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Effects of cold atmospheric plasma on mackerel lipid and protein oxidation during storage

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Abstract

The present study investigated the effects of cold atmospheric plasma on the shelf-life stability of lipids and proteins of commercially packaged mackerel fillets. The results showed no significant effects on lipid oxidation between the control samples and those treated at 80 kV for extended treatment times of 5 minutes using an in-package plasma system. In addition, no significant changes ($p>0.05$) were found in the fatty acid composition or nutritional quality indices after treatment. Finally, the formation of carbonyls was accelerated by plasma treatment at the end of the storage period for 4 °C and 8 °C. These results suggest that cold atmospheric plasma technologies are potentially suitable as processing technologies for the fish industry.

Keywords: Cold atmospheric plasma, mackerel, lipid oxidation, protein oxidation

1. Introduction

Fish and seafood products are some of the most common foods in our diet. Mackerel is a popular fish in Europe with more than 800 million tonnes consumed per year (FAO, 2018). It is considered a healthy diet option due to its high content of vitamins, high-quality protein as well as several other essential nutrients such as polyunsaturated fatty acids (Domingo, 2016; Swanson, Block, & Mousa, 2012). In addition, fatty acids present in fish are known to be an important source of essential omega-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are necessary for functions in our metabolism and their intake through the diet is important because humans cannot synthesise them. Numerous studies have highlighted the benefits of EPA and DHA (Shahidi & Ambigaipalan, 2018; Swanson et al., 2012). For example, they are essential for fetal development, and supplementation during pregnancy has also been linked to decreased immune responses in infants including decreased incidence of

allergies. The supplementation of both, EPA and DHA, has been reported to lead to optimal pregnancy length (Swanson et al., 2012). Kumar, Mastana, and Lindley (2016) suggested a diet rich in these fatty acids for patients with various chronic inflammatory diseases, like asthma, due to their anti-inflammatory properties. In addition, they have been reported to play a role in avoiding coronary heart diseases such as atherosclerosis and peripheral arterial disease (Lee, O'keefe, Lavie, & Harris, 2009), as well as Alzheimer's and dementia (Kröger & Laforce, 2016).

Despite all the benefits associated with the intake of fish products, there are challenges facing the industry. Fish quality starts to deteriorate post catch and during storage with spoilage microorganisms the primary factor causing fish spoilage (Gram & Dalgaard, 2002). In this regard, it is paramount to find effective means to ensure the safety and quality of fish products. The food industry is increasingly seeking new processing technologies capable of inactivating microorganisms whilst maintaining food quality. A particular focus has been on non-thermal processing technologies, which are effective at ambient or sub-lethal temperatures (i.e. temperatures below 50 ^{0}C) such as high hydrostatic pressure, pulsed electric field, ultrasound processing and more recently cold plasma (Jermann, Koutchma, Margas, Leadley, & Ros-Polski, 2015). These technologies have been applied to inactivate microorganisms such as viruses, spores, yeasts, bacteria and biofilms (Arvanitoyannis, Kotsanopoulos, & Savva, 2017; Daryaei, Yousef, & Balasubramaniam, 2016; Saldana, Alvarez, Condon, & Raso, 2014). Numerous studies have examined their potential for the decontamination of fish products, including high pressure treatment of mackerel, cod, and salmon (De Alba et al., 2019; Rode & Hovda, 2016), sea bass (Teixeira et al., 2014), herring and haddock (Karim et al., 2011) and smoked cod (Montiel, De Alba, Bravo, Gaya, & Medina, 2012). Ultrasound has been used to reduce the microbiological counts in mackerel, cod, hake and salmon fillets (Pedrós-Garrido et al., 2017).

The principal advantage of such technologies is their operation at low temperatures, thus minimising the negative effects often found with thermal processing (Awuah, Ramaswamy, &

Economides, 2007). Nevertheless, they too can also induce chemical/biochemical changes in food constituents, affecting the quality of the food product (Pérez-Andrés, Charoux, Cullen, & Tiwari, 2018).

Cold atmospheric plasma treatment can induce a wide array of active species including ozone, carbon and nitrogen oxides, free radicals, as well as negative and positive ions depending on the different gases used (Lu et al., 2016). Plasma for food applications is still in its infancy but has demonstrated significant potential for food decontamination and associated increases of shelf-life (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). A growing literature is addressing the unknows surrounding the efficacy and mechanisms of action for microbial inactivation (Liao et al., 2017). The effect of plasma on microbial inactivation has been investigated in different food substrates, including orange juice (Xu, Garner, Tao, & Keener, 2017), fresh and dried nut samples (*Juglans regia L.*) (Amini & Ghoranneviss, 2016), ground nuts (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017), strawberries (Misra et al., 2014) and blueberries (Lacombe et al., 2017).

Cold atmospheric plasma has also been shown to increase the shelf-life of meat products (Xiang et al., 2018), including chicken breasts (Wang, Zhuang, Hinton, & Zhang, 2016), beef jerky (Yong et al., 2017) and pork loin (Yong et al., 2017). Moreover, fish products, like dried filefish fillets (Park & Ha, 2015) and mackerel (I. Albertos et al., 2017) have been also tested.

The radical species present in the plasma are responsible for microbial inactivation although they may also lead to chemical reactions affecting the quality of the food product (Kim, Jayasena, Yong, & Jo, 2016; Misra, 2016; Pérez-Andrés et al., 2018). The challenge for all effective preservation techniques is to retain the sensory and quality attributes of the food while maximising shelf-life and safety profiles. Scale-up of plasma technology is another challenge which has recently been discussed by Cullen et al. (2018). Given the array of radical species found with atmospheric

plasma and the wide range of time-scales of their reactions, there are many unknowns surrounding the chemical reactions induced from plasma treatment of foods (Scholtz, Pazlarova, Souskova, Khun, & Julak, 2015).

The goal of this study was to contribute to the understanding of the effects of cold plasma on the lipids and proteins of mackerel during storage and to support scale-up approaches for this technology. For that reason, mackerel fillets were packaged and plasma treated in food packaging trays typically employed by the industry. Key quality markers were monitored for different storage temperatures and time. The impact of cold atmospheric plasma on the stability of mackerel fatty acids, such as EPA, DHA, PUFA, monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), as well as nutritional quality indices and lipid and protein oxidation were investigated.

2. Material and methods

2.1. Chemicals and reagents

Trichloroacetic acid, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)), EGTA (Egtazic acid), disodium sulphate, hydrochloric acid, potassium hydroxide, 1,1,3,3-tetraethoxypropane , 2-thiobarbituric acid , methanol, heptane, Supelco-37 FAME standard, tricosanoic acid methyl ester, as well as acetyl chloride, sucrose, mannitol and potassium chloride were purchased from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland). N-pentane as well as sodium chloride was purchased from Fisher Scientific (Dublin, Ireland). Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore water purification system (Millipore, Cork, Ireland). All chemical were GC grade.

