



Connective tissue Characteristics of Dark stained spot in salmon fillets:

Preliminary Results and Future proposals

PhD. Helena M. Moreno
Professor A. Javier Borderías
Technician Silvia Díaz

17th October, 2014

Samples we had

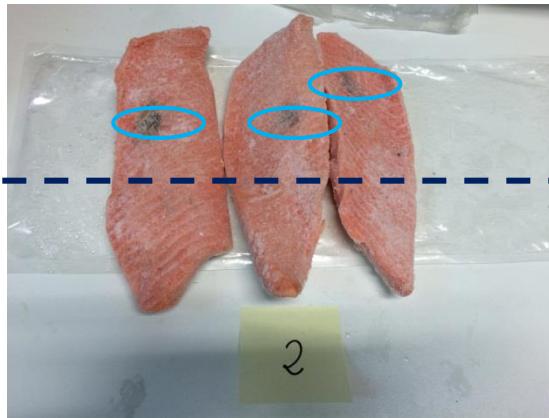
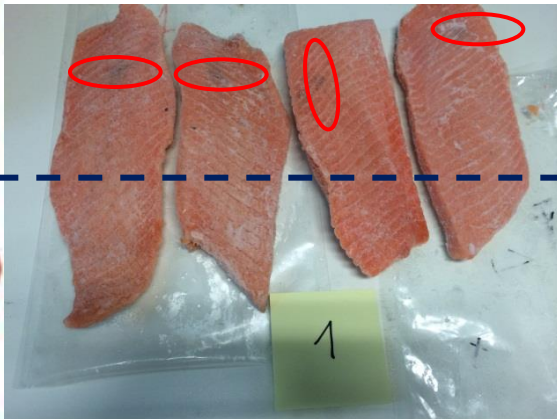
Three scores: Score 1 (4 fillets), score 2 (3 fillets) and score 4 (5 fillets)

Dark spot

Diffuse spots

Clear spots (0-3cm)

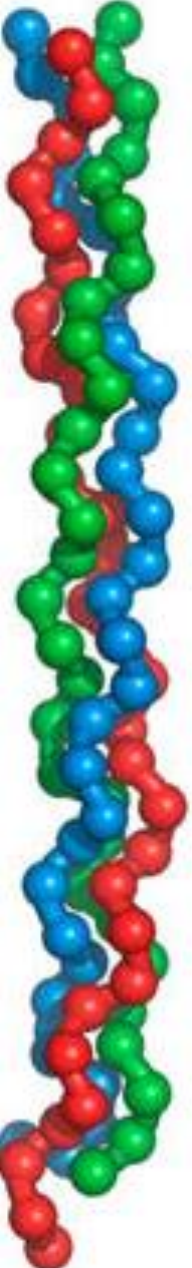
Clear spot (3-6cm)



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

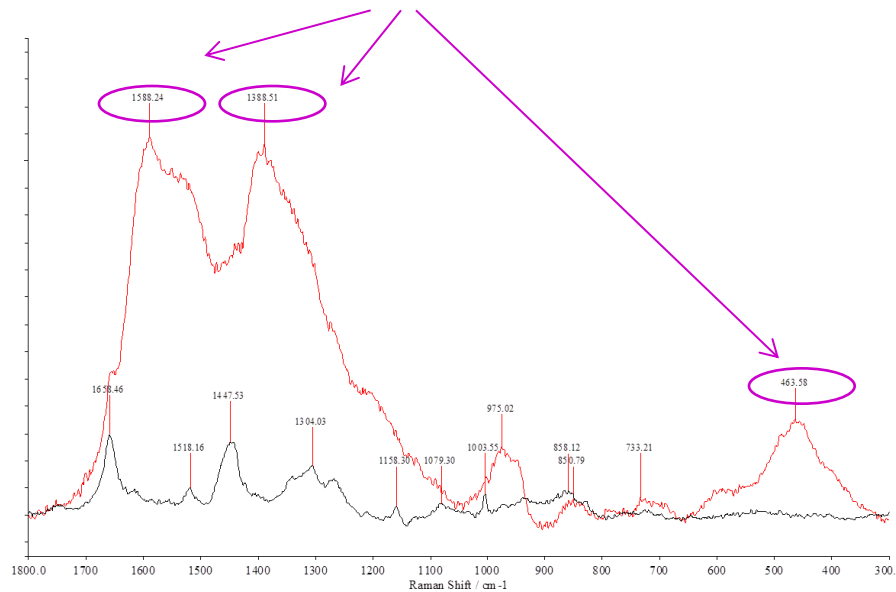
RAMAN

(780nm of 8mW power)

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

Melanin Raman Spectra

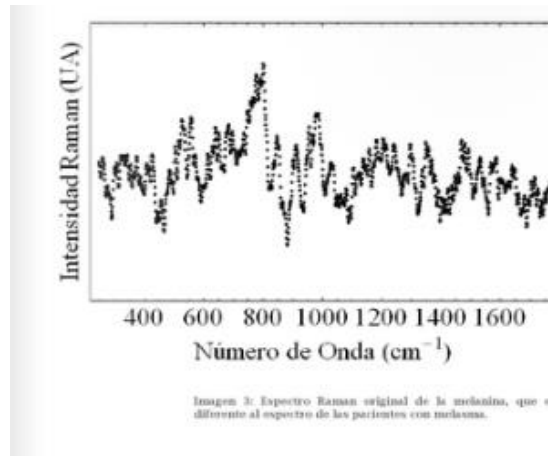
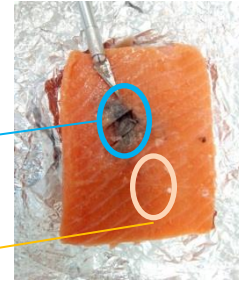
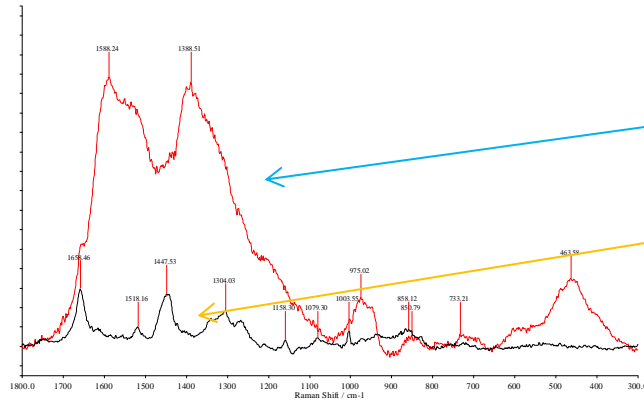
Predictors peaks of
eumelanin
 1350 cm^{-1} , 1580 cm^{-1} , 500 cm^{-1}



