



Assessment of lipid oxidation in Atlantic mackerel (*Scomber scombrus*) subjected to different antioxidant and sous-vide cooking treatments by conventional and fluorescence microscopy methods

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ABSTRACT

The aim of this study was to assess lipid oxidation development in sous-vide cooked Atlantic mackerel during chilled storage by fluorescence microscopy coupled with chemical, physical and data analysis methods. Sous-vide-cooking at 70 °C and 80 °C for 10 and 20 min was performed with and without use of antioxidants. Fluorescence micrographs of lipids extracted from the fish samples on 1st, 3rd, 7th, and 9th day of chilled storage at 4 ± 1 °C, were acquired in the wavelength region of λ_{ex} 475/40 and λ_{em} 530/50 (44 FITC filter). The obtained images were numerically processed and the resulting data correlated with 2-thiobarbituric acid reactive substances (TBARS) determined by conventional chemical method of lipid oxidation analyses. Total lipid content and color characteristics of mackerel fillets were analyzed as well. The yellowness of the fish flesh significantly correlated ($p < 0.05$, $R = 0.859$) with conjugated trienes generated from thermal polymerization of lipids during chilled storage of sous-vide cooked mackerel. Results showed that higher temperature and cooking times generally accelerated lipid oxidation in mackerel samples prepared both with and without addition of antioxidants in terms of conjugated trienes and tetraenes. However, the addition of antioxidants enhanced the lipid oxidation stability of cooked mackerel fillets.

1. Introduction

Fish has long been recognized as a valuable dietary source of high-quality easily digestible proteins, long-chain omega-3 fatty acids – docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), fat-soluble vitamins (E and D), as well as essential minerals and vitamins (Tacon & Metian, 2013). However, because fish muscle lipids are highly susceptible to oxidation due to their high content of polyunsaturated fatty acids, the sensory and nutritional quality of fish can be rapidly impaired without appropriate handling and processing, leading to rancidity and development of off-flavours. Though fish is sometimes eaten raw (e.g., sushi, sashimi), it is generally cooked prior to consumption. Cooking leads to inactivation of enzymes and pathogens, as well as enhancement of palatability. Nevertheless, to preserve sensory and nutritional quality during thermal processing of fish, careful control of technological parameters is required. Cooking procedures including boiling, frying, roasting and steaming, promote a cascade of adverse physicochemical reactions among which lipid oxidation is one of the most pronounced (Frankel, 2005; Khalil & Mansour, 1998). The

primary lipid peroxidation is followed by a number of chemical transformations including break down of hydroperoxides and formation of a wide range of secondary lipid oxidation products, including thiobarbituric acid reactive substances (TBARS) and volatile compounds, which impart off-flavours in oxidized fish (Frankel, 2005; Shahidi & Zhong, 2010). Heat treatment has been proven to induce lipid oxidation due to disruption of cell membranes and denaturation of heme-proteins (Kristinova, Mozuraityte, Aaneby, Storro, & Rustad, 2014), as well as liberation of free iron, which acts as a strong pro-oxidant promoting lipid oxidation in the product (Grunwald & Richards, 2006). This in turn impairs the nutritional and sensory properties of fish products, resulting in reduced salability (or reduced commercial or market value). The extent of quality loss depends on cooking regimes, presence of metal ions and pro-oxidants and initial quality of raw material (Hu et al., 2017; Sobral, Cunha, Faria, & Ferreira, 2018). Both temperature and cooking time have an effect on lipid oxidation reactions in fish products (Crobotova, Mozuraityte, Standal, & Rustad, 2019). The addition of antioxidants before heat treatment can reduce these negative effects (Crobotova et al., 2019; Sampels, 2015; Shahidi & Zhong, 2010).

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However, more gentle cooking methods for better preservation of bioactive compounds and lipids from heat-induced oxidation, are needed.

The growing consumer demand for minimally processed foods with extended shelf life, has resulted in the development of minimal cooking techniques, one of which is sous-vide cooking involving thermal treatment of a vacuum-packed product at lower temperatures and process times comparing to conventional cooking (Baldwin, 2012). Sous-vide cooking method has been extensively adopted by catering services and food industry to ensure better sensory and nutritional quality with a longer shelf life of the product compared to traditional thermal processing technologies (Baldwin, 2012).

Atlantic mackerel (*Scomber scombrus*) is one of the most important commercial pelagic fish species in Europe (EUMOFA, 2018) and is highly appreciated due to its high content of polyunsaturated fatty acids. This fish was ranked among the top small pelagic commodity groups both in volume and value in 2017 and 2018 in Europe (EUMOFA, 2018), playing an important role in the national economy of the countries involved in the catch and processing of these species. However, Atlantic mackerel is a very perishable fish due to the high content of long-chain omega-3 fatty acids, which are prone to oxidation leading to rancidity and to quality loss (Standal et al., 2018). Therefore, it is very important to apply a cooking technology that can preserve the nutritional and sensory quality of the fish, while reducing the rate of lipid oxidation reactions and extending the shelf life.

Although several studies on sous-vide cooking of fish have been published so far (García-Linares, Gonzalez-Fandos, García-Fernandes, & García-Arias, 2004; Gonzalez-Fandos, Villarino-Rodríguez, García-Linares, García-Arias, & García-Fernandes, 2005; Iborra-Bernad, Tárrega, García-Segovia, & Martínez-Monzó, 2014; Khalil & Mansour, 1998; Mol, Özturan, & Coşansu, 2012; Oz & Seyyar, 2016), the information on how lipid oxidation in Atlantic mackerel is affected by different cooking procedures and use of natural antioxidants, is still missing.

