# MONITORING SECONDARY STRUCTURAL CHANGES IN SALTED AND SMOKED SALMON MUSCLE MYOFIBER PROTEINS BY FT-IR MICROSPECTROSCOPY.

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**Running title: FTIR spectroscopy of smoked salmon myofibrillar proteins**

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**ABSTRACT**

Fourier transform infrared (FTIR) microspectroscopy and light microscopy were used to study changes in the myofibrillar proteins and microstructure in salmon muscle due to dry salting and smoking. Light microscopy showed that the myofibers of the smoked samples were more shrunken and their shape more irregular and edged than for the non-smoked samples. FTIR microspectroscopy showed that salting time mostly contributed in the amide I region, revealing that secondary structural changes of proteins were primarily affected by salting. The main variation in amide II region was caused by smoking. As it is known that smoke components can react with amino acid side chains and that the contribution of the side chain in the amide II region is larger than in the amide I, it is concluded that the observed differences are due to interactions between carbonyl compounds of smoke and amino acid side chains .

Keywords: FT-IR microspectroscopy, myofibrillar proteins, salmon, salting, smoking

## INTRODUCTION

Traditionally, cold smoked salmon is a highly valued product. Cold smoking is a process by which the fish is submitted to salting before being smoked at temperatures around 25ºC without further heat treatment. It is categorized as "lightly preserved" product and thus there is a need for improved knowledge about which processing parameters influence product quality.

Salting is the first step in the smoking process and is crucial to obtain high shelf-life, good quality and yield of the product. The salt may be added through dry salting, brine salting, or injection. Dry salting is the most common technique for processing cold smoked salmon (*1*). Salting time varies between smoke houses from hours to several days (*2*). Salting is known to influence texture properties and water holding capacity (*3*). Barat and co-workers (*4*) suggested that during salting some of the proteins are denatured and precipitate as a result of the high ionic forces in the media, giving rise to textural changes in the product. A recent study by Böcker and co-workers (*5*) investigated the effects of brine salting (16% NaCl) on protein structure in Atlantic salmon muscle tissue with respect to raw material variation. The highest salt uptake was achieved for frozen/thawed (4.1%), followed by post-rigor (3.0%) and pre-rigor (2.2%) salmon, respectively. They observed that samples with a lower salt uptake experienced less swelling of the myofibers.

It is known that the smoking process increases the shelf-life of fish as a result of the combined effects of dehydration, antimicrobial (*6*), and antioxidant activity (*7*) of several of the smoke components such as formaldehyde, carboxylic acids and phenols (*8*-*9*). Few studies have addressed the effect of the smoking process in salmon myofibrillar proteins. Most of the authors suggested that salt is the most important factor for textural and microstructural changes. Sigurgisladottir and co-workers (*10*) demonstrated that salmon muscle fibers shrink during the smoking process. It was also shown that the amount of salt-soluble proteins in

salmon is reduced by smoking (*11-12*). Previous studies report that interactions between smoke components and amino groups of proteins exist, especially formaldehyde as a smoke component, with the e-amino group of lysine (*13-17*).

Although it is known that the smoking process evokes reactions with amino-acid side chains, the influence of the smoking process on protein secondary structure and textural properties is still a rather unexplored field. Fourier Transform Infrared (FT-IR) spectroscopy has been widely used to study protein secondary structure (*18*) and in recent years it has become an increasingly powerful tool for the analysis of protein secondary structure in intact food tissues (*5, 19-24*). The main advantage of FT-IR microspectroscopy is that it can be easily combined with histological investigations, as it requires a similar sample preparation, and thus, specific features can be studied on parallel sections both spectroscopically and histochemically. Kirschner and co-workers (*19*) used FTIR-microspectroscopy to monitor heat-induced denaturation in beef muscle. Recently, this technique has been used to characterize processing-induced changes (salting and heating among others) on myofibrillar protein structure of pork muscle and salmon fillets (*5, 20-24*).

The objetive of this paper was to investigate structural changes in salmon muscle during salting and smoking by FT-IR microspectroscopy and light microscopy. While the effect of salting on protein structure has been previously studied by FT-IR microspectroscopy, we intended to investigate if this technique can detect changes of protein secondary structure as a function of smoking.

## MATERIALS AND METHODS

**Sample preparation.** 9 farmed salmon (*Salmo salar*) of different size (small, 3-4 kg; medium, 4-5 kg; and large, 5-6 kg) were acquired from a commercial fish company (Bremnes Seashore

AS, Bremnes, Norway). After gutting and cleaning, they were manually filleted pre-rigor on the day of harvest. To study the influence of the salting time,: 6 of the 9 salmons were dry salted at 4 ºC, their left fillets for 24 hours, and right fillets for 8 hours. After the salting and an equilibration period of 4 days at 4ºC, half of the samples were smoked: 3 fillets from each type of salting were smoked in a smoking chamber (Unimatic Smoking Unit equipped with a Unitronic SC 2000 Control Unit, Doleschal, Austria) with smoke generated from beech wood for 5.5 hours at 28 ºC. Finally, 3 of the 9 salmons were used for collecting unprocessed samples. Moreover, all individual fillets were weighed out before and after each process to know the weight loss caused by salting or smoking.

This resulted in a total of 5 different groups; (1) unprocessed samples (n=3), (2) samples salted for 8 hours (n=3), (3) samples salted for 24 hours (n=3), (4) samples salted for 8 hours followed by smoking (n=3) and (5) samples salted for 24 hours followed by smoking (n=3). Sampling for chemical analyses (salt and fat determination) was done for each condition at head and tail regions. Additionally, for light microscopy and FTIR microspectroscopy, samples from skinside (approximately 2 mm from the skin) and inside locations (approximately 30 mm from the skin) were also taken at head and tail regions for each individual, respectively. Blocks of about 0.7 cm x 0.7 cm x 0.2 cm were excised and embedded in O.C.T. compound (Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA) and then snap-frozen in liquid $N_2$. The samples were stored at -80°C until cryo-sectioning for light microscopy and FT-IR measurements. 10 μm thick cryosections were cut on a Leica CM3050S Cryostat (Leica, Nussloch, Germany).

