



OXFORD SILK GROUP
DEPARTMENT OF ZOOLOGY
OXFORD UNIVERSITY
OXFORD, OX1 3PS, UK

Small Molecules in Silks

Tom Gheysens

Oxford, United Kingdom, 2011

Supervisors

Prof. D. Knight

Prof. F. Vollrath

Small molecules in silk

Thesis abstract

Tom Gheysens

Jesus College
Trinity 2011

Oxford Silk Group
Department of Zoology

Dissertation submitted to the Boards of the Faculty of Life Sciences, in fulfilment for the degree of Doctor of Philosophy in Zoology

Whereas most silk research is focused on silk proteins, the focus of this thesis is on small molecules present in silk and why they are so important for anyone working in this field with silk.

Silks are known to perform a range of different functions in nature, whereas for people, silk can be used in a wide variety of applications. A major disadvantage of using silk in these applications however, is its large variation in properties. In this thesis, the importance of small molecules will be discussed in light of this variation.

I present for the first time that calcium oxalate monohydrate is present in the cocoons of Wild Silkmoths resulting in the inability of reeling these cocoons. Furthermore, a method was developed for removing this mineral coating resulting in that these cocoons could be reeled for the very first time, what has vast commercial implications. Additionally, I found that the cocoon spinning between *B. mori* and *G. postica* was significantly different resulting in a mineral-free cocoon for *B. mori*.

Further, an unknown polyphenol in dragline and egg sac silk was identified responsible for the yellow coloration of these silks. An enzymatic method for the synthesis of this molecule was found *in vitro* and was believed to happen similarly *in vivo*. Whereas the function of this molecule is still unknown, the extended hydrogen bonding capabilities suggest it will bind to the silk peptide.

Finally we highlight the importance of small molecules in silk and point at the significance of the food of silkmooths and spiders as the origin of these small molecules, which has never been controlled for in any research or application.

Acknowledgements

This thesis is a tribute to a magnificent research environment I am proud to be part of in, consisting of inspiring, kind, knowledgeable, and helpful people from all backgrounds and disciplines. It all started with a simple email to Prof. Fritz Vollrath who immediately gave me the opportunity to further my work on silk in one of his spin-off companies, Oxford BioMaterials Ltd. (OBM). Here I met Prof. David Knight, Dr. Nick Skaer and Ms. Tracy Callaway. Although the first year was hard (of which I partly lived in a tent), I did learn a great deal about silk and its properties, gaining new insight and inspiration to undertake a PhD.

Therefore I would like to thank my two supervisors: Prof. Fritz Vollrath for the great opportunity, mentoring and your unfaltering belief in me. Also, I am extremely grateful for the freedom I got in my research, the discussions we had and the very well timed ‘prods’ I needed for starting, ending and writing things up. To Prof. David Knight, I would like to thank you for your constant support, mentoring, proof reading and commenting on my thesis. As it has been mentioned before, you truly are a ‘living encyclopaedia’ (I.C. Dicko, *personal comm.*). Thanks for your never-ending enthusiasm and the inspiring and insightful chats we had.

To Prof. David Porter for the numerous chats we had, mostly concerning the ‘yellow peril’ and for time to time putting my feet back on the ground. Your approach was particularly good in forcing me to think outside the box and to try on other people’s hats.

My special thanks go to Dr. Chris Holland as my closest colleague and buddy who was always there to help and support (even in my darkest hours) and much more, so much that if I do need to write it all down would end up with another chapter of this thesis. Also Dr. Cedric Dicko for letting me continue one of his projects, namely the yellow pigment, and all the help with where my chemistry lacked. Also a very special thanks for his unenthusiastic comments on the first draft of my PhD-proposal (I recall the description ‘crap’) which gave me the fuel to turn my PhD application into a successful one!!

Dr. Andy Collins from Bristol University for all the extensive chemistry we did and for his enthusiasm and unlimited energy he put into the project.

Further I would like to thank the whole Oxford Chemistry department (CRL) who was at one stage completely involved in the challenge of the identification of the yellow pigment. In specially would like to thank James McCullagh who enthusiastically took on the project and further I would like to thank Tim Claridge, Barbara Odell, David Watkin, Amber Thompson for their perseverance in identifying this compound.

A big thank you to my examiners, Dr. Ann Terry and Dr. Giuliano Freddi, for a most enjoyable viva that raised some interesting questions and new areas of future work.

A big thank you for helping me with reeling, rearing silkworms and general help goes to the students Catherine, Mat, Oona, Jennifer and Caroline. For help with the HPLC when I was away I would like to thank Bjoern Greving, Maxime Boulet-Audet and Benjamin Loloju. For the invaluable help with the layout I would like to thank Osnat Hakimi. Thank you to Dr. Alison Parkin for the help with the chemistry equipment. A thank you goes to Stephanie Lesage for taking OBM off my shoulders. To conclude I would like to thank all the people of the OSG and the ABRG (Anna, Rich, Simon and Rob) for the many chats, coffees, etc.

I would also like to extend my gratitude to the people of the Graduate office, namely Lynne Bradley, Heather Green and Victoria Forth, and not to forget John Iles for their support in my research and finances. For funding I would like to thank the BBSRC/EPSRC-CASE studentship, ERC and AFSOR.

On a personal note, I would like to thank my family and especially my mother, who has been a constant support from the very beginning and also my partner Kachi Liu and daughter Kato for their patience and support during the writing-up phase. Finally, I would like to thank my HPLC for “lasting as long” as it did, even though sometimes it looked like it didn’t want to be saved from the skip at all.

Publications

- 2011 T. Gheysens, A. Collins, S.K. Raina, F. Vollrath, D.P. Knight. 2011. Demineralization enables reeling of Wild Silkmoth cocoons. *Biomacromolecules*. 12 (6), pp 2257–2266
- 2011 J. Kingdon, B. Agwanda, M. Kinnaird, T. O'Brien, C. Holland, T. Gheysens, M. Boulet-Audet, F. Vollrath. 2011. A poisonous surprise under the coat of the African Crested Rat. *Proc. R. Soc. B.* (published online)
- 2011 W. Huang, R. Begum, T Barber, V. Ibba, N.C.H. Tee, M. Hussain, M. Arastoo, Q. Yang, L.G. Robson, S. Lesage, T. Gheysens, D. Knight, J.V. Priestley. 2011. Regenerative potential of silk conduits in repair of peripheral nerve injury in adult rats. *Biomaterials*. 33 (1), pp 59-71.

In preparation:

- Gheysens, T., Holland, C., Chen, F., Vollrath, F. and Knight, D. Behavioural aspects of mineralising the silkmoth cocoon.
- Gheysens, T., Dicko, C., Claridge, T., McCullagh, J., Odell, B., Vollrath, F. and Knight, D. The golden standard: the yellow pigment in *N. edulis*.
- Gheysens, T., Claridge, T., McCullagh, J., Odell, B., Vollrath, F. and Knight, D. Enzymatic synthesis of the yellow pigment in *N. edulis*.

Patents

- Tom Gheysens, David Knight, Andrew Collins and Fritz Vollrath. 2010. A method for demineralizing wild silk cocoons to facilitate reeling. GB1018269.9.
- Tom Gheysens, David Knight and Fritz Vollrath. The use of 7-nitro xanthurenic acid as an inhibitor for β -sheet aggregation in spider silk and its implications for amyloids (in preparation).

Table of content

Chapter 1 Introduction	1
Abstract and motivation	3
1.1. <i>Silk</i>	3
1.2. <i>Composition of silk</i>	5
A. Van Der Waals forces.....	7
B. Ionic bond (electrovalent linkages)	7
C. Hydrogen bonds	8
D. Covalent bonding.....	9
1.3. <i>Applications of silk</i>	11
1.4. <i>Problem with the industrial exploitation of silks: silks' variability</i>	13
1.4.1. Silkmoth silks	15
A. Relevance.....	17
B. Aim.....	18
1.4.2. Spider silks	19
A. Relevance.....	19
B. Aim.....	20
1.5. <i>Thesis outline and motivation</i>	21
Chapter 2 Mineralisation of Wild Silkmoth cocoons and a technique for its selective removal	23
Abstract and motivation	25
2.1. <i>Introduction</i>	26
2.2. <i>Materials and Methods</i>	28
2.2.1. Samples.....	28
2.2.2. Treatments	29

2.2.3.	Analysis of mineral removal.....	30
2.2.4.	Reeling	32
2.2.5.	Tensile testing.....	32
2.3.	<i>Results</i>	34
2.3.1.	SEM observation of the mineral and its removal.....	34
2.3.2.	Spectrographic evidence for identification of the mineral and its removal	39
2.3.3.	Reeling	42
2.3.4.	Tensile properties	44
2.4.	<i>Discussion</i>	48
2.5.	<i>Conclusion</i>	50
Chapter 3 Practical implications of demineralizing Wild Silkmoth cocoons.....		51
Abstract and motivation		53
3.1.	<i>Technical field</i>	54
3.2.	<i>Background of the invention</i>	54
3.3.	<i>Brief description of the invention</i>	58
3.4.	<i>Detailed description of the invention</i>	59
3.5.	<i>Protocol 1: demineralizing and degumming Wild Silk cocoons.</i>	64
3.6.	<i>Protocol 2: tensile testing of Wild Silks</i>	64
3.7.	<i>Efficiency of reeling</i>	64
3.8.	<i>Results of tensile testing</i>	65
3.9.	<i>Claims</i>	68
3.10.	<i>Conclusion</i>	71

Chapter 4 Comparison of the behavioural aspects of mineralisation in <i>B. mori</i> and <i>G. postica</i>	73
Abstract and motivation	75
4.1. Introduction	75
4.2. Material and methods	76
4.2.1. Material	76
4.2.2. Methods	77
4.3. Results and discussion	79
4.3.1. Cocoon spinning behaviour	79
A. <i>Gonometa postica</i>	79
B. <i>Bombyx mori</i>	82
C. Comparison	83
4.3.2. Cocoon shell analysis	85
4.4. Conclusion	87
Chapter 5 Identification of the yellow pigment in <i>Nephila edulis</i>	89
5.1. Introduction and motivation	91
5.2. Material and methods	93
5.2.1. Samples	93
5.2.2. Reagents	94
5.2.3. Methods	94
5.3. Results	96
5.3.1. High Pressure Liquid Chromatography (HPLC)	96
A. Dragline pigment	96
B. Egg sac pigment	96
C. Summary	97

5.3.2.	Nuclear Magnetic Resonance (NMR)	98
A.	General.....	98
B.	Dragline pigment	99
C.	Egg sac pigment	102
D.	Comparison with xanthurenic acid	104
E.	Summary	108
5.3.3.	Mass spectrometry (MS).....	109
A.	Dragline pigment	109
A.1.	Exact mass and structural formula determination	109
A.2.	Fragmentation pathway: structure determination	111
B.	Egg sac pigment	116
C.	Summary	117
5.3.4.	Fourier Transform Infra-Red Spectroscopy (FTIR)	118
A.	General.....	118
B.	Dragline pigment	118
C.	Egg sac pigment	120
D.	Summary	120
5.3.5.	Ultra-violet spectroscopy (UV-spectroscopy)	121
A.	Prediction of the UV-spectrum of the yellow pigment: Woodward-Fieser rules.	121
B.	Summary	122
5.4.	<i>Conclusion</i>	123
Chapter 6 <i>In vitro</i> synthesis of the yellow pigment		125
6.1.	<i>Introduction</i>	127
6.2.	<i>Material and method</i>	129
6.2.1.	Reagents	129
6.2.2.	Samples	129
6.2.3.	Preparations.....	130

