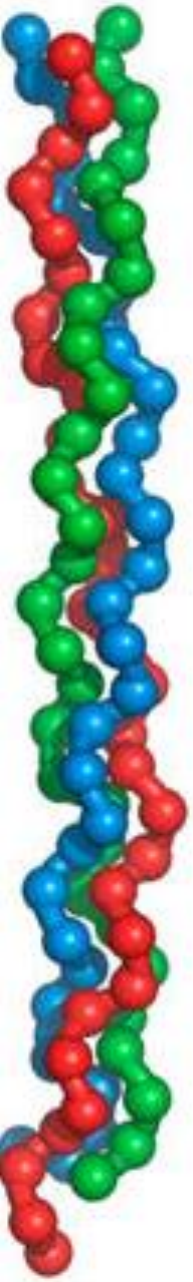


Connective tissue Characterictics of Dark stained spot in salmon fillets:

Preliminary Results and Future proposals

PhD. Helena M. Moreno

Professor A. Javier Borderías

Technician Silvia Díaz

17th October, 2014

Samples we had

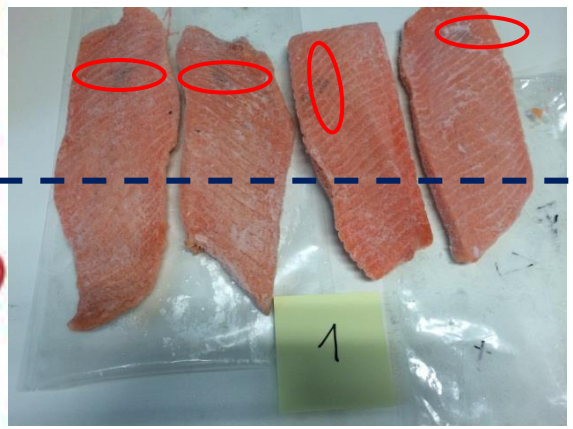
Three scores: Score 1 (4 fillets), score 2 (3 fillets) and score 4 (5 fillets)

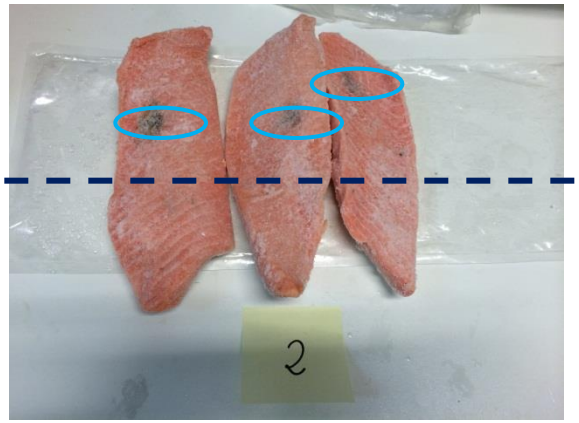
Dark spot

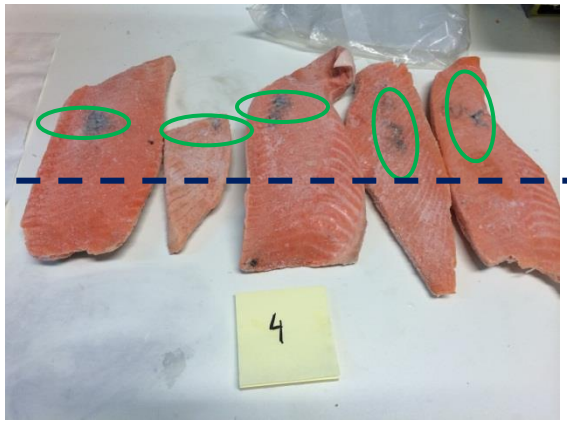
Diffuse spots

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 miscroscopy (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)

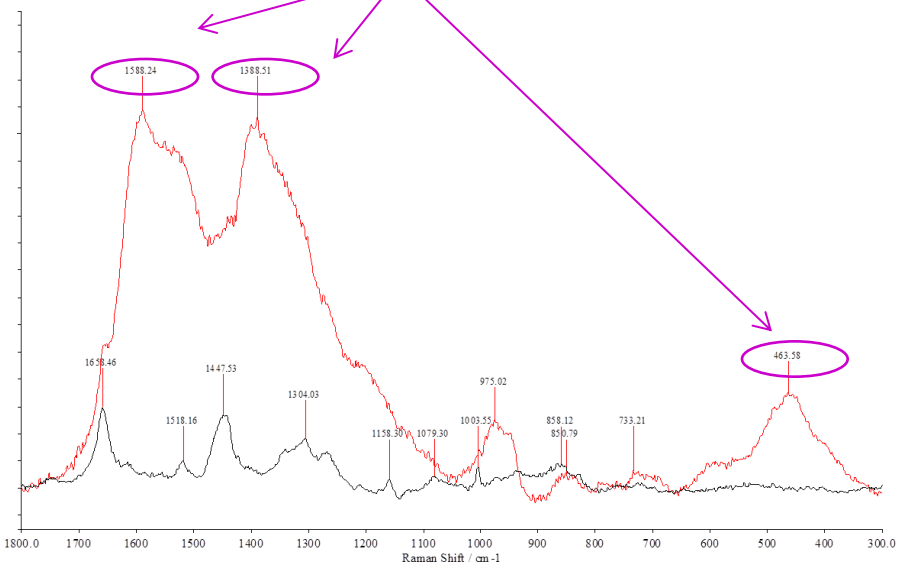
Samples		Total fillets	Clean muscle	Dark spot
			Raman	Raman (8 spectra of each dark spot)
Score 1	fillet 1,1	4	1	8
	fillet 1,2		1	8
	fillet 1,3		1	8
	fillet 1,4		1	8
Score 2	fillet 2,1	3	1	8
	fillet 2,2		1	8
	fillet 2,3		1	8
Score 4	fillet 4,1	5	1	8
	fillet 4,2		1	8
	fillet 4,3		1	8
	fillet 4,4		1	8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

Melanin Raman Spectra

Predictors peaks of

eumelanin

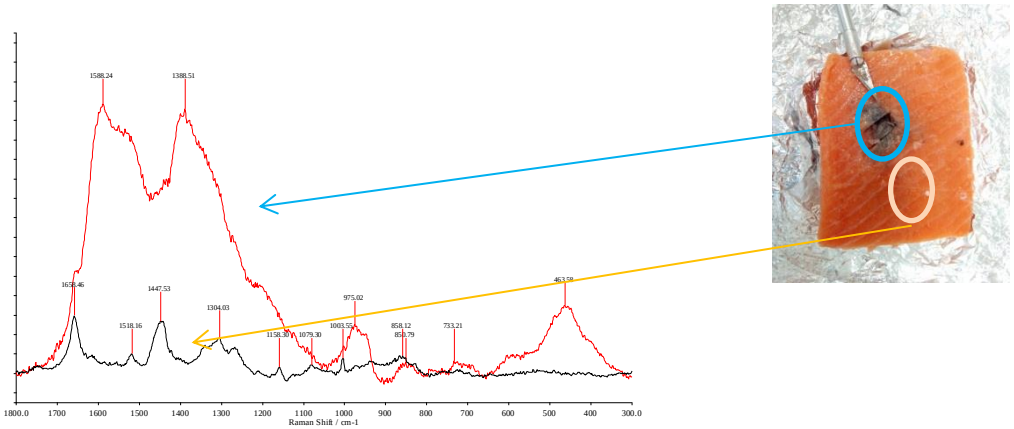
1350 cm^{-1} ,1580 cm^{-1},500 cm^{-1}



***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)



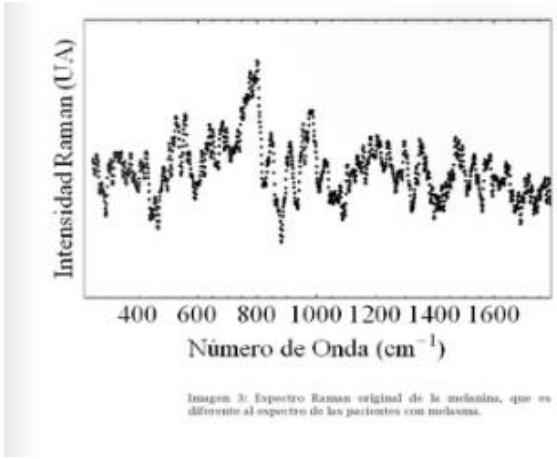


Imagen 3: Espectro Raman original de la melanina, que es diferente al espectro de los pacientes con melasma.

