Microstructural characteristics of turbot (*Scophthalmus maximus*) muscle: effect of salting and processing

De-Yang Li\textsuperscript{a,b}, Ying Huang\textsuperscript{a,b}, Ke-Xin Wang\textsuperscript{a,b}, Xiu-Ping Dong\textsuperscript{a,b}, Da Yu\textsuperscript{a,b}, Li-Hong Ge\textsuperscript{a,b}, Da-Yong Zhou\textsuperscript{a,b}, and Chen-Xu Yu\textsuperscript{a,b,c}

\textsuperscript{a}School of Food Science and Technology, Dalian Polytechnic University, Dalian, People’s Republic of China; \textsuperscript{b}National Engineering Research Center of Seafood, Dalian, People’s Republic of China; \textsuperscript{c}Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, Iowa, USA

**ABSTRACT**

The effect of salt concentration on the processing and characteristics of turbot muscle was investigated in this study. The turbot muscle samples were salted with 1%, 5%, 10%, 15%, or 20% w/w salt at 4°C for 3 h. Low-field nuclear magnetic resonance was utilized to characterize water distribution and water holding capacity in the samples. Nuclear magnetic resonance transverse (\(T_2\)) relaxation identified three water components (\(T_{21}\), \(T_{22}\), and \(T_{23}\)) which all exhibited characteristics correlated with water holding capacity. Textural analysis indicated that hardness and elasticity increased with increasing salt concentration. Histological imaging showed that with the increase of salt concentration, the muscle fiber diameter and area increased first and then decreased. The porosity of salted samples was higher than that of fresh ones. It was shown that salting at lower salt concentrations would lead to partial degradation of the fibrin in the tissue samples and the swelling of proteins. In treated samples, 5% w/w salt was shown to produce the best results. This study provides theoretical basis for the development of salted turbot meat products.

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**Introduction**

Cultivation of turbot (*Scophthalmus maximus*) is a fast-growing aquaculture industry. It is in high demand in countries where consumers prefer white, firm, and flavorful fishes.\textsuperscript{[1]} Turbot is a commercially important marine fish for aquaculture in northern China.\textsuperscript{[2–4]} The harvest of flounder fish was 118,009 tones; it was no. 3 maricultural fish in China in 2016.\textsuperscript{[5]}

Salting is one of the oldest processing and preservation methods for foods. During salting, foods are brought into contact with solid salt, or soaked in brine for extended time. Salting results in the reduction of water activity in foods, which subsequently leads to the inhibition of spoilage. In addition, salting also improves the functional characteristics of proteins in meaty foods\textsuperscript{[6]} and improves the texture and flavor of meat products.

Studies have shown that quality of “pickled” fish is dependent on the level of salt added\textsuperscript{[7–9]} As the concentration of salt increases, more protein denaturation and fiber shrinkage occur, leading to a decrease in muscle water holding capacity (WHC).\textsuperscript{[10]} The WHC of meat can be defined as its ability to retain its own water as well as added water,\textsuperscript{[11]} and it directly affects meat hardness, tenderness, and yield, thereby influencing meat economic value.\textsuperscript{[12]} Due to its importance, research works have been conducted to better understand and improve WHC.\textsuperscript{[13,14]} Although much information has been gained, there is still a need for better understanding the foundational mechanism of bulk water holding within meat,\textsuperscript{[15]} and the effects of the addition of salt on water distribution and mobility within the protein filament lattice.\textsuperscript{[16]} In recent years,
low-field nuclear magnetic resonance (LF-NMR) has been employed to characterize water mobility and
distribution within meat.\[17\] The relaxation data have been shown to correlate well with WHC.\[16\]

During salting, the microstructure of meat goes through complicated changes that correlate with the
loss of water and the changes in texture, and the quality of meat is determined to a large extent by it.\[18–20\]
However, experimental procedures that allow an objective way to quantitatively describe morphological
differences and to relate them with macroscopic attributes are generally lacking.\[21\] For pickled fishes,
currently the relationship between processing parameters and quantitative microstructure characteristics
remains to be fully explored. Microstructures of fish like turbot observed under microscope, due to its
disorderly shape, are not easy to describe in an objective and quantitative way.\[22–25\]

To our knowledge, no work has been reported on the relationship of salting conditions and
changes in microstructural characteristics of turbot. Therefore, in this work, we attempted to
describe the quantitative microstructural changes of salted turbot by means of imaging analysis of
paraffin sections. In addition, these changes were correlated with the characteristics of salted turbot,
as explained by changes in low-field NMR $T_2$ relaxation times, texture, and WHC by principal
component analysis (PCA). A thorough comprehension of this relationship is essential to achieve
better control of deep processing of turbot fish for quality products.

