlorate that precipitated was removed by filtration using a Whatman 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.

A Jasco 2080-PU intelligent pump chromatograph (Japan) equipped with a Jasco 2070-UV intelligent UV-Vis detector and a Jasco AS-2057 Plus intelligent auto sampler was used for all analyses. The separation of a 20 µL aliquot of the stored samples was achieved on an EC 250/4.6 Nucleosil 100-5 C18 column (Macherey-Nagel, Germany), using a mobile phase of 0.04 mol/L potassium dihydrogen orthophosphate and 0.06 mol/L dipotassium hydrogen orthophosphate dissolved in Milli-Q purified distilled water, at a flow rate of 0.8 mL/min. The peaks of the ATP breakdown products (ATP,

ADP, AMP, IMP, Hx, and Ino) obtained at 254 nm and the K value was calculated using the Equation 3-1.

5.2.7 Statistical analyses

Mean values of 3 independent determinations were reported and the statistical significance of differences among treatment means was evaluated by analysis of variance (ANOVA) followed by the Tukey test at 95% significance level. Data were evaluated statistically using the software STATISTICA version 7.0 (StatSoft Inc. 2004, USA).

5.3. Results and discussion

5.3.1 Glazing loss

The weight of glazing lost during storage of salmon samples treated with water/chitosan solutions and three different coating uptakes was evaluated in order to determine which coating had a higher loss rate (Figure 5-3).

The equations representing the trend lines obtained for each treatment are presented in Table 5-1.

As can be seen by the positive slope of the trend lines (Table 5-1), the amount of coating lost increased steadily during the storage period for all treatments. Analysing each type of coating applied, it was clear that the higher the coating uptake, the higher the coating loss, except for samples treated with 0.25 % chitosan solution. In that case, the weight of coating lost for 6 % and 8 % coatings was very similar.

The fact that higher weight loss occurred with higher coating uptakes may be related with coating thickness. In thicker coatings, water molecules on the surface are more

distant from the center of the product, where the temperature is lower, being more susceptible to temperature fluctuations and eventual phase transitions.

Table 5-1 - Equations of trend lines for coating loss (y, in g) of salmon samples during storage time (x, in weeks), for 14 weeks at -5 °C.

	Trendlines		
Treatment	**6%**	**8%**	**11%**
water	y = 0.0584x + 0.0767 R^2 = 0.8817	y = 0.0852x + 0.0414 R^2 = 0.9024	y = 0.0887x + 0.095 R^2 = 0.9008
0.25% chitosan	y = 0.0392x + 0.0749 R^2 = 0,8553	y = 0.0347x + 0.1114 R^2 = 0.8007	y = 0.0514x + 0.1799 R^2 = 0.8431
0.50% chitosan	y = 0.034x + 0.1104 R^2 = 0.6502	y = 0.0448x + 0.0613 R^2 = 0.9432	y = 0.0533x + 0.1518 R^2 = 0.9112
0.75% chitosan	--	y = 0.0575x + 0.0491 R^2 = 0.9341	y = 0.0756x + 0.0762 R^2 = 0.9477

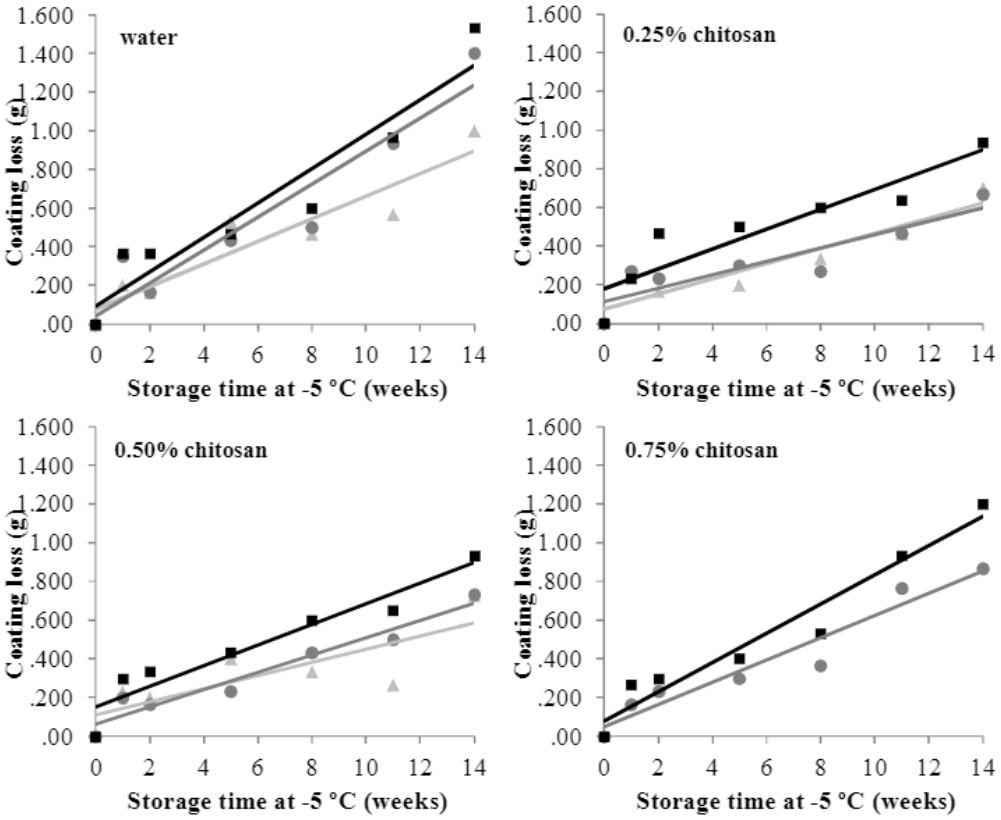

Figure 5-3 - Coating loss (g) and corresponding trend lines for salmon samples coated with water glazing; 0.25 %, 0.50 %, and 0.75 % chitosan solutions and 6 % (▲), 8 % (●), and 11 % (■) coating uptakes during 14 weeks of storage at -5 °C.

Comparing the different types of coating for the same coating uptake, the higher loss corresponded to water and 0.75 % chitosan coatings. The use of 0.25 % and 0.50 % chitosan coatings seemed to be the best option among the treatments studied to decrease the rate of coating loss, especially when 6 % and 8 % of coating were applied.

The lower percentage of coating loss in samples treated with chitosan solutions might be due to the rheological properties of the polymer. According to Hwang & Shin (2001), the viscosity of chitosan solutions increases with polymer concentration, which may have increased the toughness of chitosan coatings. However, a direct relation between the increase in chitosan concentration and coating loss was not identified. Although the coatings applied were only partially lost (1.0 ± 0.2 g in the worst case, which represents a loss of 19.4 ± 3.0 %) it does not ensure that the salmon samples remained completely protected. According to Johnston *et al.* (1994b), the corners and edges of glazed fish pieces are more susceptible to dehydration and can be damaged long before the overall weight loss reaches the weight of glaze applied. Coating application does not allow the elimination of fish dehydration, it just retards its occurrence.

Table 5-2 shows the predicted time to reach 50 % of coating loss for all treatments, considering a linear trend for the 14 weeks analysed. According to these forecasts, the samples coated with 6 % of water solution would be the first losing 50 % of coating applied, in about 42 weeks, whereas 0.25 % chitosan solution with 8 % coating uptake would retard this effect up to 92 weeks. A relation between the delay of coating loss and the chitosan concentration or amount of coating applied was not clearly identified, although the coating loss was retarded for water and 0.75 % chitosan coatings by increasing the coating uptake and for 6 % coatings by increasing chitosan concentration.

Table 5-2 - Predicted time to reach 50 % of coating loss.

	Time to 50 % coating loss (weeks)		
Treatment	**6%**	**8%**	**11%**
water	42	48	60
0.25 % chitosan	58	92	82
0.50 % chitosan	74	87	82
0.75 % chitosan	--	56	60

Figure 5-4 shows the weight loss of salmon samples from the control group (uncoated) during storage. After 14 weeks of frozen storage, salmon pieces lost about 0.7 ± 0.2 % of their initial weight.

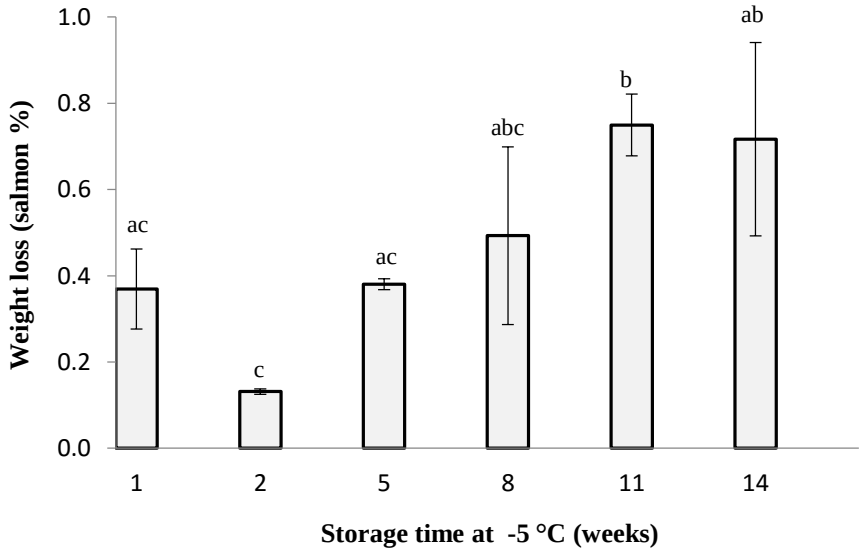

Figure 5-4 - Weight loss (%) of salmon samples from the control group during 14 weeks of storage at -5 ºC. Each bar represents the mean ± standard deviation of three replications. Different letters indicate a statistically significant difference (Tukey test, $p < 0.05$).

Although this is a reduced value, it is important to underline that weight loss was evaluated with salmon in a frozen state. After thawing, loss of water from the fish muscle (drip loss) also occurs, leading to negative changes in texture and colour (Blond & Mestre, 2004). According to Johnston *et al.*, (1994b), the rate of weight loss might vary with several factors such as temperature, temperature fluctuation, humidity, and

shape and size of the product. Usually, moisture loss is more pronounced when temperature fluctuations occur (Gonçalves & Gindri Junior, 2009) therefore, the low values of weight and coating loss obtained might be explained by a well-controlled storage temperature. During the 14 weeks the salmon pieces were stored at -5.0 ± 0.6 °C with maximum temperature amplitude of 2 °C.

5.3.2 Total viable counts

Microbial activity is the main factor limiting the shelf-life of fresh fish, and estimations of the total viable counts (TVC) have been used as an acceptability index in standards, guidelines and specifications (Ólafsdóttir *et al.*, 1997). The initial total viable count (TVC) value of salmon was 3.8 ± 0.5 log CFU/g and the evolution of this index during storage is shown in Figure 5-5.

Slight variations in TVC occurred during the storage period for all treatments. However, the microbiological limit of 5×10^5 CFU/g (5.7 log CFU/g) recommended by (ICMSF, 1986) for frozen fish of good quality was never exceeded.

There was no evidence that the type of coating applied influences the microbiological growth as well as the amount of coating applied. Microbiological growth is known to be inhibited by freezing temperature. According to Jay et al. (2005), the minimal reported growth temperature for foodborne microbial species is -5 °C for Vibrio spp. and *Cladosporium cladosporiodes*. The slight variations observed during the 14 weeks might be related with the variability inherent to fish samples.

5.3.3 pH value

Changes in pH values during storage for 6 %, 8 % and 11 % coating uptake are shown in Figure 5-6.

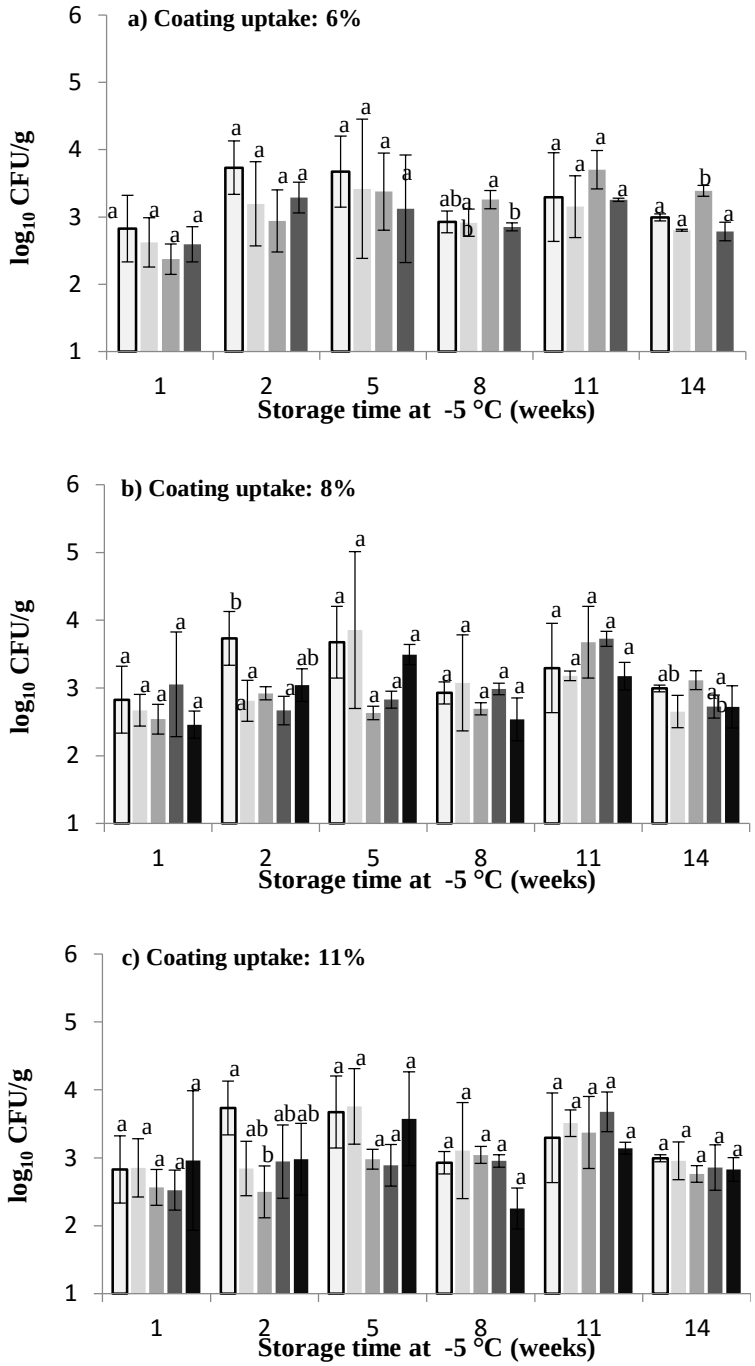

Figure 5-5 - Total viable counts (log_{10} CFU/g) for salmon samples of control group (□) and coated with water (▫), 0.25 % chitosan (▪), 0.50 % chitosan (■), and 0.75 % chitosan (■) during 14 weeks of storage at -5 °C, for different glazing percentages a) 6 %, b) 8 % and c) 11 %. Each bar represents the mean ± standard deviation of three replications. Different letters at the same week indicate a statistically significant difference (Tukey test, $p < 0.05$).

The initial pH value of salmon samples was 6.27 ± 0.15. After 14 weeks of frozen storage, the pH of uncoated samples was 6.14 ± 0.02, whereas for samples coated with water the pH values were 6.09 ± 0.04, 6.18 ± 0.07, and 6.21 ± 0.02 for 6 %, 8 % and 11 % of coating uptake respectively. Salmon samples coated with chitosan revealed final pH values slightly lower than samples coated with water and uncoated samples. The amount of coating applied did not show a significant influence on the evolution of pH. According to (Singh & Balange, 2005) the decrease in pH of fish samples might result from protein breakdown and release of phosphoric and lactic acids occurring during freezing and thawing processes. However, if these processes had occurred, the pH of uncoated samples should have decreased too. Thus, the reduction of pH values of samples coated with chitosan may be related with migration from the coating itself, which has an acid pH value (2.6), to fish muscle or with the inability to completely remove the coating before pH measurement. A study performed by Sathivel *et al.* (2007) also demonstrated that initial pH value of salmon fillets did not vary for uncoated samples when stored at -35 °C for 8 months. In addition, samples uncoated and coated with distilled water had a pH value slightly higher than samples coated with lactic acid and 1 % chitosan.

5.3.4 Thiobarbituric acid value

At temperatures below 0 °C, oxidation rather than microbial activity becomes the major spoilage factor and particularly important for shelf life. The TBA assay has been widely used to evaluate lipid oxidation in food (Guillén-Sans & Guzmán-Chozas, 1998; Ólafsdóttir et al., 1997).

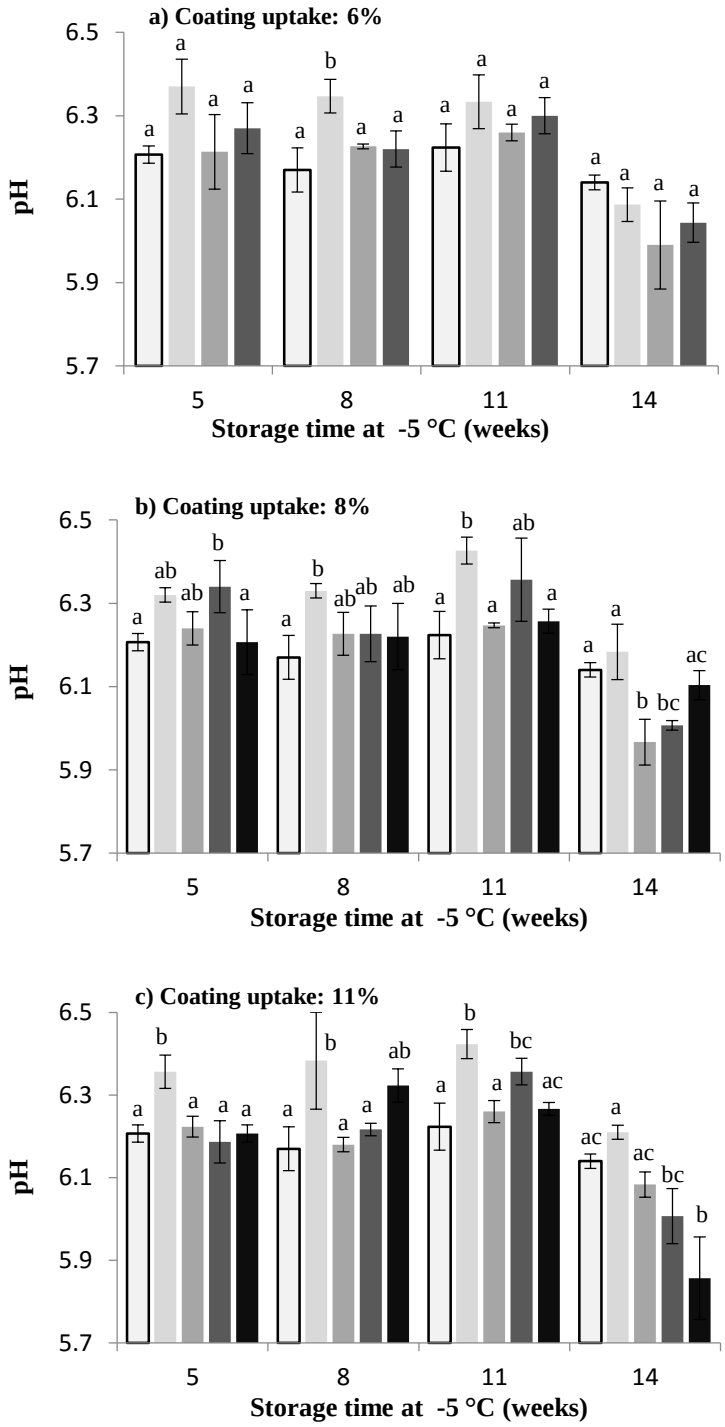

Figure 5-6 - pH values for salmon samples of control group (□) and coated with water (▫), 0.25 % chitosan (▪), 0.50 % chitosan (■), and 0.75 % chitosan (■) during 14 weeks of storage at -5 °C, for different glazing percentages a) 6 %, b) 8 % and c) 11 %. Each bar represents the mean ± standard deviation of three replications. Different letters at the same week indicate a statistically significant difference (Tukey test, $p < 0.05$).

The initial TBA value of salmon was 0.03 ± 0.01 mg MA/kg and changes of this parameter during storage for 6 %, 8 % and 11 % coating uptake are shown in Figure 5-7.

Although in general this index remained stable during the storage period, in the last week the TBA value doubled. A study performed by (Sathivel *et al.*, 2007) demonstrated that distilled water and 1 % chitosan coatings were effective in reducing lipid oxidation in salmon fillets stored at -35 °C for 8 months when compared with uncoated samples. Both coatings were resistant to oxygen diffusion retarding lipid oxidation, however, the protective effect of chitosan was more pronounced, perhaps due to its antioxidant properties reported by (Shahidi *et al.*, 1999). The thickness of coating applied had no influence on lipid oxidation control, which might mean that it is not necessary to use high thickness of coatings to inhibit lipid oxidation.

5.3.5 Total volatile basic nitrogen

The total volatile base nitrogen (TVB-N) is an indicator of the presence of nitrogenous materials resulting from the action of proteolytic bacteria (Kilincceker *et al.,* 2009). The measurements of this parameter are used as an acceptability index for certain fish species (Commission of the European Communities, 1995).

The initial TVB-N value was 7.2 ± 1.3 mg nitrogen/100 g salmon. After 14 weeks of frozen storage, the TVB-N of the control group was 6.8 ± 1.1 and for coated samples the TVB-N values are presented in Table 5-3. The TVB-N values remained stable for all treatments far below the 35 mg nitrogen/100 g fish established as limit of acceptability of salmon by EU Directive 95/149.