A.	Chemical nitration of xanthurenic acid	130
B.	Enzymatic nitration of xanthurenic acid	130
C.	Large scale preparation of nitrated xanthurenic acid.....	132
6.2.4.	Methods	132
6.3.	<i>Results</i>	133
6.3.1.	Synthesis	133
A.	Preferred position of a substituted nitro group on xanthurenic acid.....	133
B.	Chemical synthesis	134
B.1.	Analytical separation	134
B.2.	Preparative separation	137
B.3.	Summary	138
C.	Enzymatic synthesis	139
C.1.	Effect of sodium nitrite and hydrogen peroxide concentration	139
a)	General	139
b)	Products	142
c)	Summary	144
C.2.	Effect of pH	145
a)	General	145
b)	Products	148
c)	Summary	150
D.	Conclusion	151
6.3.2.	Identification of the enzymatic produced dragline pigment	153
A.	HPLC: large scale preparation.....	153
B.	FTIR of the purified synthesized product.....	155
C.	Nuclear magnetic resonance (NMR).....	155
D.	Mass spectrometry (MS)	158
E.	Summary.....	162
6.4.	<i>Conclusion</i>	163

Chapter 7 Summary and future work.....	165
Abstract and motivation.....	167
7.1. <i>General discussion</i>	167
7.1.1. Calcium oxalate.....	167
A. Origin	167
B. Mechanism	169
C. Function	170
D. Behavioural and evolutionary aspects: comparison with <i>B. mori</i>	171
7.1.2. Yellow pigment (7-nitro xanthurenic acid)	173
A. Origin	173
B. Mechanism	176
C. Function	177
C.1. Tryptophan metabolism and metabolites.....	177
C.2. 7-nitro xanthurenic acid	178
7.1.3. Overall significance	180
7.2. <i>Practical implications</i>	180
A. Calcium oxalate.....	180
B. 7-nitro xanthurenic acid (yellow pigment)	182
7.3. <i>Future work</i>	184
A. Calcium oxalate monohydrate (Whewellite)	184
B. Yellow pigment (7-nitro xanthurenic acid)	184

Appendix A	Supplementary information	185
A.1.	Media coverage	187
a)	Oxford university website	187
b)	European commission.....	188
c)	New York Times	189
A.2.	Detailed protocol 1 and 2 of Chapter 3	190
a)	Protocol 1: demineralizing and degumming Wild Silk cocoons.	190
b)	Protocol 2: tensile testing of Wild Silks.....	191
A.3.	Comparison xanthurenic acid and yellow pigment: HPLC and UV	192
A.4.	The effect of pH on the NMR ¹ H spectrum of 7-nitro xanthurenic acid	194
A.5.	FTIR spectrum and interpretation of Xanthurenic acid.....	196
A.6.	The FTIR-spectrum of the purified egg sac pigment fraction	198
A.7.	Raw data mass spectrograms of the yellow pigment.....	199
A.8.	Mass spectroscopic data of Xanthurenic acid	200
A.9.	The effect of substituted groups on Liquid Chromatography (HPLC).....	205
a)	A hydroxyl group.....	205
b)	A nitro-group.....	206
c)	Conclusion.....	207
A.10.	Detailed analysis of the FTIR-spectrum of the enzymatically synthesized compound.....	208
A.11.	Raw data of the mass spectrometric fragmentation pattern of the enzymatic synthesised product ..	210
Appendix B	References	213

List of Figures

Figure 1-1. The glandular origin, function, and material properties of the different silks of the golden orb weaving <i>Nephila</i> species. Note the wide variation of mechanical properties each optimised for a specific use (taken from ¹³).	4
Figure 1-2. Types of molecular bonding occurring in silks and their relative strength. Whereas most interactions are reversible, covalent bonding is irreversible having vast implications in silk processing ²⁶⁻²⁸	6
Figure 1-3. Different covalent bonding types occurring in silk assembled from references ^{29, 42, 46, 49} . a) Di-sulfide cross-linking between two cystinyl residues of two adjacent peptides. b) Dityrosine cross-linking between two adjacent tyrosyl residues. c) An example of a cross linking between peptides by a polyphenol, in this case gentisic acid, as a cross-linker between two amino acids of two different peptides. d) No covalent cross-linking between peptides.	10
Figure 1-4. Schematic drawing of relations and variations introduced in silkmoth and spider silks during spinning and post-spinning. The gray box represents the weak reversible interactions whereas the brown box represents the covalent bonding process. The colour code for the reactions is as follows: black for the factors influencing spinning, brown for the factors involved in tanning, blue for the importance of water and red indicating the focus of this thesis.	14
Figure 1-5. Flowchart of the outline of this thesis.	20
Figure 2-1 (on next page). High magnification pairs of SEM pictures (500x, scale bar 50µm) of the same untreated (mineralized; left-hand side) and treated (demineralized; right-hand side) cocoon shell for some selected silkmoth species: <i>Antheraea frithi</i> (a), <i>Antheraea mylitta</i> (b), <i>Antheraea pernyi</i> (c), <i>Attacus atlas</i> (d), <i>Bunaea alcinoe</i> (e), <i>Hyalophora cecropia</i> (f), <i>Philosamia cunninggii</i> (g), <i>Saturnia pyri</i> (h). All other wild silk species studied exhibited similar results.	34
Figure 2-2. Low and high magnification SEM pictures of quadrants of cocoon discs of <i>G. postica</i> . Untreated (natural) cocoon disc, (a) low magnification (scale bar 500 µm) and (b) detail of covering mineral crystals (scale bar: 50 µm) with arrows indicating embedded fibers. Demineralized cocoon disc (c) with arrows indicating visible fibers in the cocoon shell network (scale bar: 500 µm) and (d) detail of cocoon disc surface showing fibers and gum, with arrows indicating crystal imprints (scale bar: 50 µm). A degummed cocoon disc (e) low magnification (scale bar: 1mm) showing the unravelling	

of the disc and (f) a detail of the fibers with arrows indicating imprints showing that brins were deformed where they crossed one another in forming the network (scale bar: 50 μm). 37

Figure 2-3. Shows the ranking of the species according to the estimated approximated degree of mineralization (see method section) and the ease of reeling these cocoons according to this ranking. 39

Figure 2-4. Spectra of the extracted mineral powder (blue trace), its presence in the natural cocoon shell (black trace) and its removal (red trace) from the cocoon shell by the demineralizing treatment. The EDX shows that the mineral powder consists of calcium, oxygen and carbon (a, blue trace), while the XRD spectra of the extracted powder (b, blue trace) of the *G. postica* cocoon was identified as, and indexed against whewellite (JCPDS card 01-077-1160). The EDX (a) and XRD (b) spectra both show that the peaks characteristic for calcium oxalate disappeared after the demineralization treatment (black line, natural cocoon disc; red line, demineralized cocoon disc). The FTIR spectra (c) showed that the extracted powder from the cocoons (2, blue line) matches the calcium oxalate hydrate spectra (1, orange line) bought from Sigma. The untreated (black line) cocoon discs (3) matched the calcium oxalate spectra (1, orange line) and that almost no mineral was present on the inside of the untreated disc (4). After demineralizing (red line) the calcium oxalate was completely removed on the outside (5) as well as on the inside (6) of the cocoon with no traces of EDTA (7, green line). 40

Figure 2-5. (a) The maximum continuous length of reeled silk fiber per cocoon, each column representing a new try after a fiber breakage. (b) Stress-strain graphs are shown from *G. postica* silk with different treatments: Treated with pineapple juice, carded and unraveled from hand spun yarn (blue); Silk fibers extracted by reeling after demineralizing the cocoon before (black); and after sodium carbonate degumming (red) ($n = 4$ cocoons, averaged representative curves). Figure 2-5 (c-d) SEM pictures of demineralized (c) and sodium carbonate degummed (d) fibers after reeling (scalebar: 10 μm). 45

Figure 3-1: A comparison of the X-ray scattering spectra of the same *Gonometa postica* cocoon outer shell section before (shown in black)) and after demineralisation (shown in red). The peaks due to the crystalline mineral are entirely absent in the demineralised material. 63

- Figure 3-2: Stress-strain graphs from *G. postica* fibres extracted according to the prior art which involves treating with pineapple juice and carding (a), and fibres extracted by reeling after demineralising the cocoon (b) without degumming (black) and with degumming (red)..... 65
- Figure 3-3: Box plots comparing the effect of demineralisation (red), demineralisation/ sodium carbonate degumming (green) and pineapple degumming (blue) on the tensile properties of the silk filaments: break stress (a); the break load (b); the cross sectional area (c); the Young's modulus (d; break strain (e); and breaking energy (f). The top and bottom of the boxes represent $\pm$ the standard deviations while the vertical bars represent the minimum and maximum values. The mean value is represented by a dot in the box while the line in the box represents the median; n = 150 in each case. 67
- Figure 4-1. a) Cocoon construction time lines for *A. pernyi*, *S. cecropia*, *G. postica* and *B. mori* showing the percentage time (%) spend on each spinning phase (total time: ± 48 h) mentioned in section 4.3.1.A. (CEB: cocoon expansion behaviour). b-c) High magnification SEM pictures (scale bar: 100 μ m) of the cross-section of the cocoon shell of *G. postica* (b) and *B. mori* (c), split in an upper and lower half, showing the natural and demineralised cocoon respectively with superimposed the EDX mapping for oxygen (yellow) and calcium (red). d-e) High magnification pairs of SEM pictures (500x, scale bar 50 μ m) of the same untreated (natural; left-hand side) and treated (demineralized; right-hand side) cocoon shell of *G. postica* (d) and *B. mori* (e). 81
- Figure 4-2. a-b) The EDX (a) and XRD (b) of the outer (black) and inner (red) layer of the cocoon in *G. postica* in comparison with the outer (blue) and inner (green) layer of the cocoon in *B. mori* indexed to calcium oxalate (JCPDS card 01-077-1160). Some traces of calcium carbonate were seen in the XRD-spectra of *B. mori* but were absent in the EDX-spectra. The FTIR-spectra (c) indicated that calcium oxalate was present in the outer (1) and inner (2) layer of *G. postica* and not in the outer (3) and inner (4) layer of *B. mori*. Also the impregnation fluid of *G. postica* (8) and the secretion (9) and poo (10) of *B. mori* during spinning were compared with pure calcium carbonate (5), calcium oxalate (6) and oxalate ion (7). 84
- Figure 4-3. Column graph of the mean percentage amino acid composition of the natural cocoon shell of *G. postica* (green, n: 5), after demineralizing (red, n: 5) and after demineralizing/degumming (blue, n: 5) with the standard deviation indicated by lines. 87

