Treball de Fi de Grau

Grau en Enginyeria Química

Antioxidant activity of pecan nut (*Carya illinoinensis*) in fish

MEMÒRIA

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ABSTRACT

The effects of pecan nut (*Carya Illinoinensis*), roselle flower (*Hibiscus Sabdariffa*) and moringa leaves (*Moringa Oleifera*) as antioxidant and antimicrobial agents on shelf life extension of fresh sardine (*Sardina pilchardus)* and Gilt-head Sea Bream (*Sparus Aurata)* stored at 4 ± 1 ºC were evaluated over a period of time until they were no longer edible. Four experiments were performed with different treatments and concentrations. In all cases the samples were compared to a negative control (with no treatment) and to a positive control (a sample containing butylated hydroxyanisole (BHA), a currently used artificial antioxidant).

The main goal of this work was to evaluate the potentials of pecan nut as a natural antioxidant to be used as an effective food preservative for the food industry. Although roselle and moringa also have antioxidant properties, they were aimed to act as antimicrobial agents, to complement the activity of pecan nut, and discover any possible synergic effects of combination of treatments. Radical scavenging activity and total phenolic content assays of the three natural compounds were performed through DPPH and Folin-Ciocalteu analyses to have an idea of their radical scavenging power.

To assess the effectivity of the treatments on the fish samples physicochemical (thiobarbituric acid reactive substances (TBARS), fatty acids, hexanal and biogenic amines), sensory and microbiological characteristics of fish samples were periodically analyzed.

All treatments showed some difference in comparison to the control, although similar effectiveness to BHA was found in samples containing concentrations of 5% w/w or higher of either pecan nut or roselle. Although moringa showed promising results in TBARS analysis, it was quickly discarded due to its green color, which conferred a non-agreeable aspect to the samples that could lead to consumer's rejection. Among all treatments 10% w/w pecan nut showed the highest effectivity in preservation of the fish samples. Treatments with presence of roselle and moringa reduced microbial growth as compared with either treatments with pecan nut or the control.

Results could therefore indicate that addition of a natural preservative with a combination of pecan nut and roselle may be a promising method to extend shelf life of fresh fish during chilled storage while maintaining its quality indexes. These results are promising for the food industry, since there is a raising concern from consumers to avoid the use of artificial antioxidants and find healthier alternatives.

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1. GLOSSARY

ABTS: 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid

ADI: acceptable daily intake

Amu: atomic mass unit

BA: Biogenic amines

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CAD: Cadaverine

CFU: Colony-forming units

CHD: Coronary heart disease

CVD: cardiovascular disease

DHA: cis-4,7,10,13,16,19-docosahexaenoic acid

DO: Dopamine

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DW: Dry Wieght

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Administration

EPA: cis-5,8,11,14,17-eicosapentaenoic acid

ETSEIB: Escola Tècnica Superior d'Enginyeria Industrial de Barcelona

EU: European Union

EUMOFA: European Market Observatory for Fishering and Aquaculture

FA: Fatty Acids

FAME: Fatty Acids Methyl Ester

FDA: Food and Drug Administration (USFDA)

FID: flame ionization detector

Folin: Folin-Ciocalteu Analysis

FRAP: Ferric reducing antioxidant power

FW: Fresh weight

GAE: Gallic Acid equivalent

GC: Gas chromatography

HIS: Histamine

HPLC: High-pressure líquid chromatography

HS-GC/MS: Headspace gas chromatography mass spectrometry

L: Lipid radicals

LC: Liquid chromatography

LDL: Low density lipoprotein (cholesterol)

LOO·: Lipid peroxides

LOOH: Lipid hydroperoxides

MDA: Malonaldehyde

MOAI: Mono amine oxidase inhibitor

MUFA: monounsaturated fatty acids

n3: n3-polyunsaturated fatty acid

n6: n6-polyunsaturated fatty acid

OC: Octopamine

OPT: O-ophtalaldehyde

ORAC: Oxygen Radical Absorbance Capacity

PAC: Proanthocyanidins

PN: Pecan Nut

PTFE: Polytetrafluoroethylene

PUFA: polyunsaturated fatty acids

PUT: putrescine

RDM: Reagent delivery module

ROS: Reactive oxygen species

RSA: Radical scavenging activity

SD: Standard deviation

SEM: Standard error of the mean

SER: Serotonine

SFA: Saturated fatty acids

SPD: Spermidine

SPM: Spermine

SW: Sample weight

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

TBQH: Tert-butylhydroquinone

TCA: Trichloroacetic acid

TE: Trolox equivalent

TPC: Total polyphenol content

TRP:Ttryptamine

TSA: Tryptone soya agar

TYR: Tyramine

UB: Universitat de Barcelona

UPC: Universitat Politècnica de Catalunya

USA: United States of America

UV/VIS: Ultraviolet-visible spectroscopy

v/v: volume volume

w/w: weight weight

2. FOREWORD

2.1. Origin of the project

Additives are present in most of the food consumed nowadays. Antioxidants are used to prolong the shelf-life of food products, making possible its commercialization locally and worldwide, facilitating consumers the possibility to shop less often and diminishing food waste by delaying food spoilage. Nevertheless, research has shown that most of the artificial antioxidants currently used could promote and/or cause multiple health threatening conditions.

2.2. Motivation

Since no previous experiments have been found in literature about pecan nut (*carya illinoinensis*) as a fish preservative or combinations between pecan nut and roselle (*Hibiscus Sabdariffa*) or moringa (*Moringa Oleifera*), there is room to research on the potentials of these combinations and their effectivity, as well as to create a new product for the food industry.

3. INTRODUCTION

3.1. Objectives

The main goal is to research the antioxidant and antimicrobial activity of pecan nut (*carya illinoinensis*) to assess its effectivity as a natural food preservative either alone or in combination with roselle (*Hibiscus Sabdariffa*) and moringa (*Moringa Oleifera*).

This goal can be disaggregated into the following concrete points:

1. Bibliographic study of currently existing research on use of pecan nut as a food preservative and more specifically for fish
2. Research about pecan nut's antioxidant activity. Folin-Ciocalteu and DPPH analysis to assess its radical scavenging power
3. Determination of pecan nut's effectivity as an antioxidant and antimicrobial fish preservative through evolution of TBARS analysis and recount of mesophilic bacteria respectively. Detection of lipid and protein oxidation by-products such as hexanal and biogenic amines
4. Determination of roselle and moringa's antioxidant and antimicrobial activity and possible combinations with pecan nut through performance of the aforementioned analysis

5. Evaluation of success in comparison to negative (without treatment) and positive controls (current commercial antioxidants)
6. Acceptability of designed product, sensory analysis, preference among treatments and possible applications to food industry

3.2. Scope of the project

Food additives serve many purposes including preserving food, improving its texture, flavour or appearance and prolonging its shelf life. Antioxidants are used to increase the shelf life of food products by protecting them against deterioration caused by oxidation, such as fat rancidity and colour changes. Antioxidants used as food preservatives can be obtained from natural sources or synthetically manufactured. Many studies - mostly carried out in animals but also some in humans - have shown that artificial additives could cause health problems such as skin conditions, allergy, stomach problems, asthma, weight gain, headache, behaviour changes and cancer (Ito *et al.,* 1983; Goodman *et al.,* 1990 and Reus *et al.,* 2000). Most of these substances are regulated by health organisations such as the FCA (U.S. Food and Drug Administration) or the EFSA (European Food Safety Authority) so health threatening concentrations are never reached. This is done through the ADI (Acceptable Daily Intake) which is the maximum amount of each additive that can be added to a food product. The problem is that these substances could be dangerous even at low concentrations, and that they are present in so many food products that it is difficult to prove that ADIs are not surpassed. Some of the most common synthetic antioxidants used nowadays in the food industry as TBHQ (Tert-butylhydroquinone), BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene), Propyl gallate, Octyl gallate and Dodecyl gallate have shown the adverse reactions after consumption mentioned before (Race, 2009).

Due to the potential risks of these artificial antioxidants, there is an increasing demand from consumers to buy food with natural additives and without artificial ones or a reduced amount of them. This has created a suitable environment to investigate on possible natural preservatives. Many naturally occurring compounds such as phenols (phenolic acid, polyphenols, tannins), and organic acids (acetic, lactic, citric) have been considered in this context. Many spices, herbs and extracts possess antimicrobial activity (Zhang *et al.*, 2016).

3.3. Why are antioxidants needed for food preservation?

Lipid oxidation is a natural process which occurs in all foods, but especially in those containing fat. This reaction, triggered by the contact between oxygen and lipids decreases the quality of food, promotes rancidity, off-flavour and taste and, also, generates free radicals Reactive Oxygen Species (ROS) which have been associated with development and promotion of cancer (Falowo *et al.*, 2014).

Both fish and meat are products which get easily oxidized due to the high amount of polyunsaturated fatty acids (PUFA) they contain. Lipid oxidation is difficult to control, because once it has been started a chain of reactions occur, resulting in a wide range of products. The oxidation leads to the formation of lipid radicals (L) which react to lipid peroxides (LOO·) and hydroperoxides (LOOH). The first ones will form products such as aldehydes, alkanes and conjugated dienes. Oxidation can be initiated due to presence of oxygen, light, heat, metal ions and radicals (Samples, 2013).

Lipid oxidation reduces product stability and shelf storage time and is an inconvenience for the food market. Therefore, antioxidants are used to limit the scope of the reaction. They work by getting reduced in contact with oxygen, avoiding the oxidation in the food itself.

Antioxidants are naturally produced in living cells to protect them against free radicals. That is why they can be found in spices, seeds, herbs, essential oils, fruits, vegetables and leaves (Zang *et al.*, 2017) These antioxidants can be used in a lot of different forms such as pure extracts, powders, in films, in coatings or as a blend of active components among others. In the case of fish and meat, these antioxidants can be applied through the feed of the animal or post-mortem in the processing, two very wide fields which also present different outcomes. This work focuses on post-mortem application. Many of the natural antioxidants have been found to have health promoting benefits such as anti-inflammatory properties, which gives an added value to food products where they are present, even with the potential to turn them into functional foods.

Previous studies have shown the antioxidant power of nuts, as well as successful results in their use as antioxidants for meat -mostly in the form of walnut (*Juglans regia L.*) (Vinson *et al.*, 2011). Therefore, in this study their use as food preservatives for fish will be investigated, as well as their potential to be used as a regular additive for the food industry.

3.4. Selection of the nut type

There is currently a big database of research about different natural antioxidants and antimicrobials to be used as food preservatives such as rosemary, tea polyphenol, oregano oil, thymol, grape seed extract or chitosan (Ramziia *et al.,* 2018; Falowo *et al.,* 2014 and Li *et al.,* 2012).. Previous studes have only been found assessing the effects of walnut leaves on fish preservation (Bello *et al.,* 2013), but nut kernels haven't yet been used as antioxidants for fish burgers, which is the aim of this project.

Phytochemicals are chemical compounds produced by plants to help defend themselves against competitors, predators or pathogens. The name comes from the Greek word *python* which means plant. Because there are many different types of nuts, one of them should be selected according to its phytochemicals content and the antioxidant activity they provide.

According to Bolling (2011) Figure 1 shows the classification of tree nut phytochemicals, the presence and amount of each component depends on the type of nut, cultivar location and post-harvest conditions among others:

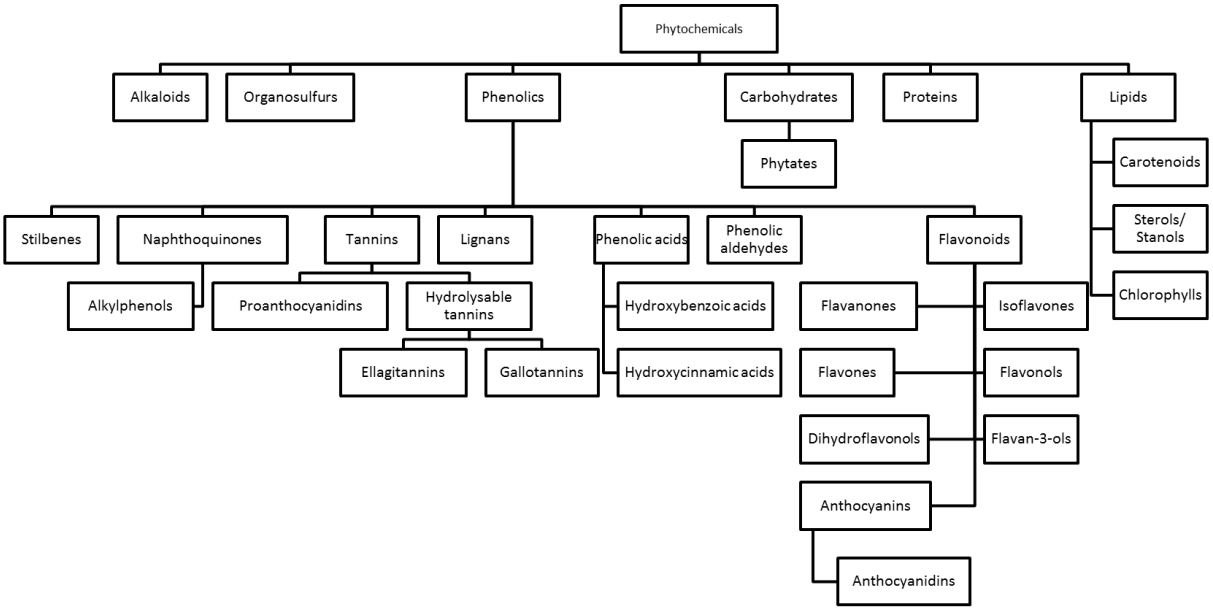


Figure 1: Classification of tree nut phytochemicals (Bolling *et al.*, 2011)

Bolling reviewed different databases for phytochemical contents and antioxidant capacity of almonds, Brazil nuts, cashews, hazelnuts, macadamias, pecans, pine nuts, pistachios and walnuts:

- **US Department of Agriculture phytochemical databases**: total phenols, flavonoids, isoflavones, total proanthocyanidins (PAC)
- **Phenol-Explorer phytochemical database:** flavonoids, phenolics, stilbenes and PAC
- **US Department of Agriculture 2009 National Nutrient Database for Standard Reference:** phytosterol

In all of them pecan nut shows the higher values in most of the phytochemicals in current literature. The values are collected in Table 1:

Phytochemical (mg/100g)	Amount
Alkaloids (ng/g)	ND
Phytates	851,60
Chlorophylls	ND
Lignans	21,00
Alkylphenols	ND
Naphthoquinones	41,03
Hydrolysable tannins	ND
Sphingolipids	373,45
Total phenols	1588
Carotenoids	55
Phenolic acids and aldehydes	2052
Flavonoids	2713,49
Proanthocyanidins	493,90
Sterols	233,52
Stilbenes	ND

Table 1: Phytochemicals present in pecan nut according to US Department of Agriculture phytochemical databases, Phenol-Explorer phytochemical database and US Department of Agriculture 2009 National Nutrient Database for Standard Reference. Values displayed are means from all available in literature. ND= Not determined in the literature.

In the same study by Bolling a comparison between the different types of nuts can be found. In Figure 2 it can be clearly seen that pecan nut almost invariably has the higher content of phytochemicals among all nut types.

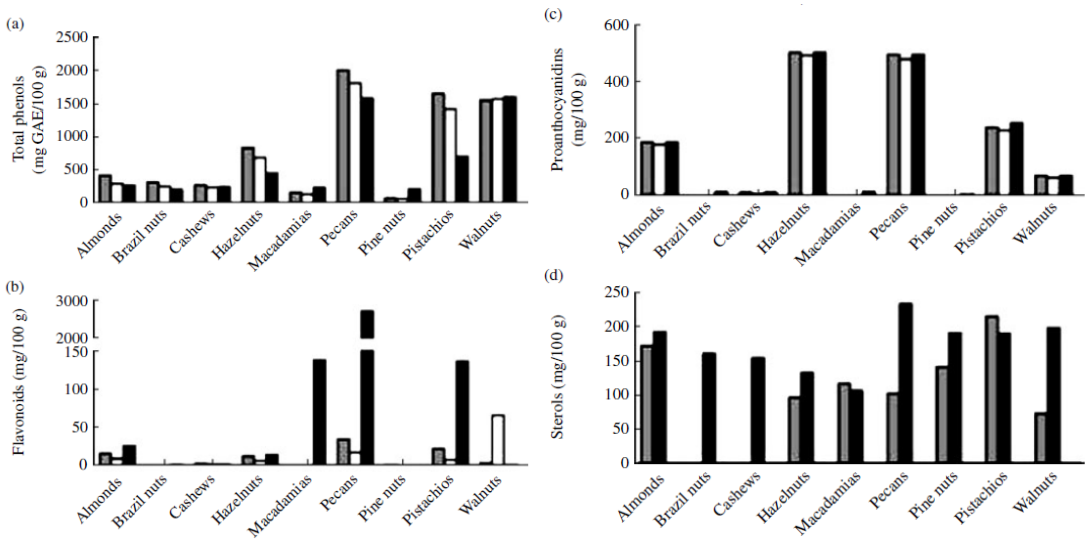


Figure 2: Values for (a) total phenols, (b) flavonoids, (c) proanthocyanidins and (d) sterols. Grey = Phytochemical database values for tree nuts relative to current literature. Black= US Department of Agriculture. White= Phenol-Explorer; GAE, gallic acid equivalents.

Because in many studies (Alasalvar *et al.,* 2009; Bolling *et al.*, 2011 and Alasalvar *et al.*, 2014) pecan nuts have shown the stronger antioxidant activity within all nuts, they have been chosen to perform the experiment.

3.5. A review on Mexican pecan (*Carya illinoinensis*) phytochemicals and their antioxidant activity

De la Rosa (2011) performed a thorough study on the phenolic compounds and antioxidant activity of kernels and shells of Mexican pecan (*Carya illinoinensis*) from Chihuahua, Mexico and found high concentrations of total extractable phenolics, flavonoids and proanthocyanidins in kernels and 5-20 fold higher concentrations in shells. Table 2 shows their results on three different sampling locations.

	Phenolic compounds (mg GAE/g FW)		**Flavonoids (mg CE/g FW)**		**Proanthocyanidins (mg CE/g FW)**	
Growing area	Kernel	Shell	Kernel	Shell	Kernel	Shell
1	11,7 ± 0,3	86,4 ± 7,1	5,9 ± 0,7	33,1 ± 1,8	20,6 ± 1,7	396,0 ± 30,2
2	12,5 ± 0,2	65,3 ± 6,9	6,4 ± 0,8	26,3 ± 2,6	26,7 ± 4,5	316,1± 17,3
3	11,9 ± 0,3	92,5 ± 9,0	6,8 ± 0,8	36,1 ± 1,8	20,3 ± 0,5	464,4± 38,0

Table 2: Phenolic compounds, Flavonoids and Proanthocyanidins of Pecans grown in the State of Chihuahua, Mexico (De la Rosa *et al.*, 2011).

Many methods have been used to calculate the antioxidant and radical scavenging activity, finding that both kernel and shells are highly effective scavengers -the shell is three times more effective- mainly due to the presence of phenolic compounds. Table 3 details the radical scavenging activity of pecans grown in the State of Chihuahua, Mexico.

	ORAC (ROO* scavenging) (μmol TE/g FW)		DPPH* scavenging Flavonoids (μmol TE/g FW)		ABTS*$^{-}$ scavenging (μmol TE/g FW)		HO* scavenging (mmol GAE/g FW)	
Growing area	Kernel	Shell	Kernel	Shell	Kernel	Shell	Kernel	Shell
1	231,2 ± 15,0	859,5 ± 180,8	104,4 ±8,3	655,1 ± 49,9	83,4 ± 1,2	594,5 ± 83,6	12,8 ± 1,6	37,0 ±3,1
2	261,5± 37,6	680,3 ± 66,8	108,7 ± 9,0	537,8 ± 33,8	81,8 ± 3,0	518,4 ± 80,7	11,9 ± 0,5	30,2 ± 2,2
3	227,0 ± 50,1	1350,3± 85,9	102,6 ± 9,3	720,3 ± 50,2	75,9 ± 11,8	644,2 ± 62,2	13,0 ± 1,9	41,7 ± 5,8

Table 3: Radical Scavenging Activity of Pecans Grown in the State of Chihuahua, Mexico (De la Rosa *et al.*, 2011).

3.6. Antimicrobial and antifungal compounds

Fish and fish products are highly perishable and develop many bacteria and fungi during storage. A common practice to prolong shelf-life of these products is the use of antibiotics in livestock (Katakweba *et al.,* 2012 and Ferber, 2003), their counterpart being that bacteria develop resistance to them and a higher amount is the needed. Instead, there are different plants known to have antibacterial and antifungal properties. Other methods of preservation consist in reducing water activity through salting, smoking or drying, all of which change the structure, texture and taste of the fish products.

Different studies have researched the antimicrobial and antifungal properties of *Moringa Oleifera* (Vinoth *et al*., 2012) as well as proved it an effective compound against development of bacteria in fish (Adeyemi *et al.*, 2013; Onyuka *et al.*, 2013 and Bijina *et al.*, 2011).

