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Combined effect of sous-vide and natural preservatives on shelf-life extension of Mackerel (*Scomber colias*) fillets

Faculdade de Ciências e Tecnologia

2023

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Mestrado em Aquacultura e Pescas

(Especialidade em Aquacultura)

Trabalho efetuado sob a orientação de:

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída

Carlos António Duarte Casanova Parreira

Acknowledgments

I would like to thank my supervisors, Prof. Dr. Eduardo Esteves and Prof. Dr. Jaime Aníbal for the opportunity of working in this project, and for all their support and availability demonstrated during this past year. I would like to thank the laboratory technicians Eng. Clarisse Ramalho and Eng. Vera Gonçalves for all the help and patience they had to teach me how to work with the different tools and compounds used during testing.

I would also like to thank my friends and family for all the support and kind words given throughout the process, and finally I would like to give a loving thank you to my girlfriend for all her help, support, love and encouragement to keep my composure during these stressful times, giving me the strength to keep doing the best that I could possibly do during this period.

Abstract

Mackerel, *Scomber colias* is one the most important commercial pelagic species in Portugal and Europe, being very sought after by consumers and playing an important role inside the marine food web, being mostly found in temperate or tropical waters like open oceans, coastal regions, and some semi-enclosed seas.

Mackerel has become the object of study in recent years, with farming efforts using aquaculture systems being most notably localized in Nigeria and in Japan, with the objective of supplying local markets ever-growing demands. It is also a very sought-after species due to its high content in PUFA, however, it is also a very perishable fish due to the high content of long-chain omega-3 fatty acids, making mackerel prone to oxidation leading to the development of rancidity and loss of quality.

These negative traits make it imperative to find a solution, so we decided to apply sous-vide cooking, to preserve sensory and nutritional quality of the fish, and to reduce the rate at which lipid oxidation reactions occur to extend its shelf-life.

We opted for two traditional processing and complementary methods alongside the application of sous-vide cooking, a brining method, and marinating in olive oil and garlic, which were tested against a control group that was applied the sous-vide technique to test different parameters.

The sampling was done in two separate instances, instrumental (color, hardness, pH, and water activity) and physicochemical analysis (TBARS, TVNN).

We obtained positive results in our test parameters. The TBARS test remains inconclusive due to the possibility of results being skewed due to the lack of sensitivity of the performed test. Even with the TBARS test being inconclusive we obtained enough data that can confirm some of the benefits of the sous-vide cooking technique and the use of natural preservatives in improving shelf life of the mackerel.

Keywords: *Scomber colias*, Mackerel, sous-vide, sustainability, brining and marinating

Resumo

A cavala, *Scomber colias,* é uma das espécies comerciais de peixe pelágico mais importantes em Portugal e na Europa, sendo encontrada em zonas costeiras, oceano aberto ou mares semi-fechados em climas temperados ou em climas tropicais. É uma espécie muito procurada pelos consumidores devido ao elevado grau nutricional e palatibilidade, A cavala também tem um papel importante dentro das teias alimentares marinhas ocupando uma posição mais centralizada na mesma.

Em anos recentes, a cavala tem-se tornado um objeto de estudo na área da Aquacultura, com os esforços de cultivo mais notáveis na Nigéria e no Japão, tendo como objetivo principal, responder ao aumento da procura de cavala nestes mercados combatendo o aumento continuo da procura da espécie nestes mercados. Recentemente, na cidade de Sakaiminato, localizada na prefeitura de Tottori, Japão, foram efetuados os primeiros cultivos bem-sucedidos de cavala com o auxílio de sistemas RAS (Recirculating Aquaculture System).

A cavala é um peixe muito procurado devido ao elevado conteúdo de ácidos gordos polinsaturados (PUFA) e ómega-3, que torna a cavala um peixe muito perecível sendo muito propenso a processos oxidativos que levam ao desenvolvimento de rancidez e perda de qualidade.

As características da cavala levam a que seja imperativo desenvolver uma solução de modo a abrandar a perda de qualidade da cavala. Ao longo dos anos, vários métodos e técnicas foram desenvolvidos de modo a combater e evitar o desperdício alimentar, ou aumentar o tempo de prateleira de um produto, em geral, estes métodos incluem os métodos mais tradicionais, tais como, secar, salgar, congelar e fumar, como alguns exemplos. Temos também métodos de processamento térmico, como por exemplo enlatados, e novos métodos tais como alta pressão hidrostática ou radiação ionizante.

Estes métodos são particularmente eficazes e benéficos devido à sua ação inibitória de desenvolvimento de organismos específicos à degradação e deterioração alimentar, e também pela eliminação de certos tipos de agentes patogénicos. Além destes fatores existem também fatores externos tais como, manuseamento incorreto, condições de armazenamento e processamento também contribuem para a degradação que muitas vezes se mostra como rancidez e sabores não normais.

Para corresponder ao desejo do consumidor de obter alimentos frescos de alta qualidade com tempo de prateleira elevado é necessário aplicar um conjunto de normas de boas práticas, boas práticas de higiene e controlo de pontos críticos na análise de perigo e a criação de novas estratégias de modo a preservar ou prolongar o tempo de prateleira de um produto e garantir a segurança do consumidor.

Para combater e tentar retardar as reações oxidativas na cavala decidimos aplicar a técnica de sous-vide. Sous-vide é o nome atribuído à técnica de cozinhar sob condições de vácuo, esta técnica consiste em cozinhar os materiais crus, ou então, em cozinhar os materiais crus e outros intermediários a temperaturas e tempos controlados dentro de pacotes termicamente estáveis em que os conteúdos estão em condições de vácuo. O vácuo previne a rancidez causada pela oxidação lipídica e também previne a degradação pela ação de microrganismos aeróbios, que são os principais intervenientes no aparecimento de sabores não nativos durante a refrigeração e armazenamento. O embalamento a vácuo permite a preservação dos produtos no seu interior, mantendo a sua qualidade e aparência durante um maior período, e por isso, esta técnica tem vindo a ganhar tração e tornou-se um tópico de investigação derivado da necessidade de minimizar o desperdício alimentar e responder à crescente procura de artigos de mar. A evolução desta técnica levou ao seu uso em diferentes espécies, tais como, *Mytillus galloprovinciallis* (mexilhão-do-Mediterrâneo), que foi cozinhado numa solução de NaCl a 90 ºC e obteve uma extensão de tempo de prateleira de 30 dias. Outro caso de sucesso foi o caso do achigã (*Micropterus salmoides)* que revelou um maior conteúdo de água imobilizada, estabilização da estrutura secundaria de proteínas e oxidação lipídica reduzida.

No presente estudo optou-se por dois métodos tradicionais de confeção em conjunto com a aplicação da técnica de sous-vide, o método de salmoura e o método de marinada de azeite, alho e orégãos, o grupo de controlo usado foi apenas submetido ao processo de sous-vide sem nenhum tratamento prévio.

Realizaram-se dois ensaios com as preparações. No primeiro, efetuaram-se os testes com parâmetros instrumentais, cor, dureza, pH e atividade da água, e no segundo foram

efetuados os testes com parâmetros físico-químicos, em que foram testadas substâncias reativas ao ácido tiobarbitúrico (TBARS) e azoto básico volátil total (TVBN).

Nos testes instrumentais, obtivemos resultados positivos que são indicativos dos efeitos da técnica de sous-vide e dos tratamentos aplicados às amostras. Os testes de cor mostraram que as nossas amostras reagiram aos testes efetuados, mantendo-se os parâmetros L, a, e b relativamente estáveis durante o período da experiência. O pH das amostras manteve-se maioritariamente estável durante o período da experiência, de acordo com os dados de outros autores noutros estudos semelhantes. Quanto a dureza das amostras, segundo os nossos resultados, tanto o método de sous-vide como os tratamentos usados não tiveram impacto na dureza da amostra, que, todavia, diminui com o tempo do ensaio. Nos testes de atividade da água, os nossos resultados sugerem que tanto o tempo como o tratamento a que as amostras são submetidas intervêm na quantidade de moléculas de água livres existem na amostra.

No que toca aos parâmetros físico-químicos, para o teste de TVBN os resultados apresentaram significância estatística nos fatores tratamento e tempo, demonstrando um abrandamento da degradação proteica nos dois tratamentos em comparação com o grupo de controlo, estando dentro dos limites propostos pela regulamentação europeia, passando esses limites no dia 11 e no dia 43 de amostragem (azeite e alho, e salmoura respetivamente), podendo obter melhores resultados usando amostras em melhor estado de conservação ou frescas. No caso dos resultados do TBARS não houve nenhuma significância estatística, mas obtiveram-se valores bastante elevados e indicativos de oxidação lipídica. Segundo alguns autores, a concentração em TBARS não tem muita sensibilidade e pode reagir com outros compostos envolvidos no processamento, ao que os resultados serem no nosso caso, uma sobrestimação da oxidação lipídica na amostra.

Obtivemos resultados positivos nos parâmetros testados. O teste TBARS permanece inconclusivo, devido à possibilidade dos resultados obtidos sejam errados, devido à falta de sensibilidade do teste TBARS efetuado. Apesar do teste TBARS ser inconclusivo, obtivemos dados suficientes que confirmam alguns dos benefícios da técnica culinária de sous-vide e o uso de preservantes naturais no tempo de prateleira nos filetes de cavala

Termos chave: *Scomber colias*, Cavala, sous-vide, sustentabilidade, redução de desperdício

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1. Introduction

1.1. Aquaculture production

World population continues to rise, and proportionally, so will the pressure for global food items demand. Fish has long been recognized as a viable dietary source due to the high quality and digestibility of the nutrients present in them. Fish stocks get depleted easily with no time to recover and human consumption for fish products is on the rise, with an annual increase of 3.1%, contrasting with 2.1%/year for other animal protein (FAO, 2022). Aquaculture follows this trend and has steadily been increasing global production reaching 122.6 million tons in live weight, a 2.7% increase when compared to 2019 (FAO, 2022).

1.2. Mackerel

Mackerel (*S. colias*) is one of the most important commercial pelagic species in Europe, mainly distributed along the western European continent, from the west coast of Portugal and Bay of Biscay to the north of Norway, into the Arctic Circle (Bolster, 1974; Borchers et al., 1997; Uriarte & Lucio, 2001; Iversen, 2002; Borja et al., 2002).

Mackerel is an important food source for marine mammals and other fish species whilst also being a commercially important fish species (ICES, 2015). This species has become the object of study in recent years in Nigeria and Japan to farm it in aquaculture systems to be able to supply the local markets ever-growing demands.

The species is also a very sought-after species due to its high content (± 25.7 g/100 g of fillet) of polyunsaturated fatty acids (PUFA) (Regulska-Ilow et al., 2013). However, it is also a very perishable fish due to its high content in long-chain omega-3 fatty acids making it prone to oxidation leading to rancidity and to quality loss (Standal et al., 2018), making it imperative to apply cooking technology that can preserve sensory and nutritional quality of the fish, and also to reduce the rate of lipid oxidation reactions whilst extending the shelf-life (Cropotova, Mozuraityte, Standal, & Rustad, 2019a).

1.3. Aquaculture products' freshness and spoilage

Health organizations have been increasingly recommending seafood and related products for consumption due to their rich nutritional composition, these items present a good source of protein, long-chain omega-3 fatty acids (DHA and EPA), fat-soluble vitamins (E and D) and essential minerals and vitamins (Tacon & Metian, 2013). The

increased demand for fishery and aquaculture products in recent decades has been accompanied with on-growing quality, safety, and nutritional awareness, as well as waste reduction and by-product valorization (Esteves et al., 2016).