Lipid degradation has generally been assessed by conventional methods of analysis focusing mainly on detection of the primary (hydroperoxides, conjugated dienes, etc.) and secondary (TBARS, etc.) oxidation products (Frankel, 2005). However, it has been also shown that both types of lipid oxidation products may react with other food constituents (phospholipids, free amino acids, peptides, etc.) to produce fluorescent compounds (Aubourg, 1999; Leake & Karel, 1985; Schaich, 2008). Oxidized lipids and fats emit light in the near UV-region. This specific autofluorescence has mostly been studied spectrophotometrically to estimate the oxidation rate of foods containing polyunsaturated lipids (Leake & Karel, 1985; Schaich, 2008). Therefore, investigation of such interaction structures by fluorescence spectrometry methods can become a complementary tool for estimation of lipid oxidation (Schaich, 2008; Aubourg, 1998 and, 1999; Crobotova & Rustad., 2017).

The main benefits of fluorescence spectroscopy methods are their high speed of implementation (taking only a few minutes), low sample requirements, high sensitivity, non-invasive assay, as well as low cost and simplicity as compared to conventional analytical methods of analysis (Aubourg, 1999). Regardless a big number of studies on spectrofluorometric analysis of oxidized oils, to the best of our knowledge, there is no information available on the use of fluorescence microscopy technique to rapidly assess the extent of oxidized lipids in seafood products during storage.

Fluorescence microscopy is a powerful and commonly used technique used in many areas of life sciences including food chemistry, biology and medicine. Due to its inherent selectivity and the large spectral range of different fluorophores, fluorescence microscopy allows imaging of cellular, subcellular and molecular structures simultaneously (Lichtman & Conchello, 2005). In addition, fluorescence microscopy techniques have been shown to be efficient for non-invasive assessment of texture changes in fish muscle (Crobotova, Mozuraityte,

Standal, & Rustad, 2018) and monitoring of lipid peroxidation in model lipid membranes (e.g. liposomes) and cell membranes (Greene, Lincoln, & Cosa, 2017; Takahashi, Shibata, & Niki, 2001). However, in most of the studies the visualization and control of lipid oxidation can only be performed with the use of special fluorescent dyes (Chotimarkorn, Ohshima, & Ushio, 2005; Krumova, Greene, & Cosa, 2013; Takahashi et al., 2001). A direct fluorescence microscopy analysis was carried out with 3-PeDPP (Chotimarkorn et al., 2005) and DPPH oxide (Takahashi et al., 2001) to monitor lipid oxidation in tuna oil and in the membrane of live cells, respectively. These studies showed that the fluorescence intensity of 3-PeDPP and DPPH oxide could be successfully used in the quantitative analysis of hydroperoxides in a good correlation with the lipid peroxidation extents determined by conventional methods of analyses. However, a significant drawback limiting the use of conventional oxidation-sensitive dyes for visualization and quantification of reactive oxygen species (ROS) in the living cells by fluorescence microscopy is that either the non-oxidized form (for example, fluorescein, cis-PnA, or BODIPY-FL) nor the oxidized form (in case of resorufin derivatives), is detected. As a result, important corrections for dye uptake and differentiation cannot be performed (Krumova et al., 2013). Another challenge is that many fluorescent dyes used for staining have fluorescence excitation and emission wavelengths inside the UV region, causing lipid peroxidation by itself (Chotimarkorn et al., 2005). Therefore, new possibilities for non-invasive fluorescence microscopy control of lipid peroxidation based on auto-fluorescence signal coming from lipid oxidation products, should be explored.

The aim of this work was to study and exploit capabilities of fluorescence microscopy in terms of sensitivity and precision to assess lipid oxidation stability of Atlantic mackerel subjected to antioxidant treatment followed by sous-vide cooking and chilled storage on the basis of intrinsic fluorescence of lipid oxidation products.

Hence, the aim of the present study was to elucidate the effect of different cooking regimes (time and temperature) and natural antioxidants (rosemary extract and rosemary extract with ascorbyl palmitate) on lipid oxidation stability of Atlantic mackerel during chilled storage determined by conventional and novel fluorescence microscopy methods.

2. Materials and methods

2.1. Sample preparation and sous-vide cooking

The Atlantic mackerel fillets (*Scomber scombrus*) used as raw material in the present study were supplied frozen from Pelagia AS (Selje, Norway) in January 2017. Average weight of the resulting skin-on fillets was 89 ± 9.6 g, with an average length of 17 ± 1.4 cm. A total of 45 fishes (90 mackerel fillets) were involved in the study. Fish fillets were thawed at 0 °C for 5 h. Two fillets were placed in polyamide/polyethylene bags having the following parameters: thickness of 90 µm, gas permeability (O₂, N₂ and CO₂) of up to 60 cm³/m²*d*bar and water vapor permeability of up to 4 g/m²*d (BST-090 type bags) from “Three Seal Bags” series (Rolf Bayer Vacuumverpackung GmbH, Veitsbronn, Germany), and further heat-sealed using a vacuum sealing machine (Webomatic Vacuum packaging system, Super max, 3000 sensor, Frankfurt, Germany) with extent of vacuum 99.6%. Temperature data loggers type SL52T (Signatrol, Tewkesbury, UK) with an embedded thermocouple probe were manually inserted into the vacuum bags along with the fish fillets to monitor fluctuations of temperature over the sous-vide cooking and chilling experiment. The antioxidant-treated and untreated fish fillets were sous-vide cooked by applying different time-temperature treatments and subjected to further chilled storage according to the planned experimental design displayed in Table 1. Two types of commercial antioxidants were used: TR25 (rosemary extract and mix of tocopherols) and RPT40 (rosemary extract, α-tocopherol and ascorbyl palmitate), (commercial antioxidant supplier, Herentals, Belgium). Due to an agreement between the authors and the

Table 1
Experimental design for sous-vide cooking and chilled storage of mackerel samples.