*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



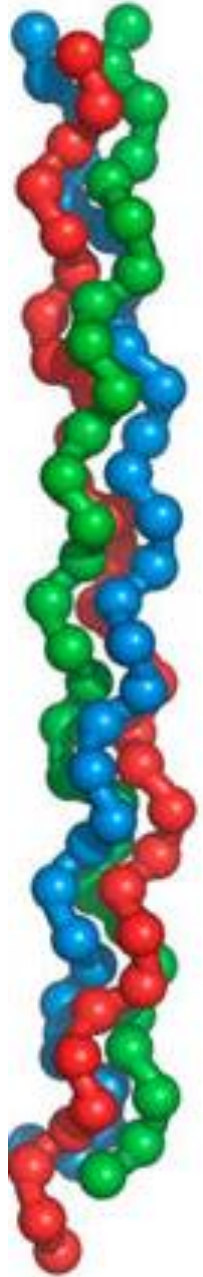
Washed with cold water



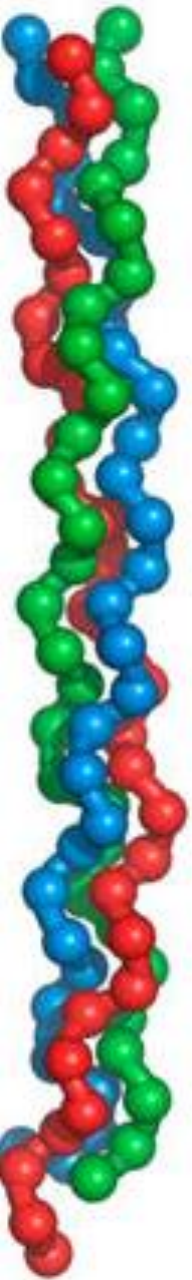
Connective tissue

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

CT Analyses



Preliminary Results

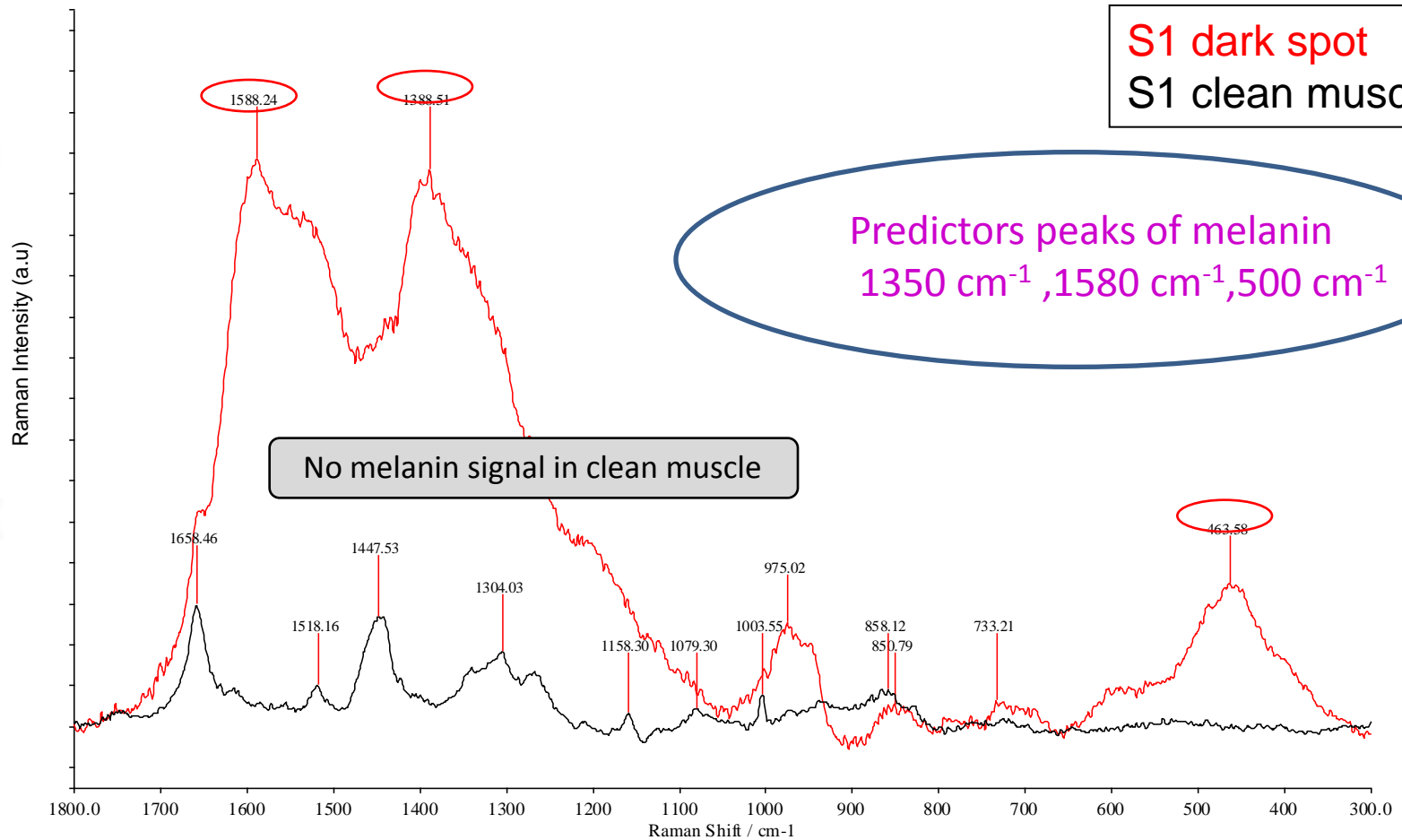
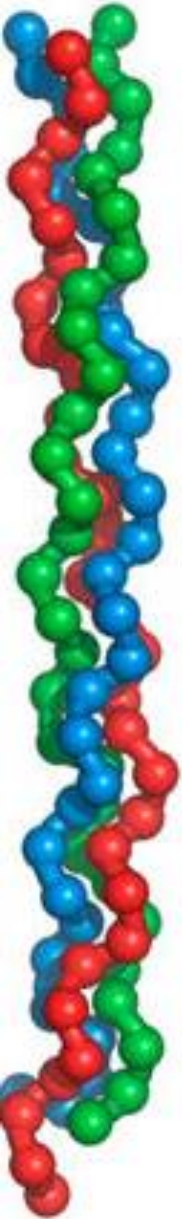


Analyses over Salmon Muscle

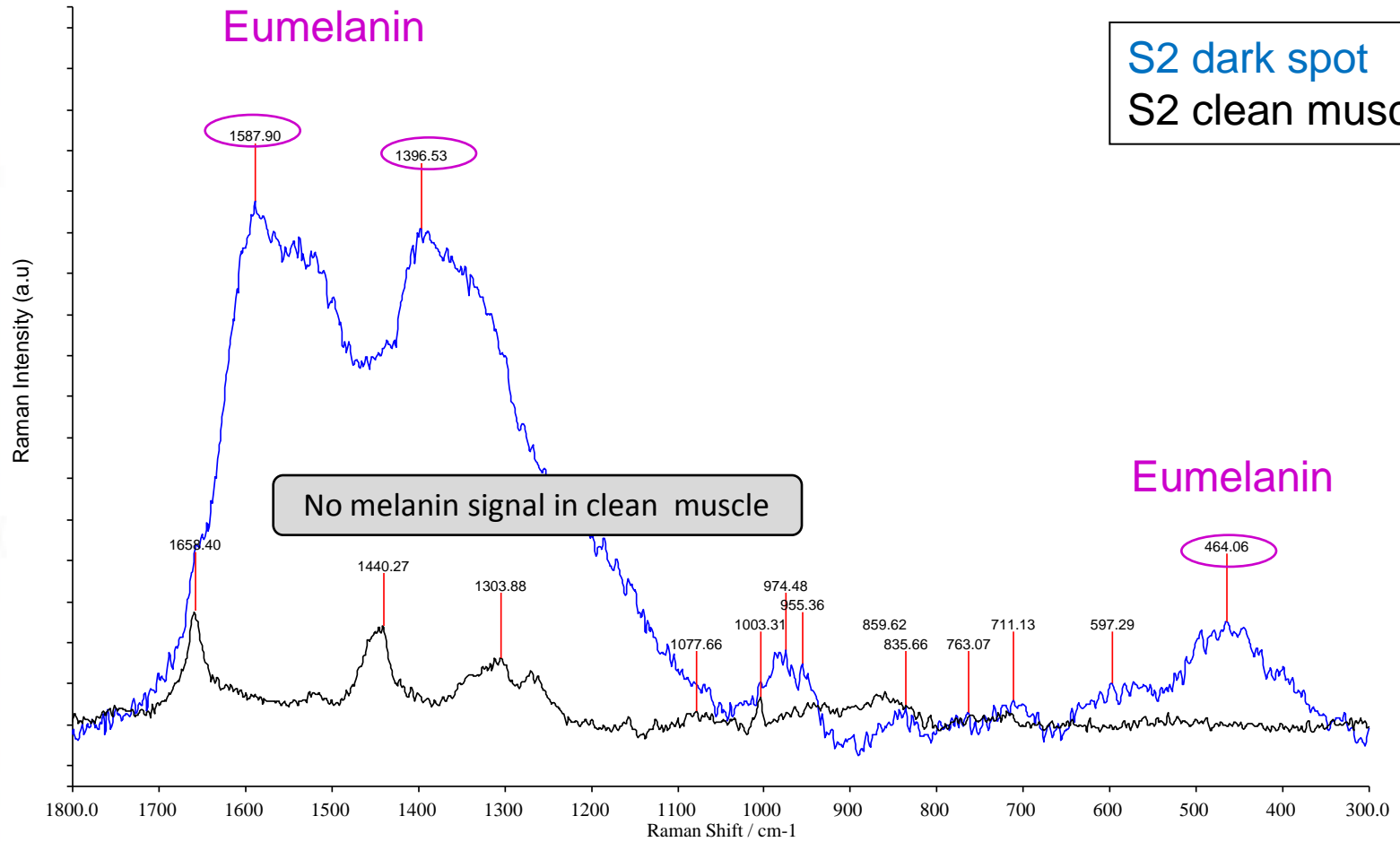
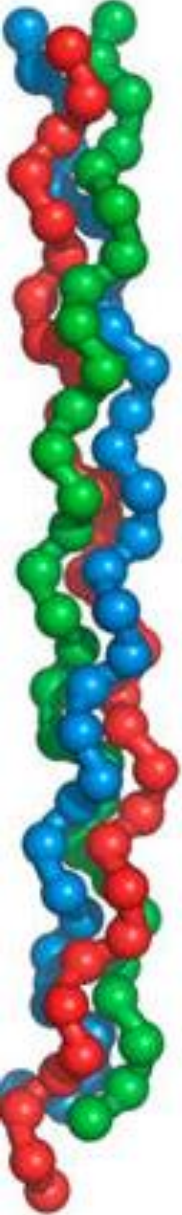
Raman from clean muscle and
dark spot.