**Chemical Analysis.** NaCl content in the samples was determined as water soluble $Cl^-$ by titration with a Corning Salt Analysator 926 (Chloride analyzer 926 Corning, Corning Medical and Scientific, Halstead, England) (*25*). The fat content was measured using a low-field $^1$H-NMR instrument (Maran Ultra, 23 MHz, Oxford 5 Instruments, UK). The instrument was calibrated with refined salmon oil prior to analysis. Except for temperature

standardization, no further sample preparation was performed. The weight of the fillets was registered before (w1) and after (w2) each process to calculate the weight loss according to the following equation (1):

$$\text{Weight loss } \% = (w1-w2)/w1 \times 100 \quad [1]$$

The results given in table 1 for salt and fat content are based on duplicated determinations and are presented as average values together with their standard deviations. In addition, the significance of the differences was determined by use of *t* test.

**Light Microscopy.** For light microscopy, cryosections were stained with 1g/100ml Toluidine Blue (Sigma-Aldrich Norway AS, Oslo, Norway) to elucidate the general structure of the muscle samples. The sections were examined with a Leica DM 6000B microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and images were acquired at 10 x magnification with an Evolution MP 5.0 CCD Camera (Media Cybernetics, The Imaging Experts Silver Springs, Maryland, USA). The images taken as light micrographs correspond to the same areas from which spectra were collected by FT-IR microspectroscopy on parallel cryo-sections.

**FT-IR microspectroscopy and pre-processing of the FT-IR spectra.**

For FT-IR microspectroscopy, the cryosections were thaw-mounted on 3 mm thick ZnSe slides and were stored overnight in a desiccator at room temperature before the measurements. The FT-IR measurements were carried out with an IRscope II combined with an Equinox 55 FT-IR spectrometer (both Bruker Optics, Germany). Spectra were collected from single myofibers in transmission mode in the frequency range from 4000 to 1000 $cm^{-1}$ using a mercury cadmium telluride (MCT) detector. For each spectrum, 256 interferograms were co-added and averaged at a resolution of 6 $cm^{-1}$. The microscope and the spectrometer were purged with dry air to reduce spectral contributions from water vapor and $CO_2$. A

background spectrum of the ZnSe substrate was recorded before each spectrum was measured in order to account for variations in water vapor and $CO_2$.

For every sample two different blocks were analyzed. From every block one crysection was prepared and three spectra were acquired from three different myofibers per section resulting in total in 180 spectra: 3 spectra from each cryosection x 4 locations (skinside and inside at head and tail regions, respectively) x 5 conditions (unprocessed, salted for 8 h, salted for 24 h, salted for 8 h with subsequent smoking and salted for 24 h with subsequent smoking) x 3 biological replicates (individual fishes) per condition. Spectra were pre-processed by taking second derivatives applied a nine point Savitzky-Golay filter (*26*), followed by extended multiplicative signal correction in the spectral range 3200-1000 $cm^{-1}$ (*27*).

**Data Analysis.** The 180 FT-IR spectra from each block were averaged resulting in just one spectrum per experimental condition, in total 60 mean spectra. The data analysis was carried out using Principal Component Analysis (PCA) and Partial Least Squares Regression (PLSR). PCA was used for the extraction and interpretation of systematic variance in multidimensional data sets by means of a small number of non-correlated variables. PCA finds directions in the data that explain most of the variation (*28*). These new orthogonal directions are linear combinations of the original ones and are ordered with respect to the amount of explained variance. In this paper, PCA was used to investigate the variation in amide I and II regions as a function of salting and smoking processes Multivariate analysis was done both on mean-spectra and on non-averaged spectra, resulting in the same variation patterns.

In PLSR only that part of the main variation in a data matrix **X** is extracted that at the same time maximizes the variation in a data matrix **Y**, i.e. PLSR aims at using only the most relevant part of the variation in **X** for the regression of **Y**, while the unstable or irrelevant variation in **X** is left out of the calculation. In this study, the design variables were used as the **X**-matrix and the measured variables (FT-IR data) were used as **Y**-matrix (*28*). The results of

PLSR were then studied using so-called correlation loading plots. The correlation loading plot shows the correlation of the **X** and **Y** variables to the PLSR components (scores). Since in second-derivative spectra minima are referring to spectral bands, the spectra were multiplied by -1 before analysis by PLSR in order to facilitate interpretation.

All preprocessing and data analysis was performed using an in-house program written in Matlab version 7.3 (The MathWorks, Natick, MA) and using The Unscrambler® version 9.2 (Camo Process AS, Norway).

## RESULTS AND DISCUSSION

### Chemical Analysis and microstructure.

**Table 1** shows the results obtained for weight loss, salt and fat content in processed samples, expressed as percentage values, together with their standard deviation and the significance level between samples. As expected, salt contents are significantly higher in samples salted for 24 hours compared to the samples salted for 8 hours. Moreover, the highest salt contents are found in the tail samples. A significant effect of salt concentration on weight loss ($P < 0,05$) is observed, being in average 6,45%±0,26 and 4,86%±0,64 for samples salted for 24 hours and 8 hours respectively. As it could also be observed in **Table 1**, the fat distribution on studied fillets is not homogeneous, as it has been demonstrated by other authors (*5, 29*), decreasing from the head region (17,1%±1,9) towards the tail (6,0%±1,3). In addition, the flesh near the tail is thinner than in the head region. Therefore the salt penetrates more deeply in the tail part. Several authors have reported that salt uptake in muscle is reduced by increasing fat content (*30-31*).

Moreover, smoking shows significant effects on weight loss ($P < 0,001$), which can be ascribed to the dehydration of the flesh after the smoking process. Therefore salt concentrations are higher in smoked samples than in non-smoked ones. In addition, salt

content and weight loss values for smoked fillets show that samples salted for 24 hours contained generally more salt than the samples salted only for 8 hours. The weight loss of the fillets decreases from 9,18%±0,51 to 8,03%±0,99 as a function of salting time.

