DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

EFFECTS OF PLASMA-ACTIVATED WATER (PAW) ON OXIDATION OF LIPIDS IN SEAFOOD

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ACRONYMS AND ABBREVIATIONS

APPJ	Atmospheric pressure plasma jet
CFU	Colony-forming unit
COPS	Cholesterol oxidized products
DBD	Dielectric barrier discharge
FAME	Fatty acids methyl esters
FID	Flame ionization detector
GC	Gas chromatography
MUFA	Monounsaturated fatty acid
NTP	Non-thermal plasma
ORP	Oxidation-reduction potential
PAW	Plasma-Activated Water
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SD	Sterile distilled
SDS	Sodium Lauryl Sulfate
SFA	Saturated fatty acid
TBA	Thiobarbituric acid
TBARS	2-thio-barbituric acid reactive substances
TMS	Trimethylsilyl

INTRODUCTION AND AIM OF THE THESIS

With the growth of population and demand for food security and safety, food preservation plays a crucial role in the development of the food industry. Over the past two decades, thermal preservation such as sterilization, pasteurization, or high-temperature drying has been utilized to extend the shelf life of food. Although these thermal methods are effective in prolonging the shelf-life of foods, the drawback of these techniques that they cause several alterations in texture, aroma, and color as well as nutritional losses, and chemical modification. In order to meet customers' need for safety, enhance the shelf life while maintaining quality, avoiding the undesirable effects generated by heat treatments, emerging technologies in the food industry need to be developed and implemented (Coutinho et al., 2018). In this regard, some non-thermal emerging techniques such as pulsed light, ionizing radiation, high-intensity ultrasound, high hydrostatic pressure, ultraviolet light, pulsed electric fields, and cold plasma must be mentioned (Olatunde & Benjakul, 2018).

Seafood has become the main source of food that is consumed and favored all over the world. Especially, sardine and mussels are widely consumed and rich in nutrients. These seafood has excellent sources of vitamins and minerals such as Vitamin B12, Vitamin C, Vitamin D, Selenium, Phosphorus, Calcium, Iron, etc. which have been proven that bring various positive effects on human health. However, the concern about the safety of seafood also is growing rapidly due to raw consumption and the insufficiency of hygienic food practices. Moreover, the natural composition of seafood is a main factor regarding its safety. According to the Centers for Disease Control and Prevention, microorganisms such as *Listeria monocytogenes*, *Salmonella*, *Clostridium perfringens*, and *Staphylococcus aureus* are the major species causing several illnesses and death every year in the world. Unfortunately, these microorganisms have been isolated in seafood and seafood processing areas. Recently, a newspaper from the Chinese Center for Disease Control and Prevention reported that SAR-CoV-2 contamination was found in frozen seafood. Therefore, the application of non-thermal technologies is imperative for assuring the food safety of seafood and increasing its shelf-life with minimal modification of organoleptic properties.

Cold plasma is one of the advanced technologies that attract attention in food application. Cold Plasma technology has been proven as an effective one in microbial decontamination, cancer treatment, wound healing, and so forth (Graves, 2014; Shaw, Shama, & Iza, 2015; von Woedtke, Reuter, Masur, & Weltmann, 2013). In food technology, it has been used for the preservation of food such as meat (Misra & Jo, 2017), fish (Olatunde, Benjakul, & Vongkamjan, 2019), or milk (Coutinho et al., 2019). There are several applications of cold plasma on substance and one of the applications that have gained a lot of importance for its efficacy against microbiological preservation of food is Plasma-Activated Water (PAW). However, extensive research

on the impact of cold plasma treatments on macronutrients is still lacking. For example, in the treatment of foodstuffs with cold plasma, lipid oxidation and protein denaturation are expected. These consequences can adversely affect the shelf-life, sensorial properties, and quality of products. Several research reported the oxidative impact of cold plasma on food ingredients. For examples, different studies reported that this emerging technology has a negative effect on the fatty acids of pork (Jayasena et al., 2015; Kim et al., 2011), chicken (H. Lee et al., 2016), beef (Bauer et al., 2017; Jayasena et al., 2015; Wang et al., 2021).

Objective:

The main goal of this thesis was to investigate if PAW could cause any undesirable effects on lipids such as the oxidation or degradation of cholesterol leading to the formation of oxidized compounds. To do so, we first applied PAW to samples fillets of *Sardina pilchardus* and *Mytilus Galloprovincialis* and then evaluated the lipid oxidation level through the quantitative and qualitative analysis of fatty acid composition and oxysterols in lipid extraction from samples treated with plasma-activated water (PAW).

CHAPTER 1: LITERATURE REVIEW

1.1. Cold plasma

Plasma is considered as the fourth state of matter apart from solid, liquid, and gas (Misra, Schlüter, & Cullen, 2016; Figure 1-1). Plasma is the predominant state of matter in the known universe, but not on our planet because of the conditions of pressure and temperature (Tabares & Junkar, 2021). If we add energy to the gas, we will remove electrons from the atoms or molecules and in this way, we achieve a new state of matter, plasma, which contains an array of reactive species such as electrons, free radicals, ions, etc. (Chizoba Ekezie, Sun, & Cheng, 2017). The energy required to ionize the gas can come from electricity, heat, radio waves, or microwaves. Langmuir used the term 'Plasma' when he observed oscillations in ionized gas and described it as the 'region containing balanced charges of ions and electrons'(Langmuir, 1928). Plasma is an electrically 'quasi-neutrally' charged gas that is partially or fully ionized. Plasma states can be found in natural phenomena like lightning bolts and aurora or the man-made sources such as the fluorescent or neon lights, plasma televisions etc.

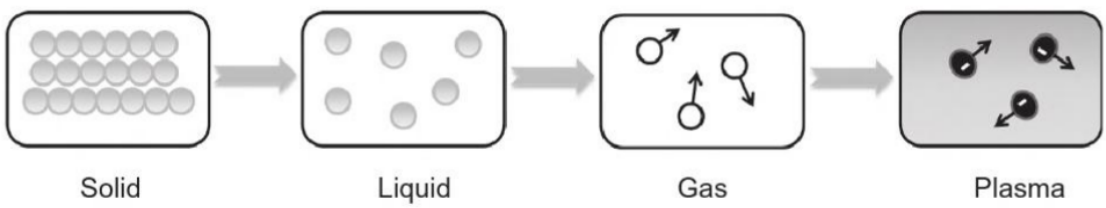

Figure 1-1: Pictorial representations of the four states of matter (Misra et al., 2016)

Plasma can be sorted on the basic kind of generation into two types, equilibrium (Thermal plasma) and non-equilibrium (Low thermal plasma). Thermal plasma in which air is heated around 20 000 K, consists of ions, electrons and gas molecules in thermodynamic equilibrium. The low-thermal plasma further categorized into quasi-equilibrium plasma (100-150°C) and non-equilibrium plasma (< 60°C) (Mandal, Singh, & Pratap Singh, 2018). The non-equilibrium plasma, where the temperature of electrons is extremely high but the temperature of ions and unionized species is around room temperature following the thermodynamic non-equilibrium between them and the gas remains near room temperature. For this reason, the non-equilibrium plasma is also called as Non-thermal plasma (NTP) or Cold plasma.

The energetic electrons ionize, dissociate and excite the atoms and molecules in the gas. This creates reactive species. These can also interact with each other or with the surrounding gas to form new reactive species (Whitehead, 2016). Depending on the gas present in the environment, different reactive oxygen and nitrogen

species are formed in the plasma. These reactive oxygen and nitrogen species are responsible for the chemical and biological effects of cold plasma.

Plasma can be generated by various sources including corona discharge, dielectric barrier discharge, glow-discharge, radio-frequency discharge, atmospheric pressure plasma jet, micro-hollow, gliding arc discharge, and plasma needle where all have been used for food processing applications. Among these, dielectric barrier discharge and atmospheric pressure plasma jet are more likely to be used in this field. Most of these devices have limited application on large-scale uses due to the direct application on food stuff. The direct application leads to the inability to treat foods of large size, exclusively irregular shape food. This problem can lead to inhomogeneous treatment on food and also miss some hidden spots that might contain microorganisms. Therefore, Plasma-Activated Water (PAW) was invented to solve these obstacles. Therefore, Plasma-Activated Water (PAW) was developed to solve these obstacles. PAW can decrease these negative effect caused by applying direct plasma air on food surfaces such as loss of color, biodegradation of bioactive compound (Misra, 2016) and is able to treat entire surface of the foods on a large scale.

1.2. Plasma-activated water (PAW)

1.2.1. PAW generation

PAW is generated by applying plasma treatment to water with the plasma plume above or underneath water surface. Water molecules interact with the plasma-generated reactive particles and create several chemical reactions, which produce a mixture of biochemical reactive species called PAW. Depending on the chemical environment, generation mode, device set up and voltage, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be formed. The species can be formed in the liquid and at the gas-liquid interface (Rahman et al., 2022; Figure 1-2). There are several NTP-water reaction systems, such as dielectric barrier discharge (DBD), pulsed corona discharge, atmospheric pressure plasma jet (APPJ) and gliding arc discharge.

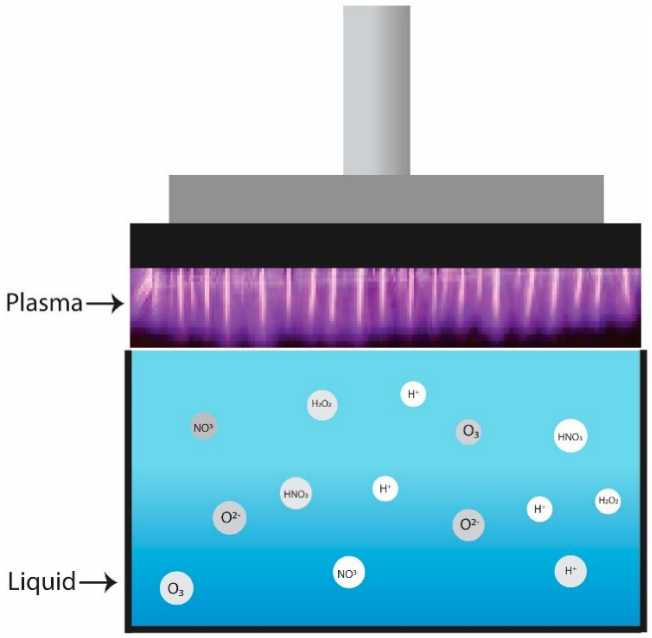

Figure 1-2: Plasma-activated water generation (Rahman et al., 2022)

The plasma source used in this thesis was pulsed corona discharge. The term "corona discharge" refers to a partly self-sustaining discharge of gas in a non-uniform electric field that is characterized by a strong inhomogeneous electric field, ionization, and luminescence surrounding the electrode. The majority of the energy was introduced into the plasma, like in other non-thermal plasmas, by the acceleration of electrons in an electric field. Additionally, background gas was dissociated, ionized, and excited by collisions with these energizing electrons into a variety of active species (including ions, excited molecules, atoms, and radicals). Corona discharge is hence advantageous for high chemical reactivity and energy efficiency, and it is frequently employed in surface treatment for gas purification. In general, the two main types of corona discharge are glow discharge and streamer discharge. It has been shown that these two discharge modes may co-exist in the same discharge process even if their energy states are very different. On the other hand, at high applied voltage and high current, both corona discharge types are quickly changed into spark discharge or arc discharge. However, the reduced applied voltage and current will limit the power injected and discharged, resulting in poor performance in particular applications. Therefore, pulsed corona discharge has been suggested as a solution to deal with this issue (D.-W. Lee, Lee, Chun, & Lee, 2003; Y.-H. Lee et al., 2003; Vandenbroucke, Morent, De Geyter, & Leys, 2011). Within dozens of nanoseconds, a pulse power source initiates and sustains a pulsed corona discharge, which produces a quick rise in high voltage. Figure 1-3 shows schematic diagram of corona discharge plasma to generate the active water