Adeyemi performed a study with 1%, 2% and 3% concentrations of moringa added to smoke-dried African catfish (*Clarias gariepinus*) stored at room temperature (37 ± 2 ºC). The three levels proved to be useful in diminishing the load of microbial and fungi compared to the control, with the 3% concentration being the more effective one.

Onyuka researched the antibacterial activities of salt (chloride solution), chlorinated solution, moringa n-hexane extract and moringa ethanol extract concentrations in O. *niloticus* and *R*. *argentea* fish samples at 0 h and after 8 h of treatment duration. The results showed that all

work properly as antimicrobials. Salt was also effective because it diminishes the water activity in the sample, thus creating a less suitable environment for bacteria to grow.

Roselle (*Hibiscus Sabdariffa*) contains many different phytochemicals with antioxidant properties. Aqueous extract of hibiscus has high tannin (4420,87 ± 110,7 mg CE/100g) and anthocyanin (205,76 ± 3,4 mg c-3-QE/100g) contents and shows high ferric reducing antioxidant power (FRAP) (2883,23 ± 218,7 μmoles Fe (II)/100g). Aqueous and ethanol extracts also present antimicrobial activity against food-borne pathogens *Salmonella typhimurium* and *Staphylococcus aureus* (Mak *et al.*, 2012 and Mohamed Radwan Afify, 2016). Even so, a study carried out on *sucuk* (Turkish dry-fermented sausage), showed that this extract is less effective than other plant extracts such as *Urtica dioica* and also less effective than current commercially available chemical preservatives like nitrite, nitrate and BHT (Karabacak *et al.*, 2007).

All these studies show potential for moringa (*Moringa Oleifera)* and roselle (*Hibiscus Sabdariffa)* to be used as natural preservatives together with pecan nut (*Carya illinoinensis*) in order to develop new healthier and functional food with increased shelf life. Because salt has also been found to delay bacterial spoilage, it will also be added in small amounts to the samples.

To enlarge the shelf live and preservation of fish products, the synergic effect between the natural antioxidant of pecan nut (*Carya illinoinensis*) and two different antimicrobial and antifungal compounds, namely moringa (*Moringa Oleifera)* and roselle (*Hibiscus Sabdariffa*) will be investigated.

3.7. Health promoting benefits of natural antioxidants

Natural antioxidants have a double functionality; they can be used to substitute chemical additives, but they often also work as healthy additions to foods, turning them into functional products.

Free radicals are an important cause for diseases like CVD, cancer, Alzheimer's, arthritis, diabetes, cataract, premature senility, arteriosclerosis and Parkinson's (Sun *et al.*, 2018 and Valko *et al.,* 2007). They destroy the internal redox balance; therefore, consumption of natural antioxidants is crucial to maintain the homeostasis (internal balance).

In these last years, where natural antioxidants have been gaining importance in the food market, different studies have been carried out proving they are effective for the treatment of

diseases. Li (2014) investigated the main natural antioxidants, where they can be found (vegetables, fruits, nuts, tea, oils…) and their targeted diseases.

Many studies (Jiménez-Colmenero *et al.*, 2010; Ercoskun *et al.,* 2009; Serrano *et al.,* 2007; Serrano *et al.,* 2006 and Danut Mocanu *et al.,* 2015) have been performed using walnut as a substitute for meat fat, creating a functional products with more MUFAs, PUFAs and phenolic compounds instead of saturated fatty acids and cholesterol, which are considered promoters of diseases like CVD or obesity.

Jiménez-Colmenero assessed the effect of meat-based functional foods with an added 21% walnut, through a 5-week study with volunteers presenting increased risk of cardiovascular disease. Instead of regular meat, the volunteers consumed meat products with added walnut, which meant a consumption of 19,4 g of walnut/day at the end of the week, which is more or less 70% of the suggested amount by the FDA (2009). The result after the study was a reduction of intermediate clinical markers of CHD (such as total and LDL cholesterol), improvement in antioxidant status and reduction in thrombogenesis markers. The conclusion is, therefore, that nuts can work as functional meat derivatives and have to be considered for future developments of functional foods as well as promoting their current function as natural antioxidants.

3.8. Selection of the fish type

Initially gilt-head sea bream (*Sparus Aurata*) was chosen to perform the experiments. This fish is easily available in Spain and throughout all Europe; landings of seabreams in Europe in 2014 amounted to 38 thousand tonnes (EUMOFA, 2016). Nevertheless, during the process of the work, it was apparent that the low percentage of fat present in this species was not ideal to be able to extract clear results. Therefore, even though the initial experiment was performed with Gilt-head Sea Bream, sardine (*sardine pilchardus*) was used for the following ones. It was chosen because of its recognized content of fat, enough information in literature could be found and moreover because it is an important fish species in Europe and specifically in Spain.
Its production in Europe reached 175 thousand tonnes in 2014, with a value of 161 million euro (EUMOFA, 2016).

3.9. Hexanal formation

Foods with high fat content such as sardine are susceptible to rancidity because of peroxidation of their lipid fraction. The oxidation of unsaturated fatty acids generates hydroperoxides, highly reactive substances which rapidly decompose into volatile and non-volatile compounds such as hydrocarbons, alcohols, acids, aldehydes and ketones (Sanches-Silva, 2004). These are called secondary lipid oxidation products and contribute to flavor and taste deterioration. Hexanal is a by-product of the reaction of lipid oxidation and it is mainly generated due to the oxidation of w-6 fatty acid peroxides, mostly from linoleic acid through 13-hydroperoxide (Figure 3). Arachidonic acid is also involved in creation of hexanal (Varlet *et al.,* 2007).

The most commonly used method for the determination of lipid oxidation is 2-thiobarbituric acid reactive substances test (TBARS), which allows a rapid assessment of lipid oxidation crucial to the food industry. Even so, it has received much criticism because its lack of sensitivity and specificity (Melton, 1983; Wang *et al.,* 1997 and Goodridge, 2003) because compounds not related to lipid oxidation can also react with thiobarbituric acid and because naturally colored substances can interfere with the results of the analysis (Ross, 2006).

Hexanal, being a major product of fat oxidation which increases during storage, has hence become a more trusted oxidative state indicator (Panseri *et al.,* 2011). It has been widely reported to have a "green" odor (Varlet *et al.,* 2007; Triqui *et al.*, 2003 and Ganeko *et al.*, 2008).

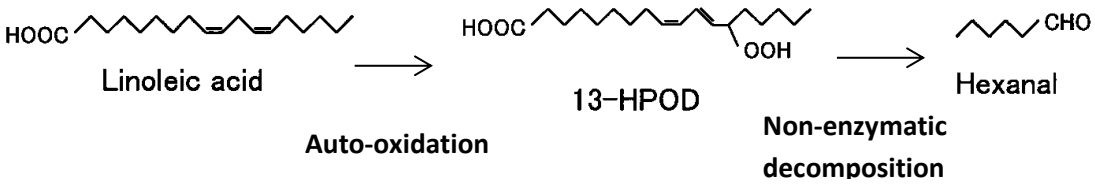


Figure 3. Generation of hexanal from Linoleic Acid via 13-hydroperoxide (Kuroda *et al.,* 2003).

3.10. Biogenic amines in food

Biogenic amines (BA) are basic nitrogenous compounds usually generated in foods and beverages by microbial decarboxylation of amino acids or amination and transamination of aldehydes and ketones (Askar *et al.,* 1986). They are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms (Brink *et al.*, 1990). The chemical structure of BA can either be: aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine).

Amines are usually formed during a decomposition or spoilage process involving formation of free amino acids through proteolysis together with bacterial production and action of amino acid decarboxylases. Amino acid decarboxylation takes place by removal of the α-carboxyl group to give the corresponding amine. Amino acid decarboxylases are found in certain *Enterobacteriaceae*, *Clostridium*, *Lactobacillus*, *Streptococcus*, *Micrococcus*, and *Pseudomonas* species, among others (Shalaby, 1996).

In non-fermented foods the presence of biogenic amines above a certain level is considered as indicative of undesired microbial activity. Therefore, the amine level could be used as an indicator of microbial spoilage (Santos, 1995). However, the presence of biogenic amines in food does not necessarily correlate with the growth of spoilage organisms, because they are not all decarboxylase-positive (Santos *et al*., 1985 and Vidal *et al*., 1990).

The best known type of food poisoning caused by biogenic amines derives from consumption of high levels of histamine. It is also referred to as “scromboid fish poisoning” because of the frequent association of this illness with consumption of scombroid fish such as tuna, mackerel, saury, bonito, seerfish and butterfly kingfish although non-scombroid fish like sardine, anchovies, pilchards, marline or herring have also been implicated in cases of histamine poisoning (Taylor, 1983). Putrescine and cadaverine, which occur in high levels in toxic fish, have been reported to potentiate the biological effects of histamine (Arnold *et al.*, 1978) up to ten times (Parrot *et al.*, 1966; Bjeldanes *et al*., 1978 and Hui *et al.*, 1985). Other biogenic amines which may increase the toxicity of histamine are tyramine, tryptamine and β-phenylethylamine (Stratton *et al*., 1991). In the European Union (EU) the legal limit for histamine levels in fish is 100 mg/kg in raw fish and below 200 mg/kg in salted fish for species belonging to the Scombridae and Clupeidae families

Another typical phenomenon is the “cheese reaction” caused by high levels of tyramine in cheese which reacts with mental depression drugs containing monoamine oxidase inhibitor

(MOAI) giving rise to hypertensive crisis (Taylor, 1983). The allowable maximum level of tyramine in foods is 100-800 mg/kg and concentrations of 1080 mg/kg are toxic for humans (Shalaby, 1996).

Amines have also been studied as carcinogenic precursors, since some amines may be nitrosated or act as precursors for other compounds capable of forming nitrosamines (Shalaby, 1996). Moreover, the presence of nitrite can induce the formation of carcinogenic N-nitrosamines from amines, and factors such as heating can turn putrescine and cadaverine into N-nitrosopyrrolidine and N-nitrosopiperidine respectively (Doyle *et al.*, 1993).

Shalaby reports histamine, tyramine, cadaverine, putrescine, agmatine, spermine and spermidine to be the amines which can develop in fish. The highest amounts found in canned sardine according to literature are 850 mg histamine /100g, 115 mg putrescine /100g and 270 mg cadaverine /100g.

4. EXPERIMENTS

4.1. Materials and methods

4.1.1. Preparation of products

Pecan nut (*Carya illinoinensis*)

Pecan nut (PN) (*Carya illinoinensis)* from USA (1,005 kg) was shredded with a mortar and liquid nitrogen, to obtain a powder texture. To defat the nut and obtain more precise results, 4 g of shredded nut were agitated for 60 min with 20 mL of n-hexane and centrifuged at 2000 rpm (Consul, Ortoalresa). The supernatant was removed and in the residue 20 mL n-hexane were added and centrifuged again for 10 min. The upper phase was again removed and the solid residue left to rest for 5 days in the darkness to ensure complete evaporation of hexane.

To extract the nut's phenolic compounds 1 g of the defatted sample was diluted in 15 mL of 1:1 v/v ethanol-water solution and agitated for 90 min at 900 rpm. Then the solution was centrifuged (Consul, Ortoalresa) at 2500 rpm for 20 min and the upper phase extracted. Then 5 mL ethanol-water solution was added and the sample agitated manually and centrifuged for 10 min. The upper phase was extracted again, resulting in a total amount of 19 mL of nut extract, which was stored at −20 ºC until analysis.

Pecan nut ethanol extract was used for DPPH radical scavenging activity and total polyphenol content assays. The nut was shredded and directly applied into the fish samples.

Moringa (*Moringa Oleifera*)

Moringa donated by the NGO Mujeres Burkina from Burkina Faso.

For the warm extraction 1 g of shredded sample was diluted in 15 mL of 1:1 v/v ethanol-water solution and agitated at medium rate for 30 min in a water bath at 50 ± 5 ºC. Then the solution was centrifuged (Consul, Ortoalresa) at 2500 rpm for 20 min and the upper phase extracted. Then 5 mL more of ethanol-water solution was added and the sample agitated manually and centrifuged for 10 min. The upper phase was extracted again, resulting in a total amount of 16,29 mL of moringa extract which was stored at −20 ºC until analysis.

Moringa ethanol extract was used for DPPH radical scavenging activity and total polyphenol content assays. Powdered leaves were directly applied into the fish samples.

Roselle (*Hibiscus Sabdariffa*)

A bag of roselle or Flor de Jamaica was purchased from the brand La Habanera, Jamaica, Colima, Col, Mexico and stored in the freezer at -20 ± 1 ºC. Before use it was shredded with a mortar and turned into powder. Roselle flower powder was weighed (1 g) and extracted with 10 mL of 70% v/v ethanol-water with 0,1% v/v HCl 37%. After that the extract was stirred for 90 min at at 60 ºC and centrifuged (Consul, Ortoalresa). The supernatants were stored at −20 ºC until analysis.

Roselle ethanol extract was used for DPPH radical scavenging activity and total polyphenol content assays. Powdered flower was directly applied into the fish samples.

4.1.2. Methods

Total Polyphenols Analysis

The Folin-Ciocalteu (Folin) analysis was used to measure the total polyphenol content of the extract using Gallic Acid (GA) as standard (Singleton *et al.*, 1998 and Santas *et al.*, 2008). Samples were analyzed both directly and in a 1:10 (v/v) dilution. For each sample, in triplicate, 20 µL of the sample, 80 µL of Folin-reagent 0,62 N, 80 µL of 4% saturated sodium carbonate and 80 µL Milli-Q water were added in a well. Then the plaque was stirred and kept in the dark at 25 ºC to react for an hour, after which the absorbance was measured at 765 nm with an UV/VIS Spectrophotometer plaque reader (FLUOstar Omega, BMG Labtech). Extraction solvents with reactive were used as blank. The standard curve was obtained by plotting the absorbance against different concentrations of GA (ranging from 0,12 to 1,73 mmol). Results are expressed as mg Gallic Acid Equivalents (GAE)/g of sample weight (SW) ± standard deviation.

DPPH radical scavenging activity assay

Radical scavenging activity of pecan nut, roselle and moringa was evaluated using the DPPH radical scavenging activity method described by Gallego (2013) with some modifications. Ethanol extracts from both compounds were diluted 1:10 (v/v) and 5,07 mM DPPH radical in methanol was prepared. An initial absorbance measurement of 200 µL DPPH reagent at time 0 was performed; afterwards 20 µL of diluted sample were added and the mix quickly measured at 517 nm every 15 min for 90 min. The antioxidant activity of the samples was determined with a trolox standard curve ranging from 0,02 to 0,5 mM. Results are expressed in µmol Trolox Equivalents (TE)/ g of sample weight (SW) ± standard deviation. The measurements

were done in triplicate of each sample. The inhibition percentage of sample was calculated using the following equation:

$$\%\ \text{inhibition of sample} = \left(\frac{A_{\ 0} - A_{\ \text{of sample}}}{A_{\ 0}}\right) \times 100$$

Where A_0 = initial absorbance of DPPH solution, A of sample = sample absorbance after determined time of reaction

Thiobarbituric acid Reactive Substances lipid oxidation analysis

Oxidative stability was determined by changes in thiobarbituric acid reactive substances (TBARS) over chilled storage during a period between 4-10 days depending on the oxidation rate. The procedure for measurement of TBARS was based on methods used by Gallego (2015) with some modifications. Procedure was as follows: triplicates of 0,5±0,02 g (0,0001 precision) of each sample were added with 0,5 mL of 0,3% w/w EDTA solution for preservation. Then homogenized in 2,5 mL of TBA reagent (prepared by mixing 43% w/w TCA solution with 0,93% w/w TBA solution, adding 10,4 mL HCl 37% and diluted with distilled water up to 500mL) with an Ultra-Turrax blender (Ika-Werke, GmbH & Co, Staufen, Germany) for a minute*. The blended samples were filtered through filtering paper and the reaction activated by inserting the simple tubes in a 100 ºC water bath for 10 minutes. The reaction was stopped by direct contact of the tubes with ice for 3 minutes and let back to room temperature before measurement. The resulting colour was measured at 532 nm in a UV/VIS Spectrophotometer. The standard curve was prepared using malondialdehyde (MDA) and results were expressed as mg MDA/kg sample. The determination was done in triplicate for each sample.

Roselle has a pink colour which interferes with the TBARS analysis; therefore, its colour before TBA reaction was also measured in order to be subtracted in the absorbance to obtain accurate results.

Microbiology Analysis

Microbiology analysis was performed to test the contamination of the samples through the presence of mesophilic bacteria, which grow at moderate temperature conditions, i.e. between 15 and 35 ºC. This analysis could also be used to have an idea of the antimicrobial activity of an additive, by comparison of the different samples with the control.

To perform the analysis each sample of fish muscle (10 g) was added with 90 mL of Ringer solution and homogenized with a Stomacher for 5 min. Depending on the level of contamination, the sample was directly analysed or diluted using 1 mL of sample with 9 mL of Ringer solution (-2). If the desired dilution was (-3), 1mL would be taken from the (-2) dilution and added 9 mL of Ringer solution, and so forward for (-4) or (-5) dilutions. Then 100 μL from the dilution were spread in a Petri plaque using Triptone Soya Agar (TSA) as the growth media for the culturing of bacteria. Then the plaques were closed with a lid and incubated at 35 ºC to promote the growth of mesophilic bacteria. After 24 h a recount of mesophilic microorganism was performed to assess the level of contamination of each sample. All the procedures were carried out in a sterilized environment.

Calculations were performed using the following equation in CFU units (Colony-forming unit):

Nº of colonies /g sardine (CFU) = (nº colonies present x dilution factor) /g of sardine

Fatty Acids Analysis

The samples were analyzed to evaluate the amount of fatty acids present and its evolution throughout the experiment. A higher amount of fatty acids in the beginning indicate that the reaction of lipid oxidation is going to occur faster and the sample is more prone to spoilage.

Fatty acids methyl ester (FAME) analysis was performed according to the method described by Viegas (2016) with modifications. Duplicates of 200 mg of each sample were weighed into glass tubes and kept in dry ice. Then 750 μL of 1:2 v/v water-methanol solution, followed by 500 μL of chloroform and 250 μL distilled water were added. After each addition the sample was vortexed for 1 min. Then the samples were centrifuged at 2000 g, 4 ºC for 20 min (Sigma 6k10). The upper layer was transferred to a 1,5 mL Eppendorf and kept at -20 ºC to be analyzed for metabolites (results not present in this work). The lower layer was transferred to opaque vials and evaporated with a stream of nitrogen (Nitrogen generator: Parker-Balston Analytical Gas Systems Filtration Access panel, Sample concentrator: Techne) until only an oil

residue was present. Then the samples were added 2 mL hexane, vortexed for 30 sec and left to rest for 5 min, to ensure a good dilution of the fat with the hexane. After, 200 μL 2 M potassium hydroxide in methanol solution were added and the samples centrifuged for 10 min at 2000 g (Consul, Ortoalresa). The upper phase was transferred into a 2 mL Eppendorf and kept at -80 ºC until gas chromatography was performed.

Fatty acids (FA) composition was analyzed using a GC-2025 with autosampler (Shimadzu, Japan), equipped with a flame ionization detector and a BPX-70 (SGE) column (30 m x 0.25 mm ID x 0.25 μm d.f.). The oven temperature was 60 ºC, held 1 min, raised to 260 ºC at the rate 6 ºC/ min, while the injector and the detector temperatures were set at 260 and 280 ºC, respectively. The sample size was 1 μL and the carrier gas was helium. The split used was 1:20. Fatty acids were identified by comparing the retention times of FAME with the standard 37 component FAME mixture (Figure 4). Two replicate GC analyses were performed and the results were expressed in GC area % as mean values ± standard deviation. The FA in the samples were analyzed at days 1 and 4 of each experiment to look for significant differences in FA compositions.

Fatty acids analysed were: Caproic Acid (C6:0), Caprylic Acid (C8:0), Capric Acid (C10:0), Undecanoic Acid (C11:0), Lauric Acid (C12:0), Tridecanoic Acid (C13:0), Myristic Acid (C14:0), Myristoleic Acid (C14:1), Pentadecanoic Acid (C15:0), cis-10-Pentadecanoic Acid (C15:1), Palmitic Acid (C16:0), Palmitoleic Acid (C16:1), Heptadecanoic Acid (C17:0), cis-10-Heptadecanoic Acid (C17:1), Stearic Acid (C18:0), Oleic Acid (C18:1n9), Linolelaidic Acid (C18:2n6t), Linoleic Acid (C18:2n6c), α-Linolenic Acid (C18:3n3), γ- Linolenic Acid (C18:3n6), Arachidic Acid (C20:0), Cis-11-Eicosenoic Acid (C20:1n9), Heneicosanoic Acid (C21:0), cis-11,14-Eicosadienoic Acid (C20:2), Cis-11,14,17-Eicosatrienoic (C20:3n6), Methyl cis-5,8,11,14-eicosatetraenoic acid (Arachidonic Acid) (C20:4n6), cis-8,11,14-Eicosatrienoic (C20:3n6), Behenic Acid (C22:0), Erucic Acid (C22:1n9), cis-5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5n3), Tricosanoic Acid (C23:0), Cis-13,16-Docosadienoic (C22:2), Lignoceric Acid (C24:0), Nervonic Acid (C24:1), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6n3).