Most fish produced by aquaculture is for human consumption, but not all the fish produced gets set on consumers' tables. Fish is a highly perishable food item prone to oxidation and formation of off flavors, resulting from the interaction of microbiological, physical, and chemical changes (Figure 1.1). The initial loss of fish freshness is normally due to its enzymatic and chemical reactions, whereas microbiological activities are involved in the entire spoilage process (Sallam, Ahmed, Elgazzar, & Eldaly, 2007).

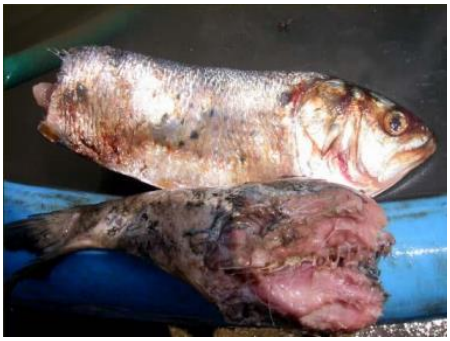

Figure 1.1 - Seafood deterioration due to high protein, non-protein nitrogen and unsaturated lipids (Source: Esteves and Anibal, 2022).

1.4. Fish processing methods

To avoid spoilage several different methods were developed to extend shelf-life of food (Figure 1.2). These methods include chilling/refrigeration and freezing, more traditional methods, such as salting, and drying for example, conventional, thermal processing methods, such as canning, and novel methods such as, high-hydrostatic pressure or ionizing radiation. These methods are especially beneficial either due to the delay of enzymatic reactions or the inhibition of specific spoilage organisms or elimination of food-borne pathogens (Esteves & Genç, 2016). External factors such as inappropriate handling, storage conditions and processing also contribute to spoilage that often shows as rancidity and off flavors (Esteves et al., 2016).

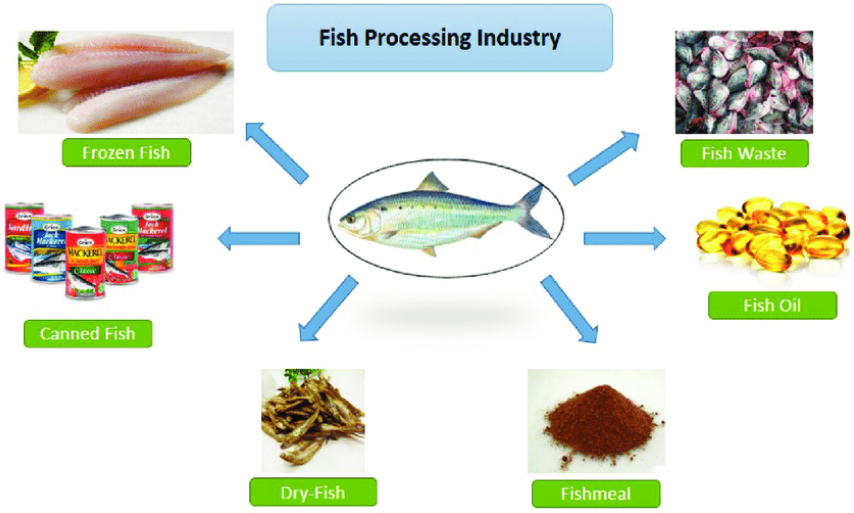

Figure 1.2 - Different items obtained from fish processing (Source: Shaik & Sarbon, 2022).

1.5. Sous-vide

Sous-vide, is the French name given to the process of cooking "under vacuum" conditions. This cooking method consists of raw materials or raw materials with intermediate foods cooked under controlled temperatures and time inside thermally stable vacuumed pouches (Schellekens, 1996; Warner et al., 2017). The vacuum prevents the rancidity caused by lipid oxidation, as well as food degradation by aerobic microorganisms, which are responsible for the development of off flavors during refrigeration storage. Also, vacuum packaging allows for the preservation of the food's constituents, thus maintaining the quality and appearance of the product for a longer period (Ayhan & Kahve, 2020).

Sous-vide cooking has become a topic under investigation derived from the necessity to minimize food waste and increasing consumer demand and as such, many different sea species have been used alongside this technique to prove its effects. Some authors have been successful in their endeavors, in the case of *Mytillus galloprovinciallis* (Mediterranean mussel) there was an extension of shelf-life of up to 30 days when sous-vide cooked in NaCl solution at 90ºC for 10 minutes (Bongiorno et al., 2018). Sous-vide cooking of *Micropterus salmoides* (Largemouth bass) revealed higher immobilized water, stable secondary structure of proteins, and minimal lipid oxidation (Wan, Cao, & Cai, 2019) and, efforts done using the sous-vide cooking technique on Atlantic mackerel (*Scomber scombrus*) at temperatures of 60, 75, and 90 ºC for 10, 15, and 20 minutes respectively and then stored at 4 ºC showed that increasing cooking temperature lead to a decrease in sarcoplasmic and myofibrillar proteins, and also a significant increase in

lipid oxidation and yellowness during storage (Cropotova, Mozuraityte, Standal, & Rustad, 2019b).

1.6. Modified atmosphere packaging

Modified atmosphere packaging or MAP (which in theory includes vacuum package) is a technology that dates to the 1930's and has become a critical area of research in terms of minimizing waste through spoilage in fish products (Farber, 1991). Recent studies have estimated that fish post-harvest losses amounted to a staggering 27% of global capture and cultured fish (FAO, 2018). Microbial activity is a major contributor for quality loss and subsequent spoilage of fish products (Alfaro et al., 2013a; Alfaro et al., 2013b). A major driving force for food spoilage minimization is consumer demand for fresh products, even though the adoption of modified, or controlled atmosphere systems in fish products has been generally slower when compared to other products due to concerns regarding *Clostridium botulinum* type E (Mireles Dewitt & Oliveira, 2016). While *Clostridium botulinum* is commonly found in various environments, type E is specifically linked to marine food products. This type exhibits the capability to generate toxins under conditions of limited oxygen, particularly within refrigerated atmospheres. Under favorable circumstances, the lowest temperature range at which *Clostridium botulinum* type E can thrive and produce toxins is between 3.0°C and 3.3°C (FAO, 2001), and is a concern when submitting marine food products to MAP conditions.

Vacuum packaging was the first MAP to be developed for commercial use, this technique consists in packaging a product in a film of low oxygen permeability. Subsequently, the package is then sealed after the evacuation of the air contained in it. When executed under optimal conditions, the process should achieve oxygen levels that are below 1% (Ruiz-Capillas & Moral, 2001). Respiring foods can have passive atmosphere generation inside a package, while active generation of atmosphere can be accomplished by use of O_2 scavengers, CO_2 absorbents, or by use of emitters, ethanol emitters and ethylene absorbers among others (Ruiz-Capillas & Moral, 2001).

Mechanical air replacement can be achieved by use of gas flushing or compensating vacuum, the first being generally achieved using form-fill-seal equipment that injects a constant gas stream, or gas mixture, into the package. After the air is replaced by the gas via dilution process, the package is then sealed (Ruiz-Capillas & Moral, 2001). Residual oxygen levels in gas-flushed vary according to the equipment used, type of packaging

and product type with typical residual oxygen levels ranging from 2% to 5% (Blackistone, 2009). Compensated vacuum is a two-stage technique where air is firstly removed by action of vacuum and then the modified gas atmosphere is introduced prior to sealing the packaging, the packaging can either be pre-formed or thermal formed, with its efficiency being much superior to that achieved by gas flushing regarding residual oxygen levels (Ruiz-Capillas & Moral, 2001).

To fulfill consumer desire for fresh chilled foods with extended shelf-life, the application of good manufacturing practices (GMP), good hygienic practices (GHP), and hazard analysis of critical control point (HACCP) are required in the production, distribution, storage, and retailing of refrigerated foods (Li et al., 2012), also creating numerous diverse preservation strategies to preserve or prolong shelf-life of fresh foods to ensure product safety (Sallam, 2007).

1.7. Thermal processing

Thermal processing, which includes "traditional" cooking, is a food sterilization technique in which the food is heated to temperatures that are high enough to destroy microbes and enzymes, it is known to induce lipid oxidation, with its driving force being the disruption of cell membranes and denaturation of heme-proteins (Kristinova, Mozuraityte, Aaneby, Storro, & Rustad, 2014), and also the liberation of free iron that acts as a strong pro-oxidant promoting lipid oxidation in fish (Grunwald & Richards, 2006), which will negatively impact fish products by affecting nutritional and sensory properties of the fish (Cropotova, Mozuraityte, Standal, & Rustad, 2019b). The extent of quality loss will depend on the initial material quality, presence of metal ions and pro-oxidants, and on the cooking regime applied (Hu et al., 2017; Sobral, Cunha, Faria, & Ferreira, 2018), this can be counter-acted with the addition of antioxidants before heat treatment (Cropotova, Mozuraityte, Standal, & Rustad, 2019b).

Thermal processing, normally in the 60-95ºC range, is still one of the most common processing methods used to obtain convenient food with an extended shelf-life. It is a challenging process because thermal processing treatments are required to inactivate target microorganisms that cause undesirable changes in their lipid and protein fractions, and in seafood, thermal processes severely degrade the quality of the product when design to have a shelf-life of several days under chilled conditions (Rosnes, Skåra, & Skipnes, 2011).

1.8. Objectives

This work aims to study the effects of application of sous-vide combined with traditional processing techniques using natural preservatives, brining and marinating (in olive oil and garlic), in terms of quality and to prolong the shelf-life of mackerel *S. colias* fillets. Although mackerel is normally only associated with fisheries, due to its high lipid and protein profiles (making the mackerel easily spoilable) and the potential for aquaculture production, our findings can be adapted to the process to other commercially farmed or captured fish species with similar composition.

2. Methodology

2.1. Study Site

The study was conducted in the laboratories of Departamento de Engenharia Alimentar (DEA), Instituto Superior de Engenharia (ISE), Universidade do Algarve, in Campus da Penha, Faro, Algarve.

Figure 2.1 – General view of the Food Processing Laboratory (ISE)

The fillets were prepared in the Food Processing Laboratory (Figure 2.1), while the determinations of physicochemical parameters were carried out in the Food Chemistry Laboratory (Figure 2.2).

Figure 2.2 – Setup for the determinations of physcochemical parameters in the Food Chemistry Laboratory (ISE)

2.2. Sample Preparation

For this study, previously quick-frozen mackerel fillets were thawed before the application of sous-vide. The control group (C) consisted of mackerel fillet subjected to sous-vide cooking, and two preparations were tested. The first consisted in brining (5%) for 30 min (SM) at a ratio of 4:1 (brine:mackerel). The second preparation consisted of marinating the mackerel fillet for 5 min in olive oil and garlic (AA) at a ratio of 1:4

(olive oil and garlic:mackerel) and some oregano and then letting them rest for 1 minute.

Then, the fillets (thawed, brined, and marinated) were vacuum packed (Figure 2.3) by use of a multipack vacuum packaging system (Henkelman Boxer 42, Germany). The packaging film was coextruded laminate composed of an exterior cast polyamide (PA) layer, a coextruded interior barrier layer containing ethylene vinyl alcohol (EVOH), and a polyethylene (PE) sealing layer. For this film, the oxygen transmission rate (OTR) is 0.5 $cm^3 m^2 d^{-1}$ bar at 23 °C and 85% relative humidity.

