Title: A non-invasive approach to assess texture changes in sous-vide cooked Atlantic mackerel during chilled storage by fluorescence imaging

Authors: Janna Cropotova[1], Revilija Mozuraityte[2], Inger Beate Standal[2], Turid Rustad[1]

Affiliations:

[1]Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway

[2]SINTEF Oceans, Trondheim, Norway

Corresponding author: Janna Cropotova

Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway

Email: janna.cropotova@ntnu.no

Abstract

The aim of this study was to investigate the potential of fluorescence microscopy coupled with chemical, physical and data analysis methods for reliable and non-invasive detection of changes in texture parameters of sous-vide cooked Atlantic mackerel during chilled storage. Fluorescence micrographs of cook loss and connective tissue of the fish samples after sous-vide treatment at 60°C and 75°C for 10, 15 and 20 minutes taken in the 1st, 3rd and 7th day of chilled storage were acquired. The obtained images were numerically processed and the resulting data was directly correlated (R=0.960) with the total collagen content determined by a chemical method. Partial least squares analysis was applied to derive statistically significant regression models revealing the influence of each of sous-vide regime parameters on changes in total collagen content and texture parameters of Atlantic mackerel during chilled storage. Results showed that both collagen integrity and firmness of mackerel flesh were significantly ($p<0.05$) affected by the temperature of sous-vide treatment and duration of chilled storage, leading to gradual softening of the fish tissue due to degradation of collagenous tissue.

Keywords: fluorescence microscopy, Atlantic mackerel, sous-vide cooking, chilled storage, collagen content

1. Introduction

Atlantic mackerel (*Scomber scombrus*) is one of the most valuable fish species in Europe for its high nutritive value and bioactive compounds such as essential long-chain omega-3 fatty acids – docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), fat-soluble vitamins (E and D) and easily digestible proteins, which all together make it a complete source of essential nutrients (Venugopal, 2009). This pelagic fish have been widely used for different products such as smoked mackerel, mackerel pates, ready-to-eat fishcakes and mackerel fillets in tomato sauce. In terms of value, their production has lately become more and more important due to the increasingly growing trend towards healthier eating and increased consumer demand for safe, nutritional and palatable food products with documented health benefits. However, conventional thermal processing of fish raw material at high temperatures, may lead to a loss of fat-soluble vitamins and omega-3 fatty acids (Garcia-Linares et al., 2004). In order to preserve these healthy components from thermal degradation, there is a need for a mild-heat treatment such as sous-vide cooking. Sous-vide cooking is defined as thermal treatment of vacuum-packed food under controlled conditions of temperature and time (Baldwin, 2012), and is a promising cooking technique aiming to better preserve nutritional and sensory quality of food. This method implies the use of lower heating temperatures (below 100°C) and longer cooking times compared to traditional cooking procedures, followed by a rapid cool-down to 0-4°C and subsequent chilled storage. The main benefits for the fish industry include a rapid and large-scale preparation of high-quality seafood due to the use of sealed vacuumized pouches aiming to keep flavor of the product and improve its palatability, texture and nutritional properties (Garcia-Linares et al., 2004).

The quality of sous-vide cooked fish depends on both the intrinsic factors such as chemical composition (water- and salt-soluble proteins, fat, water and collagen content, etc.), texture and color, and the extrinsic ones such as pre- and post-slaughter handling procedures, processing and storage conditions. The texture of fish muscle is one of the main quality attributes of freshness and mouthfeel, which depends on several parameters such as flesh juiciness, firmness and cohesiveness (Laroche et al., 1995), as well as the internal cross-linking of connective tissue and the detachment of fibers (Cheng et al., 2014). Flesh juiciness is highly affected by both initial water content and water holding capacity (WHC) influencing cook loss during heat treatment and storage (Laroche et al., 1995), while tenderness of fish muscle is mainly attributed to denaturation of connective tissue (Cheng et al., 2014). Reciprocally, the WHC is strongly influenced by the structural changes occurring in fish muscle proteins, as well as distribution of intra- and extra-cellular water and fiber shrinkage (Schnepf, 1989). Upon heating, sarcoplasmic proteins from fish flesh expand and form a gel (Baldwin, 2012), whilst myofibrillar and connective tissue proteins contract and shrink (Baldwin, 2012; Moreno et al., 2012). Collagen is the main constituent of connective tissue in raw fish muscle influencing its firmness (Hatae et al., 1986; Sato et al., 1986). This protein exists in form of fibrous sheets

called *myocommata* acting as a glue to unite the blocks of muscle tissue – *myotomes* (Kimura et al., 1988). The extent of collagen denaturation depends mainly on temperature and time of heat treatment (Moreno et al., 2012). A number of studies (Moreno et al., 2012; Belitz et al., 2009; Kimura et al., 1988) attributed the decrease in toughness during heat treatment to reduction in connective tissue strength due to collagen denaturation, causing shrinkage and further solubilization of the connective tissue into gelatine. It was previously demonstrated that structural and textural changes in fish products during traditional and sous-vide cooking were due to denaturation and gelation of collagen (Moreno et al., 2012; Belitz et al., 2009). However, these studies focused mostly on the physicochemical properties such as component, subunit construction and denaturation temperature of extracted collagen.