**Figure 1** shows selected images of transverse cryosections of samples from head region. As it can be observed, unprocessed samples exhibit a structure with the myofibers appearing well-attached. For the unprocessed sample there is no microstructural difference between the skinside and the inside. After salting, clear differences between the inside and the skinside can be seen. The inside samples are more detached and the extracellular spaces increase with the salting time. This is due to the fact that during dry salting, the salt ions diffuse through the muscle tissue by osmotic forces between the surrounding brine and the muscle, showing shrinkage of the muscle fibers. The differences between skinside and inside samples can be explained by the fact that the lipid content in the skinside is higher leading to a decrease of the salt diffusivity (*32*).

After the smoking process, the differences appear to be even bigger: After salting for 24 hours, the myofibers of the smoked samples appear to be more shrunken and their shape appears more irregular and edged than for the non-smoked samples. This is in agreement with the results obtained by Sigurgisladottir and co-workers (*10*). After salting for 8 hours, the effect of smoking was not so strong. Also the skinside part of smoked samples was subjected to only minor changes.

**FT-IR Microspectroscopy.**

**Figure 2** displays a typical spectrum of a single myofiber obtained from a fresh salmon muscle cryosection in the spectral region from 4000 to 1000 $cm^{-1}$. This spectrum represents the most typical features observed for the 5 groups investigated. The amide I (1700-1600 $cm^{-1}$) is the most dominant band in the myofiber spectrum. Of all the amide bands, of which there exist nine, the amide I was found to be the most useful for the analysis of secondary structure

of proteins because of its sensitivity to hydrogen-bonding pattern, dipole-dipole interaction, and the geometry of the polypeptide backbone (*18, 33*). It is mainly affected by the C=O stretching vibration with a minor contribution of C-N stretching and N-H bending vibrations. To enhance spectral resolution and gain insight in changes related to secondary structure of the myofibrillar proteins, the amide I regions was investigated as second derivative spectra. In the upper left corner of **Figure 2** the corresponding second derivative spectrum in the amide I region for two exemplary spectra of unprocessed and processed samples are shown. In the second derivative spectra of the amide I region we were able to identify and assign 9 bands (1694, 1682, 1667, 1658, 1653, 1639, 1628, 1619 and 1609 $cm^{-1}$). Our findings are in agreement with earlier studies on fish, pork and beef tissues (*5, 19-24*). The band assignments are listed in **Table 2**.

*Spectral differences within unprocessed samples.*

Principal Component Analysis (PCA) was performed to study the unprocessed samples in the amide I region (1700-1600 $cm^{-1}$). The score plot of the first and second principal component based on 36 myofiber spectra (inside/skinside, head/tail, 3 different sizes) did not reveal any systematic clustering of the samples according to individual or sample position (results not shown). Higher principal components were also examined and no systematic variations could be found. From these results, it can be concluded that, for the unprocessed samples, myofiber FTIR spectra from different individuals and different locations show no systematic variation with respect to local variation within each salmon fillet and individuals.

*Multivariate Analysis of myofiber spectra from processed salmon, non-smoked and smoked samples.*

***Analysis of amide I***

PCA was used to investigate non-averaged spectra of (salted) non-smoked and smoked samples in the amide I region (1700-1600 $cm^{-1}$) (shown in **Figure 3**). The explained variance

for PC1 and PC2 are 46% and 34% respectively. The first principal component showed a systematic variation with respect to depth (difference between inside and skinside), whereas the second principal component showed a systematic variation with respect to smoking. Inspecting the loading vector of component one, we found that the band near 1653 $cm^{-1}$, which is α-helical structures, contributed strongly (results not shown). It was shown that this band decreased in magnitude in inside samples of salmon fillets if we compare with skinside samples, implying a loss of α-helical components. This may be explained by the fact that the skin of the fish protects the flesh near the skin from salt and smoke components. In addition, the fat deposited in the fish muscle acts as a barrier against the diffusion of these components from the inside to muscle regions near the skin during processing; consequently the skin side locations are less accessible. In the following we treat spectra from inside and skinside locations separately.

PLSR was carried out to study the effect of the design factors on the FTIR spectra of processed samples for skinside and inside samples separately. In the analysis, the ***X*** matrix contained the design parameters while the ***Y*** matrix consisted of selected wavenumbers in the amide I region. In **Figures 4a** and **4b** the correlation loading plots of the first two PLS components of inside and skinside respectively are shown. The inner and outer circles in the figures refer to 50 and 100% explained variance, respectively. For the inside part samples (**4a**) the explained variance in **X** and **Y** for PLS component 1 and 2 are 23%, 23% for **X** and 36% and 21% for **Y**. As **Figure 4a** shows, the salting time spans out the first PLS component while smoking spans the second PLS component. Samples salted for 24 hours were highly correlated with the bands 1628, 1694, 1682 and 1667 $cm^{-1}$, whereas samples salted for 8 hours were well correlated with the band near 1653 $cm^{-1}$. This shows that longer salting periods resulting in higher salt concentrations (see **Table 1**) lead to a higher share of β-sheet structures and nonhydrogenated C=O groups and a lower share of α-helical structure in the

myofibers (see **Table 2**). These findings support the result obtained by previous studies related to different salt concentrations (*20, 34*). Böcker and co-workers (*20*) showed that in pork muscle tissue subjected to brine salting at different salt concentration (0.9, 3, 6, and 9% NaCl) the amount of α-helical structures (1653 $cm^{-1}$) was higher in samples with low salt content and the level of nonhydrogenated C=O groups (1668 $cm^{-1}$) was increased at higher salt concentrations. In another study (*22*) also in pork tissue subjected to salting at different concentration (3, 6, and 9% NaCl) revealed that salting induced an increase in aggregated β-sheet structure and a decrease in α-helical structure. A recent study by Böcker and co-workers (*5*) on brine salting (16% NaCl) of Atlantic salmon showed that the salt uptake varied due to the salmon raw material quality. The qualities which had higher salt content in the muscle presented an increase in the share of the 1668 $cm^{-1}$ band.