Materials and methods

Materials and salting

Living turbot fishes ($n = 30, 1.3–1.5$ kg, and length $38–42$ cm) were randomly selected and purchased
from a local market (Meilinyuan Market, Dalian, China). Fishes were kept on ice and transported to
a laboratory within 0.5 h of purchasing. The fishes were killed by a big bang on the head and were
skinned, and then the muscle meat was obtained. The muscle from each fish was cut into cubes of
$1.5 \times 1.5 \times 1.0$ cm$^3$ using a stainless steel scalpel. The same amount of fish was soaked in brine at
different salt concentrations (1%, 5%, 10%, 15% and 20% w/w); the ratio of fish:brine was 1:3, for 3 h
at $4 \degree$C. The raw turbot was used as control.

Measuring water holding capacity

Water holding capacity (WHC, %) was determined according to the method developed by R.M.
Uresti.\[26\] Two layers of the filter paper were put at the bottom of 80 mL centrifuge tubes. Samples of
minced fish were weighted (A1), then placed at the bottom of centrifuge tubes and on top of the
filter paper, and centrifuged at 1000 r/min×15 min at $4 \degree$C. Immediately after centrifugation, the
samples were weighted (A2), and the WHC was calculated as follows:

$$\text{WHC(%) = } \frac{A1 - A2}{A1} \times 100\%$$

Measurements were performed in quadruplicate.

Instrumental textural properties

Texture profile analysis (TPA) was performed on turbot samples using TA.XT plus texture analyzer
(Stable Micro Systems, Haslemere, Surrey, UK). The size of each muscle sample was
$1.5 \times 1.5 \times 1.0$ cm$^3$. The operating parameters were as follows: Model P50 head; pre-test speed
2.0 mm/s, test speed 1 mm/s, and post-test speed 1 mm/s; compressed depth 60%; time interval 5.0 s;
compressed times 2. Each treatment was measured with four replicates.\[27\]
**Low-field NMR measurement**

A low-field NMR analyzer (LF-NMR, NMI20-030H-I, Suzhou Niumag Analytical Instrument Co., Suzhou, China) equipped with a 30 mm diameter coil and a 0.5 T permanent magnet corresponding to a proton resonance frequency of 23.2 MHz at 32°C was used to collect \( T_2 \) transverse relaxation data for turbot samples by using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with a \( \tau \)-value (time between 90 and 180 pulses) of 200 μs. Data from 6000 echoes were acquired as four scans. The LF-NMR relaxation curves were obtained after multi-exponential fitting of CPMG decay curves using MultiExp Inv analysis software (Suzhou Niumag Analytical Instrument Co., Suzhou, China). All measurements were performed with three replicates for each sample.

**Histological analysis of microstructure**

Turbot samples (1.5 × 1.5 × 1.0 cm³) were salted for 3 h at 4°C by soaking in brine of 1%, 5%, 10%, 15%, and 20% w/w salt concentrations, respectively. Post-salting, samples were rinsed with deionized water and blotted dry. A non-treated sample was used as the control. Samples were immediately fixed in a 10% formaldehyde solution (AR, Tianjin Shigeru chemical reagent, China) for 24 h. Imaging slides for each sample were prepared by paraffin section and hematoxylin and eosin staining technique.

A microscopic imaging system (Model No. DP72, Labophot-2, Olympus, Japan) was used to examine the effect of salt concentration on fibers. Images for each sample were generated with three slides. Ten sites were selected at random for image acquisition from each slide. Images were analyzed using Image-pro plus software (Version 6.2.1, Media Cybernetics, USA) to obtain the area and diameter of each microfiber.

