



# Connective tissue Characteristics of Dark stained spot in salmon fillets:

## Preliminary Results and Future proposals

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# Samples we had

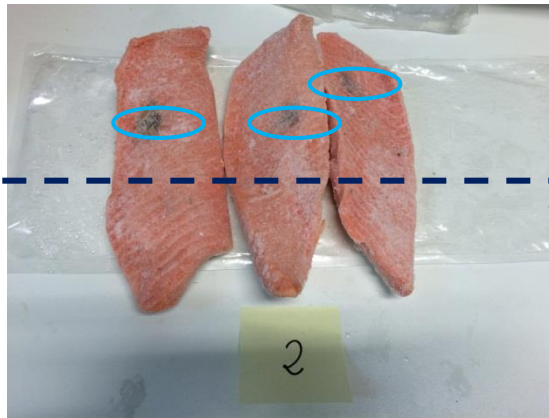
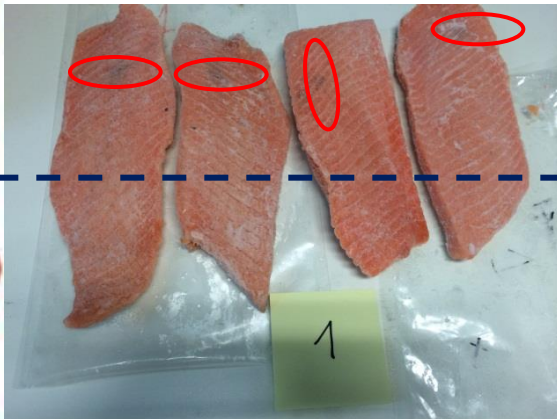
Three scores: Score 1 (4 fillets), score 2 (3 fillets) and score 4 (5 fillets)

Dark spot

Diffuse spots

Clear spots (0-3cm)

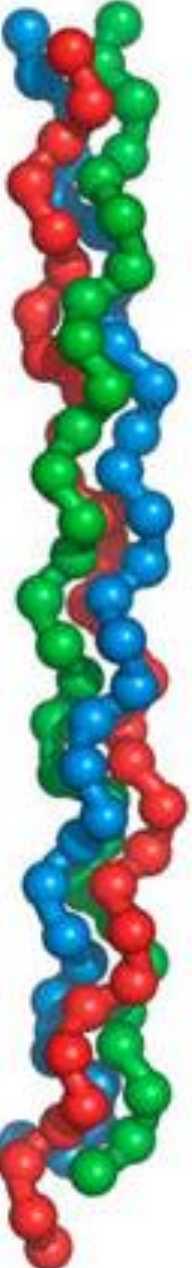
Clear spot (3-6cm)



Clean muscle

# Initial Approach to samples

## Analyses over Salmon muscle and over isolated Connective tissue



Clean salmon muscle  
Dark spot muscle

❖ Melanin Detection (presence of blood in the dark spot?) and determination: Raman spectroscopy (NIR)

Connective tissue of clean muscle and dark spot

- ❖ Amino acid composition (HPLC)
- ❖ CT fobers morphology: Scanning electron microscopy (SEM)
- ❖ CT thermal stability: Differential scanning calorimetry (DSC)
- ❖ CT molecular structure: Fourier transform infrared spectroscopy (FTIR)

# I.- Analyses over Salmon Muscle

## ❖ Melanin Determination

❖ Raman (A. Jorge Alberto Jorge García. National Science Museum, Madrid. CSIC)

❖ Dark spot

❖ Clean Muscle

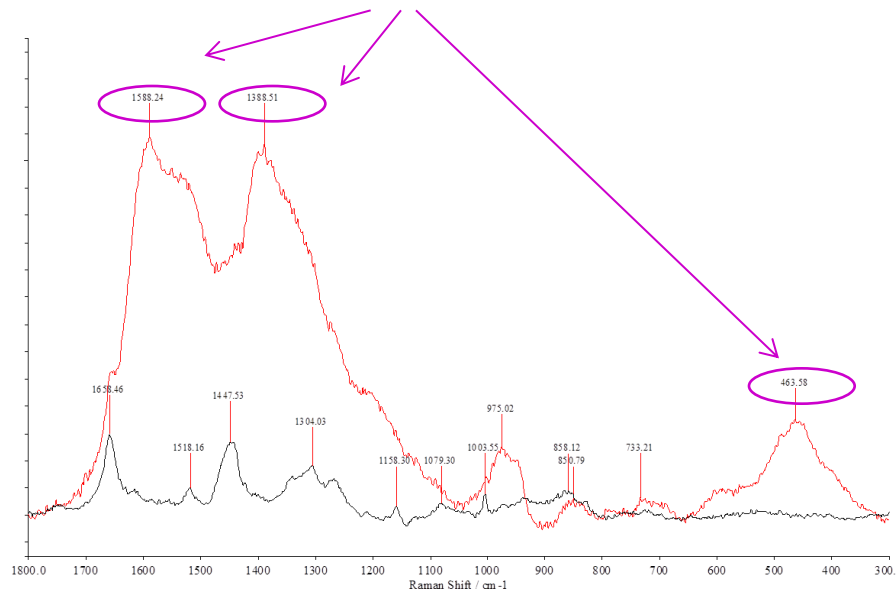
**RAMAN**

(780nm of 8mW power)

Samples	Total fillets	Clean muscle	Dark spot
		Raman	Raman (8 spectra of each dark spot)
<b>Score 1</b> fillet 1,1 fillet 1,2 fillet 1,3 fillet 1,4	4	1	8
		1	8
		1	8
		1	8
<b>Score 2</b> fillet 2,1 fillet 2,2 fillet 2,3	3	1	8
		1	8
		1	8
<b>Score 4</b> fillet 4,1 fillet 4,2 fillet 4,3 fillet 4,4 fillet 4,5	5	1	8
		1	8
		1	8
		1	8
		1	8
		<b>12</b>	<b>96</b>
<b>Total spectra: 108</b>			

# Melanin Raman Spectra

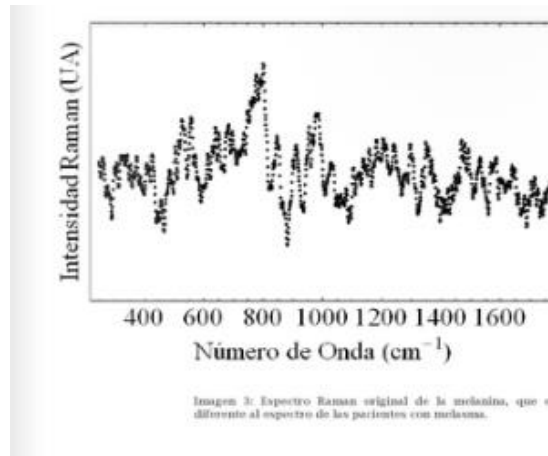
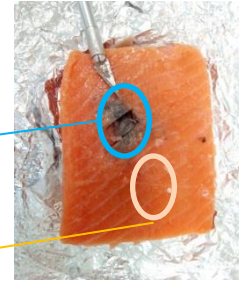
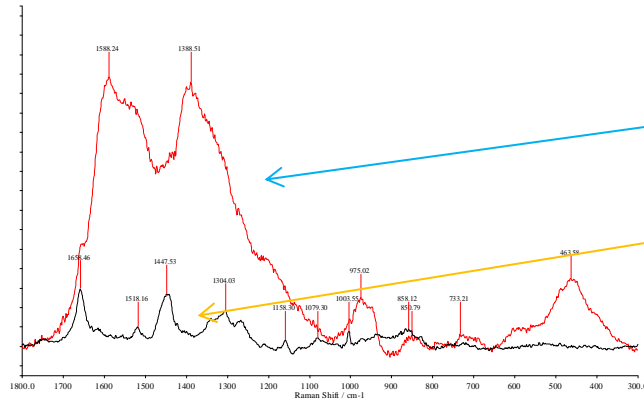
Predictors peaks of  
eumelanin  
 $1350\text{ cm}^{-1}$ ,  $1580\text{ cm}^{-1}$ ,  $500\text{ cm}^{-1}$



\*References: Huang et al. 2004. J. Biomed. Opt. 9:1198-1205; Galván et al., 2013. Acta A. Mol. Biomol Spectrosc. 110:55-59. Galván et al., 2013. Pigment Cell Melanoma res. 26:917-923