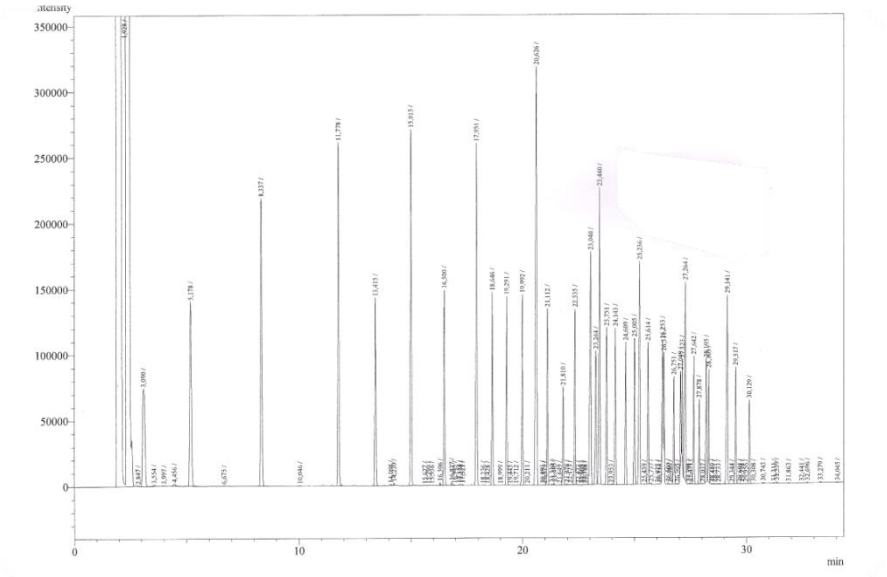


Figure 4. Chromatogram of 37 component FAME mixture standard

Determination of Hexanal by HS-GC/MS

Preparation of the samples consisted in weighting 0,5 g of minced sample and adding 1,5 mL milli-Q water in a headspace vial, which was then sealed air-tight with a PTFE septum (Andrés *et al.*, 2004). The standard curve was prepared using hexanal with concentrations from 0,005-0,434 ppm and an internal standard (Figure 5). Results were expressed in mg hexanal/ g sardine.

The vials were incubated at 80 ºC during 30 min. The analysis was performed by HS-GC/MS, by injecting 1 mL of vapor phase through a special syringe kept at 85 ºC. Equipment used consisted on a TRB-624 60 m x 0,32 mm x 1,8 mm column, with 1,8 ml/min helium flow. The injector temperature was 220 ºC with split mode injection (split flow 20 ml/min). Temperature program was 60 ºC (2 min), 8 ºC/min until 220 ºC (5 min). Interface temperature was 260 ºC and ionization source temperature 230 ºC. Ionization mode: electron ionization, SCAN mode (29-250 amu). Instrumentation: Trace GC gas chromatograph with Headspace Triplus autosampler coupled to a DSQII mass spectrometer (ThermoFisher Scientific).

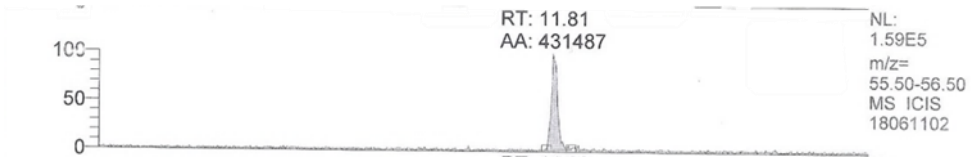


Figure 5. Hexanal standard

Biogenic Amines Analysis

Biogenic amines analysis was performed following the method described by Hernández-Jover (1996).

Reagents and Standards: Acetonitrile was of high performance liquid chromatography (HPLC) grade (SDS, Peypin, France). Other chemicals were of reagent grade. Sodium acetate, Brij-35, 2-mercaptoethanol, and o-phthalaldehyde (OPT) were obtained from Merck (Darmstadt, Germany); sodium octanesulfonate, from Romil Chemicals (Cam bridge, Great Britain); and boric acid and potassium hydroxide, from Panreac (Montplet & Esteban SA, Barcelona, Spain). Double-distilled water was obtained from the Milli-Q System (Millipore Corp., Bedford, MA).

As biogenic amine standards histamine (HIS), tyramine (TYR) , serotonin (SER), tryptamine (TRP), octopamine (OC) hydrochloride, dopamine (DO) 3-hydroxitiramine hydrochloride, cadaverine (CAD), putrescine (PUT), spermine (SPM), and spermidine (SPD) were used (Figure 6 and Figure 7). A concentrated 1000 mg/L stock solution as a free base of each biogenic amine in 0,1 N HCl was prepared. A 50 mg/L intermediate solution was prepared in 0,1 N HCl from the stock solution. Calibration standards of 0,25 mg/L for all the amines and 2 mg/L for spermine were prepared in 0,1 N HCl from the intermediate standard solution. Then, they were stored in refrigerator, and protected from light.

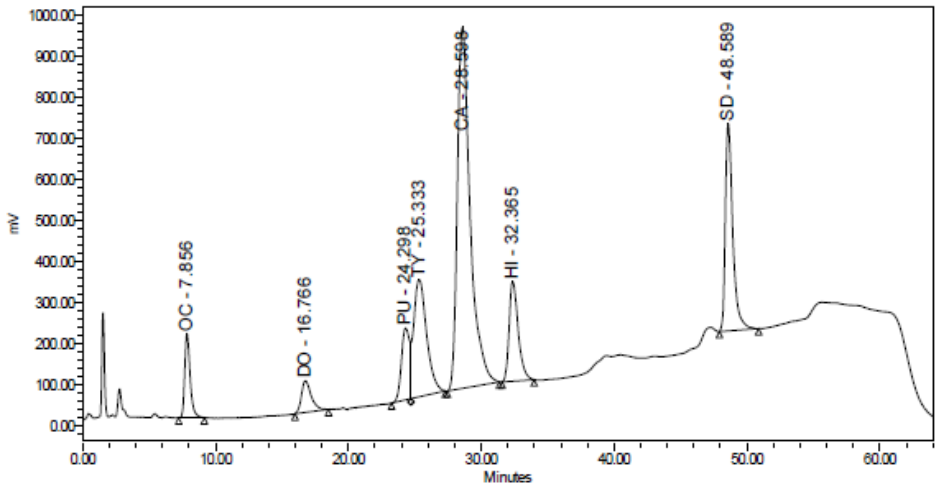


Figure 6. Chromatogram of a biogenic amine standard solution of 0,25 mg/L.

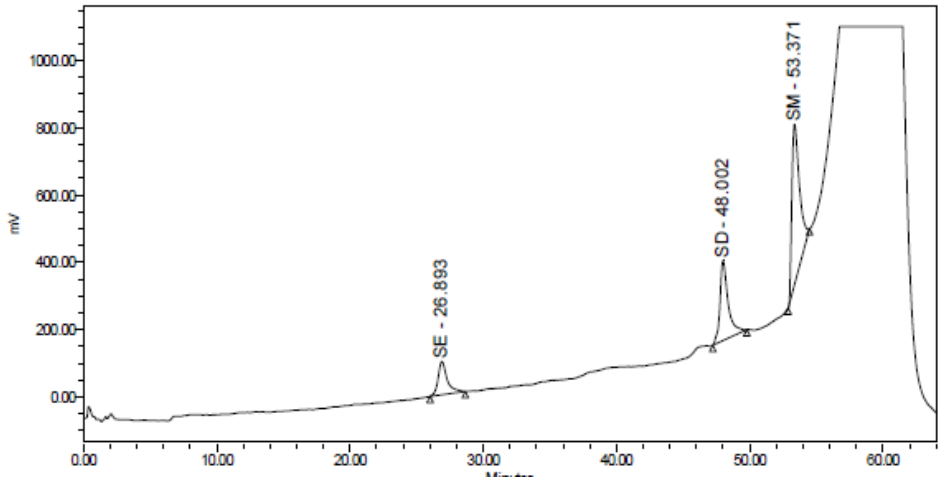


Figure 7. Chromatogram of a biogenic amine standard solution of 0,25 mg/L (SE, SD) and 2 mg/L (SM).

HPLC Analysis. The HPLC system (Waters Chromatography, Milford, MA) consisted of a Waters 600 E system controller pump, a Waters 715 autosampler, a Waters RDM (Reagent delivery module) postcolumn reaction equipment, and a Waters 470 spectrofluorometric detector. The waters RDM was connected to a zero dead volume mixing T installed between the column outlet and the detector. A coil of 200 cm long and 0,01 in. i.d. stainless steel tubing was used to connect the T with the detector. Data acquisition was accomplished by a system MAXIMA 820 (Waters). The separation was performed on a Nova Pack C18 column, 3.9x 150 mm, 4 µm particle size (Waters), with a matching guard cartridge.

Mobile Phase.: (1) *Eluent A*. A solution of 0,1 N sodium acetate and 10 mM sodium octanesulfonate was adjusted to pH 5,20 with acetic acid.

(2) *Eluent B*. Solvent B + Acetonitrile (6.6:3.4). Solvent B consisted of 0,2 M sodium acetate and 10 mM sodium octanesulfonate solution and was adjusted to pH 4,50 with acetic acid.

(3) *The gradient program* was implemented as follows: time = 0 min, 80% A, 20% B; time = 50 min, 20% A, 80% B; time= 52 min, 20% A, 80% B; time = 54 min, 80% A, 20% B; and time = 64 min, 80% A, 20% B. The two last steps were to reequilibrate the column to the initial conditions. The increase of eluent B was according to an exponential function of second order.

(4) *Postcolumn Derivatizating Reagent*. A 15,5 g sample of boric acid and 13,0 g of potassium hydroxide were dissolved in 500 mL of water. A 1,5 mL aliquot of 30% Brij-35 solution and 1,5 mL of 2-mercaptoethanol were added. Then, 0,1 g of OPT dissolved in 2,5 mL of methanol was added and the solution mixed. The derivatizating reagent was prepared fresh daily and protected from light.

(5) *The flow rate of the* mobile phase was 1 mL/min, and the flow rate of the derivatizating reagent was 0,5 mL/min. Mobile phases and the derivatizating reagent were filtered and degassed before use. The column and postcolumn reaction equipment were set at room temperature. Automatic injection of standard solutions (20 μL) or prepared samples (20 μL or 50 μL) was carried out when the eluate was alkaline (pH 10,50-11,00) and a steady base line was recorded. The eluate was monitored at 340 nm excitation and 445 nm emission wavelengths.

Sample Preparation: Samples were prepared according to the method of Komprda (2005) with some modifications. Samples with a weight of 1 g were extracted with 2 mL of HCl 0,1 M and triturated and homogenized using an Ultra-Turrax blender (Ika-Werke, GmbH & Co, Staufen, Germany) for 1 min. Then they were centrifuged (Sigma 6k10) for 15 min at 4 ºC and 3000 rpm. The supernatant was separated and the solid residue was repeatedly extracted with 2 mL of HCl 0,01 N, vortexed for 30 s and centrifuged 15 min with the same conditions. The supernatant was separated again and the combined extracts were made up to 10 mL. The samples were filtered through a 0,45 μm filter prior to LC analysis.

Preference sensory analysis

Sensory analysis was conducted by a taste panel consisting of 37 non-trained judges (21 males and 16 females) with ages between 7 and 60. The participants declared to be free of dried fruits allergy. Four fish patties were tasted which contained treatments of 5% w/w pecan nut, 10% w/w pecan nut, 5% w/w pecan nut + 5% w/w roselle and the control sample. The samples were distributed in plates and coded with a random three digit number (Figure 10). The subjects were instructed to taste each sample and grade them from 1 (most preferred) to 4 (least preferred). Results were analyzed using the tables developed by Basker (1988).

Figure 8. Preparation of patties for the sensory analysis

Figure 9. Trays with four different treatments

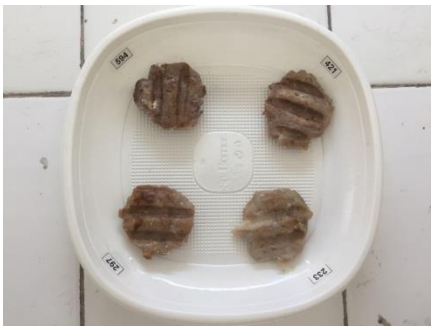


Figure 10. Dish used in the sensory analysis. Samples are coded with a three digit number.

Statistical Analysis

The mean value and standard deviation were calculated from the data obtained from the three samples for each treatment. One-way ANOVA was used to determine the significance of differences at $P < 0,05$. All statistics were performed using Minitab-16 for Windows.

4.2. Previous characterization of pecan nut, roselle and moringa

4.2.1. Results and discussion

Total phenolic content and DPPH radical scavenging activity analyses

The total phenolic content (TPC) presented no significant difference between pecan nut and roselle but was higher in moringa leaves. The radical scavenging activity (RSA) was significantly higher in moringa leaves, followed by pecan kernels and roselle (Figure 11, Figure 12 and Table 4).

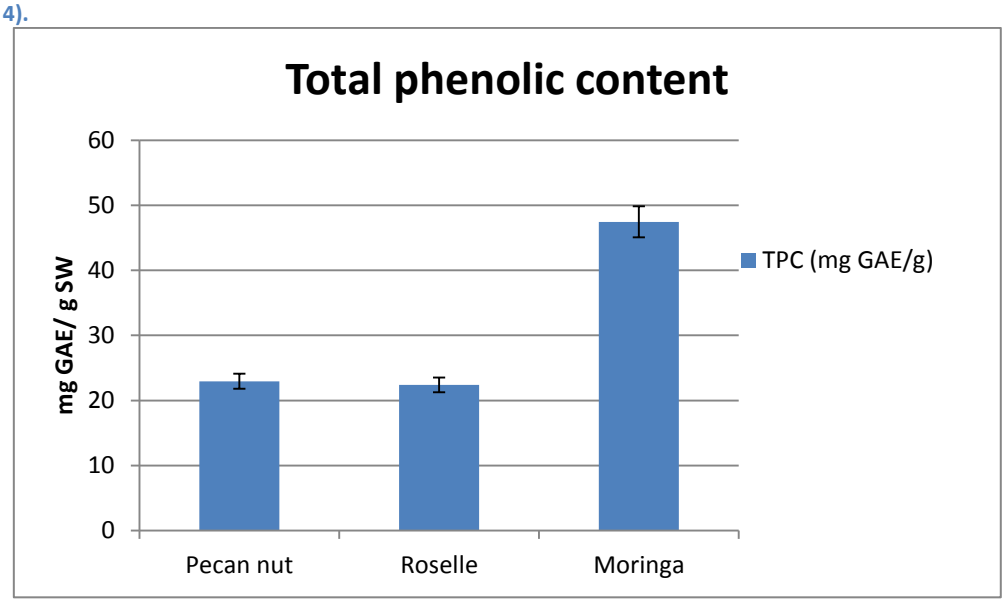


Figure 11. Comparison of total phenolic content obtained via Folin-Ciocalteu analysis of pecan nut, roselle and moringa. Results are displayed as an average of nine measures with a standard deviation.

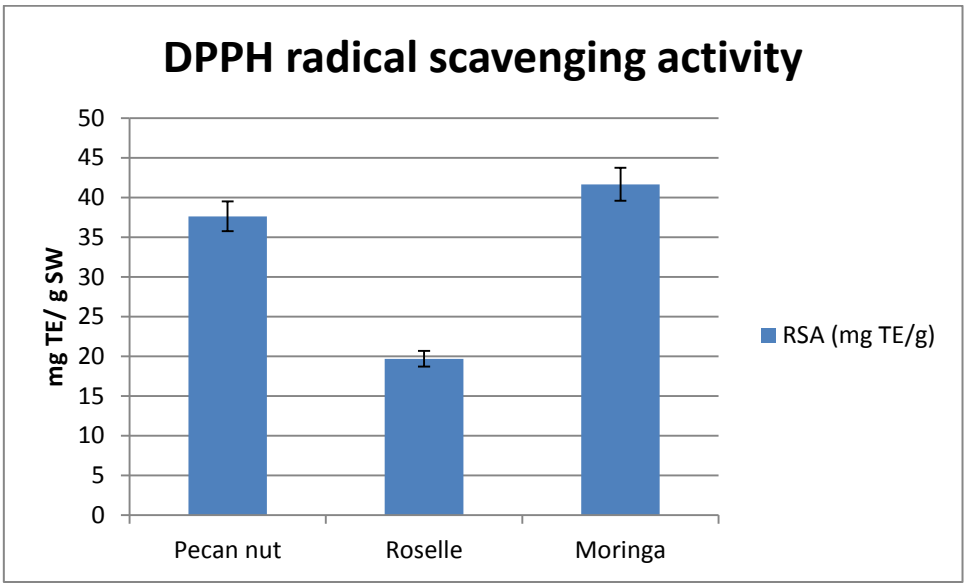


Figure 12. Comparison of radical scavenging activity obtained via DPPH analysis of pecan nut, roselle and moringa. Results are displayes as an average of nine measures with a standard deviation.

TPC content and RSA for pecan nut kernel, roselle and moringa ethanol extracts vary significantly in previous bibliography research.

Table 4 displays some values available in literature and the results obtained in this work; both present the same range of values and can hence be compared. A possible reason for the great variability could be related to the origin and cultivar of each sample.

TPC[A] (mg GAE/g SW)	RSA[B] (mg TE/g SW)	References
	Pecan nut	
22,95[b] ± 0,04	37,63[b] ± 1,08	*
20,16	-	Alasalvar *et al.,*2009
45,6 ± 1,14	97 ± 6,7	Villareal-Lozoya *et al.,*2007
15,88	55	Bolling *et al.,*2011
-	26,34 ± 0,78	De la Rosa *et al.,*2010
	Roselle	
22,40[b] ± 0,02	19,69[c] ± 0,42	*
45,98 ± 1,07	19,24 ± 0,1	Mak *et al.,*2013
2,81 ± 0,2	8,51 ± 2,45	Afify *et al.,*2016
	Moringa	
47,45[a] ± 0,06	41,65[a] ± 0,41	*
27,43 ± 4,98	47,76 ± 3,7	Pakade *et al.,*2013 – methanol extract
88,2 ± 0,34 (Nawabshah) 89,9 ± 0,33 (Jamshoro) 127,9 ± 0,29 (Mardaan) 105,4 ± 0,38 (Chakwal) 119,4 ± 0,31 (Balakot)	-	Siddhuraju *et al.,*2003
44,3 ± 0,21 (Nicaragua), 21,0 ± 0,18 (India) 38,1 ± 0,25 (Niger)	-	Iqbal *et al.,*2006

Table 4. Phenolic content and antioxidant capacity of defatted pecan kernels, roselle flower and moringa leaves. [a,b,c]The means followed by different letters in the same row indicate significant differences by Tukey's test (P < 0,05). [A]Total extractable phenolic content (Folin-Ciocalteu assay), [B]Antioxidant capacity (DPPH free radical scavenging assay), *This work.

4.3. Gilt-head Sea Bream (*Sparus Aurata*)

4.3.1. Materials and Methods

4.3.1.1. Preparation of products

Fish Sample

One fresh Gilt-head Sea Bream (*Sparus Aurata),* bred in a vivarium from Spain with an average weight of 700g was purchased in a local market and immediately transported with ice to the laboratory. To obtain the fish sample, the Sea Bream was cleaned and the head, guts, tail, spine and skin were removed. Only loins were used.

Treatments

Trays were prepared for each day and treatment and stored in the refrigerator at 4 ± 1 ºC. Five samples were analysed each day: control, 1% w/w pecan nut (PN), 2% w/w pecan nut, 4% w/w pecan nut and 1% w/w BHA.

4.3.2. Results and discussion

Note: this experiment was the first one performed, therefore it was very useful as a preliminary work, to adjust concentrations of compounds and to realise that a fish species with a higher amount of lipids was necessary to observe clearer results on effectiveness of the different treatments. As a consequence, not all of the analyses were carried out for these samples.