There is little information about modification of collagen in pelagic fish, particularly Atlantic mackerel during sous-vide cooking and its influence on texture parameters. However, changes in microstructure of collagen from connective tissue could reflect the internal subtle changes taking place in the fish muscle (Cheng et al., 2014). The muscle structure is completely modified after sous-vide treatment, affecting both the water-holding capacity and texture parameters of fish (Baldwin, 2012). Therefore, monitoring the changes in muscle tissue at the microstructure level may provide a useful information related to changes in texture parameters of fish. Fluorescence of collagen in the UVA ~335–400 nm and visible spectral regions has been largely investigated in the last few decades (Andersen & Wold, 2003; Isaksson et al, 2002; Davis, 1982). The pronounced, characteristic auto-fluorescence of collagen in the wavelength range of 370–700 nm and its alteration due to denaturation and cross-linking (Wold, 1999) is valuable for non-invasive estimation of detrimental changes occurring in connective tissue of fish (Isaksson et al., 2002). Moreover, image analysis of fluorescence micrographs can act as a reliable tool to quantitatively characterize fish muscle tissues, with a further relation to texture parameters (Andersen & Wold, 2003). Thus, measurement of collagen microstructure in fluorescence mode could provide more information for detailed interpretation of texture alterations in sous-vide Atlantic mackerel induced by external conditions such as sous-vide cooking time and temperature, as well as duration of subsequent chilled storage.

Although fish texture can be assessed through a number of sensory and instrumental techniques, it is difficult to come to an agreement on the best one, because there is no single method universally accepted for application in the fish processing industry (Cheng et al., 2014). Therefore, a novel complex approach comprising the use of fluorescence microscopy technique coupled with chemical, physical and numerical methods is proposed in the present study for reliable assessment of changes in texture parameters of Atlantic mackerel after sous-vide treatment and chilled storage.

2. Materials and Methods

2.1 Sample preparation and sous-vide cooking

The Atlantic mackerel fillets (*Scomber scombrus*) were delivered frozen from Pelagia A.S. (Selje, Norway) in January 2017. Fish fillets were defrosted at 0°C for 5h and vacuum-packaged into Rolf Bayer seal pouches type BST 90 (two fillets in each bag) with a thickness of 90 µm, heat resistance of -25°C/+100°C, O_2 permeability of <60 cm^3/m^2 at 23°C/75% RH and water vapor permeability of <4 $g/m^2 \cdot d$ at 23°C/85% RH. Temperature data loggers type SL52T (Signatrol Ltd, UK) were inserted into vacuum pouches with the mackerel fillets to control temperature fluctuations during sous-vide cooking and subsequent chilled storage. The fish fillets were subjected to sous-vide cooking in two water baths (Grant, UK) at 60°C and 75°C for 10, 15 and 20 min each. After the thermal treatment, they were rapidly cooled down and stored on ice in a cold room at 0±1°C for 1, 3 and 7 days. At each sampling day, the chilled mackerel samples were carefully pulled out from the vacuum pouches and used for experiments. Changes in water content and cook loss, collagen content and microstructure, as well as texture parameters of mackerel fillets were investigated. Analyses were performed in three replicates for each vacuum package containing two mackerel fillets.

2.2 Chemical and physical assays

Water content

Water content was determined by drying samples of 2 g at 105 °C for 24 h to a constant weight, according to the official method (AOAC 2005). The analyses were run in triplicate.

Water holding capacity (WHC)

The determination of the water holding capacity (WHC) is an established method of studying the degree of denaturation of proteins in fish muscle. WHC of sous-vide samples was determined according to the method by Skipnes et al (2007), based on the method described previously by Eide et al. (1982). This method allows determination of the ability of cooked fish flesh to withhold the water during centrifugation (Skipnes et al., 2007). Briefly, the deskinned mackerel fillets were ground and homogenized with a kitchen blender. The resulting fish mince (~2 g) was placed in the Eppendorf conical tubes of 15 ml and centrifuged at 528 g for 15 min using a Heraeus Multifuge X1 (Thermo Scientifc, USA). Dry matter content in the fish mince was determined before centrifugation as mentioned above and WHC of sous-vide cooked samples was determined, as follows (Eq. 1):

$$WHC = (V - \Delta V) / V \cdot 100\% \qquad (1)$$

where V is the water content in sous-vide cooked mackerel samples before centrifugation (g) and ΔV is the weight of the exudate separated from the sample during centrifugation (g). The analyses were run in triplicate.

Total collagen content

Determination of total collagen was conducted according to the method described by Leach (1960) as modification of method by Neumann & Logan (1950), based on the determination of hydroxyproline content in a fish muscle sample previously subjected to acid hydrolysis with 6M HCl (0,05 g/5 ml), at 105 °C for 24 h. This is a colorimetric method based on the oxidation of hydroxyproline with hydrogen peroxide in the presence of alkaline copper sulphate, followed by the destruction of excess of peroxide by heat and subsequent addition of p-dimethylaminobenzaldehyde in the presence of sulphuric acid to produce a coloured complex for spectrophotometric detection at 555 nm. To convert the amount of hydroxyproline into collagen, a factor of 11.42 was used (Sato et al., 1989) and expressed as % wet weight. The analyses were run in triplicate.