**Statistical analysis**

Statistical treatment of the results was performed using Statistical Analysis System IBM SPSS (Statistical Package for the Social Sciences) statistics v19.0 (SPSS Inc., USA). One-way analysis of variance (ANOVA) was employed to determine the significance of main effects by means of Duncan’s multiple range test with a significant level of \( p < 0.05 \). All the figures were plotted using Origin 8.5 software (Microcal, USA). PCA was conducted to find the main variations in the multivariate data and determine the relationship between the WHC, textural quality characteristics, LF NMR data, and microstructure characteristics during turbot salting by using Statistica 8.0 analysis software.\(^{28,29}\)

**Results and discussion**

**WHC**

WHC is one of the most important processing properties of fish. The WHC of salted turbot muscle increased at 1% salt concentration compared to control, but decreased with increasing salt concentration, as shown in Figure 1 (\( p < 0.05 \)). At lower salt concentration, salt increases the ionic strength in the muscle, which favors myosin B transitioning into the sol state, and may lead to the increase of WHC and the occurrence of additional water absorption. With the salt concentration continue to increase, proteins are now exposed to high levels of salt present in the brine, and the resulted protein degradation and fiber shrinkage may lead to decreased WHC.\(^{10,30}\)

**TPA**

Salted fish would dehydrate, leading to textural changes reflected in changes of sensory attributes. In this work, hardness and elasticity changes of the turbot salted at different brine concentrations were investigated, as shown in Figure 2 and Figure 3, respectively. Toyohara\(^{31}\) pointed out that the connective tissue around
fresh fish muscle cells helps maintain good texture characteristics in the muscle, while the dissolution of the gluing tissue may cause the fish to become tenderer. As shown in Figure 2, the hardness of the fish increases with an increase in salt concentration. As more salt enters the muscle, its protein denatures, and protein surface structures change, with more hydrophobic groups exposed. Hence, association between water and

**Figure 1.** Effect of salt on WHC of turbot (mean ± SD, n = 4). *Different letters indicate significant differences (p < 0.05) between treatments.

**Figure 2.** Effect of salt on hardness of turbot (mean ± SD, n = 4). *Different letters indicate significant differences (p < 0.05) between treatments.
proteins weakens, and the water film between protein molecules starts to disappear, causing more intermolecular collisions and aggregation. The fish muscle loses water in this concentration range of salt, and the microbial growth is inhibited. At the same time, the endogenous autolytic enzymes are also inhibited. Denaturation of muscle proteins at high salt content renders its structures more rigid, and increases the hardness in turn. On the other hand, salt may also cause salt-soluble proteins to dissolve and form into a sol-gel. The formation of salt-soluble protein sol-gel may lead to an increase in elasticity in the turbot, as evidenced by Figure 3.

**Proton relaxation determined by NMR**

The longitudinal relaxation time $T_1$ and the transverse relaxation time $T_2$ determined from low-field NMR spectra are time constants used to describe the proton relaxation process, especially protons (i.e., H) in H$_2$O. Compared with $T_1$, $T_2$ is more sensitive to the presence of multiphase morphology. The $T_2$ relaxation time reflects the chemical environment of the proton in the sample, which is related to the binding force and freedom of the proton. The larger the proton bound or the smaller the degree of freedom, the shorter the relaxation time of $T_2$, and the longer the $T_2$ relaxation. Differences in $T_2$ can be attributed to water state in a food sample. It can be used to distinguish between free water and crystal water that does not interact with solid particles or solvent, or chemically bound water. Therefore, the determination of water state in food is typically through the study of transverse relaxation time $T_2$. As shown in Figure 4, three peaks could be identified in LF-NMR $T_2$ spectra of turbot samples, which corresponded to three types of water: $T_{21}$ peak has a relaxation time of 1−5 ms, which corresponds to tightly bound water molecules at the fish surface; $T_{22}$ peak has a relaxation time of 50−150 ms, representing immobilized water existing between fibrils, myofibrils, and membranes; $T_{23}$ peak has a relaxation time of 700−1500 ms, characterizing free water residing in muscle fibers outside the fiber bundle. Interestingly, these three peaks appeared to be affected differently by salting. As shown in Table 1, for $T_{21}$ values, significant variations were observed among samples treated with brine of different salt concentrations of 1%, 5%, and 15%,
but no significant difference was found between samples treated with concentrations of 10% and 20%. $T_{22}$ values varied significantly between no or low salt condition (1%) and high salt conditions (>5% salt). $T_{23}$ values in contrast were significantly affected by salting, regardless of the salt concentration though. $A_{Total}$ indirectly reflected the total amount of H (i.e., total amount of H$_2$O) in the sample. Compared with the fresh turbot, the changes in $A_{Total}$ suggested that salting affected water loss in samples. The relative peak area of the sample treated with 1% brine increased, consistent with the overall increase of WHC in those samples. Jiang\[36\] proposed that the protein structure and the spatial arrangement of muscle tissue would affect water distribution. In this study, at low salt concentration of 1%, water absorption seems to occur mainly due to weak interaction between muscle proteins and water molecules that brings in mostly free water and immobilized water (i.e., $T_{22}$ and $T_{23}$).