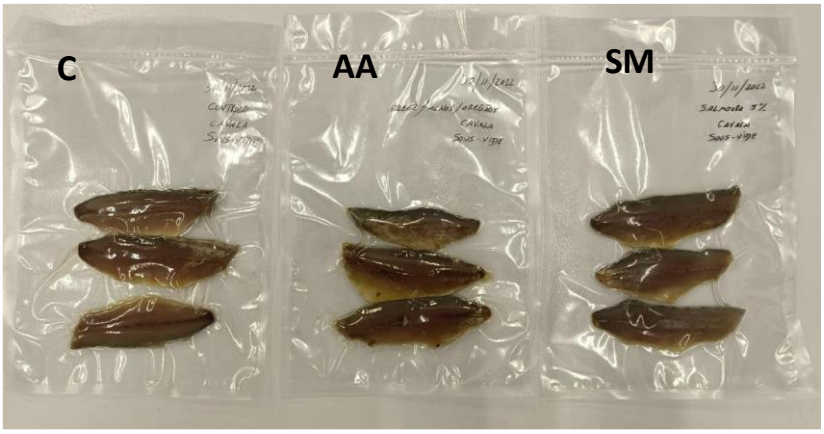

Figure 2.3 - Mackerel fillets vacuum packed, Control (C), Olive oil, garlic, and oregano (AA), Brine 5% (SM) respectively.

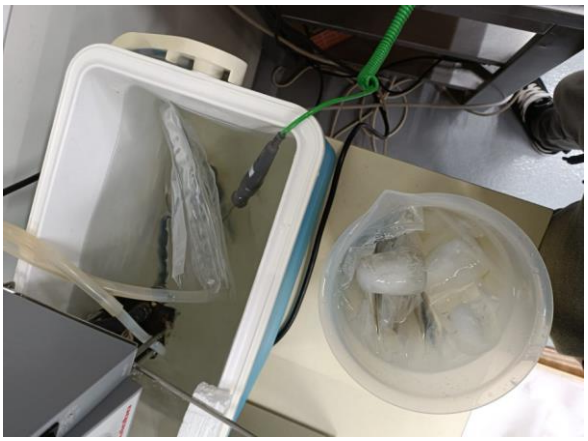

Figure 2.4 – Top view of thermostatic water bath (left) used for cooking the mackerel fillets, and water and ice bath used to cool down samples (right).

The fillets were then cooked in a water bath at 60ºC (Figure 2.4) for 3 minutes, being then refrigerated for 6 minutes in a water and ice bath kept at 2-3ºC (Figure 2.4).

After this step, sous-vide cooked samples were stored at 3ºC ± 1ºC and samples were taken out of storage and analyzed every 4-7 days according to sampling plan.

Due to the large number of samples that require preparation and quality parameters to be determined in each sampling, two sequential but interrelated trials were carried out, in which, we determined instrumental (color, hardness, pH, water activity) and physicochemical parameters (TVBN and TBARS).

2.3. Experimental Design

Trials and data collection were carried out between January and July 2023. Several quality parameters were determined, namely:

2.3.1. Color (Instrumental Analysis)

Color was recorded according to the colorimeter's (model PCE-CSM 10, PCE, USA) instructions (including calibration when necessary) (Figure 2.5), using the CIE L*a*b* color system (Figure 2.6), making 3 readings (randomly spaced) per sample on its muscle side. In CIE L*a*b* color space, L* refers to lightness (0 is black and 100 is white), a* indicates greenness ($a < 0$) or redness ($a > 0$), and b* measures blueness ($b < 0$) or yellowness ($b > 0$) of samples (Pathare et al., 2012).

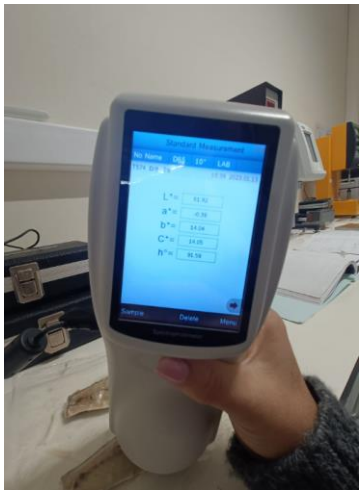

Figure 2.5 – Colorimeter model PCE-CSM 10 used to obtain readings of lightness (L), greenness-redness (a) and blueness-yellowness (b).

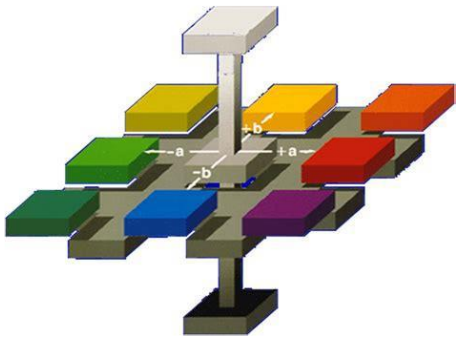

*Figure 2.6 – CIE L*a*b* color space system graphical representation (adapted from Rodgers et al., 2008).*

2.3.2. Hardness

Following the texturometer's (LFRA Texture Analyzer, Brookfield Engineering Labs Inc., USA; Figure 2.7) instructions and pre-determined properties of the compression test, a spherical inox probe with 12.7 mm-diameter (ref. TA18), was used to determine fillets' hardness. Test conditions were speed of 1 $m.s^{-1}$ and a distance corresponding to 50% deformation, two readings will be done on the anterior side of the fillet. The measurements (in kgf, where 1 kgf = 9.806 N) were analyzed using TexturePro Lite v1.1 software (Brookfield Engineering Labs Inc., USA) and the hardness (in Newtons (N)) was calculated as the peak load of the compression cycle.

Figure 2.7 - LFRA Texture Analyzer used in hardness tests.

2.3.3. pH

According to the pH digital meter (model Glp 21, Crison, Spain) instructions (including calibration, when necessary), pH was determined for each sample by inserting the semi-solids probe (Figure 2.8) directly into the muscle and making 3 readings per sample. The average of these readings was the pH of the sample.

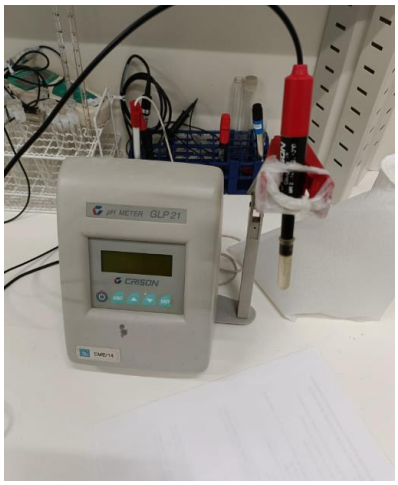

Figure 2.8 - pH meter and semi-solids probe used for pH readings.

2.3.4. Water Activity (aW)

Approximately 2 g of muscle tissue per sample was minced and evenly placed in the reading cells (Figure 2.9). The cells were incubated for 15 minutes at 25 °C and the aW measured using Hygrolab (Rotronic Instruments, UK) (Figure 10); aW will be in the range of 0-1 (or 0-100%), where 0% means the fillet would not have any free water molecules available, 100% means that all available water molecules are free being equivalent to water activity of distilled water.

Figure 2.9 - Hygrolab (Rotronic Instruments, UK) probe and readings display (left), reading cells and fillet mincing (centre and right).

2.3.5. Thiobarbituric Acid Reactive Substances (TBARS)

Determination and procedures were carried out as described in the Portuguese Standard NP 3356 (IPQ, 2009) with adaptations considering Ramanathan & Das (1992). Briefly, 2 g of fish tissue was minced and weighed (±0.0001 g), added with 100 µl of Butylated hydroxytoluene (BHT) and 25 mL of Trichloroacetic acid (TCA) and homogenize the

sample, which was then filtered using Whatman no.1 filters. We then pippeted 1 mL of extract and 4 mL of TCA in heat resistant tubes and then add 5 mL of TBA reactive solution, placed it in boiling water for 40 minutes, letting then cool to room temperature (Figure 2.10). We read its absorbance at 532 nm with the aid of a Hitachi (U-2000) spectrophotometer.

A standard curve was prepared. In a 100 mL Erlenmeyer flask of 50 µl of Triethyl phosphate (TEP) and 50 mL of HCl (0.1 N) were mixed and then be placed in boiling water for 10 minutes and then cooled. After the cooling, 2.4 mL of the solution was transferred to a 100 mL volumetric flask filling the rest with distilled water, this is the stock solution (0.1 mM MDA).

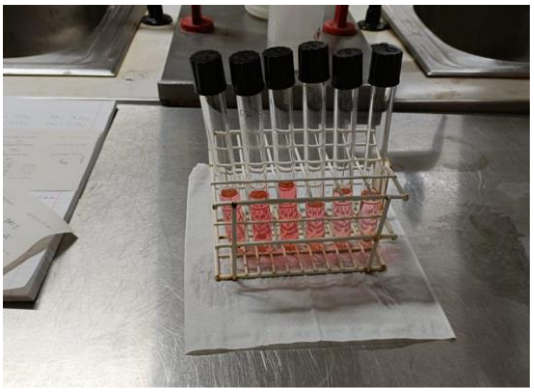

Figure 2.10 - Samples after preparation cooling down post heat treatment.

The calibration curve was prepared using the following concentrations: 0.006 mM, 0.01 mM, 0.018 mM, 0.03 mM, 0.04 mM and 0.05 mM, of which, 3 mL, 5 mL, 9 mL, 15 mL, 20 mL, and 25 mL of MDA stock solution was respectively added to 50 mL volumetric flasks with the remaining volume being filled with distilled water.

We pippeted 5 mL of each solution to a test tube and add 5 mL of TBA reactive solution. A blank would also be prepared using 5 mL of distilled water and 5 mL of TBA reactive substance.

The tubes were then be placed in a water bath at 70ºC-80 °C for 30 minutes and then let to cool to room temperature before reading their absorbance at 532 nm.

TBARS (µg MDA/kg) was calculated using:

$$TBARS = \frac{72 * MDA}{m * 1} * (25 + m * H)$$

where MDA is the concentration based on ABS and obtained from the calibration curve, m is the weight of the sample (g) and H is the moisture/water content in the sample (%).

2.3.6. Total Volatile Basic Nitrogen (TVBN)

TVBN was determined using a modified version of the micro diffusion method by Conway & Byrne (1933) as described in Portuguese Standard NP 2930 (IPQ, 2009). The first step to determine TVBN is extraction, 10 g of muscle sample was weighed (±0.0001 g) and placed in a 20 mL tube of trichloroacetic acid (TCA) was added and then homogenized with the sample being filtered using Whatman No.1 filters.
For the determination step, firstly 1 mL of boric acid along with 2 drops of indicator were pipetted in the center of the Conway cell (Figure 2.11), 1 mL of filtrate would be transferred to the inner rim of the cell using a precision pipette, adding then 1.5 mL of distilled water to the same zone semi-covering the cell with its lid, adding finally potassium carbonate and closing the cell completely and gently rotating the cell to mix the liquids that were loaded.