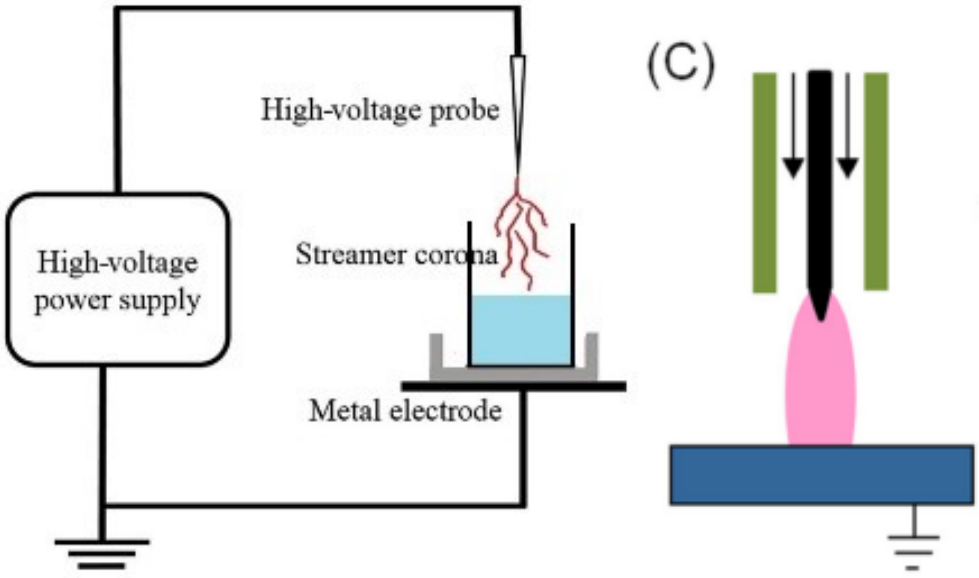

Figure 1-3: Schematic diagram of the experimental setup using corona electrical discharge plasma as a tool to generate PAW (Wu, Sun, & Chau, 2018) (left) and gas phase plasma with liquid electrode (right) (P. J. Bruggeman et al., 2016). Blue = liquid, pink = plasma, green = dielectric, black = metal electrodes

The reactive oxygen and nitrogen species (RONS) plays crucial role in the antimicrobial effects of PAW. To understand about the pathway of reactive species in water and the physicochemical properties of PAW will be

useful to investigate the oxidation mechanisms of PAW and positive effects of PAW that can further apply to food products.

1.2.1.1. Chemical properties of PAW

When PAW is generated, different reactions occur in the aqueous solution such as the gaseous species into liquid, and chemical reactions between lipid mlecules and gaseous species (P. J. Bruggeman et al., 2016). The research of Wende, von Woedtke, Weltmann, & Bekeschus (2019) shows that plasma treatment of water leads to non-equilibrium dissociation of water molecules, which results in formation of short-living species such as hydroxyl ions (OH^-), hydrated electrons. These species continues to react to create stable species, such as ozone (O_3), superoxides (O^{2-}) and hydrogen peroxide (H_2O_2). These reactions show below:

$$H_2O + e^- \rightarrow OH\cdot + H\cdot + e^- \quad (1)$$

$$H_2O + e^- \rightarrow OH\cdot + H^+ + 2e^- \quad (2)$$

$$OH\cdot + OH\cdot \rightarrow H_2O_2 \quad (3)$$

$$H\cdot + O_2 \rightarrow HO_2 \quad (4)$$

$$OH\cdot + H_2O_2 \rightarrow OOH + H_2O \quad (5)$$

$$O_2^- + H^+ \leftrightarrow OOH \quad (6)$$

$$O_2 + e^- \rightarrow O^+ + O\cdot + 2e^- \quad (7)$$

$$O_2 + O\cdot \rightarrow O_3 \quad (8)$$

Hydroxyl radicals (OH·) are short-lives species with high redox potential that can react with other moleculars in the liquids to form new radicals, leading to further reactions. For example, OH· reacts with the lipids produces lipid peroxyl radicals and lipid peroxide (Ayala, Muñoz, & Argüelles, 2014). H_2O_2 is reported as a stable species and biologically active agent that significantly contributes to antimicrobial and cytotoxic effects in plasma-activated water (Julák, Hujacová, Scholtz, Khun, & Holada, 2018). Beside reactive oxygen species (ROS), PAW also has reactive nitrogen species (RNS), such as nitric oxide NO, peroxynitrate O_2NOO^-, peroxynitrite $ONOO^-$, and nitrogen dioxide (NO_2•) radicals. The NO_2^- produced from plasma generation reacts with water to produce acids such as HNO_2 and HNO_3. This is the reason why the pH of PAW decrease and form acidic environment.

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (9)$$

$$NO_2\bullet + OH\bullet \rightarrow HNO_3 \quad (10)$$

The acidification increases the nitrite disproportionation or nitrite degradation to nitrates and H_2O_2 reacts with nitrites to form $ONOO^-$s.

$$3NO_2^- + 3H^+ \rightarrow 2NO + NO_3^- + H_3O^+ \quad (11)$$

$$2HNO_2 \rightarrow NO^{\cdot} + NO_2^{\cdot} + H_2O \quad (12)$$

$$2NO_2 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+ \quad (13)$$

$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \quad (14)$$

$$ONOOH \rightarrow NO_2^{\cdot} + OH^{\cdot} \quad (15)$$

$$ONOOH \rightarrow HNO_3 \rightarrow NO_3^- + H^+ \quad (16)$$

All of these reactions are generated in the gas, in the liquid, and at the interface between the gas and liquid, all of which contribute to the complex environment in PAW in varying degrees (Figure 1-4, Figure 1-5).

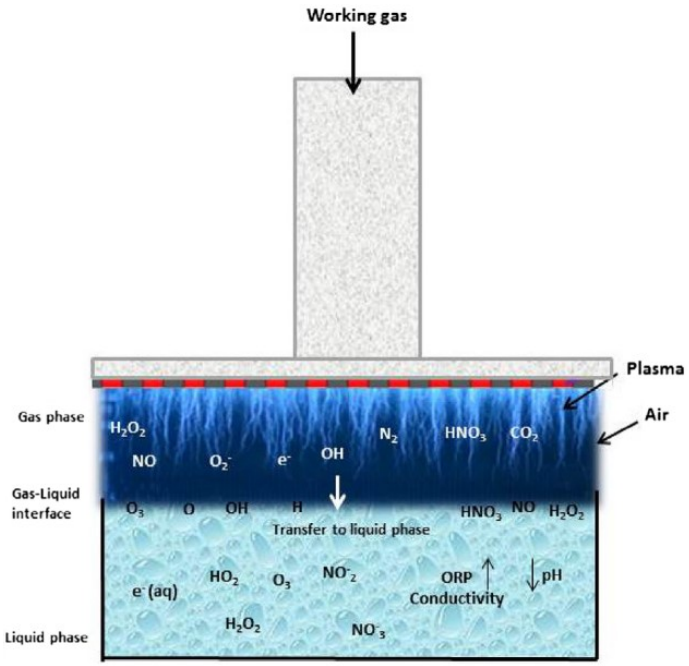

Figure 1-4: The interactions of plasma in the gaseous phase with water in the liquid phase during PAW generation(Y. M. Zhao, Patange, Sun, & Tiwari, 2020)

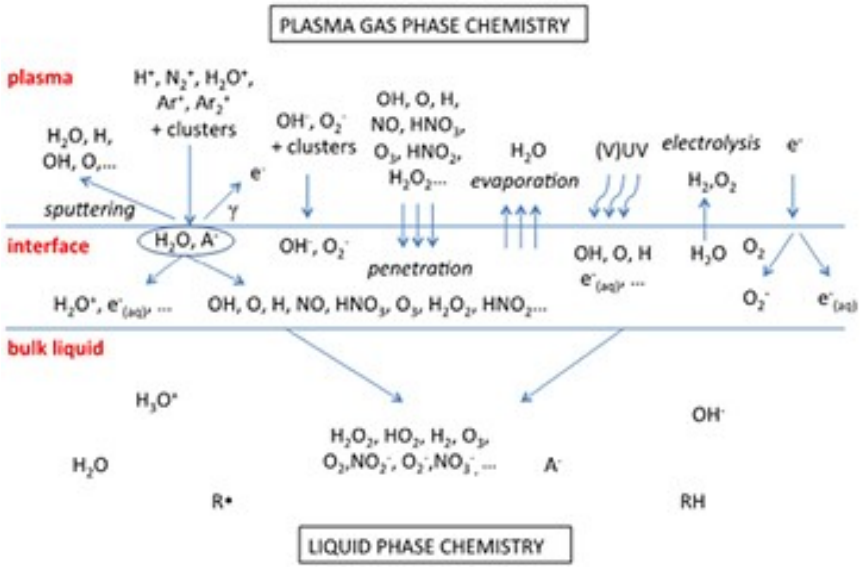

Figure 1-5:Schematic diagram of some of the most important species and mechanisms for an air plasma in contact with water (Samukawa et al., 2012)

1.2.1.2. Physical properties of PAW

As mentioned before, while generating PAW, the interaction between water and reactive species leads to the reduction in pH of the water. The absorption of NO_x from plasma generation into solution which produces

nitric acid and peroxynitrous acid. The formations of these newly chemical species in PAW are attributed to decrease in pH. This is one of the reasons for the anti-bacterial activity of PAW. The pH was reduced from 7 to 3.2 after 10 minutes of activation using atmospheric plasma jet (Ar/O_2^- working gas) in the experiment of Ma et al., (2015). Another research showed similar results in pH acidification up to 3.7 after 5 min of treatment (Zhang et al., 2016). Some authors reported continuous decrease in pH of the plasma-activated nonbuffered solution if they raised treatment time. However, after a certain time, the pH reaches a steady state and remains constant. The decrease in the pH and the generation of acidified compounds in the PAW may vary based on method and feed gas used for plasma generation and type of plasma used (P. Bruggeman & Leys, 2009). Another reason that influences the decrease of pH was found by Lukes, Locke, & Brisset (2012), dependent on the polarity of the applied voltage, where negative discharge caused a higher decrease in the pH than the positive.

Besides pH, plasma treatment also caused the electrical conductivity modification of water. The increased present of ROS and RNS in plasma-activated water impacts positively on the conductivity of PAW. The conductivity is connected to pH, as the pH drops, it increases due to the greater mobility of H^+ ion than OH^- ions (Lukes et al., 2012). Moreover, the formation of NO_2^- and NO_3^- ions also contributes to an increase in conductivity of PAW (Brisset & Pawlat, 2016).

The oxidation-reduction potential (ORP) is considered as the important factor affecting the microbial inactivation since it destroys the cell membrane and the cellular defense mechanisms of the microbes (L. B. Liao, Chen, & Xiao, 2007) and it measures the ability of a solution to oxidize or reduce another substrates. Among the ROS, H_2O_2 is the main factor to the redox reactions where it can be an oxidant or as a reductant (Lukes et al., 2012). Plasma treatment water can increase the ORP values. In Zhang et al., (2016) study, ORP values were observed a 63.3% increase of distilled water after plasma treatment of 20 min using Ar/O_2 gas plasma. Along with longer treatment time, the ORP values increase greater in Li et al., (2018) and Ma et al., (2015) studies.

1.3. Plasma-activated water application on seafood

Microbial inactivation is the principal nutritional application of Plasma-activated water. Through the synergistic effects of induced oxidation and a large pH decrease, the indirect plasma treatment of water results in the generation of numerous ROS, primarily H_2O_2 and nitrogen reactive species, such as NO_2^- and NO_3^-, as well as metal ions. These ROS have an antimicrobial impact. After plasma treatment, those substances may still be in PAW for a few days, prolonging the decontamination effect of suspended goods (Traylor et al., 2011).