**Histological characterization of microstructure**

The salted fish muscle contains large amounts of water, not only in inter-filament spaces within the myofibrils, but also in extracellular spaces and in spaces between myofibrils.\[33\] The water molecules have different mobilities, due to their different degrees of association with the proteins and salt as well. Analysis of images (Figure 5) showed that all salt concentrations caused shrinkage of muscle fibers in comparison with the fresh control. Fresh turbot muscle fiber was closely arranged with fewer voids in between. As the concentration of salt increased, the arrangement of fibers became more and more disordered. At 1% salt, the sample showed similar fiber arrangement as in fresh samples; fiber swelling and coarsening became more apparent as salt concentration increased. The swelling of turbot muscle fibers was most visible with 5% salt. Higher concentration (15% and 20% salt) of salt indeed inhibited fiber swelling. It caused more fiber fracture and fragmentation, while the difference between the 15% and 20% salt-treated samples was not significant. This is similar to the findings of Knight and Parsons who reported that varying degrees of myofibrillar swelling occur at different NaCl concentrations.\[37\]
Table 1. NMR parameters obtained from different treatments with turbot.

| Sample            | $T_{21}$ (ms) | $T_{22}$ (ms) | $T_{23}$ (ms) | $A_{21}$ (1/g) | $A_{22}$ (1/g) | $A_{23}$ (1/g) | $A_{\text{Total}}$ (1/g) |
|-------------------|---------------|---------------|---------------|----------------|----------------|----------------|----------------------------|
| Fresh turbot      | 2.01 ± 0.00$^d$ | 57.22 ± 0.00$^a$ | 1497.45 ± 229.15$^b$ | 113.39 ± 3.67$^b$ | 7118.99 ± 46.71$^d$ | 24.77 ± 2.11$^a$ | 7257.14 ± 45.68$^d$ |
| 1% Salted turbot  | 2.43 ± 0.20$^c$ | 65.79 ± 0.00$^b$ | 811.13 ± 0.00$^a$ | 132.07 ± 2.68$^c$ | 8103.23 ± 59.76$^a$ | 202.62 ± 0.62$^c$ | 8437.92 ± 61.57$^c$ |
| 5% Salted turbot  | 1.67 ± 0.13$^c$ | 91.32 ± 7.52$^b$ | 979.16 ± 80.64$^a$ | 118.95 ± 10.34$^b$ | 5727.56 ± 250.38$^b$ | 29.43 ± 2.80$^b$ | 5875.95 ± 241.20$^b$ |
| 10% Salted turbot | 1.32 ± 0.00$^b$ | 95.66 ± 7.52$^b$ | 1072.27 ± 0.00$^b$ | 74.53 ± 3.63$^a$ | 5479.69 ± 27.34$^a$ | 27.84 ± 1.34$^b$ | 5582.06 ± 26.98$^a$ |
| 15% Salted turbot | 1.00 ± 0.00$^a$ | 100.65 ± 14.01$^b$ | 892.11 ± 70.13$^b$ | 110.81 ± 7.15$^b$ | 5823.94 ± 33.21$^b$ | 52.05 ± 0.95$^d$ | 5986.81 ± 39.72$^b$ |
| 20% Salted turbot | 1.45 ± 0.11$^b$ | 95.66 ± 7.52$^b$ | 1025.71 ± 80.64$^a$ | 74.45 ± 9.53$^a$ | 6685.76 ± 52.25$^c$ | 34.36 ± 1.65$^c$ | 6794.57 ± 46.76$^c$ |