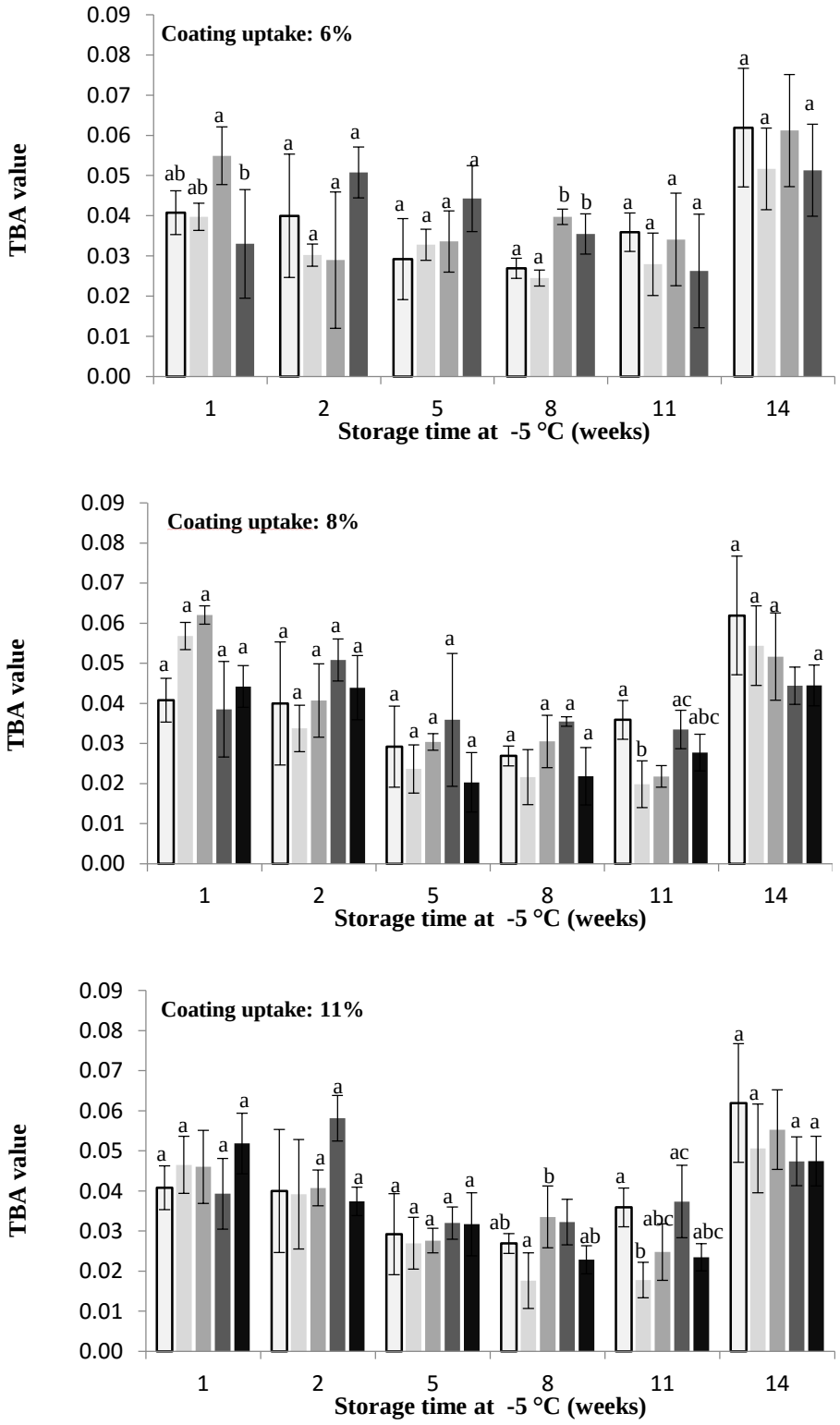

Figure 5-7 - Thiobarbituric acid (TBA) values for salmon samples of control group (□) and coated with water (▫), 0.25 % chitosan (▪), 0.50 % chitosan (■), and 0.75 % chitosan (■) during 14 weeks of storage at -5 °C, for different glazing percentages a) 6 %, b) 8 % and c) 11 %. Each bar represents the mean ± standard deviation of three replications. Different letters at the same week indicate a statistically significant difference (Tukey test, $p < 0.05$).

These low values indicate a good state of fish preservation. Gonçalves & Gindri Junior (2009) evaluated the influence of different glazing percentages on TVB-N evolution of frozen shrimp stored at -18 °C during 180 days and verified an increase only after 90 days. Probably, the time of salmon storage was not long enough to identify differences among the various coatings since the activity of spoilage bacteria and endogenous enzymes is practically stopped at low temperatures.

Table 5-3 Total volatile basic nitrogen (TVB-N) values for salmon samples after 14 weeks of storage at -5 °C; standard deviation corresponds to three replications; different letters indicate a statistically significant difference (Tukey test, $p < 0.05$).

	TVB-N (mg Nitrogen/100 g salmon)		
Treatment	**6 %**	**8 %**	**11 %**
water	5.92 ± 2.06[a]	8.80 ± 2.36[a]	6.97 ± 1.10[a]
0.25 % chitosan	5.05 ± 0.66[a]	6.08 ± 0.77[a]	5.73 ± 0.39[a]
0.50 % chitosan	6.11 ± 1.68[a]	7.10 ± 3.28[a]	6.31 ±1.32[a]
0.75 % chitosan	--	5.31 ± 1.75[a]	7.71 ± 0.85[a]

5.3.6 K-value

Adenosine triphosphate (ATP) degradation by endogenous enzymes in fish during the early stages of storage was found to be parallel the loss of fish freshness. *K* value, a measure of adenine nucleotides and their degradation products has been used as a reliable indicator of freshness that is applicable for frozen fish (Olafsdóttir *et al.*, 1997). The effect of various chitosan concentrations on K value evolution during storage for 6 %, 8 % and 11 % coating uptake is shown in Figure 5-8.

The initial K value of salmon samples was 53.8 ± 9.4 % which indicates that ATP degradation as already occurred in significant extension. Souza *et al.* (2010) reported an initial K value of 10.6 % for fresh salmon fillets of the same species. Various factors as type of muscle, stress of fish during capture, and storage temperatures affect the K value of fish (Huss, H. 1995; Souza et al., 2010).

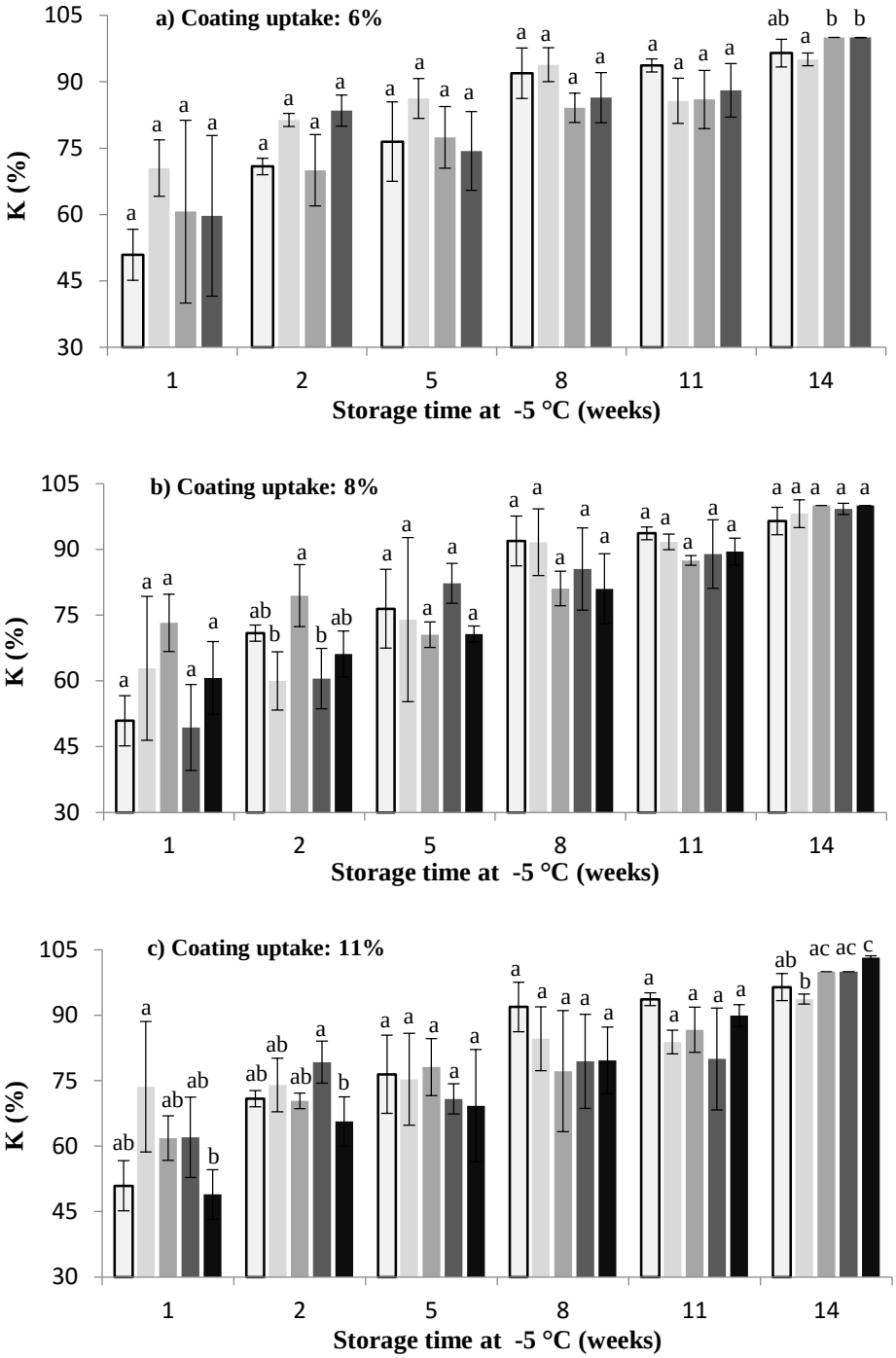

Figure 5-8 - K values for salmon samples of control group (□) and coated with water (▫), 0.25 % chitosan (▪), 0.50 % chitosan (■), and 0.75 % chitosan (■) during 14 weeks of storage at -5 °C, for different glazing percentages a) 6 %, b) 8 % and c) 11 %. Each bar represents the mean ± standard deviation of three replications. Different letters at the same week indicate a statistically significant difference (Tukey test, $p < 0.05$).

The difference between initial K values obtained in both studies might be related with fish provenance and time elapsed prior to analysis. The salmon used in this study was from aquaculture and was previously filleted, packaged and frozen whereas fish used by the mentioned authors was obtained fresh. The K index increased during the storage period nearly reaching 100 % in all treatments. Between second and eighth weeks, salmon treated with 0.75 % chitosan coating showed a slightly slower increase than the control group, however, the trend for the other coatings was very similar to that of untreated samples. The amount of coating applied for the same type of coating did not seem to affect the K value evolution. Linear trend lines were adjusted to the experimental data (Table 5-4).

Table 5-4 - Equations of trend lines for K values (*y*, in %) of salmon samples during storage time (*x*, in weeks) for 14 weeks at -5 °C.

	Equations of trend lines		
Control	y = 3.2967x + 56.986		
Treatments	**6 %**	**8 %**	**11 %**
water	y = 2.1714x + 68.196	y = 2.0684x + 64.886	y = 3.234x+ 57.077
0.25 % chitosan	y = 2.8742x + 59.165	y = 2.3431x + 64.208	y = 2.7616x + 59.254
0.50 % chitosan	y = 2.7012x + 62.143	y = 3.4545x + 54.006	y = 2.4204x + 60.868
0.75 % chitosan	--	y = 3.0597x + 56.620	y = 3.5639x + 52.055

Table 5-5 shows the time required to reach half the increase from the initial K value and 100 %, calculated based on the trend lines. A three weeks' difference was obtained between the worst situation when salmon samples were treated with 6 % of water solution and the best result with 11 % of 0.75 % chitosan solution. Generally, the higher the chitosan concentration, the higher the time required to reach the established K value. With respect to coating uptake, the effects were not clear.

Table 5-5 - Estimated time to reach a K value of 77 %.

	Time to K = 77 % (weeks)		
Treatment	**6 %**	**8 %**	**11 %**
water	4.1	6.2	5.9
0.25 % chitosan	6.4	5.5	6.4
0.50 % chitosan	5.5	6.7	6.7
0.75 % chitosan	--	6.7	7.1

5.4. Conclusions

Although the storage temperature defined (-5 °C) was much higher than that established for frozen fish preservation (-18 °C), it still inhibited microbial activity, keeping salmon samples below the maximum microbiological limit recommended for frozen fish. This prevented observing the influence of the type and amount of coating applied on the microbiological growth.

The pH value of untreated salmon during storage indicated a good preservation of muscle. The type and the amount of coating applied did not influence the evolution of pH. The TBA value of salmon samples indicated that the amount and type of coating applied had no statistically significant influence on lipid oxidation control, meaning that it is not necessary to use high amounts of coatings to inhibit lipid oxidation.

The TVB-N values remained stable for all treatments, far below the limit of acceptability established for salmon, indicating a good state of fish preservation. No differences were observed among the various treatments applied.

The K index increased during the storage period nearly reaching 100% in all treatments. The high K values indicated an advanced stage of ATP degradation. The amount of coating applied for the same type of coating did not affect the K value evolution.

Chitosan coatings showed to be a better option than water coating to protect salmon from dehydration in pilot-scale tests. In percentage of coating applied, the coating loss

of chitosan treatments was smaller. The weight of coating lost was shown to increase with the amount of coating applied.

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Chapter 6. Effects of glazing and chitosan-based coating application on frozen salmon preservation during six-month storage at industrial freezing chambers

This chapter is based in the article:

Soares, N. M. F., Oliveira, M. S. G., & Vicente, A. A. (2015). Effects of glazing and chitosan-based coating application on frozen salmon preservation during six-month storage in industrial freezing chambers. *LWT - Food Science and Technology*, *61*, 524–531. http://doi.org/10.1016/j.lwt.2014.12.009

6.1. Introduction

Freezing and glazing are techniques commonly used to reduce the incidence of fish deterioration processes. In order to find an alternative to complement freezing and replace water glazing, the present work aimed at evaluating the effect of water glazing and of 0.5 % w/v and 1.5 % w/v chitosan glazing on quality parameters of frozen fish. Both types of coatings - water and chitosan glazing - were applied directly on frozen Atlantic salmon (*Salmo salar*) and stored for 257 days at -22 °C. Several parameters such as glazing loss, weight loss, drip loss, Total Viable Counts (TVC), Total Volatile Basic-Nitrogen (TVB-N), K-value, pH and colour coordinates *L*a*b** were periodically evaluated in order to compare water glazing with chitosan glazing and uncoated control samples. Samples coated with 1.5 % w/v chitosan performed better in maintaining the colour of the salmon and controlling microbial contamination of frozen and thawed samples.

6.2. Materials and methods

6.2.1 Fish preparation

Frozen and vacuum packaged skinless Atlantic salmon (*Salmo salar*) fillets from aquaculture were 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 6-1) 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 heat 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 (set point -22 °C), for 257 days.

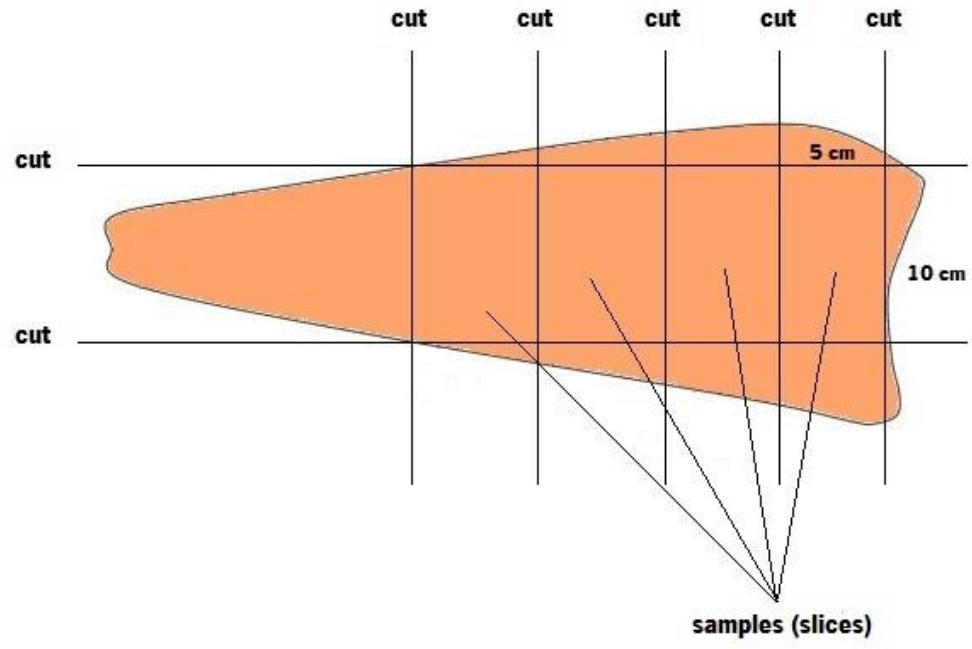

Figure 6-1 - Illustration of the salmon fillet, exemplifying the scheme of cuts used.

6.2.2 Glazing solutions

The chitosan solutions used in this study were prepared using chitosan from Golden-shell Biochemical Co. Ltd. (China) with a 91 % degree of deacetylation. In a 5 L Erlenmeyer a 2 L solution of chitosan (0.50 and 1.5 % w/v) were prepared dissolving 10 and 30 g in a 1 % lactic acid solution (90 % w/w purity) and the volume was completed up to 2 L with distilled water. The solution was stirred with a magnetic stirrer in a heating plate (VWR; Model: VMS-C7 Advanced) at 70 °C, until complete dissolution of the chitosan. The heating was then turned off and the solution remained in agitation overnight. The solution was then transferred to a closed glass container and stored at 8 °C. Distilled water was used for water glazing.

6.2.3 Sample preparation

Samples coated with chitosan

Frozen salmon samples were weighed (W_{salmon}) and divided in two groups: one group of samples was immersed in a 0.5 % w/v chitosan solution at 5.18 ± 0.49 °C during 35 s and another group of samples was immersed in 1.5 % w/v chitosan solution at 8.10 ± 0.57 °C during 10 s. The solution temperature was monitored by an infrared Pronto Plus thermometer (HANNA Instruments, HI765PW and HI99556-10, Romania). Samples were subsequently drained for 2 minutes and weighed again (W_i). The temperatures and dipping times of the different coating solutions are different because they were adjusted to achieve a similar coating uptake in all samples. These experiments were performed in a pilot-scale glazing tank with the help of a stainless steel mesh, used to collect the samples from inside the tank in order to minimize the interference with the amount of coating applied. Following Equation 5-1, the chitosan glazing uptake was calculated, where W_{salmon} and W_i 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 was obtained for chitosan solutions of 0.5 % and 1.5 %, respectively (w/v).

Samples coated with water

A similar process was followed for water glazed salmon samples. These samples were weighed (W_{salmon}), dipped in water at 0.28 ± 0.08 °C for 40 s, drained for 1 minute and weighted again (W_i). Glazing uptake was calculated using Equation 5-1. An average of glazing uptake of 8.4 ± 0.3 % was obtained.

Control samples

Samples from the control group were left untreated. These unglazed samples were used for comparison with the remaining groups of samples.

6.2.4 Sample storage

All salmon samples were individually packed in ziplock polyethylene freezer bags, inside corrugated carton boxes, and stored in an industrial freezing chamber maintained at -21.4 ± 1.6 °C (set point -22 °C), for 257 days. This temperature was monitored using a data logger (DS7922 1Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., USA). All analyses were done in triplicate.

6.2.5 Samples analyses

Glazing loss

After the storage period, coated/glazed samples were weighed again (W_f) and the coating/glazing loss was calculated using Equation 5-2.

Weight loss

The control samples left untreated do not have any coating. In this case, the uncoated samples were initially weighed (W_{salmon}) and after the storage period were weighed again ($W_{salmonf}$) and the weight loss was calculated with Equation 6-1:

$$\%\ \text{Weight Loss} = \frac{W_{salmon} - W_{salmonf}}{W_{salmon}} * 100 \qquad \text{Equation 6-1}$$

Drip loss

To calculate the drip loss, all frozen samples were removed from the freezer, kept for 22 h in the refrigerator at 5 °C, removed from the zip-lock polyethylene bags and placed on a rack for 2 min to release drip, after which thawed samples were weighed. Drip loss was calculated using Equation 6-2, were W_{dripi} indicates the weight of frozen

samples without glazing and before being placed in the refrigerator and W_{dripf} indicates the weight of thawed samples (Sathivel *et al.*, 2007).

$$\% \text{ Drip Loss} = \frac{W_{dripi} - W_{dripf}}{W_{dripi}} * 100 \qquad \text{Equation 6-2}$$

Determination of TVC

Total Aerobic Plate counts were estimated by the procedure based on the BS EN ISO 4833:2003 Standard Protocol (more detail information in section 5.2.5) (ISO, 2013). The Total Viable Counts (TVC) were estimated for frozen (-22 °C) and thawed samples (5.9 °C).

Determination of TVB-N

The value of TVB-N was determined by the method of Conway as described in NP 2930:2009 (IPQ, 2009) and presented in detail in section 5.2.6.

Determination of K-value

K-value was determined according to the method of Ryder (1985) as described by Souza *et al.* (2010). A 5 g 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, Switzerland). After standing in flaked ice for 30 min, the potassium perchlorate that precipitated was removed by filtration using Whatman nº 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 being subsequently analysed by High Performance Liquid Chromatography (HPLC). Twenty microliter aliquots of all samples were analyzed by 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. Separation of the nucleotide products was achieved using a mobile phase of 0.04 mol/L KH_2PO_4 and 0.06 mol/L K_2HPO_4 dissolved at a 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. ATP breakdown products comprising adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino), and hypoxanthine (Hx) were measured, and the K-value was calculated using Equation 3-1 described by Saito *et al.* (1959).

Determination of pH value

After removing the 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 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, Switzerland).

Determination of colour parameters

The instrumental measurement of salmon colour was performed using a colourimeter (CHROMA METER CR-400/410, AQUATEKNICA, SA, Konica Minolta, Japan). Results were expressed using the CIE *L*a*b** system. 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 *ΔE*ab* (Equation 3-2). The samples, with approximately 1 cm of thickness, were evaluated at six different points, 3 points on the right side and 3 points on the left side, to obtain an average colour value within the same sample. The equipment was calibrated using the white calibration plate available from the equipment (Figure 6-2). Salmon samples were stored in a controlled temperature

chamber at -15.8 ± 1.7 °C (set point -18 °C) and they were taken at different time points for evaluation. This temperature was monitored using a data logger (DS7922 1-Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., USA).

6.2.6 Statistical analyses

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).