Texture parameters

Hardness and cohesiveness of sous-vide cooked mackerel flesh was measured on a TA.XT2 Texture Analyzer (SMS Stable Micro Systems, Ltd., Surrey, UK) equipped with a 1 kg load cell according to the method described by Hultmann & Rustad (2002). A flat-ended cylinder of 12 mm in diameter was pierced into the fish fillet at a constant speed of 1 mm s^{-1} until it had reached 60 % of its height, carefully avoiding myocommata. The holding time between the compressions was 5 s. The maximum resistance force was recorded in Newton (N) and expressed as the average of 3 to 5 determinations per fillet. Cohesiveness, which represents the force holding the integrity of myotome blocks together, while preventing the fish flesh from gaping, was calculated as the ratio of areas delimited by the curves of the second and the first compression.

Differential scanning calorimetry (DSC)

Thermal behavior of the mackerel muscle and collagen extracted as described by Qixing et al (2014) was studied by using a differential scanning calorimeter (micro DSC VII, SETARAM, France) equipped with SETSOFT 2000. Samples were encapsulated in a hermetically sealed inert “measurement” vessel, whilst an empty vessel was used as reference to compensate for the thermal effect due to heating up. The approximate sample weight taken for the assay was 35 mg. The samples were scanned in triplicate at 1 °C/min from 20 to 90 °C under a dry nitrogen purge at 40 mL/min and the temperature (T_{peak}, °C) and enthalpy (ΔH, J/g) for each of transitions were determined as mean values with their standard deviations.

Fluorescence microscopy of collagen fibrils

Direct fluorescence visualization of solubilized collagen in fish tissue fluid of mackerel samples was performed on a Zeiss Axio Imager Upright microscope equipped with Zeiss Plan-Apochromat 20× objective and AxioCam ERc5s with a filter set 49 DAPI (EX 365/50, FT 395, EM 445/70). Spectral phasor analysis was applied to exclude interference in the UV region arising from auto-fluorescence signals of other compounds. Collagenous tissue extracted from the muscle of raw mackerel was used as a reference sample for discriminating the auto-fluorescence signal coming from collagen from connective tissue of the fish. Briefly, 10 g of the fish muscle was cut into 10 mm x 10 mm pieces with a surgical blade and soaked in 0.1 M NaOH with a sample/solution ratio of 1:30 (w/v) for 48 hours, with a change of solution every 6 hours to remove non-collagenous proteins. The resulting sample was then washed with portions of distilled water until the drained water reached a neutral pH and centrifuged at 1500 g for 10 min by using a Heraeus Multifuge X1 (Thermo Scientifc, USA) to separate the remaining lipid fraction. Free collagen fibrils collected after the treatment were directly visualized under fluorescence mode by using a Zeiss Plan-Apochromat 20× objective to aid interpretation of the auto-fluorescence signal arising from collagen in the fish tissue. The total fluorescence intensity (TFI) of collagenous tissue in the acquired images was determined by integration of auto-fluorescence signals arising from all collagen fibrils after subtraction of background. The image processing system used was a software package ImageJ, v1.51k 1 (National Institutes of Health, Bethesda, MD, USA). The measurement of integrated intensity for fluorescence quantification was performed according to the method described by Verdaasdonk et al. (2014). The method selection was based on more accurate determination of fluorescence intensity of complex and multiple structures that differ in size and shape, and have different fluorophore density (Verdaasdonk et al. 2014). In addition to conducting integrated intensity measurements, it was also important to correct for background fluorescence (Hoffman et al., 2001). This was done according to the method described by Verdaasdonk et al. (2014) and the resulting background intensity value was subtracted to calculate the intensity of the region of interest. The acquired micrographs were processed using a classical image-processing protocol of the ImageJ software, comprising background subtraction, automatic threshold and binary filter, and the resulting fluorescent areas were analyzed on the mask image (Schneider et al., 2012). The total fluorescence intensity was then assessed by using integrated densities of fluorescence per particle area.

Statistical Analysis

All physical and chemical analyses were conducted in triplicate, and data were reported as the mean ± standard deviation. Simple correlations among the studied parameters were evaluated by the Pearson

coefficient. Multiple regression analysis was performed using forward stepwise multivariate technique to reveal significant variables that could explain the susceptibility of the fish flesh to gradual softening during sous-vide cooking and subsequent chilled storage. The obtained regression model was derived by using Statgraphics Centurion XVI software, version 16.1.15. The coefficients of determinations for all parameters displayed a good fit of the generated model at the 95% confidence level. The ANOVA of the response was performed for finding the significance of variables and all insignificant terms ($p>0.05$) were rejected.

3. Results and discussion

Water content and water holding capacity (WHC)

Water content showed a high variation of values in the experimental data set (Table 1), while ranging from 57.8% to 68.1% along the sampling days. WHC of sous-vide cooked mackerel also varied significantly during chilled storage (Table 1), while being reduced with 7.4%-44% in comparison with initial WHC of raw Atlantic mackerel (92.2±1.1). The decrease in WHC of the fish flesh during cooking is probably related to denaturation of both myofibrillar and connective tissue proteins (mainly collagen), leading to increased cook loss (data not shown) and detrimental changes in texture (Suvanich et al., 2000; Ofstad et al., 1993). Thus, water-imbibing capacity of the fish myofilaments by connective tissue proteins is reduced during heat treatment, forcing the immobilized cellular water to move and flow out at lower pressure (Ofstad et al., 1993). Upon increasing the temperature of cooking, extracellular spaces in the fish flesh expand, and breakage of pericellular layers along with shrinkage of myofibrils and collagen occur. This results in emergence of intracellular gaps in the flesh, leading to impaired muscle integrity and reduction in texture parameters of the fish (Ofstad et al., 1993). Multiple regression analysis revealed that neither water content nor WHC of the studied mackerel samples were influenced by sous-vide cooking regimes, or duration of chilled storage.