Smoked and non-smoked samples were separated along PLS 2. For non-smoked samples the absorbances at the bands near 1639 $cm^{-1}$ and 1609 $cm^{-1}$ were increased. The band at 1639 $cm^{-1}$ was assigned both to antiparallel β-sheet structures (intramolecular) (*18*) and to O-H bending of water (*35*). Since smoked samples show a higher weight loss than non-smoked ones (see table 1), the decrease of absorption at 1639 $cm^{-1}$ for smoked sample may be explained by dehydration. Dehydration after smoking has been reported by several authors (*11-12*). The band at 1609 $cm^{-1}$ has frequently been discussed to be related to amino acid side chains (*36*). In previous studies on muscle fiber tissues, this band has been tentatively assigned to the amino acid tyrosine (*23-24*). Since carbonyl compounds of smoke can react with amino groups of protein chain and/or of the side chains as in a Maillard reaction, we may expect that in the present study the band at 1609 $cm^{-1}$ is decreased in smoked samples, which is confirmed by Fig. 4a.

For the skinside part samples (4b) the explained variance in **X** and **Y** for PLS component 1 and 2 was 22% and 22% for **X** and 32% and 14% for **Y**, respectively. In contrast to the inside

samples, for the skinside samples, PLS1 is mainly explained by the smoking and to a lower degree by the salting time. This means that smoking introduces larger variations in the amide I region than salting time. Apart from this, the general picture in Fig. 4b is unchanged and confirms the findings from Fig. 4a.

***Analysis of amide II***

In the second derivative spectra of the amide II region (1600-1500 $cm^{-1}$), the following bands were identified: 1584, 1574, 1567, 1555, 1544, 1534, 1526 and 1510 $cm^{-1}$. The band assignments are listed in **Table 2** together with the bands found in amide I region. These bands were assigned and discussed previously for pork muscle fiber tissue (*21*).

PLSR was performed to study the effect of the design factors on the FTIR spectra of processed samples in the amide II region for the inside and skinside parts separately. The respective correlation loading plots are shown in **Figures 5a** and **5b**. For the inside part (**5a**) the first PLS component has an explained variance of 23% and 56% for **X** and **Y**, respectively, whereas the second PLS component has an explained variance of 23% and 12% in **X** and **Y**, respectively. For the skinside part (**5b**), the first PLS component has an explained variance of 22% and 41% for **X** and **Y**, respectively, whereas the second PLS component has an explained variance of 22% and 11% in **X** and **Y**. An interesting result is that the main variation in the amide II region is due to smoking for both parts of fillet (inside and skinside). Along PLS1, smoked samples are totally separated from non-smoked samples, whereas PLS2, separated the samples with regards to salting time.

The amide II region is well known to be conformationally sensitive, although it is more difficult to interpret than the amide I region. It is dominated by N-H bending (60%) and C-N stretching (40%) vibrations. In addition, it has been suggested that the contribution of amino acid side chains to the amide II region may be larger than in amide I region (*37-39*). As it has been demonstrated in previous studies, the smoking process in some fish showed a decrease

in the availability of lysine (*15-16*). Riha and co-workers (*15*) revealed that carbonyl compounds of liquid smoke solutions undergo a condensation reaction with amino groups of proteins where lysine appeared to be the most reactive amino acid. Moreover, Sisko and co-workers (*16*) also reported a lysine loss in smoked trout compared to non-smoked trout. We may conclude that changes in amide II region could be related to interactions between smoke components and amino groups from lysine or other N terminal of side chains more than specific protein secondary structural changes. This is also confirmed by the fact that in this kind of reactions N-H groups of proteins are interacting, which contribute strongly to the amide II region.

**Comparison of light microscopy and FT-IR Microspectroscopy**

Both FTIR spectra and light microscopy images from salmon muscle tissue were compared after introducing variation due to salting and smoking. A longer salting period resulted in increased amounts of aggregated β structures and in decreased amounts of α-helical structures as identified in the amide I region. These changes were related to observations made by light microscopy where salted samples appeared to be more detached and their extracellular spaces increased. However, smoking did not affect the same IR bands as salting. As it was observed in amide I, the bands near 1639 $cm^{-1}$ and 1609 $cm^{-1}$ were decreased as a result of dehydration and interactions between smoke components and amino acid side chains. Moreover, in the amide II region, the major differences were caused by smoking. It is likely that the interaction between carbonyl compounds of smoke and amino groups from lysine or other N terminal of side chains is the main contributor to the FTIR signal in the amide II region. After smoking, light images showed that the myofibers of the smoked samples appeared to be more shrunken and their shape appeared more irregular and edged than for the non-smoked samples.

In conclusion, an understanding of the structural and chemical changes of food muscle proteins in smoking processes is crucial for the production of high quality smoked products.

Most of the major quality issues in smoked salmon, such as water holding capacity and texture, are related to myofibrillar proteins. In the present study it has been demonstrated for the first time that FTIR microspectroscopy can be used to identify the interactions between proteins and smoke components. Furthermore, it has been shown that salting time is an important parameter in the cold smoking process, affecting the secondary structure of fish proteins. Data from this study can provide an insight into the possibilities of FT-IR microspectroscopy as a non-destructive technique to monitor processing-induced changes in muscle tissues.

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**Table 1.** General sample characteristics for non-smoked and smoked salmon fillets. H indicates samples from head and T samples from the tail region of the fillet.