Determination of melanin

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

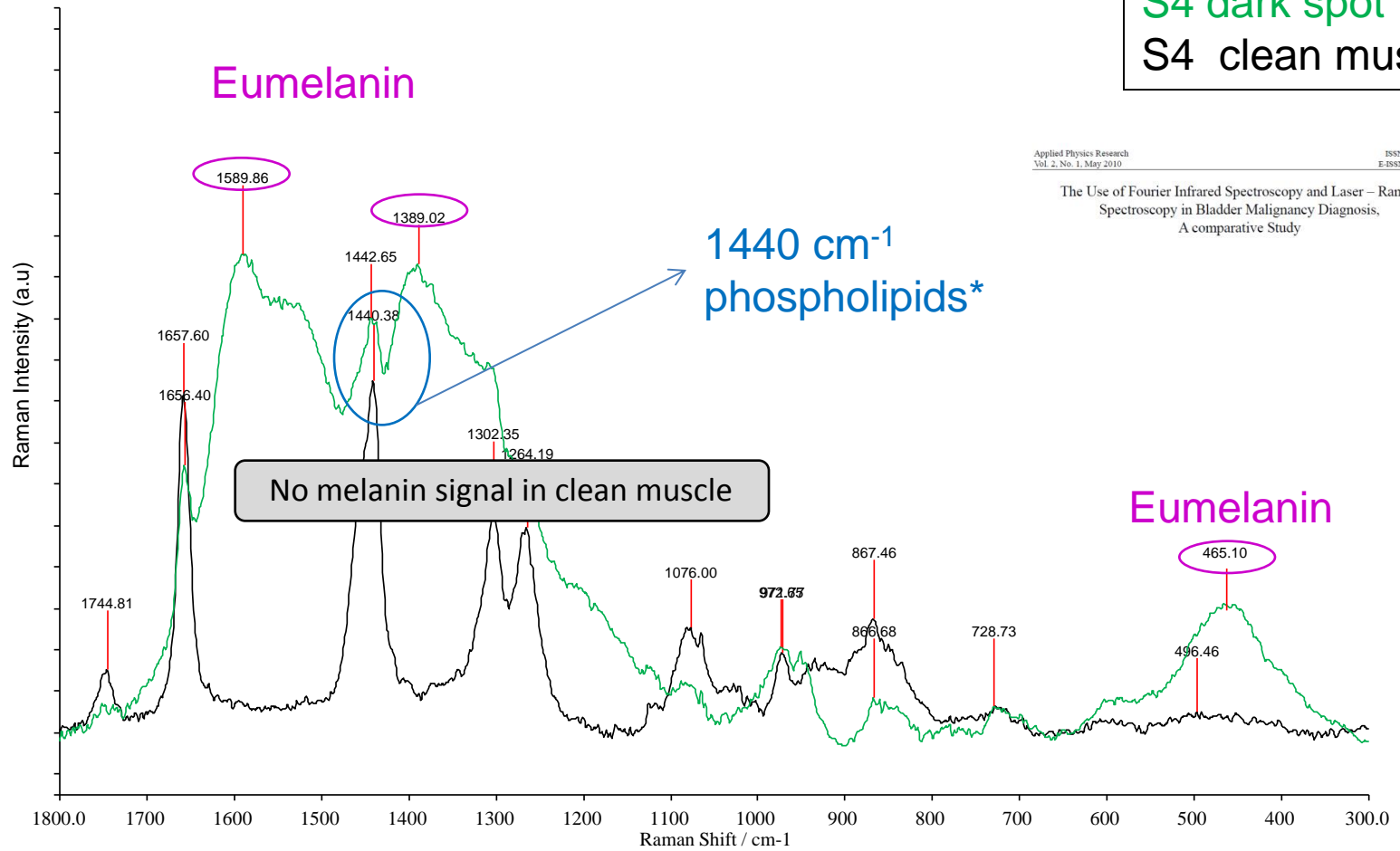


Mean Spectra from dark spot of score 2/clean muscle spectrum of S2



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

S4 dark spot
S4 clean muscle





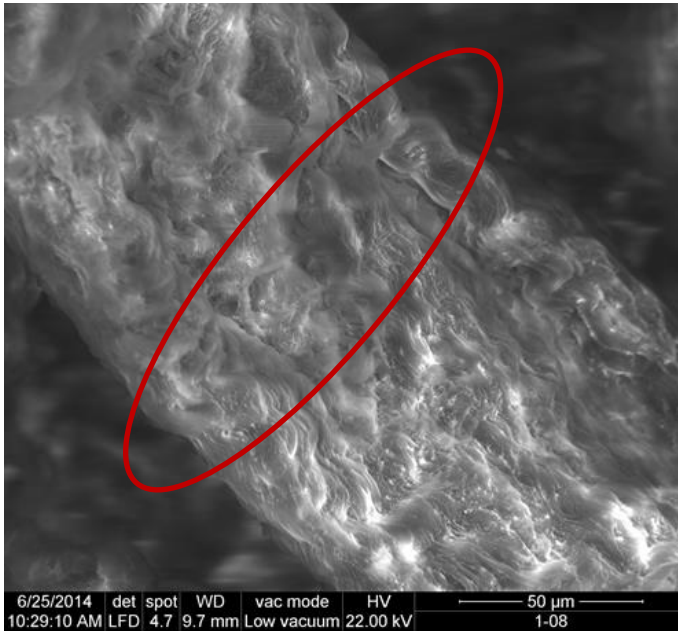
II.- Analyses over Connective Tissue

SEM clean muscle and
dark spot.

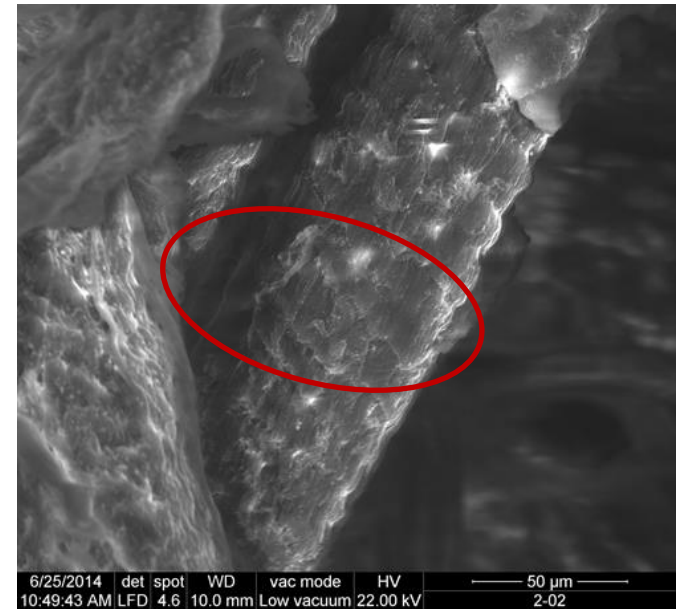
Morphology of CT fibers.