Table 1

Total collagen content

According to Table 1, total collagen content in sous-vide cooked Atlantic mackerel varied from 0.19% to 0.78% wet weight. During sous-vide cooking this collagen content was reduced by 12.5% - 78.9% in comparison to initial collagen content in raw mackerel (0.89±0.04). In order to reveal the influence of sous-vide treatment regime parameters (temperature and time) and duration of chilled storage on the rate of total

collagen decrease, a multivariate regression analysis was performed. The resulting model is described by the following regression equation (Eq. 2):

$$\Delta C = 32.13 + 0.35 \cdot \tau + 5.95 \cdot d \quad (R^2 = 0.735, \text{RMSE} = 2{,}15\%, p=0.002) \qquad (2)$$

where ΔC is relative decrease in total collagen content (%); τ and d are duration of sous-vide cooking (min) and chilled storage (days), respectively. The model contains independent variables possessing significant contribution ($p<0.05$) to collagen decrease in the fish flesh during sous-vide cooking and subsequent chilled storage: sous-vide cooking time (τ) and duration of chilled storage (d). It is well observed that both decreased the total collagen content in the fish flesh according to positive signs of their regression coefficients. The revealed decrease in total collagen content can be attributed to partial loss of solubilized collagen with fish juice (cook loss) during both sous-vide cooking and subsequent chilled storage. According to Table 1, the decrease in collagen content reached to the largest during prolonged cooking (15-20 min), which can be explained by the partial solubilization of collagen and the shrinkage of muscle fiber, allowing the juice containing a part of solubilized collagen dripping out from the fish flesh (Hatae et al., 1996). However, the main contribution is clearly exhibited by duration of chilled storage due its higher regression coefficient in comparison with duration of sous-vide cooking. This may be explained by remaining activity of some proteolytic enzymes and collagenases responsible for cleavage of collagen fibrils (Shyu et al., 2012). Some of collagenases remain active above 60°C (Baldwin, 2012) and can thereby hydrolyze the peptide bond in denatured collagen helix into three-fourth or one- fourth telopeptides which are further cleaved by other tissue proteinases (Shyu et al., 2012).

Texture parameters

Hardness of sous-vide cooked mackerel ranged from 5.62 N to 7.54 N along the sampling days (Table 1), and decreased with 14.3% - 37% in comparison to hardness of raw mackerel (8.91±0.43). Attempts to interpret the softening of sous-vide cooked mackerel fillets during chilled storage were taken by using multivariate regression analysis. The resulting model is described by the following regression equation (Eq. 3):

$$\Delta H = 19.85 + 0.19 \cdot \tau + 1.97 \cdot d \qquad (R^2 = 0.626, \text{RMSE} = 6{,}08\%, p=0.013) \qquad (3)$$

where ΔH is relative decrease in hardness denoting softening of the fish tissue (%); τ and d are duration of sous-vide cooking (min) and chilled storage (days), respectively.

From the derived model, it is clearly seen that duration of both sous-vide cooking (τ) and subsequent chilled storage (d) are the main parameters influencing tenderization of the fish flesh. However, in order to interpret this phenomenon, changes in intrinsic factors such as myofibrillar proteins and connective tissue during

sous-vide cooking followed by chilled storage, as well as their interactions, should be considered. Heat treatment affects the structural integrity of myofibrils, causing reduction of WHC (Ofstad et al., 1993). With increased cooking temperature and time, the three-dimensional network of denatured and aggregated myosin is destroyed and capillary water comes out from the network structure (Skipnes et al., 2008; Ofstad et al., 1993). Thus, WHC of Atlantic mackerel may be indirectly associated with the degree of myosin degradation, influencing the changes in texture parameters of the fish. A multivariate regression analysis was carried out to explain the influence of collagen content and WHC on the fish flesh tenderization during sous-vide cooking and subsequent chilled storage, and the following model was obtained (Eq. 4):

$$\Delta H = -58.84 + 3.59 \cdot C + 106.81 \cdot WHC \qquad (R^2 = 0.905,\ RMSE = 0{,}98\%,\ p=0.000) \qquad (4)$$

where ΔH is relative decrease in hardness denoting softening of the fish tissue (%); *C* is total collagen content (% wet weight) and *WHC* is water holding capacity (%).