116

117 *2.2 Sample preparation*

118

119 Fresh mackerel was purchased at a local fish monger in Dublin in September 2016. Each
120 mackerel was divided into two fillets and each fillet, with skin attached facing down, was packaged
121 individually (Ilpra Foodpack VG 400 Packaging Machine, Italy) in a black amorphous polyethylene
122 terephthalate (APET/PE) pack (195x155x37 mm), and sealed under atmospheric air conditions with a
123 low oxygen permeable barrier polyvinyl-chloride film (3 cm^3/m^2/24 h at Standard Temperature and
124 Pressure (STP); Versatile Packaging, Ireland), to mirror common commercial practice.

125

126 *2.3 Plasma treatment*

127

128 An in-house dielectric barrier discharge atmospheric plasma system was employed, which
129 was described previously (Pankaj, Misra, & Cullen, 2013). Each packaged tray was located between
130 two circular aluminium electrodes (outer diameter = 158 mm) separated by two polypropylene (PP)
131 dielectric layers (2 mm thickness). The distance between the dielectric layers was the height of the
132 tray employed (37 mm). Five minutes of treatment was performed at a discharge voltage of 80 kV
133 Root Mean Square (RMS). The choice of these conditions was based on a recent study in the area
134 carried out by our group (I. Albertos et al., 2017) which showed this treatment as the most effective
135 one to reduce total aerobic psychrotrophic bacteria and *Pseudomonas* counts in in-packaged plasma
136 treated mackerel fillets with no impact on colour or TBARs values. Both the control and plasma
137 samples were kept at 4 °C and held for 24 hours to allow the radical species formed to interact with
138 the food following the method of Ziuzina, Patil, Cullen, Keener, and Bourke (2013). After that,
139 samples were randomly divided into three different batches and stored at three different temperatures:
140 4 °C, 8 °C and -20 °C. Samples from each temperature were collected after 2, 5 and 7 days and in the
141 case of the -20 °C batch also after 2 weeks. For each day, samples were homogenised using a blender

(Robot Coupe-R301 Ultra ;Nisbets, Cork, Ireland) and were stored under vacuum at -80 °C until the day of the analysis. All the treatments were carried out in duplicate.

2.4 Lipid oxidation

Lipid oxidation was quantified using the TBARs method according to Maraschiello, Sárraga, and Garcia Regueiro (1999), which is based on the detection of malondialdehyde (MDA), a by-product of peroxidation of polyunsaturated fatty acids and esters, associated with off-flavours and off-aromas in meat products (Ghani, Barril, Bedgood, & Prenzler, 2017). Briefly, 1 g of blended mackerel sample was suspended in 20 mL of MilliQ water and homogenised with an Ultraturrax homogeniser (Labortechnik, Staufen, Germany) at 13,500 rpm for 30 seconds. 5 mL of 25% trichloroacetic acid solution was added and the solution which was kept at 4 °C for 15 min. After centrifugation at 3,500 rpm for 30 minutes at 4 °C using a Hettich Rotanta 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland), the solution was filtered using a Whatman number 51 filter paper (Sparks Lab Supplies Ltd., Dublin, Ireland). A 1.5 mL aliquot of the filtrate was collected and mixed with 1.5 mL 0.6% of 2-thiobarbituric acid with the reaction performed in a water bath at 70 °C for 30 minutes. A standard curve was prepared using a 1,1,3,3-tetraethoxypropane solution. Absorbance of samples and standards were measured spectrophotometrically on a UV–Vis Spectrophotometer (Shimadzu UV-1700, Columbia, USA) at a wavelength of 532 nm. Results were expressed as milligrams of malondialdehyde per kilogram of mackerel (mg MDA/ kg mackerel).

2.5 Fatty acid profile

2.5.1Microwave-assisted preparation of fatty acid methyl esters (FAMEs)

Microwave assisted FAME preparation was carried out using a MARS 6 Express 40 position Microwave Reaction System (CEM Corporation, Matthews, NC, USA) according to Brunton, Mason, and Collins (2015). Briefly, approximately 0.8 g of chilled blended mackerel was accurately weighed out into a reaction Xpress vessel. 100 µL of internal standard (C23:0 methyl ester, achieving a final concentration following extraction of 0.1 mg/mL) as well as 10 mL of potassium hydroxide (2.5%, w/v) in methanol (MeOH) solution were added to the vessel. The reaction vessels containing a 10 mm stir bar each were closed well and introduced into the MARS 6. The saponification programme consisted of bringing the temperature up to 130 °C over 4 minutes and holding at this temperature for another 4 minutes. Then, tubes were cooled down in an ice bath. For esterification, 15 mL of 5% (v/v) acetyl chloride in MeOH solution were added to the vessels. Esterification was carried out by heating the vessels to 120 °C over 4 minutes and holding at this temperature for 2 minutes. The reaction tubes were removed and cooled on ice for 5 minutes. To the cooled vessels, 10 mL of pentane was added and vessels were inverted to achieve extraction. After that, about 20 mL of saturated NaCl solution was added, and the inversion process was repeated. The separation of the organic and aqueous layer was achieved, and an aliquot of the upper pentane layer, where the FAMEs were located was transferred into GC vials (1.5 mL) containing about 0.2 g anhydrous sodium sulphate and analysed using gas chromatography.

2.5.2 Gas chromatography-flame ionisation detector analysis

Separation was carried out using a Clarus 580 Gas Chromatograph (Perkin Elmer, Massachusetts, USA) fitted with a flame ionisation detector (GC-FID). The separation of 37 FAMEs was carried out in 35 minutes employing a CP-Sil 88 capillary column (Agilent, Santa Clara, California, USA) with a length of 100 m x 0.25 mm ID and 0.2 µm film. The inlet temperature was set to 250 ºC and the injection volume was 0.5 µL. The carrier gas was hydrogen at a constant flow of 1.25 mL/min, and the split ratio was set at 10:1. The oven was set to 80 °C with an initial temperature

ramp of 6.2 °C/min to 220 °C which was held for 3.2 minutes. A second temperature ramp of 6.3 °C/min to 240 °C followed which was held for 6.5 minutes. The FID was set at 270 °C. Compounds were identified by comparing their retention times with those of authentic standard of FAMEs from the Supelco 37 FAME mix. The content of each fatty acid was calculated using following equation (Eq. (1)).

$$\text{(eq.1) FA content} = \frac{\text{Peak Area(FAME)}}{\text{Peak Area(ISTD)}} \text{x} \frac{\text{Weight(ISTD)}}{\text{Weight(Sample)}} \text{x ISTD purity x 10 x 0.96}$$

Where, FA content is the amount of a given fatty acid in the sample (mg/g), 10 is the dilution factor and 0.96 is the conversion factor for the internal standard to account for the internal standard already being presented as a FAME.

Due to the variability of the fat content, fatty acid content was normalised and results were expressed in percentages:

$$\text{(eq.2) FA content (\%)} = \frac{\text{FA content (mg/g)}}{\text{Total fat content (mg/g)}} \text{x100}$$

A full list of all calculated fatty acids in g/kg fish is provided as supplementary material. (Sup 2).