Seafood needs a longer shelf life because it is shipped over extensive distances. However, the product quality quickly deteriorates as a result of microbial activity. In a study, X. Liao et al. (2018) opted to preserve shrimp in PAW ice rather than tap water. The product could now be kept fresh for an additional 4–8 days according to the results, which showed a significant decrease in microbial development. The PAW ice treatment also slowed down the loss of color and hardness. Recently, *Pseudomonas fluorescens* cultured on fresh mackerel

fillets was reduced by 0.4 log after PAW treatment (Y. M. Zhao, Ojha, Burgess, Sun, & Tiwari, 2020). Zhao et al. (2021)'s discussion of the combined impact of PAW and other technologies came later. *Salmonella typhimurium* and *Listeria monocytogenes* were significantly reduced by PAW application on grass carp, with log reductions of 1.44 and 1.21, respectively. By lowering 1.03 log CFU/g, a different experiment on Yellow River carp fillets demonstrated the efficiency of PAW against *Shewanella putrefaciens* bacteria (Liu, Zhang, Meng, Bai, & Dong, 2021). In comparison to sterile deionized water treatment, the fillets' lightness value (L*) rose, the redness value (a*) greatly decreased, but the yellowness value (b*) remained constant. The fact that there was little to no change in the sensory and textural characteristics is a plus. Therefore, PAW could be applied within seafoods industry to treat water which is used for washing the fish during the basic processing steps such as gutting, filleting or skinning, since poor quality of water used during those fish processing steps can result in cross-contamination of the final product.

Fish curing is another intriguing use of Plasma-Activated Water that results from the nitrate production in plasma-treated water. The use of nitrates during curing process is usually applied to red meat products, to improve flavour and color as well as increase microbial stability, including prevention of growth of *Clostridium botulinum* (Gassara, Kouassi, Brar, & Belkacemi, 2016), however certain fish products, such as tuna fish, sabre-fish, salmon or shad are also cured. The use of nitrate-containing PAW can minimize or even eliminate the need for curing salt during the curing process, and through the process of plasma activated brine injection the antimicrobial effect of plasma treatment could be even more pronounced, though further study is required to confirm this.

Non-thermal plasma could also be effectively used for the treatment of wastewater from fish industry, allowing for its re-use. Ammonia, chlorine, food additives, chemical disinfectants, cleansers, and aids, as well as pathogenic bacteria, including *fecal coliforms*, are common contaminants found in effluent from the seafood production (AMEC Earth & Environmental Limited, 2003). It can take the place of conventional effluent treatment techniques like ozone or chlorine water treatment since plasma-activated water produces ROS. Numerous contaminants, including ammonia, phenols, chlorophenols, and benzoic acid, that are frequently found in wastewater from the seafood industry have been demonstrated to be completely degraded in wastewater treated by plasma (Cheng, Chen, Wu, & Ho, 2007).

The plasma-activated water has high potential to be used in almost all processing steps of seafood processing, from steps which including washing till preservation and wastewater purification. PAW allows to improve the microbiological quality of the fresh seafood and has low operation costs and high efficiency. However, in some recent research has shown the lipid oxidation of seafood treated with PAW.

1.4. Modifications of lipids by plasma-activated water

1.4.1. Lipid oxidation in food by plasma-activated water

Lipids are constituted of fatty acids-saturated, monounsaturated or polyunsaturated, basis the number of double bonds between the carbons. Lipid oxidation, which affects color, flavor, safety, and nutritional value, is a

significant contributor to quality degradation. Lipid peroxidation may result from interactions between food cells and PAW carrying ROS (Yong et al., 2018). It is being researched whether PAW affects lipid oxidation. Several methods can detect the lipid oxidation in foods by measuring the primary oxidation products (which are usually non-volatile components) or the secondary oxidation products (which often comprise volatile components) as in peroxide value (PV) and 2-thio-barbituric acid reactive substances (TBARS) assays respectively (Y. M. Zhao et al., 2021). When PAW-cured sausage was stored for 21 days, according to Jung et al., (2015), the PV value increased from 1.33, 1.57, 3.68, 4.20 meq O_2/kg in 0, 7, 14, and 21 days. However, when the storage period was prolonged to 28 days, the value fell to 3.67 meq O_2/kg compared to 21 days of storage. When loin ham was kept for two weeks, Yong et al. (2018) observed a similar trend in the PV value, PV gradually increased during the first week of storage and started to decrease in the second week. When compared to the maximum allowable value of peroxide in marine lipids, which is 5 meq O_2/kg, a recent research on PAW + Ultrasound treatment on mackerel fillets revealed a PV value of 0.5 meq O_2/kg lipid (Y. M. Zhao et al., 2021).

Additionally, X. Liao et al., (2020) report showed that there was no significant change in Thiobarbituric acid (TBA) for beef during PAW thawing, which agrees with the TBARS value of PAW-cured dry pig loins (Luo et al., 2019). In a different investigation, shrimp were kept with PAW ice for seven days by X. Liao et al. (2018). The shrimps' TBA value was decreased when examined after the storage period compared to tap water ice storage. They believed that the PAW ice treatment reduced microbial growth caused a decrease in lipid oxidation. The TBARS value of dried pork loins treated with PAW was significantly reduced, according to Luo et al., 2019, but not significantly different from that of tiger nuts after exposure to either PAW-blanching treatment or PAW only treatment (Muhammad et al., 2019) or beef (Y. Zhao et al., 2020) following PAW treatment.

1.4.2. Expected oxidation mechanisms by plasma-activated water

It is now understood that plasma-activated water might promote the oxidation of lipids. It is uncertain what the processes of lipid oxidation are and how much they contribute to the oxidation. These could differ from the traditional oxidation of lipids. In the presence of catalytic systems like light, heat, and metals (like Cu, Fe), which include free radicals or other intermediate reactive species and lead to thermal-, or auto-oxidation, food lipids are susceptible to the oxidation process. The latter is the more prevalent oxidation process, characterized as the autoxidation interaction of food lipids with oxygen via free radical chain reactions that have three separate stages: initiation, propagation, and termination (Figure 1-6).

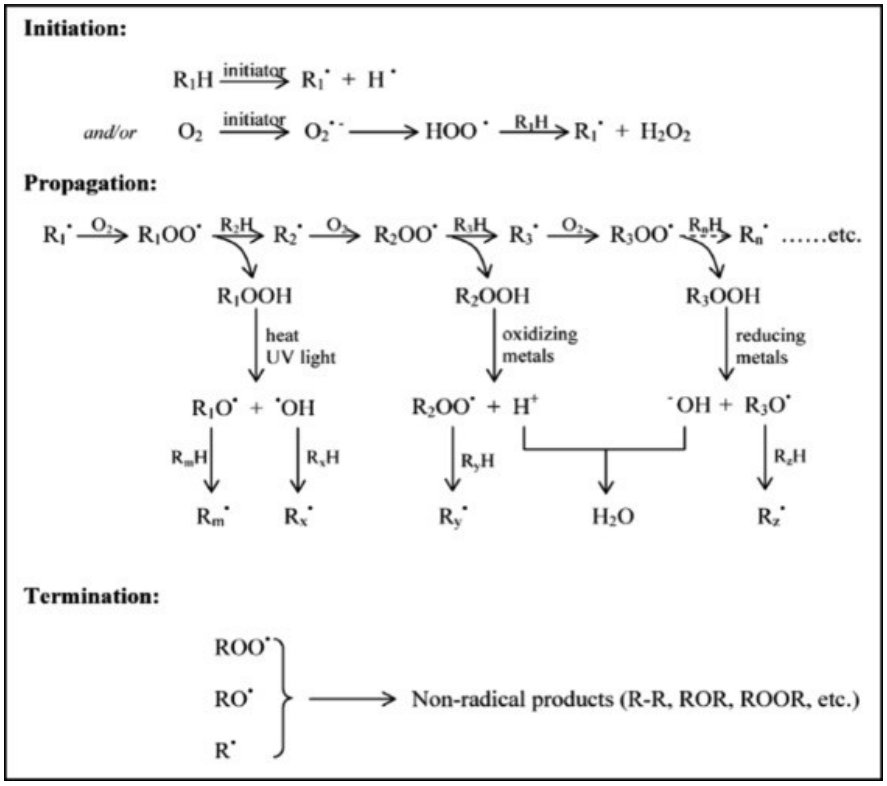

Figure 1-6: A simplified scheme for the lipid autoxidation mechanism (Shahidi & Zhong, 2010).

The reaction can be initiated in the cold plasma by radicals and UV photons (equation 1). This occurs preferentially at a double allylic or allylic carbon atom because the activation energy for removing a hydrogen atom is lower there. After the formation of fatty acid radicals, the propagation step follows. After reaction with oxygen, a peroxide radical is formed (equation 2). The peroxide radical reacts with another fatty acid, resulting in the formation of a hydroperoxide and a new fatty acid radical (Equation 3). Finally, the formed radicals react with each other to form non-radical end products in the termination step (equation 3, 4 and 5). The list of termination products is not complete

Initiation: $RH \rightarrow R\bullet + H\bullet$ (1)

Propagation: $R\bullet + O_2 \rightarrow ROO\bullet$ (2)

$ROO\bullet + RH \rightarrow ROOH + R\bullet$ (3)

Termination : $ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$ (4)

$RO\bullet + R\bullet \rightarrow ROR$ (5)

$R\bullet + R\bullet \rightarrow RR$ (6)

CHAPTER 2: MATERIALS AND METHODS

2.1. Sampling and Activated-water plasma treatments

Sardine *(Sardina pilchardus.)* and the mussel *(Mytilus Galloprovincialis.)* were selected for plasma treatment. They are both significant commercial fish species from the Mediterranean that are nutrient-rich. Sardine *(Sardina pilchardus.)* is a gregarious plankton-eating fish that lives in the open sea, at different sea depth levels and it is rich in polyunsaturated fatty acids with around 20.9% of polyunsaturated fatty acid n3 (PUFA-n3) (De Leonardis & Macciola, 2004). Mussels *(Mytilus Galloprovincialis.)* represent more than 75% of the total shellfish production. Mussels are bivalve mollusc and filter-feeding organisms which can accumulate bacteria in very high quantity are found as good source of palmitic acid with around 26% (Saghk, 1997).

Both of them were purchased from local distributor in Italy. Sardine fillets *(Sardina pilchardus.)* and mussel samples *(Mytilus Galloprovincialis)* were weighted at around 40 – 45g and weighted at 20 – 25g respectively. Therefore, they were dipped in 150mL of PAW for varying period of time to further analysis. The portion of sample/solution ratio was 1:3. All experiments were conducted in triplicate.

Pulsed corona discharge was the plasma device that has been utilized to generate plasma. A stainless steel pin electrode was linked to a high voltage microsecond pulsed generator as plasma source (Figure 2-1). Peak voltage was set to 18kV, while the pulsed repetition frequency was set at 5 kHz. The plasma plume was generated by air and above water surface. There was 500ml of water containing in glass jar and treated with plasma air in 1 minute. The type of water used is the sterile distilled (SD) which is water free from organic materials but still contain inorganic chemicals and has pH value around 3,9 (Table 2-2). Dipping times of 10, 20, and 30 minutes for sardines and 5, 10, and 15 minutes for mussels were tested. Moreover, samples are also dipped in sterile distilled (SD) water without Plasma treatment with 10, 20, and 30 minutes for sardines and 5, 10, and 15 minutes for mussels in order to compare the effect of PAW with only SD water.