Figure 5-1. Chromatographic data of the dragline extract (orange trace) and egg sac extract (red trace) with (a) the chromatograms resulting in a common peak with apex at 14.5 minutes (analytical column see methods section) and UV-absorption spectra (200 to 800nm) of the apex of both chromatograms (b).....	97
Figure 5-2. HSQC of the purified extracted dragline pigment (orange) and egg sac pigment (red) in acidified D ₂ O.	99
Figure 5-3. HMBC of the purified extracted dragline pigment (orange) and egg sac pigment (red) in acidified D ₂ O and the resulting solved structure from the ¹³ C-assignments.	100
Figure 5-4. The predicted structure from NMR data of the extracted purified dragline pigment. The ¹³ C chemical shifts are shown in red and the ¹ H shifts in blue (ppm).	102
Figure 5-5. HSQC of xanthurenic acid (Sigma) in acidified D ₂ O.	104
Figure 5-6. HMBC of xanthurenic acid (Sigma) in acidified D ₂ O and the resulting structure and ¹³ C assignments from the HMBC spectra.	105
Figure 5-7. Chemical structures of (a) the dragline pigment molecule and (b) its most closely structurally related molecule, xanthurenic acid. Carbons and hydrogens are numbered for comparison in the Table 5-2 and Table 5-4.	106
Figure 5-8. a) Mass spectra obtained from purified dragline pigment on the SYNAPT with positive ionisation electrospray and at a current of 1.24e ⁴ eV. The mass spectrogram of the relative abundance of the ions is plotted against their mass (m/z). b) Theoretical spectrum of the yellow pigment.	109
Figure 5-9. The fragmentation of the yellow pigment on the SYNAPT with positive ionisation spray of the mass at 251 (at 1.71e ⁴) and the fragments of mass 223 (at 7.61e ³) and 205 (at 2.50e ³). The raw data can be found in Appendix A.7.....	111
Figure 5-10. A hypothetical fragmentation pathway of the dragline pigment based on the experimental data. The fragmentation suggested the presence of a nitro-group.....	114
Figure 5-11. A detail of the mass region 173 to 180 of the mass spectra obtained after fragmenting masses 251 at 221 eV (a), 223 at 5.65e ³ eV (b) and 205 at 98 eV (c) indicating the loss of CNO ₃ , NO ₂ and NO respectively.	115
Figure 5-12. FTIR-spectra of purified dragline pigment with peaks indicated.....	119

- Figure 5-13. The predicted structure for the dragline pigment with its chemical properties (a) and a graphical representation (balls and sticks model) of the dragline pigment (b). 124
- Figure 6-1. The sample preparation of the two experiments for different concentrations (on the left) and at different pH (on the right). 131
- Figure 6-2. Two resonant forms of xanthurenic acid: (a) the carbonyl form at pH<4 and (b) the hydroxyl form at pH > 4, with numbers indicating the possible nitration sites in the structure (1 to 4) and tabulated next to it the energy required to nitrate this position and the dipole to overcome. 134
- Figure 6-3. Chromatographic data of injected chemically nitrated xanthurenic acid showing (a) a 3D-representation of the isogram of the run with the X-axis as retention time (0 to 25min), Y-axis as absorbance (mAU) and Z-axis as wavelength (from 200-800nm), (b) the 2D-isogram with the peak selected for the dragline pigment showing the measured UV-spectra and chromatogram (270nm) at this point (green), and (c) The spectral match of the measured UV-spectrum of the apex at 14.4 minutes (green) with the spectrum from 7-nitro xanthurenic acid (red, n:25) and the difference spectrum between both spectra (below, green). 135
- Figure 6-4. Chromatographic traces taken at 270nm of extracted dragline pigment (yellow trace), xanthurenic acid (blue trace) and chemically nitrated xanthurenic acid (red trace) with some characteristic peaks indicated with dotted lines (sodium nitrite, xanthurenic acid and 7-nitro xanthurenic acid (yellow pigment))..... 137
- Figure 6-5. Chromatogram at 270nm of attempt at chemical synthesis of nitrated xanthurenic acid run on the semi-preparative column resulting in poor separation and a massive impure peak at 19.1 minutes. 137
- Figure 6-6. The chromatograms (at 270nm with normalized Y-axis) for the three different concentrations of sodium nitrite (0.1M, 0.02M and 0.005M) grouped together for the four different concentrations of hydrogen peroxide used (0.01M, 0.001M, 0.0001M and 0.00001M). The region that was affected by sodium nitrite is indicated. The four main peaks that varied outside this region are labelled (xanthurenic acid and unknown products 1, 2 and 3). 140
- Figure 6-7. UV spectrum of the three product peaks (1, 2 and 3) in the enzymatic nitration of xanthurenic acid. Product 2 had a similar UV spectrum as 7-nitro xanthurenic acid (yellow pigment) and whereas both other spectra were unidentified spectra. Peak maxima were labelled. 141

Figure 6-8. Chromatographic traces (270nm) of one sample (0.1M NaNO ₂ and 0.00001M H ₂ O ₂) after increasing time intervals during synthesis. Xanthurenic acid and the enzymatic synthesis products are labelled.....	144
Figure 6-9 shows the averaged chromatograms (n: 5, trace at 270nm, Y-axis: 0 to 1AU) for both pH conditions (pH 6.0 and pH 7.5) at the different time intervals (15sec, 1min, 5min, 10min and 15min). The effect of sodium nitrite was shaded and labelled. The six main peaks that varied outside this region (xanthurenic acid and six product peaks) were marked.	146
Figure 6-10. UV-spectra at the apex of the different product peaks in the chromatograms shown in Figure 6-9.	147
Figure 6-11 (on next page). Chromatographic data of injected xanthurenic acid/HRP reaction mixture showing (a) a 3D-representation of the isogram with the X-axis as retention time (min), Y-axis as absorbance (AU) and Z-axis as wavelength (200-800nm), (b) the 2D-isogram with “Product 2” (the putative yellow pigment) showing the UV-spectrum and Chromatogram (270nm) at this point, and (c) The spectral match of the UV-spectrum of the apex at 14.4 minutes (green) with the spectrum from 7-nitro xanthurenic acid (n:25, red) and the difference spectrum.	151
Figure 6-12. a) The preparative chromatogram (270nm, Y-axis: 0 to 2 AU) for the enzymatic synthesized dragline pigment. The effect of sodium nitrite was shaded and labelled. The products that were found back from the analytical separation were labelled, b) purified extracted yellow pigment of dragline silk (7-nitro xanthurenic acid), and c) the purified enzymatic synthesized Product 2 (the putative 7-nitro xanthurenic acid).	153
Figure 6-13. HSQC of the purified synthesized “Product 2” fraction (shown in purple) compared to that of 7-nitro xanthurenic acid (shown in red).	156
Figure 6-14. HMBC comparison between the purified synthesized “Product 2” (shown in purple) compared to that of 7-nitro xanthurenic acid (shown in red).	157
Figure 6-15. Nano-chromatograms obtained from the TOF- (a) and uTOF-system (b) showing the trace for 7-nitro xanthurenic acid (red trace) and the purified synthetic “Product 2” (purple trace). Note that the nano-chromatogram (b) of 7-nitro xanthurenic acid was run with a different method and therefore the retention time was not reliable in these chromatograms.	158

Figure 6-16. Showing the enlarged mass spectrum from the main mass at ± 251 from 7-nitro xanthurenic acid (1), Product 1 (2), Product 2 (3) and the predicted mass spectrum based on the structural formula, $C_{10}H_7N_2O_6$ (4).	160
Figure 6-17. The mass fragmentation patterns at low current (a and b) and at a higher current (c and d) of 7-nitro xanthurenic acid from the extracted dragline (red), and Peak 1 (green) and Peak 2 (blue) from the purified synthesized "Product 2". Raw data in Appendix A.11.	161
Figure 6-18. Enzymatic synthesis of 7-nitro xanthurenic acid via sodium nitrite and hydrogen peroxide in vitro and probably also in vivo.	163
Figure 7-1. The metabolic intermediates produced via the tryptophan breakdown pathway, with tryptophan circled in green and the two small molecules of interest circled in red. ¹⁵⁷⁻¹⁵⁸	175
Figure 7-2. Figure exactly as in Figure 1-4, with the findings of this thesis added (in red) and its implications in silk processing and handling.	181

List of Tables

Table 2-1. The weight and mass density of the <i>G. postica</i> cocoon discs of the untreated cocoons (A) versus the effect of demineralization (B) and combined demineralization and degumming (C). Each box shows the mean, standard deviation and two tailed probability (ANOVA).	36
Table 2-2. Reeling data of five different <i>G. postica</i> cocoons. Measures of the cocoon (width, height and volume), number of breaks during reeling, total length of silk reeled and a visual estimate of the completeness of the reeling are tabulated for each cocoon. The pictures show the cocoon before and after reeling and the amount of reeled silk (white scale bar = 1cm).	43
Table 2-3. The effect of various treatments on the tensile properties of <i>G. postica</i> fibers: (A) demineralized and reeled; (B) demineralized, reeled and sodium carbonate degummed; (C) fibres produced by degumming with pineapple juice, carded, hand spun and unravelled. Also showing, a recalculation of the tensile properties demineralized, reeled and sodium carbonate degummed <i>G. postica</i> fibres based on the cross section of pineapple degummed fibres (see text). Each box shows the mean (top left), standard deviation (bottom left) and two tailed probability (bottom right, ANOVA).	47
Table 3-1: Reeling data of four different <i>G. postica</i> cocoons is shown. Both the total length of silk reeled from each cocoon and a visual estimate of the completeness of the reeling are tabulated.	64
Table 3-2: The effect of demineralisation, combined demineralisation and degumming according to the present method and treatment with pineapple juice according to the prior art on the tensile properties. Each box shows the mean, standard deviation and one tailed probability (ANOVA).	66
Table 5-1. The direct coupling of the ^1H to the ^{13}C was listed from the HSQC together with the long range coupling of the ^1H with the ^{13}C -carbons from the HMBC for the purified extracted dragline pigment.	101
Table 5-2. Comparison and difference between the carbon and proton shifts of the extracted purified dragline pigment and egg sac pigment. The most important and characteristic shifts for the dragline pigment are indicated in yellow.	103
Table 5-3. The direct coupling of the ^1H proton to the ^{13}C carbons were listed from the HSQC together with the long range coupling of the ^1H proton with the ^{13}C carbons from the HMBC for xanthurenic acid (Sigma).	106

Table 5-4. Comparison and difference between the carbon and proton shifts of the extracted purified dragline pigment and xanthurenic acid. The major differences between the two compounds are highlighted in yellow.....	107
Table 5-5. Predicted structural formulas for the measured accurate mass of the yellow pigment (251.0303).....	110
Table 5-6. All mass fractions obtained from the fragmentation of the dragline pigment in MS were listed and indexed (A till G) together with the mass difference from the ion fractions with the yellow pigment. For all ions the possible structural formulas were calculated and the most likely ion was tabulated next to it.....	113
Table 5-7. Mass loss obtained from the difference between the ions at 177, 176 and 175 with masses 251, 223 and 205 respectively, indicating the fragmentation of the nitro-group.	116
Table 6-1. The averaged peak areas (n: 3, at 270nm) of the four main peaks (xanthurenic acid and three products (1, 2 and 3)) were tabulated for each sample. Peaks were listed in order of elution time.	142
Table 6-2. The averaged peak areas (n:3, at 270nm) of the seven main peaks (xanthurenic acid and six products (1 to 6)) were tabulated for both pH conditions, pH6.0 and 7.5, for the different time intervals (15sec, 1min, 5min, 10min and 15min). Peaks were listed in order of elution time.	148

Chapter 1

Introduction

Abstract and motivation

Silkmoth silks as well as spider silks are getting increasingly more attention over the years as a possible “biopolymer” in applications ranging from biomaterials to food sensors. New applications for silk are found on a daily basis, however the fundamental basics of silk are still badly understood and in particular the different types of bonding between and within peptides, and the interaction with small molecules. The aim of this thesis is to provide a better understanding of the interactions between silks and small molecules together with their possible implications. Therefore, this chapter will give a brief review of the biological significance of silk and the many different types of bonding occurring in silk, and between peptides and small molecules. Furthermore the significance of these types of bonding on the processing of silks was discussed for the use in these applications. In the following chapters we will find out that one of the main variability’s in silk reeling and toxicity was due to the mineralisation of the cocoon shell in Wild Silkmoths and we will identify a new polyphenol present in dragline and egg sac silks and the synthesis thereof. The implications of these new findings are then discussed in terms of bonding types in the last chapter and linked back to this chapter to understand the implications on the applications and variability in silks.