Connective tissue normal muscle (50μm)

Score 1



Score 2

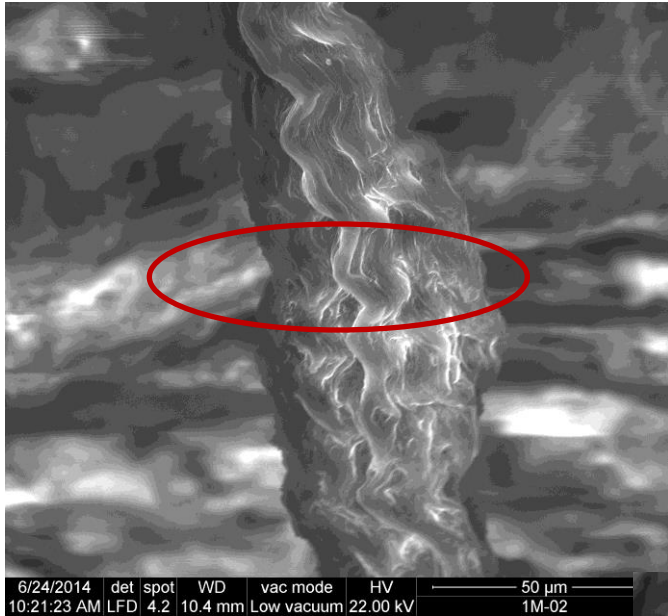


Structures formed by CT fibers
S1 > S2 > S4

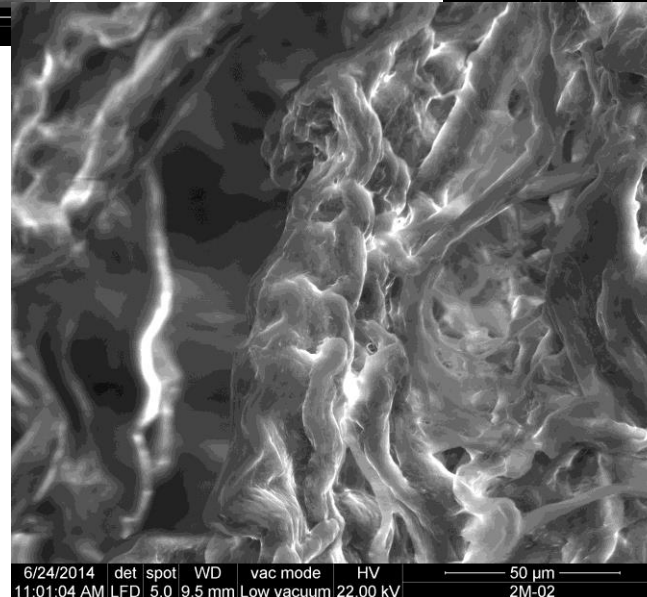
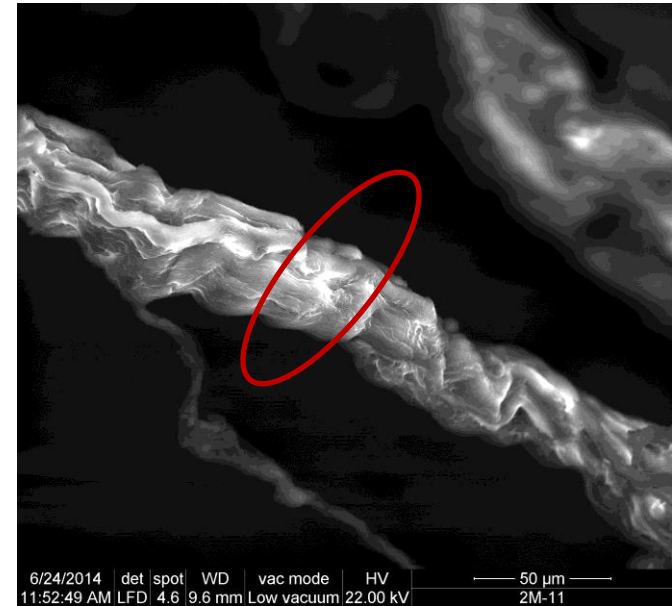
Score 4

Connective tissue Dark spot (50 μ m)

Score 1

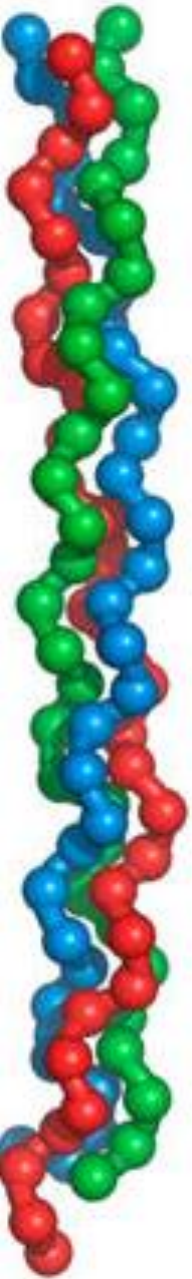


Score 2



Score 4

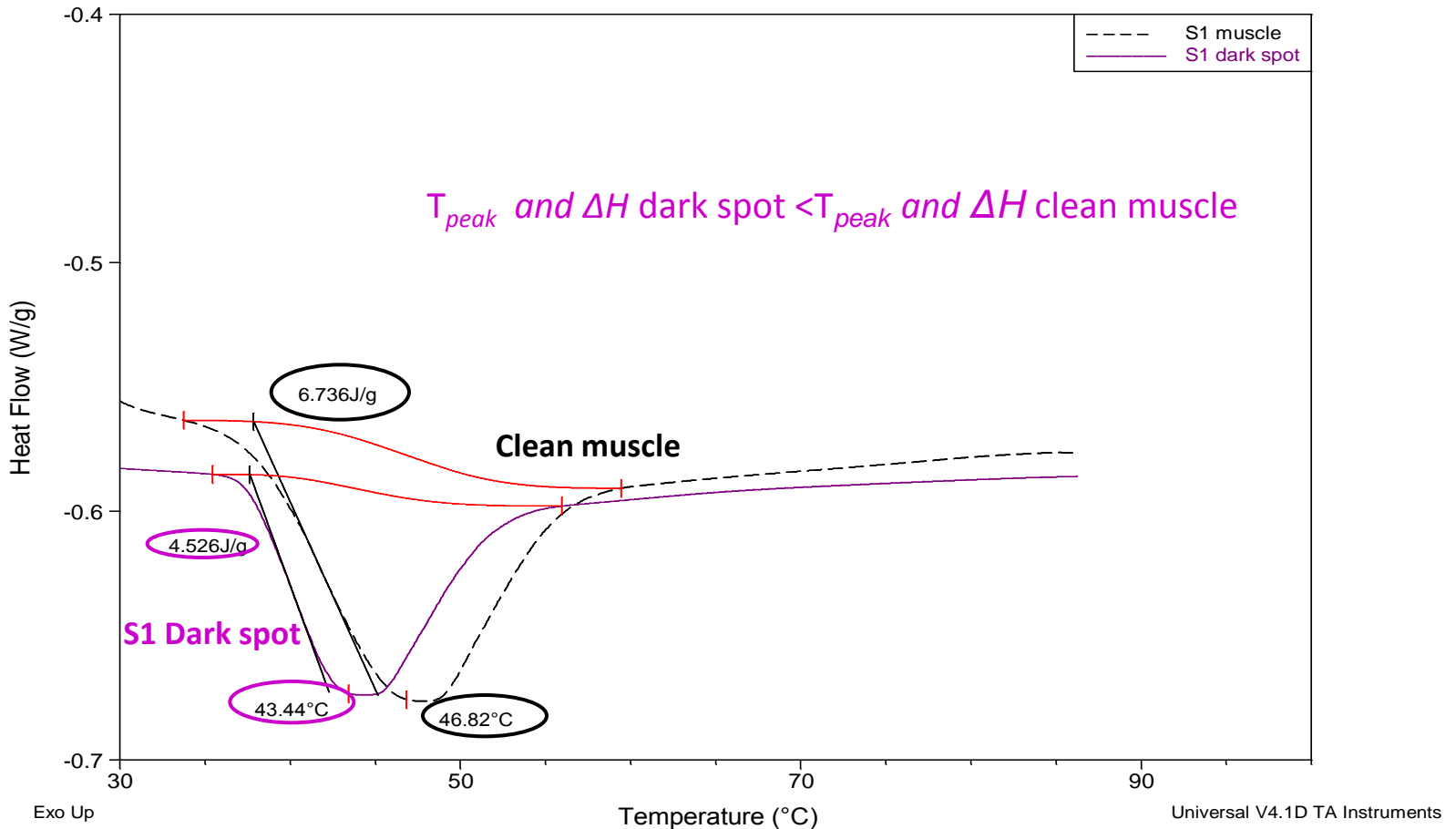
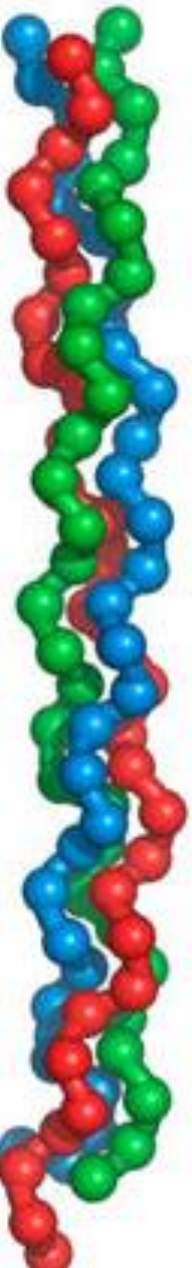
**Structures formed by CT fibers
are less organized as the presence
of melanin increases
S4 > S2 > S1**