2.5.3 Nutritional quality indices

Nutritional quality indices of plasma treated mackerel were assessed from fatty acids data. Saturation index (SI) is a carcinogenesis marker while atherogenicity (AI) and thrombogenicity (TI) are related to the incidence of coronary heart disease. They were calculated using the following equations (Ulbricht & Southgate, 1991):

$$\text{(eq.2) SI} = \frac{[\text{C14:0+ C16:0 +C18:0}]}{[\sum \text{MUFA+ } \sum \text{PUFA}]}$$

$$\text{(eq.3) AI} = \frac{[\text{C12:0}+4 \times (\text{C14:0})+\text{C16:0}]}{[\sum \text{MUFA} + \sum \text{PUFA}]}$$

$$\text{(eq.4) TI} = \frac{[\text{C14:0}+\text{C16:0}+\text{C18:0}]}{\left[0.5 \times \left(\sum \text{MUFA} + \sum \text{n6}\right)+3 \times \sum \text{n3} + \frac{\sum \text{n3}}{\sum \text{n6}}\right]}$$

2.6 Protein oxidation

Protein carbonyls are biomarkers of protein oxidation. They are generated through several different mechanisms induced during oxidative stress. The content of carbonyl groups was determined by the dinitrophenylhydrazine (DNPH) method using a Biocell protein carbonyl enzyme Immuno-assay kit (BioCell Corporation Ltd, Auckland, New Zealand) (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997). All required reagents were provided by the test kit. Protein extraction was performed according to the procedure by Devries et al. (2008). Briefly, 1 g of mackerel sample was added to 25 mL protein extraction buffer (70 mmol/L sucrose, 220 mmol/L mannitol, 5 mmol/L Hepes, pH 7.2, 1 mmol/L EGTA, pH 7.2, and 150 mmol/L KCl) and homogenised for 30 seconds using an Ultraturrax homogeniser (Labortechnik, Staufen, Germany) set to 13,500 rpm. Each homogenate was adjusted to pH 7.2 using either 1 mol/L HCl or 1 mol/L NaOH and then centrifuged for 10 min at 3,500 rpm at 4 °C using a Hettich Rotina 460R centrifuge (Fisher Scientific Ireland, Dublin, Ireland). The amount of soluble protein in the supernatant was quantified by measuring its absorbance at 280 nm using an ND-100 Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Protein carbonyl content determination was then carried out according to the Biocell protein carbonyl enzyme Immuno-assay kit manufacturer's guidelines and measured using a plate reader (FLUOstar Omega Microplate Reader, BMG Labtech GmbH, Offenburg, Germany). Protein carbonyl content was calculated using a standard curve and results expressed as nmol DNPH/ mg protein.

2.7 Statistical analysis

Analysis of variance (ANOVA) of the dependent variables was carried out using Minitab 17.1.0 (Minitab Inc, Stage College, Pennsylvania, USA). Statistics were performed using a general linear model (GLM) where the treatment and temperature were considered as fixed and days as random factors. Tukey's multiple comparison was used for post-hoc analysis on the significant factors.

3. Results and discussion

3.1 Lipid oxidation

One of the most common undesirable reactions found in food is lipid oxidation, which can affect both product taste and odour. Lipid oxidation processes occur via a free radical chain mechanism. Consequently, all radical species forming the plasma bulk could encourage these reactions (Gavahian, Chu, Khaneghah, Barba, & Misra, 2018). One of the most common products of lipid oxidation is malondialdehyde, which can be measured by TBARs analysis.

Plasma treatment was found to have no impact on lipid oxidation, as no significant differences ($p>0.05$) were found for the TBARs values between untreated and treated samples (Fig .1). However, the TBARs values significantly increased ($p<0.05$) during storage for both the control and plasma samples (Fig 1), which is in line with previous research. For example, Romotowska, Karlsdottir, Gudjonsdottir, Kristinsson, and Arason (2016) investigated lipid deterioration of mackerel during frozen storage at -18 °C and -25 °C, with TBARs formation observed over time. These results agree with the findings of I. Albertos et al. (2017) for mackerel treated with a dielectric barrier discharge at 70 and 80 kV for treatment times of up to 5 minutes. These authors observed no significant effect on

TBARs values between the treated and untreated samples for any of the applied voltages or treatment times. No significant differences in TBARs values were also reported in Atlantic herring treated at 70 kV for 5 min compared to the control samples 1 day after treatment but a significant increase of those values was observed during the storage period (Albertos et al., 2019). When Lee et al. (2016) employed a plasma treatment for chicken breasts using a flexible thin-layer dielectric barrier discharge (FTDBD) plasma, no significant differences in the TBARs values was detected. Similar results were found after plasma treatment using a dielectric barrier discharge on milk samples during a 5 and 10 minutes treatment process at 250 W (Kim et al., 2015). Contrary to these findings, several articles report that plasma induces lipid oxidation. For example, the TBARs values for plasma treated sushi products (Nigiri and Hosomaki) were found to increase after being treated at 70 kV or 80 kV for 5 min (Kulawik et al., 2018). A higher surface area and a higher concentration of unsaturated fatty acids resulted in higher levels of TBARs in Hosomaki sushi (Dawczynski, Schubert, & Jahreis, 2007; Kulawik et al., 2018). A significant increase in TBARs values has been reported by Albertos et al. (2019) in Atlantic herring treated at 80 KV for 5 min. When dried Alaska pollock shreds, semi dried squid and Gwamegi (semi-dried raw Pacific saury) were treated by corona discharge plasma jet (CDPJ), a significant increase ($P<0.05$) in TBARs values were detected (Choi, Puligundla, & Mok, 2016, 2017; Puligundla, Choi, & Mok, 2018). According to these authors, that increase during the CDPJ treatment may be attributed to the further oxidation of primary lipid oxidation products by reactive species generated by the jet plasma. Similar results were obtained by Olatunde, Benjakul, and Vongkamjan (2019b) who found an increase in TBARs values in plasma treated Asian sea bass slices with increasing treatment times that could be as a result of the ozone generated. In their study, the samples were packed in heat sealed bags filled with argon/oxygen (90:10), treated with high voltage cold atmospheric plasma (HVCAP) at 80 kV RMS for 2.5, 5.0, 7.5 and 10 min and stored at 4 °C for 1 h as post-treatment time. TBARs values were also significantly higher for plasma treated brown and white rice for all treatment conditions with significant increases found for with increasing

treatment times of up to 20 minutes (Lee et al., 2018). It has been reported that the nature of the food matrix and the way the product is handled prior to plasma exposure can affect the oxidation rate in plasma treated samples (Gavahian et al., 2018). The optimization of the process parameters is also needed in order to reduce the rate of lipid oxidation in plasma treated products.