Experimental conditions			
Cooking temperature (°C)	Cooking time (min)	Use of antioxidant ^a	Sampling day
70	10	0	1, 3, 9, 15
		A	
80	10	0	1, 3, 9, 15
		A	
70	20	0	1, 3, 9, 15
		A	
80	20	B	1, 3, 9, 15
		0	
		A	
		B	

^a 0 – no use of antioxidant, A – antioxidant TR25 (rosemary extract), B – antioxidant RPT40 (rosemary extract, α -tocopherol and ascorbyl palmitate).

antioxidant-producing company on nondisclosure of the types of antioxidants provided by the company for research purposes and used in the study, their full names are not displayed in the paper. The fish was treated with the antioxidants before vacuum-packing in the following amounts: 1000 ppm of TR25 or 2000 ppm of RPT40. The antioxidants were manually distributed over the whole fillet surface by gentle rubbing, and further left for 1 h to be absorbed by the fish flesh.

The fish samples were cooked in two water baths (Grant, UK) set at 70 °C or 80 °C for 10 or 20 min. Immediately after the heat treatment, they were rapidly chilled with solid ice and put in chilled storage (in a cold room) at 4 ± 1 °C for 1, 3, 9 and 15 days (Table 1). The chilling temperature of 4 ± 1 °C was selected in the present experiment due to previously good quality results obtained during chilled storage of sous-vide cooked Atlantic mackerel at the same temperature regime (Crobotova et al., 2019). In each sampling day, the chilled sous-vide treated mackerel samples were removed from the vacuum packages and analyzed. Control samples (raw mackerel fillets) were thawed at 0 °C overnight and vacuum-packed along with other experimental samples, and analyzed after one day of chilled storage at 0 ± 1 °C. Changes in primary and secondary lipid oxidation products as affected by different cooking regimes and storage duration, were studied. Analyses were performed in four replicates for each vacuum package containing two mackerel fillets. Due to limited amount of material and time resources, the efficiency of antioxidant RPT40 was tested only for mackerel samples subjected to sous-vide cooking for 20 min and analyzed in the first and last day of chilled storage.

2.2. Chemical and physicochemical assays

2.2.1. Lipid extraction

Lipids were extracted from raw (control) and sous-vide cooked mackerel samples by a mixture of chloroform-methanol-water by the Bligh and Dyer (1959) method. The fish fillets were previously cut into small pieces, minced with a kitchen blender (Bosch 600 W, Gerlingen, Germany) and the obtained mince was taken (10 g) for extraction of lipids. Chloroform extracts of lipids were collected and further used for determination of primary and secondary lipid oxidation products.

2.2.2. Primary and secondary products of lipid oxidation

Primary and secondary lipid oxidation products were quantified by determination of peroxide value (PV), conjugated dienes (CD), trienes (CT), tetraenes (CTr) and 2-thiobarbituric acid reactive substances (TBARS), as follows.

PV was measured by using the iodometric titration method described in AOCS official methods (Cd 8b-90) (AOCS, 2003). The end point of titration was assessed potentiometrically with an automatic titrator (TitroLine 7800, Xylem Analytics, Mainz, Germany) coupled

with a platinum electrode (Pt 62). The analysis was performed in four replicates and the results were expressed in meq active oxygen/kg lipids as a mean value \pm SD.

A modification of methods by Aubourg (1998) and Mozuraitye, Kristinova, Standal, Evensen, and Rustad (2017) was used for the spectrophotometric measurement of conjugated structures in fish lipids: CD, CT and CTr. Briefly, the absorbance of 200 μ L chloroform extracts of lipids was measured in a 96-well plate against the solvent (chloroform) with a plate reader (TECAN, Grödig, Austria) at 233, 268 and 315 nm corresponding to CD, CT and CTr formation, respectively (Igarashi & Miyazawa, 2005). If the absorbance exceeded 0.8 absorbance units (AU), lipid extracts were diluted with the solvent (1:2–1:8, v/v), and the measurement was repeated.

The amount of the conjugated structures was calculated according to Aubourg (1998), as follows:

$$CD/CT/CTr = A \cdot V/w \quad (1)$$

where A is the absorbance reading, V denotes the volume (ml) of the chloroform extract and w is the mass (mg) of the lipid material (oils) in the extract measured.

The results were expressed as CD, CT, CTr values in ml/mg. The analysis was performed in four replicates for each sample, and the average with standard deviation was calculated.

TBARS were determined according to the method described by Ke and Woyewoda (1979), by using a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA), as follows. Lipids extracted from mackerel muscle (around 10 mg) were weighed in conical-bottom glass centrifuge tubes (10 mL, KIMAX, Fisher Scientific, US). Further, 5 ml of 2-thiobarbituric acid (TBA) working solution prepared from mixing 180 ml of TBA stock solution, 120 ml of chloroform, 15 ml of 0.3 M sodium sulphite solution and 9,45 ml of 3% BHT solution in ethanol, was added to the tubes, and the obtained mixture was mixed on a whirl mixer for 15–20 s. The tubes were then incubated in a boiling water bath at a temperature of 98–100 °C for 45 min. After incubation, the tubes were cooled down in cold water, and 2.5 ml of 0.28 M trichloroacetic acid (TCA) solution was added and mixed by inversion. The tubes were then centrifuged for 5–10 min at 5000 g at room temperature to ensure a good separation of the pink water phase from the chloroform phase. The optical density of the pink-colored water phase was determined at 538 nm. The analysis was carried out in four parallels and the results were expressed in μ mol TBARS/g lipids as a mean value \pm SD.