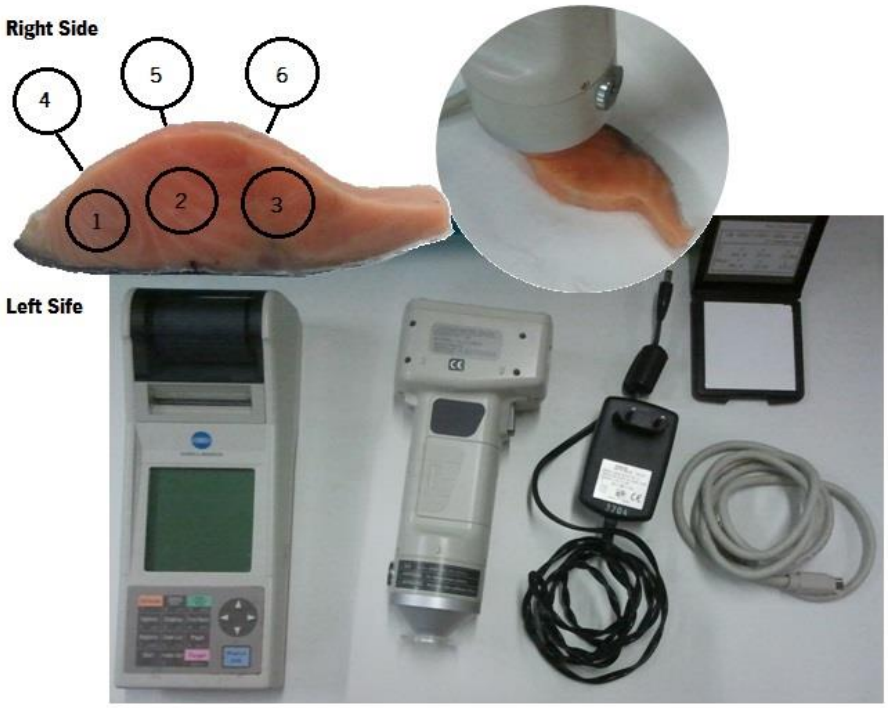

Figure 6-2 - Illustration of the methodology and equipment used in the measurement of the colour of salmon.

6.3. Results and Discussion

6.3.1 Coating loss

The effect of chitosan and water glazing and storage time on the glazing loss of salmon samples during storage at -22 °C is shown in Figure 6-3. The application of both glazing (chitosan and water) presented no statistically significant effect in the initial glazing loss values. Although stable in an initial period, after 13 and 39 days of storage the samples glazed with 0.5 % chitosan showed a tendency to have smaller glazing loss values than simple water glazed samples. This trend was clear in the subsequent period, after 68 and 125 days of storage, when this difference becomes significantly different. Samples coated with 1.5 % chitosan were also tested but only on two occasions: 68 days and 182 days of storage but no statistically significant differences between the two treatments were found.

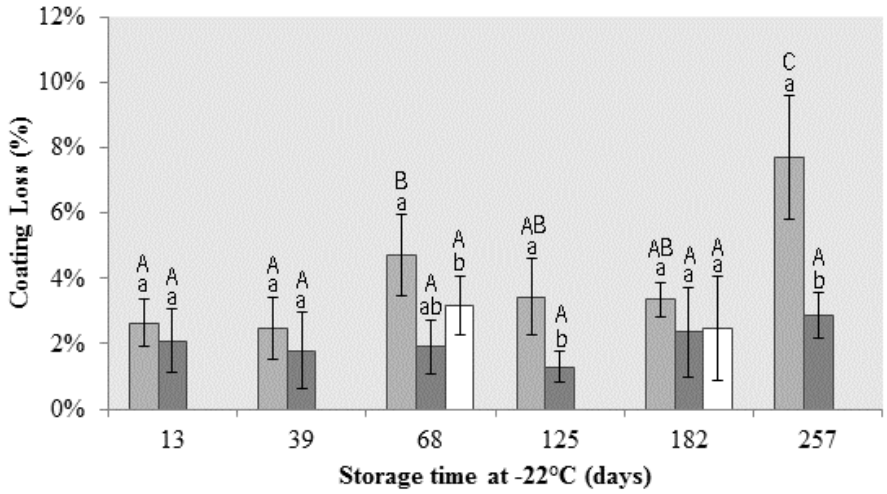

Figure 6-3 - Coating loss (%) for salmon samples glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 6 months of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same colour indicate a statistically significant difference (Tukey test, p<0.05).

The same tendency did not occur when comparing samples glazed with 1.5 % chitosan with samples glazed with 0.5 % chitosan, since the former seem to suffer an equal or

greater coating loss. In the last moment, after 257 days of storage, the glazed samples show a higher coating loss when compared with the other moments and the other coatings, while the samples coated with 0.5 % of chitosan remain stable.

At this moment, although the standard deviation is higher for the simple water glazed samples, it is even more evident the greatest coating loss of the glazed samples comparing with that of samples glazed with 0.5 % of chitosan.

According to Gonçalves *et al.* (2009), the weight loss by dehydration during freezing and storage can be reduced by two methods: covering the surface with packaging material or surrounding the product with a thin layer of ice. Kilincceker *et al.* (2009) add that if the product is subject to inadequate cold storage, glaze will evaporate instead of tissue water itself, thus protecting the product. Also, 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 sublimated. That is, while coatings lose their water by sublimation during storage, they prevent losses of food moisture. A study performed by Soares *et al.* (2013) reports a higher coating loss of frozen salmon samples during storage at -5 °C when compared to losses at lower temperatures. 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 glazing loss during storage, thus increasing fish protection.

6.3.2 Weight loss

The weight loss of salmon samples during storage at -22 °C is shown in Figure 6-4. These values showed no significant differences throughout storage, except at the last moment, where the weight loss value was statistically different from the first two initial moments. Despite being very small, these values show an increasing tendency along the entire storage period, starting at 0.08 ± 0.04 % and ending at 0.20 ± 0.07 %.

Johnston *et al.*, (1994) state 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 of less than 2 °C and due to the fact that all samples, including the control samples without coating, are stored in polyethylene bags, inside corrugated carton boxes, which also act as protection.

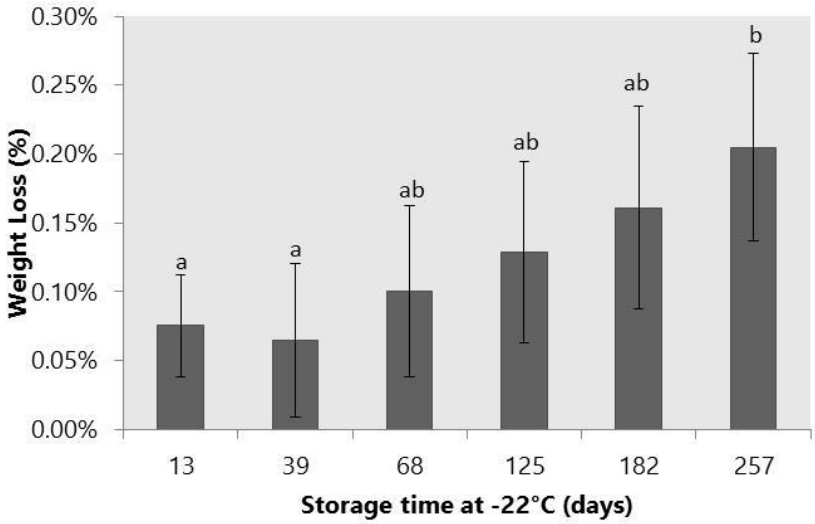

Figure 6-4 - Weight loss (%) of uncoated salmon samples from the control group (■ QS) during 257 days of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different letters indicate a statistically significant difference (Tukey test, $p<0.05$).

6.3.3 Drip loss

The drip loss of salmon samples during storage at -22 °C is shown in Figure 6-5. The different glazings did not appear to interfere in a significant way with the drip loss, since the values do not show statistically significant differences in this case. The initial drip loss value for the control sample (without glazing) was 1.7 ± 0.5 % and the values for these control samples increased throughout storage. Except in the 126 days of storage (for all glazing solutions tested) the amount of drip loss increased steadily over time.

As a whole, drip loss followed a growing trend during storage, increasing significantly (to almost twice) for all samples in the last sampling period. 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.

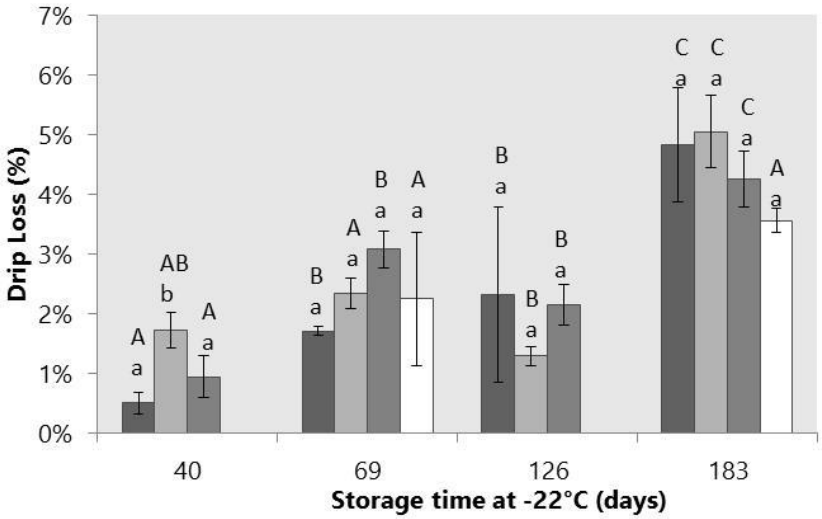

Figure 6-5 - Drip loss (%) of salmon samples from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 183 days of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same colour indicate a statistically significant difference (Tukey test, p<0.05).

6.3.4 TVC

The total viable counts (TVC) for frozen salmon samples during 250 days of storage at -22 °C are presented in Table 6-1. Data show that unglazed samples display values generally greater than glazed samples. The samples glazed with chitosan showed favorable values of TVC when compared with unglazed and simple water glazed samples. While samples glazed with 1.5 % chitosan constantly show values below 10, the same did not happen with samples glazed with 0.5 % chitosan. Both glazings -

water and chitosan - acted in the reduction or maintenance of the microbial load of the frozen samples. However, the samples glazed with chitosan showed the most promising results in microbial protection, especially when 1.5 % chitosan glazed was used. As expected freezing also has been effective since all TVC values, including for uncoated samples, are below the acceptable threshold around 10^7—10^8 CFU/g, which lies at the point of sensory rejection (Ólafsdóttir et al., 1997) and never exceeded the microbiological limit of $5X10^5$ CFU/g recommended by ICMSF (1986) for frozen fish of good quality**.**

Table 6-1 - Total viable counts (TVC) values for frozen salmon samples from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) after 250 days 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
	250	1.70E+03	8.50E+02	1.30E+05	4.42E+04	7.43E+04
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
	250	1.10E+04	7.00E+03	3.30E+03	7.10E+03	3.85E+03
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	-	-
	250	4.40E+02	4.00E+02	2.40E+02	3.60E+02	1.06E+02
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 250 days of storage at -22 °C are presented in Table 6-2. The same trend observed for frozen salmon could be confirmed. Again, all values are below the threshold of rejection and unglazed samples have higher TVC values than glazed samples. The chitosan glazed samples showed lower TVC values than the ones glazed

with simple water. Again, 1.5 % chitosan glazing 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 glazing demonstrated to be effective in protecting thawed samples at refrigeration temperatures (simulating thawing conditions of fish at consumers' homes).

Table 6-2 - Total viable counts (TVC) values for refrigerated salmon samples (5.9 °C) from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) after 250 days of storage at -22 °C; standard deviation corresponds to three replications.

TVC (5.9°C)	Storage Time (days)	Sample 1 (CFU/g)	Sample 2 (CFU/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
	250	1.60E+03	1.40E+03	7.10E+05	2.38E+05	4.09E+05
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
	250	1.00E+04	1.70E+04	5.20E+03	1.07E+04	5.93E+03
Q5	13	<10	1.00E+02	2.50E+02	-	-
	40	<10	2.40E+02	1.50E+02	-	-
	62	<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
	250	8.50E+02	9.30E+02	4.10E+02	7.30E+02	2.80E+02
Q15	62	<10	<10	<10	-	-
	181	<10	<10	<10	-	-

6.3.5 TVB-N

TVB-N is one of the parameters used to evaluated fish freshness, monitor spoilage and assess its quality, through measurements of characteristic volatile compounds (Ólafsdóttir *et al.*, 1997).

The TVB-N values for frozen salmon samples during storage are presented in Figure 6-6. The initial TVB-N value for the unglazed sample was 6.96 ± 1.01 mg Nitrogen/100 g sample. In the first moments, after 13 and also after 69 days, the TVB-N values

suffered no great changes, decreasing just slightly. However, all the samples increased at the last moment, after 188 days of storage. The same is true for the samples glazed with 1.5 % chitosan. 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 particular effect of 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 (Commission of the European Communities, 1995). It is also important to note that the storage temperature was a relevant factor, being able to inhibit changes in volatile compounds produced by bacteria and consequently maintaining the TVB-N values stable, during 188 days of frozen storage.

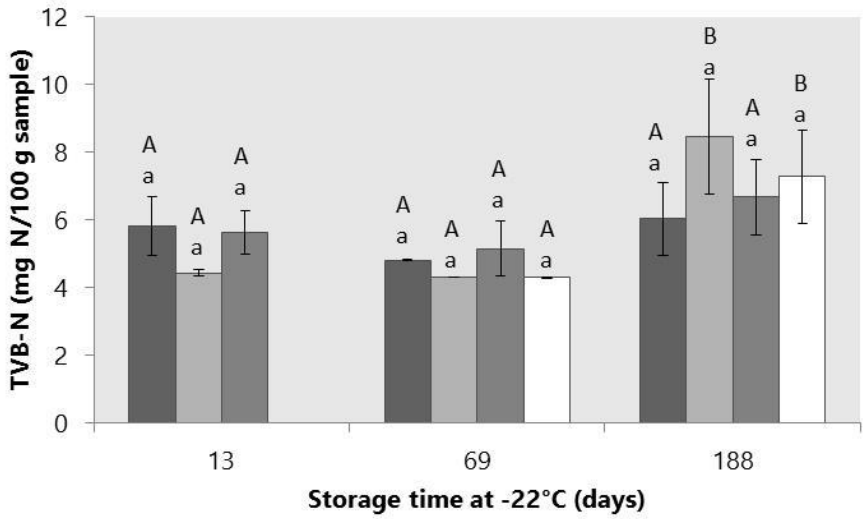

Figure 6-6 - Total volatile basic nitrogen (TVB-N) values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 188 days of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same colour indicate a statistically significant difference (Tukey test, $p<0.05$).

6.3.6 K-value

The K-value is used as an index for 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 (Souza *et al.*, 2010).

The K-values for frozen salmon samples, during 258 days of storage, are presented in Figure 6-7. 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 majority of K-values are low, never exceeding 67 %. However, it was expected that the samples could exhibit high K-value results, since they came from a processed product from 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. So it can be stated that the salmon samples indicate an adequate 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.

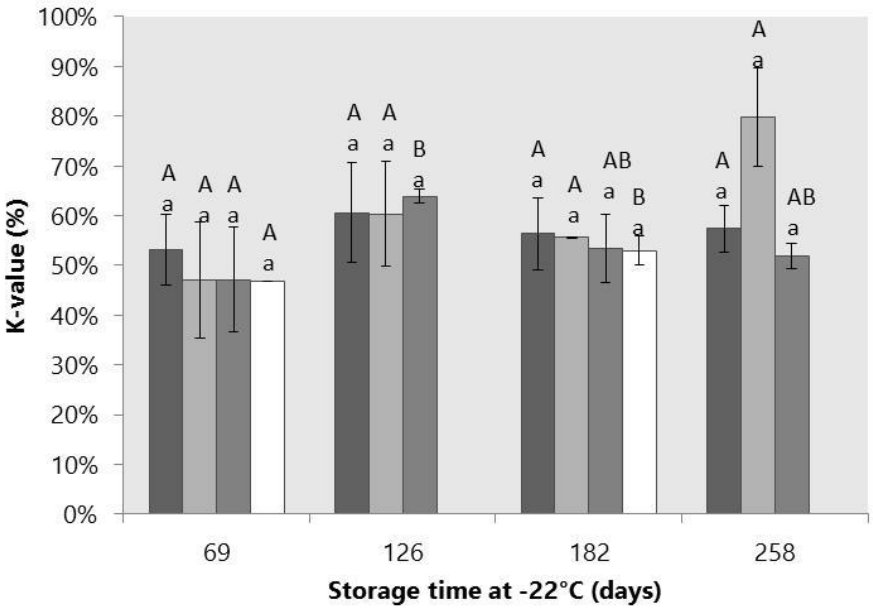

Figure 6-7 - K-values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 258 days of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same colour indicate a statistically significant difference (Tukey test, $p<0.05$).

6.3.7 pH value

pH values during frozen storage are represented in Figure 6-8. The initial pH of the uncoated sample was found to be 6.43 ± 0.05. After 13 days, the pH of all samples increased. 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).

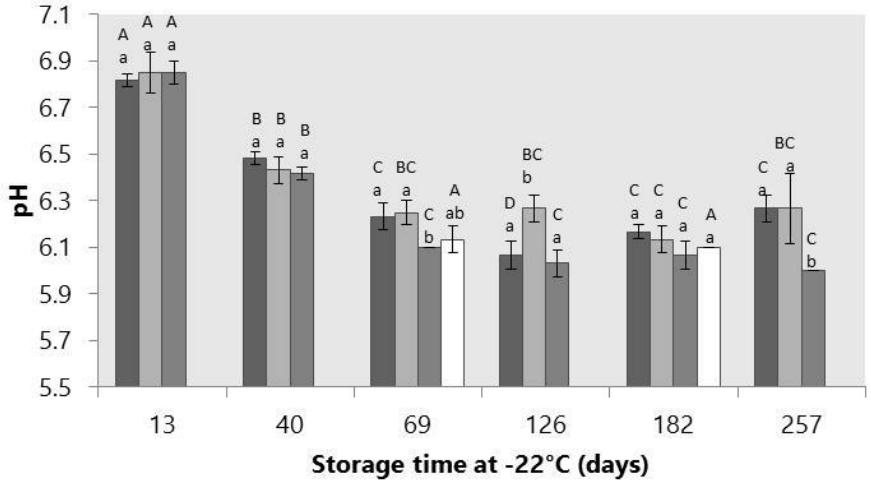

Figure 6-8 - pH values for salmon samples from the control group (■ QS), glazed with water (□ Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 257 days of storage at -22 °C. Each bar represents the mean ± standard deviation of three replications. Different small letters in the same day and different capital letters in bars with the same colour indicate a statistically significant difference (Tukey test, $p<0.05$).

However, at the last moment, after 257 days of storage, the pH of the samples glazed with 0.5 % chitosan was significantly lower than the values of the unglazed and glazed samples. This may indicate that at long-term, the chitosan coating will assist in the stability maintenance of the pH value. According to Rodriguez-Turienzo *et al.* (2011), freezing causes 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 values of all the samples were not higher than the limit of 6-6.5, recommended by Kilincceker *et al.* (2009), with the exception for the first sampling moment (13 days). Similar results were reported in a study conducted by Sathivel *et al.* (2007) during 8 months at -35 °C, where the

uncoated samples showed a pH of 6.6 ± 0.1, glazed samples a pH of 6.5 ± 0.1 and samples glazed with chitosan a pH of 6.4 ± 0.1.

6.3.8 Colour parameters

As is commonly known salmon has natural colour variations. For this reason, it was not possible to compare the colour match between different coatings, since each sample group was obtained from different salmon fillets. These variations would greatly affect the colour parameters during storage, which would invalidate conclusions. Therefore, it was only evaluated the variation of the colour parameters for each group of samples - unglazed, glazed with simple water and glazed with chitosan solution - over time, separately.

The results for $L^*a^*b^*$ obtained during the experiment did not present significant variations or any kind of trend.

Figure 6-9 shows the variation in perceived colour differences of the salmon samples during storage (ΔE^*ab). A large variability of the values is noticeable, especially for the samples coated with 0.5 % chitosan. This may have been due to the higher colour coordinates 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 storage, these samples showed greater stability presenting, for the majority of time, the lowest values of ΔE^*ab, even below the acceptable thresholds of 2 (Cruse, 2015). For this reason, this coating may be the one that better protects fish colour, since this was the coating who caused minor colour differences when applied on frozen salmon samples and consequently being better accepted by consumers.

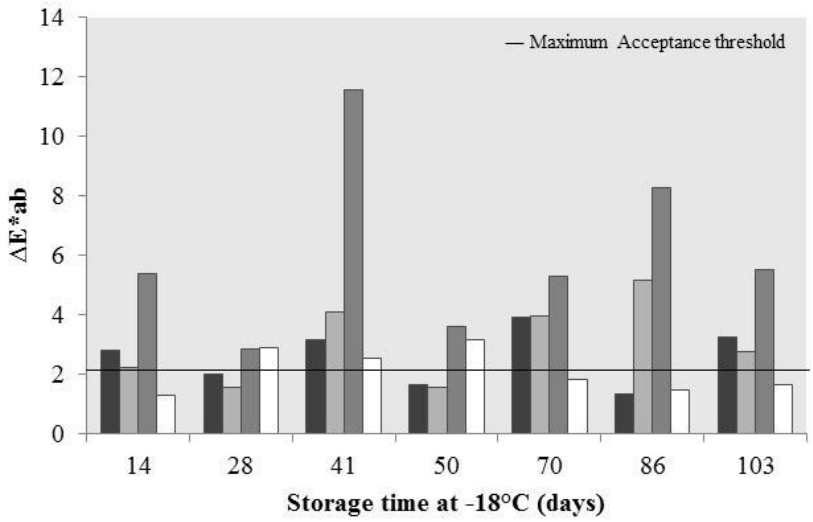

Figure 6-9 - Δ*E*ab* values for salmon samples from the control group (■ QS), glazed with water (□Q0) and glazed with 0.5 % chitosan (■ Q5) and 1.5 % chitosan (□ Q15) during 103 days of storage at -18 °C.

6.4. Conclusions

One of the main conclusions of this work was the effective protection of the 1.5 % chitosan coating in maintaining the colour of salmon and in the control of microbial activity. In fact, the samples coated with 1.5 % chitosan showed less perceptible colour differences and provided a consistent protection for both - frozen and thawed samples - against microbiological contamination. Thus, this coating is a viable alternative to simple water glazing, not affecting the colour perception of quality of consumers.