# 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



Clean  
muscle  
&  
Dark spot



Homogenization with 0.8 % NaCl



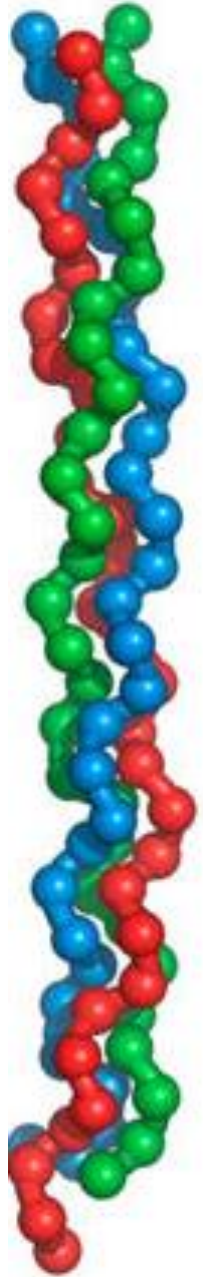
Washed with cold water



**Connective tissue**

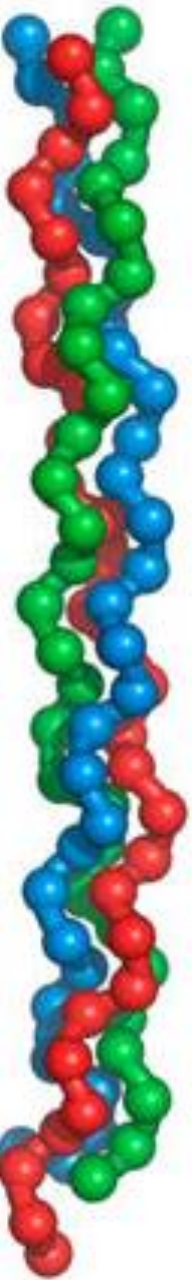
To dry connective tissue 5 mins  
consecutive washes with 50-70-85-  
96 and 100% ethanol (CT SEM  
limitation)

CT Analyses



# Preliminary Results



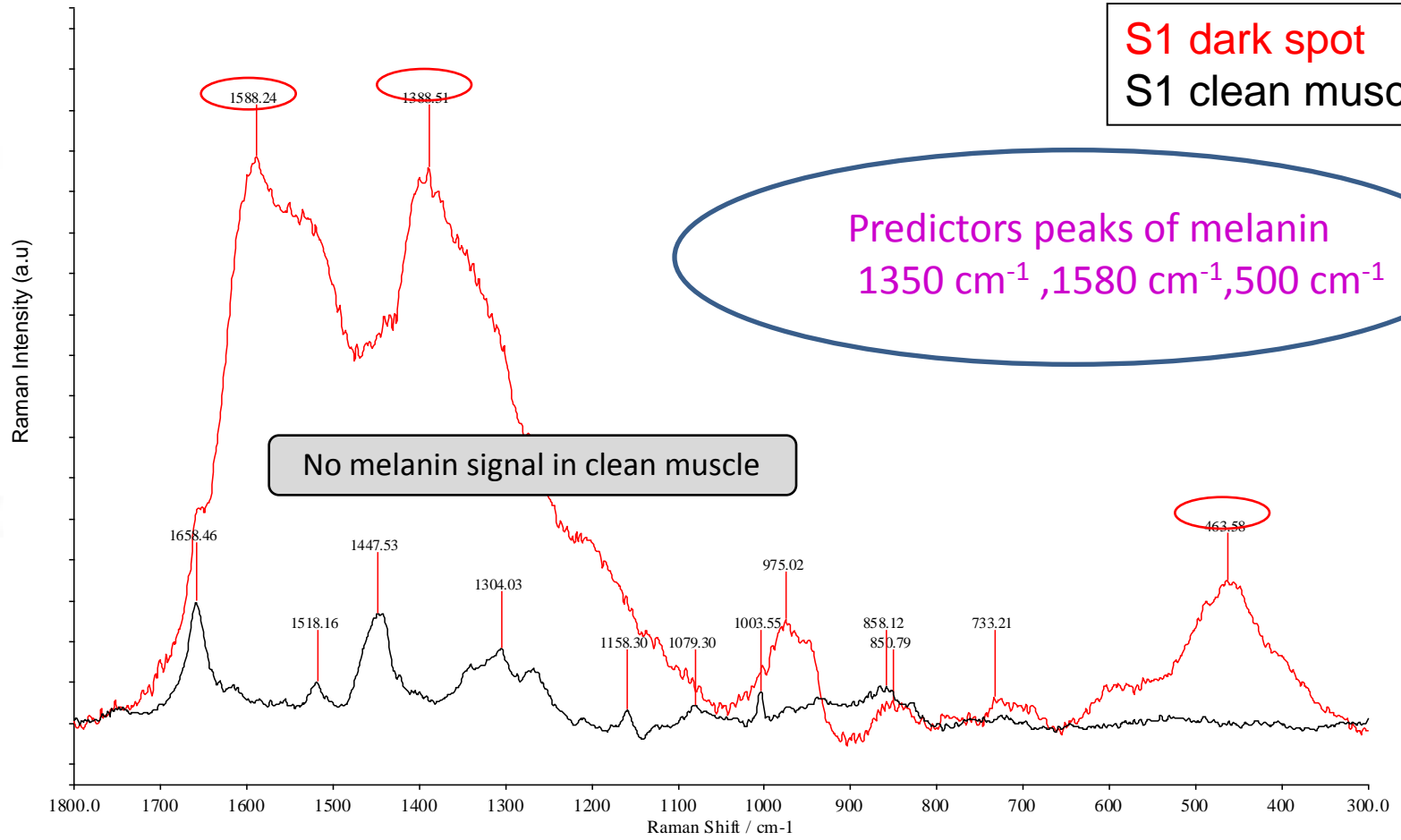
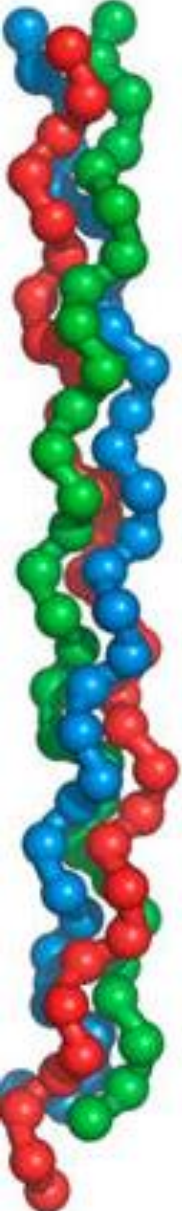