2.2.3. Color parameters

Color parameters of the fish fillets were measured instrumentally using a Minolta Chroma meter CR-400 (Konica-Minolta, Osaka, Japan). Before starting the analysis, the instrument was calibrated with a standard white plate. The measurements were performed on pre-selected locations at the surface of each mackerel fillet at room temperature. In the present paper, just yellowness recorded in color coordinates of b^* (yellowness, $b^* > 0$, blue < 0) according to the lab scale established by Commission Internationale de l'Éclairage (CIE, 2001), is reported as an indicator of visual changes in lipid oxidation stability. Yellowness was determined on each samples in three readings and the average was calculated.

2.2.4. Fluorescence microscopy of extracted lipids

According to our previous investigations (Crobotova & Rustad, 2017) conducted in agreement with other studies (Aubourg, 1999; Wold & Mielnik, 2000), fluorescence intensity in the UV wavelength region (300–400 nm) shifts towards the region of blue-green visible part of the spectrum (450–570 nm) with progress of lipid oxidation (propagation-termination stage). Moreover, according to our preliminary investigations (Crobotova & Rustad, 2017), the fluorescence intensity in the second wavelength region highly correlated with secondary lipid

oxidation products.

Therefore, in the present study we went further, aiming at studying the possibility to assess oxidation status of extracted lipids by non-invasive fluorescence microscopy approach, using just small amounts of sample.

Direct fluorescence visualization of lipids extracted from sous-vide cooked mackerel samples was performed on a Zeiss Axio Imager Upright microscope (Oberkochen, Germany) equipped with Zeiss Plan-Apochromat 20 × objective and AxioCam ERc5s with a filter set 44 FITC (EX 475/40, FT 500, EM 530/50). The mean fluorescence intensity (MFI) of extracted lipids in the acquired images was determined after subtraction of background, according to previously developed protocol (Crobotova et al., 2018). The image processing system used was the software package ImageJ, v1.51k 1 (National Institutes of Health, Bethesda, MD, USA).

2.3. Experimental design and statistical analysis

Statistical analysis and data processing were conducted using SigmaPlot software (Systat Software Inc., San Jose, California, USA), version 16.1.15. Statistical significance of the experimental data was verified by using Student's t-test and Analysis of Variance (ANOVA). To establish a relationship between certain parameters, Pearson correlations were calculated. Differences were considered significant at $p < 0.05$.

Multiple regression analysis was performed to explain the dynamics underlying the quality deterioration process in sous-vide cooked mackerel during chilled storage and to identify the contribution of each of the process parameters to detrimental changes occurring in the product.

3. Results and discussion

3.1. Total lipid content

No significant variation in lipid content was found in sous-vide cooked mackerel samples during the storage period (Fig. 1). However, it was slightly lower in almost all cooked fish samples compared to raw mackerel fillets used as control samples ($28.9 \pm 0.4\%$), probably due to some heat-induced leakage of fat during sous-vide treatment.

3.2. Primary and secondary lipid oxidation products

Peroxide value and conjugated lipid structures were used as a measure of primary lipid oxidation products in the analyzed mackerel samples.

The PV of almost all mackerel samples cooked at 70 °C displayed a significant ($p < 0.05$) increase after 1-day chilled storage compared to initial PV of raw mackerel (control samples) of 18.0 ± 0.9 meq active oxygen/kg lipids (Fig. 2A). However, the PV was significantly lower in all mackerel samples subjected to sous-vide cooking at 80 °C for 10 or 20 min (Fig. 2B) compared to the control. Moreover, none of the samples when applying a temperature of 80 °C exceeded the acceptable level of 5 meq active oxygen/kg lipids (CODEX STAN, 2017), denoting

satisfactory quality of the fillets with regard to oxidative lipid stability. However, almost all the 70 °C samples had significantly higher PV-values than those established by the CODEX STAN for fish oils after 1 and 3 days of storage. Taking into account that raw mackerel was also characterized by a significantly higher PV-value than the admissible level of 10 meq active oxygen/kg lipids (CODEX STAN, 2017), we hypothesize that this phenomenon occurred due to initially heterogeneous composition of the fish within one batch (differences in dark muscle and blood content, etc.). Thus, lipid oxidation process could be already in propagation stage in some raw mackerel fillets before sous-vide cooking experiment. Nevertheless, mackerel samples treated with antioxidants before sous-vide cooking at 70 °C for both 10 or 20 min, had significantly lower values of PV compared to untreated samples (Fig. 2A). This reveals that natural antioxidants TR25 and RPT can successfully retard primary lipid oxidation in cooked pelagic fish during storage.

A large variation of values was found in the results for amount of CD, CT and CTr in sous-vide cooked mackerel samples during chilled storage (Fig. 2C–D and 3A–D). However, conjugated structures for all mackerel samples cooked at 80 °C, and the majority of samples cooked at 70 °C displayed a significant ($p < 0.05$) increase along the chilled storage compared to CD, CT and CTr values of control samples of 0.05 ± 0.01 , 0.08 ± 0.02 and 0.29 ± 0.06 ml/mg, respectively (Fig. 2C–D and Fig. 3). At the same time, there was an insignificant increase in these conjugated lipid structures after sous-vide treatment at 80 °C compared to cooking at 70 °C. This tendency may be because at elevated temperatures lipids undergo thermo-oxidation, polymerization, hydrolysis, cyclization, and isomerization leading to formation of conjugated di-, tri-, and tetradene groups (Lalas, 2009). Thus, lipids containing methyleneinterrupted dienes or polyenes get a shift in the position of the double bond during the oxidation due to isomerization and the formation of CD or other conjugated systems such as CT and CTr (Lalas, 2009). The amount of CD increased with the oxygen uptake during the early stages of oxidation, leading to generation of secondary lipid oxidation products, along with the peroxides (Halliwell & Gutteridge, 1989).