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

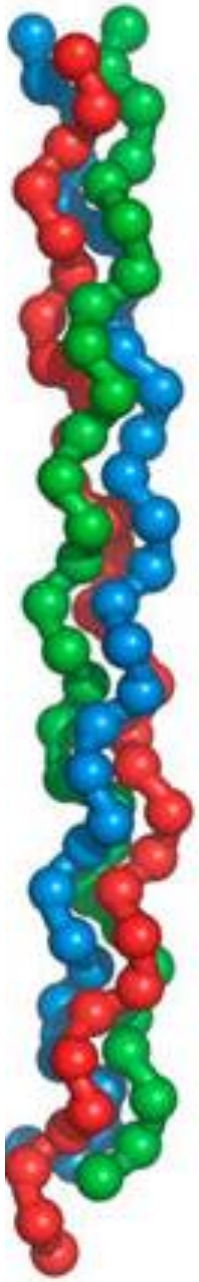
Homogenization with 0.8 % NaCL

Washed with cold water

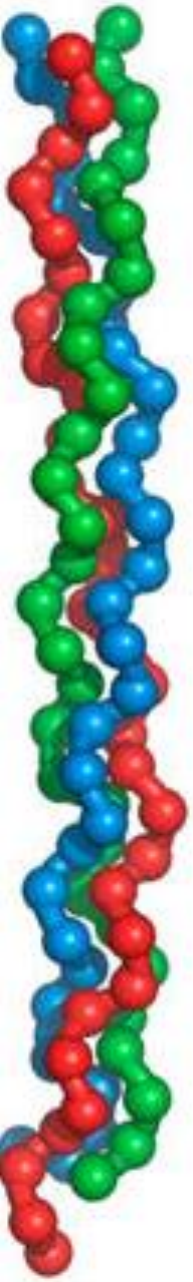
Connective tissue

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

CT Analyses



Preliminary Results

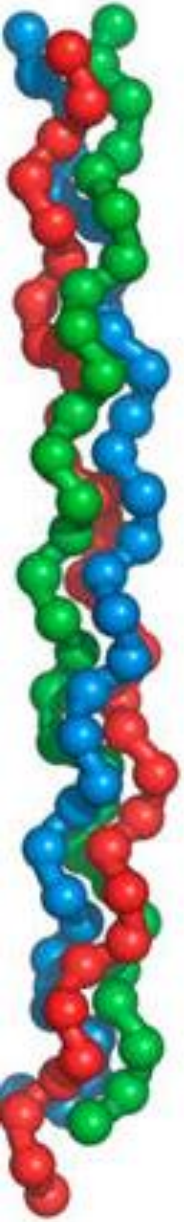


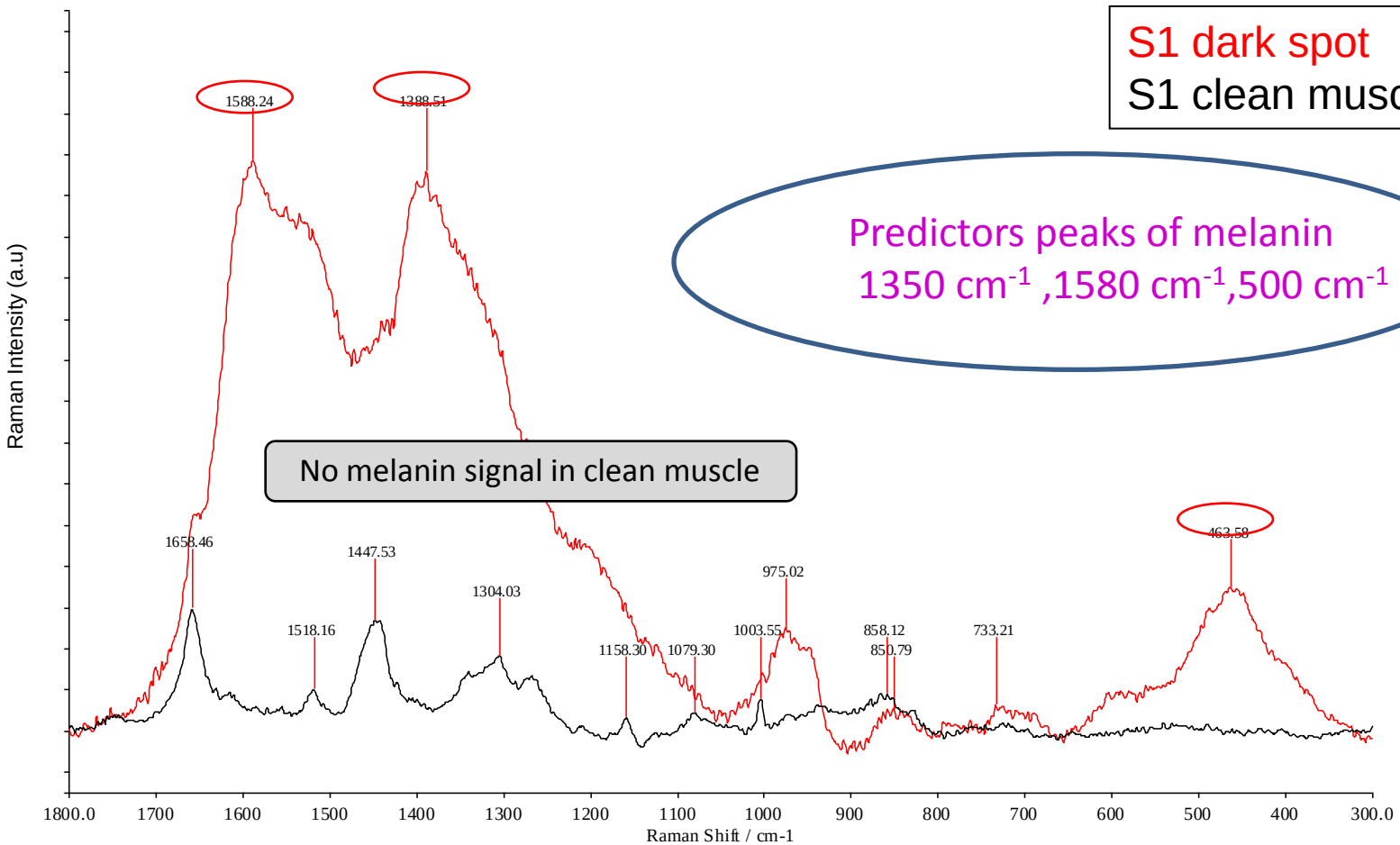
Analyses over Salmon Muscle

Raman from clean muscle and dark spot.