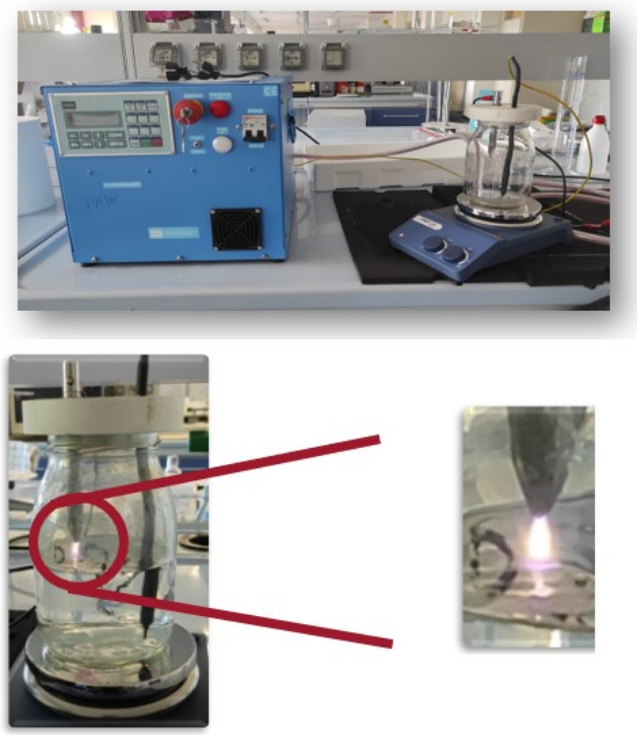

Figure 2-1: Plasma-activated water set-up with corona pulsed discharge

Table 2-1: Plasma device parameters

	Sardine samples	**Mussel samples**
Sterile distilled (SD) water volume (mL)	500	500
Peak voltage (kV)	12	12
Pulse repetition frequency (kHz)	5	5
Treatment time (min)	1	1

Table 2-2: Plasma-Activated water parameters

	pH	Hydrogen peroxide (mg/l)	Nitrates (mg/l)
Sterile distilled (SD) water volume (mL)	3.9	3 – 10	40 – 80

2.2. Total lipid extraction and total fatty acid analyses

After dipping in various PAW solutions for various lengths of time, the solutions were draining and the samples were firstly drying with paper tissue and further undergoing total lipid extraction according to Bligh, E.G. and Dyer. A sufficient volume of a 1:1 v/v chloroform/methanol combination was used to homogenize the samples, forming a monophasic system with the water present (chloroform/methanol/water ratio: 1:1:0,4 *v/v/v*). The solvent mixture was divided into two layers after being diluted with aqueous potassium chlorine 0.88% (chloroform/methanol/water 1:1:0.9 v/v/v), with the upper (chloroform layer) layer retaining the lipids. In a rotary evaporator, the solvent was extracted, and lipids were then kept at − 20 °C until analysis.

According to Tavoletti et al. (2021), fatty acids methyl esters (FAMEs) were prepared from lipid extracts by acid-catalyzed transesterification, and analyzed in a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and a TG-Polar capillary column 60 m × 0.25 mm i.d., 0.20 µm film thickness (Thermo Fisher Scientific, Waltham, MA, USA) in the operative conditions described in Ruschioni et al., (2020). A standard mixture of 37 FAMEs (Supelco-Bellefonte, Pennsylvania, PA, USA) was used for the identification of chromatographic peaks. The total fatty acid (FA) compositions (weight % of total FA) were calculated by the peak area normalization method.

2.3. Peroxide Value Analyses

The peroxide number is a measure of the primary oxidation products. Peroxide value (PV) was determined according to AOCS Official Method Cd 8b-90 and expressed as mEq O2/kg oil. In a 250 mL Erlenmeyer flask with a glass stopper, weigh 0.1 g of the sample, then add 50 mL of the 3:2 acetic acid-isooctane solution. Add 30mL of distilled water after adding 0,5mL of saturated KI solution and thoroughly shaking the mixture for 1 minute. Titrate with 0.1N sodium thiosulfate, gradually adding it until the yellow iodine color is nearly gone. 0.5mL of 10% Sodium Lauryl Sulfate (SDS) and 0.5mL of the starch indicator solution should be added. To completely liberate the iodine from the solvent layer, continue the titration with steady agitation, especially close to the end point. Drop by drop, add the thiosulfate solution until the blue color is gone.

The peroxide value is determined by measuring the amount of iodine which is formed by the reaction of peroxides (formed in fat or oil) with iodide ion.

$$2I^- + H_2O + HOOH \rightarrow HOH + 2\ OH^- + I_2$$

The base produced in this reaction is taken up by the excess of acetic acid present. The iodine liberated is titrated with sodium thiosulphate.

$$2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2\ I^-$$

2.4. Analysis of Cholesterol and Cholesterol oxidized products (COPs)

The cold overnight saponification was performed according Larkeson, Dutta, & Hansson to collect the non-volatile polar fraction of the total unsaponifiable matter. Around 200 – 250mg of the lipids were mixed well with 1mL of 5α-cholestane (1000 ppm in hexane) and 25µL of 19-hydroxycholesterol (500 ppm hexane/isopropanol 3:2 *v/v*) as internal standards for total sterols and COPs quantification, respectively. 5 mL of 2M potassium hydroxide (KOH) in ethanol was added in solution in a glass tube and left overnight (about 18h) in the dark at room temperature (25°C). After storing in 18 hours, addition of 10mL water and tube was shaken vigorously. Therefore, the water phase was removed and the organic phase was washed three times with 0,5M KOH in water, and then repeated washed with 10mL water until the solution became clear. The

solvent was dried under nitrogen, and the unsaponifiable was dissolved in 1mL hexane/diethyl ether (75:25, *v/v*), and then were injected to the column.

The COP fraction was enriched further by solid phase extraction (SPE). Silica cartridges (500mg, 6mL; Supelco-Bellefonte, PA, USA) were preconditioned with 10mL of hexane. The unsaponifiable was eluted via the column dropwise after the tube had been cleaned with an additional 2 mL of hexane/diethyl ether (75:25, *v/v*) mixture. The column was then eluted using 3 mL of a hexane/diethyl ether (60:40 *v/v*) solution, and the eluates were then discarded. 4 mL of acetone was used to elute the residual cholesterol and the COPs. A ready-to-use silanazing mixture (pyridine/chlorotrimethylsilane/ hexamethyldisilazane 10:1:2 *v/v/v*; Supelco-Bellefonte, PA, USA) was used to derivatize total unsaponifiable matter and the COPs fraction for gas chromatography (GC) analysis after the acetone was dried under nitrogen. For the gas chromatography -mass spectrometry (GC-MS) analysis of sterols and their oxidation products, a Thermo Fisher Scientific Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) in conjunction with an ISQ 7000 single quadrupole mass spectrometer and outfitted with a Phenomenex (Torrance, CA, USA) Zebron ZB-5 capillary column were used. A Dual Detector Microfluidics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to split 1:1 the injected sample between the MS and the GC-FID. By operating in constant pressure mode, the kit enables maintaining a constant split ratio (Helium 110 kPa). The transfer line, FID, and injector (in splitless mode) were all set to 300 °C, whereas the ion source was set to 320 °C. The oven temperature increased from 90 to 290 °C at the rate of 30 °C/min, then increased to 300 °C at the rate of 1 °C/min and was kept at this value for 15 min. According to the internal standard approach, electronic impact (EI) fragmentation was gathered in the range of 50-650 a.m.u. for peak identification, and the FID signal was used to quantify the unsaponifiable matter components.

2.5. Data Analysis

The experimental variables were compared between the treatments (RAW (raw samples); PAW of SD, (i.e., plasma-activated sterile distilled water samples), and (C of SD, (i.e., sample treated with SD water without plasma treatment)) using the Tukey-honest Kramer's significant difference (HSD) test. JMP® Version 10 (SAS Institute Inc., Cary, NC, USA) software was used for all statistical analyses.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Total fatty acids composition

3.1.1. Total fatty acid composition in Sardine

Sardine *(Sardina pilchardus.)* lipids have important nutritional characteristics because of their high level of ω3 fatty acids. A wide variety of fatty acids were detected among total lipids of sardine (Table 3-1)

Table 3-1: Total fatty acids composition of Sardine in varying treatments

Total FA (*w/w* %)	RAW[1]	C10	C20	C30	P10	P20	P30
C10:0	0.02 ± 0.01	0.04 ± 0.03	0.04 ± 0.01	0.05 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.08 ± 0.05
C12:0	0.31 ± 0.03	0.06 ± 0.03	0.06 ± 0.04	0.03 ± 0.00	0.06 ± 0.02	0.04 ± 0.02	0.05 ± 0.02
C13:0	0.02 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.02
C14:0	5.45 ± 0.53	1.87 ± 0.53	3.01 ± 1.86	2.50 ± 0.42	2.88 ± 0.41	2.52 ± 0.23	2.94 ± 0.32
iso-C15:0	0.01 ± 0.01	0.02 ± 0.04	0.04 ± 0.06	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.05	0.01 ± 0.00
anteiso-C15:0	0.01 ± 0.01	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.02
C14:1 Δ9	0.06 ± 0.01	0.05 ± 0.01	0.09 ± 0.06	0.06 ± 0.01	0.09 ± 0.04	0.08 ± 0.00	0.10 ± 0.05
C15:0	0.41 ± 0.25	0.60 ± 0.08	0.73 ± 0.21	0.58 ± 0.07	0.72 ± 0.11	0.67 ± 0.06	0.80 ± 0.03
C15:1 Δ 10	0.02 ±0.01	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.01	0.55 ± 0.95
C16:0	12.09 ± 0.50	24.00 ± 0.46	22.62 ± 0.78	23.09 ± 1.59	24.53 ± 2.36	23.46 ± 1.16	23.48 ± 0.83
iso-C17:0	0.16 ± 0.12	0.34 ± 0.07	0.45 ± 0.17	0.31 ± 0.04	0.38 ± 0.04	0.36 ± 0.05	0.42 ± 0.01
anteiso-C17:0	0.09 ± 0.01	0.32 ± 0.45	0.79 ± 0.78	0.14 ± 0.12	0.10 ± 0.01	0.44 ± 0.65	0.13 ± 0.06
C16:1	9.70 ± 0.30	1.31 ± 0.25	2.24 ± 1.38	3.00 ± 1.65	1.69 ± 0.26	1.61 ± 0.26	2.10 ± 0.64
C17:0	0.34 ± 0.15	0.79 ± 0.04	0.79 ± 0.09	0.69 ± 0.08	0.71 ± 0.18	0.62 ± 0.04	0.85 ± 0.04
C17:1Δ10	1.23 ± 0.25	0.65 ± 0.42	1.52 ± 1.38	0.85 ± 0.03	0.90 ± 0.00	1.06 ± 0.99	1.03 ± 0.58
C18:0	2.27 ± 0.39	4.06 ± 0.17	3.79 ± 0.56	4.19 ± 0.06	3.66 ± 0.39	3.80 ± 0.36	4.26 ± 0.28
C18:1 Δ9c	3.43 ± 0.35	6.30 ± 0.34	5.60 ± 0.74	7.02 ± 0.24	8.35 ± 0.58	6.94 ± 1.82	7.13 ± 0.89
C18:1Δ11	4.02 ± 0.7	1.78 ± 0.09	1.81 ± 0.22	2.13 ± 0.34	1.77 ± 0.07	1.63 ± 0.06	1.78 ± 0.05
C18:2 n-6	1.20 ± 0.25	1.16 ± 0.09	1.32 ± 0.20	1.22 ± 0.19	1.62 ± 0.62	1.22 ± 0.10	1.50 ± 0.08
gamma-18:3	0.40 ± 0.26	0.04 ± 0.01	0.04 ± 0.03	0.06 ± 0.04	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.03
C20:0	0.34 ± 0.05	0.14 ± 0.05	0.36 ± 0.19	0.38 ± 0.26	0.17 ± 0.01	0.15 ± 0.06	0.34 ± 0.20
alpha-18:3	0.48 ± 0.35	0.58 ± 0.11	0.66 ± 0.47	0.61 ± 0.09	0.78 ± 0.15	0.65 ± 0.10	0.70 ± 0.21
C20:1 Δ 11	0.54 ± 0.42	0.29 ± 0.03	0.53 ± 0.42	0.41 ± 0.15	0.30 ± 0.06	0.28 ± 0.02	0.33 ± 0.07
C21:0	0.03 ± 0.01	0.01 ± 0.02	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.03	0.02 ± 0.03
C20:2 Δ 11,14	0.35 ± 0.02	0.17 ± 0.02	0.21 ± 0.06	0.19 ± 0.00	0.16 ± 0.03	0.16 ± 0.03	0.22 ± 0.04
C20:3 n-6	0.47 ± 0.18	0.11 ± 0.01	0.12 ± 0.04	0.13 ± 0.02	0.10 ± 0.01	0.14 ± 0.06	0.10 ± 0.02
C22:0	1.39 ± 0.38	1.53 ± 0.18	1.37 ± 0.37	1.34 ± 0.04	1.37 ± 0.17	1.35 ± 0.14	1.33 ± 0.09
C20:3 n-3 + C20:4 n-6	0.18 ± 0.02	0.07 ± 0.02	0.12 ± 0.07	0.06 ± 0.02	0.04 ± 0.03	0.05 ± 0.03	0.04 ± 0.03