*Different letters in a column indicate significant differences ($p < 0.05$) within each treatment (ANOVA).
Each value is expressed as means ± S.D. ($n = 3$).
Values in the same row followed by different superscript letters are significantly different ($p < 0.05$).
As histological parameters (Table 2) showed, the largest amount of swelling was caused by 10% w/w salt, evidenced by the largest overall fiber area and diameter. It was significantly larger than the swelling observed with other treatments (Table 2). Histologically, samples treated with 20% w/w salt did not differ significantly from samples treated with 15% w/w salt, nor did it differ significantly from fresh control. The average fiber diameter reached the maximum in samples treated with 5% w/w salt. The swelling might be due to water being absorbed into fibrin which leads to an increase in both fiber area and fiber diameter. Earlier reports showed that  

Figure 5. Effect of salt on the longitudinal section muscle of turbot.  

| Treatments (salt concentration, % w/w) | Fresh | 1% | 5% | 10% | 15% | 20% |
|---------------------------------------|-------|----|----|-----|-----|-----|
| Area (μm²)                            | 3248.6 ± 78.0<sup>a</sup> | 4418.0 ± 777.6<sup>ab</sup> | 5476.5 ± 756<sup>b</sup> | 5503.7 ± 549.9<sup>b</sup> | 4280.2 ± 87.2<sup>a</sup> | 3731.2 ± 27.4<sup>a</sup> |
| Diameter (μm)                         | 40.1 ± 0.2<sup>a</sup> | 46.4 ± 5.2<sup>ab</sup> | 54.1 ± 4.0<sup>b</sup> | 48.9 ± 1.6<sup>a</sup> | 43.6 ± 4.1<sup>a</sup> | 40.7 ± 4.7<sup>a</sup> |
| Porosity (%)                          | 31.3 ± 1.3<sup>a</sup> | 35.4 ± 5.3<sup>ab</sup> | 43.6 ± 2.8<sup>c</sup> | 40.1 ± 1.6<sup>bc</sup> | 50.8 ± 2.6<sup>d</sup> | 46.6 ± 2.9<sup>cd</sup> |

<sup>a,b,c,d</sup>Different letters in a column indicate significant differences (p < 0.05) within each treatment (ANOVA). Each value is expressed as means ± S.D. (n = 3). Values in the same row followed by different superscript letters are significantly different (p < 0.05).  

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under <10% w/w low salt conditions, water absorption might occur, which could result in an increase in fiber porosity, consistent with the results of this study.

**Principal component and correlation analysis of low-field NMR, WHC, TPA, and histological microstructure data**

It is well known that microstructure plays a critical role in determining meat physical properties, such as WHC, but an objective method to measure such influence is still lacking due to the difficulty of quantitative description of the microstructure. In this work, we aim to establish a correlation between microstructural changes during salting and the physical characteristics of the fish.\[38\]

Correlations were found between some LF-NMR parameters, WHC, and histological variables. Analysis of the histological images (Figure 4) along with correlations between histological variables and LF-NMR parameters (Table 3) further enhanced our understanding of water