Although independent variables of the model were significantly correlated with the decrease of the flesh firmness across Pearson coefficients ($p<0.001$), their combination resulted in outstanding values of R^2 = 0.905 and p = 0.000, indicating high reliability and predictability of the model. The both regression coefficients for WHC and collagen content have positive signs in the model, denoting that all these parameters lead to fish flesh softening. However, the model displayed the highest regression coefficient for WHC, indicating its major contribution to the fish flesh softening. The presence of this parameter in the model is in full agreement with previous studies that observed a significant correlation between a decrease in hardness of fish flesh and increased water content in its muscle (Feng et al., 2017; Love & Haq, 1970). This phenomenon may be explained by the fact that during chilled storage of Atlantic mackerel after sous-vide treatment at 60°C and 75°C, some residual enzymatic activity may still take place due to the remnants of oxygen that have stayed inside vacuum pouches (Kim & Park, 2000; Diaz et al., 2011). Proteolysis leads to the splitting of peptide bonds of the fish muscle proteins, which results in a loosening of their structure and making available more water-binding sites, thus facilitating the release of denatured proteins, including solubilized collagen, from the fish flesh into the juice. Generally, application of low-temperature heat treatment (<80 °C) still allows some of proteolytic enzymes and collagenases to be active (Makinodan et al. 1987; Stoknes et al. 1993), which may further lead to denaturation and weakening of muscle and connective tissue of the fish. Although the proteolytic activity decreases with increasing the temperature of heat treatment (Makinodan et al. 1987; Stoknes et al. 1993), some remaining activity of proteolytic enzymes and collagenases can still take place, thereby worsening texture parameters of the fish. Generally, cooking at 60-75 °C may not be sufficient to ensure the safety of cooked fish subjected for prolonged refrigeration (Nyati, 2000), and quality deterioration due to bacterial spoilage and enzymatic degradation may take place.

Collagen content was the parameter with the second highest contribution in the obtained model. This may be explained by the fact that its heat solubilization and further degradation during chilled storage disintegrates and flakes off myotomes in the fish muscle, leading to gradual tenderization of the fish flesh (Belitz et al., 2009). Collagen from connective tissue of fish muscle shrinks and solubilizes at around 50°C, but more intensively above 55°C to form gelatin (Moreno et al., 2012). Thus, sous-vide cooking at 60°C-75 °C can increase the tenderness of the fish flesh by solubilizing collagen into gelatin, thereby reducing interfibre adhesion, as well as decreasing the strength of myofibrils due to remaining proteolytic activity. Moreover, total collagen content in sous-vide cooked mackerel was significantly correlated ($R = 0.948$) with hardness of the fish flesh (Fig. 1A), showing that gradual softening of the mackerel fillets during chilled storage was mainly attributed to a decrease in total collagen content. As it was mentioned above, some of collagenases remain active above 60°C and thus can significantly increase tenderness of sous-vide cooked fish flesh during chilled storage due to breakdown of collagen in connective tissue (Baldwin, 2012). Cohesiveness of sous-vide treated mackerel varied from 0.37 to 0.40 during storage. It did not display a high variation of values in the experimental data set, but was significantly correlated ($R = 0.865$) with total collagen content of sous-vide cooked mackerel during chilled storage (Fig. 1B). Cohesiveness characterizes the degree of integrity of fish myotome blocks. Its decrease during chilled storage for all sous-vide cooked mackerel samples (Table 1) shows that myotomes of the fish flesh gradually separate. Collagen fibers of the myocommata are connected to the myotomes by collagenous microtubules helping to maintain the integrity of the fish muscle, while preventing it from gaping (Bremner & Hallett, 1985). When these microtubules break due to cleavage of denatured collagen during storage, cohesiveness of the fish flesh is reduced, leading to gaping. In addition, thermal treatment weaken the collagen at the myotome-myocommata junction, decreasing the cohesiveness (Bremner & Hallett, 1985). Therefore, cohesiveness of raw mackerel is slightly higher (0.42±0.02) than for sous-vide cooked samples (Table 1).

Differential scanning calorimetry (DSC)

The DSC curve for raw mackerel muscle displayed 4 endothermic peaks (Figure 2A). According to the literature, the first two peaks correspond to denaturation of myosin (T_m) and collagen shrinkage ($T_{c.s.}$) (Skipnes et al., 2008; Howell et al., 1991; Hastings et al., 1985). Fish myosin proteins can easily be denatured by heating (Chan et al., 1992; Hastings et al., 1985). Temperature for myosin denaturation (T_m) in Atlantic mackerel is 28.2±1.7 °C. Chan et al. (1992) discovered that some regions of myosin molecule are less thermo-stable than others and have a tendency to denature before the complete denaturation of the whole myosin molecule. After denaturation of myosin, shrinkage of collagen takes place, resulting in the second endothermic peak endothermic peak at 32 ± 2°C. The third endothermic peak relates to collagen

gelation (T_g) and corresponds to the gelation peak of extracted collagen shown in Fig. 2B, being at the same time in agreement with findings of other authors (Moreno et al., 2012; Skipnes et al., 2008). This transition occurs at 49.4±1.6 °C (Fig. 2A) due to the process of collagen gelation in a hydrated environment and is caused by the breaking of internal cross-links. The onset temperature of collagen degradation of 32 ± 2°C displayed as the second endothermic peak in the DSC curve may be explained by conformational changes occurring within the fibrils before gelation, such as partial shrinkage of the fibrils (Bozec & Odlyha, 2011). These results are in agreement with data of Hastings et al. (1985), revealing two denaturation peaks at ~32 °C and ~40 °C respectively for collagen in connective tissue isolated from cod. The last peak (T_a) in the DSC diagram occurring at 68.3±2.4 °C (Fig. 2A) is due to actin denaturation (Skipnes et al., 2008; Hastings et al., 1985). Actin is one of the most thermo-stable proteins in fish muscle, which is not denatured by freezing and is more heat-stable than myosin (Hastings et al., 1985). This investigation revealed the temperature range for solubilization and further degradation of collagen in Atlantic mackerel, explaining its losses with fish juice during sous-vide cooking.