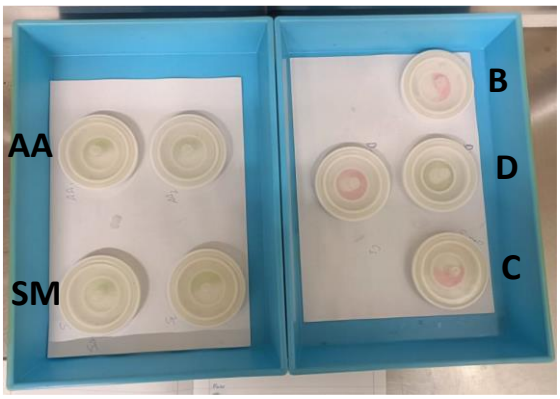

Figure 2.11 - Conway cell after heat treatment (B is blank, D is control of diffusion, C, AA, and SM are the samples).

Control of diffusion was done in the same way described above with the difference of replacing the extract volume with ammonium sulfate, the blank was also done in the same way but replacing the extract with an equal volume of distilled water.
The Conway cells were placed in the oven at 40 ºC for 1.5 h and then titrated with hydrochloric acid (0.001M) (Figure 2.12).

Figure 2.12 - Titration process used to determine TVBN.

Total basic volatile nitrogen (mgN/100 g) content was calculated using:

$$TVBN = \frac{21 * (V_2 - V_0)}{(V_1 - V_0) * V_3 * m}(20 + Fc)$$

where V_0 – Volume of the solution, in mL, of HCl used in the blank, V_1 - Volume of the solution, in mL, of HCl used in Control of Diffusion, V_2 – Volume of the solution, in mL, of HCl used in the sample, V_3 – Volume of the solution, in mL, of sample extract used in the determination, F_c – Volume correction factor (Water content available in the sample) and m – Mass (g) of the sample.

2.3.7. Statistical Analysis

All statistical tests were carried out using R software (R Core Team, 2022) via RStudio (RStudio Team, 2020). The results of quality parameters are presented as mean ± standard deviation.

All data was checked for normal distribution and homogeneity of variances, data was also log-transformed as needed to correct heteroskedasticity and/or remove outliers. The combined effects of sous-vide processing and added natural preservatives on *S. colias* fillets during the storage time were tested using two-way ANOVA, and statistical differences between groups were tested using post-hoc multiple comparisons test, Tukey HSD.

3. Results & Discussion

3.1. Color

Results show a downward trend of the sample's lightness across all test groups during the 27 days trial period (Figure 3.1).

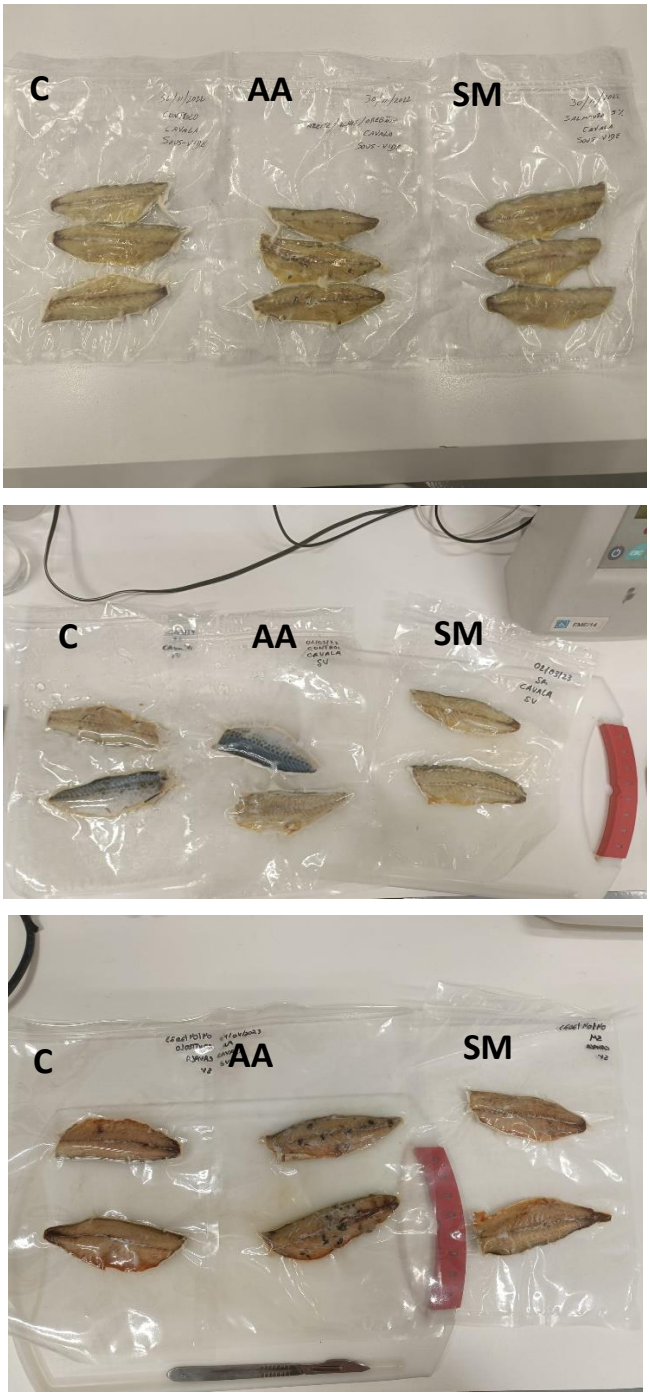

Figure 3.1 - Samples on the 1st (top), 14th. (middle) and 27th (bottom) sampling day, Control (C), Olive Oil & Garlic (AA) and Brine 5% (SM).

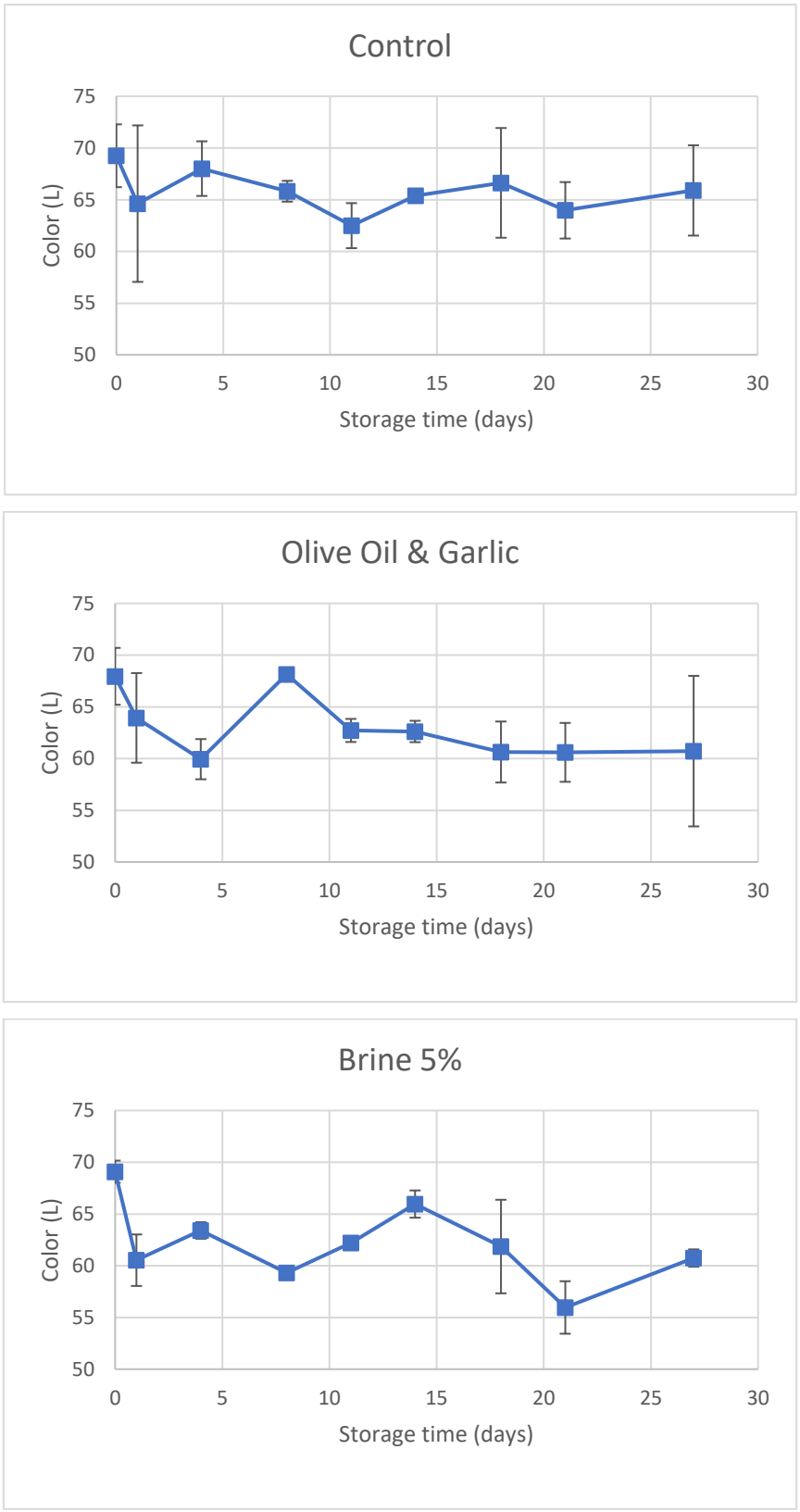

Figure 3.2 - Mean lightness (L) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

In the Control group (Figure 3.2), the highest lightness value was L= 69.25 ± 3.04 on day 0 with the lowest value being on day 21 (L= 63.97 ± 2.73), with the last trial day

showing a slightly higher value (L= 65.89 ± 4.37), this group also had the highest lightness value out of the three test groups.

For the sous-vide marinated group (Figure 3.2) we obtained the highest value on the day 8 (L= 68.15 ± 0.61) and the lowest also on day 21 (L= 60.60 ± 2.85), just marginally lower than day 27 (L= 60.72 ± 7.28).

In the sous-vide brined group (Figure 3.2) the highest value was on day 0 (L =69.09 ± 1.07), and the lowest on day 21 (L= 55.96 ± 2.54), the lowest reading across the three groups, on the final trial day the samples lightness increased (L= 60.74 ± 0.83).

These results show a reduction in the lightness of the samples during the trial period.

Time has significance (ANOVA and Tukey HSD, $P < 0.05$) in the reduction of L, the sous-vide process itself is a modified atmosphere process that eliminates most oxygen present, although oxygen levels are low, we can still expect a certain degree, albeit slower, oxidative reactions that can lead to L reduction (Ruiz-Capillas & Moral, 2001).

When testing sample groups, we also obtained statistical significance (ANOVA and Tukey HSD, $P < 0.05$), the control group obtained the highest reading of L throughout the experiment, but this is due to the nature of our treatments. The lower readings in olive oil & garlic group can be explained by its marinade, the presence of oregano can lower the L readings since the equipment covers a large area of the sample. The brine group obtained the lowest readings out of the three test groups, the brining process is known to show a slight reduction in L, as described by Agustinelli (2014).

The interaction effect of time*treatment also proved statistically significant (ANOVA, $P < 0.05$), which leads us to believe that brining and the use of antioxidants such as garlic have an impact in slowing down L reduction.