C22:1Δ13	0.14 ± 0.04	0.03 ± 0.02	0.34 ± 0.53	0.05 ± 0.02	0.05 ± 0.03	0.06 ± 0.01	0.07 ± 0.01
C22:1Δ11	1.13 ± 0.08	0.37 ± 0.02	0.48 ± 0.14	0.48 ± 0.07	0.44 ± 0.07	0.42 ± 0.04	0.47 ± 0.04
C20:5 n-3	18.64 ± 0.15	6.58 ± 0.12	7.99 ± 2.10	7.37 ± 0.55	7.75 ± 0.63	7.22 ± 0.66	7.44 ± 0.15
C23:0	0.00 ± 0.01	0.05 ± 0.08	0.66 ± 1.14	0.01 ± 0.01	0.01 ± 0.01	0.29 ± 0.46	0.02 ± 0.01
C22:2Δ13,16	0.03 ± 0.01	0.01 ± 0.01	0.54 ± 0.92	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.02
C24:0	1.74 ± 0.74	0.51 ± 0.06	0.58 ± 0.32	0.33 ± 0.07	0.43 ± 0.21	0.35 ± 0.23	0.45 ± 0.17
C24:1Δ15	1.92 ± 0.05	1.53 ± 0.43	1.37 ± 0.50	1.56 ± 0.73	1.76 ± 0.12	1.86 ± 0.18	1.90 ± 0.03
C22:5 n -3	2.38 ± 0.50	0.75 ± 0.08	0.95 ± 0.15	0.81 ± 0.13	0.71 ± 0.07	0.79 ± 0.10	1.04 ± 0.48
C22:6 n-3	28.98 ± 0.33	43.85 ± 0.79	38.72 ± 3.77	40.10 ± 1.73	38.23 ± 2.78	41.51 ± 4.51	38.17 ± 2.18

[1]*Column heads are: RAW: sample without treatment; C10,C20,C30: sample treated using SD water without plasma treatment in 10 minutes, 20 minutes and 30 minutes respectively and P10,P20,P30 samples treated with PAW (SD water type) in 10 minutes, 20 minutes and 30 minutes respectively*

There are 37 fatty acids that have been identified and quantified in Sardine sample. However, the total amount of fatty acids between various treatment were different. The C22:6 n-3 was the highest compound with nearly 40% in control and PAW samples and the anteiso-C15:0 was the lowest with only 0.01-0.02% and even non-detective in C10, C20 and P20 samples. Among them, the C16:0, C16:1, C20:5 n-2 and C22:6 n-3 have higher amount than other fatty acids, accounted for nearly 70% of total fatty acids. Therefore, 9 FAs that were the most quantitatively representative were selected, and their trend was carefully examined (Figure 3-1).

C16:0 has found at around 12.09% in RAW sample and approximately 22 – 24% in various samples treated with SD water and with PAW. The highest amount of C16:0 was the P10 with around 24.53% but there was no statistically significant difference in control and PAW samples. There results were higher (compared to C16:0 amount in raw sample) in Bandarra et al. (1997)'s study which has reported around 15 – 17% in Palmitic acid (16:0) in Sardine fish (*Sardina pilchardus.*) from Portugal. Meanwhile, in Zlatanos and Laskaridis (2007) study, the proportion of Palmitic acid was around 20 -24% in Sardine fish (*Sardina pilchardus.*) from Greek markets depends on the seasons which was compatible with concentration found from the control and PAW samples. Both of two studies also used the method of Bligh, E.G. and Dyer (1959) to extract total lipid.

9.7% of C16:1 was found in raw sample compared to only around 1-2% in samples of control and PAW treatment. In C17:0 compound, raw sample was accounted for only 0.34% while Control and PAW treated samples were higher, at around 0.6-0.8% with no statistically significant differences between them. Oleic acid (C18:1 Δ9c) were at the lowest level in RAW sample with 3.43%, follow by C20 sample with 5.60% and highest concentration in P10 sample with 8.35%. The total content of Oleic acid is consistent with data found by Zlatanos and Laskaridis (2007) in sardine (*Sardina pilchardus.*) coming from Greek markets. Vaccenic acid (C18:1Δ11) was found at highest amount in raw sample with 4.02% and 2.13% in C30 was the second highest. The lowest amount of Vaccenic acid was P20 samples, at 1.63%. The C18:2 n-6 was accounted for around 1.2% - 1.5% of total fatty acids with no significantly statistic difference between various samples. The alpha-18:3 was reported around 0.5 – 0.7% in different samples. The EPA (C20:5 n-3) concentration was highest in raw sample, accounted for 18.64% and was much lower in control and PAW samples with nearly 7-8%. These results are in agreement with the literature, where C20:5 n-2 was reported at around 17 -18% in study on the

Mediterranean sardine (Bandarra et al., 1997). Lower values of EPA in Greek sardine at around 10% were reported by Zlatanos and Laskaridis (2007). The raw sample has the lowest concentration of DHA (C22:6 n-3) at 28.98%. The amounts of DHA in the control and PAW samples were reportedly between 38% and 43%. These results are also in agreement with the literature of Zlatanos & Laskaridis (2007).

In conclusion, the compounds C16:1, C18:1Δ11, C20:5 n-3 were observed at higher concentration in raw sample than control and PAW samples while other compounds: C16:0, C17:0, C22:6 n-3, C18:1Δ9c were higher in control and PAW samples than in raw sample with significantly statistic difference.

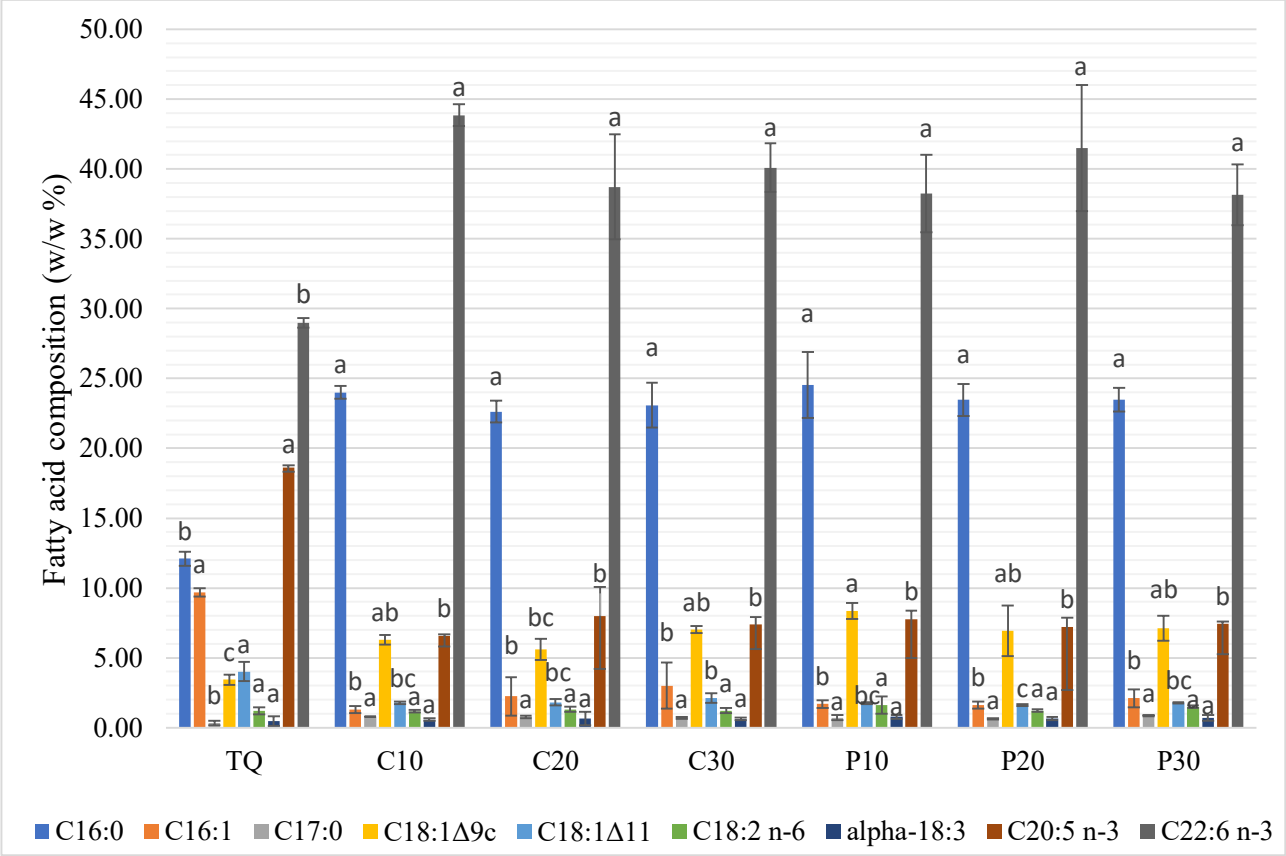

Figure 3-1: FAME composition (%) in Sardine in varying treatments

3.1.2. Total fatty acid composition in Mussels:

Table 3-2 showed 27 fatty acids have been determined and measured. The quantity of fatty acids, however, varied depending on the treatment. C20:3 n-3 + C20:4 n-6 was the lowest compound with around 0.05-0.11% of the total fatty acids, while the SFA was the largest with about 40% of them.

In Palmitic acid compound, the C10 sample had the highest content (25.09%), followed by the C5 sample (23.40%), and the lowest level was P10 sample with 20%. We noticed that samples treated with PAW had less palmitic acid than those with control and raw samples. The total content of C16:0 is agreed with data found by E. Orban et al. (2002) in mussels cultivated from Rimini (Italy), Martínez-Pita, Sánchez-Lazo, Ruíz-Jarabo,

Herrera, & Mancera (2012) in mussels came from Spain. The concentration was lower in the literature of Azpeitia, Ferrer, Revilla, Pagaldai, & Mendiola (2016) and Freites, Fernaández-Reiriz, & Labarta, (2002).

The compound C16:1Δ9 was reported around 10% of total fatty acids, with the highest quantity found in the C5 sample at 12.78% and the lowest amount found in the P15 sample at 10.59%. The same pattern was seen in C16:1Δ9, where PAW samples contained less of it than control and raw samples.

In C17:0 compound, around 0.65 – 1.02% were found in various treatment samples with no statistically significant difference. The publication of E. Orban et al. (2002) also reported the same concentration with C17:0 compound which was around 0.50 -1.00% in mussels harvested from Rimini (Italy).