The limit above which TBARs levels would indicate that secondary oxidation products could be detected by consumers have been reported to be 8 mg malondialdehyde/ kg of fish tissue (Schormüller, 1968) while Connell (1990) suggested TBARs values of 1–2 mg MDA/kg of fish flesh as the limit beyond which fish will normally develop an objectionable odour. The results obtained in the present work were much higher than these proposed limits. However, it is important to highlight that the aim of the present study was to compare non-treated and plasma treated samples rather than a quantification of MDA during storage, with no significant differences being found. Such values could be explained by the overheating that might be caused by a blender used to homogenize the samples and the penetration of oxygen and other species that could have happen while mincing. However, the results obtained in the present work are in line with those reported in mackerel by Romotowska et al. (2016). According to these authors, an increase of oxidation products may be related to the total lipid content seasonal variation, due to differences in accessibility of the feed source and also due to the geographical location of the catch seemed to exert an influence on the lipid stability. It has also been suggested that higher values of lipid oxidation products detected in mackerel could be generated during storage and transportation of the fillets (Cropotova, Mozuraityte, Standal, & Rustad, 2019).

3.2 Fatty acids profile

The fatty acid profiles for both the control and plasma treated samples during storage were analysed. The study focused on two fatty acids present in the fish which offer high nutritional values namely eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n -3). The

effects of cold atmospheric plasma on the fatty acid profiles for the mackerel fillets are summarised in Table 1. In addition, the complete results of the FAME composition in g/kg, the chromatogram of control and plasma treated samples on day 0 at 4^0C individually, and an overlay of these two chromatograms is provided as supplementary material (Sup 2 and Sup 1).

No significant differences ($p>0.05$) were found for the fatty acid profiles between the control and treated samples for the same temperature and storage day. These results agree with those reported by Kulawik et al. (2018), who described no significant differences in the content of each fatty acid studied for sushi products. In addition, there was no significant change in the fatty acid composition of beef jerky treated with a radio-frequency atmospheric pressure plasma discharge (J.-S. Kim, Lee, Choi, & Kim, 2014). However, in another study on mackerel, significant differences between the control and treated samples were observed for the content of palmitic acid (C16:0), oleic acid (C18:1, n-9), eicosapentaenoic acid (C20:5, n-3) and docosahexaenoic acid (C22:6 n-3) after treatment with a DBD operating at a discharge voltage of 70 kV and 80 kV for 1, 3 and 5 minutes (I. Albertos et al., 2017). This disagreement with our study could be due to different sample preparation methods employed. In the present work, a whole fillet with the skin attached and facing down was treated. This skin likely provided extra protection against the action of the plasma as only the upper side of the mackerel muscle was exposed and hence allowed to interact with the plasma atmosphere, possibly lessening any effect of the plasma. In addition, in our experiment, the fillet was blended after the treatment and stored. This homogenisation could mask possible localised negative effects induced by plasma.

The effect of cold atmospheric plasma on the fatty acid profile has also been studied on different matrices including soy bean oil (Yepez & Keener, 2016). A significant change of the fatty acid composition was reported after applying a plasma discharge at 90 kV for extended treatment times of 1, 1.5, 2, 4, 6 and 12 hours. They reported that α-linolenic (18:3, n-6) and linoleic (18:2, n-6)

acid significantly decreased as a function of treatment time, while oleic (C18:1, n-9), stearic (C18:0), and palmitic (C16:0) acid significantly increased. Kim et al. (2015) found a significant change of butyric (C4:0) and caprylic acid (C8:0) content in milk for plasma treatments of 5 and 10 minutes with an input power of 250 W. Surowsky, Schluter, and Knorr (2015) reported that the effect of plasma was more evident in liquid samples than solid samples due to their ability to interact with the bulk of the liquid matrix; this could explain why a significant effect of plasma treatment for oil and milk was previously reported. On the other hand, the effect of the temperature on the oxidative stability of fatty acid methyl esters has also been studied. Giua, Blasi, Simonetti, and Cossignani (2013) found that the methyl forms of conjugated linoleic acid (Me-CLA) showed the highest isomer profile modifications during a heating treatment at 180 °C for 15, 30, 45 and 60 min in order to simulate a frying process. In fact, the percentage of cis-9,trans-11 and trans-10,cis-12 CLA isomers decreased during heating time and after 60 min, 13.6% of trans,trans isomers and 4.9% of cis,cis isomers were detected. In another study, Cossignani, Giua, Simonetti, and Blasi (2014) found that methyl octanoate was the main methyl ester compound detected both for Me-CLA and for Me-LA oxidized samples after being treated at 180 °C for 15, 30, 45 and 60 min. Other studies focused on the effect of cold plasma on the fatty acid profile of chocolate milk drink and guava-flavored whey beverage when compared to conventional pasteurization has been published (Coutinho et al., 2019; Silveira et al., 2019). The chocolate milk drink subjected to intermediate cold plasma treatment conditions presented an improved fatty acid profile when compared to the pasteurized product, with a significant reduction in stearic acid and an increase in myristoleic acid, linoleic, and PUFA levels (Coutinho et al., 2019). However, when mild and severe conditions were applied, there was an increase in SFA and a decrease in both MUFA and PUFA. The changes in fatty acids were attributed to the oxygen radicals produced during plasma treatment, including ozone, that react with the unsaturated fatty acids and break down the double bonds, leading to an increase in SFA (Gavahian et al., 2018). No significant differences were found by Silveira et al. (2019) between the plasma-treated

guava-flavored whey beverage and the pasteurized product for butanoic, octanoic, decanoic, dodecanoic, myristic, and stearic acids levels. However, an increase in palmitic acid and/or a decrease in hexanoic acid, oleic acid, and MUFA were observed probably due to the reactivity of the plasma reactive species against the unsaturated fatty acids.

3.3 Nutritional indices

Nutritional indices were calculated from the fatty acid profiles of both treated and untreated samples using the equations outlined in section 2.5.3. The results are summarised in Table 2. No significant differences ($p>0.05$) were found for any index, which is to be expected given no significant differences were found for fatty acid profiles between treatments. To the best of the authors' knowledge, this is the first time that these indices have been calculated for cold plasma treated mackerel. AI relates the risk of atherosclerosis and is based on fatty acids which can increase (C12:0, C14:0 and C16:0) or decrease (ΣMUFA, ΣPUFA) the level of blood cholesterol. TI values relate to the tendency to form clots in the blood vessels, defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic fatty acids (MUFAs, n-6 PUFAs and n-3 PUFAs) (Ulbricht & Southgate, 1991). The smaller the AI and TI values, the greater health benefits derived from the product (Ulbricht & Southgate, 1991). In the present work, no significant differences ($p>0.05$) were found in the AI values in the control and plasma treated samples along the storage at the temperatures studied. Both treated and untreated samples had an AI value of 0.7 on day 0. The AI values were 0.6 and 0.7 in plasma treated samples along the 14 days at -20 °C, while the control had an AI value which varied from 0.6 to 0.9. At 4 °C, plasma treated samples showed AI values of 0.7 and 0.8 during the 7 days of storage which were in a similar range than the untreated samples. When samples were stored at 8 °C, the AI values in plasma treated and untreated samples varied from 0.6 to 0.8. On the other hand, the TI values obtained on day 0 in plasma treated samples were 0.3 which did not change ($p>0.05$) along the investigated storage period for any of the

temperatures studied. This index showed values between 0.2 and 0.4 in the control samples during the storage. AI values of 0.48 and TI values of 0.24 have been reported for mackerel by Fernandes et al. (2014) who indicated the range of the expected values for AI and TI indices to be up to 1 and 0.5, respectively. According to these authors, AI and TI values detected in the present work would be within the expected range both in plasma treated and untreated mackerel fillets and also along the storage period.