4.3.2.1. Thiobarbituric acid reactive substances (TBARS)

Start to finish date: 13/02/2018 – 23/02/2018

Day	0	1	2	3	4	6	9	10
				mg MDA/ kg				
Control	$0{,}12\,^{a}_{12} \pm 0{,}03$	$0{,}10\,^{a}_{1} \pm 0{,}03$	$0{,}11\,^{a}_{1} \pm 0{,}01$	$0{,}16\,^{a}_{12} \pm 0{,}04$	$0{,}14\,^{a}_{12} \pm 0{,}02$	$0{,}26_{2} \pm 0{,}02$	$0{,}63_{3} \pm 0{,}10$	$0{,}72\,^{a}_{3} \pm 0{,}10$
Pecan 1%	$0{,}12\,^{a}_{1} \pm 0{,}02$	$0{,}07\,^{a}_{1} \pm 0{,}00$	$0{,}09\,^{a}_{1} \pm 0{,}01$	$0{,}20\,^{a}_{1} \pm 0{,}05$	$0{,}16\,^{a}_{1} \pm 0{,}07$			$0{,}83\,^{a}_{2} \pm 0{,}13$
Pecan 2%	$0{,}08\,^{ab}_{1} \pm 0{,}01$	$0{,}11\,^{a}_{1} \pm 0{,}02$	$0{,}11\,^{a}_{1} \pm 0{,}01$	$0{,}18\,^{a}_{2} \pm 0{,}01$	$0{,}12\,^{a}_{1} \pm 0{,}01$			$0{,}55\,^{a}_{3} \pm 0{,}04$
Pecan 4%	$0{,}10\,^{ab}_{1} \pm 0{,}01$	$0{,}08\,^{a}_{1} \pm 0{,}01$	$0{,}12\,^{a}_{1} \pm 0{,}01$	$0{,}24\,^{a}_{2} \pm 0{,}05$	$0{,}10\,^{a}_{1} \pm 0{,}02$			$0{,}59\,^{a}_{3} \pm 0{,}09$
BHA 1%	$0{,}06\,^{b}_{1} \pm 0{,}01$	$0{,}09\,^{a}_{12} \pm 0{,}01$	$0{,}12\,^{a}_{12} \pm 0{,}04$	$0{,}20\,^{a}_{2} \pm 0{,}03$	$0{,}10\,^{a}_{12} \pm 0{,}02$			$0{,}69\,^{a}_{3} \pm 0{,}10$
SEM	0,02	0,02	0,02	0,04	0,03			0,09

Table 5. Changes of TBARS value (mg MDA/kg) in *Sparus Aurata* samples with different treatments in a period of 10 days. Different letters in the same column and different numbers in the same row indicate significant differences by Tukey's test (P < 0.05). SEM = Standard Error of the Mean. The values are means ± S.D. of the samples analyzed in triplicate.

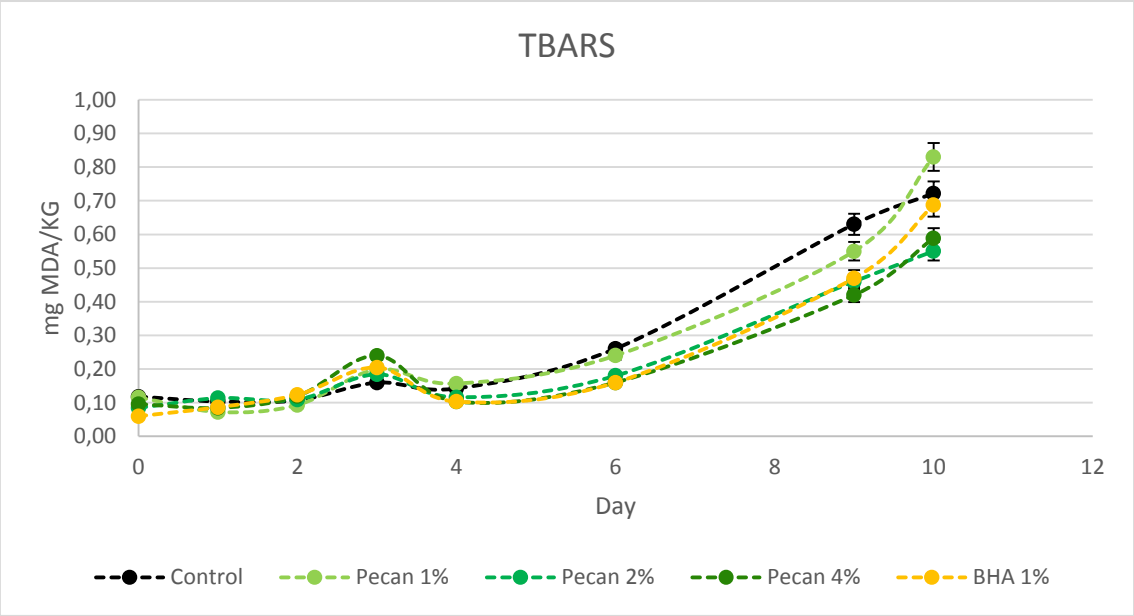


Figure 13. Evolution of TBARS value (mg MDA/kg) in *Sparus Aurata* samples with different treatments in a period of 10 days.

As it can be seen in Table 5 and Figure 13, until the 6th day the commercial antioxidant BHA had higher effectivity than any of the other treatments. After 10 days, the most effective treatments were 2% w/w pecan nut and 4% w/w pecan nut, although the fish sample was already oxidized.

4.3.2.2. Fatty acids analysis

	Day 0		**Day 4**	
Fatty Acids (%)	**Control**	**Pecan 4%**	**Control**	**Pecan 4%**
C6:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C8:0	0,07 ± 0,10	0,03 ± 0,00	0,12 ± 0,01	0,02 ± 0,02
C10:0	0,06 ± 0,08	0,01 ± 0,02	0,08 ± 0,01	0,03 ± 0,01
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,00 ± 0,00	0,04 ± 0,00	0,00 ± 0,00	0,02 ± 0,02
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	**2,31 ± 0,43**	**1,06 ± 0,08**	**2,44 ± 0,03**	**1,22 ± 0,02**
C15:0	0,26 ± 0,04	0,12 ± 0,01	0,26 ± 0,01	0,13 ± 0,00
C16:0	**19,70 ± 1,46**	**12,48 ± 0,73**	**17,67 ± 0,15**	**12,67 ± 0,22**
C17:0	0,22 ± 0,01	0,09 ± 0,01	0,21 ± 0,01	0,09 ± 0,00

C18:0	**3,62 ± 0,02**	**2,69 ± 0,06**	**3,66 ± 0,04**	**2,84 ± 0,13**
C20:0	0,13 ± 0,04	0,12 ± 0,00	0,16 ± 0,01	0,07 ± 0,00
C21:0	0,43 ± 0,04	0,16 ± 0,01	0,45 ± 0,02	0,19 ± 0,01
C22:0	0,77 ± 0,16	0,39 ± 0,02	0,61 ± 0,00	0,36 ± 0,13
C23:0	0,00 ± 0,00	0,00 ± 0,00	0,08 ± 0,00	0,01 ± 0,02
C24:0	0,31 ± 0,10	0,07 ± 0,01	0,22 ± 0,02	0,07 ± 0,00
ΣSFA	27,77	17,22	25,75	17,67
C14:1	0,18 ± 0,03	0,08 ± 0,01	0,19 ± 0,00	0,09 ± 0,00
C15:1	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C16:1	**5,04 ± 0,86**	**2,25 ± 0,22**	**5,26 ± 0,07**	**2,57 ± 0,09**
C17:1	0,27 ± 0,02	0,15 ± 0,01	0,26 ± 0,01	0,16 ± 0,00
C18:1n9t	**32,29 ± 4,20**	**45,14 ± 0,96**	**35,82 ± 0,78**	**45,09 ± 0,24**
C20:1n9	**1,05 ± 0,23**	**0,52 ± 0,02**	**1,17 ± 0,00**	**0,59 ± 0,06**
C22:1n9	**2,15 ± 0,53**	**0,64 ± 0,09**	**1,62 ± 0,07**	**0,70 ± 0,01**
C24:1	0,19 ± 0,07	0,07 ± 0,00	1,26 ± 0,03	0,50 ± 0,05
ΣMUFA	41,16	48,85	45,58	49,69
C18:2n6t	0,13 ± 0,00	0,06 ± 0,01	0,12 ± 0,00	0,07 ± 0,01
C18:2n6c	**15,94 ± 0,36**	**27,99 ± 0,77**	**16,34 ± 0,11**	**26,41 ± 0,45**
C18:3n3	0,16 ± 0,02	0,04 ± 0,00	0,10 ± 0,00	0,04 ± 0,00
C18:3n6	**1,77 ± 0,21**	**1,56 ± 0,02**	**1,82 ± 0,00**	**1,58 ± 0,01**
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:3n6	0,32 ± 0,05	0,10 ± 0,01	0,26 ± 0,03	0,11 ± 0,00
C20:4n6	0,72 ± 0,30	0,18 ± 0,03	0,45 ± 0,05	0,19 ± 0,00
C20:3n6	0,00 ± 0,00	0,02 ± 0,00	0,03 ± 0,04	0,03 ± 0,00
C20:5n3	0,25 ± 0,14	0,06 ± 0,01	0,14 ± 0,01	0,06 ± 0,02
C22:2	0,00 ± 0,00	0,02 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:6n3	**7,35 ± 2,65**	**1,82 ± 0,22**	**5,25 ± 0,28**	**2,05 ± 0,00**
ΣPUFA	26,64	31,86	24,51	30,53
PUFA/SFA	0,96	1,85	0,95	1,73
Σn6	2,93	1,93	2,68	1,97
Σn3	7,77	1,92	5,49	2,15

n6/n3	0,38	1,01	0,49	0,92
DHA/EPA	29,09	28,11	37,02	34,59
Unidentified	4,30	2,02	3,95	2,06

Table 6. Fatty acids profiles of *Sparus Aurata* muscle with different treatments at days 1 and 4 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ± S.D. of the samples analyzed in duplicate

The fatty acids (FA) present in a higher amount in Gilt-Head Sea Bream, presented in Table 6, are Myristic Acid (C14:0), Palmitic Acid (C16:0), Stearic Acid (C18:0), Palmitoleic Acid (C16:1), Oleic Acid (C18:1n9), Cis-11-Eicosenoic Acid (C20:1n9), Erucic Acid (C22:1n9), Linoleic Acid (C18:2n6c), γ- Linolenic Acid (C18:3n6) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6n3). These results are very similar to the ones obtained by Grigorakis (2002) and Mnari (2007).

In the sample with a treatment of 4% pecan nut, the most present FA are the same as in the control sample. However, the percentages of each acid vary greatly in relation to the control due to the presence in pecan nut of high amounts of Oleic Acid (C18:1n9) and Linoleic Acid (C18:2n6c) as reported in previous studies (Ryan *et al.*, 2006; T. Wakeling *et al*, 2001; Ros *et al.*, 2006 and SA Malik *et al.*, 2009).

There is no remarkable difference between FA content of samples at days 0 and 4.

4.4. Sardine (*Sardina pilchardus*)

4.4.1. Materials and Methods

4.4.1.1. Preparation of products

Fish Sample

Fresh sardine (*Sardina pilchardus*) 3 kg with an average weight and length of 27,2 ± 7,5 g and 14,8 ± 1,25 g respectively were purchased in March from a local market ("Peixateria Sunta") and transported in refrigeration to the laboratory. To obtain the fish sample, the sardine was cleaned and the head, guts, tail, spine and skin were removed. Only loins were used. After cleaning, the sardine loins were frozen and kept at –80 ºC until use, to ensure same initial conditions for all experiments.

Treatments

Trays were prepared for each day and treatment and stored in the refrigerator at 4 ± 1 ºC. In each experiment the number of samples analysed each day and the compounds present in them were different.

4.4.2. Results and discussion

4.4.2.1. Thiobarbituric acid reactive substances (TBARS)

Experiment 1

Start to finish date: 13/03/2018 – 16/03/2018

Samples: Control, 1% w/w pecan nut, 5% w/w pecan nut, 1% w/w roselle, 1% w/w moringa and 1% w/w BHA. All samples were added 1% w/w salt to delay bacterial spoilage.

Time (h)	0	23	44	66
	mg MDA/ kg			
Control	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$6{,}00\ ^{a}_{2} \pm 0{,}48$	$6{,}55\ ^{a}_{2} \pm 0{,}22$	$6{,}7\ ^{a}_{2} \pm 0{,}02$
Pecan 1%	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$3{,}16\ ^{b}_{2} \pm 0{,}46$	$5{,}52\ ^{a}_{3} \pm 0{,}07$	$5{,}74\ ^{bc}_{3} \pm 0{,}1$
Pecan 5%	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$0{,}79\ ^{cd}_{2} \pm 0{,}1$	$3{,}47\ ^{c}_{3} \pm 0{,}21$	$5{,}31\ ^{c}_{4} \pm 0{,}26$
Roselle 1%	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$1{,}25\ ^{c}_{1} \pm 0{,}14$	$4{,}35\ ^{b}_{2} \pm 0{,}6$	$5{,}79\ ^{b}_{3} \pm 0{,}13$
Moringa 1%	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$0{,}61\ ^{cd}_{1} \pm 0{,}03$	$2{,}76\ ^{c}_{2} \pm 0{,}17$	$6{,}18\ ^{b}_{3} \pm 0{,}22$
BHA 1%	$0{,}25\ ^{a}_{1} \pm 0{,}04$	$0{,}16\ ^{d}_{2} \pm 0{,}01$	$0{,}15\ ^{d}_{2} \pm 0{,}01$	$0{,}22\ ^{d}_{1} \pm 0{,}02$
SEM	0,036	0,25	0,30	0,14

Table 7. Changes of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 66 hours. Different letters in the same column and different numbers in the same row indicate significant differences by Tukey's test (P < 0.05). SEM = Standard Error of the Mean. The values are means ±S.D. of the samples analyzed in triplicate.

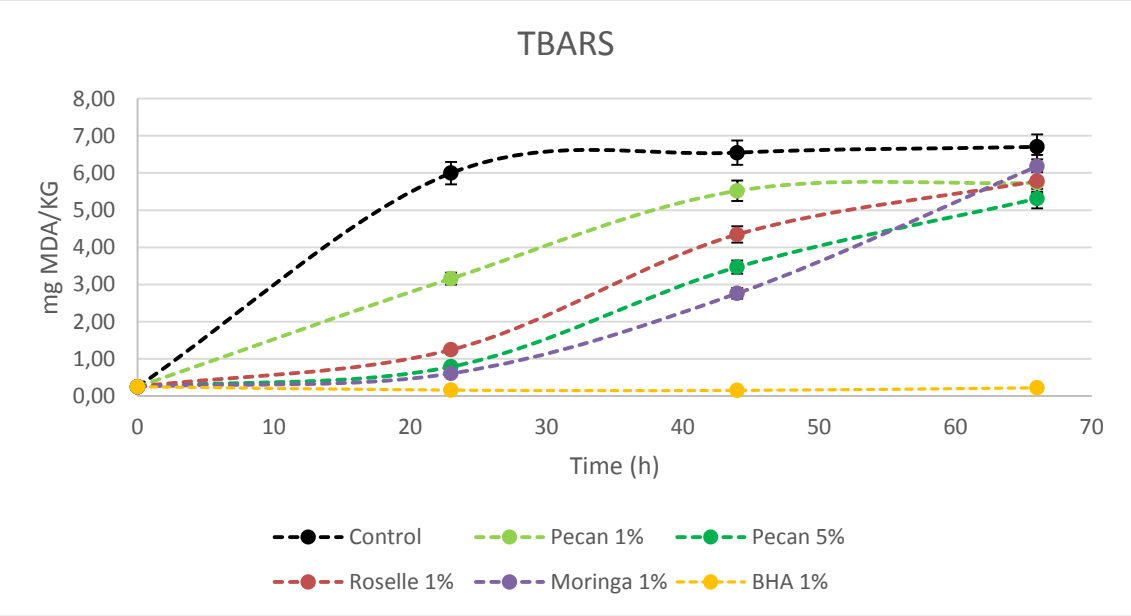


Figure 14. Evolution of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 66 hours

Results in Table 7 and Figure 14 show that after 66 hours and through the whole process the commercially used antioxidant BHA has a higher effectivity than any other treatment.

Experiment 2

Start to finish date: 19/03/2018 – 22/03/2018

Samples: Control, 5% w/w pecan nut, 10% w/w pecan nut, 5% w/w roselle, 5% w/w pecan nut + 5% w/w roselle and 0,1% w/w BHA. All samples were added 1% w/w salt to delay bacterial spoilage.

Time (h)	0	18	26	42	50	66
	mg MDA/ kg					
Control	$0{,}18\,^{a}_{1} \pm 0{,}02$	$1{,}61\,^{a}_{2} \pm 0{,}05$	$2{,}23\,^{b}_{3} \pm 0{,}01$	$3{,}56\,^{a}_{4} \pm 0{,}19$	$4{,}14\,^{a}_{5} \pm 0{,}25$	$5{,}04\,^{a}_{6} \pm 0{,}01$
Pecan 5%	$0{,}18\,^{a}_{1} \pm 0{,}02$	$0{,}52\,^{bc}_{1} \pm 0{,}13$	$0{,}53\,^{cd}_{1} \pm 0{,}15$	$1{,}81\,^{b}_{2} \pm 0{,}38$	$1{,}80\,^{b}_{2} \pm 0{,}35$	$4{,}80\,^{a}_{3} \pm 0{,}01$
Pecan 10%	$0{,}18\,^{a}_{1} \pm 0{,}02$	$0{,}33\,^{cd}_{12} \pm 0{,}03$	$0{,}42\,^{cd}_{2} \pm 0{,}04$	$0{,}40\,^{c}_{2} \pm 0{,}1$	$0{,}29\,^{d}_{12} \pm 0{,}01$	$0{,}89\,^{c}_{3} \pm 0{,}15$
Roselle 5%	$0{,}18\,^{a}_{1} \pm 0{,}02$	$0{,}27\,^{d}_{1} \pm 0{,}02$	$0{,}71\,^{c}_{2} \pm 0{,}06$	$1{,}25\,^{b}_{3} \pm 0{,}05$	$1{,}10\,^{c}_{3} \pm 0{,}08$	$2{,}33\,^{b}_{4} \pm 0{,}23$
Pecan 5% + Roselle 5%	$0{,}18\,^{a}_{1} \pm 0{,}02$	$0{,}25\,^{d}_{12} \pm 0{,}02$	$0{,}37\,^{d}_{12} \pm 0{,}04$	$0{,}65\,^{c}_{12} \pm 0{,}17$	$0{,}76\,^{cd}_{12} \pm 0{,}03$	$2{,}18\,^{b}_{3} \pm 0{,}43$
BHA 0,1%	$0{,}18\,^{a}_{1} \pm 0{,}02$	$0{,}58\,^{b}_{1} \pm 0{,}06$	$2{,}61\,^{a}_{2} \pm 0{,}25$	$3{,}59\,^{a}_{3} \pm 0{,}31$	$1{,}98\,^{b}_{2} \pm 0{,}16$	$4{,}29\,^{a}_{4} \pm 0{,}02$
SEM	0,02	0,06	0,11	0,21	0,17	0,24

Table 8. Changes of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 66 hours. Different letters in the same column and different numbers in the same row indicate significant differences by Tukey's test (P < 0.05). SEM = Standard error of the mean. The values are means ±S.D. of the samples analyzed in triplicate.

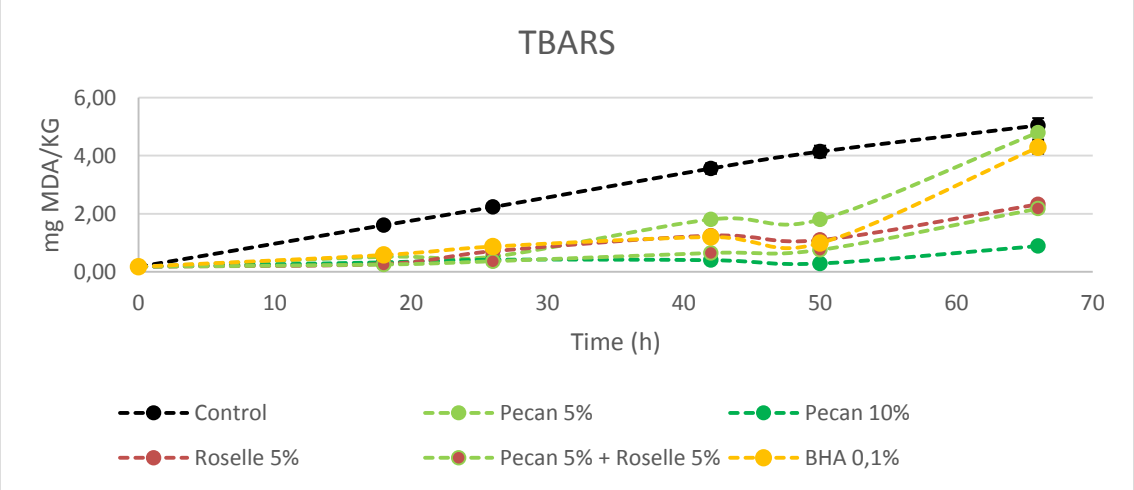


Figure 15. Evolution of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 66 hours

Results in Table 8 and Figure 15 show that after 66 hours, when all the samples are oxidized, the most effective treatment is 10% pecan nut. Pecan 5% is not very effective, while 5% roselle and 5% roselle + 5% pecan have a medium and similar effectivity. The samples with 0,1% BHA present a lot of variability, which could be explained due to the difficulty of obtaining an homogeneous product with such a small amount of the additive

Experiment 3

Start to finish date: 09/04/2018 – 12/04/2018

Samples: Control, 4% w/w pecan nut + 2% w/w Roselle, 5% w/w pecan nut + 2% w/w roselle, 6% w/w pecan nut + 2% w/w roselle and 0,1% w/w BHA. All samples were added 1% w/w salt to delay bacterial spoilage.