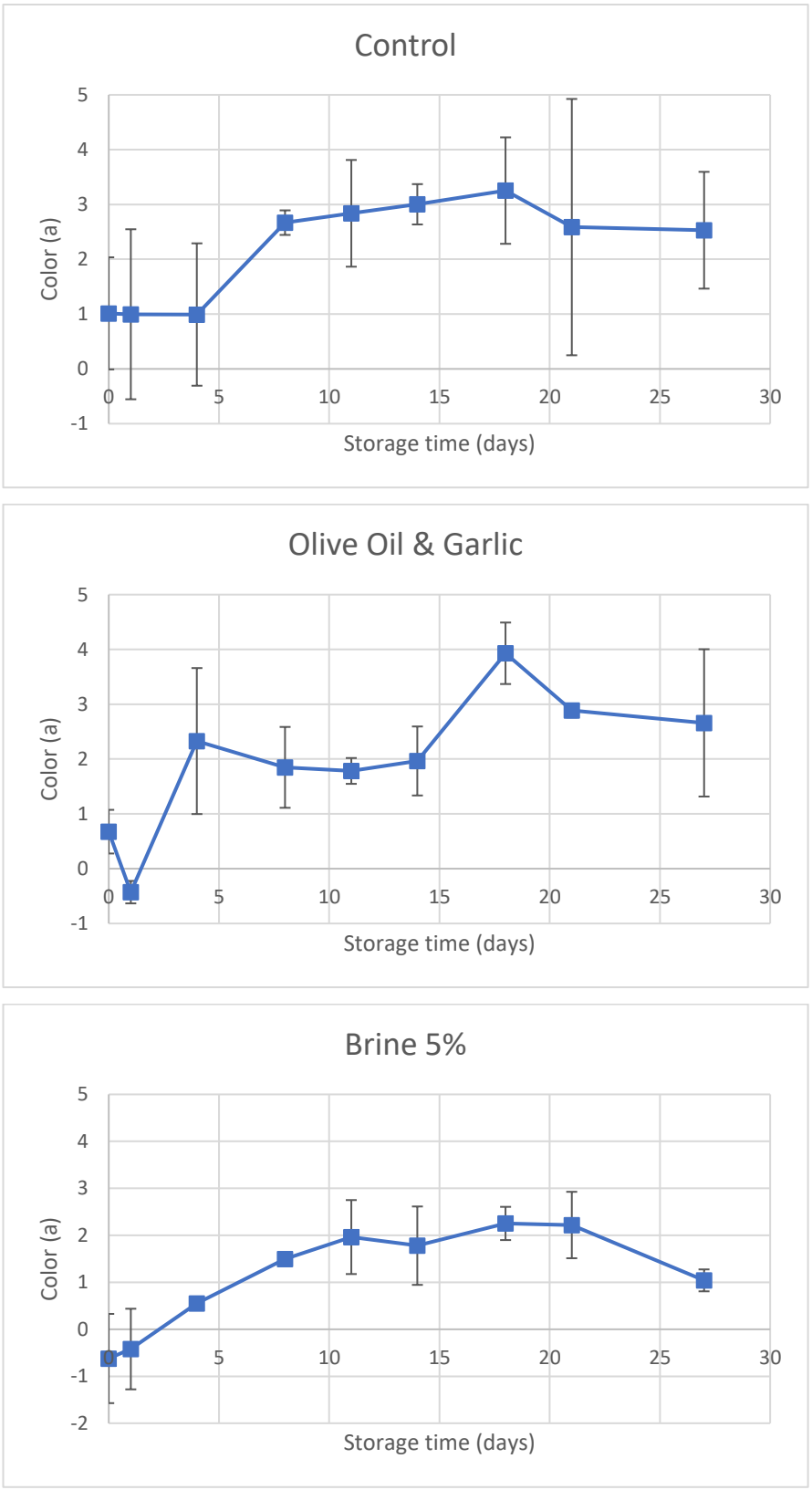

Figure 3.3 - Mean redness (a) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

As for redness (a > 0), in our control group (Figure 3.3), we can observe that redness tended to increase with time, the lowest recorded readings were day 1 (a= 0.99 ± 1.55), and the highest reading was performed on day 18 (a= 3.25 ± 0.97).

The sous-vide marinated fillets (Figure 3.3) also showed an increase redness with time, we can observe 3 distinct peaks in our data, with one of them reaching negative readings being also the lowest recorded reading on day 1 (a= -0.43 ± 0.21). The highest reading was recorded on day 18 (a= 3.93 ± 0.56), lowering redness (a= 2.89 ± 0.12) on the following sampling day and seemingly stabilizing thereon after.

The registered redness values for the brined sous-vide fillets (Figure 3.3) are the lowest of the three groups, and the increase in redness was stable during sampling. These fillets had the lowest reading on day 0 of the experiment (a= -0.62 ± 0.95), and the highest reading was on day 18 (a= 2.25 ± 0.35).

The effect of storage time on redness showed statistical significance as did sample treatment (ANOVA and Tukey HSD, $P < 0.05$). As there is still some oxygen left in sample packaging as time passes it the formation of orange/yellow chromogens (Figure 3.1) is possible by microbiological action, that might be the case for the control group, but not for the olive oil and garlic marinated and brined sous-vide fillets. The brined sous-vide group has an increase in redness and on the final sampling day a decrease, this is also described by Agustinelli (2014), the redness observed can be attributed to the presence of pigments, such as myoglobin, that can diffuse from the tissue (Corzo et al., 2006). The time*treatment interaction did not show any statistical significance (ANOVA, $P > 0.05$) on samples redness.

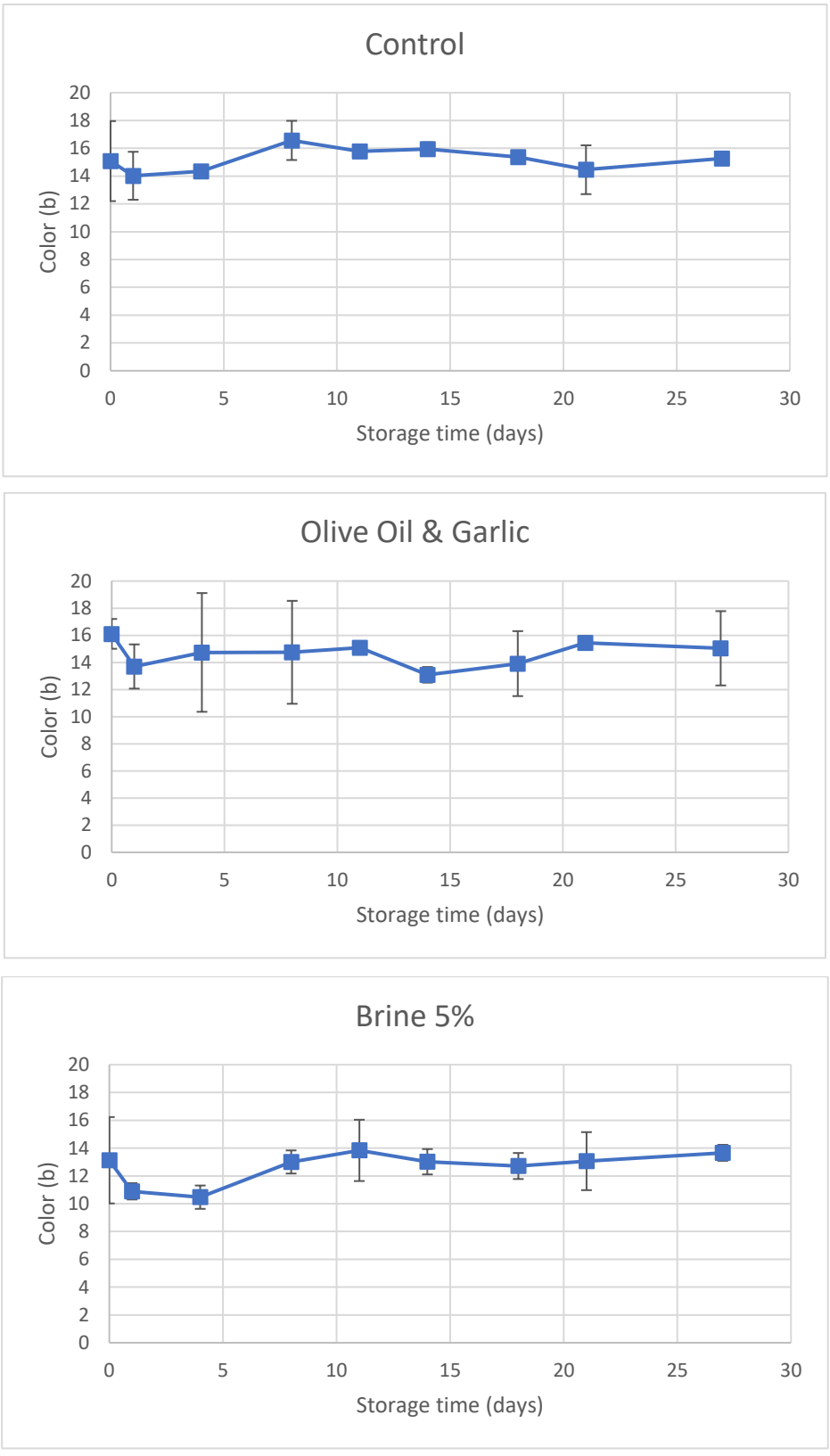

Figure 3.4 - Mean yellowness (b) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

As for yellowness (b > 0), readings on the three test groups were similar and stable, which can indicate some slight oxidation of the lipids since the first sampling day.

The lowest recorded value for the control group (Figure 3.4) was observed on day 1 (b= 14.02 ± 1.73), and the highest registered value was on day 8 (b= 16.57 ± 1.41).

The readings we obtained on the olive oil and garlic marinated sous-vide fillets (Figure 3.4), showed us our lowest reading on day 14 (b= 13.08 ± 0.58) and the highest one on day 0 (b= 16.11 ± 1.09).

As for the final group (Figure 3.4), the lowest recorded reading was on day 4 (b= 10.47 ± 0.83) and the highest one on day 11 (b= 13.84 ± 2.20).

Time did not show any statistical significance (ANOVA, P > 0.05) on yellowness, but this might be due to the storage conditions and time that they were previously subjected, presenting some frostbite from the start. Treatment proved statistically significant (ANOVA, P < 0.05), the readings of yellowness were stable throughout the whole sampling process, but this can also mean that the myoglobin released can counteract yellowness readings (Corzo et al., 2006). The interaction Time*Treatment did not show any statistical significance (ANOVA, P > 0.05).

3.2. Hardness

Hardness of mackerel fillets subjected to sous-vide cooking decreased during storage trial, but not in a linear, downward trend.