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Chapter 7. Effects of variables on the thickness of an edible coating applied on frozen fish – establishment of the concept of safe dipping time

This chapter is based in the article:

Soares, N. M., Fernandes, T. A., & Vicente, A. A. (2016). Effect of variables on the thickness of an edible coating applied on frozen fish - Establishment of the concept of safe dipping time. *Journal of Food Engineering*, *171*, 111–118. http://doi.org/10.1016/j.jfoodeng.2015.10.016

7.1. Introduction

Glazing is a technique used to retard fish quality loss during storage. This work focuses on the study of distinct variables (fish temperature, coating temperature, and dipping time) that affect the thickness of edible coatings (simple water glazing and 1.5 % chitosan glazing) applied on frozen fish. Samples of frozen Atlantic salmon (*Salmo salar*) at -15, -20, and -25 °C were either glazed with water at 0.5, 1.5 or 2.5 °C or with 1.5 % chitosan solution at 2.5, 5 or 8 °C, by dipping during 10 to 60 s. For both water and chitosan glazing, lowering the salmon and coating solution temperatures resulted in an increase of coating thickness. At the same conditions, higher thickness values were obtained when using chitosan (max. thickness of 1.41 ± 0.05 mm) compared to water (max. thickness of 0.84 ± 0.03 mm). Freezing temperature and crystallization heat were found to be lower for 1.5 % chitosan solution than for water, favouring phase change. Salmon temperature profiles allowed determining, for different dipping conditions, whether the salmon temperature was within food safety standards to prevent the growth of pathogenic microorganisms. The concept of safe dipping time is proposed to define how long a frozen product can be dipped into a solution without the temperature raising to a point where it can constitute a hazard.

7.2. Materials and methods

7.2.1 Fish preparation

Frozen skinless fillets of Atlantic salmon (*Salmo salar*) from aquaculture with proximally 1 kg (provided by Vanibru - Comércio de Produtos Alimentares, Braga, Portugal) were carefully and evenly cut in a parallelepipedic shape (Figure 7-1) with a vertical bone sawing machine (FK 32, BIZERBA, Germany) in a refrigerated room, where the temperature did not exceed 8 °C. The dimensions of the salmon pieces were approximately 6 cm x 2 cm x 2.5 cm and presented an average weight of 26.4 ± 3.4 g

for chitosan coating tests and 29.42 ± 2.01 g for water glazing tests. These samples were stored in a freezer with pre-established and fixed temperatures of -15, -20 or -25 °C, and were stabilized at least 24 h in those conditions prior to use.

Figure 7-1 - Salmon samples evenly cut.

7.2.2 Glazing solutions

The chitosan solution was prepared at 1.5 % w/v. In a 2-L flask, 22.2 mL of 1 % v/v lactic acid (90 % w/w purity) were added and the volume was made up with distilled water. Then the flask was placed in a heating plate at 45 °C under agitation. Slowly, 30 ± 0.1 g of chitosan (from Golden-shell Biochemical Co. Ltd. China, with a 91 % deacetylation degree) were added and stirred. This solution was left overnight under stirring to complete dissolution. After cooling, the chitosan solution was transferred to a closed glass container and stored in the refrigerator at 2.5, 5 or 8 °C.

In the case of glazing with simple water no preparation was required and destilled water was used.

7.2.3 Glazing application

Samples of frozen salmon were weighed (RADWAG WLC 6/A2/C/2, Poland) and subsequently immersed in the chitosan /water glazing for 10, 20, 30, 40, 50 or 60 s and left to drain for about 180 s. Then salmon was reweighed and the amount of glazing was calculated using Equation 5-1, where $W_{isalmon}$ is the weight of salmon before and W_i is the weight of salmon after application of the coating. All procedures were performed in triplicate. After coating application the salmon samples were packed in numerated zip-lock polyethylene bags and stored at -25 °C for at least 24 h.

Table 7-1 displays the various combinations of temperatures of salmon and temperatures of glazing tested. The solution temperature was monitored by an infrared Pronto Plus thermometer (HANNA Instruments, HI99556-10, Romania) with the respective probe (HANNA Instruments, HI765PW, Romania). The salmon temperature was monitored by a data logger (DS7922 1Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., USA) stored in the industrial freezer together with salmon samples.

Table 7-1 - Glazing and salmon temperatures of the tests carried out in the present work.

	Coating temperatures (°C)	Salmon temperature (°C)
Chitosan	2.4 ± 0.11 ; 4.84 ± 0.27 ; 7.75 ± 0.49	-11.90 ± 0.33
Water	0.51 ± 0.06 ; 1.58 ± 0.09 ; 2.53 ± 0.07	-17.64 ± 0.70 -22.42 ± 0.76

7.2.4 Salmon samples images

The salmon pieces described in 7.2.1 were cut into thinner slices with only a few millimeters wide using the same cutting conditions (temperature and equipment) from

sample preparation. These samples were placed in numbered zip-lock polyethylene bags and stored in the freezer at -25 °C to stabilize the temperature. Subsequently the samples were quickly transported to the laboratory and placed in a ultrafreezer (Cryocell DD86-750P) at -80 °C for at least 24 h.

Each salmon sample was placed in the center of an OLYMPUS magnifying glass plate (OLYMPUS SZ-CTV, Japan) and photographed with the 0.67 magnification using the program "Image-Pro Plus" (op+I). This allowed measuring the coating thickness using the appropriate calibration of the optical system. The coating thickness was measured at various points (Figure 7-2) and the process was repeated for the remaining samples.

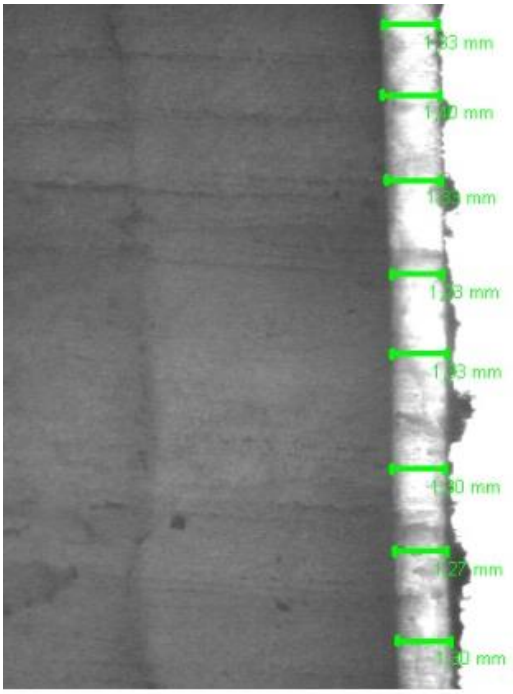


Figure 7-2 - Salmon surface after glazing and corresponding measurements.

7.2.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a PYRIS Diamond DSC (Perkin Elmer, USA). About 30 mg of 1.5 % w/v chitosan solution was placed in aluminium DSC pans. The samples were heated from -30 to 50 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere.

7.2.6 Determination of temperature profile

The temperature profile of salmon was determined using Equation 7-1 (Crank, 1975), which represents the variation of the salmon final temperature from the center to the surface.

$$\frac{T-T_W}{T_0-T_W} = \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\cos\left(\frac{(2n+1)\pi.x}{2L}\right)\exp\left(-\frac{\alpha.(2n+1)^2\pi^2 t}{4L^2}\right) \quad \text{Equation 7-1}$$

Where: T (K) = temperature at the coordinate x, T_w (K) = surface temperature, T_0 (K) = initial temperature, x (m) = depth, L (m) = half-thickness and α (m^2s^{-1}) = thermal diffusivity.

From this profile, it was possible to calculate the average temperature (T_{av}) of salmon for each dipping time, by calculating the integral of the curve corresponding to the representation of T versus x. This representation was made using a 2nd order polynomial fit, which allowed calculating the average temperature using Equation 7-2:

$$T_{av} = \frac{\int_{x_1}^{x_2} T\,dx}{\Delta x} \quad \text{Equation 7-2}$$

7.2.7 Determination of heat transferred

The heat received by salmon samples upon immersion in the coating was calculated using Equation 7-3, where Q_{salmon} (J) is the heat transferred to salmon; m_{salmon} (kg) is the initial salmon mass; $C_{p\ salmon}$ (J $kg^{-1}K^{-1}$) is the heat capacity of salmon; T_{av} (K) is the salmon average temperature; and T_i (K) is the salmon initial temperature:

$$Q_{salmon} = m_{salmon}\,C_{p\ salmon}\,(T_{av} - T_i) \quad \text{Equation 7-3}$$

7.2.8 Statistical analyses

The tests conducted for each set of parameters were performed in triplicate. For each triplicate five measurements of thickness were made, which resulted in 15 thickness values for each condition. The data were subjected to one-way analysis of variance (ANOVA) using STATISTICA 10 software (StaSoft Inc. 2013), while average values were compared by Tukey's test with the level of significance set at $p<0.05$.

7.3. Results and Discussion

7.3.1 Differential scanning calorimetry

Differential scanning calorimetry was used to determine the freezing point of the chitosan solution and its crystallization heat. The results of heat flow (Figure 7-3) regarding a 1.5 % w/v chitosan solution showed the following properties: at a freezing temperature of -0.51 ^{o}C chitosan presented an enthalpy change of 250.23 J g^{-1}, while for water an enthalpy change of 334 J g^{-1} at the freezing temperature of 0 ^{o}C was determined (both measured under atmospheric pressure).

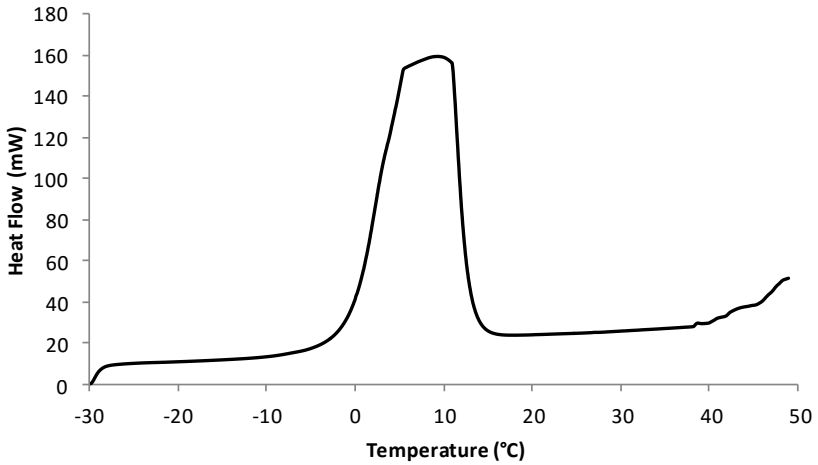


Figure 7-3 - Heat flow variation of a sample of chitosan heated from -30 to 50 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere.

7.3.2 Thickness of coatings from chitosan solutions

The application of chitosan solutions at 2.5, 5 and 8 °C on frozen fish at -25, -20 and -15 °C with different dipping times (10, 20, 30, 40, 50, 60 s) resulted in different coating thicknesses. The final thickness for each set of parameters was measured through the pictures taken to the corresponding salmon samples. The variation of coating thickness with dipping time is represented in Figure 7-4. These results show that for salmon at -25 °C, thickness increases with higher dipping time and lower coating temperature. For short dipping times (10 to 30 s), the thickness for the temperatures of 8 and 5 °C are similar and did not compensate the energy spent to lower the chitosan solution temperature. However in the case of 2.5 °C, the opposite is verified and the thickness for these conditions is consistently higher. This can be explained by the lower temperature of the coating solution (2.5 °C), which is closer to chitosan's freezing point, thus requiring a lower quantity of energy for the phase change (liquid to solid). As chitosan solution temperature drops, specific heat also decreases (less heat is required to lower the temperature) which makes the temperature of 2.5 °C more effective from a heat exchange point of view (Fellows, 2000). For longer dipping times (60 s), the coating thickness values obtained for 2.5 and 5 °C are statistically similar, reaching a maximum of 1.41 ± 0.05 mm, while at a temperature of 8 °C, lower thickness values were attained (1.24 ± 0.03 mm).

Using the same conditions of chitosan solution temperatures and dipping time, an increase of the salmon temperature in general decreases the final thickness of the coating. This can be explained based on the fact that the difference between the temperature of the salmon and that of the coating is smaller, therefore there is a lower amount of energy (in the form of heat) being transferred from the coating to the salmon. However, for longer dipping times the differences between the thickness values obtained for the three chitosan temperatures are progressively reduced, since at 60 s there is no statistically significant difference between all thickness results. In these conditions (salmon temperature of -20 °C), for the longest dipping time (60 s) the maximum value of thickness obtained was 1.08 ± 0.03 mm, a significantly lower result comparing with the salmon at -25 °C that was 1.41 ± 0.05 mm.

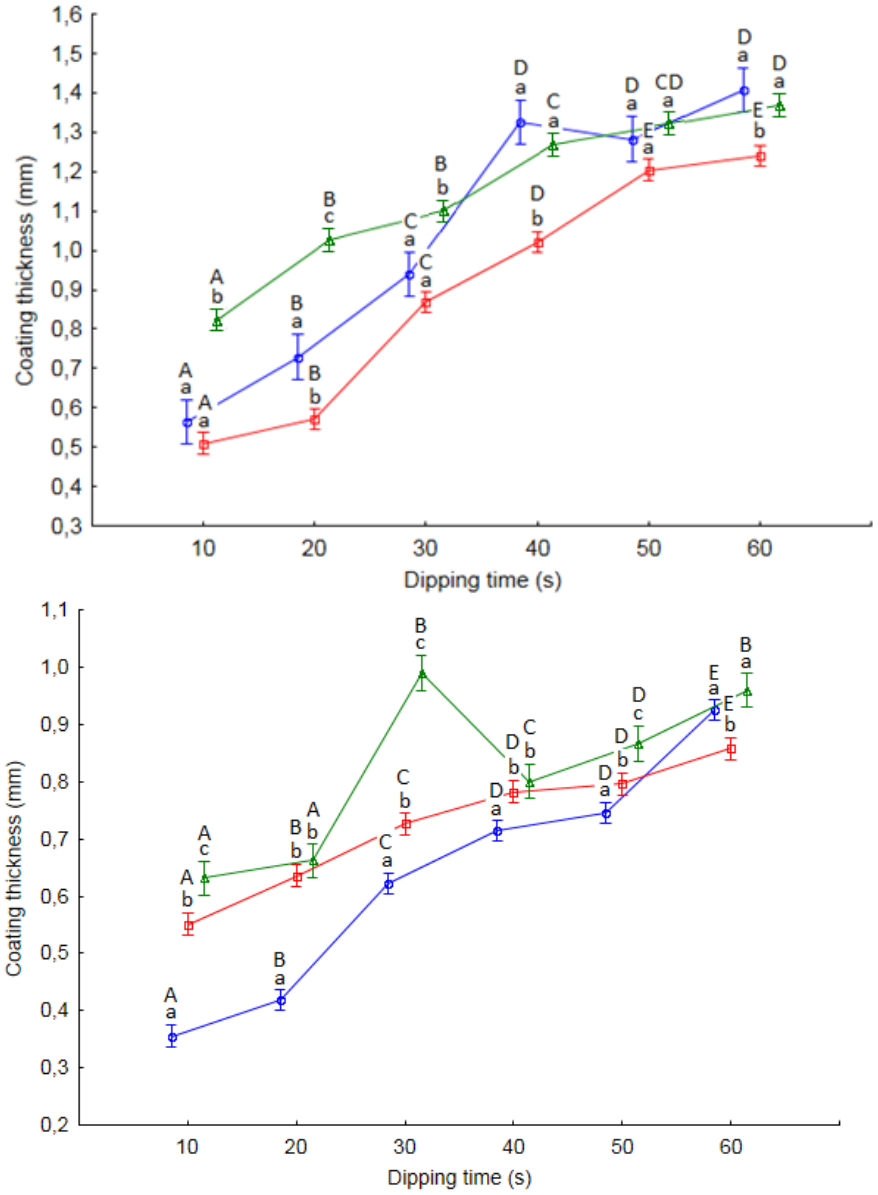
The results for salmon at -15 °C confirm the expected trend. A dipping time of 10 s together with a coating temperature of 8 °C yielded the smallest thickness of all chitosan coatings (0.36 ± 0.03 mm), being considerably smaller than the 0.55 ± 0.02 mm and 0.63 ± 0.03 mm achieved for coating temperatures of 5 °C and 2.5 °C, respectively. For longer dipping times (40 to 60 s) the difference between coatings thickness values is reduced.

A study performed by El-Hefian *et al.* (2010) showed that as chitosan solution temperature rises, the correspondent viscosity falls. A higher viscosity can assure a superior adhesion between salmon and chitosan solution leading to a higher final thickness, since it has a greater resistance to movement. The thicknesses obtained for the various chitosan solution temperatures are in agreement with these results, since even though the temperature differences are relatively small, the chitosan solution temperature which presents higher thicknesses is the lowest (2.5 °C).

7.3.3 Thickness of water glazing

The application of water at 0.5, 1.5 and 2.5 °C on frozen fish at -25, -20 and -15 °C with different dipping times (10, 20, 30, 40, 50, 60 s) results in different glazing thicknesses. The final thickness for each set of parameters was determined by measuring the images taken to the glazed salmon samples. The variation of glazing thickness with dipping time is represented in Figure 7-5.

For salmon at -25 °C, the temperature of water of 0.5 °C shows the best overall results for short dipping times. However, for longer times, the difference tends to diminish and for 60 s of dipping time the glazing thickness values obtained at the three different temperatures are statistically similar, reaching a maximum of 0.84 ± 0.03 mm. It is also to be noted that in any case a stabilization or decrease of thickness was observed, therefore 60 s is not the limiting time for the increase of thickness. All the thickness values obtained with water glazing are lower than those obtained with chitosan glazing.



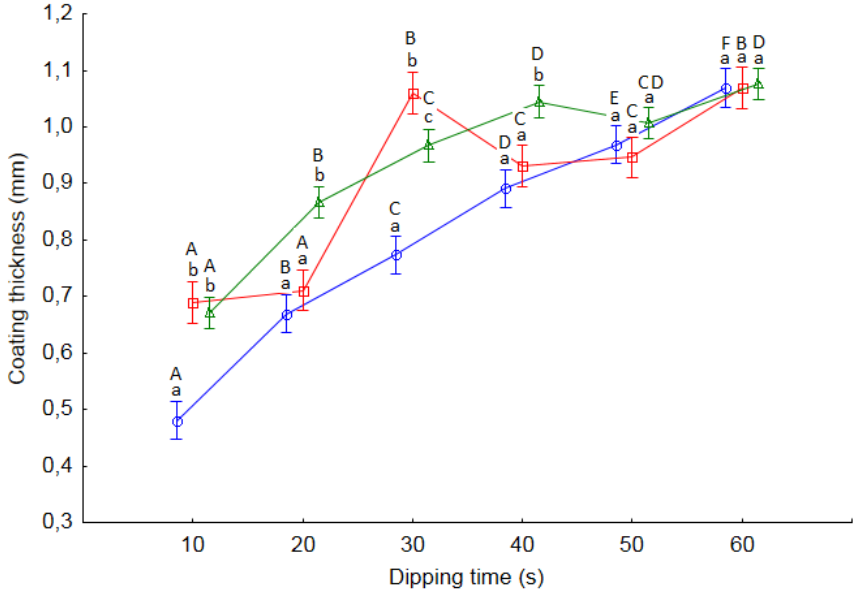


Figure 7-4 - Coating thickness as a function of dipping time for salmon at -25 °C (top left), -20 °C (top right) and -15 °C (bottom left) glazed with chitosan at 2.5 °C (△), 5 °C (□) and 8 °C (○). Each point represents the mean ± standard deviation of fifteen replications. Different small letters in the same dipping time and different capital letters in points with the same colour/marker indicate a statistically significant difference (Tukey test, $p<0.05$).

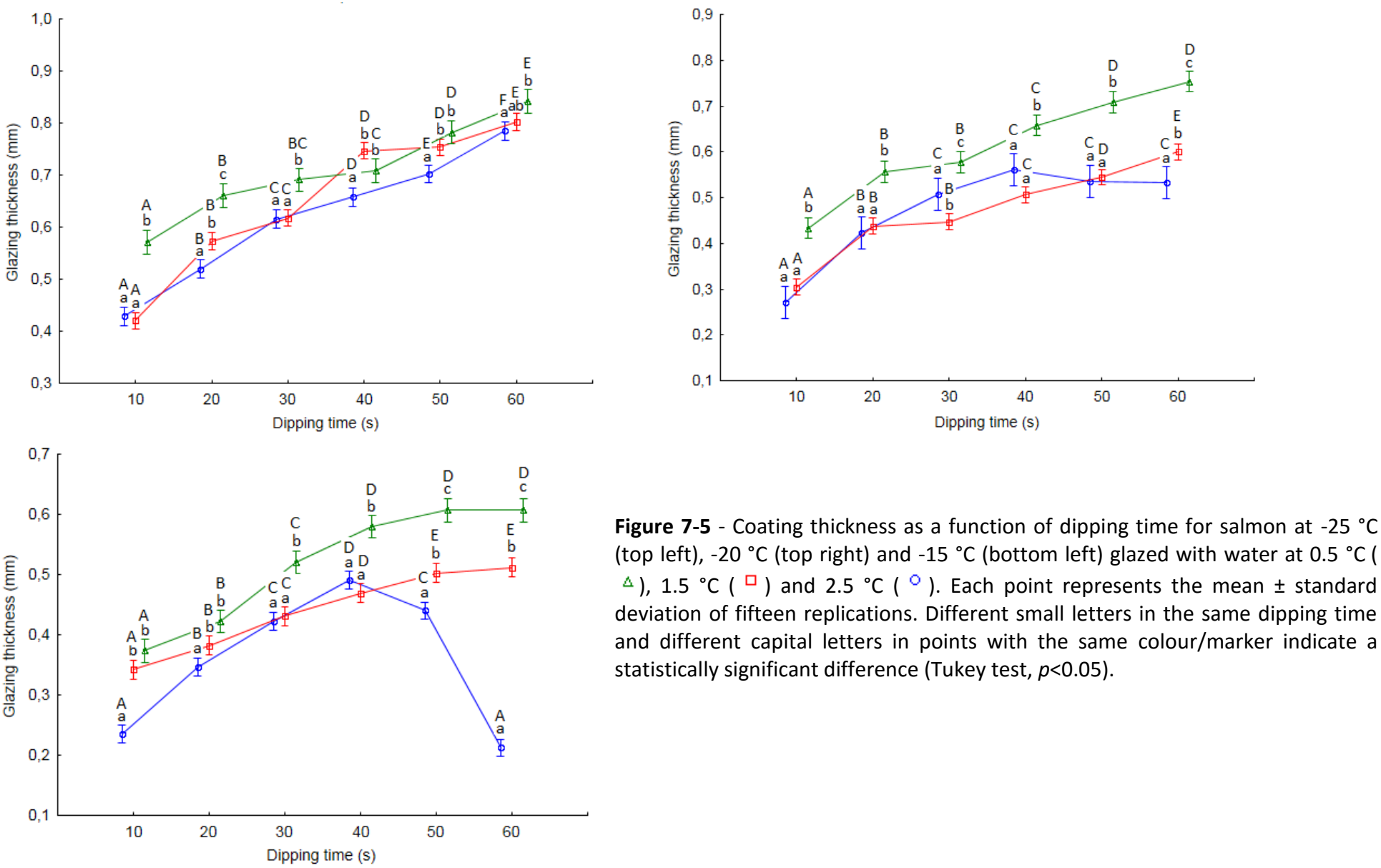


Figure 7-5 - Coating thickness as a function of dipping time for salmon at -25 °C (top left), -20 °C (top right) and -15 °C (bottom left) glazed with water at 0.5 °C (△), 1.5 °C (□) and 2.5 °C (○). Each point represents the mean ± standard deviation of fifteen replications. Different small letters in the same dipping time and different capital letters in points with the same colour/marker indicate a statistically significant difference (Tukey test, $p<0.05$).