Time (h)	0	16	23	41	48	65
	mg MDA/ kg					
Control	$0{,}76\,^{a}_{1} \pm 0{,}12$	$4{,}13\,^{a}_{2} \pm 0{,}3$	$4{,}53\,^{a}_{2} \pm 0{,}23$	$5{,}76\,^{a}_{2} \pm 0{,}09$	$5{,}85\,^{a}_{2}$	$6{,}09\,^{a}_{2} \pm 1{,}03$
Pecan 4% + Roselle 2%	$0{,}76\,^{a}_{12} \pm 0{,}12$	$0{,}58\,^{b}_{2} \pm 0{,}1$	$0{,}45\,^{c}_{2} \pm 0{,}09$	$0{,}54\,^{c}_{2} \pm 0{,}11$	$1{,}17\,^{bc}_{12} \pm 0{,}4$	$1{,}62\,^{b}_{2} \pm 0{,}48$
Pecan 5% + Roselle 2%	$0{,}76\,^{a}_{1} \pm 0{,}12$	$0{,}43\,^{b}_{1} \pm 0{,}12$	$0{,}59\,^{c}_{1} \pm 0{,}16$	$0{,}49\,^{c}_{1} \pm 0{,}06$	$0{,}66\,^{bc}_{1} \pm 0{,}16$	$0{,}89\,^{b}_{1} \pm 0{,}23$
Pecan 6% + Roselle 2%	$0{,}76\,^{a}_{1} \pm 0{,}12$	$0{,}56\,^{b}_{1} \pm 0{,}08$	$0{,}42\,^{c}_{1} \pm 0{,}13$	$0{,}38\,^{c}_{1} \pm 0{,}02$	$0{,}55\,^{c}_{1} \pm 0{,}09$	$1{,}10\,^{b}_{1} \pm 0{,}57$
BHA 0,1%	$0{,}76\,^{a}_{1} \pm 0{,}12$	$0{,}80\,^{b}_{1} \pm 0{,}06$	$1{,}36\,^{b}_{1} \pm 0{,}04$	$1{,}27\,^{b}_{1} \pm 0{,}43$	$1{,}41\,^{b}_{1} \pm 0{,}09$	$1{,}56\,^{b}_{1}$
SEM	0,12	0,14	0,14	0,17	0,24	0,65

Table 9. Changes of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 65 hours. Different letters in the same column and different numbers in the same row indicate significant differences by Tukey's test (P < 0.05). SEM = Standard Error of the Mean. The values are means ±S.D. of the samples analyzed in triplicate.

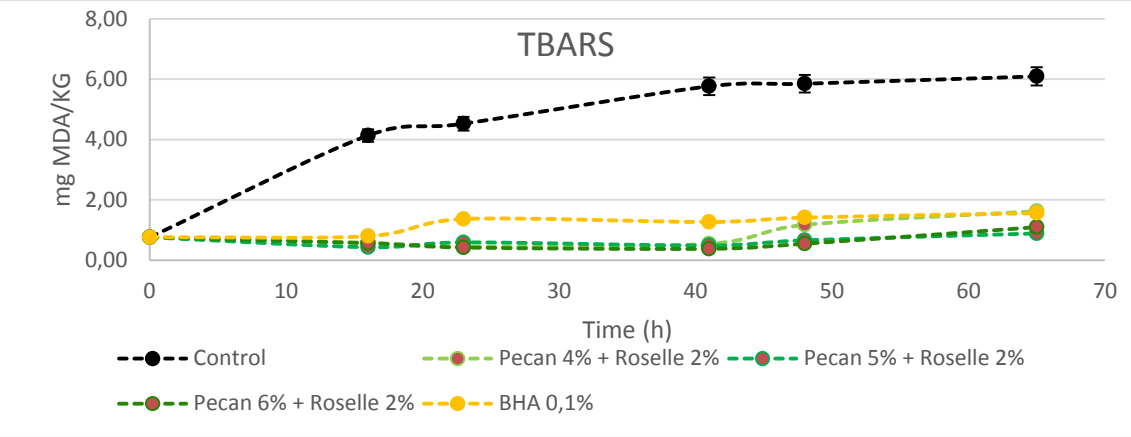


Figure 16. Evolution of TBARS value (mg MDA/kg) in *Sardina pilchardus* samples with different treatments in a period of 66 hours

Results in Table 9 and Figure 16 mainly show that all treatments applied to the fish sample are working. From the very beginning of the experiment there is a big difference between the oxidation rate of the control and all of the treatments. It can also be observed that among all the treatments the most and almost equally effective ones are the mixtures of 5% pecan + 2% roselle and 6% pecan + 2% roselle**.** The sample with 4% pecan + 2% roselle stops showing the same effectivity after 40h, where it prevents oxidation at the same rate as 0,1% BHA. In this

experiment, the mixture of natural antioxidants is more effective than a commercial concentration of the artificial antioxidant BHA.

4.4.2.2. Microbiological analysis

The main goal of this analysis was assess the antimicrobial properties of both roselle and pecan nut and reject initial contamination of the samples. Results have been analyzed in a qualitative way, because the main goal is to determine the order of magnitude of the contamination. It was observed that the presence of roselle, moringa and BHA acted as antimicrobial agents. These results support previous works in which roselle extracts have successfully been used to disinfect carrots, tomatoes (Gutiérrez-Alcántara *et al.*, 2016), romaine lettuce, alfalfa sprouts (Jaroni *et al.*, 2012) and Hass avocado (Gómez-Aldapa *et al.*, 2017). Moringa extracts have been reported as effective antimicrobials for *R. argentea* and *O. niloticus* fish species (Adeyemi *et al.*, 2013) and smoke-dried African catfish (*Clarias gariepinus*) (Onyuka *et al.*, 2013).

These results could indicate that roselle has potential to be used to supplement the antioxidant activity of pecan nut, hence obtaining a food preservative with both antioxidant and antimicrobial properties.

Experiment 1

	day 0	day 4
Control	+	++
Pecan 1%	+	++
Pecan 5%	+	-
Roselle 1%	+	-
Moringa 1%	+	-
BHA 1%	+	-

Table 10. Presence of microbiological colonies in the samples. – indicates less than 30 CFU, + an amount between 30 and 100 CFU, ++ indicates 100-200 CFU

Because all the samples showed presence of colonies at day 0, the samples for the analysis at day 4 were too diluted and a proper recount of CFU was not possible (Table 10).

Experiment 2

	day 1	day 3
Control	-	+
Pecan 5%	-	+
Pecan 10%	-	+
Roselle 5%	-	-
Roselle 5% + Pecan 5%	-	-
BHA 0,1%	-	-

Table 11. Presence of microbiological colonies in the samples. – indicates less than 30 CFU, + an amount between 30 and 100 CFU

Results in Table 11 show that roselle is performing effectively as an antimicrobial compound for mesophilic bacteria. Even so, the samples with presence of pecan nut are also not very contaminated after three days, which may indicate that it is has also antimicrobial properties.

Experiment 3

	day 0	day 7
Control	+	+++
Roselle 2% + Pecan 4%	+	++
Roselle 2% + Pecan 5%	+	++
Roselle 2% + Pecan 6%	-	-
BHA 0,1%	+	++

Table 12. Presence of microbiological colonies in the samples. – indicates less than 30 CFU, + an amount between 30 and 100 CFU, ++ indicates 100-200 CFU and +++ indicates >200 CFU.

Because in previous experiments the samples weren't highly contaminated, in this case the microbiological assay was performed at day 7. Therefore, the samples contain many more CFU than in sardine experiments 1 and 2. In comparison with the control sample, the other ones contain less CFU, which indicates all treatments are working for this purpose (Table 12).

4.4.2.3. Fatty acids analysis

Experiment 1

Although different authors (Saini *et al.,* 2014 and Moyo *et al.,* 2011) have reported α-linolenic acid to be the FA in highest proportion in moringa leaves, there is no remarkable difference in the amount of this FA in samples with and without moringa (Annex A, 26 and Table 27).

Experiment 2

	Control	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	2,54^{a} ± 0,10	2,98^{a} ± 0,22
C16:0	24,29^{a} ± 0,71	26,44^{a} ± 0,06
C18:0	**3,82^{b} ± 0,19**	**5,87^{a} ± 0,18**
ΣSFA	**33,89^{b} ± 0,86**	**38,39^{a} ± 0,40**
C16:1	1,72^{a} ± 0,04	1,91^{a} ± 0,24
C18:1n9	15,03^{a} ± 1,19	19,20^{a} ± 1,09
C22:1n9	5,76^{a} ± 0,06	4,65^{b} ± 0,20
ΣMUFA	23,44^{a} ± 1,39	27,04^{a} ± 1,49
C18:2n6c	6,57^{a} ± 0,56	8,32^{a} ± 0,23
C18:3n3	**0,17^{b} ± 0,02**	**0,70^{a} ± 0,01**
C22:6n3	**22,98^{a} ± 0,27**	**15,14^{b} ± 1,11**
ΣPUFA	**31,89^{a} ± 0,40**	**25,04^{b} ± 0,81**

	Pecan 5%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	0,63^{a} ± 0,14	0,43^{a} ± 0,14
C16:0	11,40^{a} ± 0,76	10,50^{a} ± 1,11
C18:0	2,52^{a} ± 0,08	2,65^{a} ± 0,11
ΣSFA	15,19^{a} ± 0,96	14,04^{a} ± 1,54
C16:1	0,39^{a} ± 0,09	0,28^{a} ± 0,10
C18:1n9	48,49^{a} ± 2,03	49,98^{a} ± 4,35
C22:1n9	0,96^{a} ± 0,20	0,53^{a} ± 0,25
ΣMUFA	50,23^{a} ± 1,68	51,17^{a} ± 3,13
C18:2n6c	28,15^{a} ± 0,40	29,73^{a} ± 0,48
C18:3n3	0,67^{a} ± 0,92	1,23^{a} ± 0,01
C22:6n3	3,59^{a} ± 0,35	2,03^{a} ± 1,06
ΣPUFA	33,29^{a} ± 0,01	33,20^{a} ± 0,57

	Pecan 10%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	0,29^{a} ± 0,06	0,11^{a} ± 0,16
C16:0	9,33^{a} ± 0,59	9,00^{a} ± 0,54
C18:0	2,35^{a} ± 0,07	2,46^{a} ± 0,00
ΣSFA	12,30^{a} ± 0,80	11,83^{a} ± 0,43
C16:1	0,20^{a} ± 0,03	0,17^{a} ± 0,02
C18:1n9	52,62^{a} ± 1,83	52,69^{a} ± 3,46
C22:1n9	0,46^{a} ± 0,12	0,29^{a} ± 0,05
ΣMUFA	53,60^{a} ± 1,61	53,46^{a} ± 3,03
C18:2n6c	29,32^{a} ± 0,70	31,30^{a} ± 1,85
C18:3n3	1,19^{a} ± 0,02	1,28^{a} ± 0,06
C22:6n3	1,75^{a} ± 0,57	1,04^{a} ± 0,20
ΣPUFA	32,50^{a} ± 0,17	33,81^{a} ± 2,13

	Roselle 5%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	2,49^{a} ± 0,24	2,84^{a} ± 0,08
C16:0	24,75^{a} ± 0,71	24,72^{a} ± 0,55
C18:0	3,35^{a} ± 0,19	3,81^{a} ± 0,23
ΣSFA	33,40^{a} ± 0,73	34,85^{a} ± 0,84
C16:1	1,65^{a} ± 0,19	1,89^{a} ± 0,05
C18:1n9	16,24 ± 0,35	15,96^{a} ± 1,27
C22:1n9	5,70^{a} ± 0,33	5,85^{a} ± 0,11
ΣMUFA	24,82^{a} ± 1,16	24,81^{a} ± 1,30
C18:2n6c	7,25^{a} ± 0,10	6,73^{a} ± 0,73
C18:3n3	0,39^{a} ± 0,39	0,67^{a} ± 0,04
C22:6n3	23,79^{a} ± 1,11	22,92^{a} ± 0,53
ΣPUFA	33,28^{a} ± 1,05	31,17^{a} ± 0,26

	Roselle 5% + Pecan 5%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	0,71^{a} ± 0,05	0,67^{a} ± 0,21
C16:0	12,04^{a} ± 0,38	11,28^{a} ± 1,42
C18:0	2,54^{a} ± 0,05	2,50^{a} ± 0,05
ΣSFA	16,13^{a} ± 0,48	15,20^{a} ± 1,81
C16:1	0,49^{a} ± 0,01	0,42^{a} ± 0,12
C18:1n9	46,1^{a} ± 1,37	47,56^{a} ± 4,59
C22:1n9	1,34^{a} ± 0,04	1,07^{a} ± 0,38
ΣMUFA	48,32^{a} ± 1,04	49,47^{a} ± 3,87
C18:2n6c	27,55^{a} ± 0,57	28,29^{a} ± 0,76
C18:3n3	1,27^{a} ± 0,02	1,24^{a} ± 0,01
C22:6n3	4,87^{a} ± 0,44	4,12^{a} ± 1,35
ΣPUFA	34,01^{a} ± 0,17	33,93^{a} ± 2,12

	BHA 0,1%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	3,22^{a} ± 0,78	2,76^{a} ± 0,70
C16:0	26,01^{a} ± 0,58	22,83^{a} ± 1,41
C18:0	3,95^{a} ± 0,38	3,58^{a} ± 0,21
ΣSFA	36,87^{a} ± 3,23	32,21^{a} ± 2,62
C16:1	2,12^{a} ± 0,42	1,80^{a} ± 0,38
C18:1n9t	15,62^{a} ± 3,14	25,27^{a} ± 2,53
C22:1n9	6,39^{a} ± 0,53	4,26^{a} ± 0,66
ΣMUFA	25,61^{b} ± 1,37	32,52^{b} ± 1,02
C18:2n6c	6,22^{a} ± 2,18	12,01^{a} ± 1,75
C18:3n3	0,53^{a} ± 0,41	0,43^{a} ± 0,61
C22:6n3	**22,68^{a} ± 0,31**	**13,12^{b} ± 0,51**
ΣPUFA	31,15^{a} ± 3,09	26,78^{a} ± 0,94

Table 13. Comparison of FA profiles of different treatments on *Sardina Pilchardus* muscle between days 0 and 4 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ± S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

Table 13 shows that there are generally no significant differences on the amount of each acid in day 1 and 4 for the same treatment. The only treatment which presents some significant differences is the control, in which the amounts of Myristic Acid (C14:0), α-Linolenic Acid (C18:3n3), Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) and the total amounts of SFA and PUFA vary significantly between the first and fourth day of the experiment. The following analysis of the percentages of FA for each treatment will be therefore performed without distinction between days.

The FA present in a higher amount in the control sample are Myristic Acid (C14:0), Palmitic Acid (C16:0), Stearic Acid (C18:0), Palmitoleic Acid (C16:1), Oleic Acid (C18:1n9), Erucic Acid (C22:1n9), Linoleic Acid (C18:2n6c), α-Linolenic Acid (C18:3n3) and Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3). The fatty acid compositions range from 33,89-38,39% saturated (SFA), 23,44-27,04% monounsaturated (MUFAs) and 25,04-31,89% polyunsaturated acids (PUFAs). All these results are very similar to the ones obtained in previous studies with sardine muscle (Özogul *et al.*, 2007; Shirai *et al.*, 2001 and Tarley *et al.*, 2004). Even so, in all these studies the amounts of FA in sardine vary significantly depending on the origin and season. Although the most present FAs remain the same, Özogul and Shirai report higher amounts of Myristic Acid (C14:0), Palmitoleic Acid (C16:1) and Cis-5,8,11,14,17-eicosapentaenoic Acid (EPA, C20:5n3), while amounts of Oleic Acid (C18:1n9), Erucic Acid

(C22:1n9), Linoleic Acid (C18:2n6c) and Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) are much lower.

In the sample with a treatment of 5% pecan nut, the most present FA are the same as in the control sample. However, the percentages of each acid vary significantly in relation to the control due to the presence in pecan nut of high amounts of Oleic Acid (C18:1n9) and Linoleic Acid (C18:2n6c) as reported in previous studies (Ryan *et al.*, 2006; Wakeling *et al*, 2001; Ros *et al.*, 2006 and Malik *et al.*, 2009). The sample with 5% pecan nut has almost fourfold the amount of these acids in the control and therefore the percentage of the other acids present in the sample is reduced to half the amount in the control or even less. As a consequence of this, the sum of SFA is halved, MUFA doubled and PUFA remains more or less the same, which has the side effect of increasing the total amount of the healthier types of FA (MUFA and PUFA).

The sample which contains 10% pecan nut, presents the exact same characteristics explained in the case of 5% pecan nut. The amounts of Oleic Acid (C18:1n9) and Linoleic Acid (C18:2n6c) are even a little bit higher than in the previous sample, but no significant changes occur.

The sample with 5% roselle presents FA amounts which bare no significant difference to the control, in both cases Palmitic Acid (C16:0), Oleic Acid (C18:1n9) and Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) are the acids with a higher percentage within the sample. These results match with the study by Ahmad (1979) in which amounts of Myristic (2.1%), Palmitic (35.2%), Palmitoleic (2.0%), Stearic (3.4%), Oleic (34.0%) and Linoleic (14.4%) acids are reported in roselle seeds.

The sample with 5% roselle + 5% pecan nut, shows a FA profile very similar to the one with 5% Pecan Nut, with the no significant difference in relation to it. The percentages of Palmitic Acid (C16:0) and Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) are 1% higher than in the 5% Pecan Nut sample due to the presence of roselle, which has higher amounts of these acids.

The sample with 0,1% BHA presents no significant difference to the control sample as expected, since the additive does not contain fatty acids. Only the amount of Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) varies significantly between the first and fourth day.

Other relevant data is that all treatments present low levels of Arachidonic Acid (C20:4n6, 0,03-0,58%) which may have antagonistic effects to the health benefits of the n3 FA (Kinsella,

1986). Moreover, the UK Department of Health recommends a maximum ratio of n6/n3 of 4.0, which is much higher than in any of the present treatments (0,04-0,07) (HMSO, 1994). A minimum value of PUFA/SFA ratio recommended is 0.45 (HMSO,1994), which is lower than those obtained from all fish treatments (0,65-2,86).

Palmitic acid (C16:0) was the primary saturated FA, contributing 9,33-26,44% of the total SFA content of lipids for all treatments. Oleic acid (C18:1n9t) was the most represented of the MUFAs, accounting for 15,03-52,69% of total MUFAs and Linoleic Acid (C18:2n6c) and Cis-4,7,10,13,16,19-docosahexaenoic Acid (DHA, C22:6n3) were the major FA identified as PUFAs accounting for 6,22-31,30% and 1,04-22,98% respectively.

The complete analysis can be found in Tables 28 and 29 in Annex A, which give the % as a mean value of 36 FA for each treatment on *Sardina pilchardus* muscle.