Fluorescence microscopy of collagen fibrils

The acquired micrographs of fish tissue and isolated collagenous fibrils shown in Figure 3, displayed the bright blue fibrous structures corresponding to collagen fractions. The disappearance of the elongated shape of intact collagen from collagenous tissue of raw mackerel (Fig. 3A-C) is clearly observed in all micrographs captured in fluorescence mode for sous-vide cooked mackerel (Fig. 3D-U). This phenomenon may be explained by collagen denaturation both at 60°C and at 75°C due to its thermal denaturation at temperatures above 40°C, as it was revealed by DSC. During heat treatment above 50°C, collagen from connective tissue of the fish solubilizes and becomes less structured, tending towards a circular shape (Raub, 2008). It is well observed (Fig 3D-S) that circularity of collagen fibrils is growing with both temperature and duration of sous-vide cooking in accordance with collagen denaturation. This tendency was explained by Lewis & Purslow (1989) as the loss of fibre undulations existing in raw connective tissue due to shortening of collagen fibres. Indeed, thermal treatment deeply modifies the structure and mechanical properties of collagen: upon heating at a temperature above 50°C, it contracts and become an insoluble elastic gel. During this transformation, collagen fibres acquire the structure of a random network due to cleavage of intramolecular hydrogen bounds (Moreno et al., 2012). However, as we can notice from the micrographs of sous-vide cooked mackerel on the 3rd and 7th day of chilled storage, these intermolecular reticulation bounds can also be broken, probably due to remaining activity of some proteolytic enzymes and collagenases (Shyu et al., 2012). Thus, as we can see from the acquired fluorescence microscope

391 images, collagen fibrils were substantially cleaved into collagen monomers and further to collagen peptides
392 and amino acids on the 3rd and 7th day of chilled storage (Fig. 3E-U). Final texture parameters of cooked
393 connective tissue then depend on both collagen content and reticulation characterizing the integrity of the
394 fish muscle. As mentioned above, sous-vide cooking of fish leads to collagen denaturation and
395 solubilization, while subsequent chilled storage decreases its content due to detrimental activity of intrinsic
396 enzymes (Makinodan et al. 1987; Stoknes et al. 1993). Therefore, the quantification of connective tissue
397 degradation during these processes is quite important for the correct interpretation and rapid control of the
398 fish texture. In this regard, the study aimed at applying optical fluorescence microscopy and subsequent
399 image processing to evaluate a possible relationship between total fluorescence emitted by collagen fibrils
400 and total collagen content determined by chemical method, as well as total fluorescence and texture
401 parameters of sous-vide cooked mackerel.
402 After image processing of the obtained micrographs, a direct relationship between total collagen content
403 and total fluorescence intensity of collagenous tissue fractions (Fig. 4A), as well as hardness of mackerel
404 flesh and total fluorescence intensity were obtained (Fig. 4B). The both collagen content and hardness
405 significantly ($p<0.05$) correlated with total fluorescence intensity of collagenous tissue in sous-vide cooked
406 mackerel ($R = 0.960$ and $R = 0.961$, respectively). This suggests that fluorescence microscopy opens up the
407 way to the fast, non-destructive and reliable control of collagenous tissue degradation in fish products on
408 the basis of intrinsic fluorescence of collagen and may thus be used for indirect texture analysis in the
409 future.
410

411 **4. Conclusion**

412 The present study has revealed that textural changes in connective tissue of sous-vide cooked Atlantic
413 mackerel during chilled storage may be successfully assessed by a novel complex approach involving
414 chemical, physical and fluorescence imaging methods. Fluorescence microscopy allows reliable
415 identification of detrimental changes in the structure of collagenous tissue and texture of sous-vide cooked
416 mackerel, as well as decrease in its collagen content based on intrinsic auto-fluorescence of collagen
417 molecules in the wavelength range of 300-500 nm under DAPI fluorescence mode. The regression models
418 describing the influence of each of sous-vide cooking regimes on the changes in total collagen content and
419 firmness of the fish tissue showed high correlation coefficient ($R = 0.948$). The role of water holding
420 capacity and collagen content in the susceptibility of Atlantic mackerel flesh to gradual softening during
421 sous-vide cooking and chilled storage was demonstrated as well in the study. However, the study also
422 emphasized the necessity of investigation of the influence of collagenases and collagen-degrading proteases
423 in the enzymatic dissociation of collagen from a fish tissue after different sous-vide treatment regimes and
424 during subsequent chilled storage.

5. Acknowledgments

The study displayed in the paper is based upon work supported by the JPI project ProHealth "Innovative processing to preserve positive health effects in pelagic fish products", RCN 259582/E50.