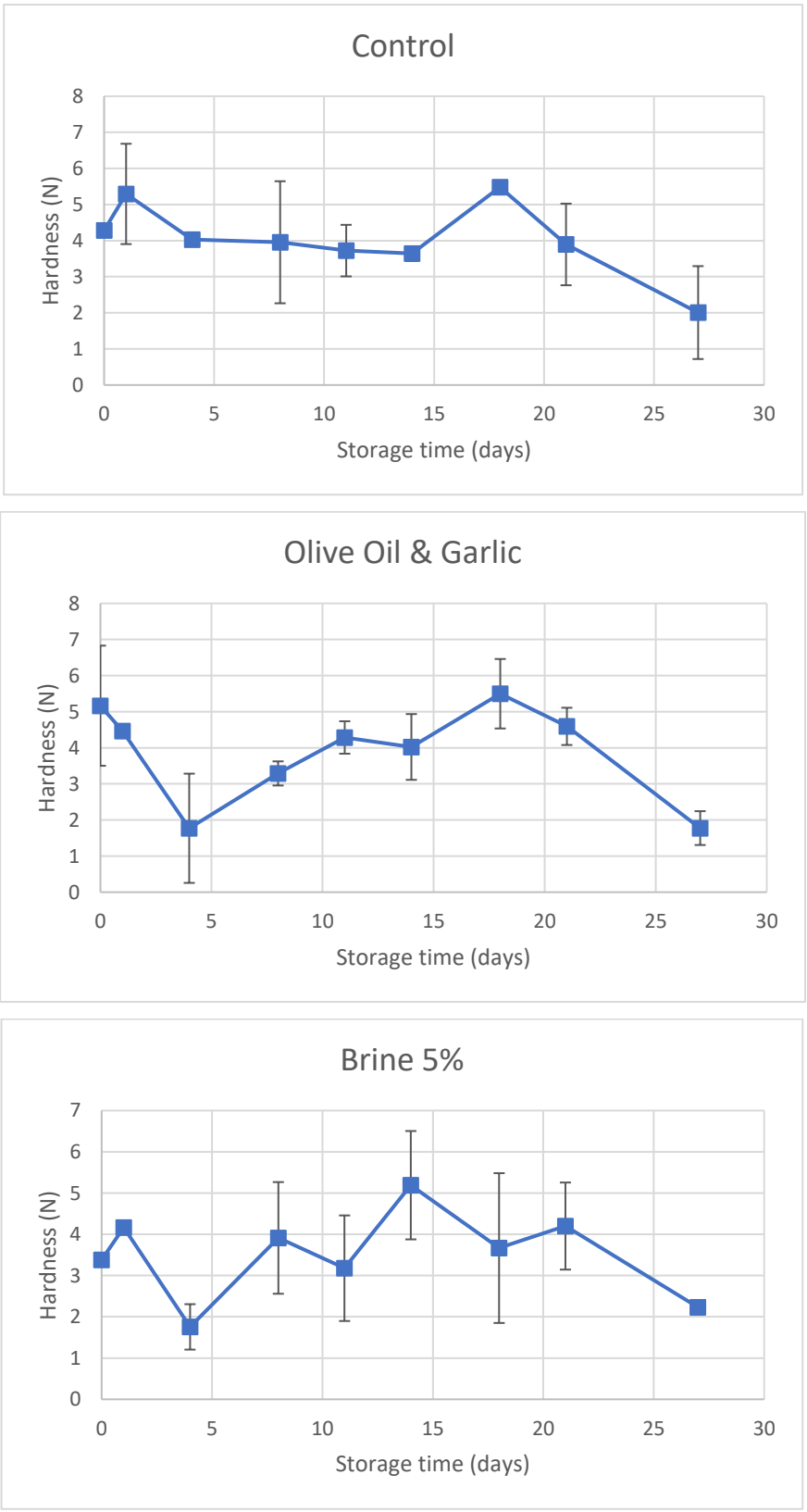

Figure 3.5 - Mean hardness (N) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

In the Control group (Figure 3.5) we observed the highest hardness on day 18 of the experiment with a hardness of 5.48 N (± 0.18), but it was also the largest sample of the group, in counterpart, on day 0 and day 1 of trials we obtained 4.28 N (± 0.04) and 5.29 N (± 1.39) respectively, with these samples being relatively the same size as all the other samples. On the last trial day, we obtained the lowest hardness value of the group with 2.01 N (± 1.29) of force exerted on the sample.

In sous-vide marinated group (Figure 3.5) we obtained 5.17 N (± 1.66) and 1.78 N (± 0.47) on day 0 and day 27 of the experiment respectively, with special note to day 4, the sample was noticeably smaller than the rest of the other samples with a hardness of 1.77 N (± 1.51), and to day 18 which coincidentally was larger than the rest of sample in the group and we obtained 5.50 N (± 0.97) of force required to press down on 50% of the sample.

Brined and sous-vide fillets (Figure 3.5) showed the best results out of the three test groups being relatively stable across samples with its only variation being the size of the specimen tested. On day 0 of the experiment, we obtained 3.38 N (± 0.11) of force required to press down on 50% of the sample and on the last day we obtained 2.23 N (± 0.13). On day 14 we observed the highest hardness value of 5.19 N (± 0.31).

Only time showed statistical significance (ANOVA, $P < 0.05$) in the hardness test. Sample hardness seems to be related to time elapsed since preparation. Degradation processes dimmish cell wall integrity that will lead to lower hardness across our samples. Neither treatment nor the interaction time*treatment had any statistical significance (ANOVA, $P > 0.05$) in this test.

3.3. pH

During our tests we obtained similar results with pH within the 6-6.1 range across all test groups, while having a longer sampling period to accommodate to our sampling needs.

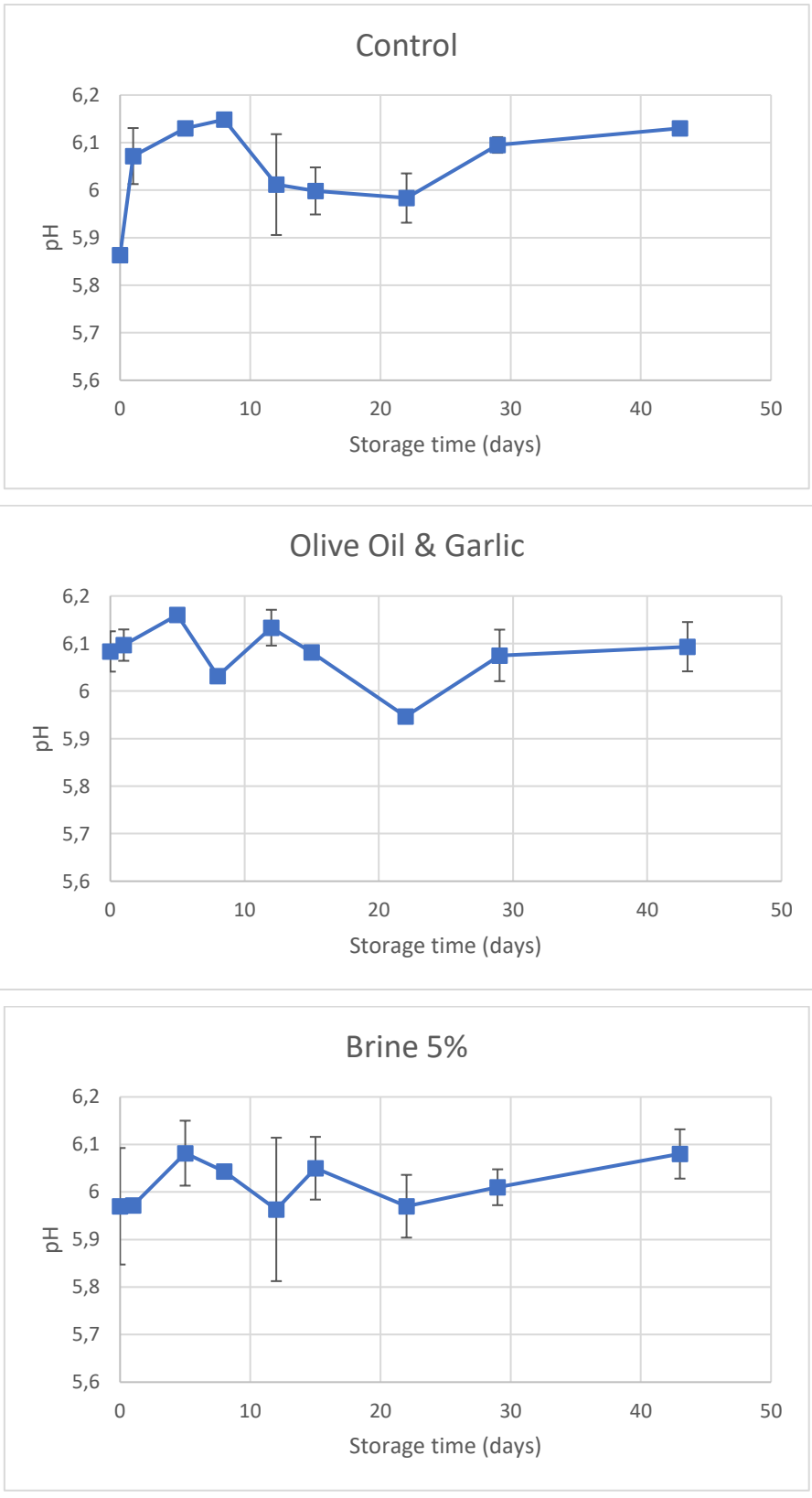

Figure 3.6 - Mean pH (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

On control group (Figure 3.6) we observed a decrease in pH up until day 22, which then started increasing. Day 0 had a pH value of 5.86 (± 0.01), which was the lowest, whilst the highest pH was recorded on days 5 and 43 with a pH of 6.13 each (± 0.01; 0.01).

The sous-vide marinated group followed a pretty stable pH trend. The tests (Figure 3.6) show a 6.13 (± 0.04) maximum on day 12 and 5.95 (± 0.00) minimum on day 22.

It is to note that tests show a slight increase in pH up to day 22 but analyzing the days that follow, we can observe a slight decrease in the rate of change of pH.

In the sous-vide brined group (Figure 3.6) of the experiment, pH slowly began to rise after day 12 which was also the lowest recording in this group with pH of 5.96 (± 0.15), the maximum pH obtained in this group was on day 5 and day 43 with 6.08 (± 0.05) pH.

Factors time, treatment, and time*treatment interaction showed statistical significance (ANOVA, $P < 0.05$) upon pH changes. The initial pH values of fish fillets before sous-vide cooking are typically around 6.05 to 6.12 (Kerimoglu et al., 2020) and during the initial storage period, the pH is consistently low, contributing to increased shelf-life (Iniesto et al., 2015). However, as the storage period progresses, the pH starts to increase (to values of ca. 7 when spoiled). This increase in pH reflects the production of alkaline bacterial metabolites in spoiling fish and coincides with the increase in Total Vola=le Basic Nitrogen (TVBN) (Mol et al., 2012, Moon et al., 2020). Despite the statistically significant changes, the average pH remains within the 5.8-6.2 range across all treatment groups, which means that the sous-vide cooking technique and to some extent, the treatments applied might have inhibitory ac=on towards the microorganisms responsible for the spoilage processes.

3.4. Water Activity (aW)

The results show us that all three test groups have diminished water activity as sampling occurred (Figure 3.7). The control group shows maximum water activity on day 1 (aW=0.932 ± 0.002) and a minimum on day 18 (aW=0.895 ± 0.016). The sous-vide marinated group treatment shows us a peak on day 8 (aW=0.948 ± 0.005) and a minimum on day 27 (aW=0.909 ± 0.003). The sous-vide brined shows similar results, with its water activity maximum on day 8 (aW=0.954 ± 0.001) and a minimum on day 21 (aW=0.907 ± 0.003).