Experiment 3

	Control	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	2,78^{a} ± 0,14	2,88^{a} ± 0,20
C16:0	23,53^{a} ± 4,34	27,70^{a} ± 3,45
C18:0	3,10^{a} ± 0,35	3,88^{a} ± 0,53
ΣSFA	32,62^{a}	38,98^{a}
C16:1	1,83^{a} ± 0,10	1,84^{a} ± 0,29
C18:1n9	20,21^{a} ± 7,26	14,31^{a} ± 1,11
C22:1n9	5,54^{a} ± 0,69	4,04^{a} ± 0,76
ΣMUFA	29,26^{a}	24,21^{a}
C18:2n6t	0,11^{a} ± 0,16	2,12^{a} ± 1,77
C18:2n6c	9,93^{a} ± 4,81	5,47^{a} ± 0,29
C18:3n3	0,40^{a} ± 0,50	1,60^{a} ± 1,38
C22:6n3	21,23^{a} ± 4,35	13,86^{a} ± 2,58
ΣPUFA	**33,08^{a}**	**25,81^{b}**

	Roselle 2% + Pecan 4%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	1,15^{a} ± 0,63	0,83^{a} ± 0,08
C16:0	13,80^{a} ± 2,40	11,52^{a} ± 1,05
C18:0	2,54^{a} ± 0,04	2,16^{a} ± 0,53
ΣSFA	19,21^{a}	18,34^{a}
C16:1	0,70^{a} ± 0,33	0,47^{a} ± 0,11
C18:1n9	37,30^{a} ± 9,98	34,89^{a} ± 11,09
C22:1n9	2,19^{a} ± 1,10	1,62^{a} ± 0,05
ΣMUFA	41,86^{a}	41,45^{a}
C18:2n6t	0,88^{a} ± 1,11	2,84^{a} ± 3,30
C18:2n6c	21,89^{a} ± 7,31	20,42^{a} ± 5,67
C18:3n3	1,29^{a} ± 0,08	2,35^{a} ± 1,77
C22:6n3	9,13^{a} ± 4,10	7,25^{a} ± 0,94
ΣPUFA	34,86^{a}	35,00^{a}

	Roselle 2% + Pecan 5%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	0,42 ± 0,00	1,53 ± 0,00
C16:0	11,37 ± 0,00	14,48 ± 0,00
C18:0	2,35 ± 0,00	2,72 ± 0,00
ΣSFA	14,69	20,57
C16:1	0,29 ± 0,00	0,96 ± 0,00
C18:1n9	48,69 ± 0,00	41,77 ± 0,00
C22:1n9	0,77 ± 0,00	2,20 ± 0,00
ΣMUFA	50,03	45,63
C18:2n6t	0,07 ± 0,00	0,01 ± 0,00
C18:2n6c	29,47 ± 0,00	23,20 ± 0,00
C18:3n3	0,04 ± 0,00	1,19 ± 0,00
C22:6n3	3,31 ± 0,00	6,11 ± 0,00
ΣPUFA	34,38	30,87

	Roselle 2% + Pecan 6%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	**1,11^{a} ± 0,16**	**0,46^{b} ± 0,01**
C16:0	12,27^{a} ± 2,05	10,36^{a} ± 0,58
C18:0	2,25^{a} ± 0,44	2,45^{a} ± 0,12
ΣSFA	**19,03^{a}**	**13,84^{b}**
C16:1	0,66^{a} ± 0,21	0,30^{a} ± 0,02
C18:1n9	34,52^{a} ± 7,19	48,62^{a} ± 1,14
C22:1n9	1,89^{a} ± 0,37	0,76^{a} ± 0,06
ΣMUFA	38,92^{a}	50,15^{a}
C18:2n6t	2,30^{a} ± 3,12	0,02^{a} ± 0,00
C18:2n6c	21,61^{a} ± 5,87	28,99^{a} ± 0,39
C18:3n3	2,28^{a} ± 1,43	1,33^{a} ± 0,03
C22:6n3	**7,23^{a} ± 0,60**	**3,10^{b} ± 0,33**
ΣPUFA	35,35^{a}	33,77^{a}

	BHA 0,1%	
Fatty Acid (%)	**Day 0**	**Day 4**
C14:0	2,87^{a} ± 0,02	1,34^{a} ± 1,07
C16:0	25,37^{a} ± 0,16	14,07^{a} ± 5,10
C18:0	3,63^{a} ± 0,35	3,20^{a} ± 0,62
ΣSFA	35,17^{a}	19,92^{a}
C16:1	1,88^{a} ± 0,04	0,89^{a} ± 0,70
C18:1n9	15,03^{a} ± 1,72	40,47^{a} ± 9,35
C22:1n9	**6,06^{a} ± 0,00**	**0,25^{b} ± 0,36**
ΣMUFA	24,47^{a}	42,23^{a}
C18:2n6t	3,57^{a} ± 2,90	0,46^{a} ± 0,64
C18:2n6c	5,37^{a} ± 1,14	23,23^{a} ± 7,37
C18:3n3	0,45^{a} ± 0,37	0,72^{a} ± 0,68
C22:6n3	**23,67^{a} ± 0,94**	**6,16^{b} ± 4,49**
ΣPUFA	34,51^{a}	32,72^{a}

*It was not possible to obtain a duplicate from the FA analysis for the sample containing Roselle 2% + Pecan 5%, hence the standard deviation is zero and significant difference cannot be found between days 0 and 4.

Table 14. Comparison of FA profiles of different treatments on *Sardina Pilchardus* muscle between days 0 and 4 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ± S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

Results follow the same tendency explained in Experiment 2, where the highest presence of FA is found for Myristic, Palmitic, Stearic, Palmitoleic, Oleic, Erucic, Linoleic and α-Linolenic Acids, as well as DHA and in the case of pecan nut presence, the amounts of Oleic and Linoleic Acids increase significantly.

The complete analysis is presented in Tables 30 and 31 in Annex A, which give the % as a mean value of 36 FA for each treatment on *Sardina pilchardus* muscle.

4.4.2.4. Determination of Hexanal by HS-GC/MS

Experiment 1

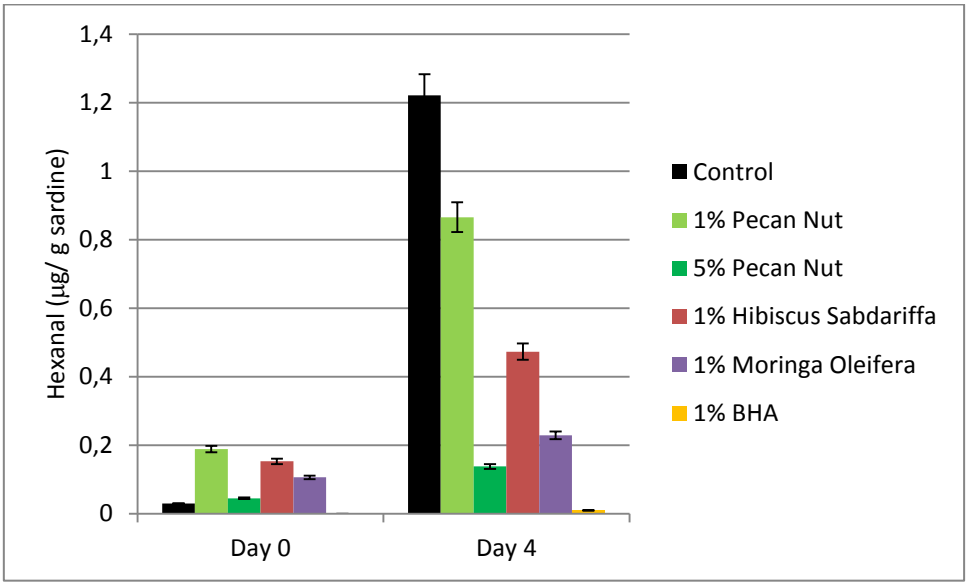


Figure 17. Hexanal content at days 0 and 4 of the experiment in samples of *sardina pilchardus* with different treatments. Results are expressed in µg hexanal/g sardine and at day 0 the amount of hexanal in the sample with treatment of 1% w/w BHA is under the limit of detection. The values are means with standard deviation of the samples analyzed in triplicate.

The variation of the hexanal content between days depends greatly on the treatment (Figure 17). The higher increase is found in the control sample, which in day 4 has more than 40-fold the amount of hexanal in relation to day 0. The treatment consisting of 1% pecan nut has little effect on preventing hexanal formation. The sample with 1% roselle in day 4 contains a significantly smaller quantity of hexanal than the control in the same day. Gibis (2014) reported hibiscus to be highly effective in preventing the generation of hexanal in liposomes containing 1% lecithin and 0,8% w/v hibiscus extract. 5% w/w pecan nut and 1% moringa have a similar and good effectiveness, while 1% BHA is the most effective treatment; in day 4 the amount of hexanal present in the sample is almost not detectable, therefore the reaction of lipid oxidation is being highly prevented.

Experiment 2

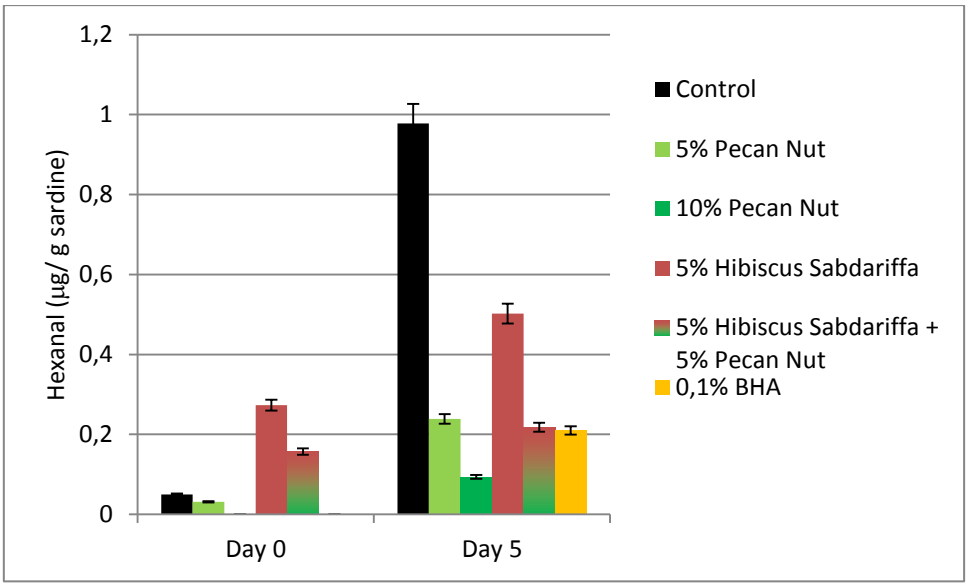


Figure 18. Hexanal content at days 0 and 5 of the experiment in samples of *sardina pilchardus* with different treatments. Results are expressed in µg hexanal/g sardine and at day 0 the amount of hexanal in the samples with treatments of 10% w/w pecan nut and 0,1% w/w BHA are under the limit of detection. The values are means with standard deviation of the samples analyzed in triplicate.

The variation of the hexanal content between days depends greatly on the treatment (Figure 18). The higher increase is found in the control sample, which in day 5 has almost 20-fold the amount of hexanal in relation to day 0. The smaller increase occurs with the sample containing a 10% of pecan nut, where the amount in day 0 is not detectable and has only slightly increased in day 5. Results from this analysis suggest that 10% pecan nut is the most effective treatment in preventing formation of hexanal as a by-product of lipid oxidation. Samples with 5% pecan nut and 0,1% BHA have a similar increase, which could imply that 5% of pecan nut is enough to equal the effects of current artificial antioxidants. The presence of roselle in the sample seems to have a positive effect on the prevention of hexanal formation, although both samples containing it, present more hexanal in the beginning.

Experiment 3

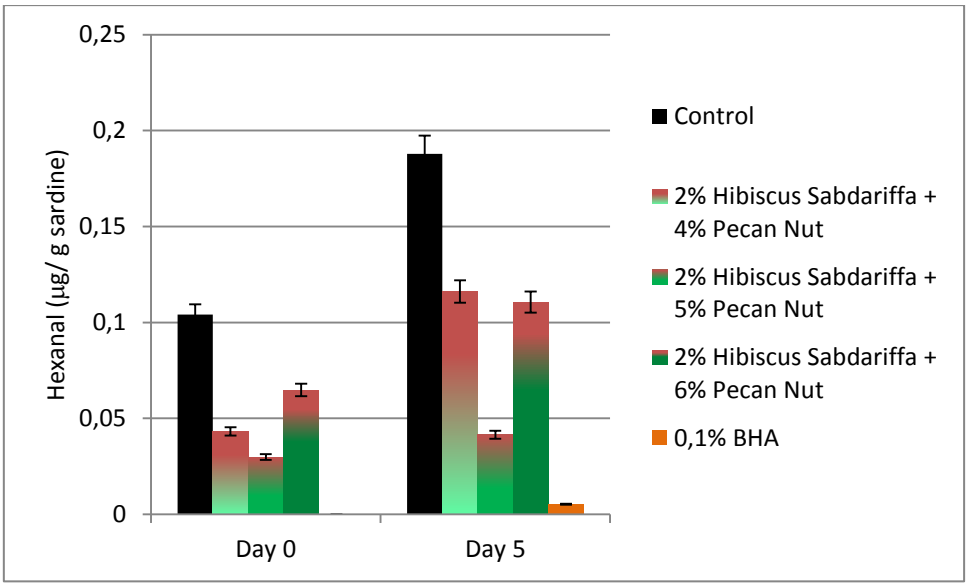


Figure 19. Hexanal content at days 0 and 5 of the experiment in samples of *sardina pilchardus* with different treatments. Results are expressed in µg hexanal/g sardine and day 0 the amount of hexanal in the sample with treatment of 1% w/w BHA is under the limit of detection. The values are means with standard deviation of the samples analyzed in triplicate.

The variation of the hexanal content between day 0 and 5 is very similar for all the treatments except 0,1% BHA. In all cases the hexanal in day 5 ranges from 1 to 3-fold the amount on day 0. No synergic effect is observed between pecan nut and roselle. The most effective treatment is 0,1% BHA; the content at day 0 is under the limit of detection and at day 5 it has only slightly increased (Figure 19).

4.4.2.5. *Biogenic Amines analysis*

Experiment 2

	Control	Pecan 5%	Pecan 10%	Roselle 5%	Pecan 5% + Roselle 5%	BHA 0,1%
OC	8,02 ± 7,83	5,93 ± 1,91	5,94 ± 3,68	4,48 ± 4,57	9,12 ± 7,59	6,76 ± 8,74
DO	4,73 ± 3,10	0,68 ± 0,50	1,36 ± 0,00	8,27 ± 0,98	10,26 ± 3,62	7,57 ± 6,25
PUT	3,34 ± 0,83	3,19 ± 1,86	1,26 ± 0,36	22,76 ± 5,10	19,92 ± 0,20	5,97 ± 4,00
TYR	4,61 ± 0,76	4,97 ± 0,88	1,14 ± 0,55	7,92 ± 1,24	8,24 ± 0,39	2,13 ± 1,30
CAD	1,03 ± 0,20	0,78 ± 0,18	0,10 ± 0,03	0,99 ± 0,76	1,08 ± 0,73	0,81 ± 0,37
SER	11,80 ± 4,45	12,41 ± 1,57	4,51 ± 3,33	30,39 ± 5,71	21,91 ± 17,55	15,78 ± 2,08
HIS	1,62 ± 1,11	1,53 ± 0,12	0,90 ± 0,58	14,08 ± 1,23	11,69 ± 2,88	4,49
SPD	7,28	6,14	6,55 ± 2,47	ND	ND	7,17
TRP	6,64	5,42	2,01 ± 1,62	ND	ND	10,34
SPM	ND	ND	6,48	ND	ND	ND

Table 15. Biogenic amines present in sardina pilchardus meat samples with different treatments at day 5. Results are expressed as mean ± standard deviation in mg/100g sardine. ND = not detected.

The amount of each biogenic amine varies significantly depending on the treatment (Table 15). Other authors have reported similar results for the quantification of biogenic amines in sardine in a period between 3-6 days while stored in refrigeration (Özogul *et al.*, 2010 and Özyurt *et al.,* 2012). As for the effectiveness of the treatments, it can be observed that samples containing 10% of pecan nut have much lower amounts of BA than the control and any other treatment. Since BA form as a consequence of protein decomposition, obtained results indicate that pecan nut is working properly as a preservative for sardine meat and has a much better performance than the artificial food preservative BHA. Samples containing 5% pecan nut show also better results in preventing the formation of most BA than BHA. Samples with the antimicrobial compound roselle present amounts of BA either similar to the control sample or higher. Karabacak (2007) reported histamine, putrescine and tyramine concentrations in sucuk batters during the ripening period. In this study, the BA concentrations of the samples containing roselle were similar to (or smaller) than the control, although the samples containing 300 and 600 mg soluble solid/kg of roselle at the fourth day doubled the amount of tyramine present in relation to the control.

Experiment 3

	Control	Roselle 2% + Pecan 4%	Roselle 2% + Pecan 5%	Roselle 2% + Pecan 6%	BHA 0,1%
OC	2,07 ± 1,68	47,55 ± 56,72	40,86 ± 57,43	15,26 ± 19,37	15,89 ± 1,89
DO	7,86 ± 3,76	22,31 ± 15,22	38,16 ± 10,02	13,34 ± 11,11	1,72 ± 0,27
PUT	3,14 ± 2,41	12,62 ± 12,92	9,58 ± 9,24	11,58 ± 4,55	5,28 ± 0,76
TYR	4,48 ± 1,03	22,01 ± 7,16	12,96 ± 2,93	5,65 ± 0,38	5,21 ± 0,97
CAD	0,68 ± 0,17	1,38 ± 0,16	3,93 ± 0,71	4,86 ± 1,47	1,54 ± 0,60
SER	5,94 ± 1,82	31,00 ± 23,69	28,80 ± 8,74	28,73 ± 10,48	18,79 ± 4,69
HIS	0,64	14,04 ± 6,11	13,93 ± 7,51	9,52 ± 3,16	2,23 ± 0,82
SPD	6,03	ND	ND	ND	ND
TRP	3,13	ND	ND	ND	ND
SPM	22,08	29,25	ND	ND	ND

Table 16. Biogenic amines present in sardina pilchardus meat samples with different treatments at day 4. Results are expressed as mean ± standard deviation in mg/100g sardine. ND = not detected

Results shown in Table 16 indicate that presence of roselle in sardine meat increases the concentrations of all biogenic amines. Addition of pecan nut has de opposite effect, as with the increase of concentration, a decrease on the presence of BA in the samples can be observed. No literature has been found to explain this effect of roselle in promoting the formation of BA. This leaves space for further studies.

4.4.2.6. Sensory Analysis

Baker's tables establish a significant difference for 28,5 points. It can be observed in Table 17 that there is no such difference between any of the samples; therefore no treatment can be established as the preferred one. Lower punctuation means higher taster's acceptability. General assessor's (Figure 20) comments point out that roselle gives an acid shade and boosts the fish taste of the sample. The other three treatments are either reported to be very similar or with an effect of pecan nut of softening the fishy flavor and even making the sample taste like meat.

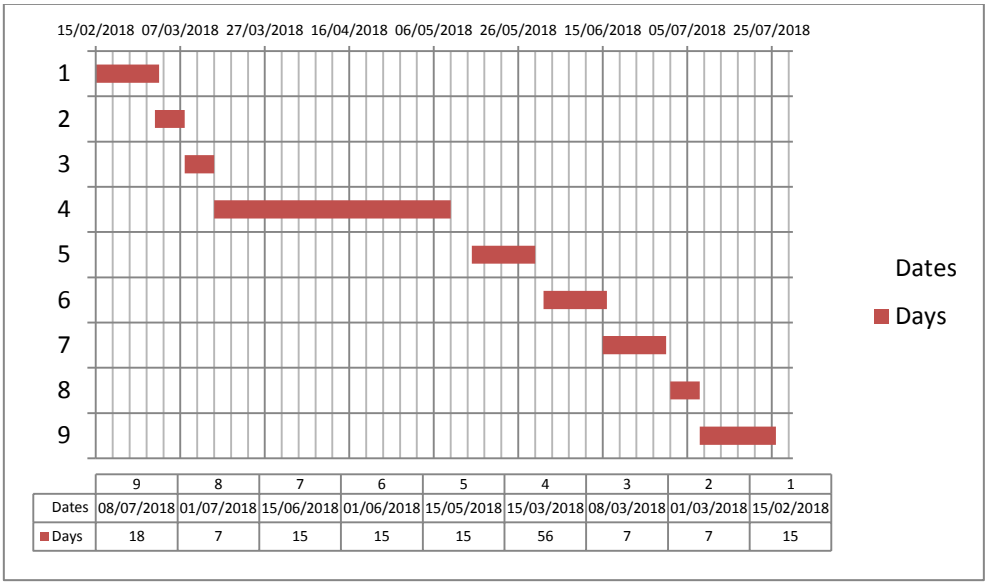
Treatment	Control	Pecan 5%	Pecan 10%	Roselle 5% + Pecan 5%
Rank total	99[a]	91[a]	80[a]	98[a]

Table 17. Rank total of treated samples according to assessor's perception and analysis with Baker's tables for 37 assessors and 4 products

Figure 20. First panel of assessors performing the sensory analysis of the samples

TIME PLANNING AND COSTS

Time planning is approximate and has changed throughout all the experiments along with the obtainment of results. Figure 21 shows the main tasks which have been carried out, their order and approximate duration in days.



	9	8	7	6	5	4	3	2	1
Dates	08/07/2018	01/07/2018	15/06/2018	01/06/2018	15/05/2018	15/03/2018	08/03/2018	01/03/2018	15/02/2018
Days	18	7	15	15	15	56	7	7	15

Figure 21. Diagram to illustrate an indicative work sequence of the project

Tasks	Content
1	Literature research
2	Preliminary DPPH and Folin-Ciocalteu assays
3	Previous experiments
4	Lipid oxidation and microbiology analysis on Gilt-Head Sea Bream and Sardine
5	FAME analysis
6	Biogenic Amines analysis
7	Hexanal analysis
8	Sensory analysis
9	Treatment of results and conclusions

Table 18. Correlation between numbered tasks in Gantt diagram (Figure 21) and the work associated to them

Costs of the work are detailed in Table 18, Table 19, Table 20, Table 21, Table 23 22, and Table 24. The main costs are attributed to food-products, reagents, equipment depreciation, services in external laboratories and cost of staff. The total cost of the project amounts to 23382,08 €.