6. References

1) Andersen, C.M., & Wold J.P. (2003). Fluorescence of Muscle and Connective Tissue from Cod and Salmon. *Journal of Agricultural and Food Chemistry, 51* (2), 470-476.
2) Baldwin, D.E. (2012). Sous Vide cooking: A review. *International Journal of Gastronomy and Food Science 1,* 15-30.
3) Belitz, H.D., Grosch, W., Schieberle, P. Food chemistry. 4.ed. Berlin: Springer, 2009.
4) Bozec, L., & Odlyha, M. (2011). Thermal Denaturation Studies of Collagen by Microthermal Analysis and Atomic Force Microscopy. *Biophysical Journal, 101*(1), 228-236.
5) Bremner, H. A. & Hallett, I. C. (1985). Muscle Fiber–Connective Tissue Junctions in the Fish Blue Grenadier (Macruronus novaezelandiae). A Scanning Electron Microscope Study. *Journal of Food Science, 50,* 975–980.
6) Chan, J.K., Gill, T.A. & Paulson, A.T. (1992). Cross-Linking of Myosin Heavy Chains from Cod, Herring and Silver Hake During Thermal Setting. *Journal of Food Science, 57*(4), 906-912.
7) Cheng, J.-H., Sun, D.-W., Han, Z. & Zeng, X.-A. (2014). Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review. *Comprehensive Reviews in Food Science and Food Safety, 13,* 52–61.
8) Davis, H. K. (1982). Fluorescence of fish muscle: Description and measurement of changes occurring during frozen storage. *Journal of the Science of Food and Agriculture,* 33, 1135-1142.
9) Diaz, P., Garrido, M.D., & Banon S. (2011). Spoilage of sous vide cooked salmon (Salmo salar) stored under refrigeration. *Food Science and Technology International, 17*(1), 31-37.
10) Eide, O., Børresen, T., & Strøm T. (1982). Minced fish production from capelin (Mallotus villosus). A new method for gutting, skinning and removal of fat from small fatty fish species. *Journal of Food Science, 47*(2), 347-349.
11) Feng, X., Fu, C., & Yang, H. (2017). Gelatin addition improves the nutrient retention, texture and mass transfer of fish balls without altering their nanostructure during boiling. *LWT - Food Science and Technology*, 77, 142-151.

12) Garcia-Linares, M.C., Gonzalez-Fandos, E., Garcia-Fernandes, M.C. & Garcia-Arias, M.T. (2004). Microbiological and nutritional quality of sous-vide or traditionally processed fish: Influence of fat content. *Journal of Food Quality, 27,* 371–387.

13) Hastings, R.J., Rodger, G.W., Park, R., Matthews, A.D. & Anderson, E.M. (1985). Differential Scanning Calorimetry of Fish Muscle: The Effect of Processing and Species Variation. *Journal of Food Science, 50*(2), 503-506.

14) Hatae, K., Tobimatsu, A., Takeyama, M. & Matsumoto, J.J. (1986). Contribution of the connective tissues on the texture difference of various fish species. *Nippon Suisan Gakkaishi, 52,* 2001–2007.

15) Hatae, K., Nakai, H., Tanaka, C., Shimada, A., & Watabe, S. (1996). Taste and texture of abalone meat after extended cooking. *Fisheries Science, 62,* 643–647.

16) Hoffman, D.B., Pearson, C.G., Yen, T.J., Howell, B.J., Salmon E.D. (2001). Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Molecular Biology of the Cell*, 12(7), 1995–2009.

17) Howell, B.K., Matthews, A.D., & Donnelly, A.P. (1991). Thermal stability of fish myofibrils: a differential scanning calorimetric study. *International Journal of Food Science & Technology, 26*(3), 283-295.

18) Hultmann, L., & Rustad, T. (2002). Textural changes during iced storage of salmon (Salmo salar) and cod (Gadus morhua). *Journal of Aquatic Food Product Technology, 11* (3-4), 105-123.

19) Isaksson, T., Swensen, L.P., Taylor, R.G., Fjæra, S.O., & Skjervold, P.O. (2002). Nondestructive texture analysis of farmed Atlantic salmon using visual/near-infrared reflectance spectroscopy. *Journal of the Science of Food and Agriculture, 82,* 53-60.

20) Kim, B., & Park, J. (2000). Surimi and Surimi Seafood. New York: Taylor and Francis.

21) Kimura, S., Zhu, X.-P., Matsui, R., Shijoh, M. & Takamizawa, S. (1988). Characterization of Fish Muscle Type I Collagen. *Journal of Food Science, 53,* 1315–1318.

22) Laroche, M., Rousseau, F., Dumont, J.P. (1995). Relations entre les résultats d'analyse sensorielle et de mesures instrumentales pour des filets de carpe. *Pisciculture Française d'Eau Vive et d'Etang Saumâtre et Marine,* (121), 22-26.

23) Leach AA. (1960). *Appendix*—Notes on a modification of the Neuman & Logan method for the determination of the hydroxyproline. *Biochemical Journal, 74* (1), 70-71.

24) Lewis, G. J. & Purslow, P. P. (1989). The strength and stiffness of perimysial connective tissue isolated from cooked beef muscle. *Meat Science, 26,* 255-269.

25) Liu, Y., Andarawis-Puri, N., Eppell, S.J. (2016). Method to extract minimally damaged collagen fibrils from tendon. *Journal of Biological Methods, 3*(4), e54.

26) Love, R. M., & Haq, M. A. (1970). The connective tissues of fish. IV. Gaping of cod muscle under various conditions of freezing, cold-storage and thawing. *International Journal of Food Science & Technology*, 5, 249–260.

27) Makinodan, Y., Yokoyama, Y., Konoshita, M. and Toyohara, H. (1987). Characterization of an alkaline proteinase of fish muscle. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 1041-1046.