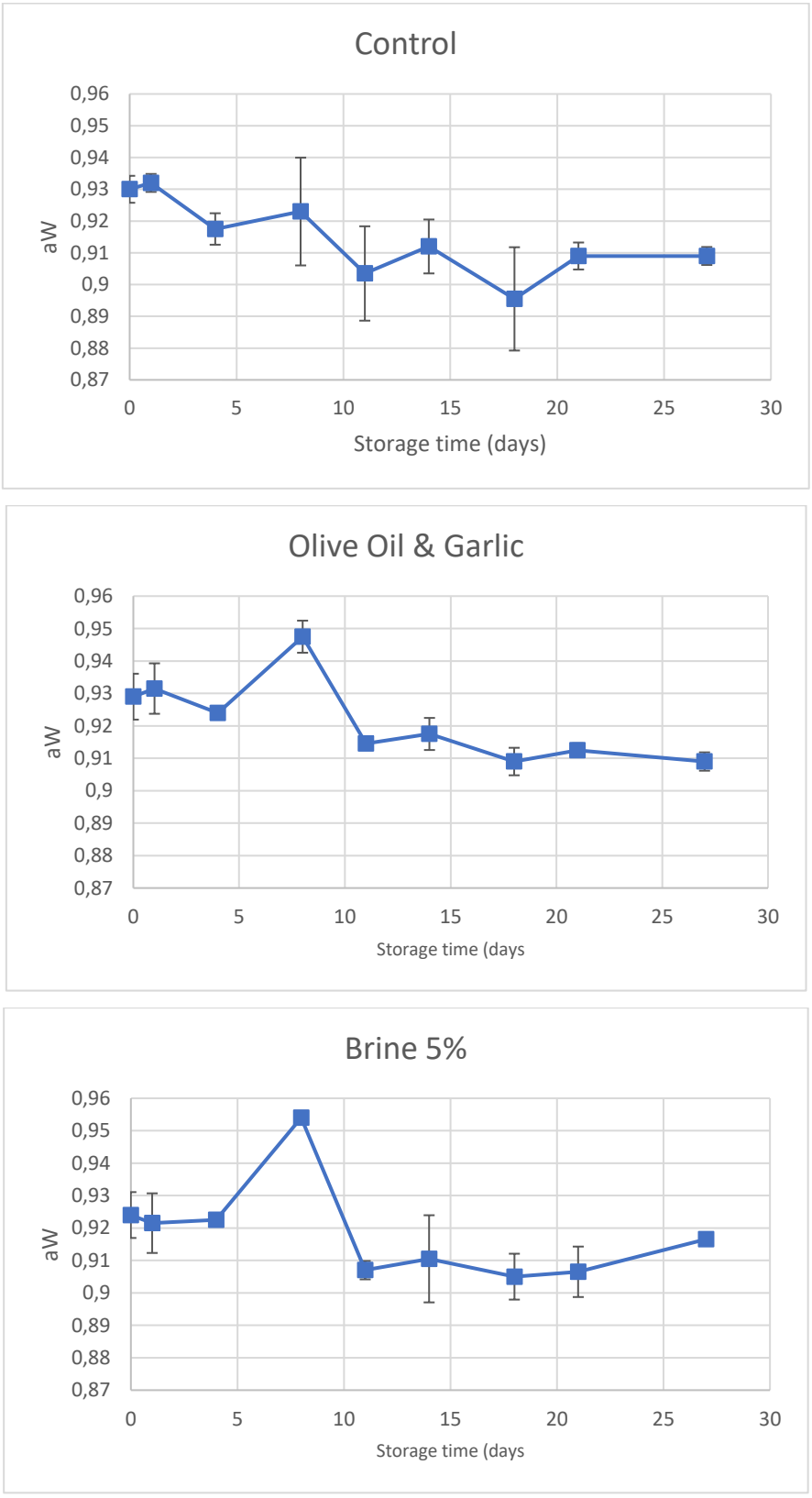

Figure 3.7 - Mean water activity (aW) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

There is statistical significance for time (ANOVA, $P < 0.05$), as seen by our results, water activity became lower as sampling occurred, once again, spoilage processes will interfere with cell wall integrity making the loss of free water inevitable. Treatment also has statistical significance (ANOVA, $P < 0.05$), more traditional methods of preparation such as salting are known to reduce water activity and inhibit microbial growth (Dong et al., 2020), and so can our treatments, which can explain the stabilization in our readings across sampling days. The interaction time*treatment did not have statistical significance (ANOVA, $P > 0.05$).

3.5. Thiobarbituric Acid Reactive Substances (TBARS)

Results show that across all treatment groups there was a significant decline in TBARS but also a rapid increase from day 0 to day 1 in all the test groups.

In the control group (Figure 3.8), we can observe a gradual decline in TBARS after hitting the maximum at day1 with 49.51 (± 2.28) µg MDA/kg and its minimum at day 22 with a value of 9.29 (± 1.01) µg MDA/kg.

In the sous-vide marinated group (Figure 3.8) showed an improvement when compared to the control group with its highest TBARS content being on day 1 with 41.76 (± 0.76) µg MDA/kg and lowest being on day 15 of the experiment with 6.89 (± 0.91) µg MDA/kg.

The sous-vide brined group (Figure 3.8) yielded the best results although with a similar trend of its highest TBARS values being on day 1 of the experiment with 54.67 (±16.03) µg MDA/kg and the lowest values being on day 15 with a TBARS content of 5.65 (± 0.97) µg MDA/kg.

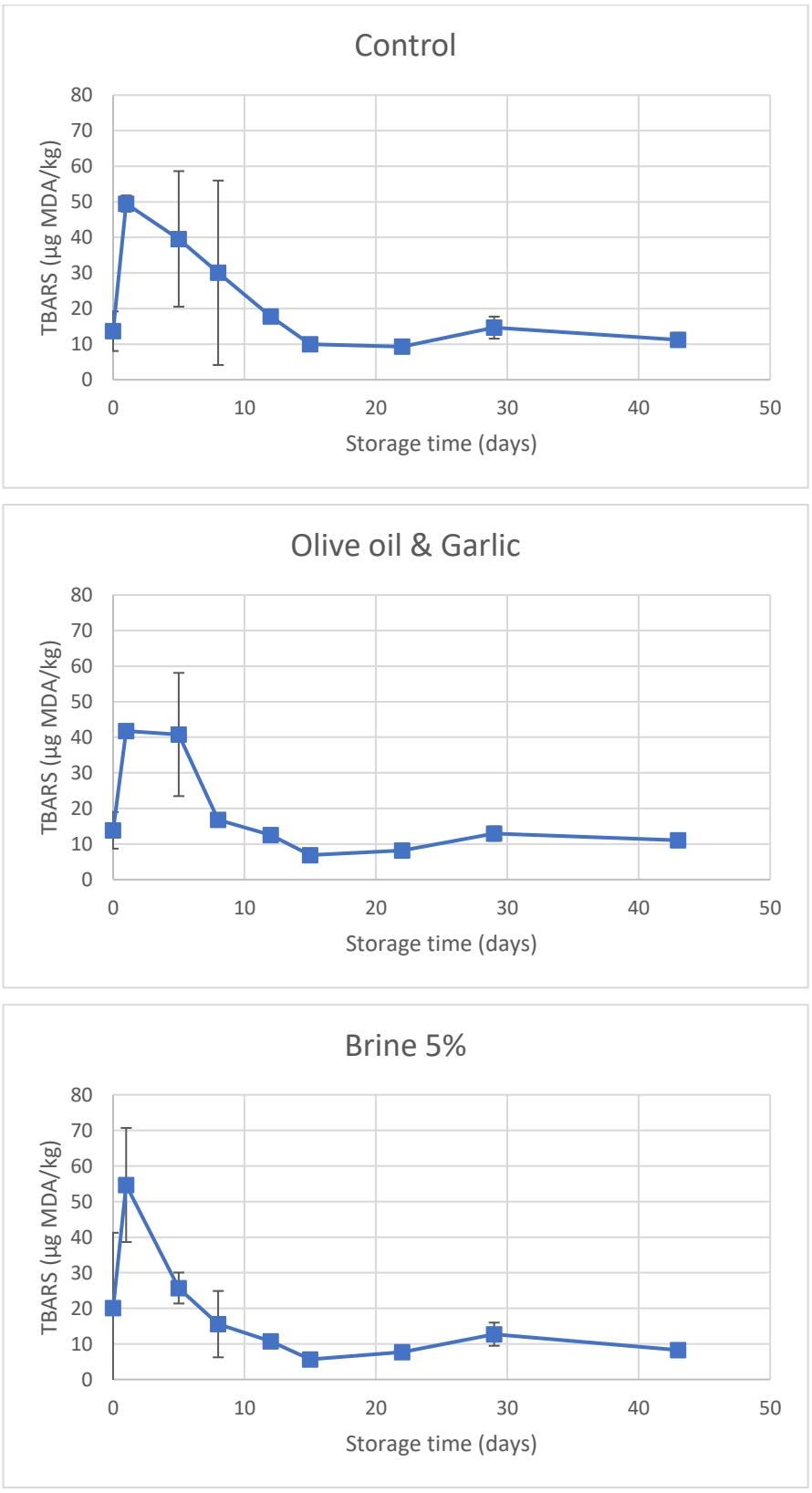

Figure 3.8 -Thiobarbituric reactive substances (TBARS) (µg MDA/kg) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

In this test our results show no statistical significance (ANOVA, $P > 0.05$) when testing treatment groups and the interaction time*treatment, but our results can also have become overestimated due to the characteristics of the TBARS test. TBA can react with other oxidized compounds apart from malondialdehyde (MDA) and can produce an overestimation of lipid oxidation (Hodges et al., 1998.; Jung et al., 2016; Kunyaboon et al., 2021). The production of yellow or orange chromogen (Figure 3.1) is another factor that can lead to overestimation of oxidation, the chromogen is a result of secondary of TBA reacting with sugars, water-soluble proteins, peptides, and free amino acids. Phenylpropanoid-type pigments can also show an absorbance at 532 nm, which also results in an overestimation of oxidation (Kunyaboon et al., 2021). MDA produced under assay conditions presents cross-reactivity with non-MDA substrates such as bile and aldehydes (Yeo et al., 1994). MDA can also be converted into other organic compounds with storage leading to low MDA values (Aubourg, 1999) which can explain the drop in TBARS content from day 4 of the experiment. Some authors have criticized the TBARS test, not only for its lack of sensitivity but also due to its high inaccuracy (Squires, 1990) due to the factors mentioned above.

Time was statistically significant (ANOVA, $P < 0.05$), as time goes by oxidative processes occur to lipids, and this can explain the drop in TBARS content as sampling occurs.

Although not regulated it is proposed a limit of 5-7 µg MDA/kg (IPQ, 2009a), and closely analyzing the results we can see that the samples presented values well above the suggested limit with some authors reporting values exceeding 29 µg MDA/kg in hake on the 8th testing day (Ruiz-Capillas & Moral, 2001) and reaching 250 µg MDA/kg on sardine by the 15th day (Nunes, Batista, & Campos, 1992).

3.6. Total Volatile Basic Nitrogen (TVBN)

Results show an increase in basic volatile nitrogen content in the fish tissue during the 43-day trial period.