The lowest level of compound C18:19, 1.76%, was found in the P5 sample and the highest level, 2.48%, in the C10 sample. The results in C18:1Δ9 were in agreement with literatures: Azpeitia et al. (2016) and Martínez-Pita et al. (2012) with mussels from Spain.

γ-18:3 compound was found at the highest amount of 0.13% in C5 sample and lowest in P10 sample with 0.05%. In general, the PAW samples had lower percentage of γ-18:3 compound at 0.27% in total compared to 0.30% in total of control samples.

Table 3-2: Total fatty acids composition of Mussel in varying treatments

Total FA (*w/w* %)	RAW[2]	C5	C10	C15	P5	P10	P15
C14:0	3.27 ± 0.44	4.11 ± 0.60	3.52 ± 0.47	3.06 ± 0.36	3.29 ± 0.24	2.98 ± 0.44	3.26 ± 0.32
C15:0	0.75 ± 0.07	0.82 ± 0.02	0.74 ± 0.13	0.70 ± 0.02	0.66 ± 0.02	0.67 ± 0.15	0.70 ± 0.05
C16:0	23.51 ± 1.26	23.40 ± 0.93	25.09 ± 1.10	22.39 ± 0.78	20.70 ± 0.41	20.00 ± 1.51	21.97 ±1.30
iso-C17:0	0.17 ± 0.01	0.19 ± 0.01	0.19 ± 0.02	0.16 ± 0.01	0.15 ± 0.00	0.14 ± 0.02	0.13 ± 0.03
anteiso-C17:0	0.44 ± 0.13	0.54 ± 0.10	0.55 ± 0.09	0.50 ± 0.04	0.52 ± 0.06	0.36 ± 0.13	0.54 ± 0.07
C16:1Δ9	11.05 ± 1.08	12.78 ± 0.99	11.96 ± 2.02	10.85 ± 0.87	10.83 ± 1.25	10.60 ± 2.40	10.59 ± 1.05
C17:0	0.95 ± 0.07	0.65 ± 0.43	1.02 ± 0.19	0.88 ± 0.07	0.87 ± 0.09	0.87 ± 0.12	0.97 ± 0.10
C17:1Δ10	0.10 ± 0.07	0.13 ± 0.04	0.16 ± 0.09	0.09 ± 0.07	0.17 ± 0.11	0.06 ± 0.05	0.05 ± 0.02
C18:0	5.53 ± 1.05	6.25 ± 1.03	7.53 ± 1.43	5.83 ± 0.60	5.66 ± 0.28	6.14 ± 0.85	5.98 ± 0.34
C18:1Δ9	2.14 ± 0.14	1.99 ± 0.13	2.48 ± 0.73	2.23 ± 0.39	1.76 ± 0.39	2.18 ± 0.31	2.05 ± 0.52
C18:1Δ11	3.69 ± 0.27	3.55 ± 0.09	3.08 ± 0.41	3.51 ± 0.17	3.10 ± 0.32	3.40 ± 0.42	3.12 ± 0.22
C18:2 n-6	1.60 ± 0.10	1.67 ± 0.06	1.32 ± 0.28	1.50 ± 0.30	1.42 ± 0.07	1.31 ± 0.21	1.44 ± 0.27
γ-18:3	0.11 ± 0.03	0.13 ± 0.01	0.07 ± 0.01	0.10 ± 0.05	0.11 ± 0.01	0.05 ± 0.03	0.11 ± 0.04
α-18:3	1.40 ± 0.10	1.48 ± 0.27	1.12 ± 0.21	1.36 ± 0.37	1.22 ± 0.13	0.98 ± 0.21	1.50 ± 0.36
C20:1Δ11	3.36 ± 0.76	3.88 ± 0.16	3.17 ± 0.45	3.79 ± 0.34	3.31 ± 0.14	2.72 ± 0.92	3.30 ± 0.35
C21:0	0.16 ± 0.06	0.19 ± 0.02	0.17 ± 0.06	0.17 ± 0.02	0.12 ± 0.03	0.08 ± 0.06	0.14 ± 0.01
C20:2Δ11,14	0.82 ± 0.29	1.03 ± 0.07	0.75 ± 0.25	0.41 ± 0.26	0.97 ± 0.09	0.67 ± 0.18	0.74 ± 0.02
C20:3 n-6	0.26 ± 0.02	0.25 ± 0.06	0.31 ± 0.16	0.27 ± 0.04	0.21 ± 0.05	0.18 ± 0.08	0.22 ± 0.06
C22:0	4.46 ± 0.62	4.34 ± 0.14	3.16 ± 1.04	3.92 ± 0.54	3.80 ± 0.03	4.03 ± 0.77	3.70 ± 0.54
C20:3 n-3 + C20:4 n-6	0.07 ± 0.04	0.07 ± 0.07	0.00 ± 0.01	0.11 ± 0.04	0.05 ± 0.06	0.00 ± 0.01	0.06 ± 0.01
C22:1Δ13	0.05 ± 0.04	0.11 ± 0.03	0.14 ± 0.03	0.04 ± 0.05	0.11 ± 0.03	0.07 ± 0.05	0.10 ± 0.02
C22:1Δ11	0.35 ± 0.02	0.35 ± 0.06	0.29 ± 0.10	0.36 ± 0.05	0.30 ± 0.01	0.30 ± 0.10	0.32 ± 0.04
C20:5 n-3	15.26 ± 0.87	14.39 ± 0.37	14.67 ± 0.39	15.91 ± 0.80	18.27 ± 1.29	20.32 ± 1.02	17.79 ± 0.39
C22:2Δ13,16	0.06 ± 0.07	0.02 ± 0.01	0.13 ± 0.14	0.12 ± 0.11	0.04 ± 0.05	0.01 ± 0.01	0.04 ± 0.02

C24:0	1.57 ± 0.12	1.58 ± 0.12	1.19 ± 0.12	1.51 ± 0.14	1.47 ± 0.17	1.55 ± 0.06	1.42 ± 0.14
C24:1Δ15	0.71 ± 0.18	0.53 ± 0.04	0.75 ± 0.30	0.78 ± 0.41	0.72 ± 0.43	0.64 ± 0.05	0.51 ± 0.02
C22:5 n-3	1.59 ± 0.16	1.67 ± 0.21	1.98 ± 0.96	1.61 ± 0.29	2.23 ± 0.91	1.70 ± 0.21	1.53 ± 0.21
C22:6 n-3	16.60 ± 1.34	13.91 ± 2.01	14.48 ± 1.35	17.83 ± 2.24	17.94 ± 1.65	18.00 ± 1.03	17.73 ± 0.96
SFA	40.79 ± 1.10	42.06 ± 1.49	43.16 ± 2.12	39.11 ± 0.72	37.2 ± 40.90	36.82 ± 2.92	38.81 ± 0.72
MUFA	21.45 ± 1.43	23.31 ± 1.10	22.02 ± 2.50	21.66 ± 0.82	20.30 ± 1.72	19.97 ± 2.95	20.04 ± 1.31
PUFA	37.77 ± 1.72	34.63 ± 2.10	34.82 ± 0.41	39.23 ± 1.02	42.46 ± 2.40	43.22 ± 1.49	41.15 ± 1.62
n-3 PUFA	34.85 ± 1.98	31.45 ± 2.01	32.24 ± 0.65	36.72 ± 1.70	39.66 ± 2.53	41.00 ± 1.63	38.55 ± 1.56

[2]*Column heads are: RAW: sample without treatment; C5, C10, C15: sample treated using SD water without plasma treatment in 5 minutes, 10 minutes and 15 minutes respectively and P5, P10, P15 samples treated with PAW (SD water type) in 5 minutes, 10 minutes and 15 minutes respectively*

Mussels were very rich in Saturated fatty acid (SFA), Monounsaturated fatty acid (MUFA) and Polyunsaturated fatty acids (PUFA). C10 of SFA compound had the highest level at 43.16% and lowest concentration at 36.82% was found in P10 sample. The PAW samples were observed lower percentages comparing to control and raw samples. MUFA was accounted for around 19.97% to 23.31% in different samples. For PUFA compound, the highest concentration was P10 sample with 43.22% and lowest level at 34.63% in C5 sample. The concentration of samples were in agreement with studies of Stratev et al. (2017) with Mussels coming from Bulgaria.

In figure 3-2, The Fatty acids methyl esters (FAME) group was the most abundant fatty acids, accounted for nearly 70% of total fatty acids in mussels. EPA (C20:5 n-3) compound was observed at the lowest level around 14.39% in C5 sample and highest percentage in 20.32% in P10 sample. The result was consistent with the reported of Martínez-Pita et al. (2012) with Mussel coming from Spain. As we observed, the EPA compound was higher in PAW treated samples than control and raw samples. DPA (C22:5 n-3) amount was found the highest amount at 2.23% in P5 sample and lowest level was at 1.53% in P15 sample but there is no significant statistic different between samples. DHA (C22:6 n-3) percentage was recorded at 13.91% in C5 which was the lowest concentration and at 18% in P10 which was the highest amount. P10 sample had the highest proportion in n-3 PUFA with 41% and C5 had the lowest proportion with 31.45%. The literature of Azpeitia et al. (2016) observed a higher proportion of n-3 PUFA while Stratev et al. (2017) with Mussel from Bulgaria and Martínez-Pita et al. (2012) with Mussel from Spain had similarity with our results. The percentage of n-3 PUFA was lower in raw and control samples than PAW treated samples.

In conclusion, the fatty acid compounds: C16:0, C16:1Δ9, C18:1Δ9 and γ-18:3 were found at higher concentration in raw and control samples than in PAW treated samples. In contrast, SFA, PUFA, n-3 PUFA, C20:5 n-3 and C22:6 n-3 decreased in raw and control samples comparing to PAW treated samples.

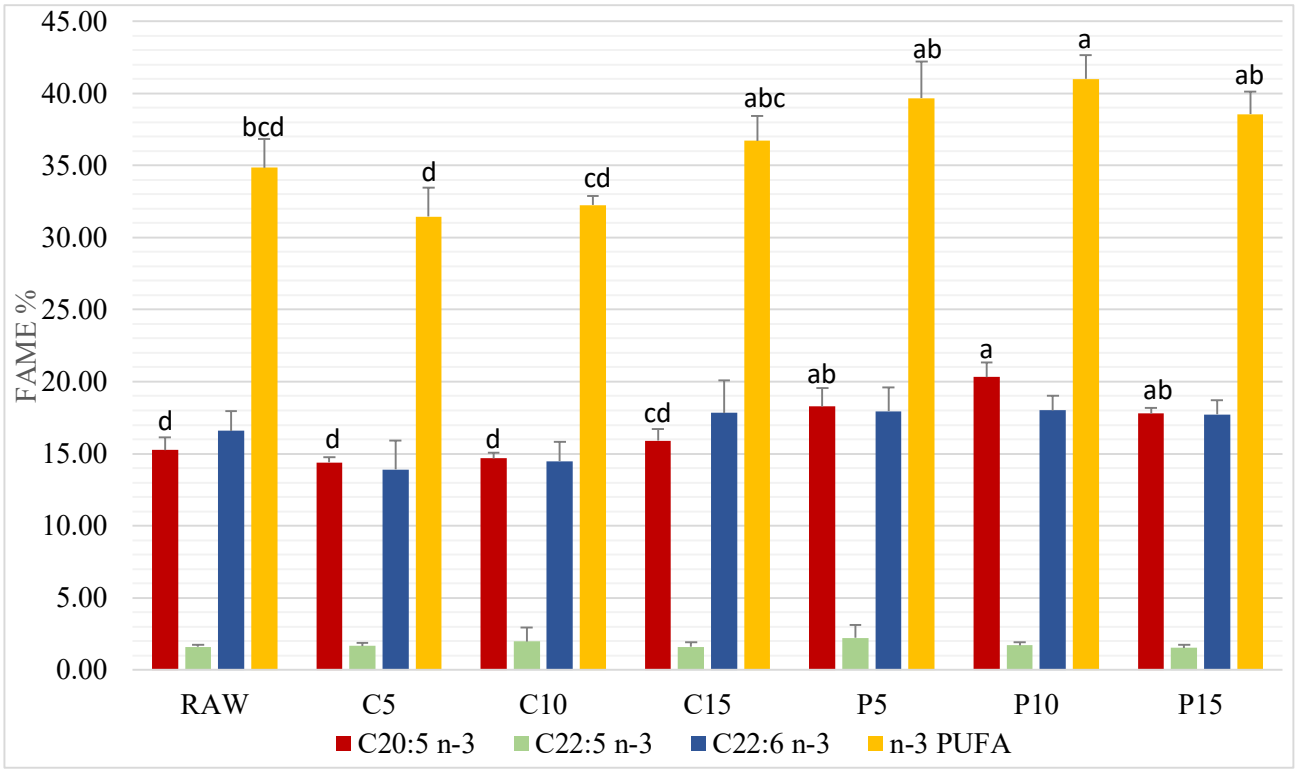

Figure 3-2: FAME composition (%) in Mussel in varying treatments

When comparing the fatty acids between Mussels and Sardine, C22:6 n-3 was observed the same trend both in Sardine and Mussel where the PAW treated samples had higher concentration than raw and control samples. Meanwhile, C16:0 was lower in raw sample than other samples in Sardine but was higher than other samples in Mussels. In C18:1Δ9, Sardine samples had lower level in raw samples than other samples while Mussels had a higher percentage in raw samples than PAW treated ones. C20:5 n-3 also had the same trend with lower concentrations in PAW treated samples comparing to raw ones in Sardine while in mussels, it had higher proportion in PAW treated samples than in raw and control sample.