1.1. Silk

Silks can generally be regarded as a group of structural proteins that are secreted by some Arthropods in which the stored protein solution in the gland is converted into a solid by a pultrusion process generally known as “spinning”¹. They are produced by some Insecta, Arachnida, Myriapoda and Crustacea where they perform different functions²⁻⁵. The two main groups of silk producers however are the Lepidoptera and the Arachnida¹. The best known

silk is of course the silk used in textiles obtained from the silk cocoon of the silkmoth *Bombyx mori*. This species has been cultivated for over 4000 years and this resulted in the great Silk Road between Asia and Europe⁶. Besides *B. mori* there are many other silkmoth species that also produce large cocoons in which to pupate. These species are usually referred to as Wild Silkmoths because they are not domesticated or used commercially, with a few exceptions such as *Antheraea pernyi*, *Antheraea mylitta* and *Antheraea assamensis* in which the cocoons are gathered from the wild^{7, 8}. The largest and best known group of silk producers next to the Lepidoptera are the Arachnida. In contrast to the Insecta and Myriapoda (one silk for one purpose), individuals of the Arachnida can have up to seven different types of silk^{9, 10} that can be used for many different purposes (see Figure 1-1) like: shelter, web, egg sac, ballooning, dragline, etc.^{11, 12}.

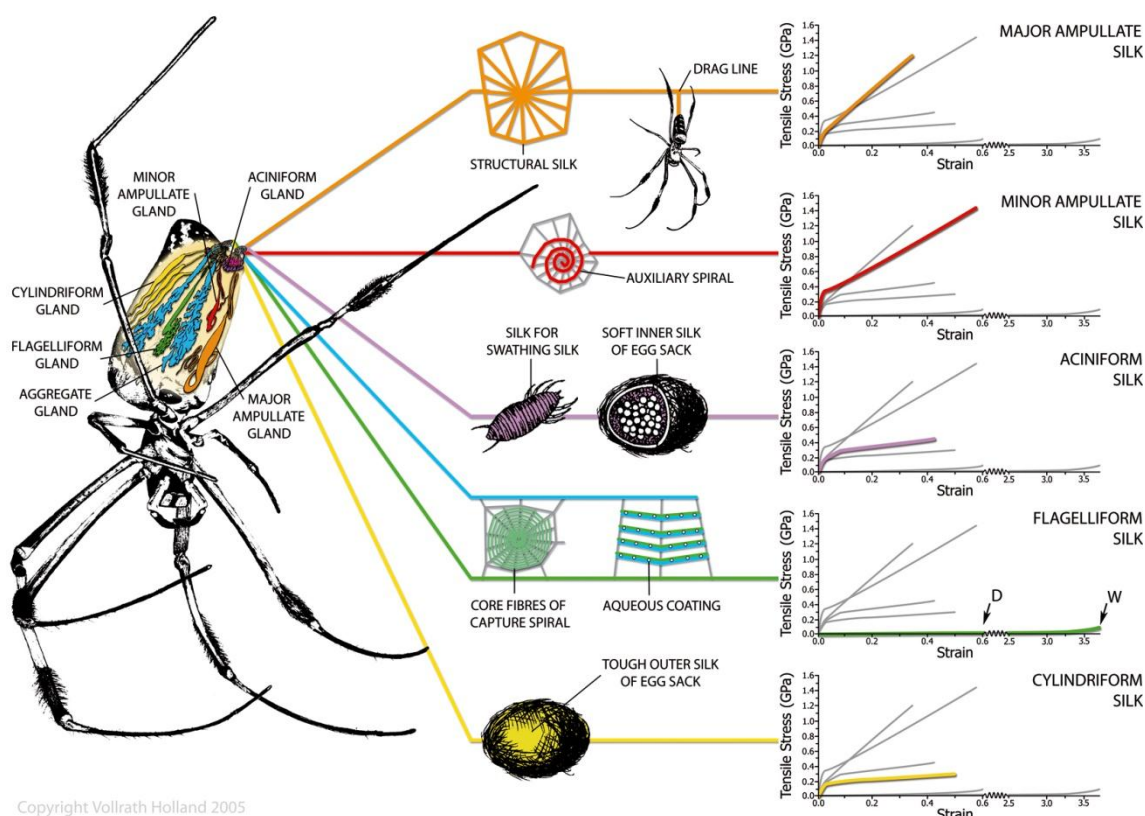


Figure 1-1. The glandular origin, function, and material properties of the different silks of the golden orb weaving *Nephila* species. Note the wide variation of mechanical properties each optimised for a specific use (taken from¹³).

1.2. Composition of silk

The difference in silk types originates in the primary structure (amino acid sequence) of the main structural proteins making up these fibres¹⁴. These proteins were named fibroins in silkmoth silks and spidroin in spider silks. These primary structures result in diverse secondary structures and ultimately in different tertiary structures in silk proteins. The resulting folded proteins making up the fibre help to determine the mechanical properties of these fibres¹⁴⁻¹⁶.

Silks are known for their high tensile toughness which can be defined as the energy (or work) required to break the fibre in tension and originates from a combination of high breaking stress and high breaking strain, though other sorts of toughness exist including compressive and shearing toughness. Researchers are now agreed that the high breaking stress of the silk fibres is attributed to the crystalline regions in the fibre, whereas the elasticity of the fibre comes from the amorphous regions in the fibre¹⁷⁻¹⁹. The crystalline regions in spider and silkmoth silks are made up of beta-sheet structures which consist of multiple repeats of alanine, glycine-alanine or glycine-alanine-serine^{20, 21}.

The amorphous regions in silk are usually made up from hairpin regions containing β -turns, and helical structures made up of GGX and sometimes GPGXX repeats. The non-repetitive regions in the N- and C-termini of the proteins might play a role in the controlled stress-induced aggregation of the silk proteins²²⁻²⁵.

It is now clear that a different primary sequence will lead to a different secondary and tertiary structure which will in turn lead to a different organisation and composition of crystalline and amorphous regions, resulting in a vast range of mechanical properties of fibres. Although these structures do help to explain the mechanical properties, the underlying stabilizing forces

necessary to organize and maintain these structures are as vitally important in obtaining and maintaining these properties. At each polypeptide link along the molecular backbone there is a $>C=O$ and $>N-H$ that is involved in linking the amino acids of the chain and sometimes in hydrogen bonding between chains or segments of chains whereas the side chains can be positively (Arg, His and Lys) or negatively (Asp and Glu) electrically charged, polar uncharged (Ser, Thr, Asn and Gln), hydrophobic (Ala, Ile, Leu, Met, Phe, Trp, Tyr and Val) and some special cases (Cys, Gly and Pro)¹⁶.

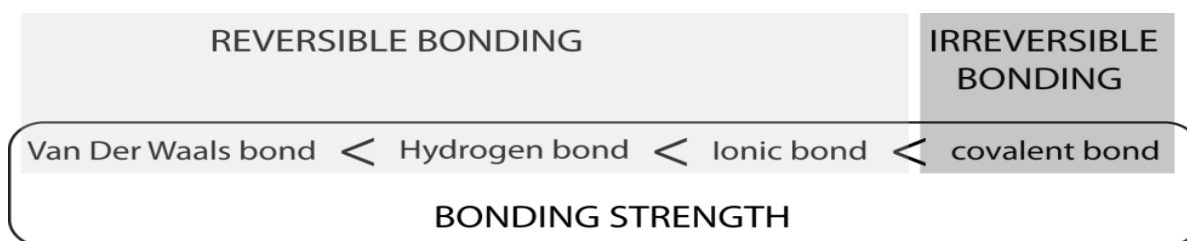


Figure 1-2. Types of molecular bonding occurring in silks and their relative strength. Whereas most interactions are reversible, covalent bonding is irreversible having vast implications in silk processing²⁶⁻²⁸.

The main interactions of the backbone and side groups within the same protein or with other proteins, other molecules or its environment can be roughly divided in four types of bonding²⁷⁻²⁹.

- Van Der Waals attractive forces
- Hydrogen bonding
- Ionic bonding (electro-valent bonding)
- Covalent bonding

A. Van Der Waals forces

The Van Der Waals force is the sum of weak temporarily attractive or repulsive forces between atoms or non-polar molecules caused by their dipoles over short periods of time. The interaction can happen between two permanent dipoles (Keesom force), between a permanent dipole and an induced dipole (Debye force) and between two instantaneously induced dipoles (London dispersion force)³⁰.

In silks this force is thought to be less important than the other forces like hydrogen bonding, electrostatic interaction and covalent bonding (Figure 1-2). However it is believed that these hydrophobic and hydrophilic forces occur in the interaction between the side chains in the crystalline and amorphous regions of the silk peptides^{31, 32} and would assist the assembly of the large spidroin peptide³³. This formation is thought to exist in an intermediate liquid crystalline state during spinning³⁴.

At a macroscopic level these forces are responsible for the silk adhering to the surface in the form of attachment discs of spiders which originate from the piriform glands³⁵. In silkworm silks this is probably also happening, in which the silk fibre is less crystalline in some regions where it is attached to a surface (personal communication David Knight).

B. Ionic bond (electrovalent linkages)

An ionic bond is an electrostatic attraction between a cation, usually a metal (Ca^{2+} , Na^+ , K^+ or MG^{2+}), and an anion, usually a non-metal (O^{2-} , N^{3-} and Cl^-) or polyatomic ion (NH_4^+) and is stronger than Van Der Waals forces (Figure 1-2). In proteins, ionic binding within the peptide is expected to happen between adjacent $-\text{NH}_3^+$ and $-\text{COO}^-$ side chains like in Lysine, Arginine, Aspartic acid and Glutamic acid however these are probably less important than the interaction of the silk peptides with its environment, like with small molecules and salts³⁶.

C. Hydrogen bonds

The hydrogen bond is an attractive interaction between a hydrogen atom from a molecule or a molecular fragment X–H in which X is more electronegative (typically O) than H, and an atom or a group of atoms in the same or a different molecule, in which there is evidence of a bond formation³⁷.

These interactions are weaker than ionic and covalent bonding (Figure 1-2) but are believed to be the most important binding forces in proteins, present in huge numbers, contributing to their exceptional toughness and as vitally important for the folding of the peptide. Many possible hydrogen bonds can be formed intra-molecular, inter-molecular or with molecules in the environment the peptide is in (water, solvent, small molecules), and are thought to be vitally important in the folding of the protein in an aqueous environment^{38, 39}. Hydrogen bonds are most commonly formed with and between carboxylic acid groups, carboxylate groups, hydroxyl groups, carbonyl groups and hydrogen²⁹.