## Analyses over Salmon Muscle

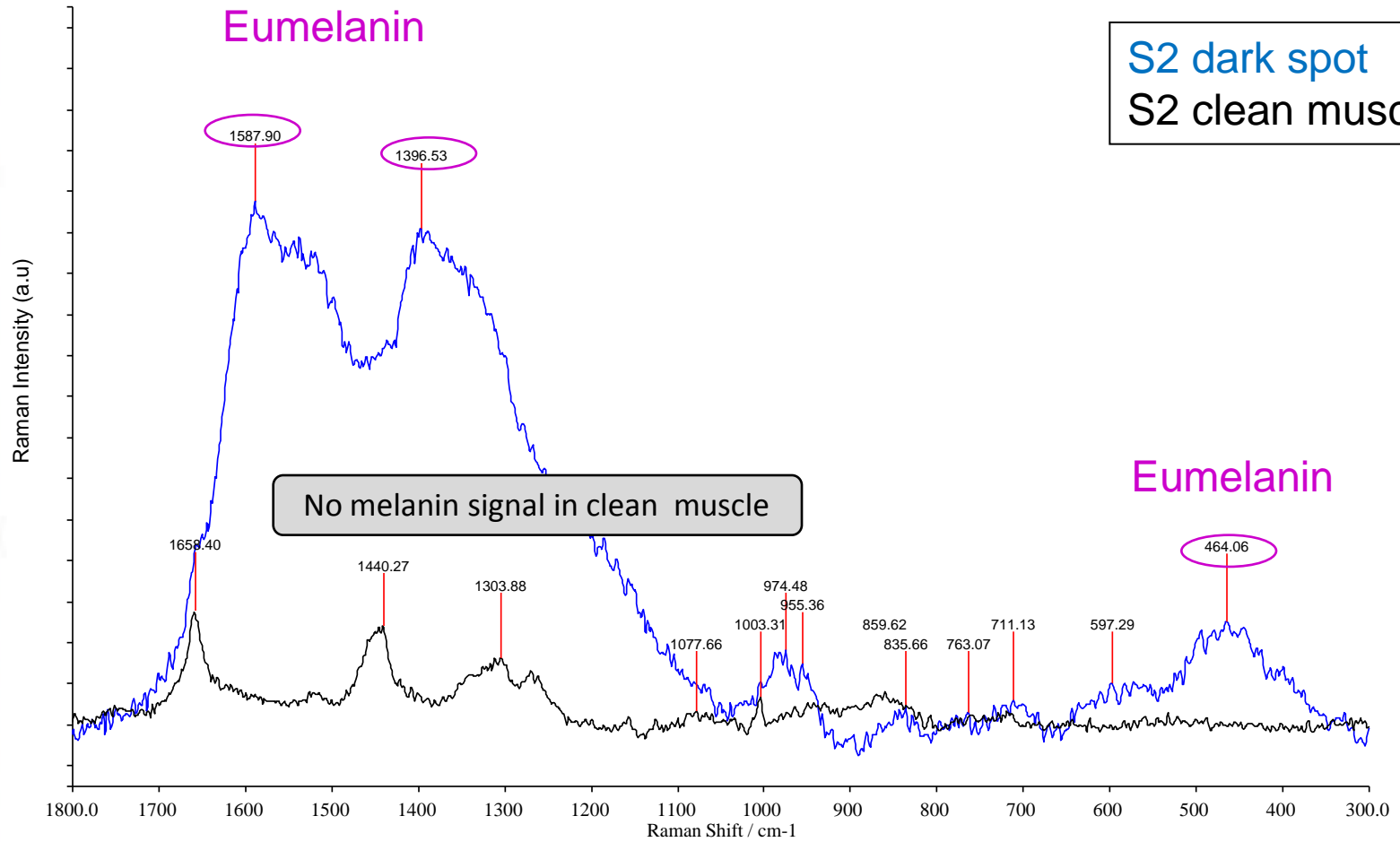
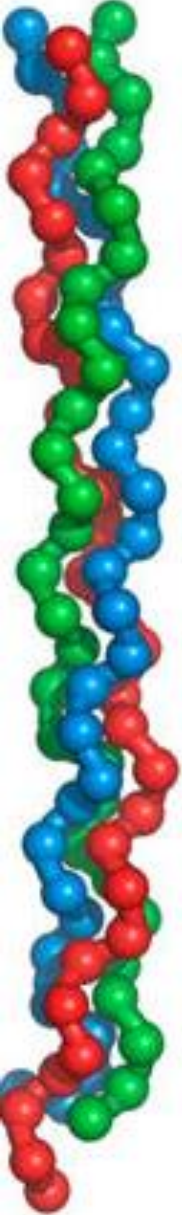
Raman from clean muscle and  
dark spot.

Determination of melanin

# Mean Spectra from dark spot of score 1/clean muscle spectrum of S1

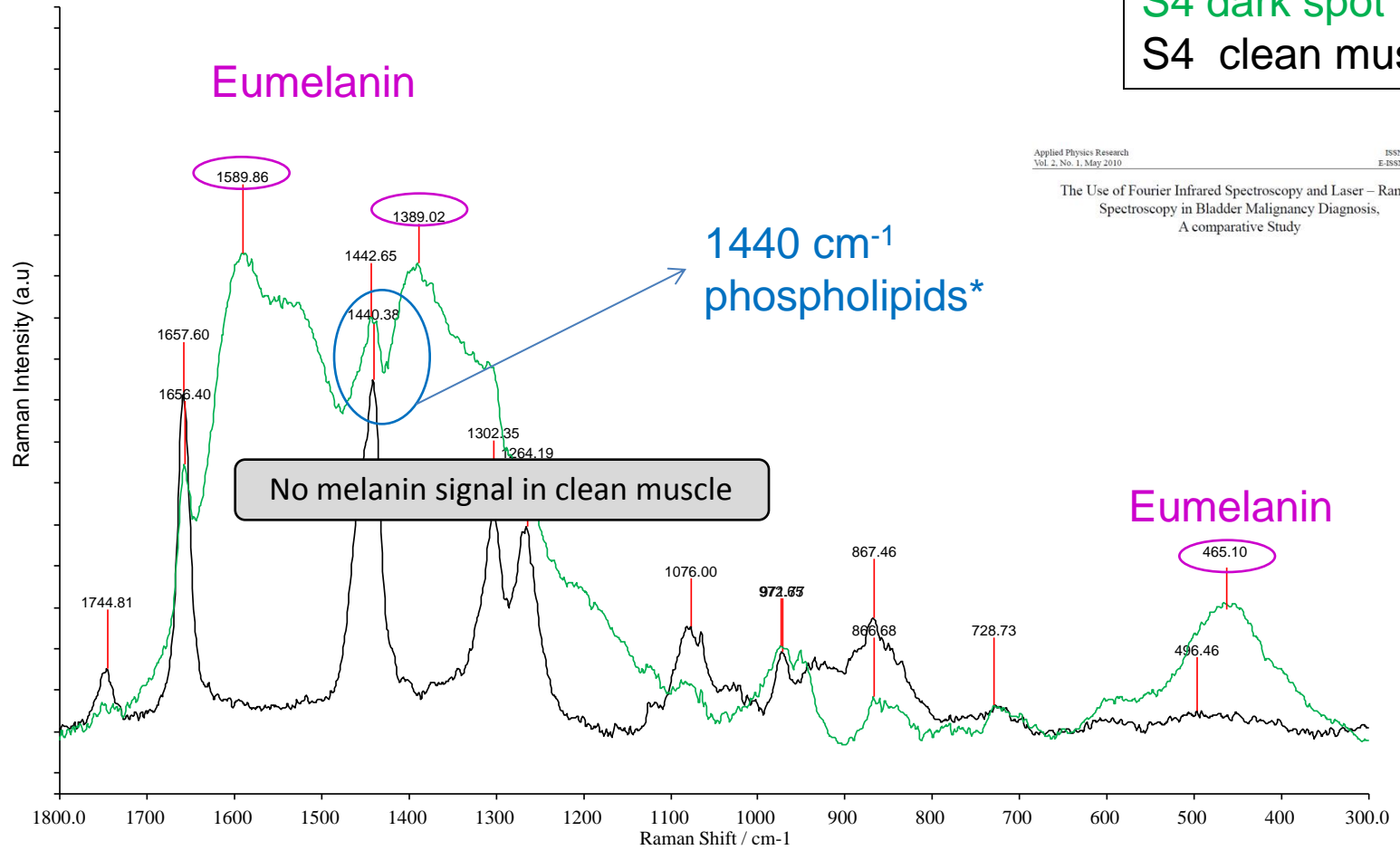


# Mean Spectra from dark spot of score 2/clean muscle spectrum of S2



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

S4 dark spot  
S4 clean muscle





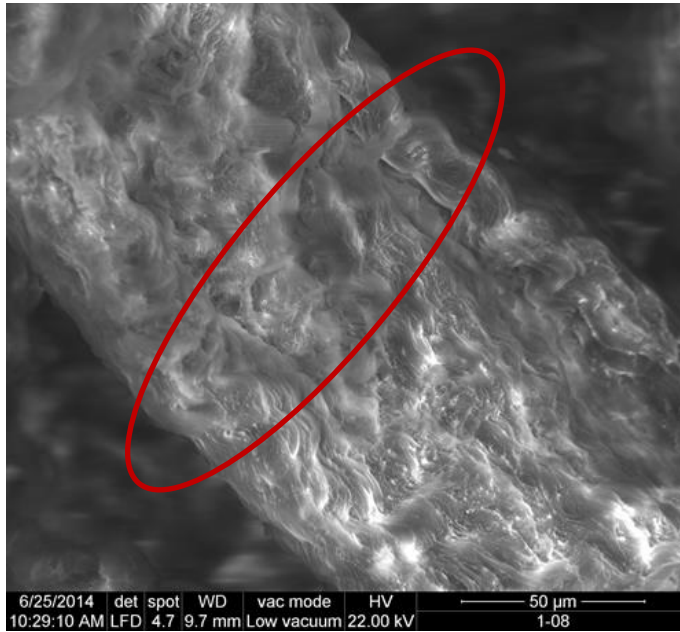
## II.- Analyses over Connective Tissue

SEM clean muscle and  
dark spot.