| | Size (Location) | % Weight Loss | | % NaCl (s) | | % fat | |
|---|---|---|---|---|---|---|---|
| | | *24 hours* | *8 hours* | *24 hours* | *8 hours* | *24 hours* | *8 hours* |
| **Non-smoked** | **small** | 6,36 | 5,50 | | | | |
| | **(H)** | | | 2,47±0,07 | 1,57±0,03** | 18,9±0,4 | 14,4±0,1 |
| | **(T)** | | | 3,88±0,16$^{c}$ | 2,29±0,06**$^{,c}$ | 4,3±0,2 | 4,9±0,1 |
| | **medium** | 6,24 | 4,85 | | | | |
| | **(H)** | | | 2,46±0,07 | 1,63±0,03** | 16,2±0,4 | 17,9±0,1 |
| | **(T)** | | | 3,28±0,12$^{b}$ | 1,79±0,04**$^{,b}$ | 7,0±0,4 | 5,4±0,2 |
| | **large** | 6,74 | 4,23 | | | | |
| | **(H)** | | | 1,91±0,04 | 1,76±0,03* | 17,8±0,1 | 18,1±0,1 |
| | **(T)** | | | 3,32±0,12$^{c}$ | 2,29±0,06**$^{,c}$ | 8,3±0,0 | 5,1±0,4 |
| **Smoked** | **small** | 9,77 | 9,17 | | | | |
| | **(H)** | | | 3,57±0,07$^{C}$ | 2,05±0,07**$^{,C}$ | 15,6±0,5 | 16,7 ±0,0 |
| | **(T)** | | | 4,74±0,22$^{b,B}$ | 2,87±0,09**$^{,c,B}$ | 5,4±0,1 | 6,1 ±0,1 |
| | **medium** | 8,91 | 7,41 | | | | |
| | **(H)** | | | 2,47±0,07$^{A}$ | 1,52±0,07**$^{,B}$ | 19,6±0,4 | 16,1±0,5 |
| | **(T)** | | | 2,54±0,08$^{a,C}$ | 2,46±0,07$^{ns,c,C}$ | 5,8±0,0 | 4,9±0,6 |
| | **large** | 8,86 | 7,51 | | | | |
| | **(H)** | | | 3,17±0,07$^{C}$ | 1,95±0,07**$^{,B}$ | 19,8±0,2 | 14,3±0,5 |
| | **(T)** | | | 4,08±0,18$^{b,B}$ | 2,50±0,07**$^{,c,A}$ | 7,0±0,4 | 7,8±0,3 |

(s) Significance level. Between salted for 24 h and 8 h: ns, not significant; * $p < 0.05$; ** $p < 0.01$. Between head and tail samples: a, not significant; b, $p < 0.05$; c, $p < 0.01$. Between non-smoked and smoked samples: A, not significant; B, $p < 0.05$; C, $p < 0.01$.

**Table 2.** IR region, FT-IR frequencies and approximate descriptions of vibrational modes.

| Region | Frequency ($cm^{-1}$) | Tentative band assignment |
|---|---|---|
| *Amide I* | 1694 | Aggregated β-sheet structures (intramolecular) |
| | 1682 | Antiparallel β-sheet structures (intramolecular) |
| | 1667 | Non- hydrogenated C=O groups |
| | 1658 | Loop structures |
| | 1653 | α-helical structures |
| | 1639 | Antiparallel β-sheet structures / O-H bending of water |
| | 1628 | Aggregated β-sheet structures (intramolecular) |
| | 1619 | Aggregated β-sheet structures (intermolecular) |
| | 1609 | Amino acid side chains (tentatively) |
| *Amide II* | 1584 | α-helical structures |
| | 1574 | Unspecified |
| | 1567 | Residue and/ or possibly aggregated β-sheet structures |
| | 1555 | Unspecified |
| | 1544 | α-helical structures |
| | 1534 | Denatured random structures |
| | 1526 | Aggregated β-sheet structures (tentatively) |
| | 1510 | Tyrosine (tentatively) |

**FIGURE CAPTIONS**

**Figure 1**. Transverse sections of salmon muscle stained with Toluidine Blue. **S** refers to skin side and **I** to inside, **U** to unprocessed samples, **S8** refers to salted for 8 hours, **S24** salted for 24 hours, **B8** salted for 8 hours followed by smoking and **B24** salted for 24 hours followed by smoking . All images shown were derived from the head region of the salmon.

**Figure 2**. Typical FTIR spectrum of a single myofiber obtained from unprocessed salmon muscle cryosection showing the spectral range from 4000-1000cm$^{-1}$. (Inset) Positive second-derivative spectra of the same unprocessed sample together with a processed sample in the amide I region.

Figure 3. PCA score plot of non-averaged samples (144 samples) using the selected wavenumbers of the inverted 2$^{nd}$ derivative in the amide I region as variables. Marked in red: salted; in blue smoked; S and I represent skinside and inside samples, respectively. The explained variance by PC1 and PC2 is 46% and 34%, respectively.

**Figure 4a/b.** Correlation loading plots of PLSR with design parameters as X and selected wavenumbers of the inverted 2$^{nd}$ derivative in the amide I region as Y. The inner and outer circles in the figure refer to 50 and 100% explained variance, respectively. For the inside part samples (**4a**) the explained variance in **X** and **Y** for PLS component 1 and 2 are 23%, 23 % for **X** and 36% and 21% for **Y**. For the skinside part samples (**4b**) the explained variance in **X** and **Y** for PLS component 1 and 2 are 22% and 22% (for **X**) and 32% and 14% (for **Y**), respectively.

**Figure 5a/b.** Correlation loading plots of PLSR with design parameters as X and selected wavenumbers in the amide II region of the negative 2$^{nd}$ derivative spectra as Y. The inner and outer circles in the figure refer to 50 and 100% explained variance, respectively. For the inside part (**5a**) the first PLS component has an explained variance of 23% and 56% for **X** and **Y**, respectively, whereas the second PLS component has an explained variance of 23% and

12% in **X** and **Y**. For the skinside part (**5b**), the first PLS component has an explained variance of 22% and 41% for **X** and **Y**, respectively, whereas the second PLS component has an explained variance of 22% and 11% in **X** and **Y**.