The β -sheet crystals in silk are stabilised by intra-sheet hydrogen bonds which occur between the amide hydrogen's and carbonyl oxygen's on the adjacent or same peptide chains³².

The strength of the hydrogen bond is considerably dependent on the ionic content of the environment the peptide is in. So silk, which is mainly held together by these forces, will only maintain constant properties in a constant environment if not stabilized by covalent bonds²⁹.

A special form of hydrogen bonding is the water cathrates by which ordered water hydrogen bonds encapsulate a hydrophobic molecule, like silk, that does not contribute to

the hydrogen bonded structure. This could explain why such a hydrophobic molecule like silk is soluble in water⁴⁰.

D. Covalent bonding

Covalent bonding is characterized by the sharing of pairs of electrons between atoms and other covalent bonds. These bonds are stronger than Van Der Waals interactions, ionic or hydrogen bonds and include pi-bonding, sigma bonding, metal-metal bonding, etc.

In proteins, covalent bonding is best known as disulphide bonding between cysteine residues (Figure 1-3, a). In the silk of *B. mori* this covalent bonding happens intramolecularly between the cysteine residues of the L (light) and H (heavy) chain of the fibroin and is vital for the spinning of the fibre^{41, 42}. This same N-terminal, and possible C-terminal, assembly of dimers may also occur in Wild silks²¹. Although cysteine residues are present in the spidroin of spider silks, there is no evidence that they form cysteine cross-links⁴³.

Another common covalent bonding in silkmoth silks was the dityrosine cross-linking (Figure 1-3, b). This bonding type was found to occur in Wild Silkmoth species but was not found in *Bombyx mori*⁴⁴, possibly explaining the extensive use of *Bombyx mori* in the silk industry and research (see further). In spider silks however this type of bonding is absent and so cannot contribute to spider silks' superior strength³³.

Besides the stabilization of proteins by intra inter-peptide covalent bonding, proteins can also be stabilized by small molecules like phenols or polyphenols, which are able to hydrogen bond in specific ways to the peptide or can even cross-link themselves with the proteins making strong covalent bonds, a process known as tanning^{42, 45-48} (Figure 1-3, c). This type of bonding has significant effects on the stability of the proteins which is reflected in the

peptides properties e.g. higher mechanical strength, insolubility, resistance to degradation^{29, 42, 45, 46, 49}.

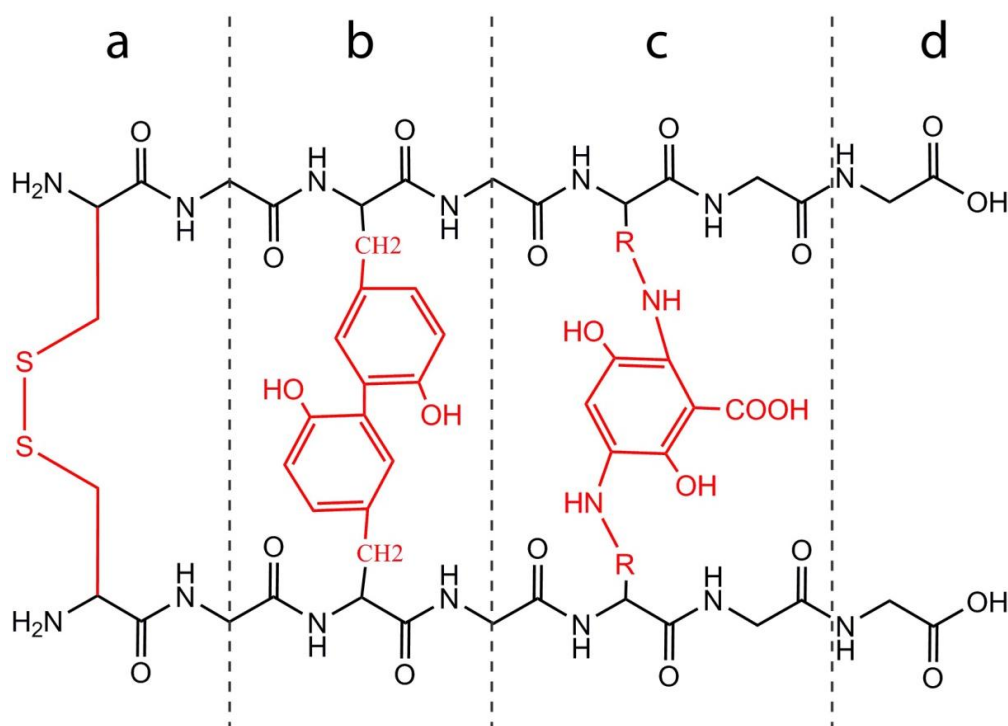


Figure 1-3. Different covalent bonding types occurring in silk assembled from references^{29, 42, 46, 49}. a) Di-sulphide cross-linking between two cystinyl residues of two adjacent peptides. b) Dityrosine cross-linking between two adjacent tyrosyl residues. c) An example of a cross linking between peptides by a polyphenol, in this case gentisic acid, as a cross-linker between two amino acids of two different peptides. d) No covalent cross-linking between peptides.

Although the forces mentioned above are important in the formation of the silk fibre, they also play a vital role in the composites made by silkworms and spiders. While spider webs are made up of different types of silk, the mechanics of the web as a whole is rather complex⁵⁰. In contrast, the non-woven silk fibres in the silkmooths cocoon are held together by sericin and the mechanism for the loss of stiffness in a cocoon can be seen as a gradual loss of connectivity of the sericin bonding between load bearing silk fibres⁵¹. Wild silkmooths, in contrast with *B. mori*, coat their cocoon with a watery secretion from the Malpighian tubules which then solidifies on drying, making the cocoon exceptionally more rigid than *B. mori*⁵²⁻⁵⁴.

Evidence is presented in Chapter 2 of this thesis that calcium oxalate mineral, crystallising out and bonded between the sericin coatings of the silk fibres on drying, could function as a filler particle in the outer shell of the cocoon. This could increase the modulus and in addition toughen the outer shell of wild silk cocoons by increasing the work required for crack propagation as in other systems⁵⁵. Thus, mineralisation with calcium oxalate in wild silkworms may augment the role of the sericin in toughening the matrix by crazing as previously suggested for the cocoon of *B. mori*⁵⁶.

1.3. Applications of silk

Because silks are made up of proteins they have huge advantages over man-made polymers in that they are bio-compatible⁵⁷, biodegradable⁵⁸, have excellent mechanical properties^{1, 59} and have a low and in some cases negative carbon footprint (personal communications R. Carter). Silk has a long history of being used in its purest form as a fibre in textiles and as sutures. However, when silk is still in the liquid state before it is spun, it can be used like any other polymer by which it can be processed differently⁶⁰. This liquid silk, in its native state can be obtained straight from the silk gland by means of dissection⁶¹. Because dissecting silk worms takes a lot of time and relatively small quantities are stored in each gland, the amount of silk obtained is small and its collection must be timed before the silkworm has started to spin making this method not viable for obtaining silk polymer solution commercially.

An alternative approach is the use of recombinant DNA technology to express silk spidroin proteins in a variety of organisms like yeast⁶², bacteria⁶³, insect cells⁶⁴, potato and tobacco plants⁶⁵, goats (Nexia) and even silkworms⁶⁶. Although most of these organisms are capable of expressing silk protein analogues, the processes for extracting them from the recombinant organism or its secretions, using solvents and other techniques damage the protein. This

probably contributes to the very poor quality fibre produced by artificial extrusion of recombinant silk protein analogues⁶³. Another, and more widely used, approach is dissolving the silk fibres back to their “native” liquid state by means of salts (like LiBr, LiSCN, etc.), a process known as “reconstituting” silk⁶⁰. However, these obtained reconstituted silk solutions do not have the same properties as the native silk dope in the spider or silk gland, and is thought to be caused by breakup of the long silk proteins into smaller fragments^{61, 67}. Also, re-establishing the original folding of the protein and the correct inter- and intra-molecular bonding as in the gland, after solubilising the silk is proven extremely difficult⁶⁸.

Nonetheless, reconstituted silk has been used in many different applications. From its liquid form, reconstituted silk can be processed in many different ways to form fibres, films, hydrogels, sponges, films, micelles, etc.^{26, 60} with potential for bone matrixes⁶⁹, cartilage scaffolds⁷⁰, neural conduits⁷¹, sutures⁷², optical wires⁷², food sensors⁷³, drug delivery systems^{73, 74}, etc.

Despite the inferior mechanical properties of the silk of *B. mori*, it is by far the most used for making reconstituted silk solutions because this silk is produced commercially in large quantities and is the only silkworm so far known to be capable of being dissolved under relatively mild conditions. In contrast, spider silks are more difficult to handle and obtain in useable quantities, however their properties are the gold standard for the silk research as they exhibit exceptional mechanical properties⁷⁵.

1.4. Problem with the industrial exploitation of silks: silks' variability

The problem in using silks lies in the huge variability and inconsistency in their properties⁷⁶. While some silks have useful mechanical properties but are very difficult to reel, degum, dissolve and are cytotoxic, others are easy to reel, degum, dissolve, are not cytotoxic but are weak making it extremely difficult for use in silk based materials⁴⁹. This variability is not only inter specific but also intra specific and can even change within silk of the same spider or cocoon^{47, 76} leading to differences in mechanical properties in fibres and to differences in the ease in which they can be dissolved to form “reconstituted” silk solutions⁴⁷. Much of the inter-specific variation can be attributed to the difference in the primary silk fibroin sequences between species. However, the intra specific variation and even the variation at an individual basis, points to variations that are introduced either at the silk spinning stage or post spinning which ultimately come down to the stabilizing bonding in silk⁷⁷ illustrated in Figure 1-4.

Reeling speed^{78, 79}, body temperature⁷⁸, pH^{80, 81}, ion concentrations^{81, 82} and water removal⁸³ in the natural spinning process are likely to influence the Van Der Waals, ionic and hydrogen bonding in silk proteins and will in this way contribute in the variation of their mechanical properties⁸⁴. One of the largest factors in variability in silks rises from the humidity at which they are extruded and reeled. This varies the extent to which spider silks super-contract⁸⁵ and silkmoth silks swell resulting in differences in the mechanical properties of the silk.