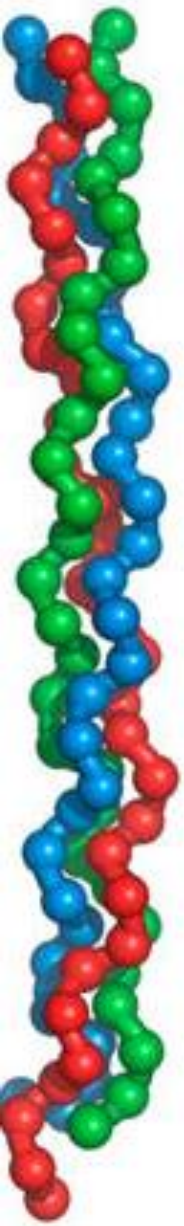
Determination of melanin

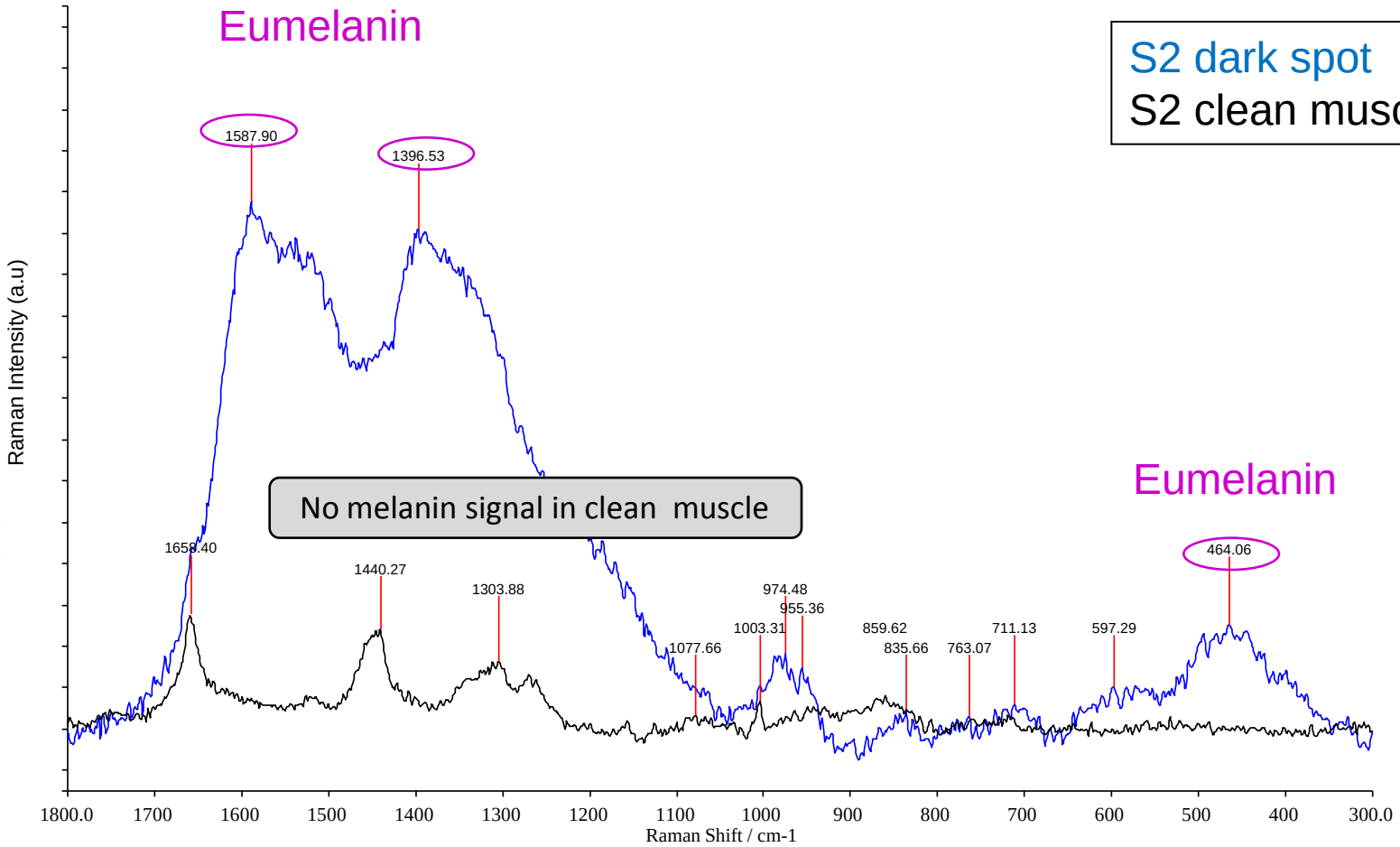
MeanSpectra from dark spot of score 1/clean muscle spectrum of S1



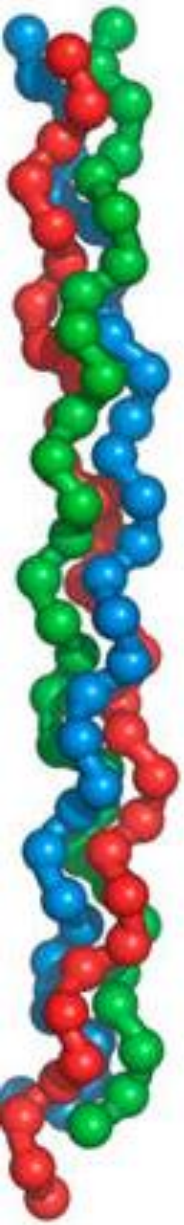


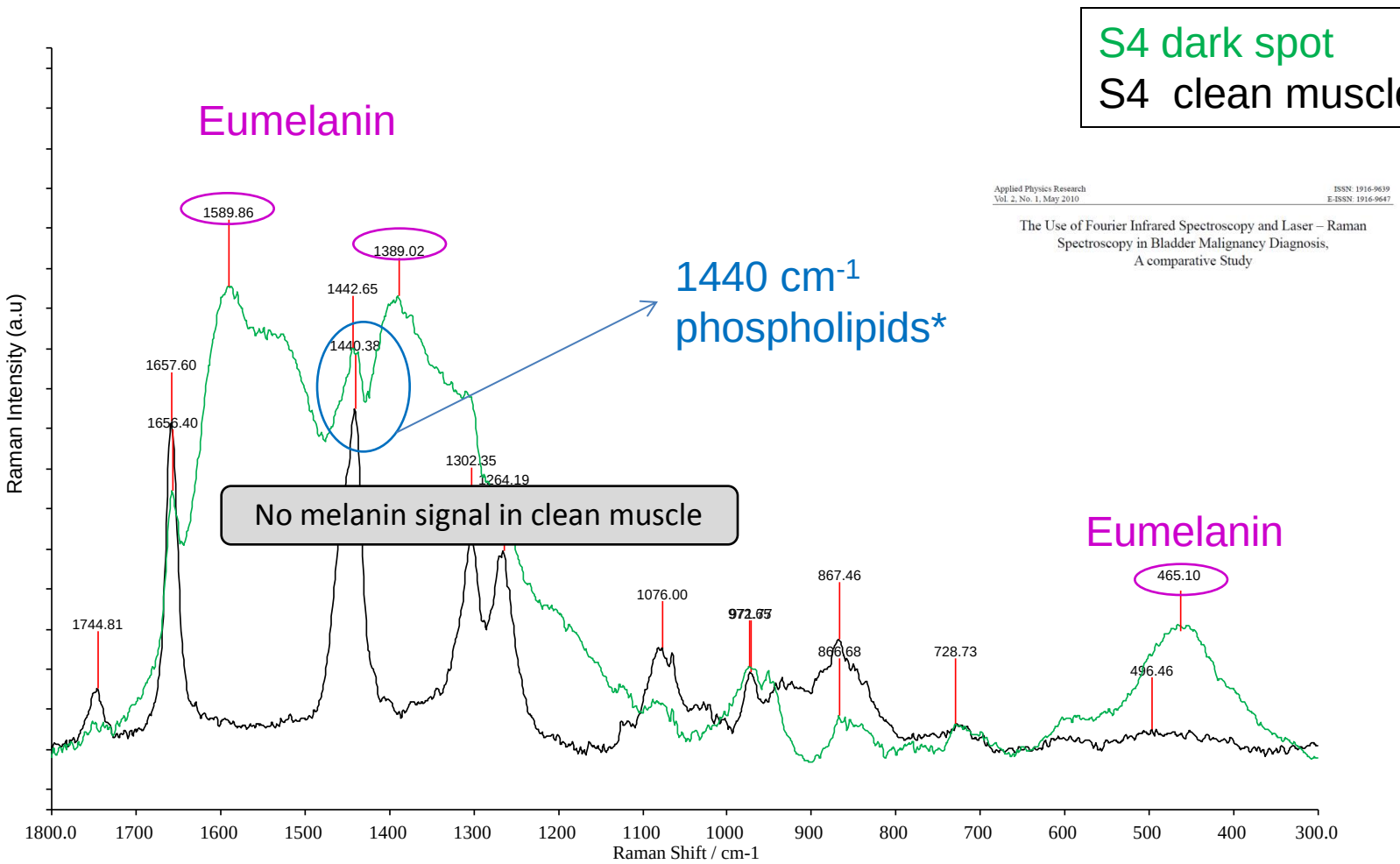
Mean Spectra from dark spot of score 2/clean muscle spectrum of S2





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





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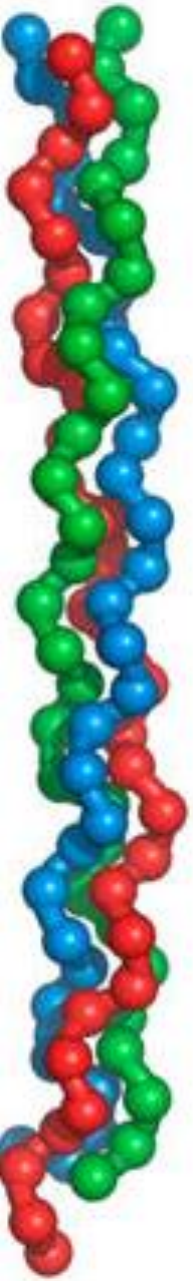
The Use of Fourier Infrared Spectroscopy and Laser – Raman Spectroscopy in Bladder Malignancy Diagnosis, A comparative Study