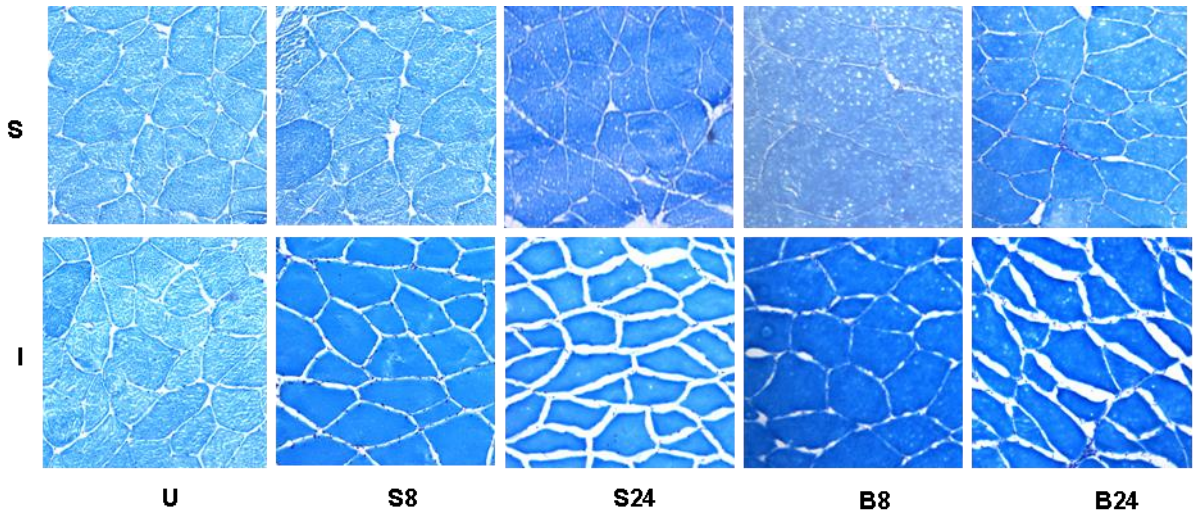


Figure 1

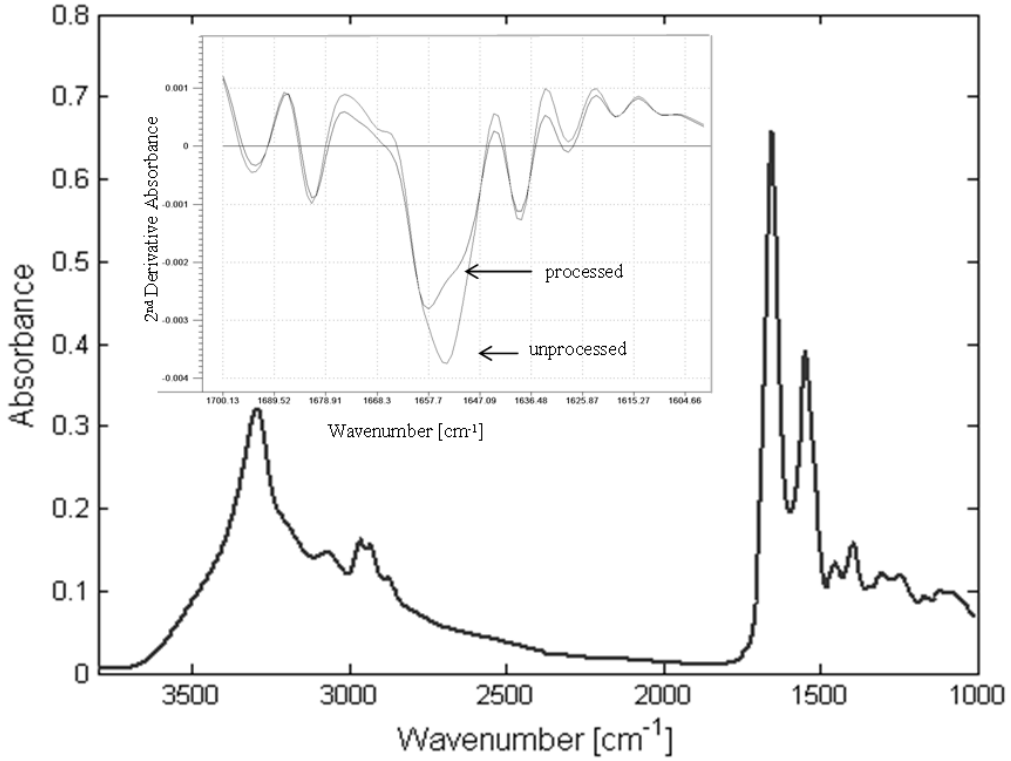
Absorbance
Wavenumber [cm-1]
2nd Derivative Absorbance
processed
unprocessed
Wavenumber [cm-1]

Figure 2

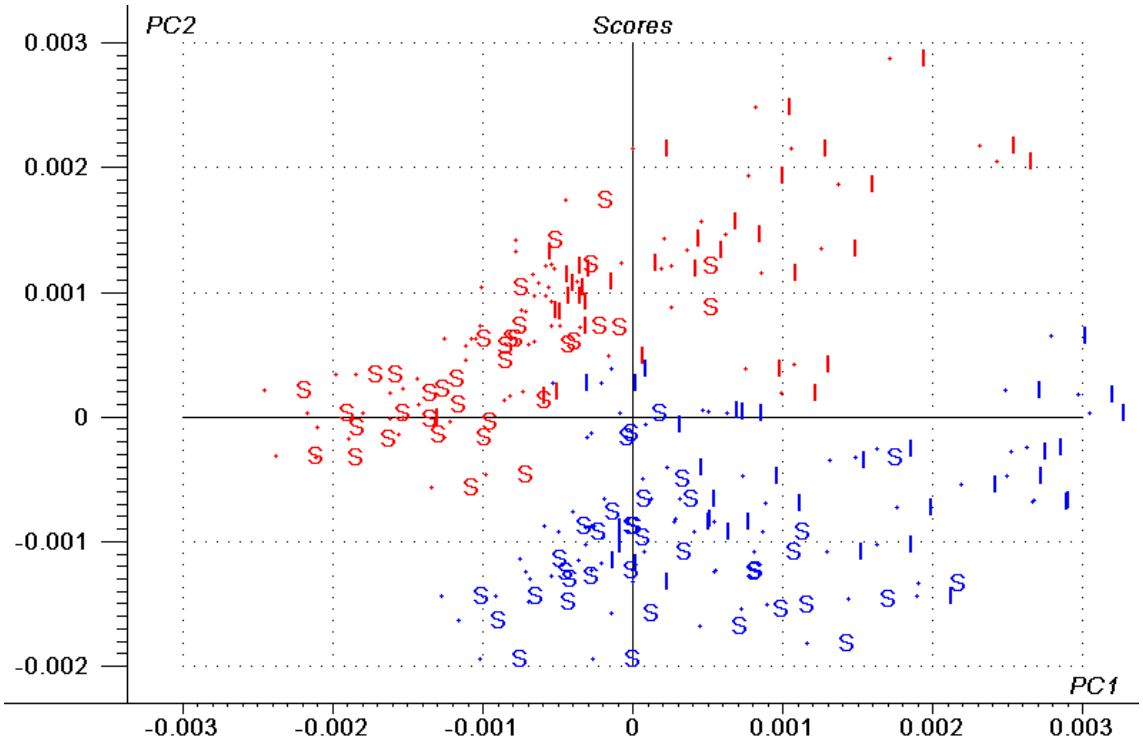
PC2
Scores
0.003
0.002
0.001
0
-0.001
-0.002
PC1
-0.003
-0.002
-0.001
0
0.001
0.002
0.003