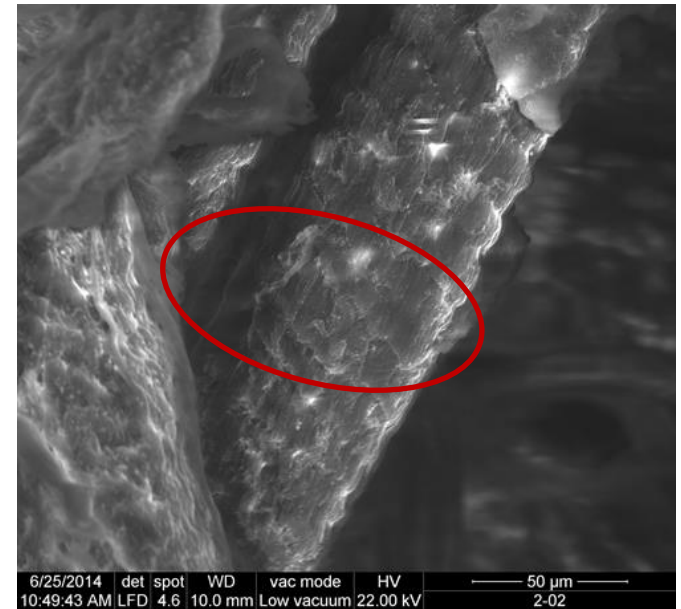
Morphology of CT fibers.

# Connective tissue normal muscle (50μm)

Score 1



Score 2

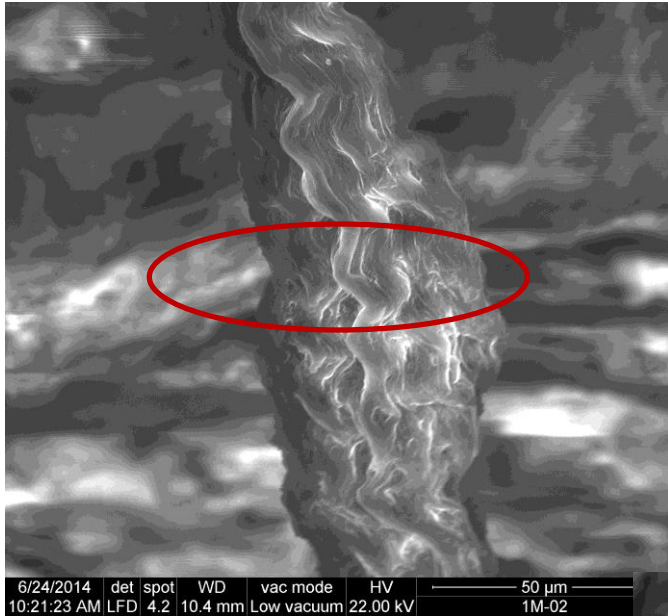


Structures formed by CT fibers  
S1 > S2 > S4

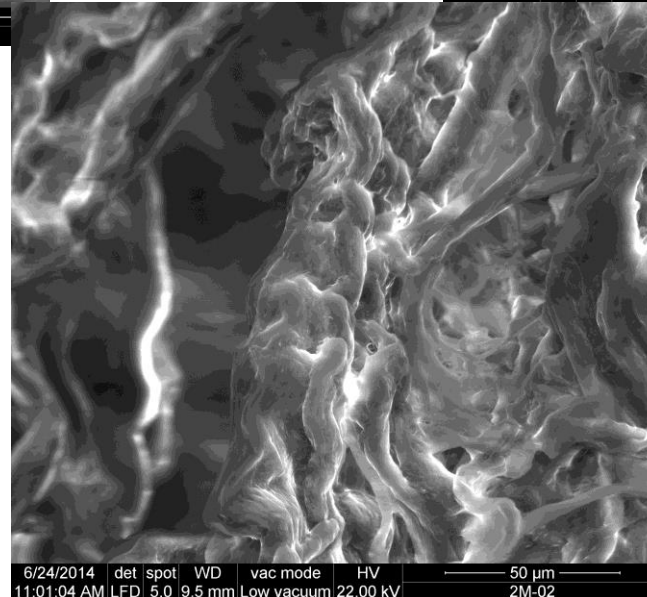
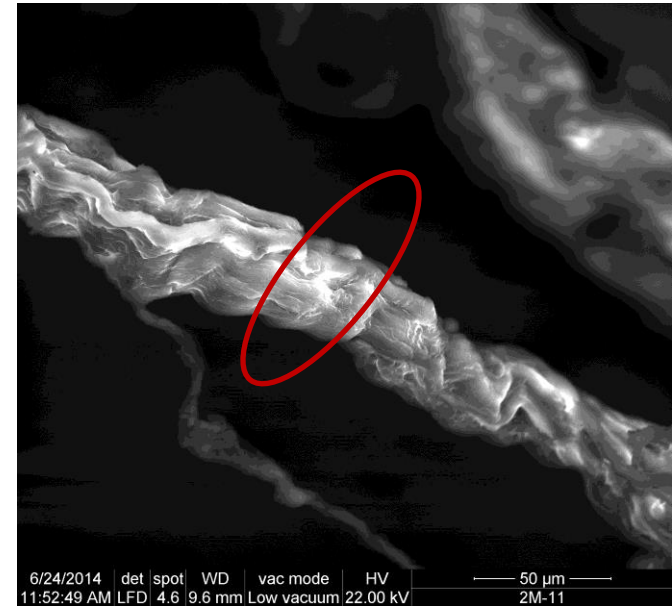
Score 4

# Connective tissue Dark spot (50 $\mu$ m)

**Score 1**

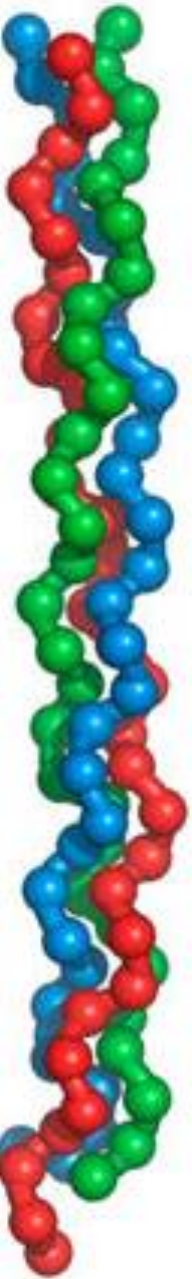


**Score 2**



**Score 4**

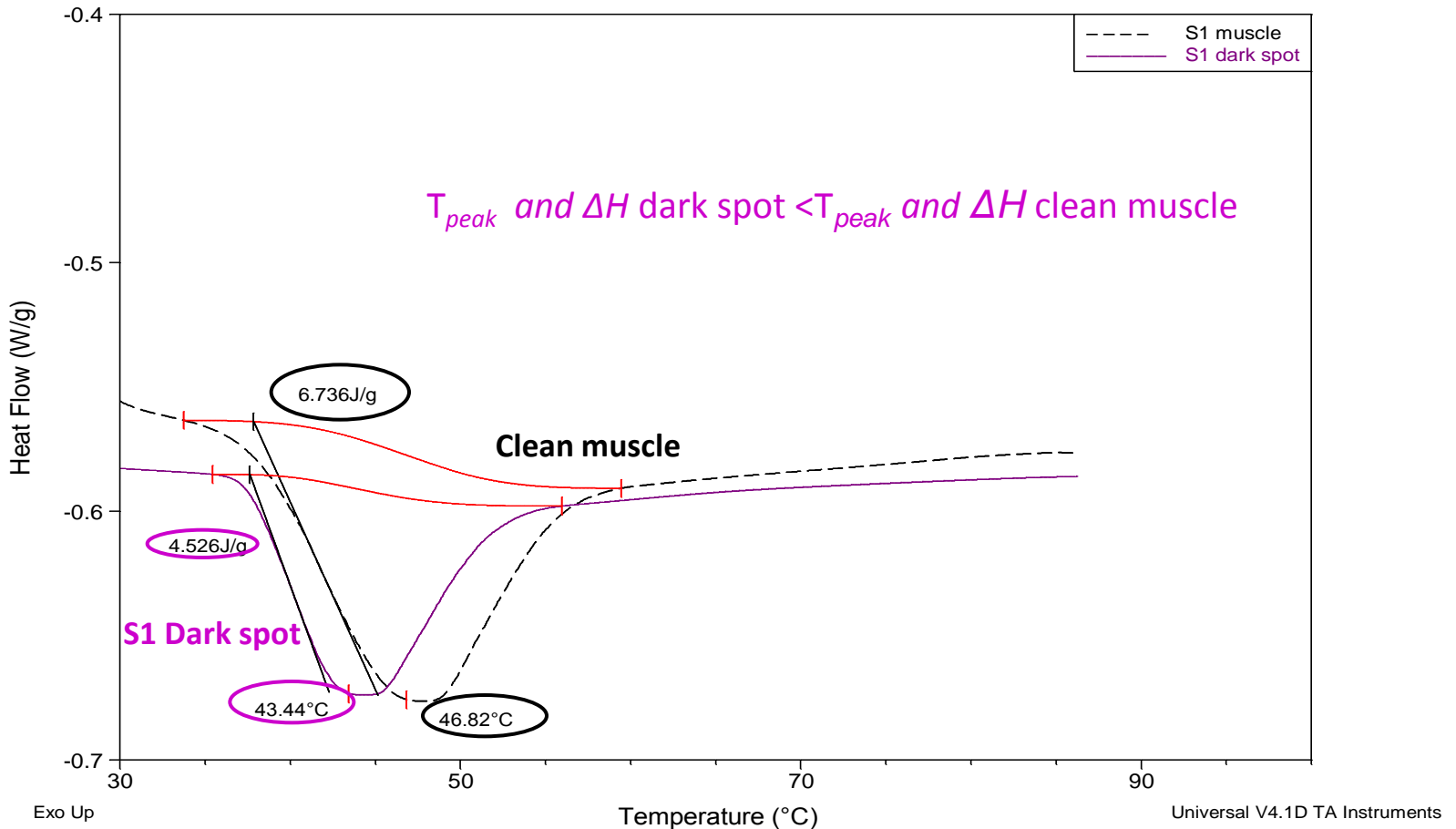
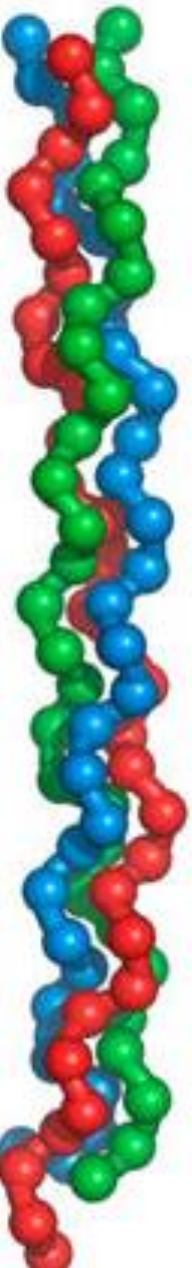
**Structures formed by CT fibers  
are less organized as the presence  
of melanin increases  
S4 > S2 > S1**