Product	**Price (€)**
Gilt-head Sea Bream	7
Sardine	70
Pecan Nut	20
Roselle	10
Moringa	10
Total	**117**

Table 19. Cost of food-products

Product name	**Supplier**	**Price**	**Amount**	**Price of used reagent (€)**
Methanol	Panreac	16,40€/L	3 L	49,20
Folin-Ciocalteu reagent	Panreac	41,30€/250mL	100 mL	16,52
Sodium carbonate	Panreac	27,6€/500g	40 g	2,21
Gallic Acid	Sigma-Aldrich	47,70€/100g	0,3 g	0,14
DPPH	Sigma-Aldrich	59,40€/g	10 mg	0,59
Trolox	Sigma-Aldrich	129€/5g	0,3 g	7,77
Ethanol	Sigma-Aldrich	88,5€/500mL	10 mL	1,77
EDTA	Sigma-Aldrich	25,25€/100g	1,1 g	0,28
TBA	Sigma-Aldrich	59€/25g	1,7 g	4,01
TCA	Sigma-Aldrich	34,60/100g	77,5 g	26,82
			Total	**109,30**

Table 20. Cost of reagents

Equipment	Depreciable amount (€)	Service life (years)	Time of use (days)	Depreciation (€)
Analytical balance (COBOS aw 220)	1300	10	45	16,03
Centrifuge (Orto-Alresa)	4200	10	15	17,26
Magnetic agitator (SBS Multipoint)	456	6	2	0,42
Spectrophotometer (FLUOStar Omega)	19600	10	45	241,64
Ultra-Turrax (Ika-Werke, GmbH & Co)	1000	10	45	12,33
			Total	**287,68**

Table 21. Cost of equipment's depreciation

Method	Price/hour	Total Price
Gas chromatography (FID)	12,32 €/hour	739,2
Gas chromatography (MS/EI)	33,84 €/hour	676,8
HPLC-C	14,69€/hour	602,7
	Total	**2018,7**

Table 22. Cost of services in external laboratories

To calculate the total staff costs human resources (HR), gross annual income (GAI) and social security (SS) costs must be taken into account. Staff expenses are therefore calculated with the following equation:

$$Staff\ Costs\ (€) = HR\ (h \cdot person) \cdot \frac{GAI + SS\ \left(\frac{€}{person \cdot year}\right)}{\left(\frac{h}{year}\right)}$$

The XVI General Agreement of the Chemical Industry defines 8 professional groups. Here are detailed the responsibilities of the two groups involved in this project:

Professional group nº 5. Required to coordinate and supervise the execution of several tasks and/or collaborators. It also includes tasks which don't involve orders but have an intermediate content of intellectual activity and human relationships. Ex: Person in charge of the project or author of the project

Professional group nº 7. Includes functions which involve complex activities with a high degree of exigence, autonomy and responsibility. Direction of a set of functions which require specialized technical or professional knowledge. Ex: Director of the project.

Professional group	€/person·year	€/person·h
5	20185,52	11,52
7	28699,97	16,38

Table 23. Minimum gross salary of the different professional groups

With the information in Table 23, which details the minimum gross salary of the different professional groups , staff costs can be calculated:

	Nº of people	Nº of hours	Salary (€/person · h)	Salary (€)	SS	Staff Cost
Project author	1	900	11,52	10368	3317,76	13685,76
Intermediate tutor	1	400	11,52	4608	1474,56	6082,56
Project director	1	50	16,38	819	262,08	1081,08
					Total	**20849,4**

Table 24. Staff costs

ENVIRONMENTAL IMPACT

Because this project is food-related there are almost no products which can have a negative impact in the environment. Table 25 details the different types of residues which can be harmful to the environment and/or living organisms. For this reason, during the project, special attention has been paid to the classification, deposition and/or recovery of the different waste generated. It can be concluded that no impact to the environment has derived from this project**.**

Residue	Use	Classification
Methanol	Extractions	Non chlorinated organic solvents
Tungsten and molybdenum	Folin-Ciocalteu assay	Solutions with heavy metals
DPPH radical in aquatic basis	DPPH assay	None (*)

Table 25. Summary of residues. *Free radicals don't need any specific treatment since they have a lifespan of hours

CONCLUSION

Pecan nut contains high amounts of phenolic compounds and has a strong radical scavenging activity. It can therefore effectively delay the reaction of lipid oxidation and formation of secondary by-products such as hexanal. Concentrations of 5% w/w have a similar effectivity as currently used artificial antioxidants such as BHA and 10% w/w shows even more successful results. Pecan nut contains high amounts of healthy mono and polyunsaturated fatty acids which are beneficial to improve general lipid profile on humans, diminish LDL cholesterol and avoid CVD and other diseases derived from free radicals. Pecan nut can also help prevent proteolysis and formation of biogenic amines as a consequence of free amino acids degradation. Even though it has previously been reported to act as an antimicrobial agent, no specific results in that sense have been found in this work. Although in the sensory test no significant difference between treatments was found, pecan nut received the highest punctuation and most assessors perceived patties containing it as agreeable and with the effect of diminishing the fishy taste of sardine.

Roselle and moringa also possess high antioxidant activity reflexed in the DPPH and Folin-Ciocalteu assays. Moringa showed promising results in the TBARS analysis, but was discarded because it conferred a green colour to the samples which could cause consumer's rejection. Even so, because its high content of polyphenols and radical scavenging activity, further experiments could be carried out to use it as a food preservative. Roselle had a good performance as antimicrobial agent for mesophilic bacteria and helped delay lipid and protein oxidation, although it was less effective than pecan nut. In the sensory test, it was reported to confer an acidic flavour which was preferred by some tasters and rejected by others.

Sardine is a fatty fish with similar and high contents of saturated, mono and polyunsaturated fatty acids which gets very easily oxidized even under refrigerated storage. It can therefore benefit from antioxidant compounds to prevent its fast deterioration.

The final conclusion of this work is that pecan nut can be effectively used in different concentrations as a food preservative to delay spoilage of fish and extend its shelf life. It is a promising natural antioxidant which contains many health benefiting compounds and which could be used to substitute currently available synthetic antioxidants. A food preservative composed by pecan nut and roselle could be even more effective in preventing both oxidation and bacterial growth.

This work produced barely no environmental impact, had a rough cost of 23382,08 € and was performed within a timespan of six months.

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A FATTY ACIDS COMPLETE RESULTS

Experiment 1: Fatty Acids analysis values

DAY 0						
Fatty Acids (%)	**Control**	**Pecan 1%**	**Pecan 5%**	**Roselle 1%**	**Moringa 1%**	**BHA 1%**
C6:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,15 ± 0,21
C8:0	0,00 ± 0,00	0,10 ± 0,14	0,01 ± 0,01	0,09 ± 0,13	0,09 ± 0,12	0,03 ± 0,04
C10:0	0,00 ± 0,00	0,07 ± 0,11	0,02 ± 0,02	0,20 ± 0,08	0,17 ± 0,04	0,02 ± 0,03
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,07 ± 0,10	0,10 ± 0,14	0,00 ± 0,00
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C15:0	0,67 ± 0,05	0,55 ± 0,00	0,09 ± 0,03	0,69 ± 0,01	0,65 ± 0,01	0,25 ± 0,22
C16:0	**25,97 ± 1,02**	**22,23 ± 0,15**	**10,23 ± 0,52**	**25,15 ± 0,24**	**24,60 ± 0,83**	**10,25 ± 8,83**
C17:0	0,53 ± 0,06	0,45 ± 0,02	0,12 ± 0,02	0,54 ± 0,03	0,54 ± 0,00	0,23 ± 0,20
C18:0	**3,80 ± 0,08**	**3,92 ± 0,15**	**2,33 ± 0,04**	**3,88 ± 0,00**	**4,24 ± 0,17**	**1,76 ± 1,36**
C20:0	0,77 ± 0,58	0,53 ± 0,55	0,09 ± 0,01	0,64 ± 0,68	1,09 ± 0,01	0,50 ± 0,44
C21:0	0,14 ± 0,01	0,06 ± 0,09	0,02 ± 0,01	0,15 ± 0,00	0,15 ± 0,01	0,00 ± 0,00
C22:0	0,87 ± 0,12	0,69 ± 0,04	0,09 ± 0,03	0,91 ± 0,04	0,86 ± 0,02	0,35 ± 0,31
C23:0	0,51 ± 0,39	0,46 ± 0,25	0,08 ± 0,06	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C24:0	0,76 ± 0,02	0,48 ± 0,00	0,03 ± 0,04	0,74 ± 0,03	0,65 ± 0,12	0,30 ± 0,26
ΣSFA	34,03	29,38	13,08	32,78	32,86	13,64
C14:1	0,14 ± 0,02	0,05 ± 0,08	0,01 ± 0,01	0,07 ± 0,10	0,15 ± 0,01	0,00 ± 0,00
C15:1	0,06 ± 0,08	0,05 ± 0,08	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C16:1	**2,57 ± 0,16**	**2,14 ± 0,07**	**0,36 ± 0,11**	**2,64 ± 0,14**	**2,50 ± 0,02**	**1,07 ± 0,94**
C17:1	0,12 ± 0,02	0,21 ± 0,15	0,04 ± 0,05	0,15 ± 0,03	0,19 ± 0,10	0,14 ± 0,00
C18:1n9	**15,27 ± 2,67**	**23,67 ± 0,85**	**49,90 ± 5,07**	**16,51 ± 0,92**	**17,22 ± 1,11**	**7,85 ± 5,80**
C20:1n9	0,75 ± 0,47	0,65 ± 0,38	0,22 ± 0,02	0,82 ± 0,59	0,39 ± 0,00	0,15 ± 0,13
C22:1n9	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	6,88 ± 0,05	6,68 ± 0,10	2,77 ± 2,40
C24:1	0,58 ± 0,30	0,46 ± 0,20	0,06 ± 0,00	0,60 ± 0,24	0,55 ± 0,21	0,18 ± 0,10
ΣMUFA	19,49	27,23	50,59	27,66	27,68	12,16

C18:2n6t	0,05 ± 0,08	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C18:2n6c	**5,81 ± 1,51**	**11,98 ± 0,65**	**30,47 ± 2,02**	**6,63 ± 0,33**	**7,27 ± 0,59**	**59,31 ± 34,07**
C18:3n3	0,50 ± 0,35	0,56 ± 0,51	0,02 ± 0,00	0,55 ± 0,34	0,96 ± 0,01	0,33 ± 0,18
C18:3n6	0,47 ± 0,47	0,54 ± 0,54	1,35 ± 0,07	0,49 ± 0,48	0,17 ± 0,02	0,12 ± 0,02
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:3n6	0,08 ± 0,11	0,00 ± 0,00	0,00 ± 0,00	0,08 ± 0,12	0,00 ± 0,00	0,00 ± 0,00
C20:4n6	0,54 ± 0,04	0,42 ± 0,01	0,05 ± 0,02	0,51 ± 0,01	0,49 ± 0,01	0,19 ± 0,17
C20:3n6	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:5n3	**6,96 ± 0,60**	**5,51 ± 0,17**	**0,73 ± 0,24**	**0,44 ± 0,17**	**0,42 ± 0,19**	**0,20 ± 0,18**
C22:2	0,09 ± 0,13	0,09 ± 0,13	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:6n3	**22,57 ± 1,92**	**17,02 ± 0,63**	**2,23 ± 0,75**	**21,22 ± 0,36**	**20,33 ± 0,69**	**8,32 ± 7,22**
ΣPUFA	37,07	36,13	34,87	29,92	29,64	68,47
PUFA/SFA	1,09	1,23	2,67	0,91	0,90	5,02
Σn6	1,14	0,96	1,43	1,08	0,66	0,31
Σn3	30,03	23,09	2,97	22,21	21,71	8,85
n6/n3	0,04	0,04	0,48	0,05	0,03	0,03
DHA/EPA	3,24	3,09	3,04	48,49	48,78	40,70
Unidentified	5,75	4,12	0,92	5,64	5,96	4,09

Table 26. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 0 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ±S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

DAY 4

Fatty Acids (%)	**Control**	**Pecan 1%**	**Pecan 5%**	**Roselle 1%**	**Moringa 1%**	**BHA 1%**
C6:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C8:0	0,65 ± 0,52	0,24 ± 0,11	0,06 ± 0,02	0,15 ± 0,21	0,33 ± 0,02	0,18 ± 0,04
C10:0	0,23 ± 0,32	0,06 ± 0,08	0,00 ± 0,00	0,18 ± 0,02	0,22 ± 0,09	0,09 ± 0,02
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,06 ± 0,09	0,07 ± 0,09	0,04 ± 0,00	0,21 ± 0,06	0,26 ± 0,14	0,08 ± 0,00
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00

C15:0	0,71 ± 0,13	0,38 ± 0,07	0,11 ± 0,01	0,74 ± 0,03	0,75 ± 0,09	0,28 ± 0,10
C16:0	**26,14 ± 2,33**	**17,95 ± 1,84**	**10,46 ± 0,51**	**26,28 ± 0,19**	**28,13 ± 0,62**	**10,71 ± 3,68**
C17:0	0,58 ± 0,15	0,36 ± 0,05	0,15 ± 0,02	0,61 ± 0,05	0,57 ± 0,08	0,23 ± 0,09
C18:0	**4,44 ± 0,54**	**3,32 ± 0,40**	**2,53 ± 0,12**	**4,13 ± 0,11**	**3,74 ± 0,06**	**1,70 ± 0,63**
C20:0	1,03 ± 0,07	0,50 ± 0,11	0,13 ± 0,04	1,09 ± 0,01	1,14 ± 0,01	0,50 ± 0,17
C21:0	0,08 ± 0,12	0,00 ± 0,00	0,01 ± 0,01	0,17 ± 0,00	0,08 ± 0,12	0,02 ± 0,03
C22:0	0,86 ± 0,18	0,45 ± 0,08	0,11 ± 0,03	0,89 ± 0,04	0,84 ± 0,17	0,38 ± 0,13
C23:0	0,36 ± 0,10	0,14 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C24:0	0,63 ± 0,12	0,38 ± 0,05	0,08 ± 0,03	0,66 ± 0,20	1,04 ± 0,32	0,35 ± 0,17
ΣSFA	34,90	23,54	13,62	34,77	36,56	14,26
C14:1	0,18 ± 0,01	0,00 ± 0,00	0,01 ± 0,01	0,17 ± 0,02	0,10 ± 0,14	0,07 ± 0,03
C15:1	0,06 ± 0,09	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C16:1	**2,81 ± 0,51**	**1,54 ± 0,33**	**0,45 ± 0,10**	**2,87 ± 0,00**	**3,00 ± 0,06**	**1,09 ± 0,39**
C17:1	0,13 ± 0,04	0,07 ± 0,10	0,07 ± 0,00	0,13 ± 0,01	0,17 ± 0,03	0,08 ± 0,06
C18:1n9	**18,7 ± 4,52**	**34,91 ± 3,04**	**47,71 ± 0,46**	**17,45 ± 0,28**	**13,72 ± 0,36**	**6,61 ± 0,59**
C20:1n9	0,41 ± 0,05	0,32 ± 0,04	0,24 ± 0,01	0,44 ± 0,04	0,40 ± 0,04	0,17 ± 0,06
C22:1n9	0,00 ± 0,00	0,00 ± 0,00	0,80 ± 0,21	5,90 ± 0,01	6,43 ± 0,08	2,85 ± 0,99
C24:1	0,66 ± 0,12	0,30 ± 0,17	0,09 ± 0,03	0,73 ± 0,01	0,77 ± 0,01	0,32 ± 0,11
ΣMUFA	22,95	37,13	49,38	27,69	24,59	11,20
C18:2n6t	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C18:2n6c	**7,76 ± 2,83**	**21,72 ± 3,70**	**31,13 ± 2,15**	**6,93 ± 0,18**	**4,98 ± 0,17**	**58,13 ± 14,87**
C18:3n3	0,13 ± 0,00	1,13 ± 0,10	1,30 ± 0,07	0,81 ± 0,03	0,86 ± 0,06	0,55 ± 0,13
C18:3n6	0,80 ± 0,01	0,14 ± 0,00	0,11 ± 0,00	0,16 ± 0,01	0,08 ± 0,11	0,32 ± 0,15
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:3n6	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,02 ± 0,03
C20:4n6	0,45 ± 0,05	0,23 ± 0,04	0,07 ± 0,02	0,48 ± 0,00	0,53 ± 0,01	0,25 ± 0,12
C20:3n6	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:5n3	**5,45 ± 0,81**	**2,85 ± 0,63**	**0,06 ± 0,03**	**0,23 ± 0,06**	**0,27 ± 0,14**	**0,08 ± 0,02**
C22:2	0,05 ± 0,07	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:6n3	**15,55 ± 2,31**	**7,94 ± 1,48**	**2,54 ± 0,73**	**18,19 ± 0,33**	**21,75 ± 0,25**	**9,51 ± 3,37**
ΣPUFA	30,19	34,01	35,23	26,79	28,47	68,86

PUFA/SFA	0,86	1,44	2,59	0,77	0,78	4,83
Σn6	1,25	0,37	0,20	0,64	0,61	0,59
Σn3	21,13	11,92	3,90	19,22	22,88	10,14
n6/n3	0,06	0,03	0,05	0,03	0,03	0,06
DHA/EPA	2,85	2,78	44,40	79,45	80,80	125,39
Unidentified	7,10	2,70	1,07	6,31	6,01	3,89

Table 27. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 4 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ± S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

Experiment 2: Fatty Acids analysis values

DAY 0

Fatty Acids (%)	**Control**	**Pecan 5%**	**Pecan 10%**	**Roselle 5%**	**Roselle 5% + Pecan 5%**	**BHA 0,1%**
C6:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C8:0	0,20 ± 0,28	0,02 ± 0,02	0,01 ± 0,01	0,17 ± 0,23	0,02 ± 0,03	0,00 ± 0,00
C10:0	0,18 ± 0,26	0,02 ± 0,02	0,02 ± 0,02	0,13 ± 0,19	0,02 ± 0,03	0,06 ± 0,09
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,00 ± 0,00	0,01 ± 0,02	0,01 ± 0,01	0,00 ± 0,00	0,02 ± 0,02	0,06 ± 0,08
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	**$2,54^{a}$ ± 0,10**	**$0,63^{b}$ ± 0,14**	**$0,29^{b}$ ± 0,06**	**$2,49^{a}$ ± 0,24**	**$0,71^{b}$ ± 0,05**	**$3,22^{a}$ ± 0,78**
C15:0	0,54 ± 0,01	0,12 ± 0,02	0,06 ± 0,01	0,54 ± 0,06	0,14 ± 0,01	0,72 ± 0,15
C16:0	**$24,29^{a}$ ± 0,71**	**$11,40^{bc}$ ± 0,76**	**$9,33^{c}$ ± 0,59**	**$24,75^{a}$ ± 0,71**	**$12,04^{b}$ ± 0,38**	**$26,01^{a}$ ± 0,58**
C17:0	0,45 ± 0,03	0,14 ± 0,01	0,10 ± 0,00	0,47 ± 0,05	0,17 ± 0,00	0,64 ± 0,20
C18:0	**$3,82^{a}$ ± 0,19**	**$2,52^{b}$ ± 0,08**	**$2,35^{b}$ ± 0,07**	**$3,35^{a}$ ± 0,19**	**$2,54^{b}$ ± 0,05**	**$3,95^{a}$ ± 0,38**
C20:0	0,82 ± 0,06	0,14 ± 0,04	0,07 ± 0,00	0,20 ± 0,10	0,19 ± 0,01	0,57 ± 0,81
C21:0	0,07 ± 0,10	0,01 ± 0,02	0,01 ± 0,01	0,12 ± 0,01	0,01 ± 0,02	0,19 ± 0,04
C22:0	0,67 ± 0,04	0,13 ± 0,05	0,05 ± 0,01	0,68 ± 0,11	0,16 ± 0,00	0,83 ± 0,23
C23:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C24:0	0,69 ± 0,10	0,10 ± 0,01	0,04 ± 0,04	0,80 ± 0,15	0,16 ± 0,04	0,67 ± 0,01
ΣSFA	33,89	15,19	12,30	33,40	16,13	36,87