Figure 3

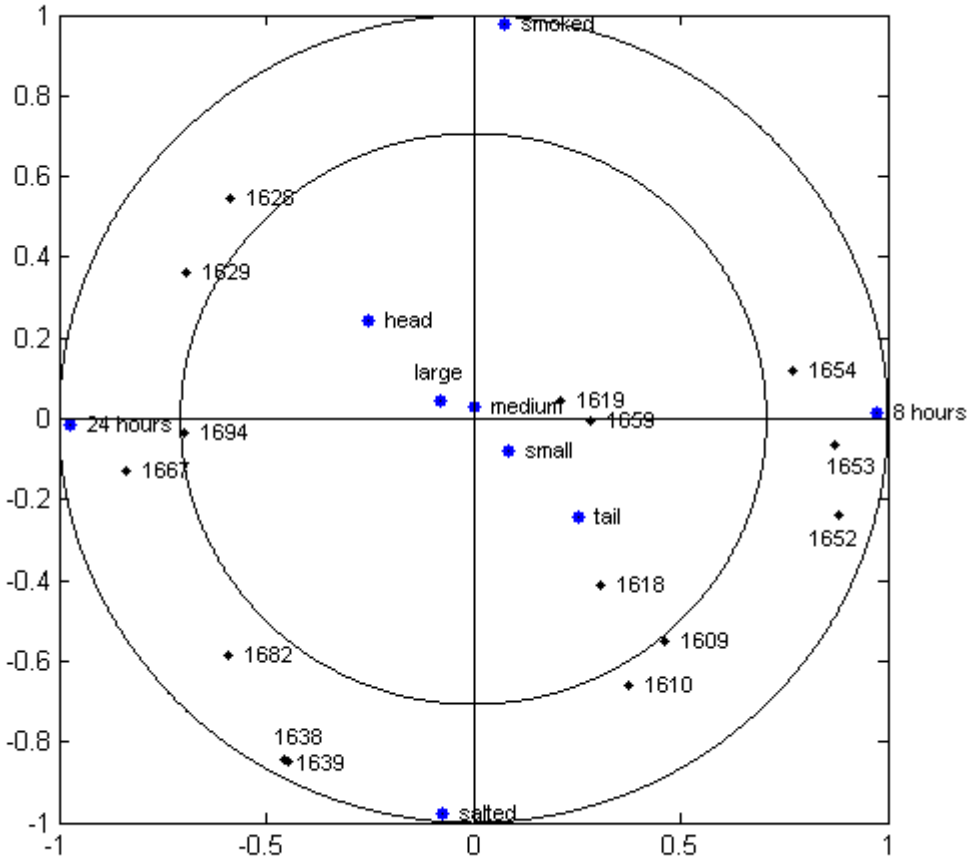
smoked
1628
1629
head
large
medium
1619
1659
1654
8 hours
24 hours
1694
1667
small
1653
tail
1652
1618
1609
1682
1610
1638
1639
salted
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1
-0.5
0
0.5
1

Figure 4a

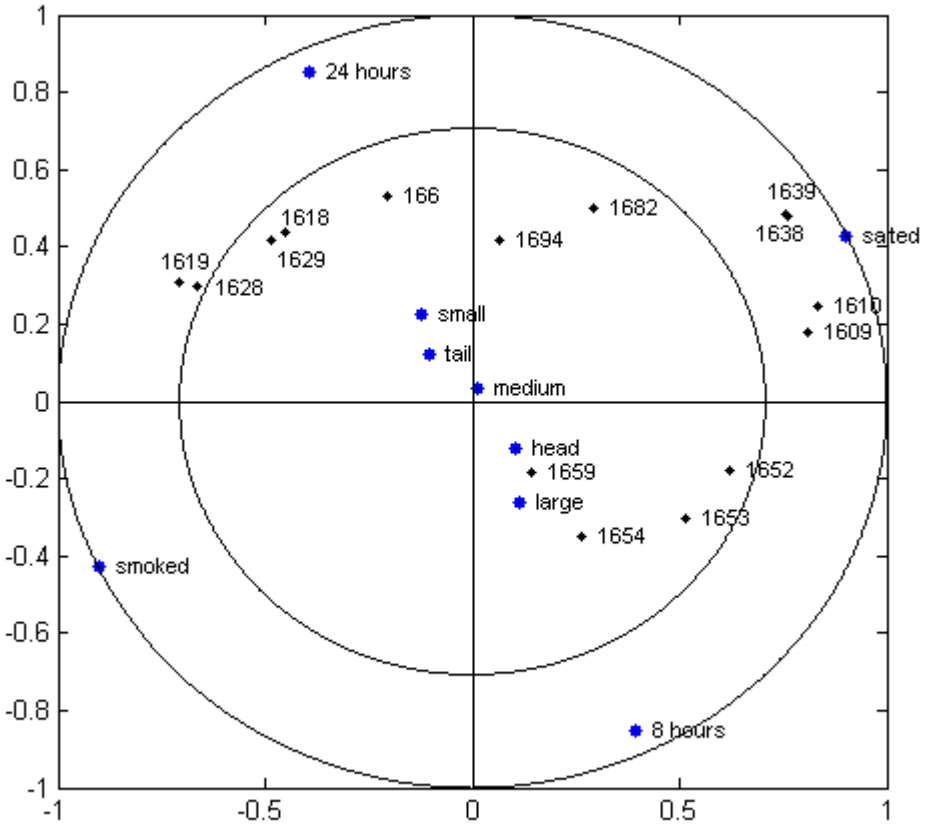
24 hours
166
1618
1629
1619
1628
1682
1694
1639
1638
sated
1610
1609
small
tail
medium
head
1659
1652
large
1653
1654
smoked
8 hours
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1
-0.5
0
0.5
1