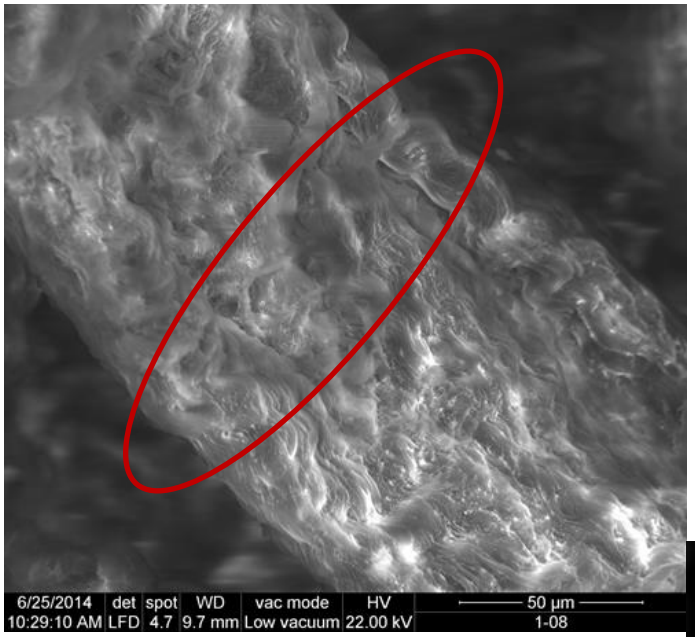
II.- Analyses over Connective Tissue

SEM clean muscle and dark spot.

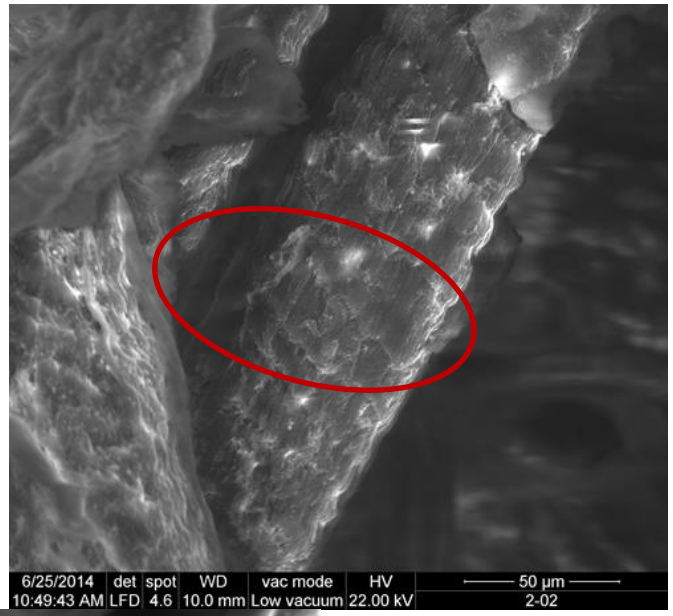
Morphology of CT fibers.

Connective tissue normal muscle (50µm)

Score 1



Score 2

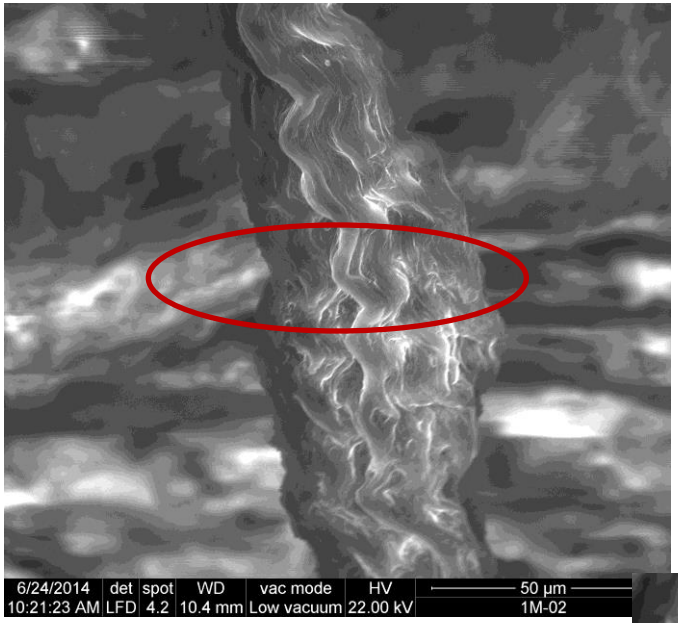


Structures formed by CT fibers
S1> S2 > S4

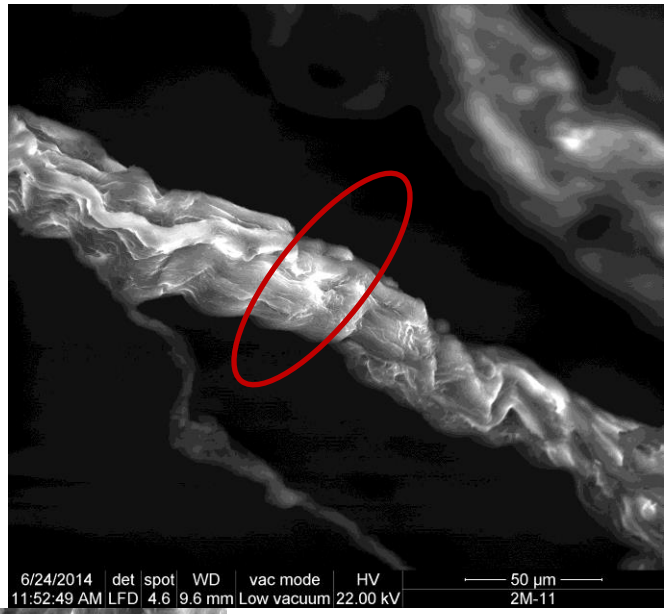
Score 4

Connective tissue Dark spot (50µm)

Score 1

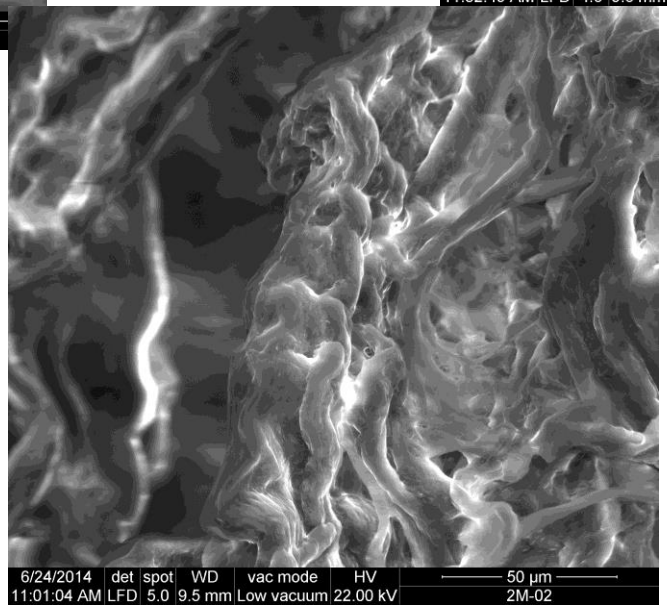


Score 2

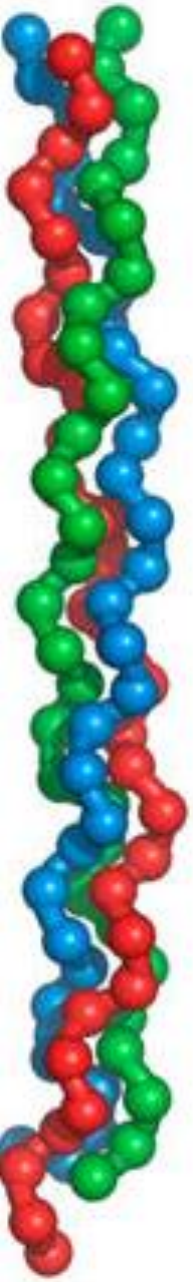


Structures formed by CT fibers are less organized as the presence of melanin increases