Mackerel would be considered a healthy food because the ratio PUFA/SFA was above 0.45 and the plasma treatment did not decrease the ratio below that recommended value (Hmso, 1994). Moreover, it can be observed that this ratio did not decrease during the storage for any of the temperatures studied. SI indicates the relationship between the sum of saturated fatty acids (C14:0, C16:0 and C18:0) (pro-thrombogenic) and unsaturated fatty acids (anti-thrombogenic). It has been reported that myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) are associated with an increased risk of coronary heart disease (Zong et al., 2016). Although to authors' knowledge, there is no numerical values assigned to SI, a food with lower values of these SFA compared to unsaturated fatty acids would be considered a healthier food. In the present work, the SI value in untreated mackerel varied from 0.5 to 0.6 and it was at the same level ($p>0.05$) in plasma treated samples during the storage period for all the temperatures studied. The n-6/n-3 ratio is considered a good indicator of nutritional value of dietary fat. Diets containing higher amount of n–6 PUFA or high n–6/n–3 ratio have been reported to promote the pathogenesis of cancer, inflammatory and cardiovascular diseases (Simopoulos, 2002, 2008). According to the nutritional recommendations, the n-6/n-3 ratio should not be higher than 4.0 (Hmso, 1994). In our study, the values of this ratio were within this range for plasma treated mackerel, both immediately after the treatment, and along the storage period for all the temperatures studied. Two studies focused on the effect of cold plasma on nutritional indices of chocolate milk drink and guava-flavored whey beverage compared to the pasteurised product has been published (Coutinho et al., 2019; Silveira et al., 2019). According to

Coutinho et al. (2019), the chocolate milk drink submitted to mild plasma conditions had similar AI and TI indices ($p>0.05$) when compared to the pasteurized product. However, when mild and severe plasma treatment conditions were applied, higher AI and TI indices were obtained, highlighting the importance of optimizing the process conditions to minimize negative impacts. In another study, a plasma treated guava-flavored whey beverage showed higher AI and TI values than the pasteurised product (Silveira et al., 2019).

3.4 Protein oxidation

Like lipid oxidation, protein oxidation is also initiated by free radicals and can be measured by the loss of sulfhydryl groups (S−H) or by the formation of protein carbonyls which are both formed during this oxidation process. Radical species can lead to the scission of the peptide backbone, the conversion of one amino acid to a different one, the formation of crosslinking as well as oxidative changes of the amino acid side chains (Estévez, 2011). For instance, an increase in the carbonyl content could lead to the formation of crosslinking, resulting in a decrease of the juiciness, tenderness and quality traits of meat products (Estévez, 2011). Hence, it is important to study if plasma treatments encourage this undesirable reaction.

The carbonyl content in mackerel ranged from 0.5-1.5 nmol/mg protein for the control samples, to 0.5-2.5 nmol/mg of protein for the plasma treated samples. The results indicated that the plasma treatment significantly accelerated the formation of carbonyl groups ($p<0.05$) for all storage temperatures studied (Fig.2); i.e. -20^0C (Fig. 2A), 4^0C (Fig. 2B) and 8^0C (Fig. 2C). In addition, the carbonyl content increased significantly for treated and untreated samples during storage independent of the temperature. Moreover, in the present study the storage temperature had a significant impact ($p<0.05$) on carbonyl formation, resulting in greater formation at higher temperatures.

A formation of protein carbonyls in mackerel mince during a storage study at 5 ^{0}C has been also reported by Eymard, Baron, and Jacobsen (2009) and at -18^{0}C Ozen and Soyer (2018). Eymard et al. (2009) found values much higher than the ones presented in this manuscript (from 2 nmol of carbonyl per mg of protein up to 10 nmol/mg after 92 hours). This increase reported by Eymard et al. (2009) could be due to the mincing of the mackerel (before the treatment), making it possibly easier for oxygen to penetrate the matrix, possibly resulting in accelerated oxidation. However, in another study, Babakhani, Farvin, and Jacobsen (2016) reported that the initial content of carbonyl groups in mackerel mince was 1 nmol/mg protein and it increased up to 1.4 nmol/mg protein after 192 hours of storage at 5 °C. The reason of this low values can be explained because the mackerel mince was packaged under vacuum. This may have slowed down the oxidation due to the lack of potential gases such as oxygen which can facilitate the oxidation of the food components.

The effect of high voltage cold atmospheric plasma (HVCAP) generated with the mixture of argon and oxygen (90:10) for 5 min at 28 °C with the addition of two different antioxidants such as ascorbic acid and ethanolic coconut husk extract on the quality of sea bass, has been investigated by Olatunde, Benjakul, and Vongkamjan (2019a). In this study, heat-sealed bags containing sea bass slices with and without antioxidants, filled with 90% argon and 10% oxygen using a vacuum-packaging machine, were treated at 16 kV RMS for 5 minutes using a dielectric barrier discharge system. A significantly higher ($p<0.05$) carbonyl content of HVCAP treated samples compared to the control ones was reported, regardless the addition of the antioxidants, concluding that HVCAP could lead to the oxidation of proteins.

There is a lack of data in the literature to compare with and to explain in more detail the effects of cold atmospheric plasma on protein oxidation in food matrices. To date, most studies have examined the effects of plasma on protein solutions. A whey protein isolate solution (2% (w/v) in 50 mmol/L phosphate buffer, pH 6.8) was treated by cold atmospheric plasma at 70 kV for periods of 1,

5, 10, 15, 30 and 60 minutes and reported a significant increase in the carbonyl content in plasma treated samples compared to the untreated ones (Segat, Misra, Cullen, & Innocente, 2015). They suggested that the formation of carbonyls could be attributed to the modifications of a number of amino acid side chain groups, especially with NH− or NH_2 or by peptide bond cleavages. On the other hand, K. H. Lee et al. (2018) found a decrease in the total free sulfhydryl groups (–SH) for peanut protein isolate solutions treated by cold plasma which is related to the oxidation of these groups to form disulphide bonds. Moreover, a decreased in the content of sulfhydryl groups was also reported all the treatment times (1, 5, 10, 15, 30, and 60 minutes) after applying a voltage of 70 kV on whey protein isolate solution (Segat, Misra, Cullen, & Innocente, 2015). A significant processing time dependant increase ($p<0.05$) in carbonyl content has been detected in the crude protease extract from squid mantle treated by a dielectric barrier discharge system at 60 kV for different treatment times (Nyaisaba et al., 2019). These authors also found a decrease in the total sulfhydryl group ($p<0.05$) (up to about 40%) as exposure time extended which was attributed to the formation of disulfide through cross-linking of sulfhydryl groups influenced by reactive species generated by cold plasma (Segat et al., 2015). Similar results were reported by Sharifian, Soltanizadeh, and Abbaszadeh (2019) who found an increase ($p<0.05$) in the carbonyl content of beef myofibrillar proteins after the DBD plasma treatment which was higher at increased processing times. The carbonyl formation has been attributed to the modification of certain amino acid side chains with $–NH_2$ or –NH, or to the cleavage of peptide bonds (Segat et al., 2015). Sharifian et al. (2019) reported a significant increase ($p<0.05$) in free sulfhydryl groups in beef myofibrillar proteins after 10 min of atmospheric cold plasma (ACP) treatment compared to the untreated samples and those treated for 5 min. According to these authors, the alteration of the terciary structure of the myofibrillar proteins caused by 10 min plasma treatment, could have facilitated the hidden sulfhydryl groups to become exposed at the protein surface and consequently, be more vulnerable to the treatment. ACP has been reported to

induce the loss of −SH groups from amino acid cysteine present in the protein structure (Segat et al., 2015).