Similarly to PV, TBARS as a measure of secondary lipid oxidation products, also increased after sous-vide cooking at 70 °C and 3-day chilled storage (Fig. 4A–B) of mackerel samples. Also, TBARS of control samples had significantly ($p < 0.05$) higher values (2.17 ± 0.27 nM/g muscle) than mackerel fillets subjected to sous-vide treatment at 80 °C for 10 or 20 min (Fig. 4B).

As previously suggested, this phenomenon can be explained by initially higher values of lipid oxidation products generated in some mackerel samples during storage and transportation of the fillets before sous-vide experiment. Moreover, a significant correlation between PV and TBARS in mackerel samples ($p < 0.05$, $R = 0.985$) shown in Fig. 5A, added up to some additional evidence in support of the theory on generation of secondary oxidation products from the breaking down of lipid peroxides (Frankel, 2005).

Nevertheless, from the experimental data shown in Fig. 4B, it is clearly seen that even highly oxidized mackerel samples after sous-vide treatment at 70 °C for 10 or 20 min, had significantly ($p < 0.05$) lower values of TBARS if they were treated with antioxidants. The antioxidant

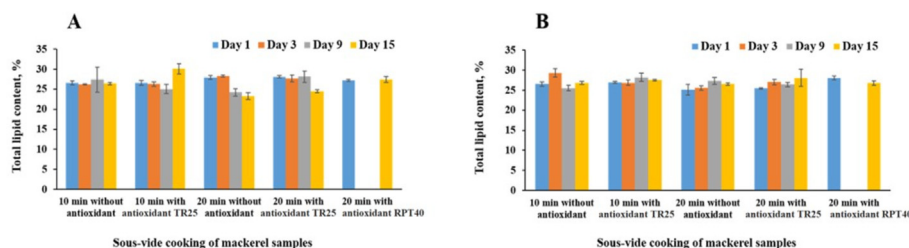


Fig. 1. Total lipid content of mackerel samples subjected to sous-vide cooking at (A) 70 °C and (B) 80 °C.

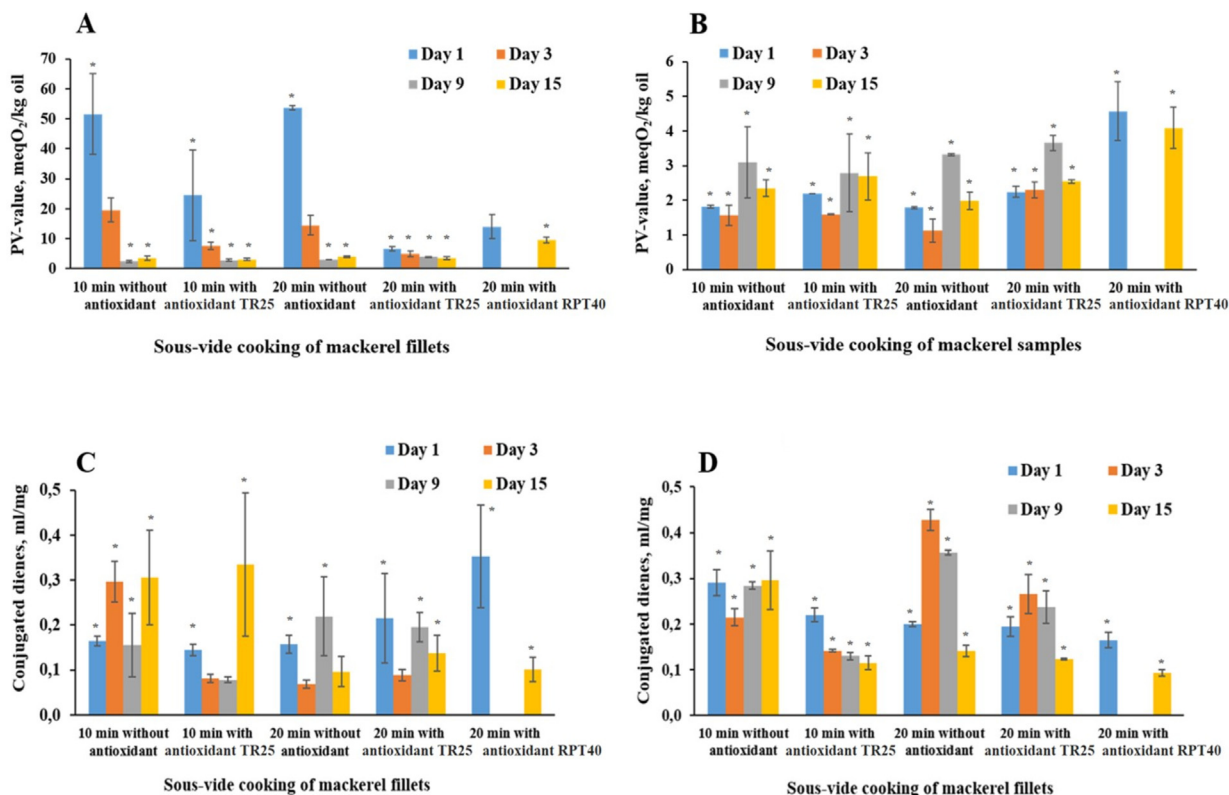


Fig. 2. Primary products of lipid oxidation formed during chilled storage of fish: amount of lipid peroxides expressed as PV-value in mackerel samples subjected to sous-vide cooking at (A) 70 °C and (B) 80 °C; conjugated dienes in mackerel samples subjected to sous-vide cooking at (C) 70 °C and (D) 80 °C. The asterisk indicates that the values is significantly different ($p < 0.05$) from the mean of the control sample (raw mackerel).