S4> S2 > S1



Score 4

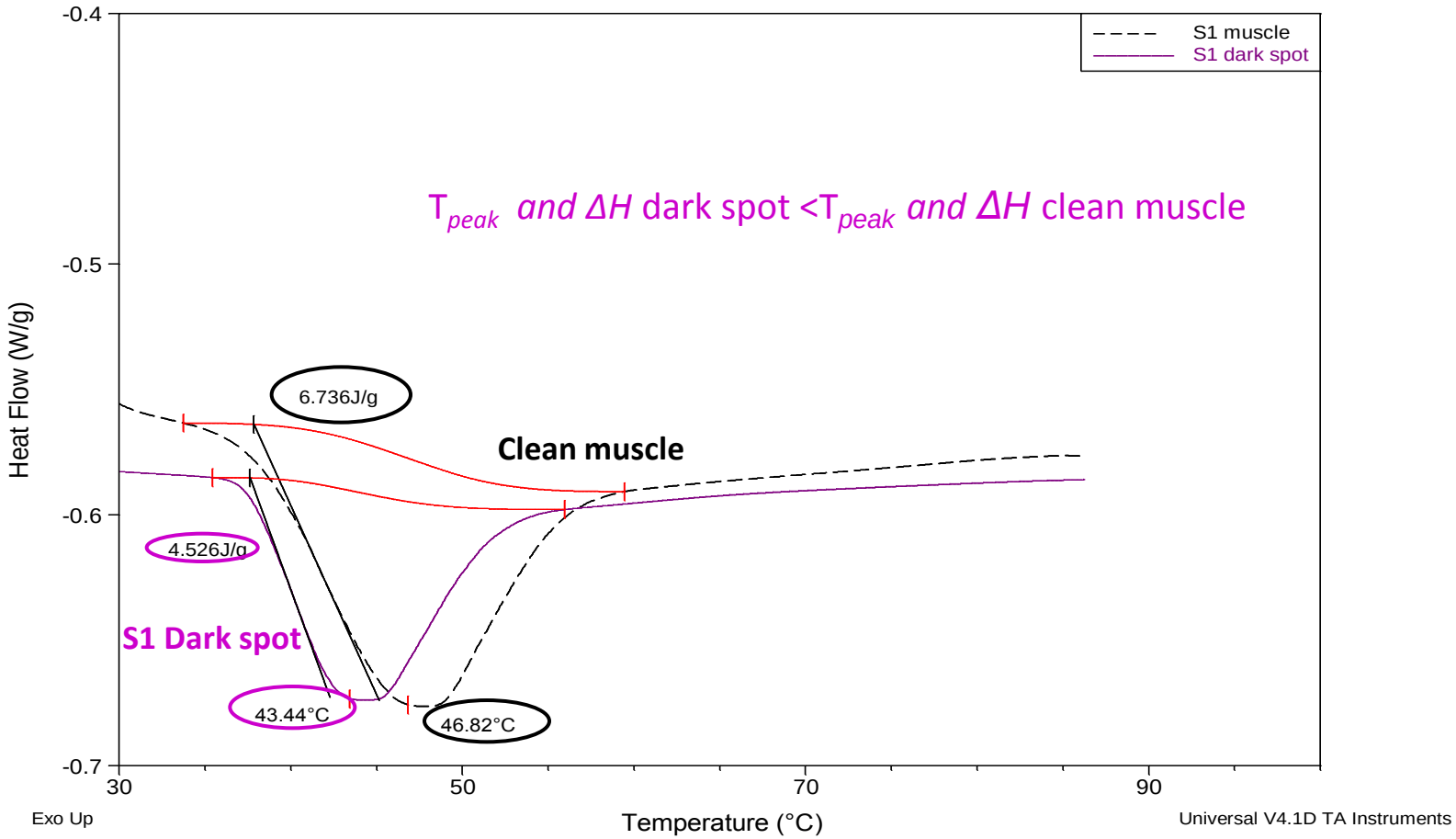


III.- Differential Scanning Calorimetry (DSC).

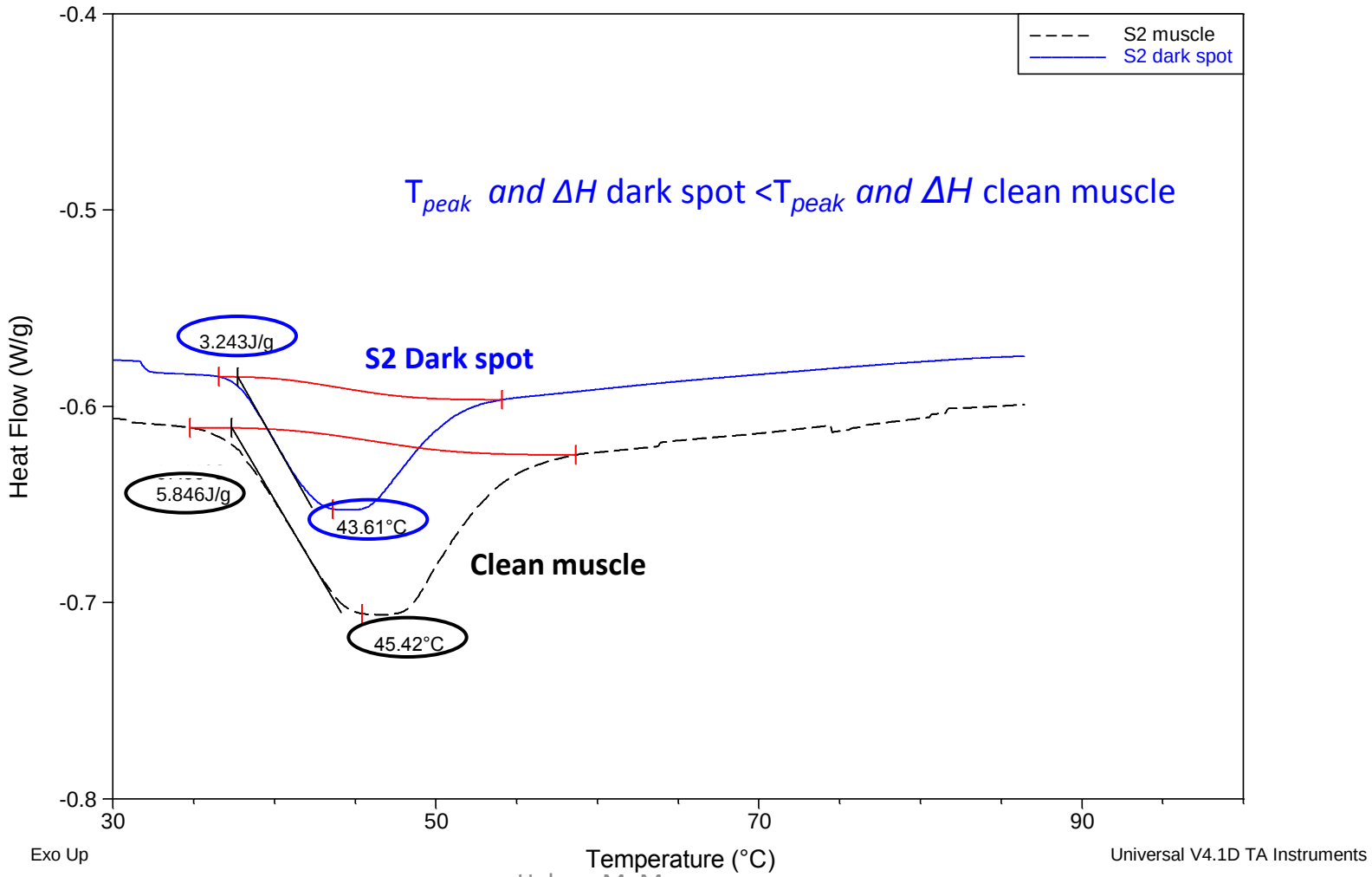
Thermal stability of Connective tissue

Helena M. Moreno

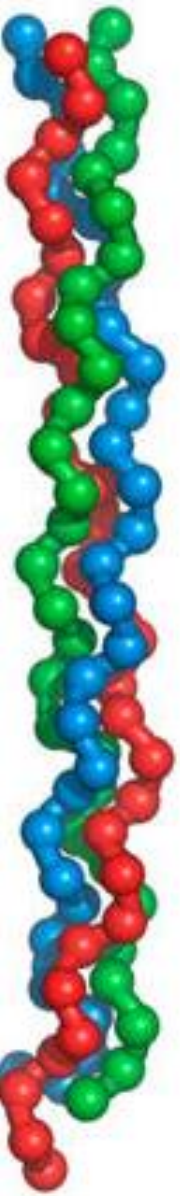
Spectra from dark spot *vs.* clean muscle spectrum of score 1