The data obtained showed that cold atmospheric plasma did not encourage observable undesirable reactions such as lipid oxidation to the bulk of the treated samples. In addition, the stability of the fatty acid composition of mackerel was not affected by the treatment, along with their nutritional quality indices. However, cold atmospheric plasma could accelerate the formation of carbonyls which are related to protein oxidation. Further research is required on the impact of cold plasma technology on the quality of seafood products in order to be implemented by the fish industry.

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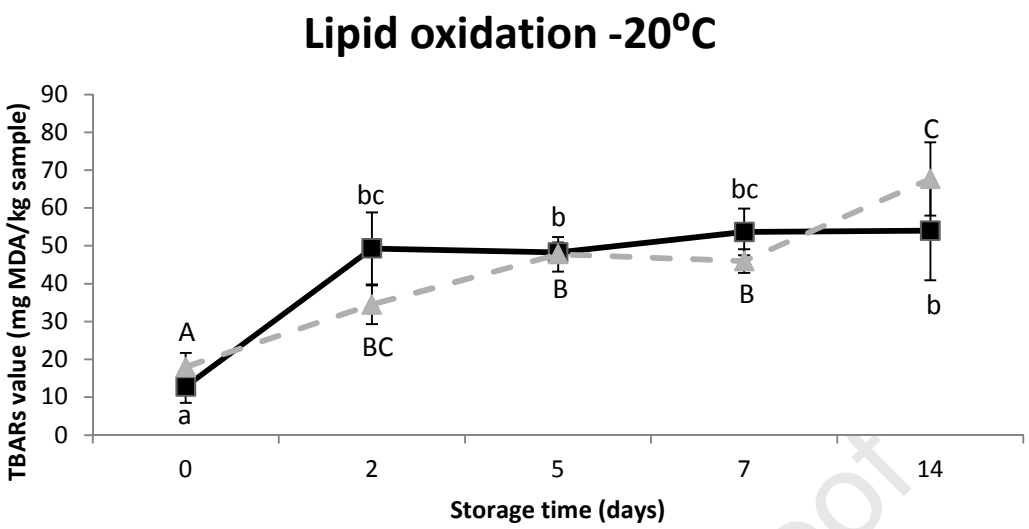
Temperature (^{0}C)	Treatment	Time (days)	SFA (%)	MUFA (%)	PUFA (%)	EPA (%)	DHA (%)
-20	Control	0	34.3 ± 0.2	30.7 ± 2.7	35.0 ± 2.8	9.9 ± 1.5	16.5 ± 1.3
		2	37.5 ± 5.9	30.2 ± 3.4	32.3 ± 2.5	8.4 ± 0.7	14.6 ± 1.3
		5	33.8 ± 1.3	27.8 ± 1.4	38.4 ± 1.3	11.8 ± 0.3	18.6 ± 1.2
		7	36.7 ± 0.5	30.1 ± 0.2	33.2 ± 0.5	8.1 ± 0.2	16.2 ± 0.3
		14	35.0 ± 0.3	29.3 ± 4.1	35.6 ± 3.8	9.8 ± 1.1	16.7 ± 2.1
	Plasma	0	35.1 ± 0.3	27.8 ± 1.4	37.1 ± 1.5	10.2 ± 0.6	18.0 ± 0.7
		2	36.4 ± 2.4	14.3 ± 8.3	39.3 ± 6.6	11.5 ± 0.7	20.9 ± 5.8
		5	34.7 ± 1.4	28.9 ± 0.5	36.4 ± 0.9	10.3 ± 0.6	17.7 ± 0.6
		7	34.2 ± 0.6	27.3 ± 1.1	38.6 ± 0.5	11.7 ± 1.0	17.6 ± 0.6
		14	34.1 ± 0.9	31.3 ± 0.9	34.7 ± 1.4	9.5 ± 0.2	17.2 ± 1.0
Temperature (^{0}C)	Treatment	Time (days)	SFA (%)	MUFA (%)	PUFA (%)	EPA (%)	DHA (%)
4	Control	0	34.3 ± 0.2	30.7 ± 2.7	35.0 ± 2.8	9.9 ± 1.5	16.5 ± 1.3
		2	33.7 ± 0.4	29.4 ± 0.3	36.9 ± 0.5	10.1 ± 0.4	18.0 ± 0.8
		5	34.3 ± 2.0	26.2 ± 4.4	39.5 ± 2.9	11.5 ± 0.9	20.8 ± 3.3
		7	35.1 ± 1.4	31.5 ± 1.4	33.4 ± 1.8	9.0 ± 0.3	15.7 ± 0 .4
	Plasma	0	35.1 ± 0.3	27.8 ± 1.4	37.1 ± 1.5	10.2 ± 0.6	18.0 ± 0.7
		2	34.1 ± 0.5	32.8 ± 0.3	33.1 ± 0.3	9.1 ± 0.1	15.8 ± 0 .2
		5	33.4 ± 2.5	31.9 ± 7.1	34.8 ± 4.8	8.6 ± 1.6	16.0 ± 1 .9
		7	36.9 ± 2.0	25.2 ± 1.8	37.9 ± 1.6	11.9 ± 1.3	18.2 ± 1.0
Temperature (^{0}C)	Treatment	Time (days)	SFA (%)	MUFA (%)	PUFA (%)	EPA (%)	DHA (%)
8	Control	0	34.3 ± 0.2	30.7 ± 2.7	35.0 ± 2.8	9.9 ± 1.5	16.5 ± 1.3
		2	36.2 ± 6.4	30.0 ± 5.4	33.8 ± 11.8	9.3 ± 3.5	17.7 ± 7.3
		5	33.7 ± 0.6	30.9 ± 2.5	35.4 ± 3.0	8.9 ± 0.7	17.8 ± 2.5
		7	36.1 ± 0.2	30.0 ± 4.1	34.0 ± 4.3	9.0 ± 1.6	15.6 ± 2.3
	Plasma	0	35.1 ± 0.3	27.8 ± 1.4	37.1 ± 1.5	10.2 ± 0.6	18.0 ± 0.7
		2	34.7 ± 1.1	30.3 ± 2.3	35.0 ± 3.2	9.63 ± 2.4	15.4 ± 0.9
		5	33.1 ± 2.6	34.1 ± 2.1	32.8 ± 0.6	9.5 ± 0.7	15.9 ± 0.8
		7	33.9 ± 0.	29.7 ± 0.5	36.4 ± 0.3	10.3 ± 0.6	18.3 ± 0.9

Table 1: Fatty acid profile during storage study. No significant differences (p> 0.05) were found for any lipid class. The differences are measured separately for 4 ^{0}C, 8 ^{0}C and -20 ^{0}C.