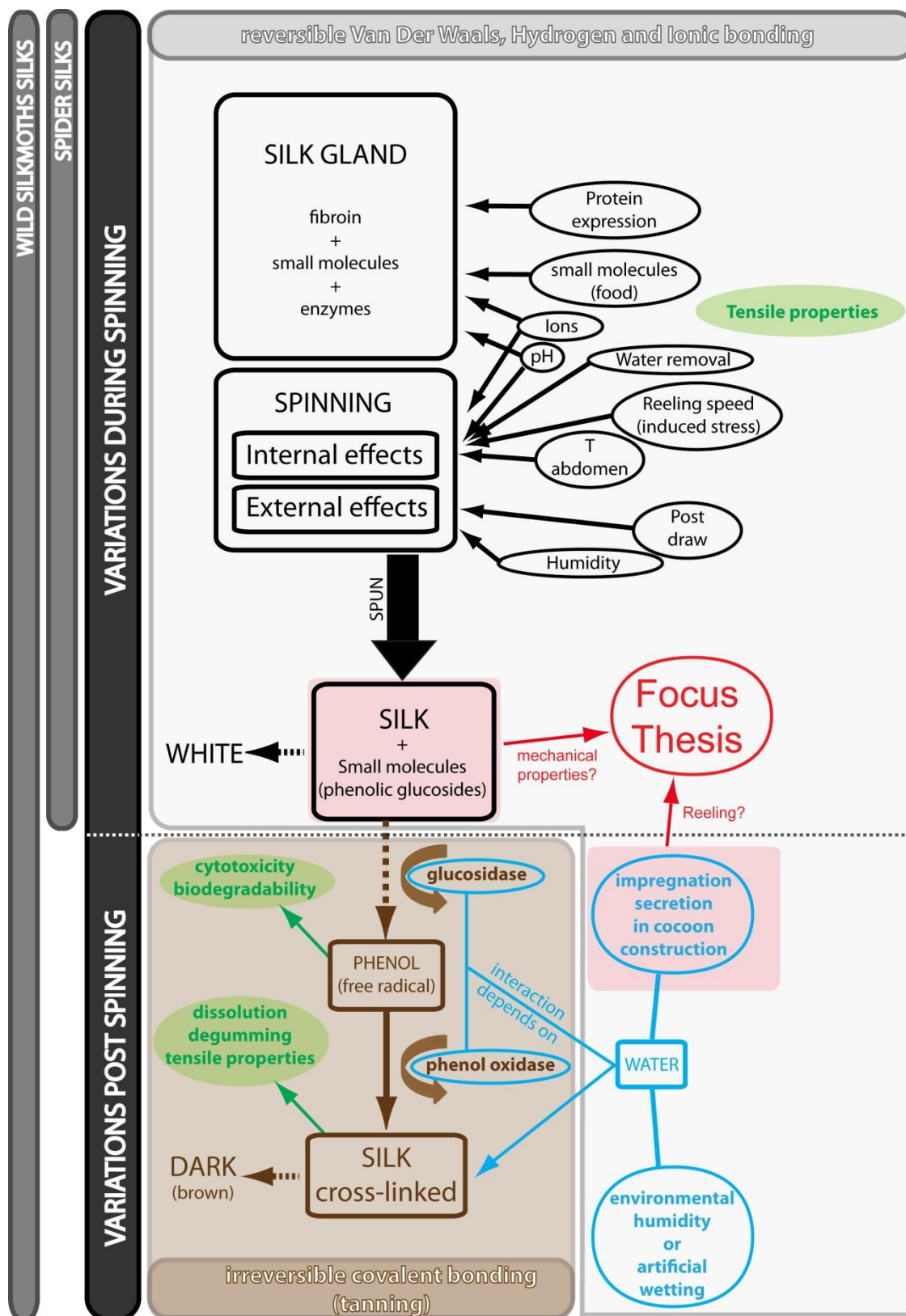


Figure 1-4. Schematic drawing of relations and variations introduced in silkmoth and spider silks during spinning and post-spinning. The grey box represents the weak reversible interactions whereas the brown box represents the covalent bonding process. The colour code for the reactions is as follows: black for the factors influencing spinning, brown for the factors involved in tanning, blue for the importance of water and red indicating the focus of this thesis.

As most silks are stabilized by weak forces (non-covalent bonding) in the natural spinning process it is very unlikely these factors contribute to the variation in dissolution and reeling seen in silks, mainly silkmoth silks. It is also unlikely that variations in the primary structure of silk proteins will have a direct effect on these two properties. However, proteins are not the only molecules present in silk fibres. Small molecules, in the form of tanning agents⁴⁷ and pigments⁸⁶⁻⁸⁸, have already been characterized and found in many different silk species and have been related to the relative insolubility of some silks in chaotropic agents⁴⁷. Nonetheless, they may very well influence the biocompatibility of silks.

1.4.1. Silkmoth silks

Whereas pure secreted silk proteins are white, like the silk of *B. mori*, the silk of Wild Silkmoth cocoons come in a wide range of different colours; green, yellow, orange, brown, black, grey, etc. Some of these colours are caused by pigments, like the green colour in *Antheraea yamamai*⁸⁹ and the yellow in the yellow strain of *B. mori*⁸⁷. Brunet⁴⁷ however noticed that most coloured silks were actually pure white when freshly spun and only turn dark after a period of time (Figure 1-4, brown). He found that this was due to tanning of the silk, which in contrast to pigmentation is a covalent bonding between silk peptides and small molecules (Figure 1-3). It was found that silks do not solely consist of fibroin and sericin but also contain enzymes and phenols⁴⁷. Two different enzymes are thought to play a part in oxidative phenolic tanning; a beta-glucosidase that cleaves a low molecular weight phenolic glycoside to unmask the tanning agent, and a phenol oxidase, that forms covalent inter-chain cross-links in the fibroin derived from the phenolic tanning agent (Figure 1-4, brown)⁴⁷. Two phenolic compounds were found namely the O-glucoside of 3-hydroxyanthranilic acid and the 5-O-glucoside of gentisic acid (dihydroxy benzoic acid) in different concentration in *A. pernyi*, *Samia cynthia*, *Hyalophora cecropia*, *Actias selene*.

Whereas the 3-hydroxyanthranilic acid glucoside is derived from dietary tryptophan, gentisic acid is being taken up from the food-plant as a conjugate, with the phenolic moiety being used in the silk gland. The phenolic molecules could themselves link two peptides together (Figure 1-3, c) or two tyrosyl residues of the peptides could be linked together by the enzymes, known as dityrosine cross-linking (Figure 1-3, b).

This tanning reaction is greatly dependent on the moisture content of the fibres allowing the enzymes and phenols to interact⁴⁷ (Figure 1-4, blue). Because most Wild Silkmoths live in pretty dry environments⁹⁰ this wetting is never assured by the humidity in the air, therefore I think they impregnate their entire cocoon with a watery secretion from the anus to guarantee the post spinning processes. This impregnating substance does not only contain water but also chalky-white crystals and is excreted exactly when the outer layer of the cocoon is finished^{52, 53}. When this secretion dries the cocoon gradually turns white-brown transforming the cocoon shell into a stiff shell filled with white crystals^{52, 53}. These crystals are thought to be calcium oxalate and have been found in other Wild Silkmoths^{52, 53, 91}. Yet except for these sporadic observations of the presence of calcium oxalate in the cocoon, nobody has actually looked at how this mineral could stabilize the cocoon shell. Wild Silkmoths in nature seem to use two main mechanisms for reinforcing their cocoons, namely tanning and mineralisation. In this thesis we will show that the mineralization of most Wild Silkmoth cocoons prevents cocoons from being reeled (Chapter 2 and Chapter 3) and that the main difference between the cocoons of *B. mori* and Wild Silkmoths was due to a behavioural difference involved with this mineralisation (Chapter 4).

It has been shown that dark fibres are generally more difficult to dissolve than white fibres as a result of this cross-linking of the fibroin with tanning agents ⁴⁷. Also an increase in mechanical strength of silk fibres before and after natural cross-linking tannins in silk has been noticed by Brunet⁴⁷ which will result in a lowered biodegradation and a pronounced cytotoxic effect. The increase in cytotoxicity could be due to the formation of a free radical (phenol) when splicing off the glucoside moiety of the molecule (Figure 1-4, green).

A. Relevance

Whereas most research of today is focussed on the structure-property relationships of silk, to understand the conversion of a liquid silk into a solid fibre and the reverse (dissolving fibres back to the “native” solution, reconstituting silk), researchers tend to forget that they are actually working on a completely variable starting product (the cocoon) and that they need to control for the cocoon’s history as well, e.g. food, spinning and storage of the cocoon’s.

The variations introduced in the cocoon silks may depend on many factors:

- different up take of phenolic compounds through the food (different compounds or amounts)
- different concentrations of phenolic agents and enzymes together with different timings of interaction
- amount of water in the cocoon and duration of the wetting that induces the tanning
- content beside water of the secretion fluid of the Malpighian tubules in the impregnation behaviour
- interaction between tanning and exudate of the Malpighian tubules in impregnation behaviour

If these factors are not controlled for, a large variation will be introduced in generated covalent and ionic bonds in the silk fibres or resulting cocoons what explains the huge variation in:

- reeling these cocoons
- making reconstituting silks
- mechanical properties of the fibres
- biodegradability
- biocompatibility/cytotoxicity

As this variation is also reflected in the literature, a better understanding of the interactions of these small molecules with the silk and cocoon will not only lead to a better control of this variation but also in less variable results of the research build on these silks, ranging from applications (biomaterials) to the structure-property relation studies. Moreover, it will enable people to select silks for given applications and to tune the properties of these silks as desired.

B. Aim

One of the aims of this thesis is to understand the implications of the last variation introduced in silkworm cocoons, namely the impregnation fluid that the silkworm uses to coat their cocoons, on the properties of the silk and the cocoon as a whole (Figure 1-4, red). Until today, no attention has been given to this fluid and its possible implications on tensile properties, cytotoxicity, reel ability and solubility.

1.4.2. Spider silks

In contrast to silkmoth silks, most spider silk types are recycled by the spider (e.g. major-ampullate, minor-ampullate, viscid and flagelliform silk) and therefore cross-linking of these silks is avoided⁹². Although no proof has been found of spider silk cross-linking³³, many small molecules have been found in spider silks (Figure 1-4, grey box (small molecules)). The largest variety has been found in the sticky spiral thread in spider webs, for example in *Araneus diadematus*, *Araneus cavaticus*, *Argiope aurantia* and *Argiope trifasciata*: GABamide (gamma-aminobutyramide), N-acetyl-aurine, choline, betaine, isethionic acid, Cysteic acid, lysine, serine, potassium nitrate, potassium dihydrogenphosphate, pyrrolidone, Glycoproteins and fatty acids^{93, 94}. These small molecules are thought to play a role in keeping these fibres wet and sticky so they keep their function, which is catching prey in dry environments.

In the other silk types this abundance of compounds is far less. In the major ampullate and tubuliform/cylindriform silk of *Nephila* only a yellow pigment is found in large amounts^{11, 95}. The yellow pigment extraction was found to consist of three substances namely xanthurenic acid (in low amounts), a hydroxylated benzoquinone or naphthoquinone (yellow), and another unstable unknown yellow molecule in larger quantities⁸⁶. These types of substances are thought to have anti-feedant properties⁹⁶ or antibacterial effects like in gonyleptidine⁹⁷ however the true function of these pigments remained unknown.

A. Relevance

Although no cross-linking occurs in the dragline silk of the genus *Nephila*, this silk is still stronger than any silkmoth silk and one of the toughest materials known on earth¹. Small molecules found in dragline and egg sac silk of *N. edulis* are from the same family as polyphenolic compounds found in the cross linking process of silkmoth silks^{47, 86}, however to

date no enzymes, beta-glucosidase and phenol oxidase, were found in these silks like in Wild Silkmoth silks. Because covalent cross-linking of phenols is one of the least common reactions in nature⁴⁵, it is believed that these molecules play a role in the folding and strength of these silk fibres, by forming many weak interactions (Figure 1-4, grey) within or between peptide chains.

B. Aim

The second aim of this thesis was to identify the yellow pigment substance in the extracted dragline and egg sac silk to enable us to make further hypothesis about its possible role in protein folding, cytotoxicity, tensile properties (Figure 1-4, red). The initial aim of this thesis was to understand the function of the pigment but due to the low abundance of the extracted yellow pigment and the difficulty of identifying this compound, this thesis will focus on the identification and synthesis of this compound for future research and applications.