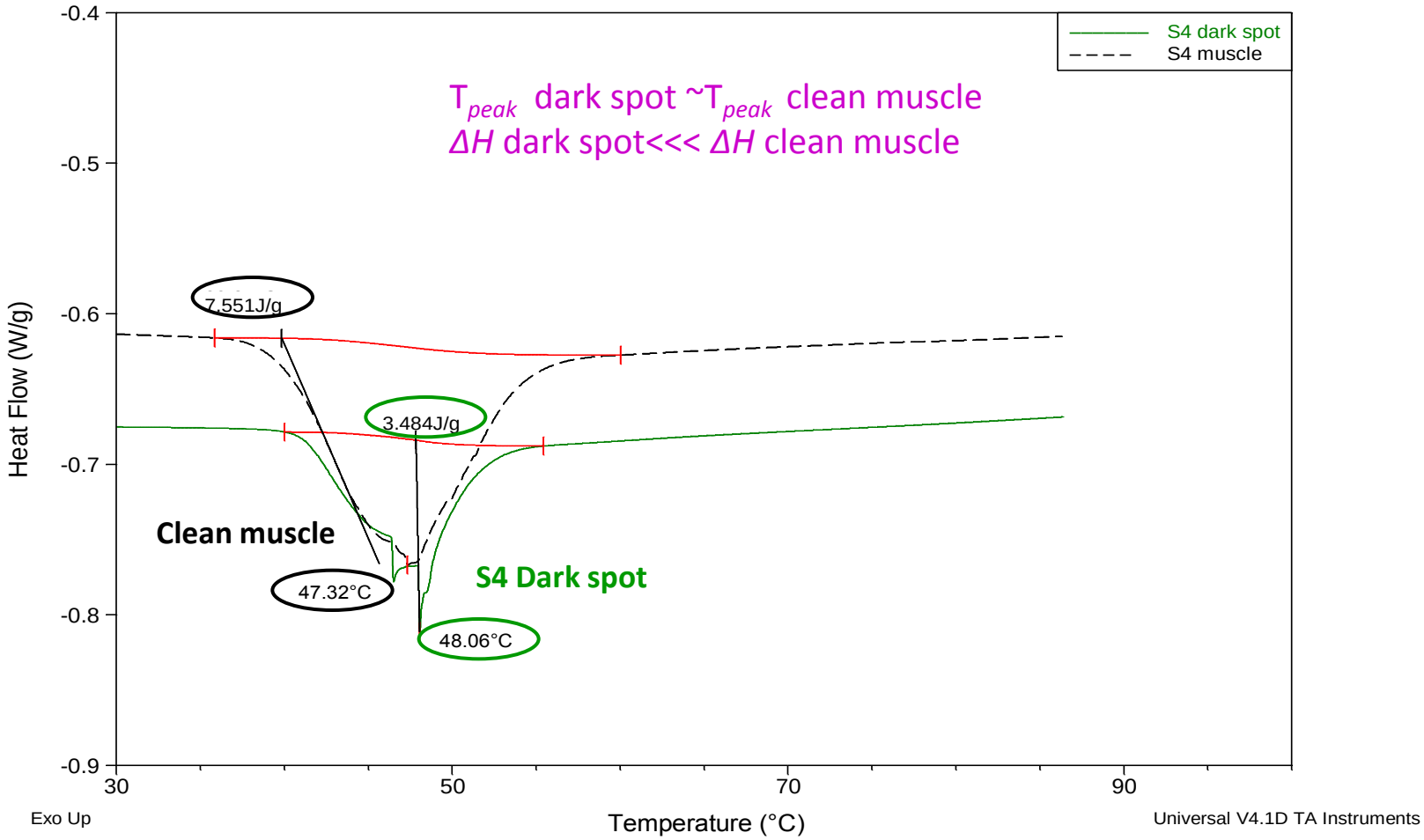


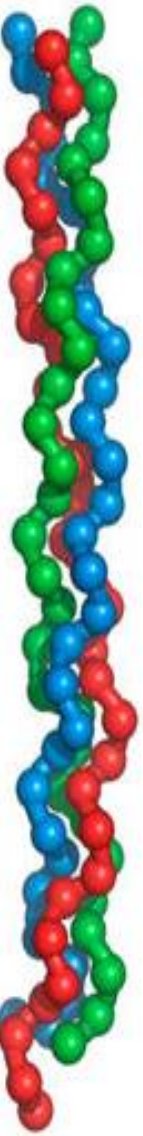
Spectra from dark spot *vs.* clean muscle spectrum of score 2



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

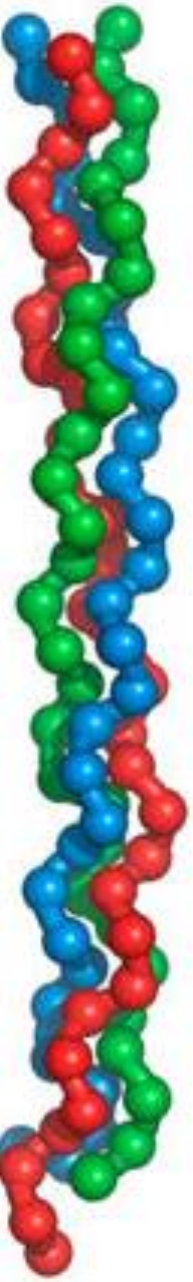






Parameters	Samples	Dark spot	Clean muscle
T_{peak}(ºC)	Score 1	43,44	46,82
	Score 2	43,61	45,42
	Score 4	47,06	47,32
ΔH (ºC)	Score 1	4,526	6,736
	Score 2	3,243	5,864
	Score 4	3,484	7,555

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

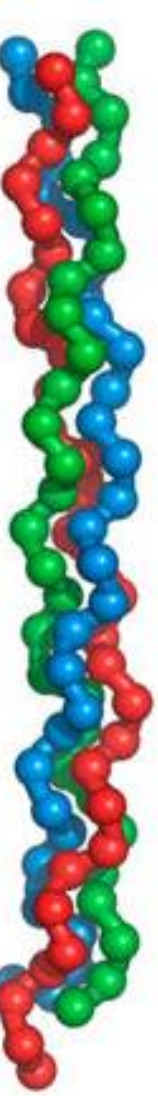


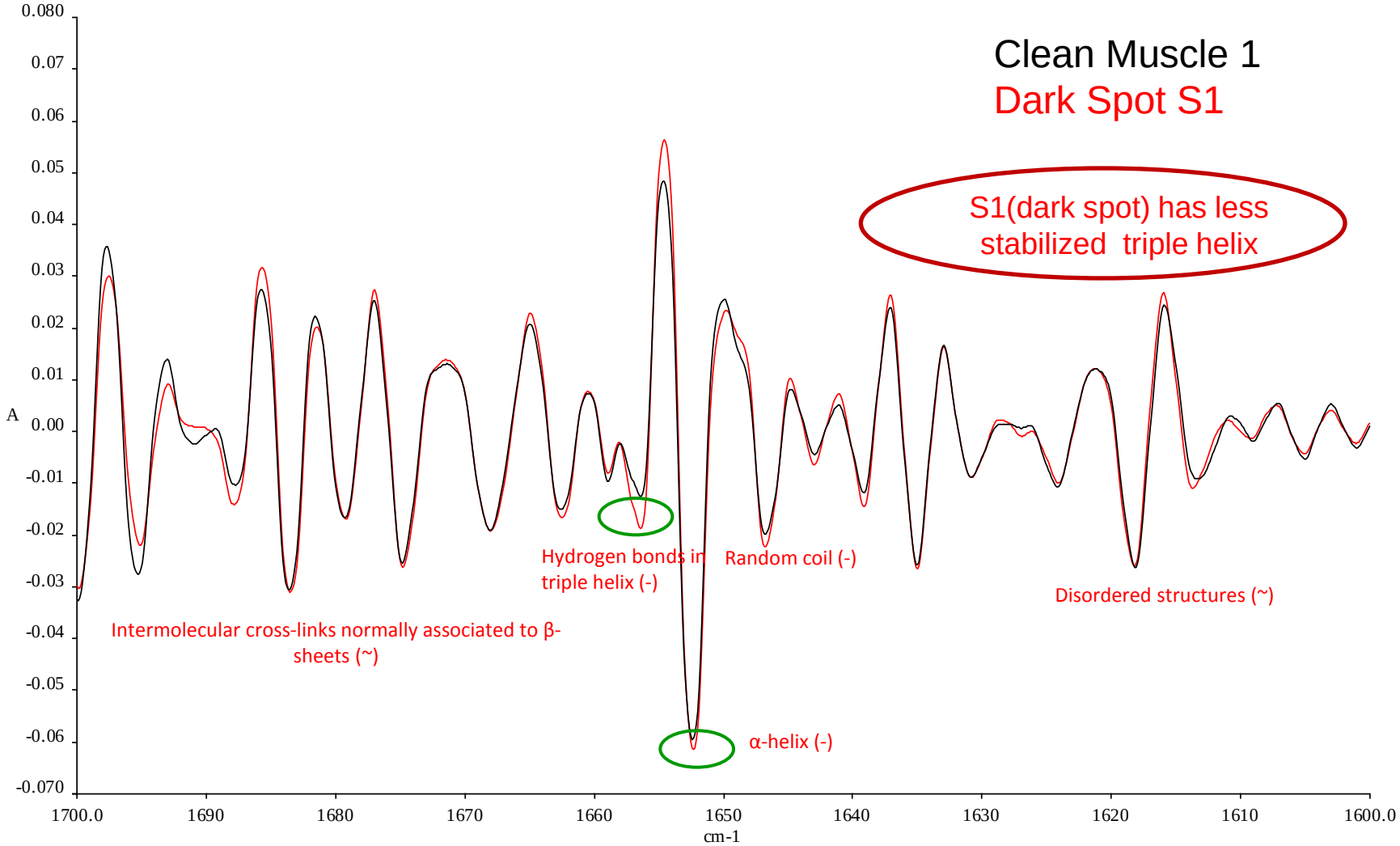
IV.- Fourier Infrared Spectroscopy (FTIR).