With the increase of the salmon temperature to -20 °C the thicknesses of the glazing obtained for various water temperatures decreased. As previously observed with chitosan solutions' coatings, the cases in which this effect is clearer are those of higher water temperatures (1.5 and 2.5 °C). The glazings obtained with water at these two temperatures have very similar thicknesses for all dipping times, with no statistically significant differences between the results. At the temperature of 0.5 °C, despite being the least affected by the increase of salmon temperature, it is also observable a decrease in the final thickness of the glazing, and its maximum value is 0.75 ± 0.03 mm. At water temperature of 2.5 °C the glazing thickness stabilizes after 40 s while at 1.5 and 0.5 °C a 60 s dipping time does not limit the thickness growth.

Using the same conditions of glazing water temperatures and dipping times, the increase of the salmon temperature consistently decreases the final glazing thickness, as expected. When salmon was at -15 °C, the maximum thickness value (0.61 ± 0.02 mm) was achieved for water glazing at 0.5 °C. It was possible to observe that after 40 s, the thickness of glazing at 0.5 and 1.5°C appeared to stabilize, with no significant differences after this dipping time. At the higher temperature of 2.5 °C, the thickness increased up to 40 s of dipping time, decreasing steeply for higher dipping times. Finally, the coating temperature of 2.5 °C may be considered as limiting to salmon temperatures higher than -25 °C because glazing thickness stabilizes after only 30-40 s. Also, since the use of conditions that do not allow an increase in thickness and may effectively lead to an excessive increase in the salmon temperature is not at all advisable, the temperature of -15 °C for salmon can be limiting because after 40 s the thickness is maintained or even lowers.

The thickness values resulting from the application of chitosan and water at the same temperature (2.5 °C) in the same frozen salmon conditions show that for all tested temperatures of salmon the thickness achieved by application of chitosan solution is always significantly higher than that obtained by water glazing. The main reason is the lower heat of crystallization (as mentioned in 7.3.1). As this energy is lower for chitosan solution than for water, a larger amount of chitosan solution changes phase and adheres to the salmon. At a salmon temperature of -25 °C, the maximum thickness reached by the water glazing at 60 s is still lower than the minimum thickness achieved

by chitosan coating at 10 s (Figure 7-6). The same is true for salmon temperatures of -20 and -15 °C, being the maximum thickness of the water glazing always lower than the minimum thickness of the chitosan coating. It can be clearly concluded that chitosan solutions have a greater ability to change phase and adhere to the frozen salmon.

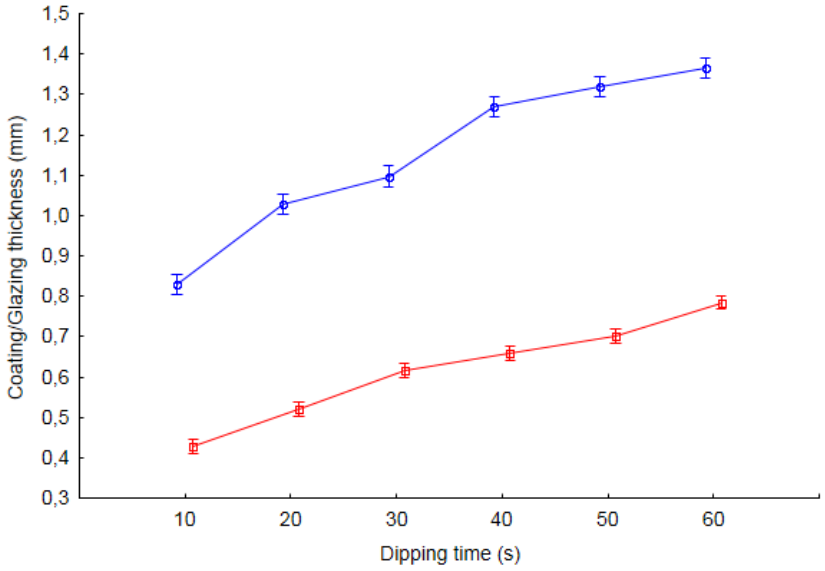


Figure 7-6 - Comparison of glazing thickness variation along dipping time for salmon at -25 °C glazed with water (□) and chitosan solution (○) at 2.5 °C. Each point represents the mean ± standard deviation of fifteen replications.

A final analysis was carried out (Figure 7-7) to compare the best results presented by water glazing (salmon temperature of -25 °C and water temperature of 0.5 °C) with the worst result presented by chitosan glazing (salmon temperature of -15 °C and chitosan temperature of 8 °C). These results show that despite until 30 s of dipping the water presents higher thicknesses, from that dipping time onwards both thicknesses are similar, with chitosan showing a better result for dipping times of 60 s.

The highest operational costs in the frozen fish processing industry come from the use of electrical power required to maintain very low temperatures in glazing baths, cold chambers and equipments used to lower the products' temperature before entering in the production line (*e.g.* cooling tunnels). It can thus be inferred that by replacing common water glazing (where salmon can be kept at -25 °C or below that, and the

glazing water bath is usually at 0.5 °C) by the application of the chitosan coating solution (at 8 °C, which is a common room temperature in this type of industry, with the salmon at -15 °C – regular freezers can be used for this temperature – as tested in the present work), significant energy savings are to be expected. However, when considering an overall strategy, any energetic aspect should be considered along with quality features (*e.g.* lipid oxidation) and their dependence on storage temperature.

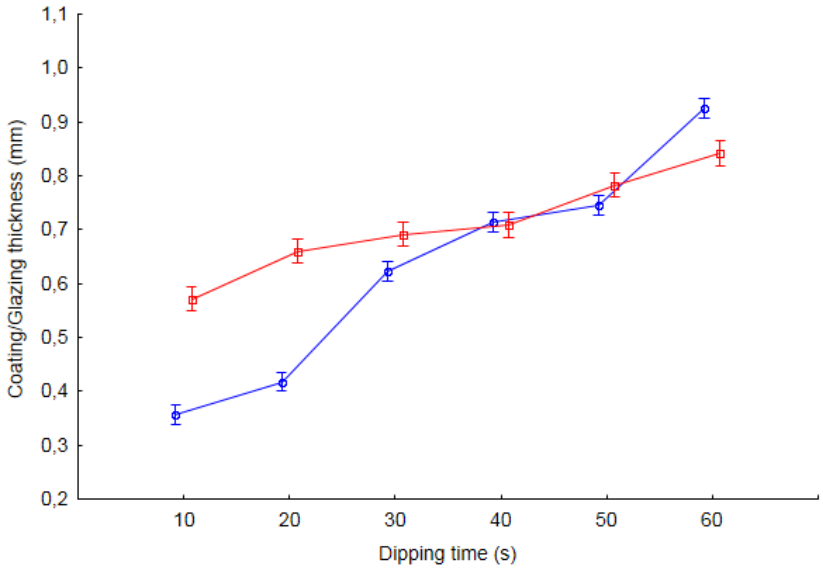


Figure 7-7 - Comparison of glazing thickness variation along dipping time for salmon at -25 °C glazed with water at 0.5 °C (□) and salmon at -15 °C glazed with chitosan at 2.5°C (○). Each point represents the mean ± standard deviation of fifteen replications.

7.3.4 Temperature profiles and the concept of safe dipping time

The temperature of -5 °C is the minimum temperature necessary for growth of pathogenic bacteria associated with fish (FDA, 2011b; Huss *et al.*, 2004; Jay, 2012). In this context, the availability of temperature profiles for frozen salmon is very important in order to determine the salmon temperature at any point for different dipping times and to check if during the glazing process salmon does not exceed the

critical temperature of -5 °C in most of its points. Furthermore, it is important to avoid temperature fluctuations, since causes melting of ice crystals leading to surface dehydration and consequently freezer burns. Every time the temperature drops again, existing ice crystals increase their size, resulting in the loss of fish quality (Fellows, 2000).

During the time that the product is dipped in the glazing solution its temperature raises. A temperature profile is established from the surface to the centre of the product that depends on the solution/product temperature, product thickness and dipping time (besides, of course, the thermal properties of product and surrounding fluid, which cannot be controlled). Temperature is a critical variable for the safety of a food product and therefore we introduced the concept of *safe dipping time* (SDT). SDT can be defined as the maximum period of time that a frozen product may be dipped in a solution until which the (raising) product temperature does not constitute a hazard.

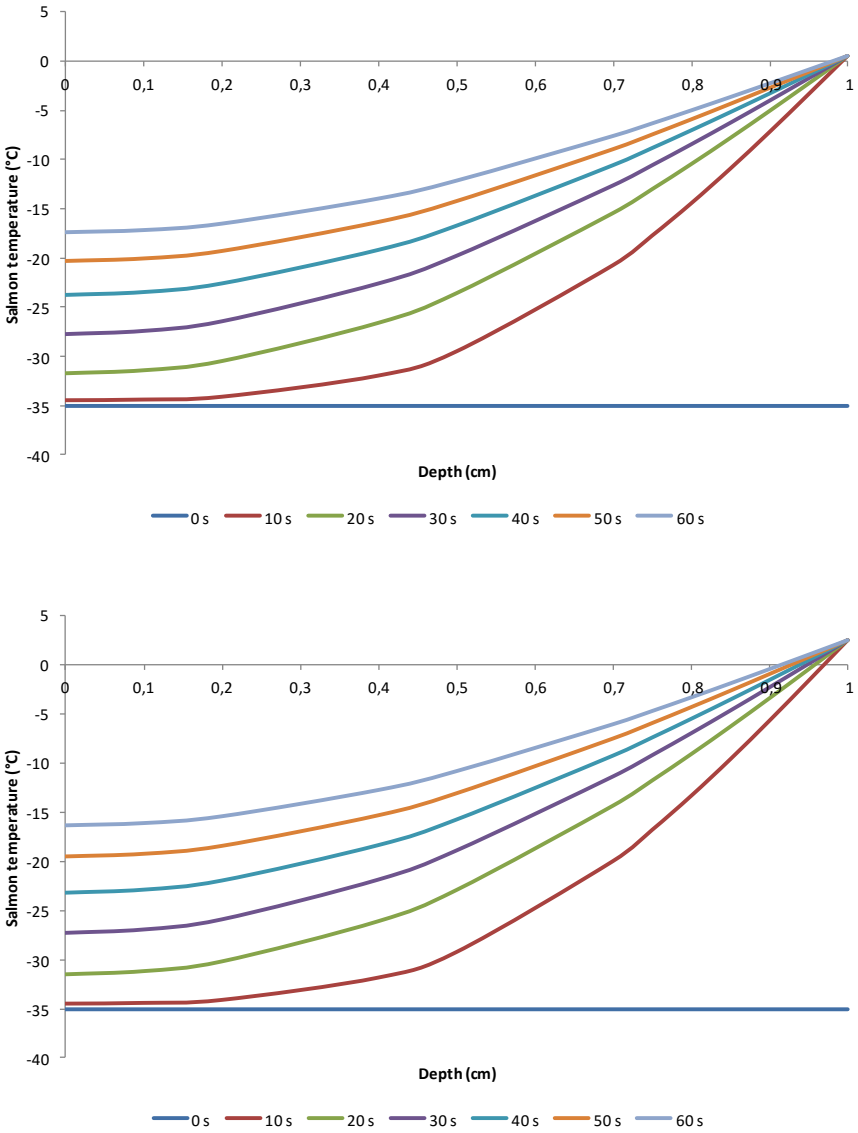
In order to correctly evaluate the SDT it will be necessary to know a) the particular conditions of glazing application and b) the conditions to which the product will subsequently be subjected. Regarding a), during glazing operations in the frozen fish industry it is inevitable that the temperature of some parts of the product (certainly the surface and some volume below it) rise above -5 °C, since glazing solutions are typically between 0.5 and 1.5 °C. This means that it is crucial to know how long it takes to have the whole product below -5 °C again after glazing application, thus ensuring that pathogenic microorganisms will not have conditions to grow, *i.e.* it is crucial to consider also the conditions regarding b): the production room temperature, the time (after glazing) that the product takes to return to a freezing chamber and the freezing chamber temperature, which are the most important factors in that case. The conjugation of a) and b) will determine the safety threshold to be used when calculating the SDT, being such threshold defined in terms of the volume of the product that can be kept above -5 °C during the whole process (*a)* + *b)*). For the particular conditions of this work, it was defined that such threshold would correspond to 20 % of the total samples volume; this value is only an estimation based on the experience and particular conditions of Vanibru, Lda. and has been used just to allow demonstrating how does the SDT concept works. From Figure 7-8 it is possible to check

the salmon temperature in each one of its points after being immersed in water at various temperatures for various dipping times.

In order to simplify the analysis of the temperature profiles of the salmon samples, Table 7-2 and Table 7-3 summarize the theoretical SDT for different salmon and coating temperatures. As expected, the maximum time that salmon may be dipped in both coatings increases with the decrease of salmon temperature and coating temperature. In general, as the temperatures used are lower in the water glazing than in chitosan coating, the safe dipping times are always higher in water.

The definition of safe dipping time for the various dipping conditions allowed the comparison of the maximum coating thickness where the salmon is still within the limits of food safety. Thus the glazing conditions to favour are those that present the longer dipping time and higher temperatures in order to reduce energy costs while continuing to be safe. When comparing the safe dipping time obtained for chitosan solution and water at the same coating/glazing temperature it can be concluded that in both cases this time is 30 s, but the resulting thickness for chitosan coating is much higher reaching 1.10 ± 0.02 mm, while water glazing only reaches 0.61 ± 0.03 mm.

The greatest thickness obtained for water glazing was 0.71 ± 0.02 mm for a salmon temperature of -25 °C, water temperature of 0.5 °C and dipping time of 40 s. In comparison, the application of chitosan solution at 5 °C in salmon at -20 °C for 10 s leads to a thickness of 0.69 ± 0.04 mm. The thickness values obtained in both cases are quite similar, however in the case of chitosan coating both chitosan solution and salmon are at higher temperatures, resulting in energy savings. As for dipping time, a reduction from 40 to 10 s leads to obvious savings in processing time.



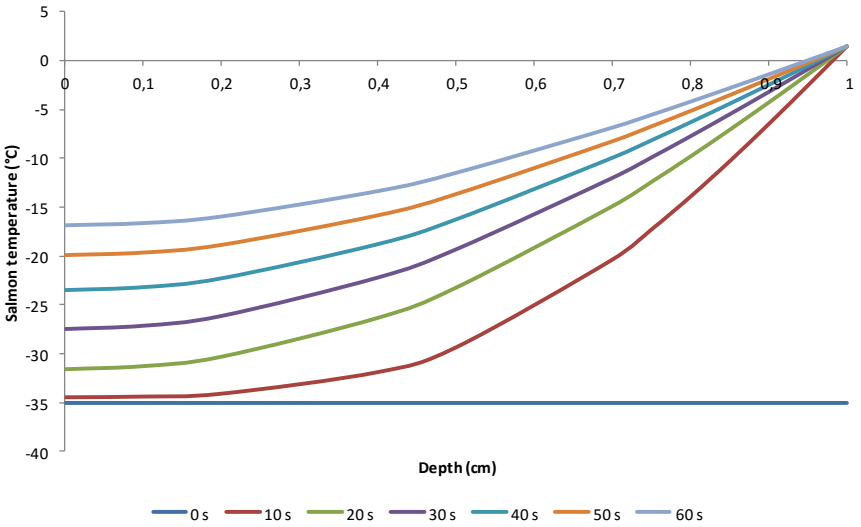


Figure 7-8 - Temperature profile showing the temperature variation from the center (depth = 0) to the surface (depth = 1) of a sample of salmon initially at -35 °C after applying a water glazing at 0.5 °C (top left), 1.5 °C (top right) and 2.5 °C (bottom left). Each curve corresponds to a different dipping time.

Table 7-2 - Maximum theoretical SDT for chitosan glazing.

Salmon temperature (°C)	Chitosan temperature (°C)	Safe dipping time (s)	Thickness (mm)
-25	2.5	30	1.10 ± 0.02
	5	20	0.57 ± 0.03
	8	10	0.56 ± 0.06
-20	2.5	20	0.87 ± 0.03
	5	10	0.69 ± 0.04
	8	10	0.48 ± 0.04
-15	2.5	10	0.63 ± 0.03
	5	5	0.55 ± 0.02
	8	5	0.36 ± 0.02

Table 7-3 - Maximum theoretical SDT for water glazing.

Salmon temperature (°C)	Water temperature (°C)	Safe dipping time (s)	Thickness (mm)
-25	0.5	40	0.71 ± 0.02
	1.5	30	0.62 ± 0.02
	2.5	30	0.61 ± 0.03
-20	0.5	30	0.58 ± 0.02
	1.5	20	0.44 ± 0.02
	2.5	20	0.42 ± 0.04
-15	0.5	20	0.42 ± 0.02
	1.5	10	0.34 ± 0.02
	2.5	10	0.24 ± 0.01

7.4. Conclusions

From the analysis of the coating thicknesses resulting from the application of chitosan solutions on frozen salmon samples it can be concluded that the lower the salmon/chitosan solution temperature used, the higher the final coating thickness: when the chitosan coating temperature was lowered from 8 °C to 2.5 °C there was an 80 % increase in coating thickness (for salmon temperature of -25 °C and 20 s dipping time). It was also possible to conclude that the decrease in coating temperature had a more significant effect for the lower dipping times; in these cases the average increase in coating thickness for salmon at -25 °C (when the chitosan coating temperature was lowered from 8 °C to 2.5 °C) was 51 % for a dipping time of 30 s and only 14 % for dipping times between 40 s – 60 s. Lowering the salmon temperature by 10 °C resulted in an average increase of the coating thickness (considering all coating temperatures) of 40-43 %. Finally, it was observed that thickness always increased with dipping time, being 60 s not a limit to the thickness increase.

The evaluation of thickness resulting from the application of water glazing showed that, when compared with chitosan solution, water alone has less ability to freeze on salmon surface. The values of thickness obtained for salmon at -25 °C and glazing/coating at 2.5 °C shows that after 60 s, the thickness of water glazing is smaller than chitosan coating at 10 s.

The dipping time of 60 s is not a limiting factor for chitosan coated samples, although it was, in some cases, for the water-glazed samples. In the particular case of salmon at -15 °C and water at 2.5 °C, the coating thickness even starts to decrease after 40 s dipping time.

This work introduces two new challenges for a frozen fish industry that aims to be more ethical and safe: one is the evaluation of glazing/coating by its thickness (and not by weight percentage, as it is presently done) aiming at defining objectively the amount of solution that is necessary to guarantee the protection of the product during shelf-life (independently of product shape or operational conditions); the other is the concept of *Safe Dipping Time* that is also related to the process of glazing but until now

somehow is neglected by the industry, that is: how much the temperature of the solution raises the product temperature and if it will be enough to allow pathogenic microorganisms to grow. Further research should be done in these areas in the next years in order to implement the use of these concepts by the frozen fish industry, especially regarding the establishment of a model to determine the safety threshold to be used in the calculation of SDT.

7.5. References

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Chapter 8. Comparing the physicochemical and microbiological effects of glazing and chitosan-based coatings applied on frozen salmon over a 70-day storage period under thermal stress

This chapter is based in the article:

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8.1. Introduction

Maintaining adequate temperature during the entire supply chain of frozen food products is one of the most nuclear objectives to guarantee food safety. Achieving that goal implies large investments of all the organizations in the market. Along the extensive entire supply chain of frozen fish the product is inevitably subjected to temperature fluctuations. There are many loads and unloads, multiple waiting's for control or processing, and mainly the time that the product is in retail stores where the product is more exposed to manipulation and ambient air. Therefore, it was significant to assess how simple water glazing and chitosan coating compare when subjected to temperature oscillation.

The protective properties of a chitosan solution at 1.5 % (w/v) were studied and compared to those of unglazed and water glazed samples, under thermal stress conditions, with temperature varying between -15 ^{o}C and -5 ^{o}C, during 70 days. Assessed parameters included pH, Total Volatile Basic Nitrogen (TVB-N), Total Viable Count (TVC) values, as well as coating loss and colour parameters.

Microbiological analyses showed that chitosan had an anti-microbiological effect on the salmon samples, reducing the number of microorganisms present, while TVB-N values were lower than regulatory standards in EU.

Colour differences and pH values did not present significant differences between different coatings, with the colour difference values increasing with the duration of the test for all samples.

Regarding glazing losses, results show that chitosan glazing offers better protective properties than a water glazing, maintaining 50 % of its coating after 70 days under thermal stress conditions, while water glazed samples lost more than 80 % of their glazing.

8.2. Materials and methods

8.2.1 Fish preparation

Frozen Atlantic salmon (*Salmo salar*) from aquaculture supplied by the company Vanibru – Comércio de produtos alimentares, Braga, Portugal) was used. Each whole salmon was cut in several pieces, with about 2 cm of thickness, using a vertical bone sawing machine (FK 32, BIZERBA, Germany). This process was carried out in a refrigerated room (with temperature between 5 ^{o}C and 8 ^{o}C) in order to reduce temperature uptake and fluctuation in the samples. The samples were separated according to the intended use and intended coating and stored in plastic bags in an industrial freezing chamber (-25 ^{o}C) until further use or transportation.

8.2.2 Coating solutions

The chitosan solutions used were prepared using chitosan from Golden-shell Biochemical Co. Ltd. (China) with a degree of deacetylation of 91 %. A 2 L solution of chitosan 1.5 % (w/v) was prepared in a 5 L Erlenmeyer flask dissolving 30 g ± 0.01 g of chitosan with 22.2 mL of a 1 % lactic acid solution (90 % w/w purity) and the volume was completed up to 2 L with distilled water. The solution was stirred with a magnetic stirrer in a heating plate (VWR; Model: VMS-C7 Advanced) at 70 ^{o}C, until complete dissolution of the chitosan. The temperature control was then turned off and the solution remained in agitation overnight. The solution was then transferred to a closed glass container and stored at 8 ^{o}C.