28) Moreno, H.M., Montero, M.P., Gomez-Guillen, M.C., Fernadez-Martin, F., Mørkøre, T., & Borderías, J. (2012) Collagen characteristics of farmed Atlantic salmon with firm and soft fillet texture. *Food Chemistry*, 134, 678–685.

29) Neuman, R.E., & Logan, M.A. (1950). The determination of hydroxyproline. *The Journal of Biological Chemistry, 184* (1), 299–306.

30) Nyati, H. (2000). An evaluation of the effect of storage and processing temperature on the microbiological status of sous vide extended shelf-life products. *Food Control, 11,* 471-476.

31) Ofstad, R., Kidman, S., Myklebust, R & Hermansson, A.M. (1993). Liquid holding capacity and structural changes during heating of fish muscle: cod (Gadus morhua L.) and salmon (Salmo salar). *Food Structure, 12*,163-174.

32) Qixing, J., Zhengran, M., Shuoshuo, W., Yanshun, X., Fengyu, T., Xueqin, X., Peipei, Y., & Wenshui, X. (2014). Effect of Temperature on Protein Compositional Changes of Big Head Carp (Aristichthys nobilis) Muscle and Exudates. *Food Science and Technology Research, 20* (3), 655-661

33) Raub, C. B., Unruh, J., Suresh, V., Krasieva, T., Lindmo, T., Gratton, E., Tromberg, B.J, George, S. C. (2008). Image Correlation Spectroscopy of Multiphoton Images Correlates with Collagen Mechanical Properties. *Biophysical Journal, 94*(6), 2361–2373.

34) Sato, K., Yoshinaka, R., Sato, M., & Shimizu, M. (1986). Collagen content in the muscle of fishes in association with their swimming movement and meat texture. *Nippon Suisan Gakkaishi, 52,* 1595–1600.

35) Schneider, C.A., Rasband, W.S., Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9, 671-675.

36) Schnepf, M. (1989). Protein–water interaction. In: Hardman T.M. (ed.), Water and Food Quality. Elsevier Applied Science, London, 135–168.

37) Shyu, D. J. H., Tzen, J. T. C. & Jeang, C. L. (2012). Enzyme Activities, in Food Biochemistry and Food Processing, Second Edition (ed B. K. Simpson), Wiley-Blackwell, Oxford, UK.

38) Skipnes, D., Østby, M.L., & Hendrickx, M.E. (2007). A method for characterising cook loss and water holding capacity in heat treated cod (Gadus morhua) muscle. Journal of Food Engineering, 80 (4), 1078-1085.
39) Skipnes, D., Van der Plancken, I., Van Loey, A. and Hendrickx, M.E. (2008). Kinetics of heat denaturation of proteins from farmed Atlantic cod (Gadus morhua). Journal of Food Engineering 85: 51–58.
40) Stoknes, I, Rustad, T. & Mohr, V. (1993). Comparative studies of the proteolytic activity of tissue extracts from cod Gadus morhua) and herring (Clupea harengus). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 613-619.
41) Suvanich, V., Jahncke, M.L. & Marshall, D.L. (2000). Changes in Selected Chemical Quality Characteristics of Channel Catfish Frame Mince During Chill and Frozen Storage. *Journal of Food Science, 65*, 24–29.
42) Venugopal, V. (2009). Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds from the Ocean. Boca Raton: CRC Press/Taylor & Francis.
43) Verdaasdonk, J.S., Lawrimore, J., Bloom K. (2014). Determining absolute protein numbers by quantitative fluorescence microscopy. *Methods in Cell Biology*, 123, 347-65.
44) Wold, J. P., Lundby, F., & Egelandsdal, B. (1999). Quantification of connective tissue (hydroxyproline) in ground beef by autofluorescence spectroscopy. *Journal of Food Science, 64*, 377-383.

Figure captions

Figure 1. Influence of total collagen content in connective tissue of sous-vide cooked Atlantic mackerel on its texture parameters: hardness (A) and cohesiveness (B) during chilled storage.

Figure 2. DSC curve of raw Atlantic mackerel (A) and collagen extracted from its connective tissue (B).

Figure 3. Fluorescence micrographs of (A) tissue fluid of raw Atlantic mackerel at magnification 20×, (B) isolated collagenous tissue of raw mackerel at magnification 10×, (C) extracted collagen at magnification 20× and fish tissue fluid of sous-vide cooked Atlantic mackerel (D-U) imaged at magnification 20× under DAPI filter cube (EX 365/50, FT 395, EM 445/70), with the following cooking-storage regimes (t-τ-d): (D) 60-10-1, (E) 60-10-3, (F) 60-10-7, (G) 50-15-1, (H) 60-15-3, (I) 60-15-7, (J) 60-20-1, (K) 60-20-3, (L) 60-20-7, (M) 75-10-1, (N) 75-10-3, (O) 75-10-7, (P) 75-15-1, (Q) 75-15-3, (R) 75-15-7, (S) 75-20-1, (T) 75-20-3, (U) 75-20-7.

Figure 4. Change in collagen total fluorescence versus total collagen content (A) and hardness of sous-vide cooked mackerel flesh (B).

Tables

Table 1. Quality parameters of sous-vide cooked mackerel during chilled storage