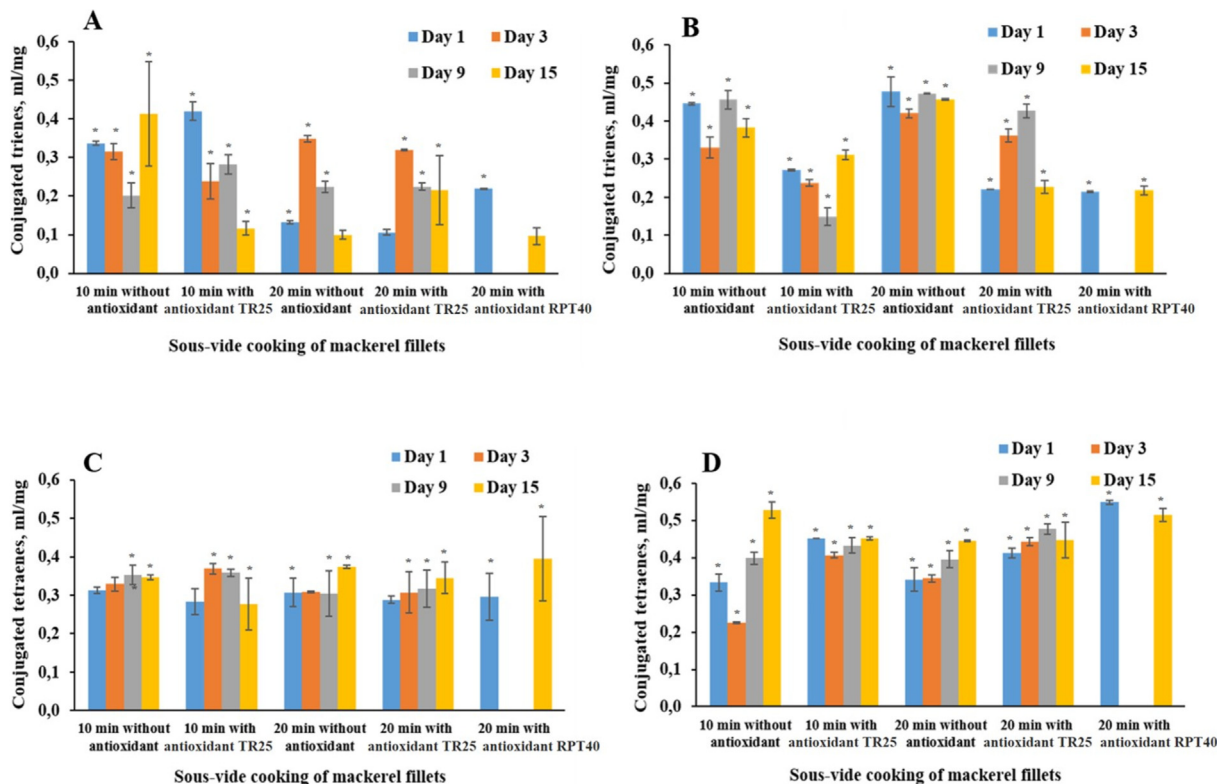


Fig. 3. Conjugated tri- and tetraene structures formed during chilled storage of fish: amount of conjugated trienes in mackerel samples subjected to sous-vide cooking at (A) 70 °C and (B) 80 °C; conjugated tetraenes in mackerel samples subjected to sous-vide cooking at (C) 70 °C and (D) 80 °C. The asterisk indicates that the values is significantly different ($p < 0.05$) from the mean of the control sample (raw mackerel).