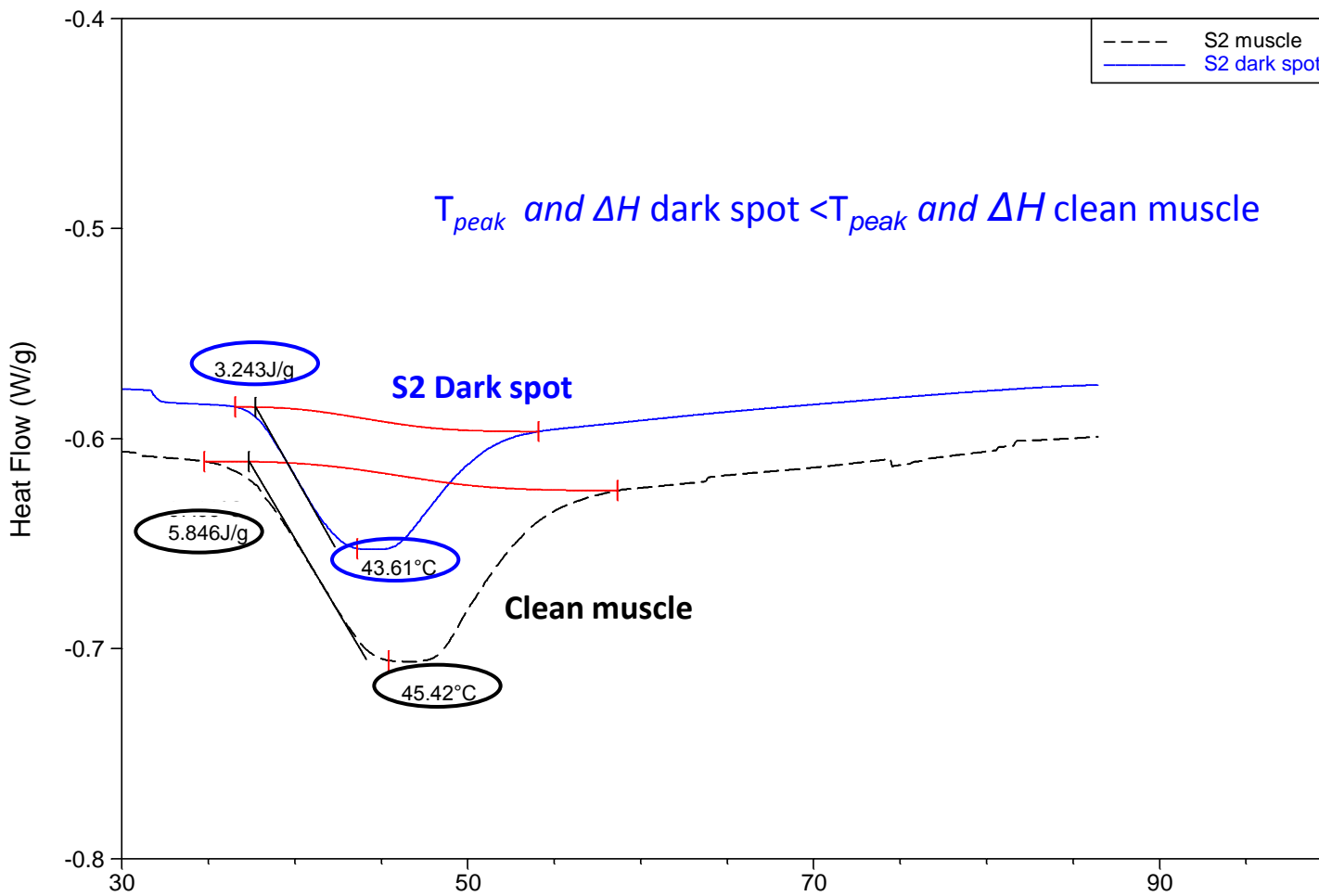
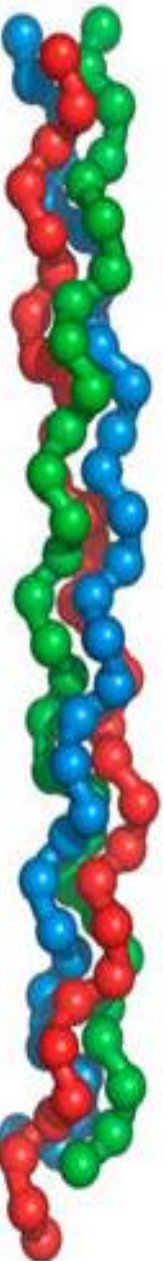
III.- Differential Scanning Calorimetry (DSC).