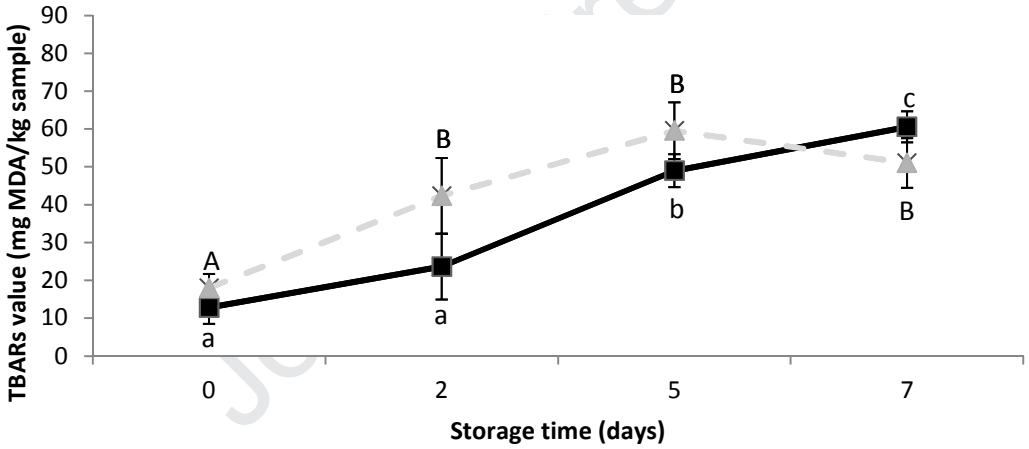
Temperature (^{0}C)	Treatment	Time (days)	Saturation index (SI)	Atherogenic index (AI)	Thrombogenic index (TI)	n-6/n-3	PUFA/SFA
-20	Control	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		2	0.6 ± 0.2	0.9 ± 0.2	0.4 ± 0.2	0.1 ± 0.0	0.9 ± 0.2
		5	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.1 ± 0.1
		7	0.5 ± 0.0	0.9 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.9 ± 0.0
		14	0.5 ±0.0	0.8 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
	Plasma	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
		2	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		5	0.5 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		7	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.1 ± 0.0
		14	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0. 1
Temperature (^{0}C)	Treatment	Time (days)	Saturation index	Atherogenic index	Thrombogenic index	n-6/n-3	PUFA/SFA
4	Control	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		2	0.5 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
		5	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	1.1 ± 0.1
		7	0.5 ± 0.0	0.8 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.1
	Plasma	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
		2	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.0
		5	0.5 ± 0.1	0.8 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.04 ± 0. 0
		7	0.5 ± 0.0	0.8 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
Temperature (^{0}C)	Treatment	Time (days)	Saturation index	Atherogenic index	Thrombogenic index	n-6/n-3	PUFA/SFA
8	Control	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		2	0.6 ± 0.2	0.7 ± 0.2	0.3 ± 0.0	0.1 ± 0.1	0.9 ± 0.4
		5	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		7	0.5 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.1
	Plasma	0	0.5 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
		2	0.5 ± 0.1	0.8 ± 0.2	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		5	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1
		7	0.5 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.0

Table 2: Nutritional indices values during storage study. No significant differences ($p > 0.05$) were found for any nutritional class. The differences are measured separately for 4 ^{0}C, 8 ^{0}C and -20 ^{0}C.

A)


Lipid oxidation -20°C
TBARs value (mg MDA/kg sample)
90
80
70
60
50
40
30
20
10
0
A
a
bc
BC
b
B
bc
B
C
b
0
2
5
7
14
Storage time (days)

B)


Lipid oxidation 4°C
TBARs value (mg MDA/kg sample)
90
80
70
60
50
40
30
20
10
0
A
a
B
a
B
b
c
B
0
2
5
7
Storage time (days)

C)