8.2.3 Sample preparation

Samples of frozen salmon were removed from the industrial freezing chamber and were weighed (RADWAG WLC 6/A2/C/2, Poland), and dipped in a 1.5 % (w/v) chitosan

solution at 8 ^{o}C (measured using an infrared Pronto Plus thermometer (HANNA Instruments, HI99556-10, Romania) with the respective probe (HANNA Instruments, HI765PW, Romania)) during 10 s and then drained for 2 min, before being weighed again and stored in the industrial freezing chamber until further use. The dipping process was performed with a pilot-scale glazing tank, previously built for this effect with a stainless steel mesh. The salmon samples intended for water glazing were weighed before dipping in distilled water for 40 s and then drained for 1 min, before being weighed again and stored in an industrial freezing chamber until further use. The dipping process was performed with the pilot-scale glazing tank and mesh mentioned above. The control samples did not require any additional treatment other than the cutting of the salmon and storage in an industrial freezing chamber.

8.2.4 Samples storage

The salmon samples were stored in an industrial freezing chamber, in which temperature fluctuations between -15 ^{o}C and -5 ^{o}C where forced by an automatic power switch every 6 h, in individual zip-lock polyethylene bags, until further use. The storage temperature of these samples was recorded using a data logger (DS7922 1Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., USA) stored inside the industrial freezing chamber. Samples for physicochemical tests were analyzed in the University of Minho facilities (Laboratory of Industry and Processes) and the samples used for the microbiological/chemical tests were maintained in the industrial freezing chamber under thermal stress until they were sent to laboratory.

8.2.5 Samples analyses

Percentage of glazing

In order to calculate the percentage of glazing, the salmon pieces were weighed before (W_{salmon}) and after (W_i) being dipped. Percentage of glazing or coating was then calculated using Equation 5-1.

Coating and Glazing loss

In the thermal stress experiment the loss of coating and glazing in response to the temperature fluctuation that the salmon samples were suffering was verified. Measurements were performed every two weeks during the first month of the experiment, and every three weeks after the first month, for a total of ten weeks.

Before each measurement, the salmon samples were inspected for ice buildup and, if necessary, ice was removed. The samples were then weighted (W_f), and coating or glazing loss calculated according to Equation 5-2.

Weight loss

During the thermal stress experiment, weight loss was controlled, in order to verify its variation with the temperature fluctuation.

In order to accomplish this, the control samples (without any coating), were weighed in the beginning of the experiment (W_{salmon}).

Then in every moment of analysis, initially every two weeks, and after a month every three weeks, the samples were weighed again ($W_{salmonf}$), and the weight loss was calculated according to Equation 6-1.

Determination of colour

In order to assess the effects of thermal stress in the salmon colour, and the effect of the simple water glazing and chitosan glazing in relation to the uncoated samples, instrumental measures of the colour of the samples were made, using a colourimeter (CHROMA METER CR-400/410, AQUATEKNICA, SA, Konica Minolta, Japan).

The results were obtained in the CIE $L^*a^*b^*$ system, in which the parameters observed were L^* for luminosity (L^*=0 corresponds to black and L^*=100 corresponds to white), a^* (-a^* for green and +a^* for red) and b^* (-b^* for blue and +b^* for yellow) for the colour coordinates.

At the beginning of the experiments, each differently coated sample was used as a control for the respective coating, allowing for the calculation of the ΔE^*ab for the remaining coatings and moments.

Determination of pH

During the thermal stress experiment, pH measurements of the salmon samples were taken. In order to do so the glazing was removed from the samples, and the samples were left to thaw in the refrigerator during 18 h at 4 ^{o}C. After that period, 5 g of the sample were taken and ground in a coffee grinder (Tristar, Netherland). Subsequently, 50 ml of Mili-Q purified distilled water were added to those 5 g of sample. That solution was then shaken in an orbital shaker (Edmund Bühler, Germany) for 30 min at 420 rpm, after which the solution pH was measured using a pH meter (Metrohm 620 pH meter, Switzerland).

Determination of Total Volatile Based Nitrogen (TVB-N)

The TVB-N values for coated and uncoated samples, were determined by the Conway method, as referenced in the NP 2930:2009 standard (IPQ, 2009) and presented in detail in section 5.2.6. The results for all salmon samples, coated or uncoated, were expressed in mg of nitrogen per 100 g of sample.

Determination of Total Viable Count

The determination of Total Viable Count was estimated and performed according to the procedure based on the ISO 4833-1:2013 standard (ISO, 2013) presented in detail in section 5.2.5. Samples of coated salmon, glazed salmon, and uncoated salmon were analyzed in quadruplicate. The results were reported as the number of microorganisms per gram of sample.

8.2.6 Statistical analyses

Experiments were performed at least in triplicate, and in some cases more. The mean values of those independent determinations were calculated for each treatment at every moment. The statistical significance of differences among treatments was evaluated by a factorial ANOVA test followed by the Tukey HSD test with significance at $p<0.05$. Data were evaluated statistically using the software STATISTICA version 10.0 (StatSoft Inc. 2011).

8.3. Results and Discussion

8.3.1 Temperature profile

The temperature profile of the thermal stress tests can be seen in Figure 8-1. In order to collect this data a data logger (DS7922 1Wire® Thermochrom® iButton®, Dallas Semiconductor Inc., USA) was used, stored inside the industrial freezing chamber containing the frozen fish. The temperature fluctuations were created using an automatic power switch. As shown in Figure 8-1, temperature fluctuated generally between -15 ^{o}C and -5 ^{o}C, in the desired interval to conduct the thermal stress test.

8.3.2 Physicochemical analyses

Percentage of glazing uptake

The percentages of glazing uptake obtained for the samples used were 9.9 ± 0.7 % for simple water glazing and 13.6 ± 0.8 % for chitosan glazing. The values for glazing percentages are in line with those reported in previous works, although higher percentages, especially for chitosan, were found (Soares *et al.*, 2015).

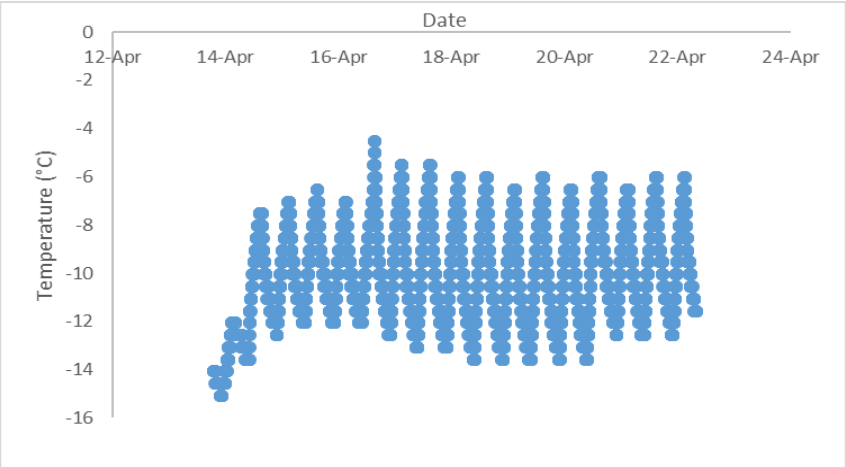

Figure 8-1 - Temperature profile for the first two weeks inside the freezing chamber during the thermal stress test.

pH values

The pH values obtained during frozen storage for 70 days are shown in Figure 8-2. The initial and final values do not seem to reflect a substantial variation for uncoated, water glazed and chitosan coated samples. The type of treatment applied does not seem to show a significant influence on the evolution of the pH values.

The lack of variation of the pH values can possibly be attributed to the duration of the thermal stress test, as in previous works a significant change in the value of pH only occurred after 14 weeks of storage, although under slightly different conditions (Soares *et al.*, 2013). The results also show that the thermal stress endured by the salmon samples has not accelerated changes in their pH values. With the exception of the mean pH value of chitosan-coated samples after 49 days of storage, all samples are within the minimum and maximum recommend limits of 6 and 6.5, found in previous works (Kilincceker *et al.*, 2009).

Colour

In order to try to reduce the normal colour variation in salmon, the same lot of salmon was used for all of the samples; despite of that, variation among samples is still

present, which is clear when observing the Δ*E*ab* value between the control sample and the uncoated sample at the initial moment.

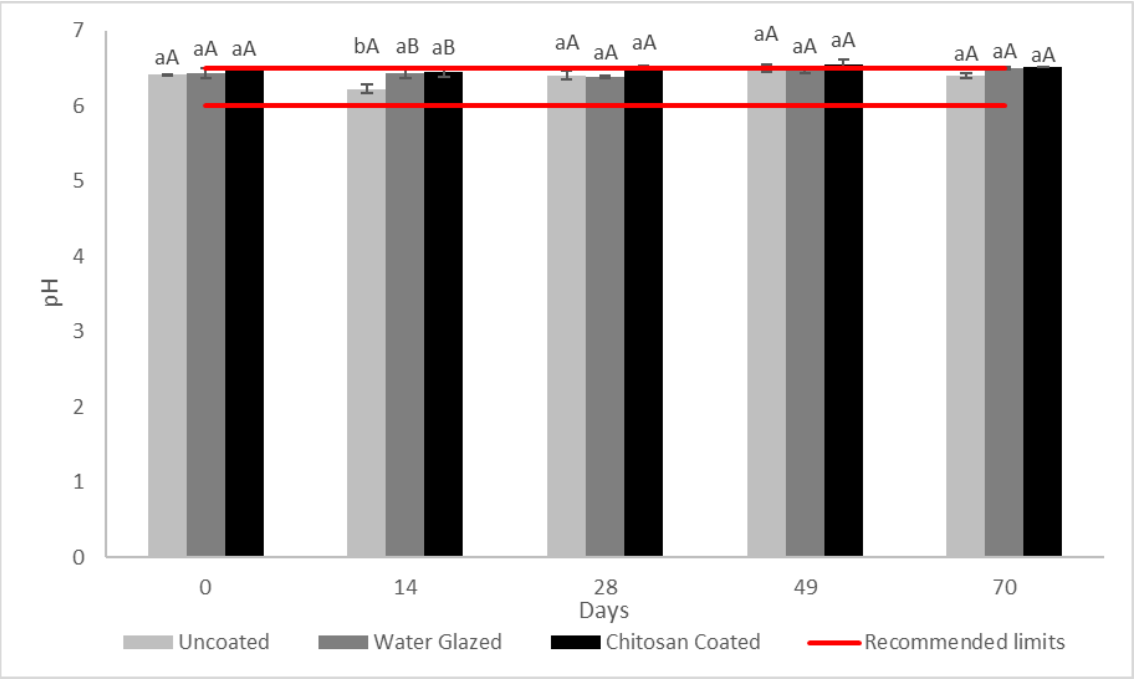

Figure 8-2 - pH values for salmon samples during 70 days of storage between -15 ºC and -5 ºC; standard deviation corresponds to three replications; different lower case letters in the same sample type, and different upper case letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

This is represented in graphs by a dotted line (see *e.g.* in Figure 8-3), which represents the natural differences between samples; such natural differences are quite significant, with similar values to those of perceived differences by an untrained observer. Nevertheless, a comparison between different types of coatings was still tested.

Regarding perceived colour differences, which were calculated as the difference between the assessed sample and a sample with the same coating at the initial moment of the experiment (Δ*E*ab* value), Figure 8-3 shows how that value varied during the 70 days of storage for the differently coated samples. These perceived colour differences follow an increasing trend in almost all of the samples analyzed, which is to be expected as they are the result of longer periods of time subjected to temperature fluctuations.

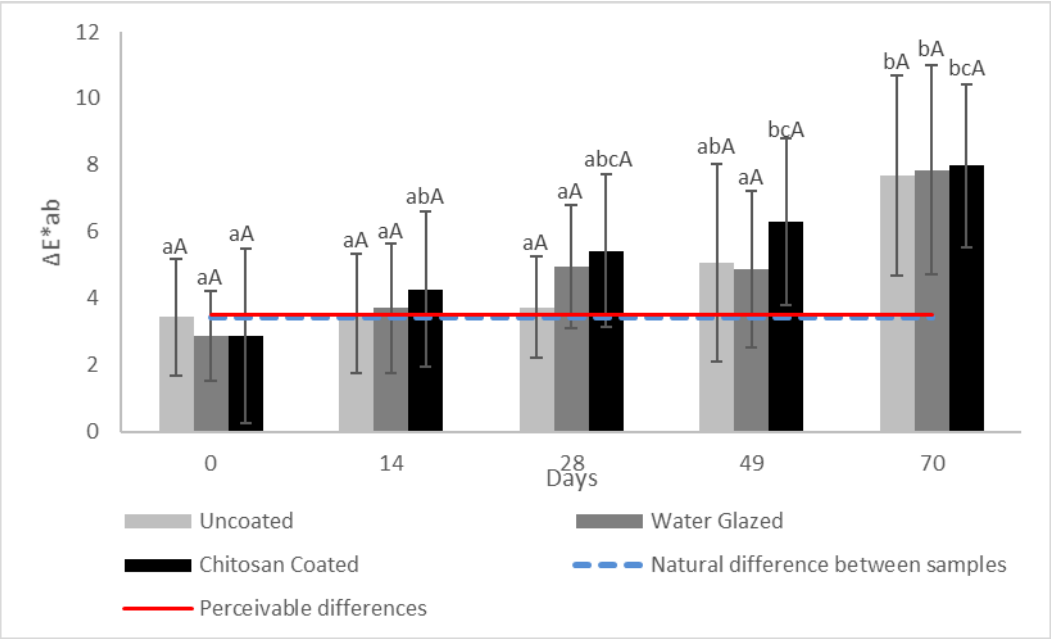

Figure 8-3 - Δ*E*ab* values for salmon samples during 70 days of storage between -15 ^{o}C and -5 ^{o}C; standard deviation corresponds to eighteen replications; different lower case letters in the same sample type, and different upper case letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

Weight, glazing and coating loss

When fish is not protected by a glazing or coating, tissue water will start sublimating promptly from the moment samples are placed at freezing temperatures, leading to a reduction in weight. Figure 8-4 shows the weight loss, in percentage, of the control samples (the uncoated samples) during the 70 days of the thermal stress test. After 70 days of storage, the control samples lost 3.7 ± 1.0 % of their initial weight.

When compared with those found in previous works (Soares *et al.*, 2013, 2015), these results confirm that temperature fluctuation has a great impact in weight lost during frozen storage and reinforce the importance of using a coating that performs better under these conditions.

Figure 8-4 also shows the percentage of water glazing or chitosan coating lost by the salmon samples under thermal stress. The loss of coating or glazing follows an

increasing trend, as it was expected based on previous works, although the order of the values are extremely different due to the temperature fluctuation in this test (Soares *et al.*, 2013, 2015).

In the first 49 days no statistically significant differences were found between glazed samples and chitosan coated samples. In the last period, however, there was a significant difference between chitosan coated (48.5 ± 5.5 %) and water glazed (80.9 ± 11.1 %) samples. Such a difference, especially taking into consideration that almost all of the glazing was lost, indicates that, under thermal stress conditions, the chitosan coating proved to be more effective than the water glazing in protecting the salmon from exposure. One of the possible reasons for the lower loss observed in chitosan coated samples may be related to the rheological properties of chitosan. Its viscosity is significantly higher than that of water and increases with chitosan concentration, which may result in a higher resistance to temperature fluctuations (Hwang & Shin, 2001; Sathivel *et al.*, 2007).

It is also worth noting that while chitosan clearly resists better than water glazing, it is expected that it may not completely protect the entire salmon, due to the fact that the edges and corners are more easily dehydrated. In this sense it is fair to mention that coating salmon with chitosan does not eliminate fish dehydration, but it does help to retard it (Johnston *et al.*, 1994).

Figure 8-4 also displays exponential trend lines that help determining the moment in which water glazing and chitosan coating would completely disappear.

Water glazed samples (left trend line equation) would lose all of their glazing after 80 days of storage under thermal stress, while chitosan coated samples (right trend line equation) would last around 108 days, 28 more days than water glazed samples, which would represent a 26 % increase in shelf-life time (regarding coating loss) under thermal stress conditions.

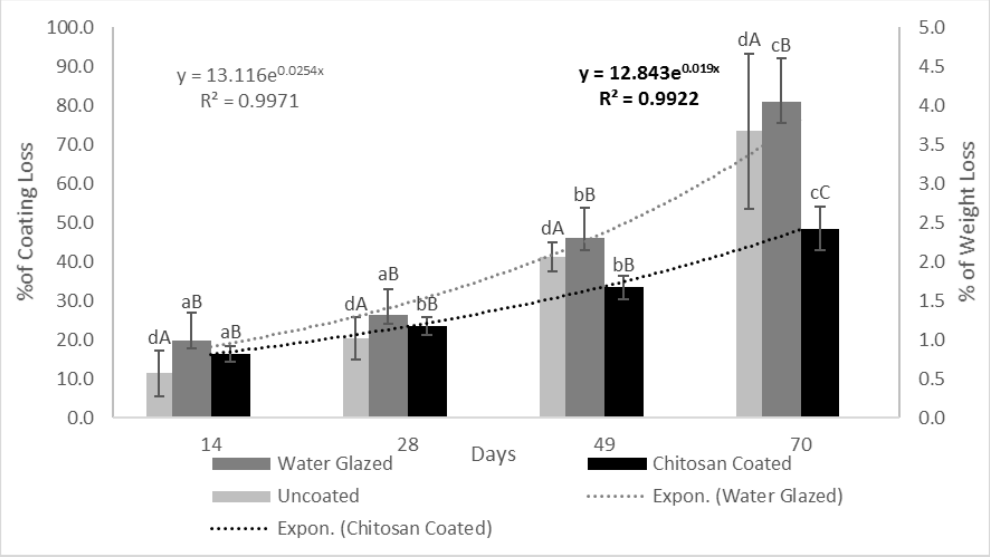

Figure 8-4 - Salmon weight, water glazing and chitosan coating losses of salmon samples during 70 days storage between -15 ^{o}C and -5 ^{o}C; water glazing exponential equation in the left of the graphic, and the chitosan coating exponential equation to the right of the graphic; Chitosan and glazing loss values can be read in the left axis, and weight loss values can be read in the right axis; standard deviation corresponds to four replications; different lower case letters in the same sample type, and different upper case letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

8.3.3 Chemical analyses

TVB-N

Figure 8-5 represents the results of the TVB-N tests performed. The control sample presented a TVB-N value of 11.2 ± 1.3 mg of nitrogen/100 g. Although with a slightly decreasing tendency, the uncoated, chitosan coated and water glazed samples did not vary greatly throughout the 70 days of storage, and did not present statistically significant differences between them. After 70 days uncoated samples present a decreased value of 9.9 ± 0.5 mg of nitrogen/100 g; water glazed samples have a value of 11.1 ± 0.9 mg of nitrogen/100 g at 14 days of storage, decreasing slightly to a value of 8.5 ± 0.8 mg of nitrogen/100 g after 70 days; chitosan coated samples present a value of 9.6 ± 0.9 mg of nitrogen/100 g at 14 days of storage, that decreases to 9.5 ± 0.9 mg of nitrogen/100 g after 70 days of storage. All of these values are well below

the 35 mg nitrogen/100 g fish established as the acceptable limit for salmon by the EU Directive 95/149 (Official Journal of the European Communities, 1995). Previous studies have shown that an increase in the TVB-N values only seems to happen after 90 days of storage, which can explain the lack of differences among the various coatings found in the present work, since the activity of spoilage bacteria and enzymes is slowed down at lower temperatures. Another possible reason for the lack of variation is the initial quality and good condition of the salmon, which is supported by the low TVC values found in next section (Gonçalves & Gindri Junior, 2009).

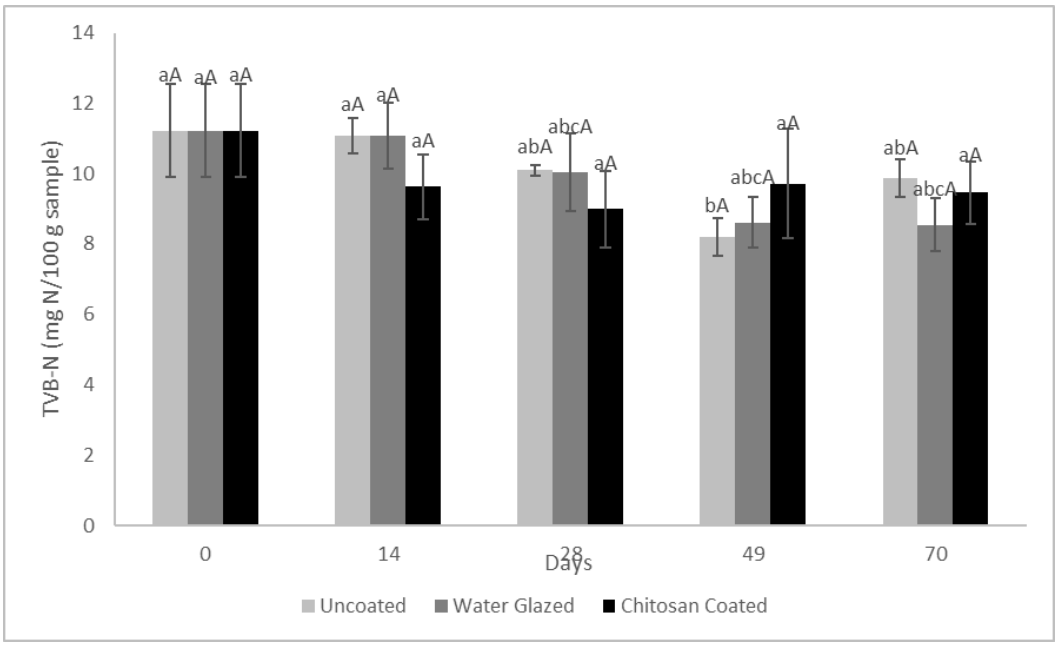

Figure 8-5 - TVB-N values for salmon samples during 70 days of storage between -15 ^{o}C and -5 ^{o}C; standard deviation corresponds to four replications; different lower case letters in the same sample type, and different upper case letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

8.3.4 Microbiological analyses

TVC

Table 8-1 presents the TVC values of salmon samples used and stored during 70 days in an industrial freezer, under thermal stress. TVC values for the uncoated samples

increased with storage time, achieving the highest value of $1.3x10^3$ CFU/g, and a similar behavior was observed for the water glazed samples, with a slight lower value of $9.2x10^2$ CFU/g. All of the values are greatly influenced by the natural variation of TVC values for fish. TVC values for the water glazed and uncoated samples remain at all times clearly higher than the TVC values of the chitosan coated samples. Chitosan coated samples clearly present the best results at all moments, with most of the samples being below the detectable value of the test (below 10 CFU/g).