C14:1	0,00 ± 0,00	0,01 ± 0,02	0,00 ± 0,00	0,05 ± 0,08	0,01 ± 0,02	0,08 ± 0,11
C15:1	0,07 ± 0,10	0,01 ± 0,02	0,00 ± 0,00	0,06 ± 0,08	0,01 ± 0,02	0,07 ± 0,10
C16:1	**1,72^{a} ± 0,04**	**0,39^{b} ± 0,09**	**0,20^{b} ± 0,03**	**1,65^{a} ± 0,19**	**0,49^{b} ± 0,01**	**2,12^{a} ± 0,42**
C17:1	0,18 ± 0,06	0,05 ± 0,04	0,07 ± 0,01	0,14 ± 0,04	0,03 ± 0,00	0,10 ± 0,14
C18:1n9	**15,03^{b} ± 1,19**	**48,49^{a} ± 2,03**	**52,62^{a} ± 1,83**	**16,24^{b} ± 0,35**	**46,10^{a} ± 1,37**	**15,62^{b} ± 3,44**
C20:1n9	0,23 ± 0,10	0,22 ± 0,00	0,21 ± 0,02	0,45 ± 0,41	0,22 ± 0,01	0,67 ± 0,21
C22:1n9	**5,76^{a} ± 0,06**	**0,96^{b} ± 0,20**	**0,46^{b} ± 0,12**	**5,70^{a} ± 0,33**	**1,34^{b} ± 0,04**	**6,39^{a} ± 0,53**
C24:1	0,46 ± 0,23	0,09 ± 0,06	0,03 ± 0,05	0,54 ± 0,25	0,11 ± 0,05	0,56 ± 0,37
ΣMUFA	23,44	50,23	53,60	24,82	48,32	25,61
C18:2n6t	0,10 ± 0,14	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,01 ± 0,01	0,12 ± 0,04
C18:2n6c	**6,57^{b} ± 0,56**	**28,15^{a} ± 0,40**	**29,32^{a} ± 0,70**	**7,25^{b} ± 0,10**	**27,55^{a} ± 0,57**	**6,22^{b} ± 2,18**
C18:3n3	**0,17^{a} ± 0,02**	**0,67^{a} ± 0,92**	**1,19^{a} ± 0,02**	**0,39^{a} ± 0,39**	**1,27^{a} ± 0,02**	**0,53^{a} ± 0,41**
C18:3n6	0,64 ± 0,02	0,66 ± 0,78	0,10 ± 0,01	0,71 ± 0,02	0,11 ± 0,00	0,44 ± 0,39
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:3n6	0,31 ± 0,26	0,05 ± 0,08	0,00 ± 0,00	0,06 ± 0,08	0,00 ± 0,00	0,07 ± 0,10
C20:4n6	0,24 ± 0,34	0,04 ± 0,06	0,04 ± 0,01	0,58 ± 0,15	0,12 ± 0,01	0,51 ± 0,00
C20:3n6	0,00 ± 0,00	0,01 ± 0,02	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:5n3	0,87 ± 0,45	0,13 ± 0,08	0,07 ± 0,03	0,51 ± 0,17	0,09 ± 0,02	0,58 ± 0,49
C22:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:6n3	**22,98^{a} ± 0,27**	**3,59^{bc} ± 0,35**	**1,75^{c} ± 0,57**	**23,79^{a} ± 1,11**	**4,87^{b} ± 0,44**	**22,68^{a} ± 0,31**
ΣPUFA	31,89	33,29	32,50	33,28	34,01	31,15
PUFA/SFA	0,94	2,19	2,64	1,00	2,11	0,84
Σn6	1,29	0,76	0,16	1,35	0,24	1,14
Σn3	24,03	4,39	3,02	24,68	6,22	23,78
n6/n3	0,05	0,17	0,05	0,05	0,04	0,05
DHA/EPA	26,38	27,60	25,08	47,02	55,97	39,33
Unidentified	10,40	1,26	1,58	8,20	1,50	6,31

Table 28. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 0 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ±S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

DAY 4

Fatty Acids (%)	**Control**	**Pecan 5%**	**Pecan 10%**	**Roselle 5%**	**Roselle 5% + Pecan 5%**	**BHA 0,1%**
C6:0	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C8:0	0,84 ± 0,17	0,12 ± 0,05	0,04 ± 0,01	0,63 ± 0,06	0,08 ± 0,04	0,86 ± 0,37
C10:0	0,47 ± 0,03	0,06 ± 0,04	0,03 ± 0,01	0,34 ± 0,01	0,01 ± 0,01	0,22 ± 0,31
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,00 ± 0,00	0,05 ± 0,03	0,03 ± 0,01	0,11 ± 0,15	0,07 ± 0,01	0,19 ± 0,07
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	**2,98^{a} ± 0,22**	**0,43^{b} ± 0,14**	**0,11^{b} ± 0,16**	**2,84^{a} ± 0,08**	**0,67^{b} ± 0,21**	**2,76^{a} ± 0,70**
C15:0	0,60 ± 0,02	0,08 ± 0,03	0,04 ± 0,01	0,59 ± 0,00	0,11 ± 0,03	0,54 ± 0,13
C16:0	**26,44^{a} ± 0,06**	**10,50^{b} ± 1,11**	**9,00^{b} ± 0,54**	**24,72^{a} ± 0,55**	**11,28^{b} ± 1,42**	**22,83^{a} ± 1,41**
C17:0	0,51 ± 0,01	0,12 ± 0,02	0,09 ± 0,01	0,55 ± 0,01	0,16 ± 0,03	0,45 ± 0,08
C18:0	**5,87^{a} ± 0,18**	**2,65^{c} ± 0,11**	**2,46^{c} ± 0,00**	**3,81^{b} ± 0,23**	**2,50^{c} ± 0,05**	**3,58^{b} ± 0,21**
C20:0	0,84 ± 0,02	0,07 ± 0,03	0,03 ± 0,01	0,72 ± 0,00	0,12 ± 0,04	0,74 ± 0,19
C21:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,13 ± 0,00	0,03 ± 0,01	0,07 ± 0,09
C22:0	0,68 ± 0,01	0,08 ± 0,04	0,03 ± 0,01	0,69 ± 0,01	0,13 ± 0,04	0,59 ± 0,24
C23:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C24:0	0,48 ± 0,01	0,06 ± 0,03	0,03 ± 0,00	0,71 ± 0,04	0,13 ± 0,07	0,46 ± 0,05
ΣSFA	38,39	14,04	11,83	34,85	15,20	32,21
C14:1	0,17 ± 0,01	0,01 ± 0,01	0,00 ± 0,00	0,11 ± 0,00	0,01 ± 0,02	0,07 ± 0,10
C15:1	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,05 ± 0,08	0,00 ± 0,00	0,00 ± 0,00
C16:1	**1,91^{a} ± 0,24**	**0,28^{b} ± 0,10**	**0,17^{b} ± 0,02**	**1,89^{a} ± 0,05**	**0,42^{b} ± 0,12**	**1,80^{a} ± 0,38**
C17:1	0,23 ± 0,04	0,10 ± 0,05	0,07 ± 0,00	0,12 ± 0,01	0,06 ± 0,04	0,30 ± 0,14
C18:1n9	**19,20^{b} ± 1,09**	**49,98^{a} ± 4,35**	**52,69^{a} ± 3,46**	**14,96^{b} ± 1,27**	**47,56^{a} ± 4,59**	**25,27^{b} ± 2,53**
C20:1n9	0,34 ± 0,00	0,21 ± 0,01	0,20 ± 0,01	0,33 ± 0,01	0,22 ± 0,00	0,35 ± 0,05
C22:1n9	**4,65^{ab} ± 0,20**	**0,53^{c} ± 0,25**	**0,29^{c} ± 0,05**	**5,85^{a} ± 0,11**	**1,07^{c} ± 0,38**	**4,26^{b} ± 0,66**
C24:1	0,54 ± 0,00	0,07 ± 0,04	0,04 ± 0,00	0,50 ± 0,27	0,13 ± 0,05	0,47 ± 0,08
ΣMUFA	27,04	51,17	53,46	24,81	49,47	32,52
C18:2n6t	0,00 ± 0,00	0,01 ± 0,01	0,02 ± 0,00	0,00 ± 0,00	0,01 ± 0,02	0,14 ± 0,20

C18:2n6c	**8,32^{bc} ± 0,23**	**29,73^{a} ± 0,48**	**31,30^{a} ± 1,85**	**6,73^{c} ± 0,73**	**28,29^{a} ± 0,76**	**12,01^{b} ± 1,75**
C18:3n3	**0,70^{a} ± 0,01**	**1,23^{a} ± 0,01**	**1,28^{a} ± 0,06**	**0,67^{a} ± 0,04**	**1,24^{a} ± 0,01**	**0,43^{a} ± 0,61**
C18:3n6	0,17 ± 0,02	0,10 ± 0,00	0,10 ± 0,00	0,14 ± 0,01	0,11 ± 0,00	0,48 ± 0,48
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:3n6	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C20:4n6	0,43 ± 0,01	0,05 ± 0,02	0,03 ± 0,00	0,54 ± 0,02	0,09 ± 0,03	0,36 ± 0,03
C20:3n6	0,00 ± 0,00	0,01 ± 0,01	0,02 ± 0,00	0,00 ± 0,00	0,03 ± 0,00	0,00 ± 0,00
C20:5n3	0,28 ± 0,03	0,04 ± 0,01	0,03 ± 0,01	0,18 ± 0,02	0,05 ± 0,00	0,22 ± 0,06
C22:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:6n3	**15,14^{b} ± 1,11**	**2,03^{c} ± 1,06**	**1,04^{c} ± 0,20**	**22,92^{a} ± 0,53**	**4,12^{c} ± 1,35**	**13,12^{b} ± 0,51**
ΣPUFA	25,04	33,20	33,81	31,17	33,93	26,78
PUFA/SFA	0,65	2,37	2,86	0,89	2,23	0,83
Σn6	0,60	0,17	0,16	0,68	0,24	0,99
Σn3	16,12	3,30	2,34	23,77	5,40	13,78
n6/n3	0,04	0,05	0,07	0,03	0,04	0,07
DHA/EPA	54,02	52,69	39,60	127,17	79,75	59,46
Unidentified	8,22	1,39	0,69	8,18	1,30	7,41

Table 29. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 4 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ±S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

Experiment 1: Fatty Acids analysis values

DAY 0

Fatty Acids (%)	Control	Roselle 2% + Pecan 4%	Roselle 2% + Pecan 5%	Roselle 2% + Pecan 6%	BHA 0,1%
C6:0	0,00 ± 0,00	0,00 ± 0,00	0,02 ± 0,00	0,00 ± 0,00	0,06 ± 0,09
C8:0	0,11 ± 0,15	0,03 ± 0,04	0,03 ± 0,00	0,00 ± 0,00	0,15 ± 0,22
C10:0	0,12 ± 0,16	0,04 ± 0,05	0,03 ± 0,00	0,00 ± 0,00	0,32 ± 0,03
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,12 ± 0,02	0,05 ± 0,02	0,04 ± 0,00	0,03 ± 0,04	0,07 ± 0,09
C13:0	0,02 ± 0,03	0,00 ± 0,00	0,00 ± 0,00	0,02 ± 0,03	0,00 ± 0,00

C14:0	**2,78^{a} ± 0,14**	**1,15^{b} ± 0,63**	**0,42^{b} ± 0,00**	**1,11^{b} ± 0,16**	**2,87^{a} ± 0,02**
C15:0	0,57 ± 0,02	0,15 ± 0,03	0,10 ± 0,00	0,49 ± 0,38	0,60 ± 0,02
C16:0	**23,53^{ab} ± 4,34**	**13,80^{ab} ± 2,40**	**11,37^{ab} ± 0,00**	**12,27^{b} ± 2,05**	**25,37^{a} ± 0,16**
C17:0	0,51 ± 0,05	0,85 ± 0,96	0,13 ± 0,00	2,07 ± 2,61	0,61 ± 0,02
C18:0	**3,10^{a} ± 0,35**	**2,54^{a} ± 0,04**	**2,35^{a} ± 0,00**	**2,25^{a} ± 0,44**	**3,63^{a} ± 0,35**
C20:0	0,52 ± 0,53	0,09 ± 0,12	0,09 ± 0,00	0,25 ± 0,03	0,46 ± 0,65
C21:0	0,14 ± 0,01	0,10 ± 0,09	0,00 ± 0,00	0,13 ± 0,12	0,06 ± 0,09
C22:0	0,76 ± 0,12	0,24 ± 0,16	0,09 ± 0,00	0,19 ± 0,01	0,74 ± 0,01
C23:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,04 ± 0,06	0,00 ± 0,00
C24:0	0,56 ± 0,12	0,24 ± 0,12	0,10 ± 0,00	0,18 ± 0,01	0,76 ± 0,03
ΣSFA	32,62	19,21	14,69	19,03	35,17
C14:1	0,13 ± 0,01	0,03 ± 0,04	0,00 ± 0,00	0,00 ± 0,00	0,06 ± 0,09
C15:1	0,10 ± 0,02	0,00 ± 0,00	0,01 ± 0,00	0,00 ± 0,00	0,06 ± 0,09
C16:1	**1,83^{a} ± 0,10**	**0,70^{b} ± 0,33**	**0,29^{b} ± 0,00**	**0,66^{b} ± 0,21**	**1,88^{a} ± 0,04**
C17:1	0,17 ± 0,08	0,80 ± 1,04	0,08 ± 0,00	0,34 ± 0,02	0,24 ± 0,09
C18:1n9	**20,21^{a} ± 7,26**	**37,30^{a} ± 9,98**	**48,69^{a} ± 0,00**	**34,52^{a} ± 7,19**	**15,03^{a} ± 1,72**
C20:1n9	0,71 ± 0,53	0,67 ± 0,63	0,19 ± 0,00	1,29 ± 1,50	0,63 ± 0,41
C22:1n9	**5,54^{a} ± 0,69**	**2,19^{b} ± 1,10**	**0,77^{b} ± 0,00**	**1,89^{b} ± 0,37**	**6,06^{a} ± 0,00**
C24:1	0,56 ± 0,16	0,18 ± 0,00	0,00 ± 0,00	0,22 ± 0,01	0,50 ± 0,24
ΣMUFA	29,26	41,86	50,03	38,92	24,47
C18:2n6t	**0,11^{a} ± 0,16**	**0,88^{a} ± 1,11**	**0,07^{a} ± 0,00**	**2,30^{a} ± 3,12**	**3,57^{a} ± 2,90**
C18:2n6c	**9,93^{a} ± 4,81**	**21,89^{a} ± 7,31**	**29,47^{a} ± 0,00**	**21,61^{a} ± 5,87**	**5,37^{a} ± 1,14**
C18:3n3	**0,40^{a} ± 0,50**	**1,29^{a} ± 0,08**	**0,04^{a} ± 0,00**	**2,28^{a} ± 1,43**	**0,45^{a} ± 0,37**
C18:3n6	0,60 ± 0,68	0,51 ± 0,58	1,32 ± 0,00	0,47 ± 0,51	0,42 ± 0,37
C20:2	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,06 ± 0,08	0,00 ± 0,00
C20:3n6	0,12 ± 0,04	0,47 ± 0,54	0,00 ± 0,00	0,77 ± 0,98	0,00 ± 0,00
C20:4n6	0,44 ± 0,09	0,18 ± 0,08	0,08 ± 0,00	0,18 ± 0,05	0,60 ± 0,02
C20:3n6	0,03 ± 0,05	0,05 ± 0,07	0,00 ± 0,00	0,08 ± 0,11	0,00 ± 0,00
C20:5n3	0,21 ± 0,01	0,19 ± 0,11	0,09 ± 0,00	0,12 ± 0,08	0,44 ± 0,22
C22:2	0,00 ± 0,00	0,25 ± 0,30	0,00 ± 0,00	0,26 ± 0,37	0,00 ± 0,00
C22:6n3	**21,23^{ab} ± 4,35**	**9,13^{bc} ± 4,10**	**3,31^{c} ± 0,00**	**7,23^{c} ± 0,60**	**23,67^{a} ± 0,94**
ΣPUFA	33,08	34,86	34,38	35,35	34,51

PUFA/SFA	1,01	1,81	2,34	1,86	0,98
Σn6	1,31	2,10	1,47	3,79	4,58
Σn3	21,84	10,61	3,44	9,63	24,56
n6/n3	0,06	0,20	0,43	0,39	0,19
DHA/EPA	100,79	48,32	38,20	59,00	53,65
Unidentified	4,82	4,00	0,82	6,66	5,31

Table 30. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 0 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ±S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).

DAY 4

Fatty Acids (%)	**Control**	**Roselle 2% + Pecan 4%**	**Roselle 2% + Pecan 5%**	**Roselle 2% + Pecan 6%**	**BHA 0,1%**
C6:0	0,00 ± 0,00	0,02 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C8:0	0,77 ± 0,09	0,03 ± 0,00	0,09 ± 0,00	0,08 ± 0,03	0,71 ± 0,49
C10:0	0,34 ± 0,02	0,03 ± 0,00	0,02 ± 0,00	0,03 ± 0,02	0,27 ± 0,13
C11:0	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	0,19 ± 0,00	0,04 ± 0,00	0,09 ± 0,00	0,05 ± 0,01	0,04 ± 0,06
C13:0	0,00 ± 0,00	0,00 ± 0,00	0,02 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C14:0	**2,88^{a} ± 0,20**	**0,42^{a} ± 0,00**	**1,53^{a} ± 0,00**	**0,46^{a} ± 0,01**	**1,34^{a} ± 1,07**
C15:0	0,58 ± 0,12	0,10 ± 0,00	0,30 ± 0,00	0,07 ± 0,03	0,25 ± 0,20
C16:0	**27,70^{a} ± 3,45**	**11,37^{b} ± 0,00**	**14,48^{ab} ± 0,00**	**10,36^{b} ± 0,58**	**14,07^{ab} ± 5,10**
C17:0	1,65 ± 1,49	0,13 ± 0,00	0,27 ± 0,00	0,13 ± 0,01	0,29 ± 0,22
C18:0	**3,88^{a} ± 0,53**	**2,35^{a} ± 0,00**	**2,72^{a} ± 0,00**	**2,45^{a} ± 0,12**	**3,20^{a} ± 0,62**
C20:0	0,77 ± 0,11	0,09 ± 0,00	0,56 ± 0,00	0,09 ± 0,00	0,36 ± 0,33
C21:0	0,22 ± 0,09	0,00 ± 0,00	0,06 ± 0,00	0,03 ± 0,01	0,00 ± 0,00
C22:0	0,55 ± 0,26	0,09 ± 0,00	0,35 ± 0,00	0,08 ± 0,04	0,06 ± 0,08
C23:0	0,05 ± 0,08	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C24:0	0,51 ± 0,49	0,10 ± 0,00	0,19 ± 0,00	0,12 ± 0,00	0,31 ± 0,22
ΣSFA	38,98	7,34	20,57	13,84	19,92

C14:1	0,14 ± 0,03	0,00 ± 0,00	0,07 ± 0,00	0,02 ± 0,00	0,00 ± 0,00
C15:1	0,00 ± 0,00	0,01 ± 0,00	0,04 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C16:1	**1,84^{a} ± 0,29**	**0,29^{a} ± 0,00**	**0,96^{a} ± 0,00**	**0,30^{a} ± 0,02**	**0,89^{a} ± 0,70**
C17:1	2,09 ± 1,77	0,08 ± 0,00	0,12 ± 0,00	0,18 ± 0,14	0,04 ± 0,05
C18:1n9	**14,31^{b} ± 1,11**	**48,69^{ab} ± 0,00**	**41,77^{ab} ± 0,00**	**48,62^{a} ± 1,14**	**40,47^{ab} ± 9,35**
C20:1n9	1,28 ± 0,84	0,19 ± 0,00	0,26 ± 0,00	0,19 ± 0,03	0,27 ± 0,07
C22:1n9	**4,04^{a} ± 0,76**	**0,77^{b} ± 0,00**	**2,20^{ab} ± 0,00**	**0,76^{b} ± 0,06**	**0,25^{b} ± 0,36**
C24:1	0,50 ± 0,10	0,00 ± 0,00	0,19 ± 0,00	0,09 ± 0,01	0,31 ± 0,24
ΣMUFA	24,21	25,02	45,63	50,15	42,23
C18:2n6t	**2,12^{a} ± 1,77**	**0,07^{a} ± 0,00**	**0,01^{a} ± 0,00**	**0,02^{a} ± 0,00**	**0,46^{a} ± 0,64**
C18:2n6c	**5,47^{b} ± 0,29**	**29,47^{ab} ± 0,00**	**23,20^{ab} ± 0,00**	**28,99^{a} ± 0,39**	**23,23^{ab} ± 7,37**
C18:3n3	**1,60^{a} ± 1,38**	**0,04^{a} ± 0,00**	**1,19^{a} ± 0,00**	**1,33^{a} ± 0,03**	**0,72^{a} ± 0,68**
C18:3n6	0,32 ± 0,26	1,32 ± 0,00	0,12 ± 0,00	0,10 ± 0,00	0,54 ± 0,63
C20:2	0,09 ± 0,12	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,01	0,00 ± 0,00
C20:3n6	1,06 ± 0,70	0,00 ± 0,00	0,04 ± 0,00	0,06 ± 0,09	0,00 ± 0,00
C20:4n6	0,56 ± 0,00	0,08 ± 0,00	0,15 ± 0,00	0,07 ± 0,01	0,03 ± 0,05
C20:3n6	0,09 ± 0,13	0,00 ± 0,00	0,03 ± 0,00	0,03 ± 0,01	0,00 ± 0,00
C20:5n3	0,13 ± 0,02	0,09 ± 0,00	0,02 ± 0,00	0,03 ± 0,00	1,58 ± 2,11
C22:2	0,52 ± 0,37	0,00 ± 0,00	0,00 ± 0,00	0,04 ± 0,05	0,00 ± 0,00
C22:6n3	**13,86^{a} ± 2,58**	**3,31^{a} ± 0,00**	**6,11^{a} ± 0,00**	**3,10^{a} ± 0,33**	**6,16^{a} ± 4,49**
ΣPUFA	25,81	17,19	30,87	33,77	32,72
PUFA/SFA	0,66	2,34	1,50	2,44	1,64
Σn6	4,15	1,47	0,36	0,29	1,03
Σn3	15,59	3,44	7,32	4,46	8,46
n6/n3	0,27	0,43	0,05	0,06	0,12
DHA/EPA	106,63	38,20	282,97	106,45	3,91
Unidentified	9,89	0,82	2,82	2,13	3,71

Table 31. Fatty acids profiles of *Sardina Pilchardus* muscle with different treatments at day 1 of the experiment. Results expressed as percentage of total fatty acid methyl esters. The values are means ±S.D. of the samples analyzed in duplicate. Different letters in the same row indicate significant differences by Tukey's test (P < 0.05).