3.2. Peroxide value

3.2.1. Peroxide value of Sardine

Because of the interaction of ROS with food lipids, lipid oxidation is one of the most significant might effects that limits the use of PAW in food commodities. Peroxide value analysis is shown in (Figure 3-3).

Surprisingly, the RAW sample had the highest value with 44.31 mEq O2/kg oil and was 4 times higher than the lowest value at 10.10 mEq O2/kg oil of P30. C30 had the second highest of peroxide value with 24.53 mEq O2/kg oil. In general, PAW treated samples had lowered PV values than raw and control samples. The results are in agreement with the study of Chaijan et al. (2021) which applying PAW in Asian sea bass (*Lates calcarifer*.) from Thailand. According to them, this may be related to the surface proteins in PAW-treated fish fillets coagulating under the influence of ROS. As a result, fish's ability to transfer oxygen inside was limited (Chaijan et al., 2021). Meanwhile, Albertos et al., (2017) observed the primary oxidation (PV) development for DBD treatment on fresh mackerel *(Scomber scombrus.*) fillets.

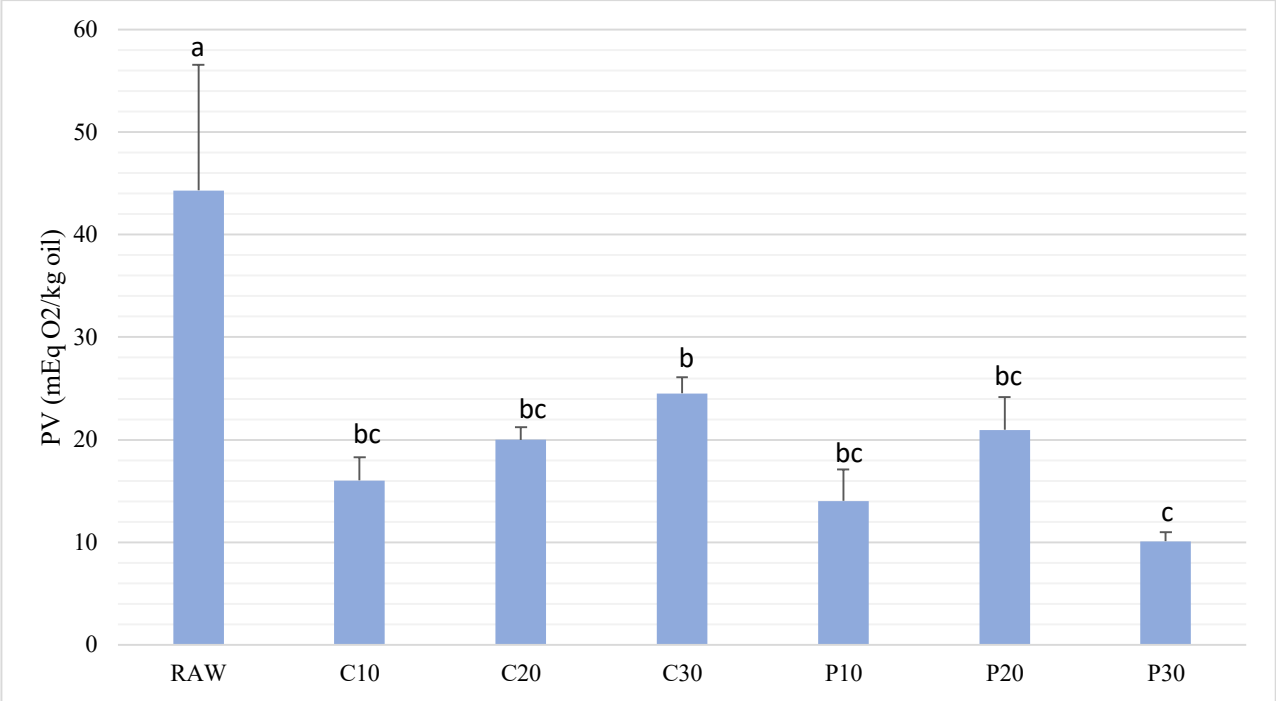

Figure 3-3: Peroxide value (mEq O2/kg oil) of Sardine in varying treatments

3.2.2. Peroxide value of Mussel

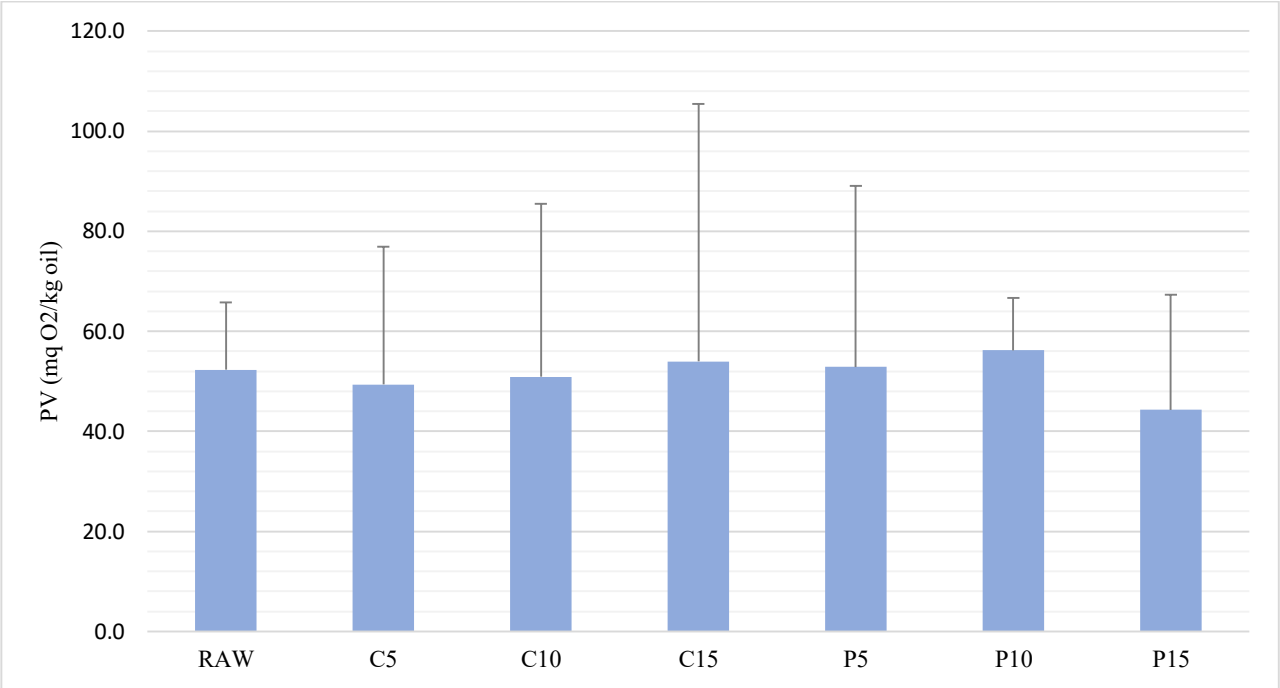

Figure 3-4: Peroxide value (mEq O2/kg oil) of Mussel in varying treatments

PV revealed a minimal degree of oxidation in the samples, which was mostly attributed to the oxidative deterioration of mussels that had already occurred prior to the plasma treatment (Figure 3-4).

In conclusion, PAW did not affect the peroxide value of mussel and even decreased the PV in Sardine samples. Therefore, the impact of PAW on primary oxidation in seafood was trivial.

3.3. Cholesterols and Cholesterol Oxidized Products (COPs)

3.3.1. Cholesterols and Cholesterol Oxidized Products in Sardine

Squalene, Cholesterols-TMS and Phytosterols-TMS were found in unsaponifiable matter components of Sardine. The highest concentration of Squalene was detected at 0.62 mg/100g fresh matter in P10 sample and the lowest value of Squalene was in P20 sample, at 0.20 mg/100g fresh matter. The Cholesterol-TMS compound was the most abundant in C10 sample with 15.05 mg/100g fresh matter. With 14.91 mg/100g fresh matter, the P10 sample had the second-highest value in Cholesterol-TMS. Only 4.56 mg/100g fresh matter was identified in the P20 sample, which had the lowest concentration. It is clear that samples treated with PAW had lower cholesterol-TMS concentrations than control samples. The concentration of the control samples for the Phytosterols-TMS compound dropped as the dipping time increased. As for the PAW-treated samples, greater Phytosterols-TMS concentrations were seen with longer treatment time. Phytosterols-TMS concentration was generally lower in the PAW-treated samples than the control samples.