Secondary structure of collagen

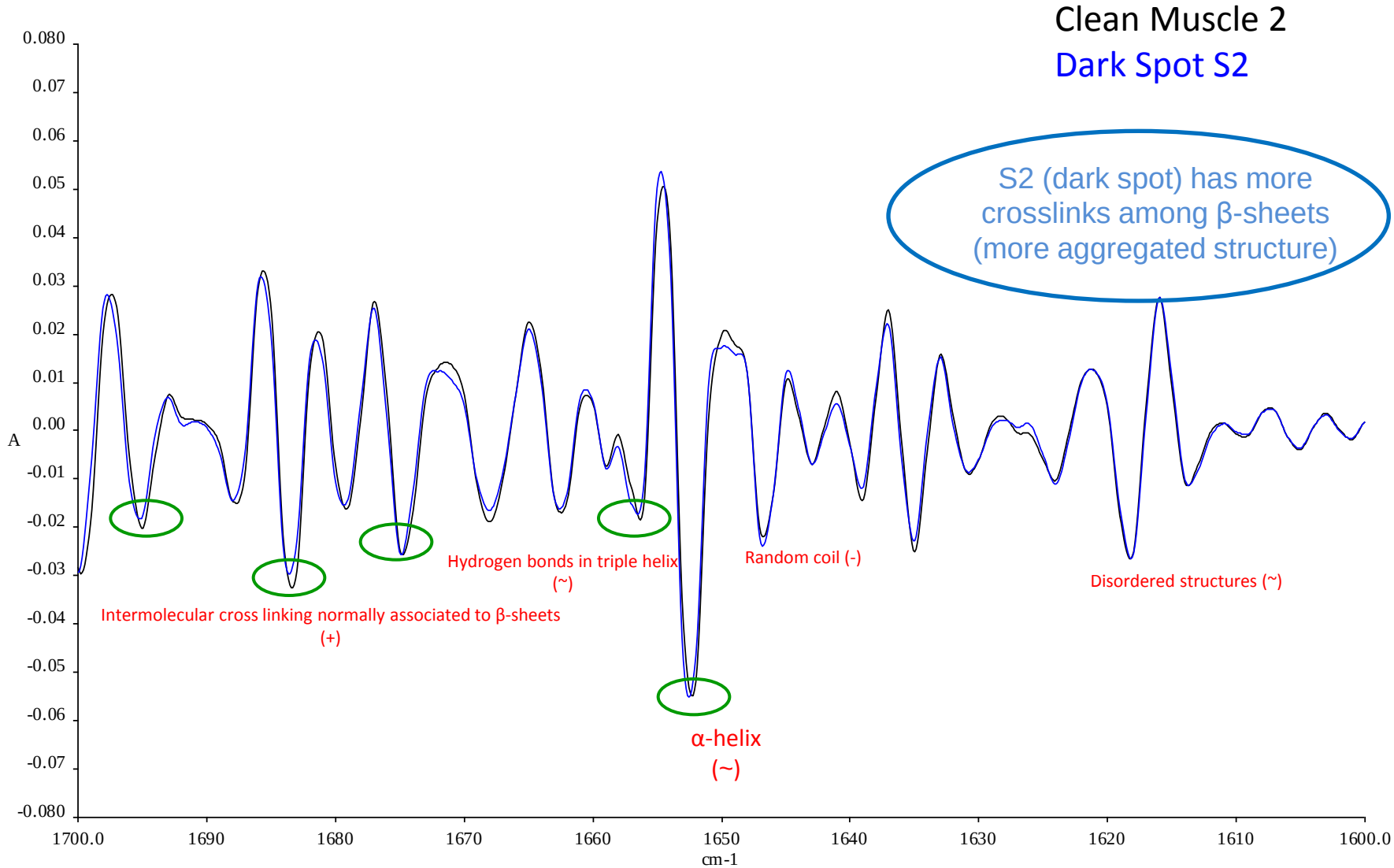
Helena M. Moreno

Amida I : Dark spot Score 1/muscle 1



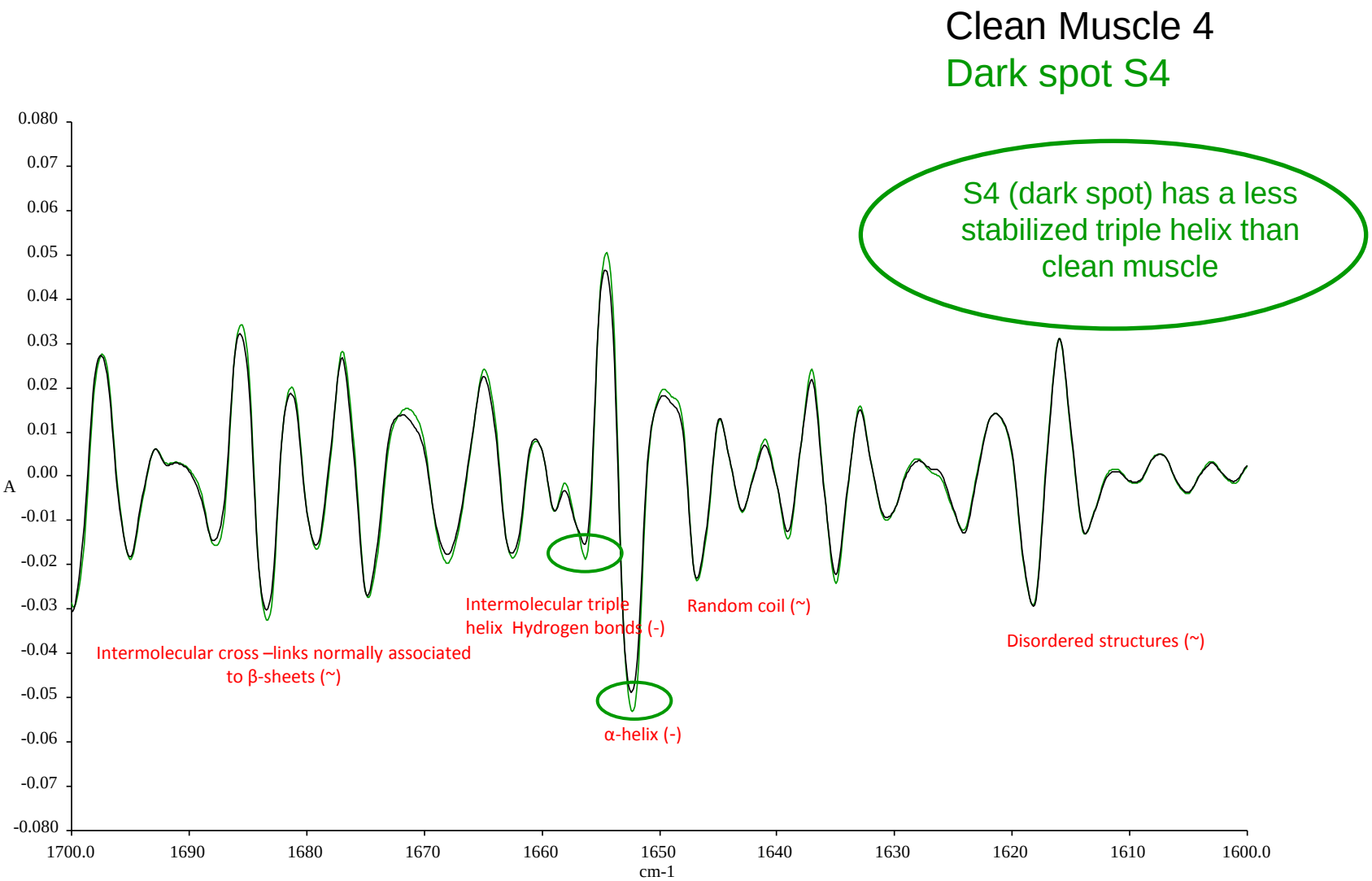


Amida I : Dark spot Score 2/muscle 2



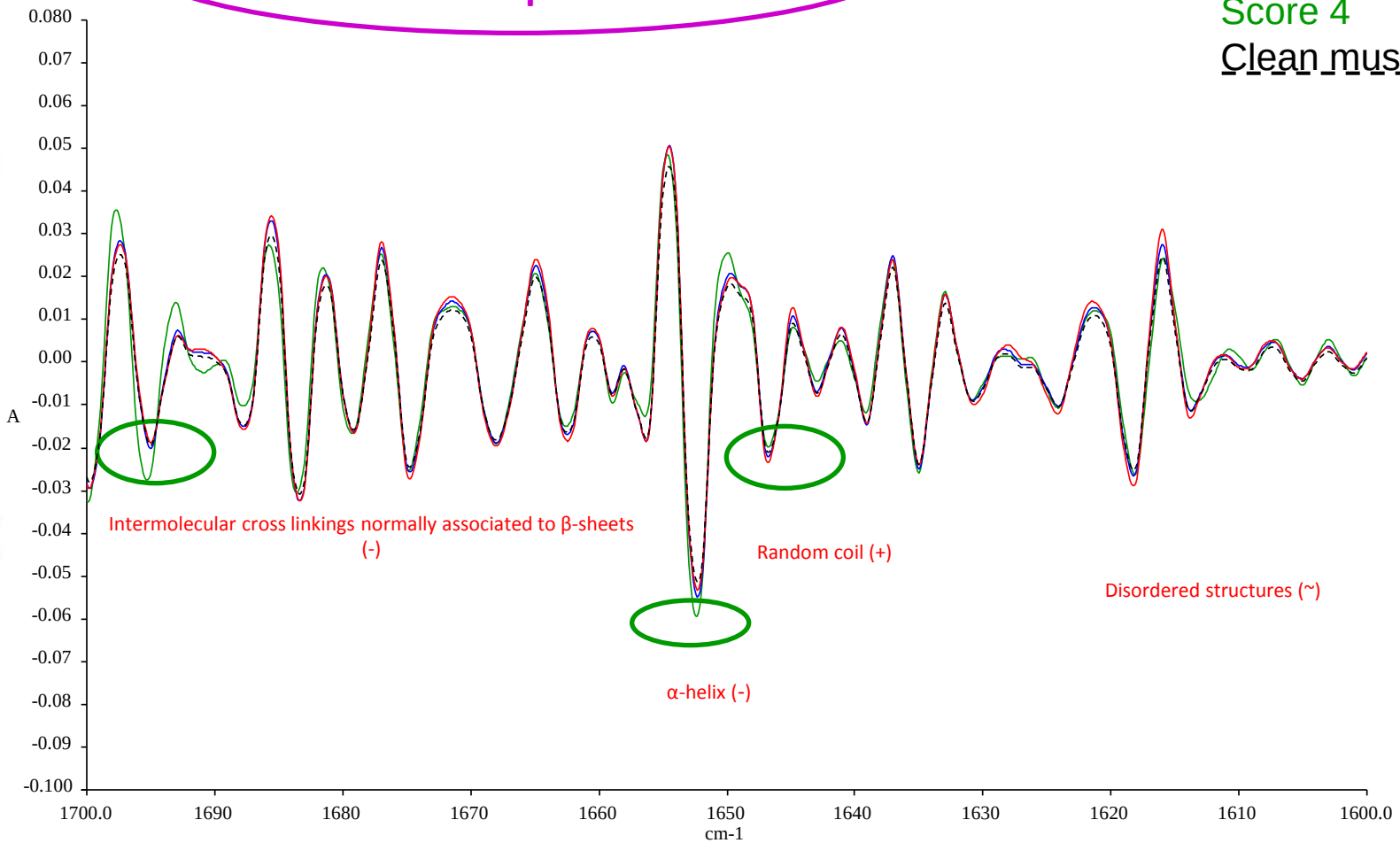
Helena M. Moreno

Amida I : Dark spot Score 4/muscle 4

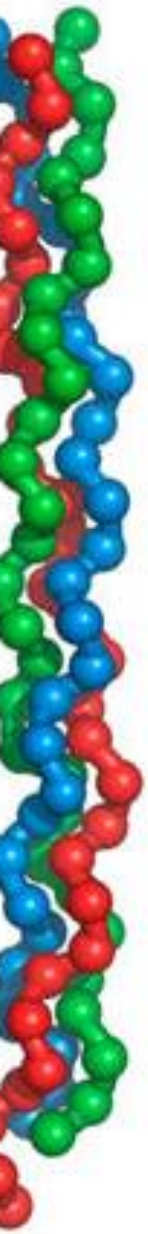


Loss of collagen typical structure with melanin presence

Score 1
Score 2
Score 4
Clean muscle



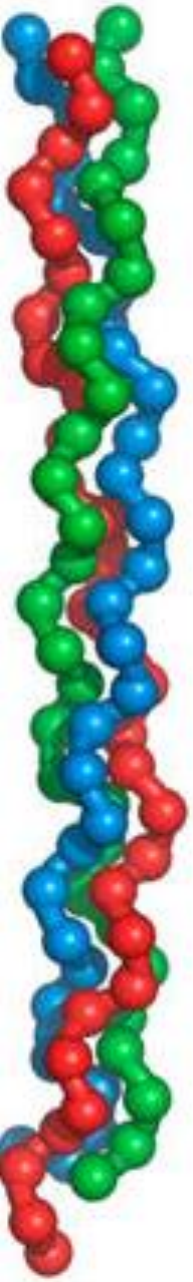
S4 lower presence of α-helix, less crosslinks stabilizing β-sheet and more random structures



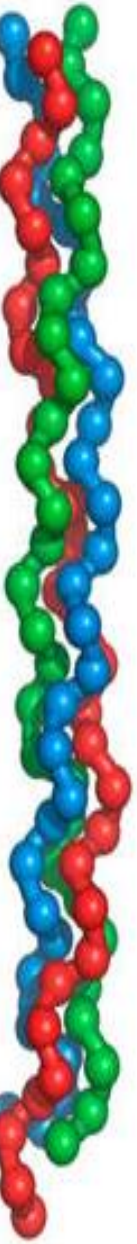
Triple helical structure preservation of Connective tissue of Dark spot and normal muscle

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 it 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 enthalphy and/or temperature of transition than clean muscle (less stabilized collagen).

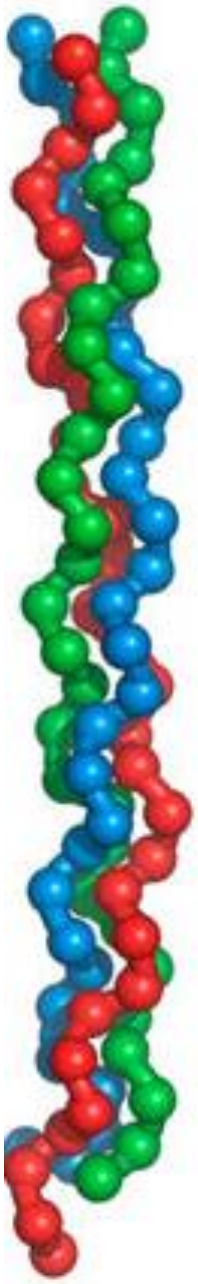
4.- Collagen with less α-helix and lower presence of Gly, Hyp and Hyl compred to clean muscle (loss of collagen typical struture).

⇩

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



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Thank you

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