Thermal stability of Connective tissue

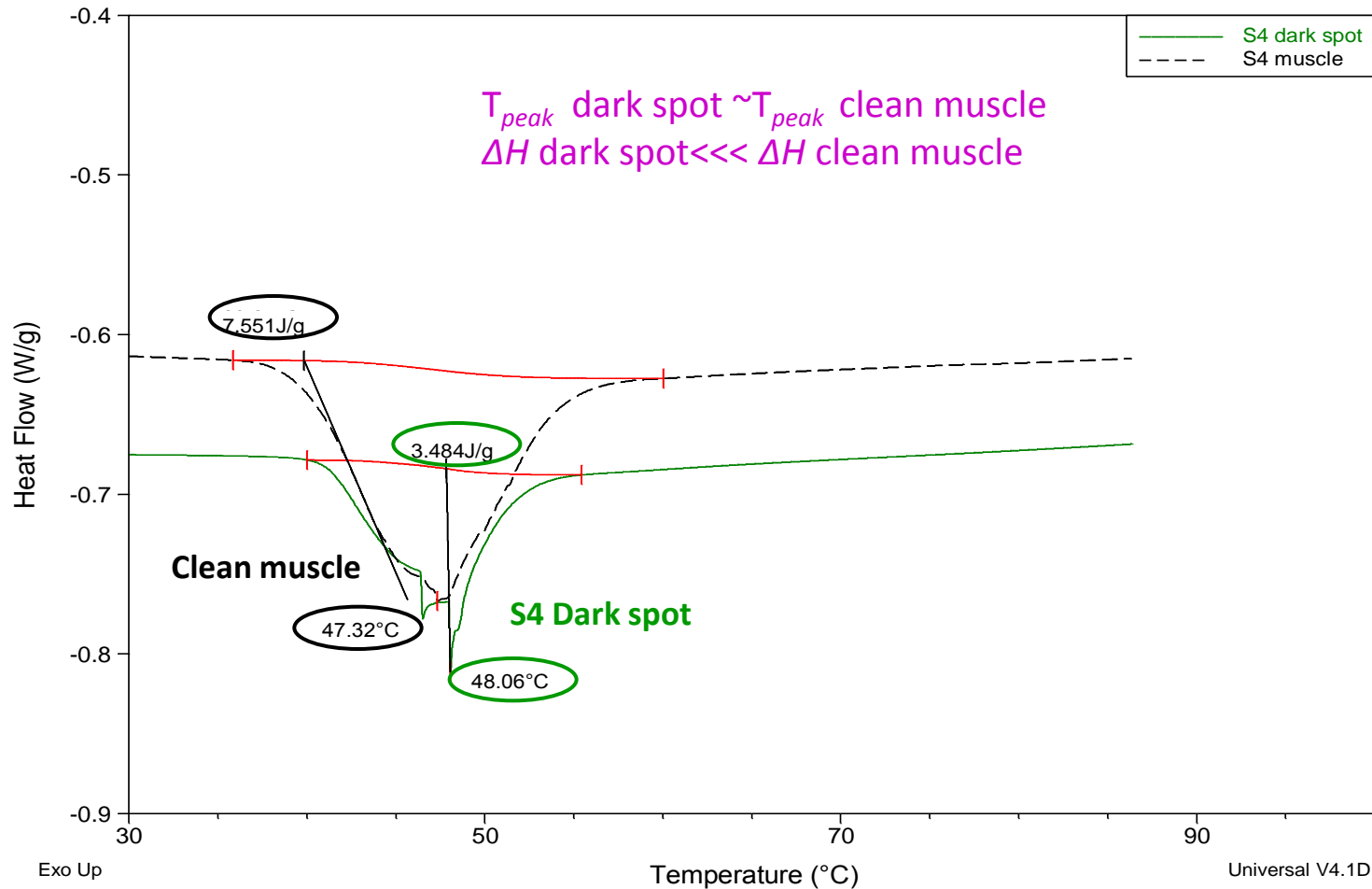
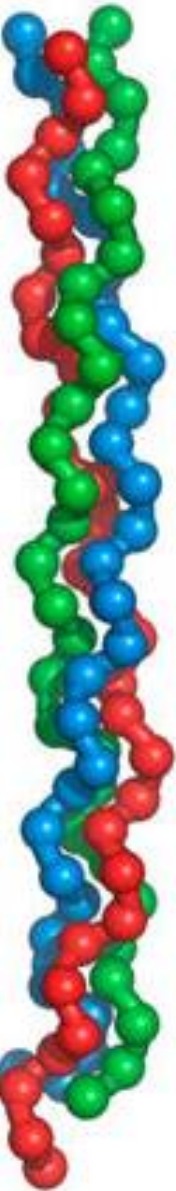
Spectra from dark spot vs. clean muscle spectrum of score 1

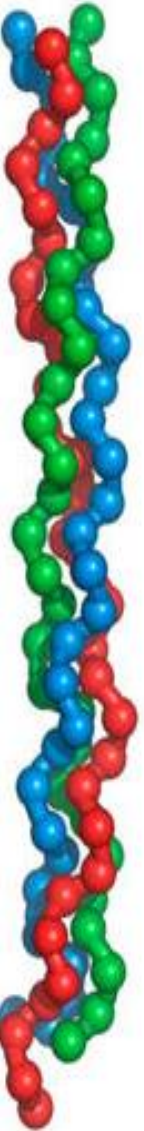


Spectra from dark spot vs. clean muscle spectrum of score 2



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

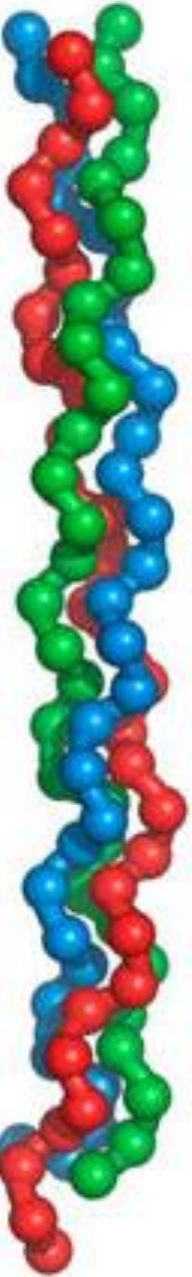




Parameters	Samples	Dark spot	Clean muscle
$T_{peak}(^{\circ}C)$	Score 1	43,44	46,82
	Score 2	43,61	45,42
	Score 4	47,06	47,32
$\Delta H (^{\circ}C)$	Score 1	4,526	6,736
	Score 2	3,243	5,864
	Score 4	3,484	7,555



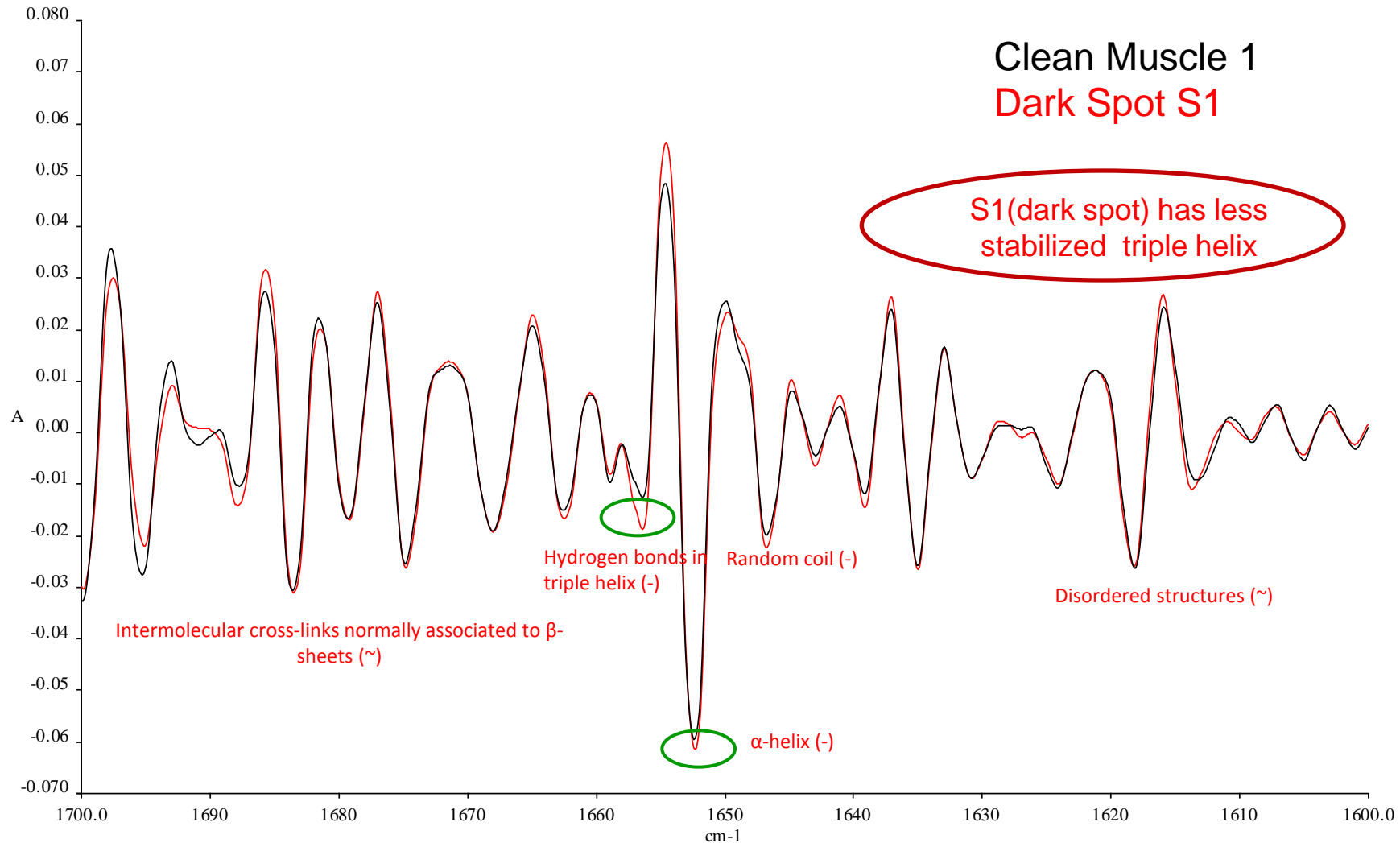
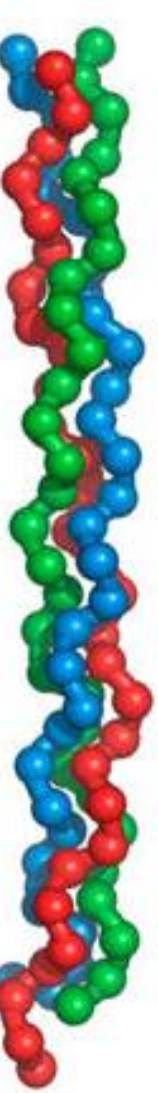
Connective tissue of dark spot has less stability to thermal treatment meaning a less stabilized structure.