Table 3-3: Cholesterols and Cholesterol Oxidized Products in Sardine (mean ± SD, n = 3)

Unsaponifiable matter components (mg/100g fresh matter)	C10	C20	C30	P10	P20	P30
Squalene	0.58 ± 0.02 [a]	0.44 ± 0.06 [c]	0.51 ± 0.01 [b]	0.62 ± 0.16 [a]	0.20 ± 0.01 [e]	0.38 ± 0.13 [d]
Cholesterol-TMS	15.05 ± 0.43 [a]	11.88 ± 1.18 [c]	9.41 ± 1.88 [d]	14.91 ± 1.13 [b]	4.56 ± 1.04 [f]	6.54 ± 1.40 [e]
Phytosterols-TMS	0.80 ± 0.75 [a]	0.63 ± 0.01 [b]	0.44 ± 0.08 [c]	0.00 ± 0.00 [f]	0.10 ± 0.00 [e]	0.38 ± 0.14 [d]
7α-OH	0.31 ± 0.08 [e]	1.77 ± 1.09 [d]	2.10 ± 0.05 [c]	0.34 ± 0.01 [e]	8.42 ± 1.14 [a]	2.34 ± 0.07 [b]
7-OH	0.47 ± 0.04 [d]	0.41 ± 0.03 [e]	2.54 ± 0.06 [b]	0.46 ± 0.11 [de]	3.39 ± 1.10 [a]	0.55 ± 0.01 [c]
4-OH	0.05 ± 0.01 [d]	0.20 ± 0.01 [c]	0.36 ± 0.04 [b]	0.06 ± 0.01 [d]	1.66 ± 1.04 [a]	0.35 ± 0.01 [b]
7-β OH	0.53 ± 0.05 [e]	2.64 ± 0.34 [d]	3.63 ± 1.50 [b]	0.48 ± 0.05 [e]	12.73 ± 2.34 [a]	3.10 ± 0.04 [c]
4-OH	0.07 ± 0.02 [d]	0.08 ± 0.00 [d]	0.39 ± 0.18 [b]	0.07 ± 0.04 [d]	0.84 ± 0.01 [a]	0.15 ± 0.02 [c]
5α,6α-epoxy	0.37 ± 0.01 [d]	0.89 ± 0.12 [c]	0.91 ± 0.02 [c]	0.15 ± 0.02 [e]	7.64 ± 0.23 [a]	1.81 ± 0.01 [b]
5β,6β-epoxy	0.42 ± 0.03 [d]	0.41 ± 0.11 [d]	2.39 ± 0.04 [b]	0.44 ± 0.01 [d]	3.93 ± 1.21 [a]	0.87 ± 0.06 [c]
x-OH	0.06 ± 0.00 [d]	0.13 ± 0.01 [c]	0.52 ± 0.05 [b]	0.05 ± 0.01 [d]	0.86 ± 0.04 [a]	0.14 ± 0.01 [c]
x-OH	0.06 ± 0.04 [d]	0.09 ± 0.01 [cd]	0.27 ± 0.01 [b]	0.09 ± 0.04 [cd]	0.66 ± 0.07 [a]	0.14 ± 0.02 [c]
6-OH	0.04 ± 0.00 [e]	0.12 ± 0.03 [d]	0.55 ± 0.02 [b]	0.06 ± 0.03 [e]	1.26 ± 0.21 [a]	0.43 ± 0.07 [c]
x-OH	0.04 ± 0.01 [c]	0.03 ± 0.00 [c]	0.12 ± 0.00 [b]	0.01 ± 0.01 [c]	0.25 ± 0.02 [a]	0.02 ± 0.01 [c]
7-Keto	0.87 ± 0.15 [e]	1.77 ± 0.82 [d]	6.01 ± 1.02 [b]	0.82 ± 0.16 [e]	11.24 ± 1.15 [a]	3.49 ± 0.19 [c]

As regard the non-volatile lipid oxidation products, nine hydroxy-sterols, one keto-sterol and two epoxy-sterols were detected. In 7α-OH compound, the greatest abundant concentration was found in P20, which was 8.42 mg/100g fresh matter. P30 had the second greatest concentration of 7-OH, which was 2.34 mg/100g fresh matter. The concentration of 7α-OH in C10 was 0.31 mg/100g fresh matter which had the lowest value. When samples were treated with PAW in comparison to control samples, it is evident that the amount of the 7-OH compound increased. P20 also reported the highest concentration in 7-OH compound, at 3.39 mg/100g fresh matter, follow by the C30 sample (2.54 mg/100g fresh matter) and 0.41mg/100g fresh matter in C20 was the lowest value of 7-OH. 4-OH compound had 1.66 mg/100 fresh matter in P20 which was the greatest amount, follow by C30 with 0.36mg/100g and P30 with 0.35 mg/100g fresh matter. The lowest concentration was found in C10 sample with 0.05 mg/100g fresh matter. PAW impacted on the concentration of 4-OH due to higher concentration was found in PAW treated samples than control samples. In 7-β OH compound, 12.73 mg/100g fresh matter was the highest amount which found in P20 samples while the lowest concentration was identified in C10 sample (0.53 mg/100g fresh matter) and P10 sample (0.48 mg/100g fresh matter). The second 4-OH compound was detected at the highest amount in P20, at 0.84 mg/100g fresh matter, follow by C30 (0.39 mg/100g fresh matter) and at the lowest concentration in C10 and P10 with same concentration: 0.07 mg/100g fresh matter. 5α,6α-epoxy compound was found at the highest amount (7.64 mg/100g fresh matter) in P20 while only 0.15 mg/100g fresh matter was found in P10 sample. P20 and P30 samples both had higher concentration of 5α,6α-epoxy compound than control samples. 5β,6β-epoxy also observed P20 as the sample with the highest concentration at 3.93 mg/100g fresh matter and lowest concentration was seen in C10 sample (0.42 mg/100g fresh matter) and C20 sample (0.41 mg/100g fresh matter) with no significant statistic

difference. In the three x-OH compounds, P20 had the highest amount (at 0.86, 0.66 and 0.25 mg/100g fresh matter) while C10 was identified with 0.06 and 0.04 mg/100g fresh matter. The same trend was observed in 6-OH compound where P20 had the highest concentration (1.26 mg/100g fresh matter) and C10 was found the least concentration (0.04 mg/100g fresh matter). 7-Keto compound was found at 11.24 mg/100g fresh matter in P20 which was the most abundant and follow by 3.49 mg/100g fresh matter in P30 sample. Meanwhile, C10 and P10 both had the smallest amount of 7-Keto with only 0.87 and 0.82 mg/100g fresh matter respectively.

Overall, it was noticeable that P20 sample contained the most COPs compounds compared to any other samples. The reason for the higher concentration than other samples could be due to the effects of PAW and the sample's own quality. Additionally, there were not much differences between the P10 samples and C10 samples in terms of nearly COPs chemicals. It shown that the 10 minutes of PAW treatment were insufficient to affect the oxidation of cholesterols.

3.3.2. Cholesterols and Cholesterol Oxidized Products in Mussels

Squalene, Cholesterols-TMS and Phytosterols-TMS were identified in the unsaponifiable matter of mussel oils by MS (Table 3-4). The squalene was detected at highest concentration in C15 samples at 0.84 mg/100g fresh matter and not found in C5 and P10 samples. The Cholesterol-TMS concentration in C5 sample was the highest value with 44.24 mg/100g fresh matter) and second highest in P10 sample with 35.79 mg/100g fresh matter. The lowest proportion was 2.59 mg/100g fresh matter in P5 sample. In general, total concentration of Cholesterols-TMS in PAW-treated samples was lower than the control samples. C5 sample had the highest proportion of Phytosterols-TMS, at 21.41 mg/100g fresh matter. The P10 sample was the second highest in the concentration of Phytosterols-TMS with 18.56 mg/100g fresh matter. The P5 sample showed the lowest level of Phytosterols-TMS with only 1.16 mg/100g fresh matter.

Table 3-4: Cholesterols and Cholesterol Oxidized Products in Mussels (mean ± SD, n = 3)

Unsaponifiable matter components (mg/100 g fresh matter)	C5	C10	C15	P5	P10	P15
Squalene	0.00 ± 0.00 ^{c}	0.06 ± 0.02 ^{b}	0.84 ± 0.10 ^{a}	0.08 ± 0.02 ^{b}	0.00 ± 0.00 ^{c}	0.09 ± 0.04 ^{b}
Cholesterol-TMS	44.24 ± 2.45 ^{a}	12.63 ± 2.02 ^{d}	26.18 ± 1.15 ^{c}	2.59 ± 0.12 ^{f}	35.79 ± 2.65 ^{b}	7.90 ± 1.06 ^{e}
Phytosterols-TMS	21.41 ± 1.54 ^{a}	6.12 ± 0.02 ^{c}	2.48 ± 0.12 ^{e}	1.16 ± 0.07 ^{f}	18.56 ± 1.42 ^{b}	4.08 ± 0.21 ^{d}
7α-OH	0.08 ± 0.01 ^{c}	0.11 ± 0.09 ^{bc}	0.12 ± 0.03 ^{bc}	0.01 ± 0.00 ^{d}	0.14 ± 0.01 ^{b}	0.24 ± 0.03 ^{a}
x-OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4-OH (tentatively)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7β-OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4-OH (tentatively)	0.68 ± 0.05 ^{d}	2.42 ± 0.50 ^{a}	0.58 ± 0.07 ^{e}	0.18 ± 0.01 ^{f}	1.10 ± 0.15 ^{b}	0.95 ± 0.02 ^{c}
5α,6α-epoxy	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5β,6β-epoxy	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
x-OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
x-OH	4.43 ± 0.43 ^{e}	14.85 ± 3.40 ^{a}	4.89 ± 1.23 ^{d}	1.53 ± 0.05 ^{f}	5.37 ± 1.34 ^{c}	5.86 ± 1.21 ^{b}
6-OH (tentatively)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

x-OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7-one	0.48 ± 0.04 [d]	1.24 ± 0.12 [a]	0.22 ± 0.01 [e]	0.05 ± 0.00 [f]	0.88 ± 0.09 [b]	0.70 ± 0.02 [c]

Samples of mussels found only 4 COPs components: 7α-OH, x-OH, 4-OH, and 7-one. x-OH compound was the most abundant compound in COPs. P15 sample had the greatest concentration of the 7-OH compound (0.24 mg/100g fresh matter), followed by P10 sample with 0.14 mg/100g fresh matter. The P5 sample had the least level of 7α-OH, which was only 0.01 mg/100g fresh matter. It is clear that an extension of the PAW treatment period may result in a rise in 7-OH concentration. In x-OH compound, C10 sample showed the highest value, at 14.85 mg/100g fresh matter. The P15 had the second highest concentration with 5.86 mg/100g fresh matter and follow by P10 with 5.37 mg/100g fresh matter. The concentration of x-OH was the smallest in P5 sample with 1.53 mg/100g fresh matter. The 4-OH compound observed the highest concentration in C10 sample, at 2.42 mg/100g. Following was the concentration of P10 sample, at 1.10 mg/100g fresh matter and P15 sample, at 0.95 mg/g fresh matter. The lowest concentration was at 0.18 mg/100g fresh matter in P5 sample. The 7-one compound had the most abundant concentration in C10 sample with 1.24 mg/100g fresh matter. The P10 sample had the second highest value, at 0.88mg/100g fresh matter and P15 had the third highest value with 0.70 mg/100g fresh matter. The lowest concentration was at 0.05mg/100g fresh matter in P5 concentration. As we noticed, the C10 sample contained the highest concentration in the three COPs components (x-OH, 4-OH and 7-one). It could be caused from the quality of sample itself that lead to higher oxidation.

In conclusion, the oxidation of cholesterols was existed in mussel samples even through in low quantities. The higher treatment time, the higher oxidation was observed.

CHAPTER 4: CONCLUSION

As a result, it was unclear how PAW affected the seafood samples (sardines and mussels), their amount was not significantly different between PAW treated and control samples. PAW did not significantly modify the overall composition of fatty acids, although an increase in some compounds (n-3 PUFA, C20:5 n-3), was noted in the PAW-treated mussel samples. As the conclusion, there is a good preservation of fatty acids compounds even treating with PAW for long period of time. Further studies are needed to understand the relationships between lipid oxidation and the operation variables (power density, exposure time). Moreover, a comparison between the plasma treatment to water with the plasma plume above or underneath water surface in term of forming RONS's composition and concentration which might affect the lipid oxidation.

The primary oxidation also did not have any development between control samples and PAW treated samples and even in Sardine, PAW treated samples reported a lower concentration in peroxide value than control and raw samples. It might cause by the denaturation of protein compounds on the surface of fish fillets with ROS which leading to the limitation of the lipid oxidation. Therefore, further investigation needed to understand also the impact of PAW also on other compounds such as amino acids, protein, etc.

The cholesterols and non-volatile oxidation profile had shown a difference between Plasma-treated water samples and control samples. The cholesterols were reduced slightly after PAW treatment and a significant increase in cholesterol oxidized products was discovered in treated samples, compared to the respective controls both in Sardine and Mussels samples.

Although there was a modification in non-volatile compounds, these findings support the safety of plasma-activated water, which is strictly required for governmental organizations to approve plasma technologies for use. Moreover, further studies on other type of seafood and food in general are needed to confirm that the impact of PAW on food is limited and insignificant.

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