Connective tissue Characteristics of Dark stained spot in salmon fillets:

Preliminary Results and Future proposals

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Samples we had

Three scores: Score 1 (4 fillets), score 2 (3 fillets) and score 4 (5 fillets)

Dark spot

Diffuse spots

Clear spots (0-3cm)

Clear spot (3-6cm)

Clean muscle
Initial Approach to samples

Analyses over Salmon muscle and over isolated Connective tissue

Clean salmon muscle
Dark spot muscle

Connective tissue of clean muscle and dark spot

- Melanin Detection (presence of blood in the dark spot?) and determination: Raman spectroscopy (NIR)
- Amino acid composition (HPLC)
- CT fibers morphology: Scanning electron microscopy (SEM)
- CT thermal stability: Differential scanning calorimetry (DSC)
- CT molecular structure: Fourier transform infrared spectroscopy (FTIR)

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## I. Analyses over Salmon Muscle

- Melanin Determination
- Raman (A. Jorge Alberto Jorge García. National Science Museum, Madrid. CSIC)
- Dark spot
- Clean Muscle

**RAMAN**
(780nm of 8mW power)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total fillets</th>
<th>Clean muscle</th>
<th>Dark spot Raman (8 spectra of each dark spot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 1 fillet 1,1</td>
<td>4</td>
<td>1</td>
<td>8</td>
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<tr>
<td>fillet 1,2</td>
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<tr>
<td>fillet 1,3</td>
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<tr>
<td>fillet 1,4</td>
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<td>8</td>
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<tr>
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<td>8</td>
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<tr>
<td>fillet 2,2</td>
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<td>8</td>
</tr>
<tr>
<td>fillet 2,3</td>
<td></td>
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<tr>
<td>fillet 4,2</td>
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<tr>
<td>fillet 4,3</td>
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<tr>
<td>fillet 4,4</td>
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<tr>
<td>fillet 4,5</td>
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<td><strong>Total</strong></td>
<td><strong>12</strong></td>
<td><strong>96</strong></td>
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</table>

<table>
<thead>
<tr>
<th>Samples Total</th>
<th>Clean muscle</th>
<th>Dark spot Raman (8 spectra of each dark spot)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>fillets Raman</strong></td>
<td><strong>4</strong></td>
<td><strong>3</strong></td>
</tr>
</tbody>
</table>

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Melanin Raman Spectra

*Predictors peaks of eumelanin*

$1350 \text{ cm}^{-1}, 1580 \text{ cm}^{-1}, 500 \text{ cm}^{-1}$


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Quantification of melanin by Raman

Three different Raman spectra (780nm of 8mW power)

Looking for a melanin commercial standard to prepare the standard curve
II.- Analyses over Connective Tissue

Isolation of connective tissue from clean muscle and dark spot

Homogenization with 0.8 % NaCl

Clean muscle & Dark spot

Washed with cold water

Connective tissue

To dry connective tissue 5 mins consecutive washes with 50-70-85-96 and 100% ethanol (CT SEM limitation)

CT Analyses

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Preliminary Results

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Analyses over Salmon Muscle

Raman from clean muscle and dark spot.

Determination of melanin

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MeanSpectra from dark spot of score 1/clean muscle spectrum of S1

Predictors peaks of melanin:
- $1350 \text{ cm}^{-1}$
- $1580 \text{ cm}^{-1}$
- $500 \text{ cm}^{-1}$

No melanin signal in clean muscle.
Mean Spectra from dark spot of score 2/clean muscle spectrum of S2

Raman Intensity (a.u)

Eumelanin

S2 dark spot
S2 clean muscle

No melanin signal in clean muscle

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Mean of Spectra from dark spot of score 4/clean muscle spectrum of S4

Eumelanin

1440 cm\(^{-1}\) phospholipids*

No melanin signal in clean muscle

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II.- Analyses over Connective Tissue

SEM clean muscle and dark spot.
Morphology of CT fibers.
Connective tissue normal muscle (50µm)

Score 1

Score 2

Score 4

Structures formed by CT fibers
S1 > S2 > S4
Connective tissue Dark spot (50µm)

Score 1

Score 2

Score 4

Structures formed by CT fibers are less organized as the presence of melanin increases:
S4 > S2 > S1
III.- Differential Scanning Calorimetry (DSC).
Thermal stability of Connective tissue

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Spectra from dark spot vs. clean muscle spectrum of score 1

$T_{peak}$ and $\Delta H$ dark spot $<$ $T_{peak}$ and $\Delta H$ clean muscle

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Spectra from dark spot vs. clean muscle spectrum of score 2

\[ T_{peak} \text{ and } \Delta H \text{ dark spot } < T_{peak} \text{ and } \Delta H \text{ clean muscle} \]
Spectra from dark spot vs. clean muscle spectrum of score 4