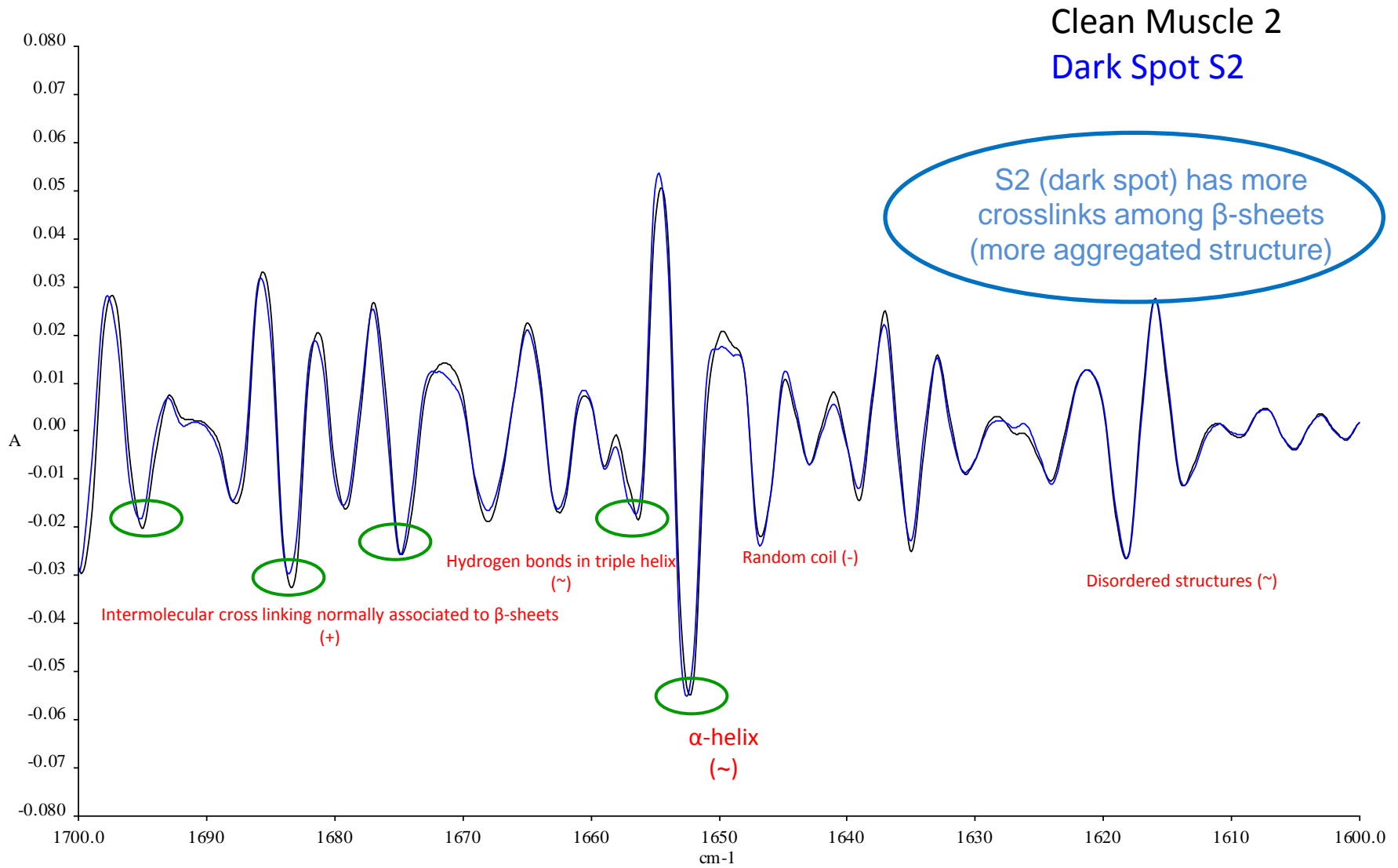
IV.- Fourier Infrared Spectroscopy (FTIR).