Figure 4b

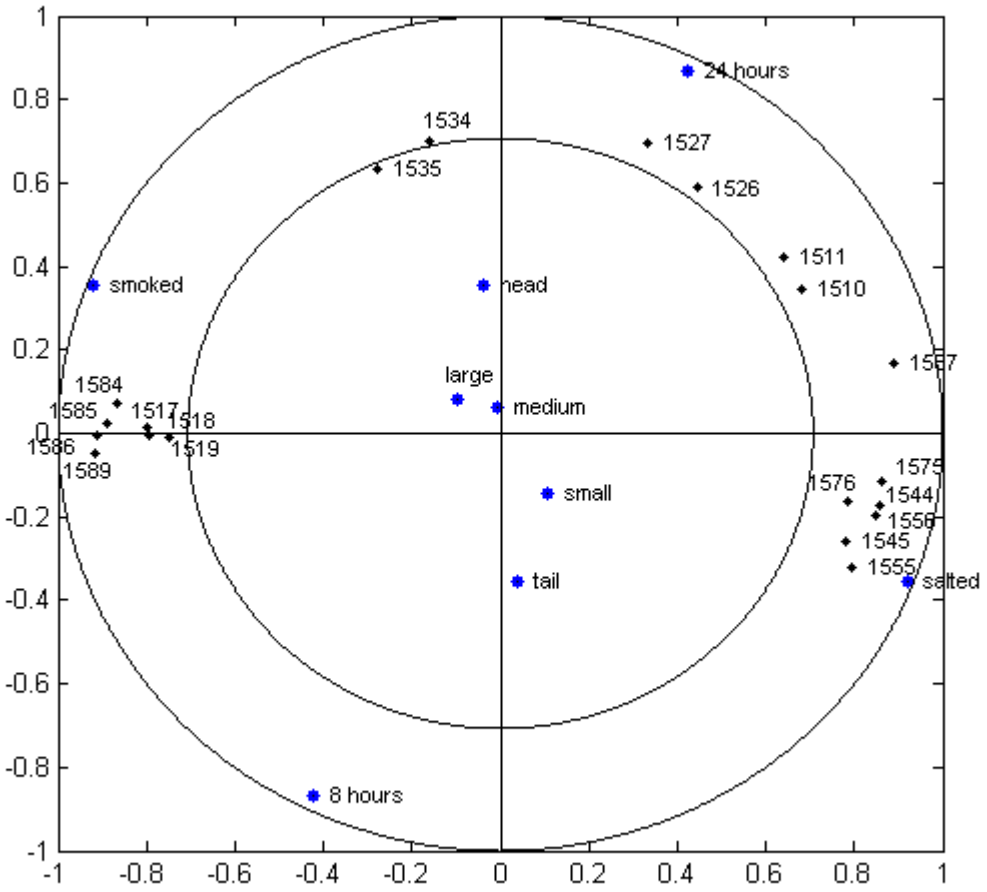
24 hours
1534
1527
1535
1526
1511
smoked
head
1510
1567
large
1584
1585
1517
medium
1518
1586
1519
1589
1575
1576
small
1544
1558
1545
tail
1555
salted
8 hours

Figure 5a

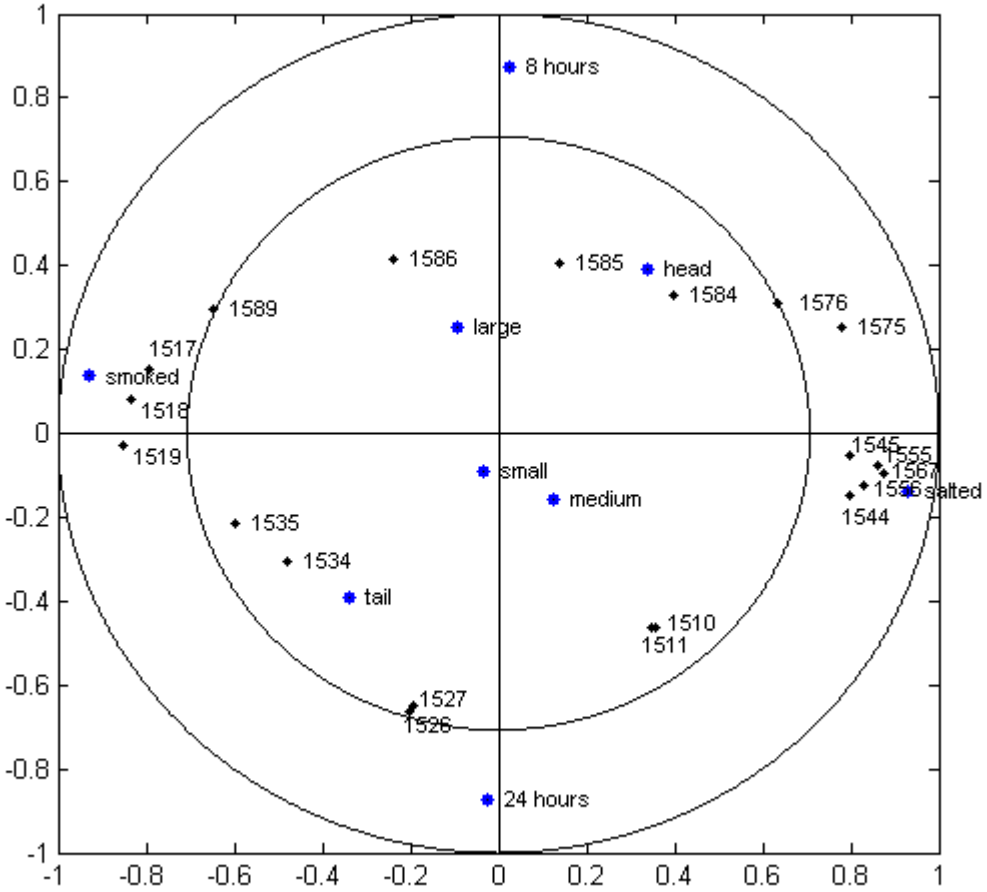
8 hours
1586
1585
head
1584
1576
1575
1589
large
1517
smoked
1518
1519
small
medium
salted
1545
1555
1567
1544
1535
1534
tail
1510
1511
1527
1526
24 hours
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 5b