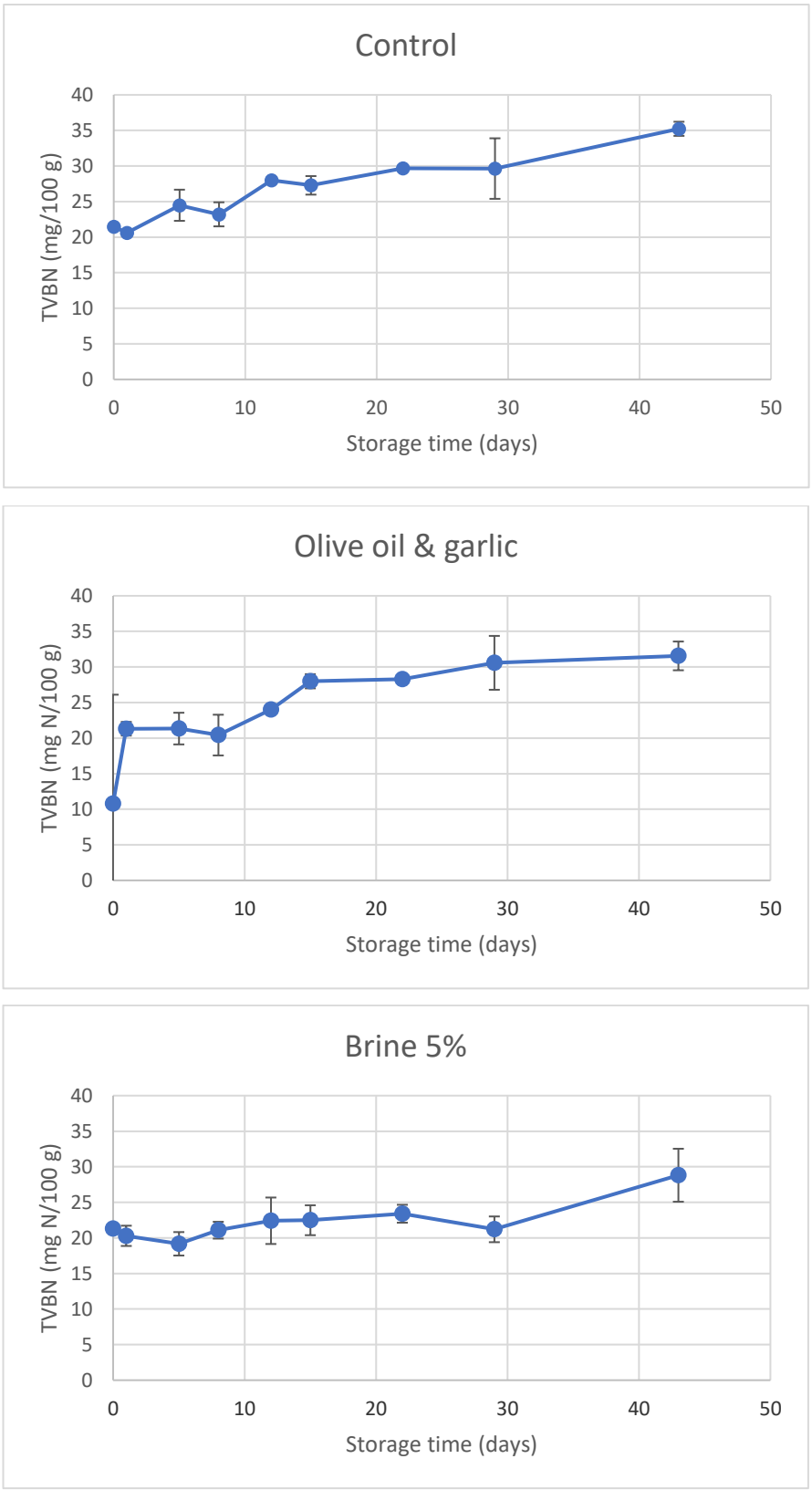

Figure 3.9 – Total volatile basic nitrogen (TVBN) (mg/100 g) (±SD) in control group (top), sous-vide marinated group (middle), and sous-vide brined group (bottom) during the experimental period.

The control group (Figure 3.9) had no type of treatment apart from the heat treatment that all groups were subjected to before packaging, and this is reflected in day 1 and day 43 of the experiment with a TVBN content of 20.61 mg N/100 g (±0.00) and 35,22 mg N/100 g (± 0.99), respectively, being the highest final TVBN content of the 3 test groups.

The sous-vide marinated group (Figure 3.9) obtained the lowest values in initial basic volatile nitrogen content in the tissue but showing an abrupt increase from day 0 with 10.81 mg N/100 g (± 15.29) to day 1 with 21.32 mg N/100 g (± 0.96), with 31.36 mg N/100 g (± 2.02) on the final day of the experiment.

The brined and sous-vide fillets (Figure 3.9) showed the lowest TVBN concentration in the sample tissue on day 1 with 20.29 mg N/100 g (± 1.42) and mostly continued its growth trend across the trial period up until day 43 with 28.81 mg N/100 g (±3.72).

Statistical analysis of our results show no significance ($P > 0.05$) when testing treatment and time*treatment, comparing our results to the Portuguese Standard and EU Regulation indication of 25-35 mg N/100 g for fish (IPQ, 2009b; UE, 2005, 2008), we observe that the control group passes this threshold on day 12 with 27,97 mg N/100 g but also has a very high starting point of 21,5 mg N/100 g which puts in question the samples freshness from the start, this is further backed from the general consensus that a fresh fish should have up to 5 mg N/100 g of nitrogen content. The sous-vide group passes the threshold on day 15 with 28.0 mg N/100 g but also shows an irregularity from day 0 to day 1 with a sudden rise of nitrogen content, this can be explained due to the variability of samples relative freshness from day 0 to day 1. In the sous-vide brined group although the starting values were high, but the samples only passed the threshold on day 43 with 28.8 mg N/100 g, these results go in accordance with the statistical significance (ANOVA, $P < 0.05$) obtained when testing time. Other studies obtained better results when also submitting mackerel to sous-vide cooking. On day 0 of their trials, they obtained a reading of 17.71 mg N/100 g (± 5.07), remaining fairly stable up to week 6 of their trials where they obtained 32.31 mg N/100 g (± 1.08) eventually reaching 36.53 mg N/100 g (± 6.32)on the last week of their trial, week 9 (Dogruyol & Mol, 2016) which is still lower than the proposed limit of 25 mg N/100 g (IPQ, 2009), which make us believe that there is some degree of success was achieved in our tests, and that our elevated readings can be related to sample freshness.

4. Conclusion

In this study, we investigated the effects of using sous-vide in combination with traditional processing techniques using natural preservatives, brining and marinating (in olive oil and garlic) on the quality and shelf-life of mackerel *S. colias* fillets. Although mackerel is usually associated only with fisheries, due to its high lipid and protein profile (making it easily perishable) and potential for aquaculture production, our findings can be applied to other commercially farmed or captured fish species with similar composition.

Sample freshness at start of trials was not optimal, but we still obtained positive results. On day 12 of the experiment, TVBN content in the control group was 27.97 mg N/100 g which is already higher than the proposed limit of 25 mg N/100 g. The olive oil and garlic marinated sous-vide fillets also passed the threshold, but on day 15 with a TVBN content of 27.99 mg N/100 g, whilst the brined and sous-vide fillets did not pass the threshold until day 43 with a TVBN content of 28.81 mg N/100 g. This leads us believe that the delay in the formation of nitrogenous compounds in the samples tissue can be related to the different treatments and, to a lesser extent, to the modified atmosphere packaging in which they were submitted.

The pH of the samples remained relatively stable in the 6-6.1 range during sampling, suggesting that the combined effect of our treatments and the sous-vide technique has had a positive impact in maintaining this parameter stable.

Water activity seems to have stabilised on day 11 for both olive oil and garlic marinated and brined sous-vide fillets, being in the 0.90-0.91 ranges, but not so much for the control group, where we can still observe some variance in our readings. Water activity decreased during the storage trial, following different patterns per treatment. Sous-vide cooking and our treatments also had a positive effect on samples, although it having the lowered L readings, the treatments and the process of cooking can lower L values, redness mostly increased, and were observed in all preparations, and yellowness remained mostly stable during the trial period. We hypothesized that it can be due to sample preparation and sample freshness.

In our results regarding TBARS, we observed a decrease in concentration but, as this is an oxidative reaction of lipids that occurs in the sample, it may simply mean that most of the lipid content in samples was already oxidized. On the other hand, there could

have been issues with the adequacy of the TBARS content as an indicator of oxidation in sous-vide fillets, that were not yet reported in the literature which is scarce on this topic.

Our tests on TVBN didn't yield any evidence that the sous-vide technique combined with the treatments have impact in the slowing down of protein degradation. However, comparing our results between samples, we can see that the treatment methods contribute to the reduction of protein degradation reactions.

To better understand this topic, more studies should be carried out at different stages of freshness and/or different species to rule out all the different factors that came into play during the experiment, with the biggest shortcoming being the freshness of the samples, this can be very promising in the matter that we can provide more suitable ways to preserve fish, promoting better sustainability and less food waste.

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6. Annex

```
Response: L
                Sum Sq  Df F value      Pr(>F)
time            781.14   8  5.6842 0.000003154 ***
treatment       393.43   2 11.4517 0.000025475 ***
time:treatment  578.11  16  2.1034      0.0114 *
Residuals      2319.00 135
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.1 – ANOVA test results for lightness (L).

```
Response: a
                Sum Sq  Df F value    Pr(>F)
time           148.925   8 11.0756 6.005e-12 ***
treatment       33.782   2 10.0496 8.538e-05 ***
time:treatment  26.469  16  0.9842    0.4775
Residuals      226.906 135
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.2 – ANOVA test results for redness (a > 0).

```
Response: b
                Sum Sq  Df F value        Pr(>F)
time             76.00   8  1.7837       0.08544 .
treatment       196.40   2 18.4385 0.00000008324 ***
time:treatment   64.32  16  0.7548       0.73322
Residuals       718.99 135
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.3 – ANOVA test results for yellowness (b > 0).

```
Response: hardness..N.
                Sum Sq Df F value    Pr(>F)
time            461743  8  5.7773 0.0002488 ***
treatment        25803  2  1.2914 0.2913349
time:treatment  191543 16  1.1983 0.3293387
Residuals       269743 27
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.4 – ANOVA test results for hardness (N).

```
Response: pH
               Sum Sq  Df F value    Pr(>F)
time           0.39490   8 21.4576 < 2.2e-16 ***
treatment      0.10521   2 22.8665 2.802e-09 ***
time:treatment 0.31001  16  8.4225 8.303e-14 ***
Residuals      0.31057 135
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.5 – ANOVA test results for pH.

```
Response: aW
                 Sum Sq Df F value        Pr(>F)
time           0.0072930  8 15.8748 0.00000002244 ***
treatment      0.0004440  2  3.8658       0.03339 *
time:treatment 0.0013373 16  1.4555       0.18926
Residuals      0.0015505 27
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.6 – ANOVA test results for water activity (aW).

```
Response: TBARS..µg.MDA.kg..AVG
               Sum Sq Df F value        Pr(>F)
time           9240.0  8 13.8533 0.00000009363 ***
treatment       161.2  2  0.9664        0.3932
time:treatment  693.2 16  0.5197        0.9133
Residuals      2251.1 27
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.7 – ANOVA test results for thiobarbituric acid reactive substances (µg MDA/kg).

```
Response: ABVT
               Sum Sq Df F value     Pr(>F)
time           1076.83  8  6.6191 0.00008624 ***
treatment        94.22  2  2.3167     0.1179
time:treatment  500.00 16  1.5367     0.1577
Residuals       549.07 27
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 6.8 – ANOVA test results for total volatile basic nitrogen (mg/100 g).