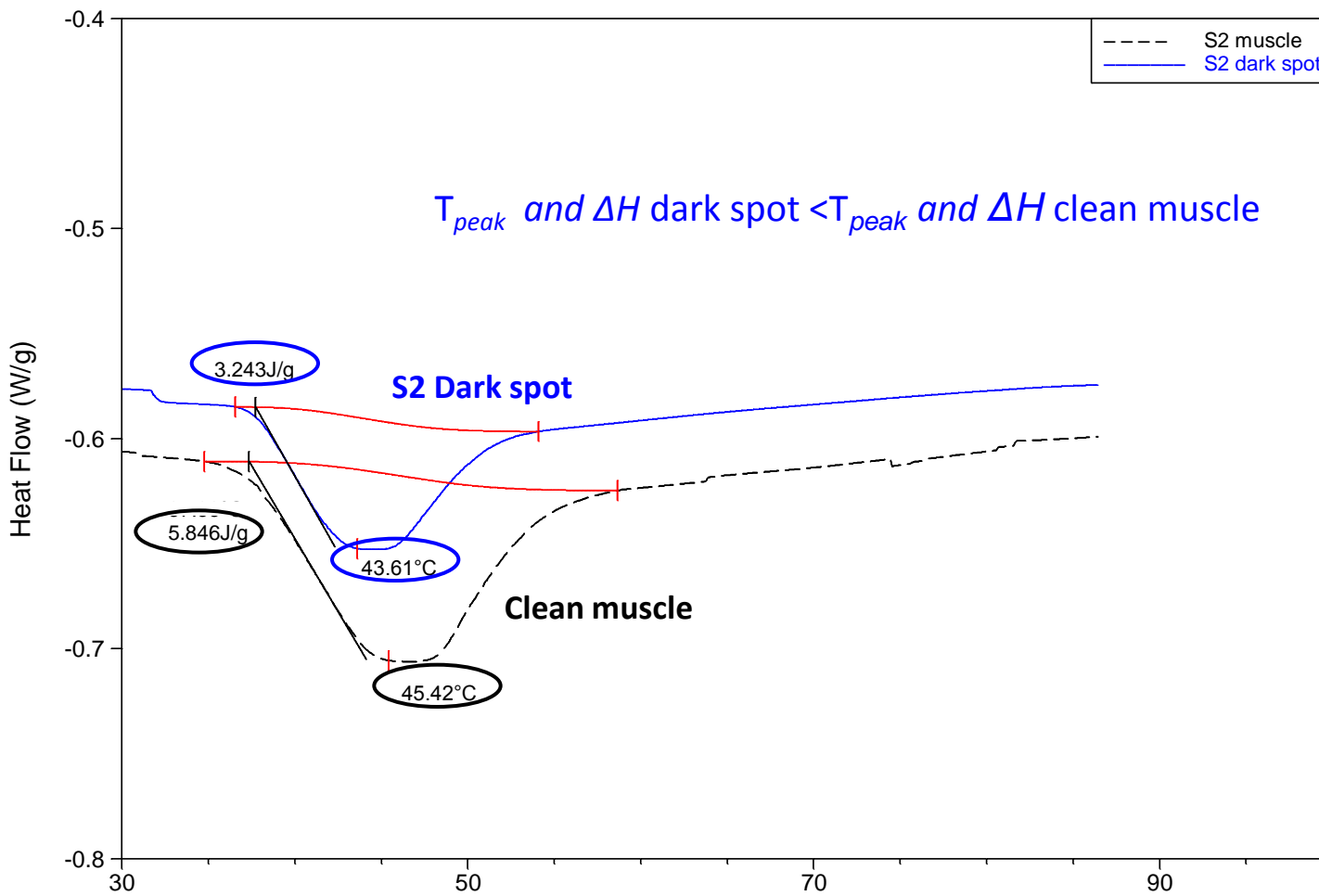
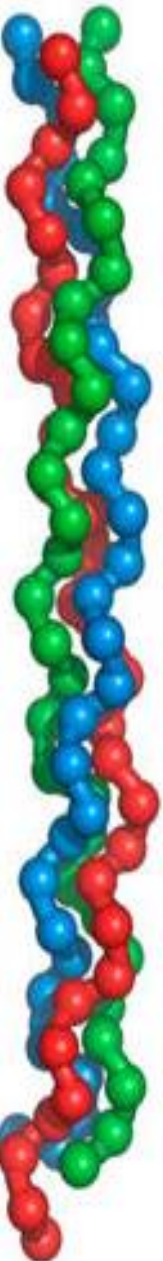
# III.- Differential Scanning Calorimetry (DSC). Thermal stability of Connective tissue