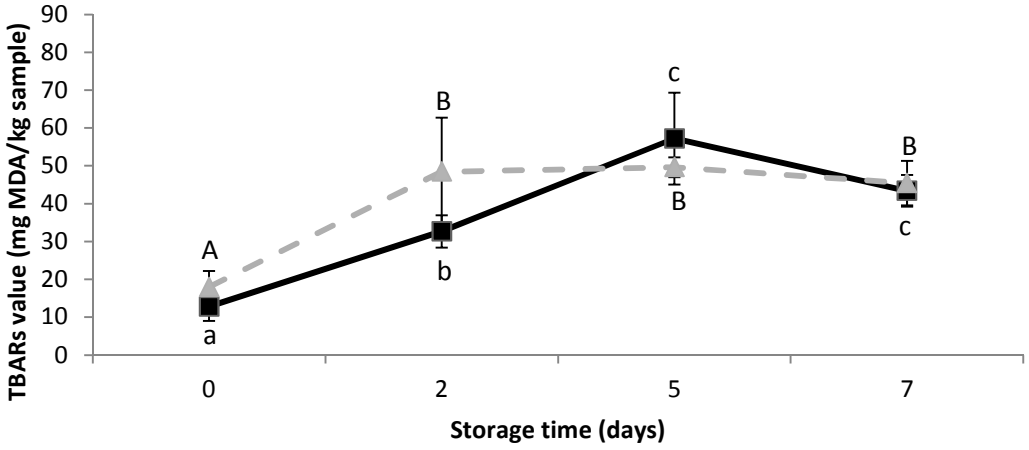
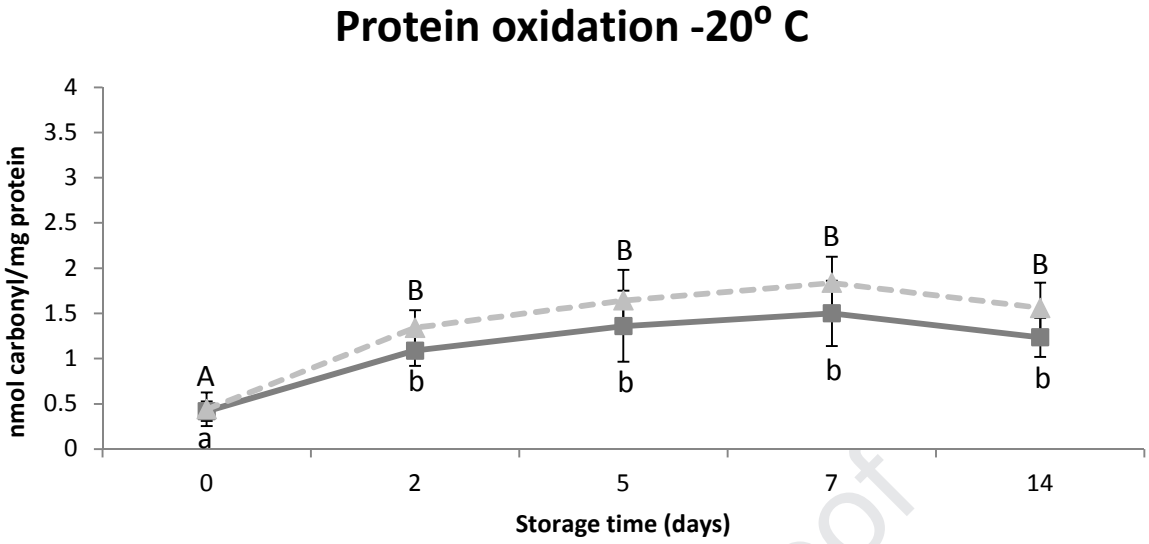
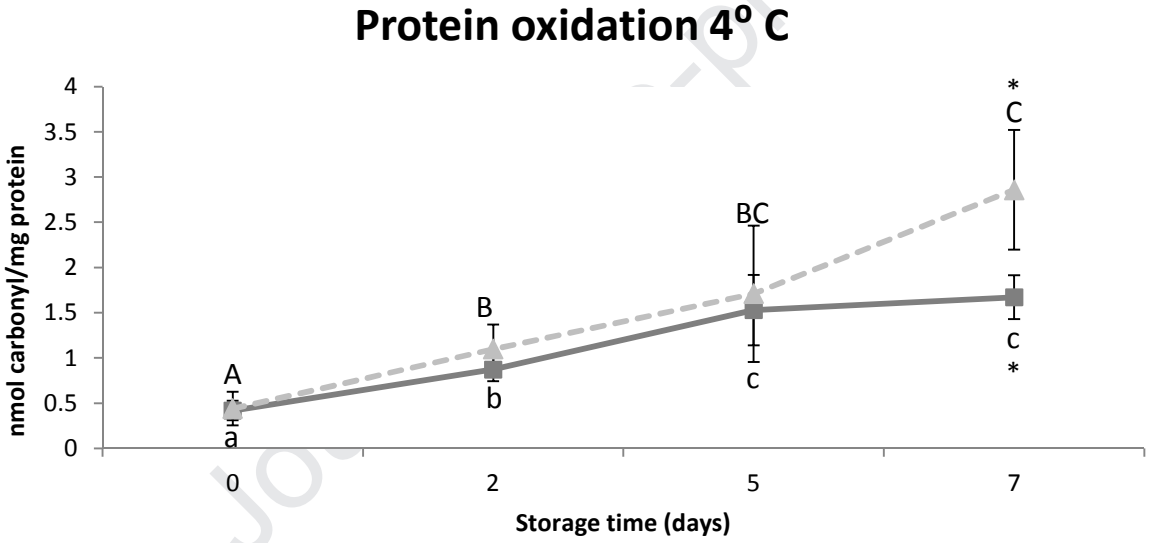

Lipid oxidation 8°C
TBARs value (mg MDA/kg sample)
90
80
70
60
50
40
30
20
10
0
A
a
B
b
c
B
B
c
0
2
5
7
Storage time (days)

Figure 1: TBARs values (mg MDA/ kg sample) for control (—■—) and plasma (--▲--) A) at -20 ºC, B) at 4 ºC and C) at 8 ºC. Different letters (lowercase for control and uppercase for plasma) indicate significant differences on the TBARs values during days of storage at the same temperature ($p < 0.05$). No significant differences between control and plasma treated samples at the same storage day were found ($p>0.05$). All the differences are measured separately for 4 ºC, 8 ºC and -20 ºC.


Protein oxidation -20º C
nmol carbonyl/mg protein
Storage time (days)
0
2
5
7
14
A
a
B
b
B
b
B
b
B
b


Protein oxidation 4º C
nmol carbonyl/mg protein
Storage time (days)
0
2
5
7
A
a
B
b
BC
c
*
C
c
*

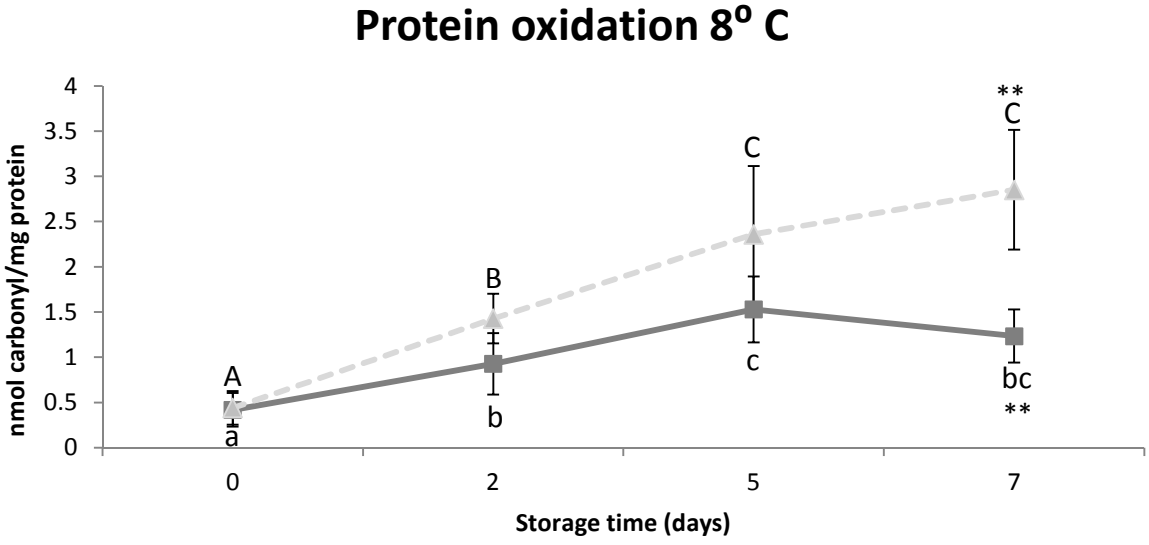

Protein oxidation 8º C
nmol carbonyl/mg protein
Storage time (days)
0
2
5
7
A
a
B
b
C
c
**
C
bc
**

*Figure 2: Carbonyl content values (nmol carbonyl/ mg protein) for control (—■—) and plasma (- -▲- -) A) at -20 ºC, B) at 4 ºC and C) at 8 ºC. Different letters (lowercase for control and uppercase for control) indicate significant differences on the carbonyl content value during days of storage at the same temperature ($p < 0.05$). Stars show significant differences between treatments at the same storage day being * ($p<0.05$) and ** ($p<0.005$). All the differences are measured separately for 4 ºC, 8 ºC and -20 ºC.*

Highlights

- No significant effect on lipid oxidation was found in mackerel at 80 kV for 5 min.
- Fatty acid profile of mackerel fillets was not significantly affected by plasma.
- Nutritional quality indices were not compromised at 80 kV for 5 min.
- CAP treatment accelerated the formation of carbonyls related to protein oxidation.