These data confirms the ability of chitosan to reduce microbial numbers on food surfaces that has been referenced by several authors over the past years (Castro & Paulín, 2012; Raafat & Sahl, 2009; Rabea *et al.*, 2003). Despite the thermal stress endured by the salmon samples used, the TVC values are well below both the maximum limit of 10^7 CFU/g for sensory detection and rejection (Ólafsdóttir et al., 1997) and the microbiological limit of $5x10^5$ CFU/g for quality of frozen fish (ICMSF, 1986).

Table 8-1 - TVC values for frozen salmon samples uncoated, glazed with water and coated with chitosan during 70 days of storage between -15 ^oC and -5 ^oC; standard deviation corresponds to four replications.

TVC -15 ^oC to -5 ^oC	Storage Time (days)	Sample 1 (CFU/g)	Sample 2 (CFU/g)	Sample 3 (CFU/g)	Sample 4 (CFU/g)	Mean (CFU/g)	SD
Uncoated Samples	0	$4.6x10^2$	$6.5x10^2$	$5.6x10^2$	$8.4x10^2$	$6.3x10^2$	140
	14	$8.1x10^2$	$6.0x10^2$	$1.1x10^3$	$5.6x10^2$	$7.7x10^2$	214
	28	$9.5x10^2$	$8.4x10^2$	$9.4x10^2$	$4.5x10^2$	$8.0x10^2$	204
	49	$6.0x10^2$	$1.4x10^3$	$5.2x10^2$	$1.5x10^3$	$1.0x10^3$	447
	70	$2.1x10^3$	$1.3x10^3$	$9.8x10^2$	$9.5\ x10^2$	$1.3x10^3$	464
Water Glazed Samples	0	-	-	-	-	-	-
	14	$6.7x10^2$	$7.3x10^2$	$8.7x10^2$	$7.9x10^2$	$7.7x10^2$	74
	28	$2.5x10^2$	$5.6x10^2$	$8.4x10^2$	$1.2x10^3$	$7.1x10^2$	350
	49	$1.9x10^2$	$3.5x10^2$	$1.8x10^2$	$4.30x10^2$	$2.9x10^2$	106
	70	$9.8x10^2$	$8.2\ x10^2$	$9.7x10^2$	$9.1x10^2$	$9.2x10^2$	64
Chitosan Coated Samples	0	-	-	-	-	-	-
	14	<10	<10	$1.8x10^2$	<10	-	-
	28	<10	$1.2x10^2$	<10	<10	-	-
	49	<10	<10	<10	<10	-	-
	70	<10	<10	<10	<10	-	-

8.4. Conclusions

Results show that, regarding the TVB-N values, neither the moment of assessment nor the type of coating had a significant impact on the results, and all values were found to be under the defined limits despite of the thermal stress conditions. TVC values showed that chitosan not only inhibited the growth of microorganisms but also reduced their number, thus offering an anti-microbiological protection when compared to water glazed samples. No prevailing tendency was found regarding pH values, and there does not seem to be an effect either from the different coatings or from the different moments of testing, once no statistically significant differences could be identified. Almost all pH values are within acceptance levels, even after the thermal stress tests.

In terms of the Δ*E*ab* values no statistically significant differences were obtained. When it comes to the protection of the frozen fish, the chitosan coating offered better results than the water glazed or the uncoated samples. Chitosan coated samples had lower losses of coating than the water glazed samples, with the water glazed samples losing over 80 % of their initial glazing at the end of the test, while the chitosan coated samples only lost less than 50 % of their initial coating. Together with the TVC results, these results point at a significantly more efficient protection provided by the chitosan coating.

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Chapter 9. Comparing the effects of glazing and chitosan-based coating applied on frozen salmon on its organoleptic and physicochemical characteristics over six-months storage

This chapter is based in the article:

Soares, N., Silva, P., Barbosa, C., Pinheiro, R. Vicente, A. A. (2016). Comparing the effects of glazing and chitosan-based coating applied on frozen salmon on its organoleptic and physicochemical characteristics over six-months storage.

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9.1. Introduction

Packaging in its classical approach is a method of providing protection to the food product. From the packaging material should not migrate to the product undesirable properties that could put in risk the safety of the consumer or the organoleptic acceptance of the product.

The effect of a chitosan solution of 1.5 % on the sensory properties of Atlantic salmon (*Salmo salar*) was studied over six months of storage. The sensory properties of the salmon were assessed through the use of a texturometer and a trained panel of judges. Microbiological and chemical parameters were studied in the form of Total Volatile Base Nitrogen (TVB-N) and Total Viable Count (TVC) tests.

Microbiological analysis showed that chitosan had an anti-microbiological effect on the salmon samples, reducing the number of microorganisms present, while TVB-N values were maitained stable during experiment.

Textural Profile Analysis (TPA) was performed and the results showed no significant differences between different coatings regarding texture. Sensory analysis by a trained panel showed that chitosan was a better choice in frozen samples, while in thawed and cooked samples no significant differences existed between chitosan-coated and glazed samples. Flavor diffusion from the chitosan coating was assessed, and analysis of the results showed no correlation between coating type and sample flavor, indicating that no flavor diffusion had occurred.

9.2. Materials and methods

9.2.1 Fish preparation

Frozen Atlantic salmon (*Salmo salar*) from aquaculture supplied by the company Vanibru – Comércio de produtos alimentares, Braga, Portugal) was used. Each whole

salmon was cut into several pieces, with about 2 cm of thickness, using a vertical bone sawing machine (FK 32, BIZERBA, Germany). This process was carried out in a refrigerated room (with temperature between 5 ^{o}C and 8 ^{o}C) in order to reduce the heat uptake and the corresponding temperature fluctuation. The samples were separated according to the intended use and intended coating and stored in plastic bags in an industrial freezing chamber (- 25 ^{o}C) until further use or transportation.

9.2.2 Coating solutions

The chitosan solutions used were prepared using chitosan from Golden-shell Biochemical Co. Ltd. (China) with a 91 % degree of deacetylation. In a 5 L Erlenmeyer a 2 L solution of chitosan (1.5 % w/v) was prepared dissolving 30 g ± 0.01 g with 22.2 mL of a 1 % lactic acid solution (90 % w/w purity) and the volume was completed up to 2 L with distilled water. The solution was stirred with a magnetic stirrer in a heating plate (VWR; Model: VMS-C7 Advanced) at 70 ^{o}C, until complete dissolution of the chitosan. The temperature was then turned off and the solution remained in agitation overnight. The solution was then transferred to a closed glass container and stored at 8 ^{o}C.

9.2.3 Sample preparation

Samples of frozen salmon were removed from the industrial freezing chamber and were weighed (RADWAG WLC 6/A2/C/2, Poland), and dipped in a 1.5 % (w/v) chitosan solution at 8 ^{o}C (measured using an infrared Pronto Plus thermometer (HANNA Instruments, HI99556-10, Romania) with the respective probe (HANNA Instruments, HI765PW, Romania)) during 10 s and then drained for 2 min, before being weighed again and stored in the industrial freezing chambe until further use. The dipping process was performed in a pilot-scale glazing tank, previously built for this effect, with the help of a stainless steel mesh used to hold the fish. The salmon samples intended for water glazing were weighed before dipping in water for 40 s and then drained for 1 min, before being weighted again and stored in an industrial freezing chamber until

further use. The dipping process was performed with the pilot-scale glazing tank and mesh mentioned above. The control samples did not require any additional treatment other than the cutting of the salmon and storage in an industrial freezing chamber.

9.2.4 Samples storage

The salmon samples were stored in polyethylene bags and separated by coating in different corrugated carton boxes.

Samples intended for sensory analysis were transported by a freezer truck to the Instituto Politécnico de Viana de Castelo – Escola Superior de Tecnologia e Gestão (IPVC-ESTG) facilities, where all of the sensory tests were performed, and where they were maintained at -18 ^{o}C until further use. The samples used for the microbiological tests were maintained in an industrial freezing chamber set to -18 ^{o}C until they were sent for microbiological analysis.

9.2.5 Samples analyses

Percentage of glazing or coating

In order to calculate the percentage of glazing or coating, the salmon pieces were weighed before (W_{salmon}) and after (W_i) being dipped. The percentage of glazing or coating was then calculated using Equation 5-1.

Determination of Total Volatile Based Nitrogen (TVB-N)

The TVB-N values for coated and uncoated samples were determined by the Conway method, as referenced in the NP 2930:2009 standard (IPQ, 2009) as explained in more detail in section 5.2.2. The results for all salmon samples, coated or uncoated, were expressed in mg of nitrogen per 100 g of sample.

Determination of Total Viable Count

The determination of Total Viable Count was estimated and performed according to the procedure based on the ISO 4833-1:2013 standard (ISO, 2013) presented in detail in section 5.2.5. Samples of coated salmon, glazed salmon, and uncoated salmon were analyzed in quadruplicate. The results were reported as the number of microorganisms per gram of sample.

Determination of texture

Texture was assessed using a texturometer (TA.XT *plus* Texture Analyser, Stable Micro Systems Ltd.) equipped with a 10 mm diameter cylinder DELRIN probe. A texture profile analysis (TPA) was performed on salmon samples (thawed and cooked chitosan coated and water glazed). Each sample was tested at least in six points, for a minimum of 18 test points for each coating or glazing.

The parameters retained with this test were the peak positive force of the first cycles, the area to positive peak of the first and second cycles, and the distance (from the beginning to the maximum peak – obtained by manually marking in the texturometer *exponent* software the points from the beginning to the top of a peak) of the first and second cycles. With this information (see section 3.1.3) it was possible to calculate the parameters Hardness, Cohesiveness and Springiness (Texture Technologies Corporation, 2015).

The TPA setup for the raw samples and for the cooked samples were very similar (Test speed: 1 mm/s; Target mode: distance; Time: 2 s), with the only difference being the distance that the probe travelled after impact, since cooking reduces the samples thickness. For raw samples it was defined as 15 mm and for cooked samples 10 mm.

Sensory analysis

a) Preparation of samples

The samples used for the sensory analysis were initially removed from the industrial freezing chamber at -18 ^{o}C and immediately evaluated. After this samples were left to thaw during 18 h at 4 ^{o}C, in two distinct ways: a) the coating was removed from the sample before thawing and b) the samples were placed inside individually marked zip-lock bags (with coating), and the coating was removed only after thawing. Cooked salmon samples were boiled in 2 L of water during 5 min, and then left to cool down for 30 min.

b) Procedure of analysis

Sensory quality evaluation was carried out performing a QDA test. Assessment sheets were previously developed by a seven judge trained panel of the IPVC-ESTG (in Apendix). Firstly the panel assessed frozen salmon samples, evaluating colour, odor and overall appearance. After thawing samples were assessed regarding the parameters of colour, odor, texture and general appearance. Lastly, cooked samples were assessed regarding four parameters, odor, texture, flavor and general appearance. All samples were rated in a structured six-point scale ranging from correct to incorrect characteristics.

9.2.6 Statistical analyses

Experiments were performed at least in triplicate, and in some cases more. The mean values of those independent determinations were calculated for each treatment at every moment. The statistical significance of differences among treatment was evaluated by a factorial ANOVA test followed by the Tukey HSD test with significance at $p<0.05$. Principal component analysis (PCA) of sensory data was performed in order to find which parameters are important to discriminate samples. The samples are labeled by time and coating type, in order to provide a better distinction between

different coatings and moments of assessment. Data were evaluated statistically using the software STATISTICA version 10.0 (StatSoft Inc. 2011).

9.3. Results and Discussion

9.3.1 Percentage of glazing and coating uptake

The percentages of glazing and coating uptake obtained for the samples used in the sensory analysis were 9.6 ± 0.7 % and 12.0 ± 0.7 % according to Equation 5-1. The values for glazing and coating percentages are in line with those reported in previous works, although higher percentages (especially for chitosan) were found (Soares *et al.*, 2015).

9.3.2 TVB-N

In Figure 9-1 it is possible to see the results of the TVB-N tests performed. The control sample presented a TVB-N value of 10.8 ± 0.9 mg of nitrogen/100 g. Although with a decreasing tendency, the samples coated with chitosan or glazed with water did not vary greatly throughout the six months of storage, and did not present statistically significant differences between them. After six months of storage, water glazed samples present a value of 9.1 ± 0.5 mg of nitrogen/100 g, while chitosan coated samples present a value of storage of 9.4 ± 0.5 mg of nitrogen/100 g, lower than the control sample, and well below the 35 mg nitrogen/100 g fish established as the acceptable limit for salmon by EU Directive 95/149 (Official Journal of the European Communities, 1995). The lack of variation in the TVB-N values and the absence of an expected increase after three months of storage can be a result of the low temperature used in the test, and the initial quality and good condition of the salmon, which is supported by the low TVC values found (next section) (Gonçalves & Gindri Junior, 2009).

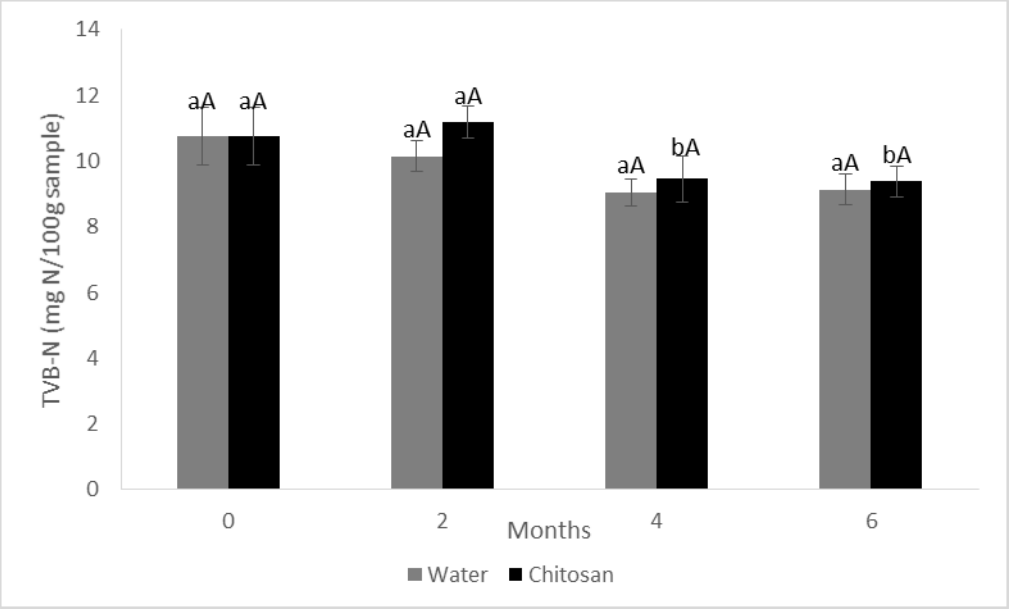

Figure 9-1 - TVB-N values for salmon samples during six months of storage at -18 ^{o}C; standard deviation corresponds to four replications; different small letters in the same sample type, and different capital letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

9.3.3 TVC

Table 9-1 presents the TVC values of the salmon samples used in sensory analysis and stored during six months in an industrial freezer set to -18 °C. Chitosan coated samples clearly present the best results at all moments, with several of the samples being below the detectable value of the test (below 10). This data on chitosan confirms ability of chitosan coatings to reduce microorganisms on food surfaces, that has been referenced by several authors over past years (Castro & Paulín, 2012; Raafat & Sahl, 2009; Rabea *et al.*, 2003). Despite the long duration of the test, the TVC values are well below both the maximum limit of 10^7 CFU/g for sensory detection and rejection (Ólafsdóttir et al., 1997) and the microbiological limit of $5x10^5$ CFU/g for quality frozen fish (ICMSF, 1986).

Table 9-1 - TVC values for frozen uncoated, glazed with water and coated with chitosan salmon samples during 6 months of storage at -18 ^{o}C; standard deviation corresponds to four replications.

Samples	Storage	Sample	Sample	Sample	Sample	Mean	SD

	Time (months)	1 (CFU/g)	2 (CFU/g)	3 (CFU/g)	4 (CFU/g)	(CFU/g)	
Control Samples	0	$1.4x10^2$	$2.7x10^2$	$4.4x10^2$	$7.5x10^2$	$4.0x10^2$	264
Water Glazed Samples	2	$8.1x10^2$	$8.8x10^2$	$4.8x10^2$	$9.5x10^2$	$7.8x10^2$	208
	4	$1.2x10^3$	$1.0x10^3$	$1.2x10^3$	$1.5 x10^3$	$1.2x10^3$	206
	6	$6.4x10^2$	$5.7x10^2$	$5.3x10^2$	$8.9x10^2$	$6.6x10^2$	162
Chitosan Coated Samples	2	$4.6x10^2$	$2.3x10^3$	$1.4 x10^2$	<10	$2.8x10^2$	190
	4	<10	$1.1x10^2$	<10	<10	-	-
	6	<10	$1.2x10^2$	$1.0x10^2$	<10	-	-

9.3.4 Sensory analyses

Frozen samples

In Figure 9-2, the sensory profiles of uncoated, water glazed and chitosan coated samples in frozen state are shown for all moments of testing. It shows that in the first two months of storage (T0 and T1) differences between the samples were negligible. On the other hand, for the time periods of four (T2) and six months (T3), differences become more pronounced, being water glazed and uncoated samples less rated, clearly indicating that, in the frozen state, chitosan coating performed better in all the parameters evaluated.

The results of the principal component analysis (Figure 9-3) for frozen samples summarize 98.67 % of the information. Factor 1 represents 83.56 % of the samples variation, while Factor 2 represents 15.11 % of that variation. This means, also according to the Eigenvalues associated with each factor (Table 9-2) that only Factor 1 is relevant to explain differences between samples.

In PCA plot, it is possible to see that the chitosan coated samples follow a similar distribution to the other samples, which indicates that no changes occurred due to the type of coating used in the samples; it is also possible to see on Factor 1 axis that the chitosan coated samples present better results until the end of the study, especially when compared to the samples without any coating (WC).

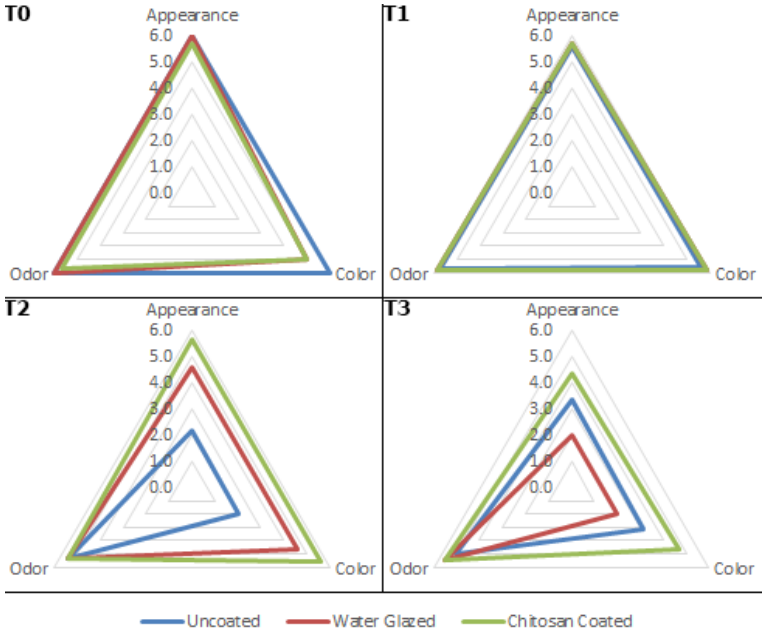

Figure 9-2 - Sensory profile of uncoated (WC), water glazed (WG), and chitosan coated (CC) frozen salmon samples, at the beginning of storage (top left), after two months of storage (top right), four months of storage (bottom left) and six months of storage (bottom right) at -20 ^{o}C.

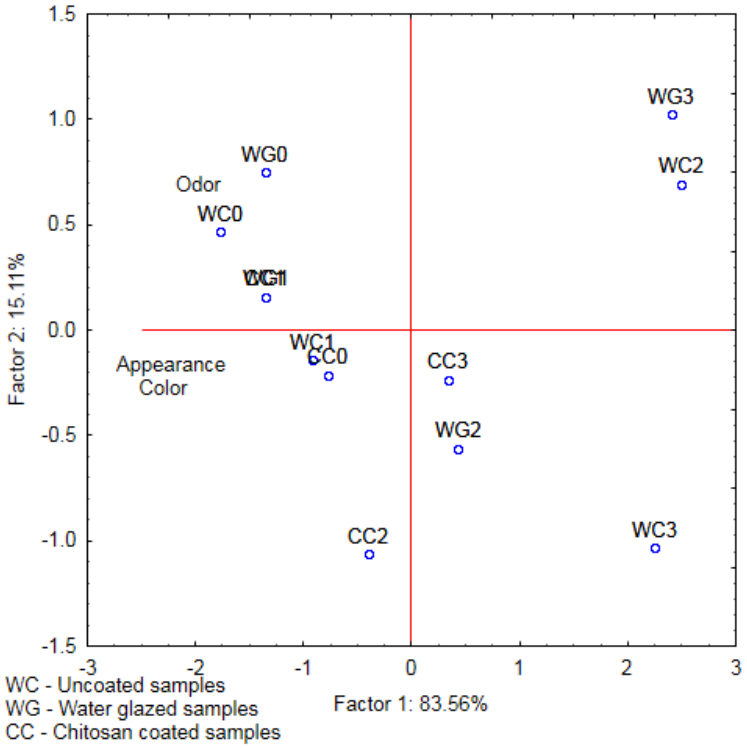

Figure 9-3 - Case projection after PCA analysis for the frozen salmon samples.

A clear pattern that is seen is that the samples assessed after four (T2) and six months (T3) tend to stand out from the rest of the samples, indicating that concerning colour, appearance and odor, chitosan coated samples (CC) have better results. This is supported by the findings in the sensory analysis profiles of Figure 9-2, where the four and six months' samples present overall lower scores when compared to samples from earlier moments of assessment. With Factor 1 being responsible for the majority of the variation (Eigenvalue >1, in Table 9-2), through the factor-variable correlation it is possible to see that the colour and appearance parameters are the ones causing higher differences between samples.

Table 9-2 - Factor-Variable correlations after PCA analysis and Eigenvalues for frozen salmon samples.