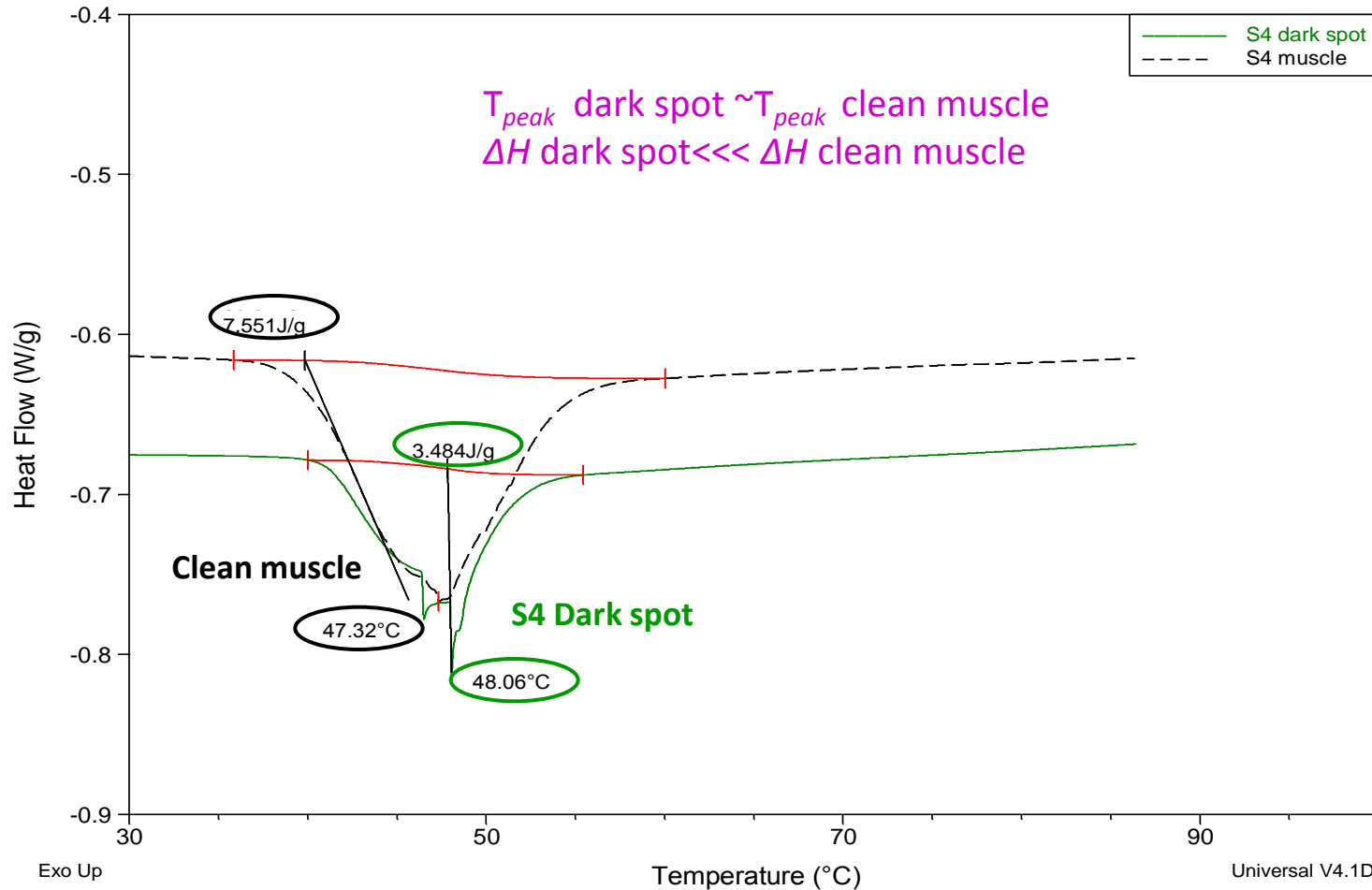
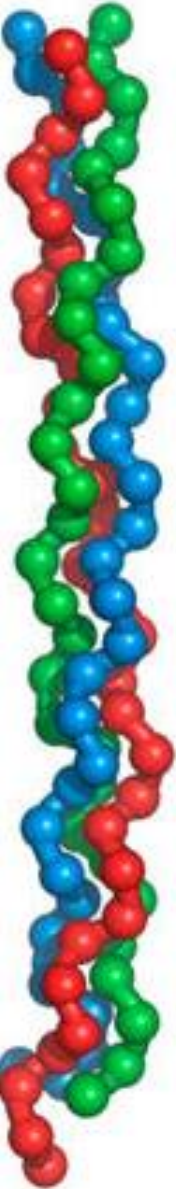
# Spectra from dark spot vs. clean muscle spectrum of score 1

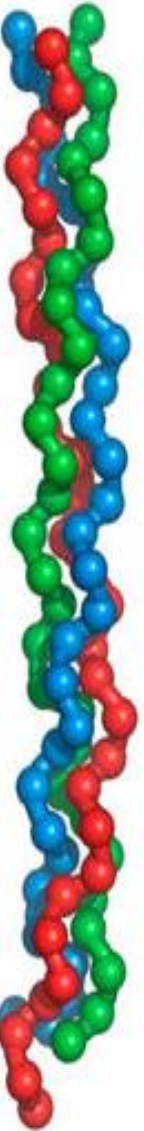


# Spectra from dark spot vs. clean muscle spectrum of score 2



# Spectra from dark spot vs. clean muscle spectrum of score 4

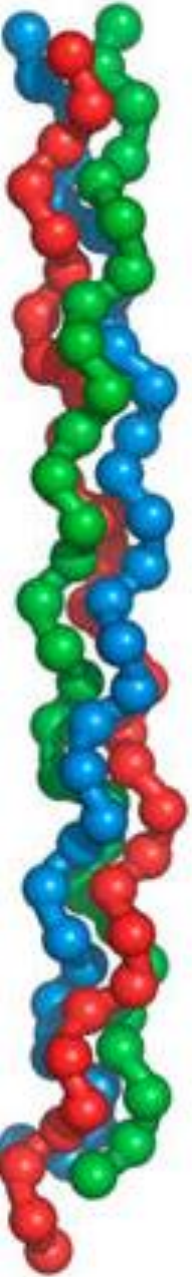




Parameters	Samples	Dark spot	Clean muscle
$T_{peak}(^{\circ}C)$	Score 1	43,44	46,82
	Score 2	43,61	45,42
	Score 4	47,06	47,32
$\Delta H (^{\circ}C)$	Score 1	4,526	6,736
	Score 2	3,243	5,864
	Score 4	3,484	7,555



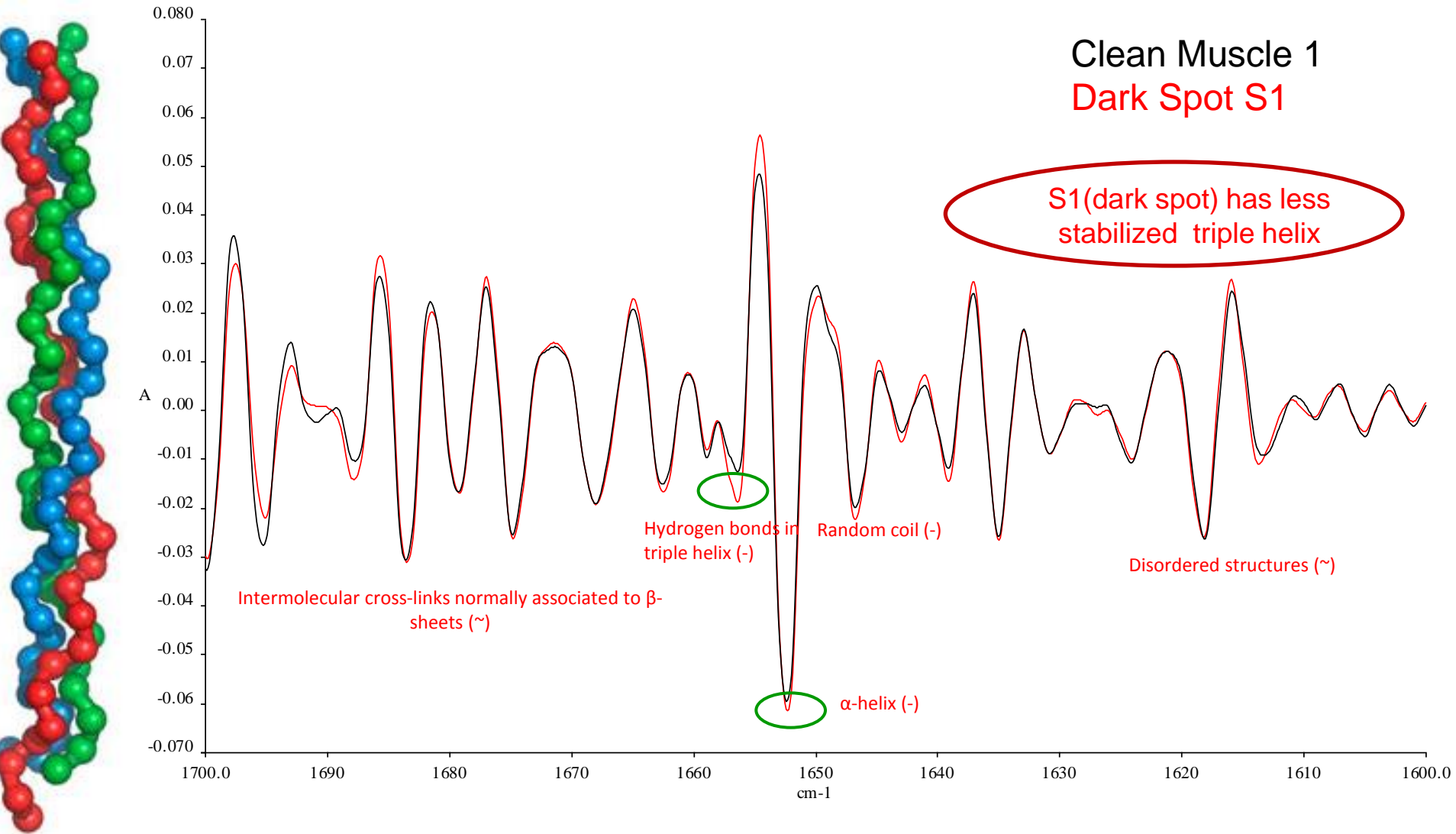
Connective tissue of dark spot has less stability to thermal treatment meaning a less stabilized structure.