**Table 3.** The correlation analysis of low-field NMR, WHC, TPA, and histology microstructure data.

|            | WHC   | Area  | Porosity | Diameter | T21   | T22   | T23   | A21   | A22   | A23   | ATotal | Hardness | Elasticity |
|------------|-------|-------|----------|----------|-------|-------|-------|-------|-------|-------|--------|----------|------------|
| WHC        | 1.000 | -0.151| -0.803   | -0.043   | 0.909 | -0.879| 0.207 | 0.714 | 0.736 | 0.585 | 0.746  | -0.859   | -0.777     |
| Area       | 1.000 | 0.142 | 0.644    | -0.249   | 0.431 | -0.419| -0.102| -0.512| -0.026| -0.485| -0.879 | -0.115   | -0.104     |
| Porosity   | 1.000 | 0.149 | 0.738    | 0.753    | 0.487 | -0.291| -0.577| -0.273| -0.566| 0.769 | 0.472  |          |            |
| Diameter   | 1.000 | -0.036| 0.337    | -0.370   | 0.223 | -0.384| 0.039 | -0.354| -0.263| -0.376|        |          |            |
| T21        |       | 1.000 | -0.821   | 0.124    | 0.578 | 0.830 | 0.655 | 0.836 | -0.696| -0.612|        |          |            |
| T22        |       |       | 1.000    | -0.422   | -0.488| -0.726| -0.395| -0.719| 0.712 | 0.566 |        |          |            |
| T23        |       |       |          | 1.000    | -0.148| 0.021 | -0.511| -0.016| -0.275| -0.054|        |          |            |
| A21        |       |       |          |          | 1.000 | 0.462 | 0.566 | 0.494 | -0.646| -0.746|        |          |            |
| A22        |       |       |          |          |       | 1.000 | 0.747 | 0.999 | -0.354| -0.362|        |          |            |
| A23        |       |       |          |          |       |       | 1.000 | 0.779 | -0.337| -0.400|        |          |            |
| ATotal     |       |       |          |          |       |       |       | 1.000 | -0.369| -0.384|        |          |            |
| Hardness   |       |       |          |          |       |       |       |       | 1.000 | 0.835 |        |          |            |
| Elasticity |       |       |          |          |       |       |       |       |       | 1.000 |        |          |            |

**Figure 6.** Results of the PCA—score plots in the PC1 vs. PC2 plane for all samples tested.
distribution within the fish as affected by salt concentration.\textsuperscript{[30]} As shown in Table 3, the porosity of muscle fibers is positively correlated with the $T_{22}$ intensity. Good correlations are also obtained between WHC and the relaxation times, the intra-myofibrillar water peak ($A_{21}$) and the extramyofibrillar water peak ($A_{22}$), with the strongest influence on WHC from immobilized water ($A_{22}$). It was previously presumed that $T_{22}$, which is an indicator of immobilized water, was closely correlated with drip loss.\textsuperscript{[39]} Our results confirmed it. WHC showed a strong, negative correlation with $T_{22}$, which implied that drip loss would be positively correlated with $T_{22}$. These results, in agreement with those of Bertram et al.,\textsuperscript{[40]} also confirmed that WHC is strongly affected by structural changes to the myofibril (i.e., porosity), which lead to changes in water distribution inside and outside of the myofibril.

A visualized comparison between samples treated differently can be made by plotting the first two PC scores calculated from a group of variables defined by the LF-NMR, WHC, TPA, and histological microstructure data. The score plot using PC1 and PC2, which accounts for 60% and 32% of the total variance, is shown in Figure 6. Clearly, samples closely clustered into groups defined by salt concentration. Furthermore, the high salt concentration samples (10%, 15%, and 20% w/w salt) all fall within the right-hand quadrant, i.e., for the scores of PC1 > 0, while the low salt concentration samples (fresh, 1% and 5% w/w salt) locate on the left-hand side. Hence, it suggests that a critical salt concentration may exist between 5% and 10% for the turbot salting operation.

\section*{Conclusion}

Findings of this study further confirm traditional WHC theories; that is, WHC is mainly dependent on the ability of the muscles to retain immobilized water; LF-NMR provided further evidence that changes in the fish muscle fiber matrix, water molecule distribution, and water mobility are all correlated with fish WHC. An increase in $T_{22}$ may be attributed to protein denaturation, and the subsequent loss of protein side chains may reduce sites for water binding. In order to have maximum WHC, intra-myofibrillar water should be increased, and extra-myofibrillar water should be decreased. This can be best achieved by salting turbot with 5% w/w brine concentration. Findings of this study could be utilized to optimize fish picking process to improve product quality.

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