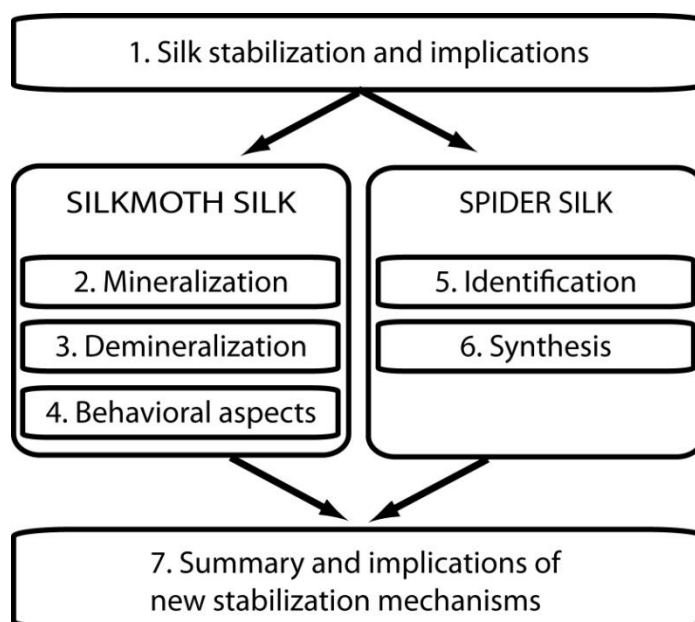


Figure 1-5. Flowchart of the outline of this thesis.

1.5. Thesis outline and motivation

The main focus of this thesis is, in contrast to most other research, not on silk proteins but on the small molecules interacting with these silk proteins. In particular, we look at the composition and implications of the impregnation fluid used in cocoons of Wild Silkmoths and at the significance of a yellow pigment found in the toughest dragline and egg sac silk in spiders (Figure 1-4, red). A flow diagram of this thesis is given in Figure 1-5.

The thesis consists of seven chapters that can be subdivided in two main parts, whereas the first part comprises of Chapters 2, 3 and 4 dealing with small molecules in silkworm silks, and the second part of the thesis consists of Chapters 5 and 6 dealing with small molecules in spider silks. This body is then prefaced with a general introduction (Chapter 1) and bundled by a summary and future work (Chapter 7) (Figure 1-5).

Chapter 1 gives a general introduction in the relationship between silks and small molecules and the motivation for the study, namely their implications on the variability in silks.

The first part deals with small molecules in Wild Silkmoth silks; however, due to biological reasons (the sericulture of Wild Silkmoths ended abruptly by a virus) we were not able to investigate the tanning process in more detail for silkworms (Figure 1-4, brown). Nonetheless, we think we addressed the second largest, if not the largest, variation introduced in wild silkworm cocoons, namely the viscous-liquid secretion used in the impregnation behaviour, as it is our belief that this together with the tanning process accounts for the main difference in silks between *B. mori* and Wild Silkmoths. In Chapter 2, the composition of the impregnation fluid was identified and a method had been developed for its selective removal, which enabled us to reel Wild Silkmoths for the very first time. This was one of the main findings of this thesis and had a global impact that was reflected in the media (Appendix A.1). Chapter 3 is

closely linked to Chapter 2 in that it is a commercial reformulation of the findings in Chapter 2 in the form of a filed patent application. Because this thesis was funded by a EPSRC-BBSRC-CASE studentship, the industrial applications of these findings were relevant. Furthermore, Chapter 4 deals with the behavioural aspects and differences of the impregnation phase in the cocoon building of Wild Silkmoths compared to that of the outlier, *B. mori*, which had been under extensive selective pressure for the ease of reeling these cocoons. In this way a very clear picture was obtained of the significance of mineralizing cocoons in light of the biological and industrial implications of the use of these silks.

The second part deals with the yellow pigment molecule found in spider silks, namely dragline and egg sac silk. Whereas all other major-ampullate silks in Araneida are colourless, the dragline silks of the *Nephila* species are golden yellow in colour. Because this coloration makes the web entirely visible for its prey and would be in this way a disadvantage to the spider, it was thought this pigmentation might play a role in the protein folding and ultimately in the tensile properties of the silk. Therefore, in Chapter 5 the yellow molecule was identified which made it possible to formulate further hypotheses about its possible function in the dragline silk and egg sac silk. This entirely new compound consisted of a very unusual substituted group and let us to suspect that it was produced enzymatically *in vivo* by the spider. This possible enzymatic process was tested *in vitro* and is discussed in Chapter 6. The identification of this compound and synthesis pathway thereof is so unusual that it puts silk and its synthesis in an new light.

Chapter 7, summarizes the findings of this thesis and addresses the newly discovered stabilizing molecules used in silk previously discussed in Chapter 1, to understand the widespread practical and biological implications of the findings in this thesis on the variation in silks.

Chapter 2

Mineralisation of Wild Silkmoth cocoons and a technique for its selective removal

Abstract and motivation

Until now most applications based on silk are commonly made up of silks of the silkmoth, *B. mori*. This is because Wild silk cocoons are much more difficult to get, handle, reel and dissolve and this is generally thought to be caused by tanning⁴⁹. However, the impregnation fluid used for tanning contains some filler particles that make the cocoon shell exceptionally tough^{52, 53}. The aim of this Chapter was to characterize the composition of the impregnation fluid and determine its implications on the properties of the silk and cocoon. Therefore the mineral was identified in a range of Wild Silkmoth species, and there after a method was constructed for the selective removal of this mineral. The mineral was identified as calcium oxalate monohydrate which is a toxic compound and could be selectively removed by an EDTA treatment. Consequently, we were able to determine the effect of mineralisation of the cocoon on reeling and discovered that cocoons could be reeled after the removal of this mineral layer. This finding was important for the use of these silks in biomedical applications and will also broaden the supply of different types of silk for future applications, discussed in Chapter 1.4.1.

The enormous commercial impact of these findings was clear because wild silks are abundant worldwide and therefore could stimulate the silk industry worldwide, whereas until now it was limited to China and India. The commercial impact of these findings was written as a filed patent application in Chapter 3. Furthermore, the difference in cocoons between *B. mori* and Wild Silkmoths will be explained according to this mineralisation phenomenon in Chapter 4. Finally, Chapter 2, 3 and 4 will be taken together in Chapter 7 for the general discussion.

2.1. Introduction

Cocoons of the Mulberry silkworm *Bombyx mori* are relatively easy to soften and reel, in comparison with most other commercially attractive silkmths⁹⁸. Indeed, perhaps this feature has led to the predominance of *B. mori* among natural silks or alternatively it is the outcome of 6000 years of selection by man for a silk with high commercial potential. After all, softening and reeling that are both easy to administer and gentle on the fiber are key to the material and its usability⁹⁹.

Typically, *B. mori* cocoons are softened by submersion in hot water. This treatment swells and partly dissolves the sericin gum, which coats and cements the fibroin filaments (brins) together in the cocoon. In contrast to the cocoons of *B. mori*, the cocoons of most other traditionally called ‘wild’ silkworm species are often heavily mineralized with calcium oxalate^{91, 100, 101}. Moreover, sometimes such ‘wild’ cocoons are additionally stabilized by oxidative phenolic tanning, dityrosine cross-linking and/or tannins derived from the caterpillar’s food plant^{44, 49}. Mineralization is probably more important than the tanning in making wild silk cocoons difficult to soften and reel.

Traditionally, two degumming approaches have been attempted to overcome this set of problems. The cocoons of Saturnid Wild Silkmths can generally be softened and degummed by boiling in concentrated sodium carbonate solutions for hours with or without the addition of soap¹⁰². However even with this relatively harsh treatment the wet reeling method used for Mulberry silk is not possible, necessitating the use of greater force obtained by dry or semi-dry reeling¹⁰³. The cocoons of several non-Saturnid wild silkworms including the *Gonometa spp.* are even more resistant to softening and require prolonged retting or treatment with pineapple extracts containing bromelain to remove the sericin¹⁰⁴.

Even with this treatment they still cannot be reeled and must be carded to be spun into a short staple yarn. Both these degumming methods tend to damage the silk fibers with the latter approach especially producing much weakened fibers capable only of short staple lengths that can only be spun into weak yarns of irregular thickness and hence considered of poor quality.

The marked contrast in the methods of obtaining silk from the cocoons of wild silkmoths with those used for the highly domesticated *B. mori* suggests a fundamental difference in cocoon composition. We formulated the hypothesis that the principal difference lay in the mineralization that is so common in wild silk moths and absent in *B. mori* and that this difference arises from the cementing together of the silk fibers in a mineralized matrix in wild silk cocoons making them impossible to wet reel. Here we first report our tests of this hypothesis by examining the effect of demineralizing a range of wild cocoons on their ability to be wet reeled. Then we focus on the Lasiocampid *Gonometa postica* because it makes an exceptionally tough mineralized cocoon containing a very interesting silk with commercial potential. Here we characterize in detail the constituent mineral added to the sericin gum and the effect of its selective removal on reeling and on the mechanical properties of the resulting silk filaments. Finally we compare the effect of the novel demineralizing method described here with that of the currently used method, pineapple juice digestion and carding, on the mechanical properties of the resulting fibers.

2.2. Materials and Methods

2.2.1. Samples

Emerged cocoons of the Saturniid family; *Attacus atlas*, *Antheraea frithi*, *Antheraea mylitta* (syn. *Antheraea paphia*), *Antheraea pernyi*, *Antheraea polyphemus*, *Antheraea roylei*, *Antheraea yamamai*, *Bunaea alcinoe*, *Callosamia promethean*, *Cricula andrei*, *Hyalophora cecropia*, *Hyalophora gloveri*, *Philosamia canningii* (syn. *Samia caningi*), *Philosamia cynthia advena* (syn. *Samia cynthia*), *Saturnia pavonia*, *Saturnia pyri* and from the Bombycid family; *Bombyx mori*, were bought from Worldwide Butterflies (UK) where they had been bred. Cocoons from the Lasiocampid *Gonometa postica* were either obtained from old collections or, for a more detailed investigation, from ICIPE in Kenya who obtained them fresh from a silk growers cooperative in Mwingi. For the comparison of silk fibres reeled using the methods described here with traditionally carded fibres, lengths of spun yarn from *G. postica* was also supplied by ICIPE having been obtained from the same batch of cocoons supplied to us. This yarn had been prepared by degumming the cocoons in a standardized protocol using a pineapple extract degumming, hand carding cocoons, and hand spinning.

For studying the removal of mineral, several samples were used from each type of cocoon, typically after the emergence of the moth and the pupal remains were removed through the emergence hole using forceps. From each cocoon, 15 discs with a diameter of 4.86 mm were pressed out of the cocoon at random locations with a sharp hole-punch. They were then subjected to demineralization with or without degumming as described below. Untreated samples were used as a control. The thickness of the discs was measured before and after treatments using a digital micrometre (Mitutoyo, IP 65) taking care not to compress the disc. During treatment, the discs and sample number engraved with a laser on a rectangular piece of plastic (polystyrene) were enclosed in small fine mesh nylon bags made with a bag sealer.