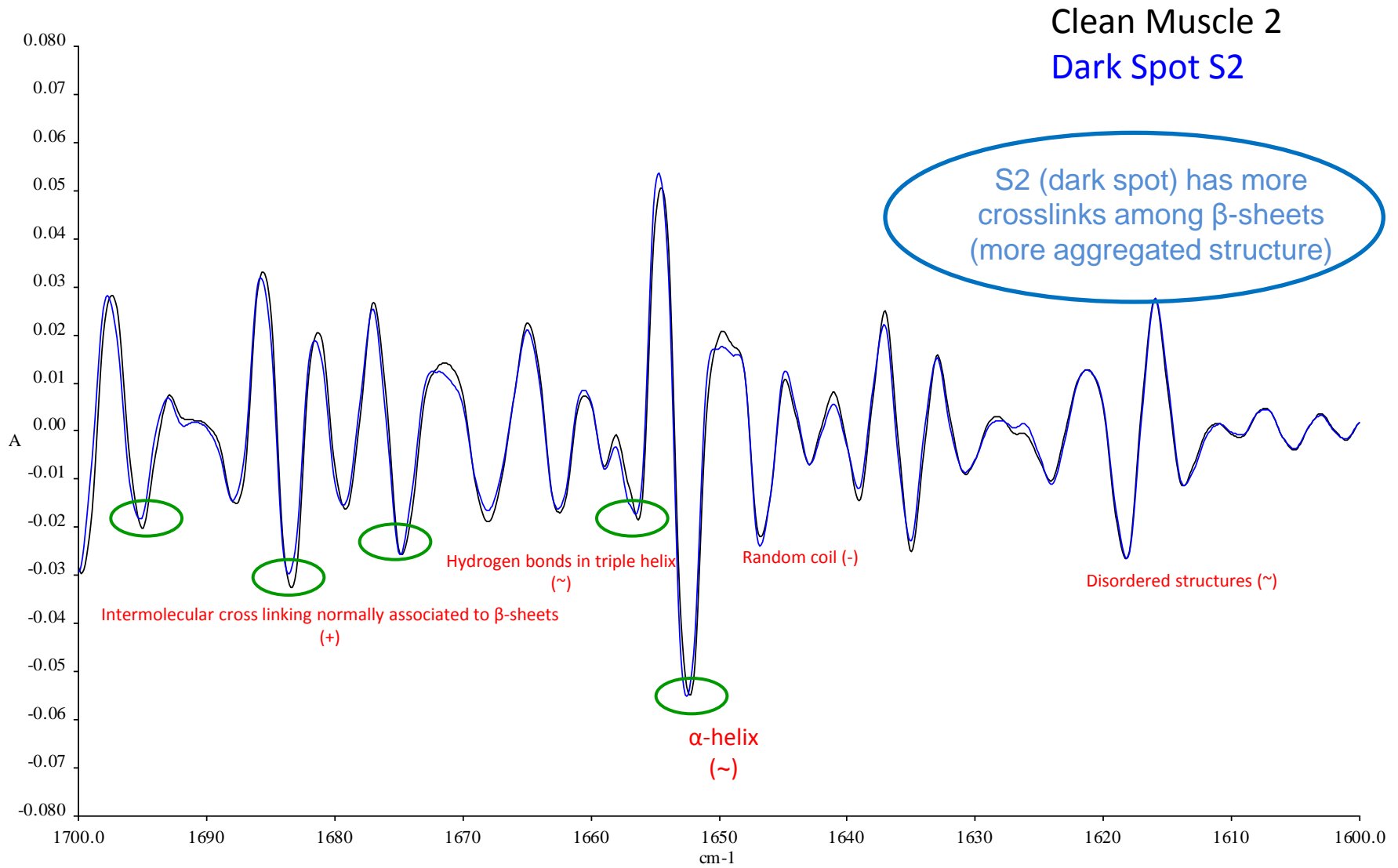
# IV.- Fourier Infrared Spectroscopy (FTIR).