	Odor	Colour	Appearance	Eigenvalues
Factor 1	-0.818	-0.952	-0.965	2.507
Factor 2	0.575	-0.274	-0.218	0.453

Thawed samples

Concerning thawed samples sensory analysis, in Figure 9-4 it can be seen that in the first four months of storage, differences between the samples were also negligible, with the exception of chitosan thawed samples, which may be due to the extra handling needed for the removal of the coating. On the other hand, after six months of storage, differences become more pronounced, with the water glazed and the chitosan coated samples having the best results compared to uncoated samples (WC) in the judges' opinion.

In Figure 9-5 it is possible to see that the chitosan coated samples follow a similar distribution as the other samples, which indicates that no significant changes were perceived by judges. The exceptions are the chitosan thawed (CT3) and the uncoated samples (WC3) assessed after six months (T3), which are distanced from the rest of the samples, indicating that they are clearly different, and in this case worse than the samples assessed at other times (as can be seen in plot T3 of Figure 9-4) revealing

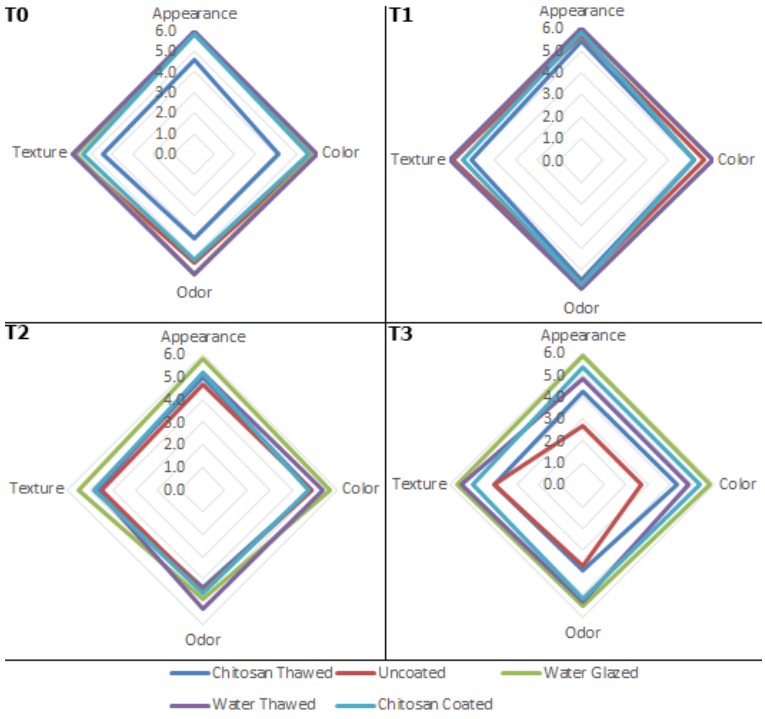

Figure 9-4 - Sensory profile of uncoated (WC), water glazed (WG), chitosan coated (CC), water thawed (WT) and chitosan thawed (CT), salmon samples after thawing, at the beginning of storage (top left), after two months of storage (top right), four months of storage (bottom left) and six months of storage (bottom right) at -20 ^{o}C.

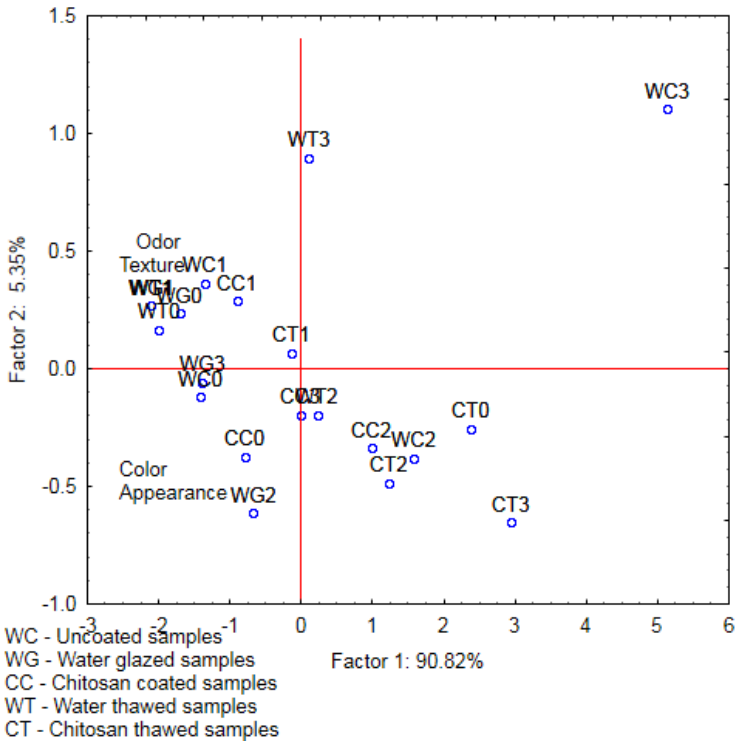

Figure 9-5 - Case projection after PCA analysis for the thawed salmon samples.

lower scores, which supports the findings in the sensory analysis, where the six months' samples present overall lower scores when compared to samples from earlier moments of assessment as seen in Figure 9-4.

PCA with thawed samples data summarizes 96.17 % of the information (Factor 1 responsible for 90.82 % and Factor 2 responsible for 5.35 %). With Factor 1 being responsible for the most part of the variation (Eigenvalue >1, in Table 9-3), through the factor-variable correlation is possible to see that all assessed parameters have a similar impact in the existing differences between samples.

Table 9-3 - Factor-Variable correlations after PCA analysis and Eigenvalues for thawed salmon samples.

	Texture	Odor	Colour	Appearance	Eigenvalues
Factor 1	-0.943	-0.939	-0.966	-0.963	3.633
Factor 2	0.213	0.254	-0.220	-0.236	0.214

Cooked samples

In Figure 9-6 the sensory profiles of uncoated, water glazed, chitosan coated, water thawed and chitosan thawed samples after cooking are shown for all moments of testing. In the first two months of storage (plots T0 and T1) differences between the samples were not significant. For the last two moments of evaluation differences are more evident. This difference is more pronounced in the case of flavor and appearance evaluation of samples with 4 months of storage. In spite of chitosan coated and chitosan thawed samples having a slight overall lower rating on appearance characteristics, in flavor and odor parameters samples have similar rating than those of the water glazed and water thawed samples, suggesting that the perceived differences are due to storage time and not so caused by coating aroma diffusion.

The results of the principal component analysis (Figure 9-7) in the cooked samples data show that 92.82 % of the variation is caused by Factor 1 and Factor 2, with Factor 1 being responsible for 78.80 % of the samples variation, while Factor 2 summarizes

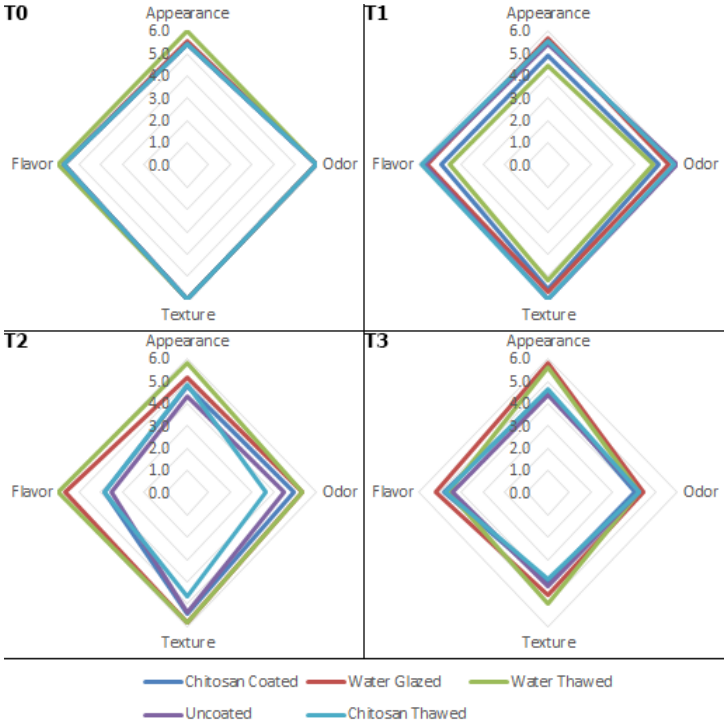

Figure 9-6 - Sensory profile of uncoated (WC), water glazed (WG), chitosan coated (CC), water thawed (WT) and chitosan thawed (CT), of cooked salmon samples, at the beginning of storage (top left), after two months of storage (top right), four months of storage (bottom left) and six months of storage (bottom right) at -20 ^{o}C.

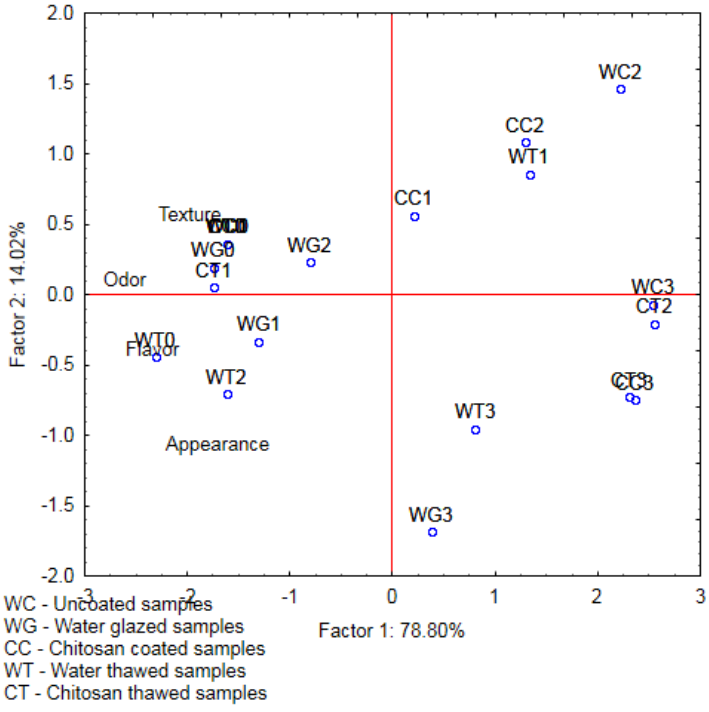

Figure 9-7 - Case projection after PCA analysis for the cooked salmon samples.

14.02 % of that variation. Table 9-4 shows the Eigenvalues associated with each factor. In the plot it is possible to see that the chitosan coated (CC) samples tend to follow a similar distribution as the other samples, which confirms that no changes occurred due to the type of coating used in the samples. A pattern that is seen is that the samples of the later assessment moments tend to be further distanced from the main cluster of samples. With Factor 1 being responsible for the most part of the variation (Eigenvalue >1, in Table 9-4), through the factor-variable correlation is possible to see that the odor parameter has a higher impact in the existing differences between samples than the other parameters.

After analyzing sensory results from all type of studied coatings and stage of the processing (raw, thawed and cooked) it can be said that the studied organoleptic properties were not significantly affected by the use of chitosan coating. Samples were quite stable over time although some alteration in flavor and odor being pointed out by judges.

Table 9-4 - Factor-Variable correlations after PCA analysis and Eigenvalues for cooked salmon samples.

	Flavor	Texture	Odor	Appearance	Eigenvalues
Factor 1	-0.900	-0.879	-0.933	-0.836	3.152
Factor 2	-0.286	0.417	0.299	-0.465	0.561

Texture analysis

The textural properties of thawed and cooked salmon were assessed by a texture profile analysis, which allowed for the determination of four parameters: hardness, cohesiveness, springiness and chewiness. The results for the thawed and cooked samples for these four parameters, during six months of storage at -18 ^{o}C can be seen in Table 9-5. Despite the differences in process and handling between the water glazed and chitosan coated samples due to difficulties in removing the chitosan coating from the samples, results do not vary significantly during the different time periods assessed

or between different coatings, for all of the parameters assessed. Although there is a slight difference between the raw and cooked samples, with the cooked samples having slightly lower values, which is to be expected given that the cooked samples suffer changes from the cooking process, among them the reduction of their thickness and changes in muscle properties. The values of all parameters are similar to those found in other studies for thawed salmon with similar conditions, although slightly higher for all moments of evaluation, suggesting that this tendency is related to the samples, rather than the coatings applied (Casas *et al.,* 2006; Martinez *et al.*, 2007). These results are in agreement with the outcome of the sensory analysis by the panel of judges regarding the texture parameter, with negligible differences between coatings.

Table 9-5 - Texture parameters for glazed and uncoated samples, for both raw and cooked states; different small letters in the same sample type, and different capital letters in the same time moment indicate a statistically significant difference (Tukey HSD test, $p < 0.05$).

Sample State	Time	Coating	Hardness	Cohesiveness	Springiness	Chewiness
Raw Samples	T0	Glazing	7.76^{aA}	0.39^{aA}	1.10^{abA}	3.86^{aA}
	T0	Chitosan	7.50^{aA}	0.38^{aA}	1.10^{aA}	3.35^{aA}
	T1	Glazing	6.05^{bA}	0.39^{aA}	1.13^{abA}	2.68^{bA}
	T1	Chitosan	6.33^{abA}	$0.34a^{bA}$	1.02^{aA}	2.38^{bA}
	T2	Glazing	6.47^{abA}	0.31^{bA}	$1.05a^{bA}$	2.92^{bA}
	T2	Chitosan	5.23^{bA}	0.32^{bA}	1.02^{aA}	1.96^{bB}
	T3	Glazing	6.09^{bA}	0.42^{aA}	1.19^{aA}	3.49^{abA}
	T3	Chitosan	6.90^{aA}	0.35^{abB}	1.07^{aB}	2.74^{abA}
Cooked Samples	T0	Glazing	7.43^{aA}	0.29^{aA}	1.00^{aA}	2.36^{aA}
	T0	Chitosan	5.71^{aB}	0.30^{aA}	1.00^{aA}	1.71^{aA}
	T1	Glazing	6.88^{aA}	0.27^{aA}	1.01^{abA}	1.82^{aA}
	T1	Chitosan	6.31^{aA}	0.30^{abA}	1.00^{aA}	1.79^{aA}
	T2	Glazing	7.00^{aA}	0.27^{aA}	1.00^{aA}	1.84^{aA}
	T2	Chitosan	5.79^{aA}	0.30^{bB}	1.02^{aA}	2.42^{aA}
	T3	Glazing	6.67^{aA}	0.29^{aA}	1.04^{bA}	2.26^{aA}
	T3	Chitosan	6.37^{aA}	0.28^{aA}	1.01^{aB}	1.80^{aA}

9.4. Conclusions

Microbiological analysis showed that the chitosan offered an anti-microbiological protection when compared with the water glazed samples, with much lower values of the microorganism's counts for the chitosan coated samples. Regarding the TVB-N values neither the time of assessment nor the type of coating had a significant impact on the results being all values below the defined limits.

Textural analysis showed no significant differences between water glazed and chitosan coated samples, in all parameters assessed. Sensory analysis conducted by judges allows concluding that the use of chitosan did not negatively influence the frozen, thawed and cooked coated samples. The flavor parameter was observed with special interest, as it is the one that can provide the most information of whether a flavor diffusion had or not occurred, and results show that no significant differences in flavor occurred between chitosan coated and water glazed samples, leading to the conclusion that no flavor diffusion from the chitosan coating was present in the assessed samples.

It should also be considered that while chitosan molecules will not diffuse from the coating, smaller molecules may diffuse, leading to an opportunity to assess the viability of encapsulation and release of and added flavor to the chitosan coating that can offer added value to the product, aside from the microbiological protection already offered.

9.5. References

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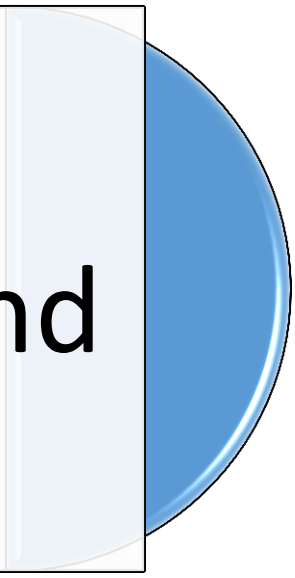
SECTION III

Conclusions and future work



In retrospective of all the work done in the last five years we believe that it can be considered to have answered positively to the original question (the one that is also the mandatory first step of the scientific method): Could a solution alternative to water be used in glazing of frozen fish that could extend the products' shelf-life? We formulated the hypothesis that chitosan solution could not only improve the ability of water glazing to protect the product from the exposure to very low temperatures but also to add other characteristics such as antimicrobial activity.

Chitosan was demonstrated to have a slower rate of coating loss, meaning that it will remain longer at the product's surface thus protecting it from the exposure to low temperatures. This is particularly important when the product is subjected to temperature fluctuations as it is commonly the case in transport and retail.

When solutions of 1.5 % chitosan were used it was concluded that they reduced the total viable count in the tested samples, often to values below the detection point. As it is of general knowledge, freezing temperatures inhibit microbial growth but the results presented in this study indicate that if the industry uses a chitosan coating it will supply clients/consumers with a product that, at the moment of consumption, will be less contaminated then when it was packed.

Organoleptic characteristics are possibly the main decision factor when buying or consuming a food product. If for water glazing it is clear that it would not change the product characteristics during storage or even after thawing, the same needed to be tested when using chitosan solutions. It was clear that the trained panel used could not perceive differences between the samples coated with water and the ones coated with chitosan. Besides that, when frozen salmon colour was assessed throughout time, the samples coated with chitosan at 1.5 % presented less colour changes and in a level that could not be perceived by regular consumers.

For the industry, besides the advantages mentioned above, the use of chitosan solution to coat frozen fish may also have significant impact in the process and in energy savings. It was shown that to obtain a coating thickness similar to that of water glazing, both product and coating temperature could be much higher (-20 ^{o}C and 8 ^{o}C) when using chitosan. This means that, besides all the advantages concluded before, the industry could also have similar protection (physical barrier) as when using water

glazing, while saving considerable amounts of energy to when lowering the temperature of the product and that of the coating solution.

This subject should be the aim of further research. It will be pertinent to analyze how a reduction of chitosan concentration below 1.5 % would impact the antimicrobial activity and also assess how it eliminates some particular pathogenic microorganisms common in seafood. Reducing the chitosan concentration would not only reduce the costs of its application but, as it reduces its viscosity, it would improve the capability of being mechanically applied, for instance, by spraying.

As it was mentioned before my goal was to promote a change in the industry paradigm of using discretionary water glazing to protect the product during storage. I cannot do it alone but I believe if, all over the world, more is done by organizations, consumers associations and regulatory authorities to fight the economic benefits of excessive glazing, more focus will be given to reduce the cost of adding coating and to the real impact of it in the products' shelf-life. The increase of fish price and the growing importance of organizations to differentiate products from the competition can also be drivers for the use of edible coatings in frozen fish.

SECTION IV

Appendix

Sensory evaluation sheet for frozen salmon samples

Panelist: _____	Date: ___ / ___ / _____
Product: **Frozen Salmon**	Code: _____

Mr(s) panelist, first judge the overall appearance of the product, then its color and finally judge its odor, following this list as presented.

Attribute	Great	Good	Average	Acceptable	Poor	Bad	Very Bad
	6	5	4	3	2	1	0
APPEARENCE	Absence of freezer burns and dehydration		Dehydration in less than 25% of the surface Slight freezer burns		Dehydration between 25% and 50% of the surface (extensive and profound freezer burns)		Strong dehydration in over 50% of the surface (extensive and profound freezer burns in all of the surface)
COLOR	Normal, characteristic of the species		Slight changes Visible in less than 25% of the surface		Changes between 25% and 50% of the surface		Abnormal Very visible and profound, affecting all of the surface
ODOR	Characteristic of the species		Neutral Identical to fresh fish preserved in a refrigerator		Characteristic odor almost imperceptible Strange odors, unpleasant, sour		Musty odor Rancid odor, unpleasant

Figure A. 1 - Sensory evaluation sheet for frozen salmon samples.

Sensory evaluation sheet for thawed salmon samples

Panelist: _____	Date: ___ / ___ / _____
Product: **Thawed Salmon**	Code: _____

Mr(s) panelist, first judge the overall appearance of the product, then its color, its odor and finally judge its texture, following this list as presented.

Attribute	Great	Good	Average	Acceptable	Poor	Bad	Very Bad
	6	5	4	3	2	1	0
APPEARANCE	Characteristic of the species		Slight visual change of the surface (less than 20%) Without evidence of freezer burns		Slight visible changes in over 50% of the surface		Total change, darkening of all of the surface
COLOR	Normal, Characteristic of the species (without changes of the initial color while fresh)		Slight changes Visible in less than 50% of the surface and flesh (slight discoloration)		Opaque flesh, without glare Discoloration of the abdominal wall		Dark flesh of purple or very brown color
ODOR	Characteristic of the species		Neutral Identical to fresh fish preserved in a refrigerator		Characteristic odor almost imperceptible Strange odors, unpleasant Uncharacteristic smell arises		Musty odor Rancid odor, unpleasant
TEXTURE	Flesh with firm consistency		Firm flesh with slight exudate		Rigid or hard flesh with exudate		Elastic or soft flesh with abundant exudate Fibrous and dry

Figure A. 2 **-Sensory evaluation sheet for thawed salmon samples.**

Sensory evaluation sheet for cooked salmon samples

Panelist: _____	Date: ___ / ___ / _____
Product: **Cooked Salmon**	Code: _____

Mr(s) panelist, first judge the overall appearance of the product, then its odor, texture and finally judge its flavor, following this list as presented.

Attribute	Great	Good	Average	Acceptable	Poor	Bad	Very Bad
	6	5	4	3	2	1	0
APPEARANCE	Flesh with a tonality characteristic of the species		Flesh with a lighter tonality (lighter pink, yellowish)		Flesh with a lightly colored tone, uncharacteristic		Flesh with an intense dark colored tone, (blackened tones, brownish)
TEXTURE FLESH COHESION	Cohesive musculature		Muscle parts stay together, but they show separation "lines" (they separate with careful manipulation)		Musculature still stays together, but separates easily		Muscles separate extremely easily Muscle don't stay together and crumble
ODOR	Fresh, normal Characteristic of the species		Slight loss, identical to fresh fish preserved in a refrigerator		Uncharacteristic smell arises		Oxidized odor, to chemical substances, acid milk, acetic acid, ammonia, oxidized fish oil and polyphosphates
FLAVOR	Fresh, normal Characteristic of the species		Loss of characteristic flavor		Uncharacteristic flavor arises		Flavor to caramel, condensed milk, metal, boiled milk

Figure A. 3 - Sensory evaluation sheet for cooked salmon samples.