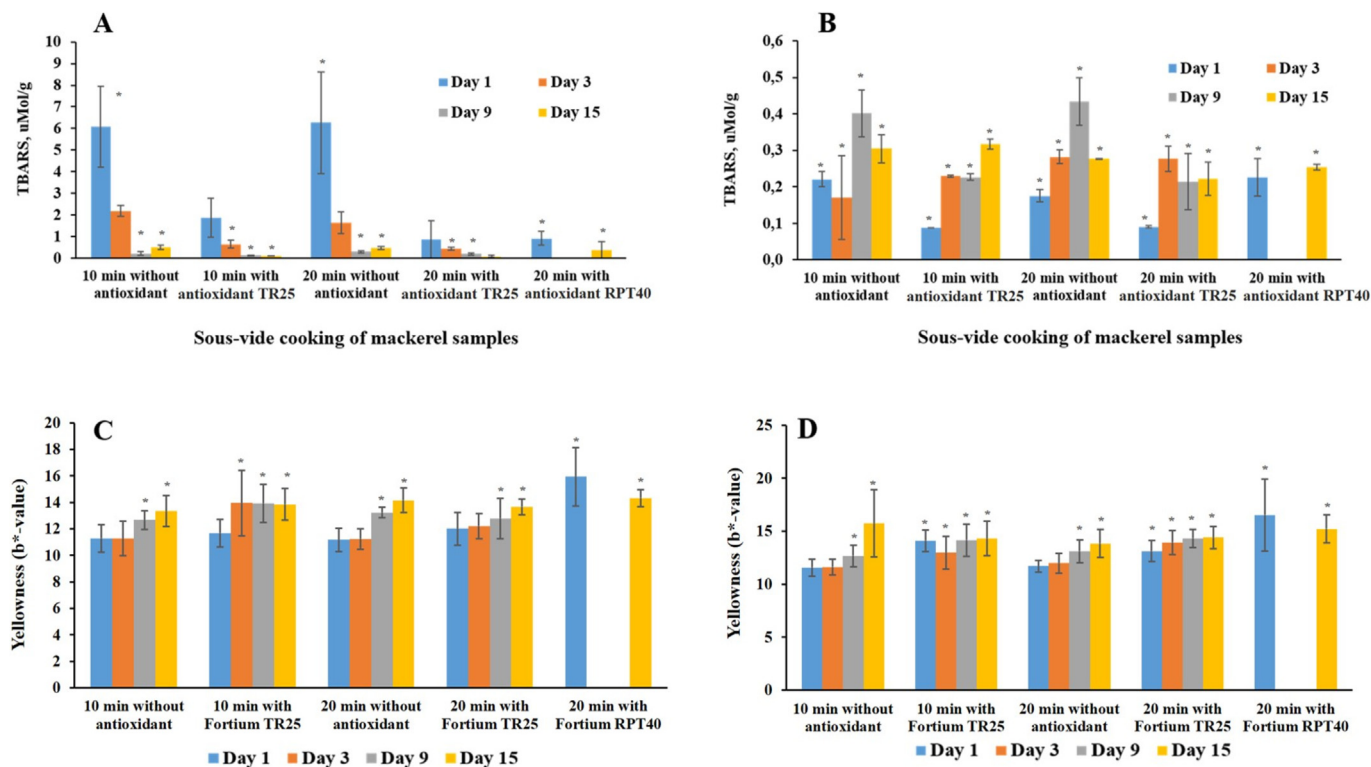


Fig. 4. Secondary products of lipid oxidation in and color change during chilled storage of fish: TBARS-values for mackerel samples subjected to sous-vide cooking at (A) 70 °C and (B) 80 °C; yellowness (b*-values) of mackerel samples subjected to sous-vide cooking at (C) 70 °C and (D) 80 °C. The asterisk indicates that the values is significantly different ($p < 0.05$) from the mean of the control sample (raw mackerel).

efficiency regarding the delay of secondary lipid oxidation is noticeable also for majority of mackerel samples after sous-vide cooking at 80 °C for 10 or 20 min (Fig. 4C). These results denote that natural antioxidants TR25 and RPT40 can be successfully applied before sous-vide

cooking of fish to delay the formation of both primary and secondary lipid oxidation products during subsequent storage.

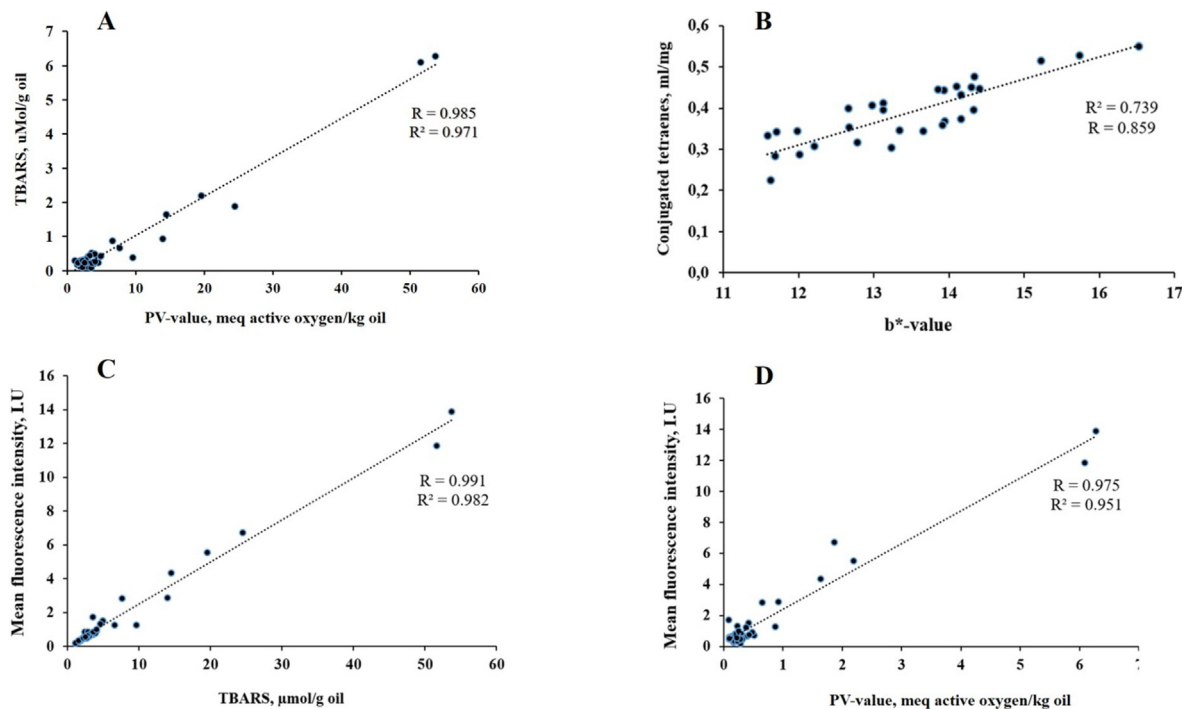


Fig. 5. Influence of primary and secondary products of lipid oxidation on color parameters and auto-fluorescence of lipids extracted from sous-vide mackerel during chilled storage: A) correlation between PV and TBARS; B) correlation conjugated tetraenes and b*-value; C) correlation between total fluorescence intensity of extracted lipids and TBARS; D) correlation between total fluorescence intensity of extracted lipids and PV.