## Secondary structure of collagen

# Amida I : Dark spot Score 1/muscle 1

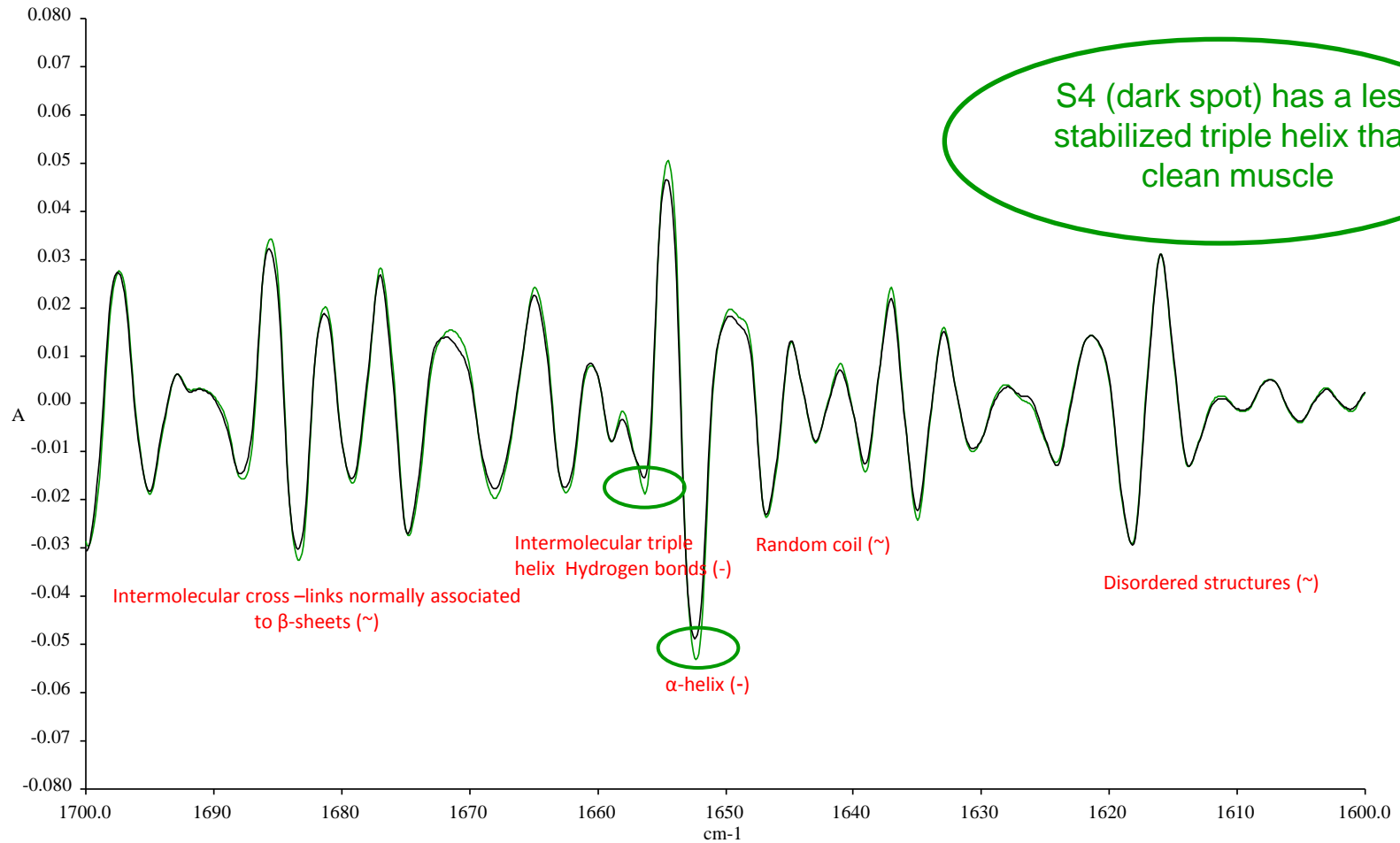


# Amida I : Dark spot Score 2/muscle 2



# Amida I : Dark spot Score 4/muscle 4

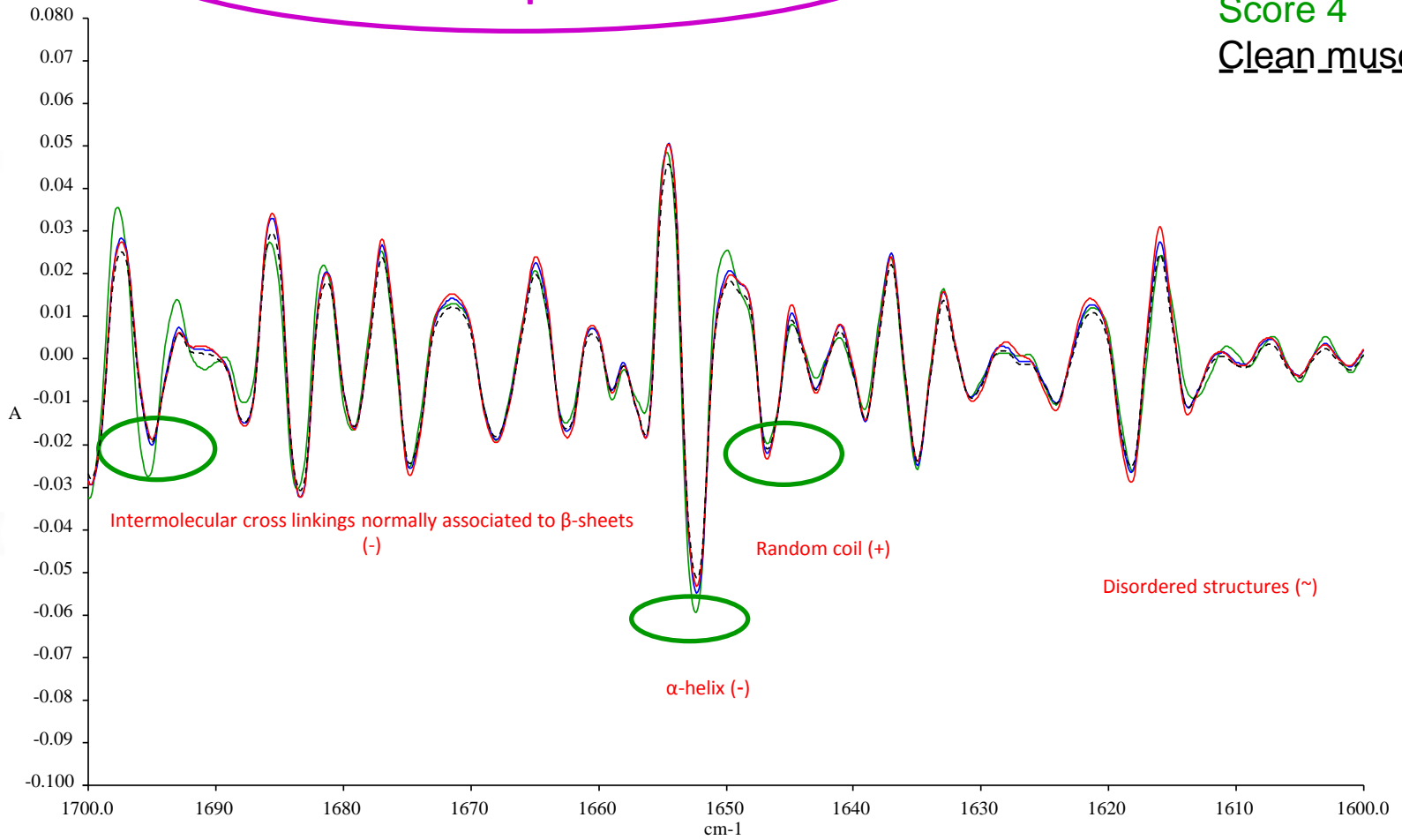
Clean Muscle 4  
Dark spot S4





Loss of collagen typical structure with melanin presence

Score 1  
Score 2  
Score 4  
Clean muscle

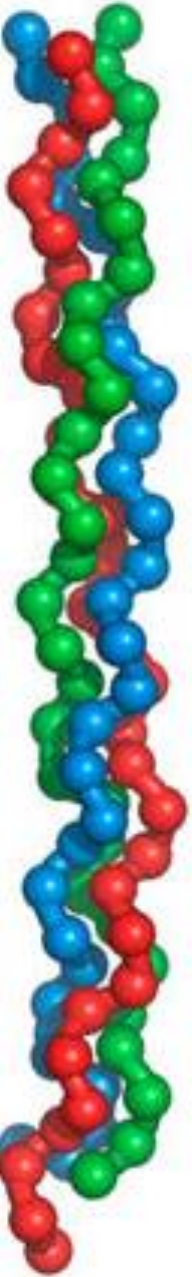


S4 lower presence of  $\alpha$ -helix, less crosslinks stabilizing  $\beta$ -sheet and more random structures

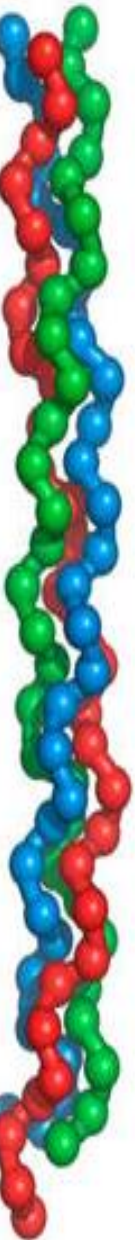
## Triple helical structure preservation of Connective tissue of Dark spot and normal muscle

Samples		Ratio 1235/1450 cm <sup>-1</sup>
Muscle	1	1,00
	2	1,01
	4	1,06
Dark spot	S1	1,03
	S2	1,02
	S4	1,04

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.



## V.- Aminoacids composition.



Connective tissue						
AA	Clean Muscle 1 residues/1000	S1 Dark Spot residues/1000	Clean Muscle 2 residues/1000	S2 Dark Spot residues/1000	Clean Muscle 4 residues/1000	S4 Dark Spot residues/1000
Asp	66,51	69,42	63,64	72,72	61,18	59,70
Thr	29,83	32,24	28,28	32,63	27,47	27,79
Ser	52,21	54,76	52,86	54,26	51,59	51,95
Glu	87,89	91,07	85,87	93,78	82,98	81,64
Gly	280,44	266,21 ↓	295,21	249,11 ↓	301,48	300,05 ↓
Ala	104,96	101,26	108,81	101,77	105,84	103,13
Cys	2,56	2,90	2,30	3,42	2,27	2,45
Val	22,76	24,14	20,17	26,09	19,44	20,04
Met	21,66	21,18	20,47	21,01	18,87	18,45
Ile	13,90	15,07	12,45	16,37	11,95	12,30
Leu	32,29	35,53	29,73	39,71	28,06	28,35
Nleu	24,62	30,36	18,67	36,16	21,93	27,97
Tyr	9,00	10,08	7,19	11,86	6,43	6,89
Phe	17,42	18,14	17,01	20,28 ↓	16,90	16,56 ↓
Hyl	10,46	9,38 ↓	9,42	7,57 ↓	9,34	9,11 ↓
His	12,28	13,39	10,99	13,48	10,06	10,14
Lys	34,72	37,70	31,51	40,03	29,00	28,45
Arg	43,53	44,53	46,06	44,16 ↓	46,84	46,66 ↓
Hyp	53,76	43,42 ↓	52,64	42,66 ↓	58,65	57,92 ↓
Pro	79,20	79,25	86,71	72,92	89,71	90,47
%Hyp/Pro	40,28	35,10	37,72	36,78	39,56	39,03
%Hyl/Lys	23,15	20,03	23,10	16,20	24,35	24,26

Less  
twister  
helix

Less  
covalent  
bonds

Less  
hydrogen  
bonds

**Dark spot collagen structure is less preserved and less stabilized than in clean muscle**

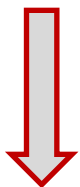
# To sum up...

## 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 enthalpy 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).

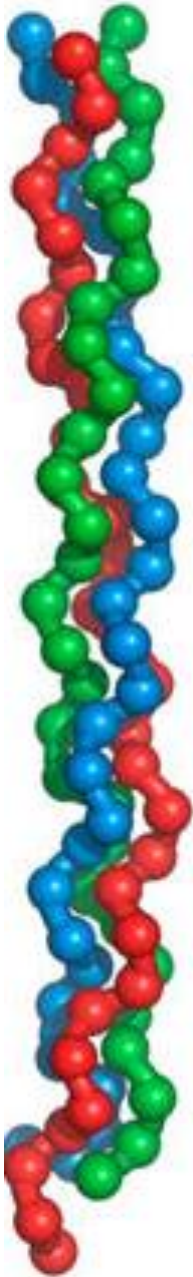


Collagen from dark spot is less stabilized, has lost part of the helical structure as compared with the collagen from the clean muscle



**Dark spots of salmon fillets had disorganized and unstable connective tissue**





**Thank you**

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