Secondary structure of collagen

Amida I : Dark spot Score 1/muscle 1

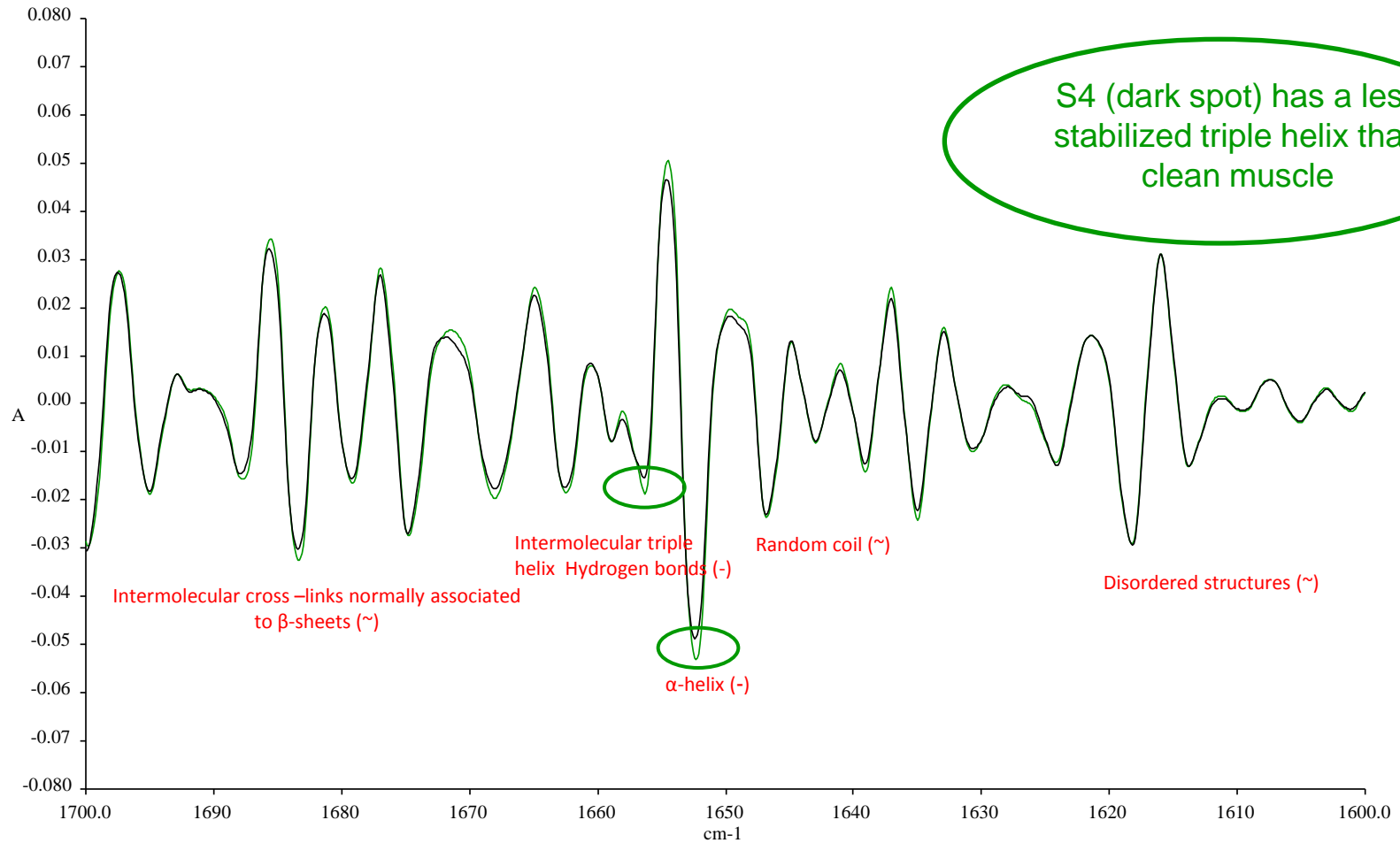


Amida I : Dark spot Score 2/muscle 2



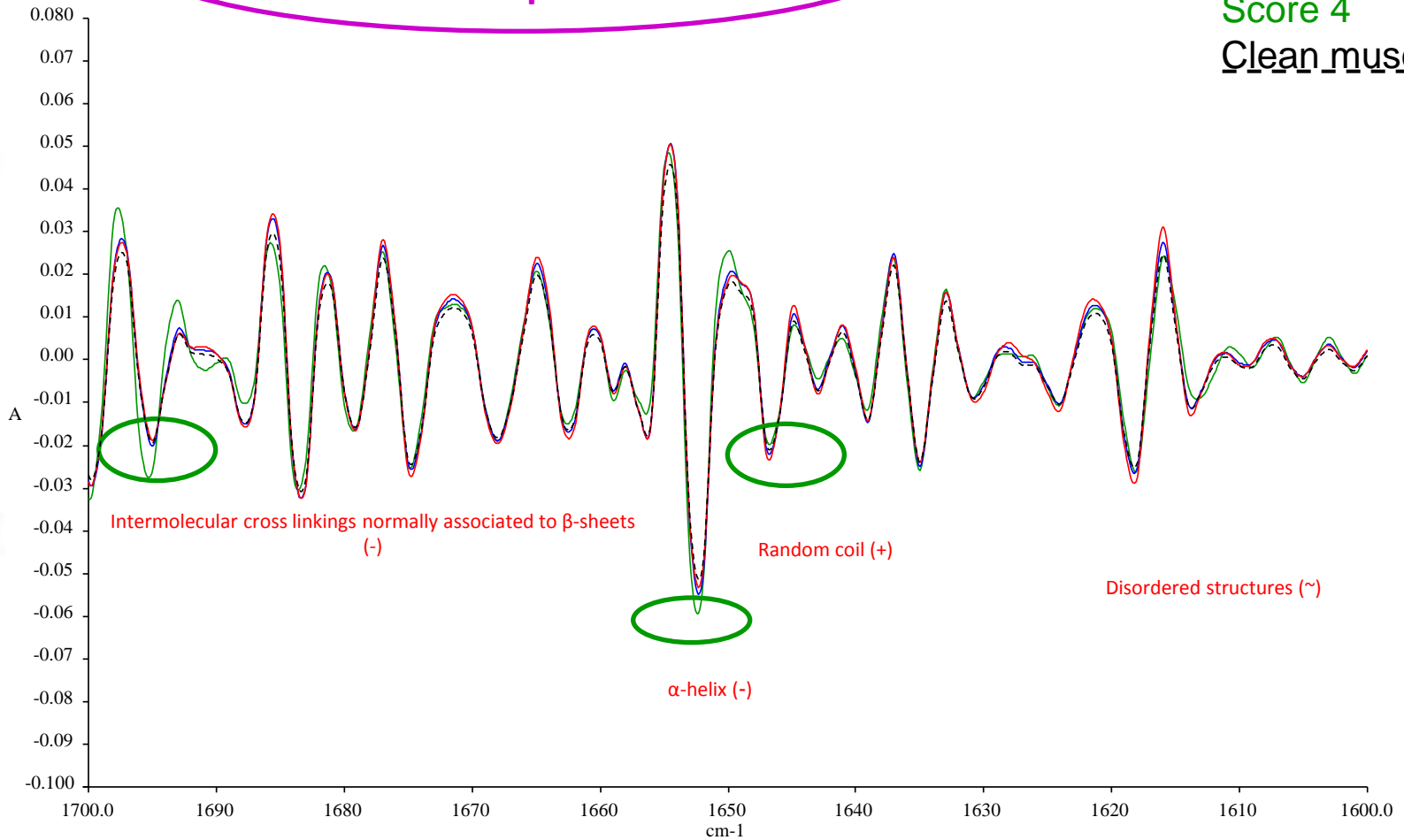
Amida I : Dark spot Score 4/muscle 4

Clean Muscle 4
Dark spot S4

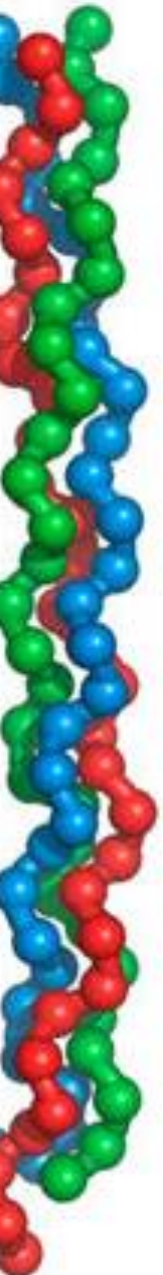


Score 1
Score 2
Score 4
Clean muscle

Loss of collagen typical structure with melanin presence



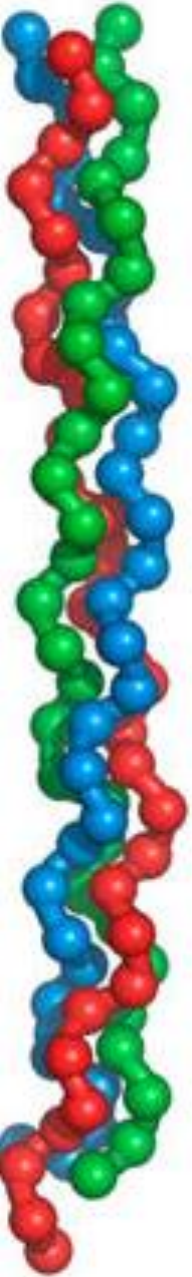
S4 lower presence of α -helix, less crosslinks stabilizing β -sheet and more random structures



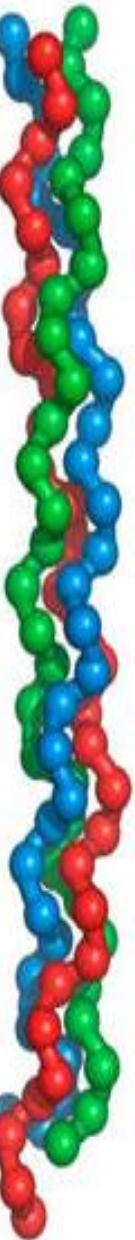
Triple helical structure preservation of Connective tissue of Dark spot and normal muscle

Samples		Ratio 1235/1450 cm ⁻¹
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

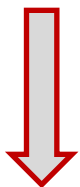
To sum up...

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 α -helix and lower presence of Gly, Hyp and Hyl compared to clean muscle (loss of collagen typical structure).

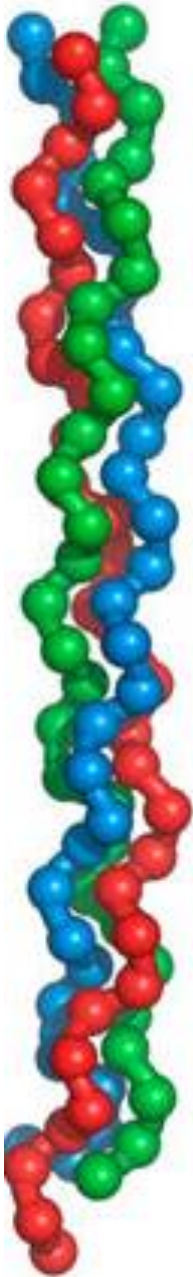


Collagen from dark spot is less stabilized, has lost part of the helical structure as compared with the collagen from the clean muscle



Dark spots of salmon fillets had disorganized and unstable connective tissue





Thank you

Helena M. Moreno
hmoreno@ictan.csic.es