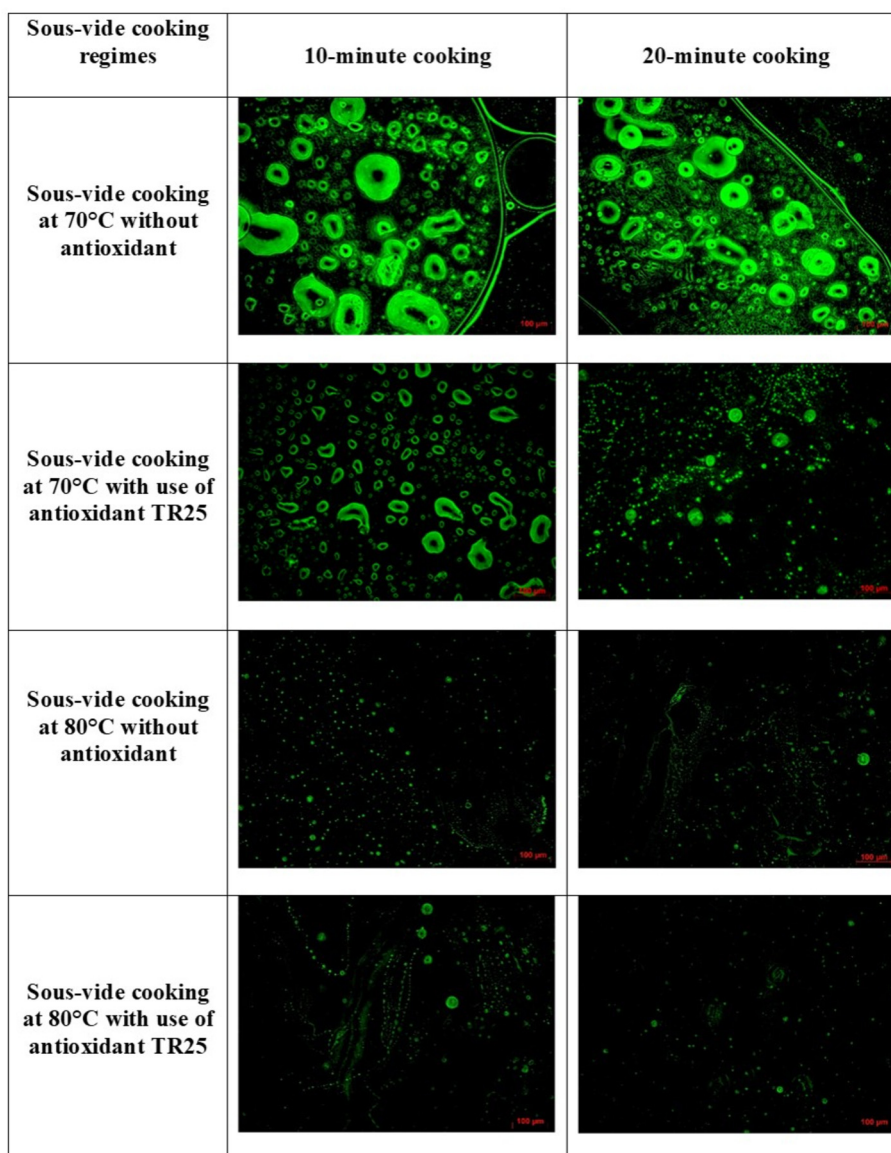


Fig. 6. Examples of selected fluorescence micrographs for lipids extracted from sous-vide cooked mackerel samples in the first day of storage.

3.3. Color parameters (yellowness)

Yellowness (b^* -values) displayed slightly higher values in all sous-vide treated samples throughout the storage period (Fig. 4C–D) compared to initial raw mackerel fillets (10.04 ± 0.94). This is in agreement with results in the studies of Karoui and Hassoun (2017) and Crobotova et al. (2019). The increase in yellowness of sous-vide cooked mackerel throughout the storage time is most likely due to accumulation of yellowish-colored compounds generated by decomposition and polymerization of primary products of lipid oxidation (Nguyen et al., 2013), giving positive correlation values (R) of 0.859 ($p < 0.05$) with conjugated tetraenes (Fig. 5B).

3.4. Fluorescence microscopy of extracted lipids

The identified auto-fluorescent species in the wavelength region of λ_{ex} 475/40 and λ_{em} 530/50 (Fig. 6) increased gradually in size and amount along with an increase in primary and secondary lipid oxidation products expressed as PV and TBARS, respectively.

Moreover, image processing of the obtained micrographs showed that fluorescence intensity of the samples significantly correlated with

TBARS data ($p < 0.05$, $R = 0.991$), as shown in Fig. 5C. It reveals the fact the conjugated 2-thiobarbituric acid reactive substances have direct influence on further formation of auto-fluorescent lipid oxidation products. At the same, the total intensity of obtained micrographs significantly correlated with primary product of lipid oxidation expressed as PV-value ($p < 0.05$, $R = 0.975$), as displayed in Fig. 5D. This phenomenon can be explained by the breakdown of both primary and secondary lipid oxidation products generating other fluorescent compounds (Frankel, 2005), which can be further quantified by fluorescence spectroscopy techniques (Aubourg, 1999). In this regard, fluorescence microscopy opens up a new way to the fast, non-destructive and reliable control of lipid oxidation stability of oils and extracted lipids, and may thus be used as indirect method of analysis applied to very small amounts of lipid samples in the future.

4. Conclusion

The present study displayed the influence of different sous-vide cooking regimes, chilled storage and use of antioxidants on lipid oxidation in Atlantic mackerel fillets. Sous-vide cooking and subsequent chilled storage negatively influence oxidative lipid stability in mackerel

fillets with regard to primary and secondary lipid oxidation products. However, it was found that the used natural antioxidants based on rosemary extract with mix of tocopherols, and ascorbyl palmitate with α -tocopherol can effectively retard lipid oxidation in cooked fish during chilled storage, and thus are suggested for application in the food industry. Also, values of CTr were significantly correlated with b*-values of the fish flesh, which suggests the contribution of thermally polymerized lipids on further yellowing of the fish flesh. A novel fluorescence microscopy method proposed in the paper also proved its high efficiency in identifying signs of auto-fluorescence of lipid oxidation products in mackerel samples. Fluorescence intensity of the samples significantly correlated with PV and TBARS values in the wavelength region of λ_{ex} 475/40 and λ_{em} 530/50.

Therefore, it can be concluded that the endogenous fluorescent species of lipid oxidation identified by fluorescence microscopy unfold new opportunities for rapid, label-free and non-invasive determination of lipid oxidation status in fish fillets.

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