$T_{\text{peak}}$ dark spot $\sim T_{\text{peak}}$ clean muscle
$\Delta H$ dark spot $<< \Delta H$ clean muscle
Connective tissue of dark spot has less stability to thermal treatment meaning a less stabilized structure.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Samples</th>
<th>Dark spot</th>
<th>Clean muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score 1</td>
<td>43,44</td>
<td>46,82</td>
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<td>T\textsubscript{peak}(^\circ \text{C})</td>
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<td>45,42</td>
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<td></td>
<td>Score 4</td>
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<td>47,32</td>
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<tr>
<td></td>
<td>Score 1</td>
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<td>ΔH (^\circ \text{C})</td>
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<tr>
<td></td>
<td>Score 4</td>
<td>3,484</td>
<td>7,555</td>
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</table>
IV.- Fourier Infrared Spectroscopy (FTIR).
Secondary structure of collagen

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Amida I: Dark spot Score 1/muscle 1

Clean Muscle 1
Dark Spot S1

S1 (dark spot) has less stabilized triple helix

Hydrogen bonds in triple helix (-)
Random coil (-)
α-helix (-)
Intermolecular cross-links normally associated to β-sheets (~)
Disordered structures (~)

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Amida I: Dark spot Score 2/muscle 2

Clean Muscle 2
Dark Spot S2

S2 (dark spot) has more crosslinks among β-sheets (more aggregated structure)

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Amida I: Dark spot Score 4/muscle 4

Clean Muscle 4
Dark spot S4

S4 (dark spot) has a less stabilized triple helix than clean muscle

Intermolecular triple helix
Hydrogen bonds (~)
α-helix (~)
Random coil (~)
Disordered structures (~)

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Loss of collagen typical structure with melanin presence

S4 lower presence of α-helix, less crosslinks stabilizing β-sheet and more random structures.
Triple helical structure preservation of Connective tissue of Dark spot and normal muscle

<table>
<thead>
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<th>Samples</th>
<th>Ratio 1235/1450 cm⁻¹</th>
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<td>Muscle</td>
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<tr>
<td>1</td>
<td>1,00</td>
</tr>
<tr>
<td>2</td>
<td>1,01</td>
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<td>4</td>
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<td>Dark spot</td>
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<tr>
<td>S1</td>
<td>1,03</td>
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<tr>
<td>S2</td>
<td>1,02</td>
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<tr>
<td>S4</td>
<td>1,04</td>
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</table>

Lower triple helix preservation of collagen of CT from Dark spot. Also collagen of CT of clean muscle from score 4 has less preserved its helical structure.

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V.- Aminoacids composition.
Dark spot collagen structure is less preserved and less stabilized than in clean muscle

<table>
<thead>
<tr>
<th>AA</th>
<th>Clean Muscle 1 residues/1000</th>
<th>S1 Dark Spot residues/1000</th>
<th>Clean Muscle 2 residues/1000</th>
<th>S2 Dark Spot residues/1000</th>
<th>Clean Muscle 4 residues/1000</th>
<th>S4 Dark Spot residues/1000</th>
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<td>Asp</td>
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<td>63,64</td>
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<td>Arg</td>
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<td>Pro</td>
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<td>79,25</td>
<td>86,71</td>
<td>72,92</td>
<td>89,71</td>
<td>90,47</td>
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<tr>
<td>%Hyp/Pro</td>
<td>40,28</td>
<td>35,10</td>
<td>37,72</td>
<td>36,78</td>
<td>39,56</td>
<td>39,03</td>
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<tr>
<td>%Hyl/Lys</td>
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<td>20,03</td>
<td>23,10</td>
<td>16,20</td>
<td>24,35</td>
<td>24,26</td>
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</table>

Less twister helix
Less covalent bonds
Less hydrogen bonds

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To sum up...

Characteristics of Dark Spot:

1. Dark color was due to melanin but not to blood.
2. Connective tissue fibers seem to form smaller structures than in clean muscle. The structures are also smaller with increasing presence of dark spot (S4 to S1).
3. Collagen with lower enthalphy and/or temperature of transition than clean muscle (less stabilized collagen).
4. Collagen with less $\alpha$-helix and lower presence of Gly, Hyp and Hyl compared to clean muscle (loss of collagen typical structure).

Collagen from dark spot is less stabilized, has lost part of the helical structure as compared with the collagen from the clean muscle.

Dark spots of salmon fillets had disorganized and unstable connective tissue.
Thank you

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