Furthermore, we tested the methods developed generically for a wide range of wild silkworms in considerable detail on one specific wild silk. We chose as a representative species *Gonometa postica* as this species (a) has been the subject of an excellent study of its ecology and behaviour¹⁰⁵, (b) has the most heavily mineralized cocoon of the 17 wild moths studied (see Figure 3) as well as (c) producing a silk that seems to have considerable commercial potential while at the same time having had the least commercial exploitation so far, due to the inability to reel silks of a consistently high quality. All together these features made *G. postica* the most challenging of the wild silks to investigate. To avoid any break in the fibre, *G. postica* cocoons were harvested before emergence of the moth, while still attached to pieces of twig, and also checked for drill-holes produced by parasitic wasps. All cocoons were stored at minus25°C for about a month prior to use. For the detailed identification of the mineral, four cocoons of *G. postica* were ground in a mortar and the resulting white powder was analysed.

2.2.2. Treatments

Demineralizing method: Many agents including aqueous solutions of ethylenediamine-tetraacetic acid (EDTA), citric acid (lemon juice, orange juice), formic acid, aluminum nitrate and phosphate ions are known to remove calcium oxalate by chelating calcium ions or directly dissolving it. Pilot experiments showed that of these reagents, warm concentrated aqueous solutions of EDTA at a pH ≥ 7 was the fastest at removing calcium oxalate from cocoons; a decrease in temperature, EDTA concentration or pH markedly increased demineralization time (unpublished results). Accordingly we used a standardized protocol for demineralization as follows. An aqueous 1M solution of ethylenediaminetetraacetic acid (EDTA) was adjusted to pH 10 with saturated sodium hydroxide solution. The samples (one cocoon or 10 discs per 600ml) were immersed in this solution for 72 hour at 40°C and gently

stirred. The cocoons and discs were kept under the surface of the solution by means of a plastic mesh, using a partial vacuum to encourage penetration of the solution.

The cocoons were then thoroughly washed with running tap water (three times 3 hours) and subsequently reeled in tap water at room temperature using the standard method for *B. mori* silk. The cocoon discs were washed in the same way and air dried overnight at room temperature. The method described here forms the basis of a patent application (GB 1018269.9, see Chapter 3).

Degumming method: Half of the reeled fibers (demineralized) and five demineralized discs of each cocoon were degummed following the industrial standard method for *B. mori* skeins; a 0.5% sodium carbonate solution (Na_2CO_3) at 80° C for one hour under stirring. Afterwards, the samples were thoroughly washed (three times) with deionized water (5l) for 2h and air dried overnight at room temperature.

2.2.3. Analysis of mineral removal

Thermal gravimetric analysis (TGA): The samples were loaded into a Q500 TGA from TA instruments and were equilibrated for 30 minutes isothermally at 26°C and a sample purge flow of 60ml/min with nitrogen (balance purge flow 40ml/min), the latter to ensure the weight change was due to mineral/gum removal and not due to water loss/gain of the sample dependent on environmental conditions. Ten samples per cocoon (n= 5) before and after demineralizing were weighed and the final weights were averaged per cocoon.

Scanning electron microscopy (SEM): Natural, demineralized and combined demineralized and degummed fibers and discs were sputter coated using an AuPd target for 180 seconds resulting in a 6-10nm layer and imaged using a Jeol Neoscope, JCM-5000 (Nikon, UK) under high vacuum at 15KeV.

Energy-dispersive X-ray spectroscopy (EDX): A JEOL JSM 6330 operating with an Oxford instruments ISIS 300 EDX unit with a working distance of 15mm and beam energy of 20Kv was used. Samples were sputter coated in 15nm of gold prior to analysis.

X-ray diffractometry (XRD): A Bruker D8 Advance powder diffractometer, with 2-theta from 5° to 50° degrees in step increments of 0.01967°/s was used.

For EDX and XRD, the heavily mineralized outer part of one disc per cocoon (n=5) was used before and after demineralizing. Representative spectra for each instrument and treatment are shown below.

Fourier Transform Infra-red Spectroscopy (FTIR): A Nicolet 6700 Fourier transform infrared spectrometer (FTIR) equipped with a liquid nitrogen cooled MCT-A detector was used with a bounce diamond attenuated total reflectance (ATR) sampling accessory (Thermo Electron Corp., Madison, WI). Spectra were acquired at 4 cm⁻¹ resolution from 4000 to 400 cm⁻¹ using a Happ-Genzel apodization, a Metz phase correction and no zero filling. Spectra were obtained from the average of 32 scans at a 5.0632 cm/s mirror speed and normalized for their intensity. All spectral operations were performed using Omnic 7.3 (Thermo Scientific, Madison, WI). Five discs of each cocoon (n= 5) were sampled three times on each side (inside and outside of the disc) before and after demineralizing. All spectra per treatment and disc side were averaged. To obtain an estimate of mineralization of the different cocoon shells, a ratio was calculated from the FTIR spectra between the absorbance of the oxalate peak at ~1312cm⁻¹ and the amide II region from 1590 cm⁻¹ to 1504 cm⁻¹.

$$Ratio_{cocoon\ shell} = \left(\frac{A_{1312cm^{-1}}}{A_{1590-1504cm^{-1}}} \right)$$

The ratio was calculated from spectra taken from the outer surface of cocoon shells (n= 5) of the different species. For comparison the same was done for calcium oxalate hydrate (Sigma)

as a reference standard for maximum mineral coverage. For *B. mori* which has no calcium oxalate and for pure calcium oxalate hydrate which has no amide II band the absorbance at this wave number was taken for the calculation. This ratio gives an estimated approximate value which depended on variables that could not be strictly standardized including the position of the sample site in the cocoon, the extent of compaction in the cocoon as received and the oxalate content of the caterpillar's food plant.

2.2.4. Reeling

Cocoons were wet reeled in water at room temperature with a reeling speed of 103mm/s. The length of reeled silk and the number of breaks during reeling were analyzed from recordings made by a Pluscom Camera (Vimicro301 Neptune).

2.2.5. Tensile testing

Samples for tensile testing were prepared from the demineralized (n=25) and from demineralized/degummed (n=25) reeled fibers of four cocoons. Samples from the commercial *G. postica* yarn made-up of multiple different cocoon filaments were obtained by gently teasing out brins (n=30). In all cases single brins were mounted without pre-load onto cardboard frames with an initial gauge length of 15 mm. They were then equilibrated at 40% RH and 26°C for five minutes prior to tensile testing under the same conditions on an Instron Universal Materials Testing Instrument at a strain rate 50% per minute using a 5 N load cell. Cross-sectional areas of the fibers were prepared by clamping orientated silk threads in a holder and cutting them across with a new razor blade before sputtering them with gold and viewing them in the SEM. Digital images of transverse sections on SEM micrographs were manually circled and analyzed with ImageJ 1.42q. Normalized cross-sectional areas were obtained by averaging 150 brins for each sample. The tensile parameters were calculated with a home designed program in Excel (Tensile Import v2.0). The J-integral method was used to

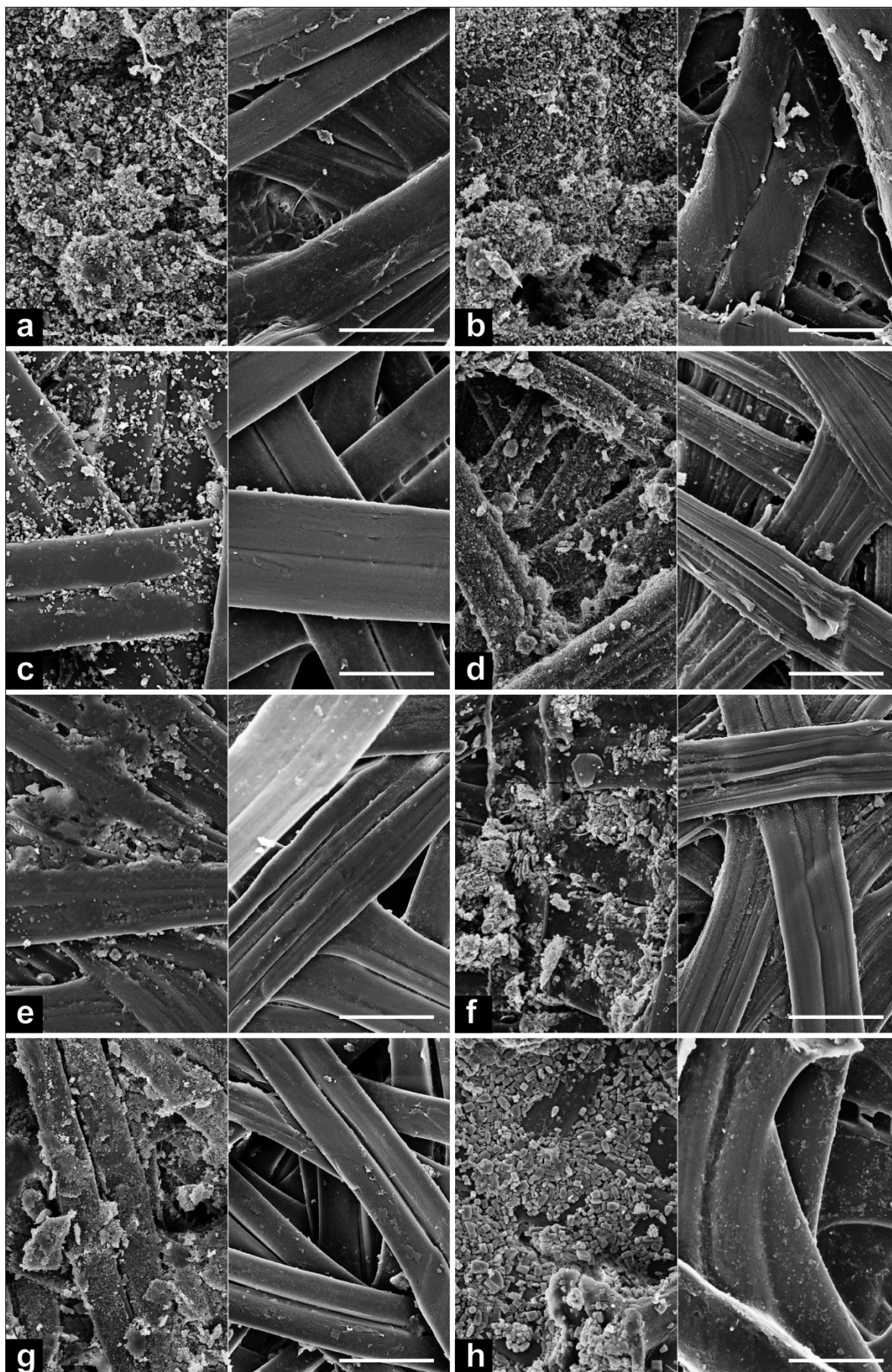
determine the energy to break. Stress strain curves were plotted using Origin Pro 8. Exlstat 7.5.2 was used for statistical analysis of the data with ANOVA (two tailed).

2.3. Results

2.3.1. SEM observation of the mineral and its removal

In general, the outer surface of the cocoons in all species studied was covered with a granular material (Figure 2-1 (left-hand half) and Figure 2-2 (a, b)). EDX and FTIR when taken together indicated that this granular material consisted predominantly of calcium oxalate (spectra were similar to Figure 2-4 (c, 3)). The cubic crystals observed in Figure 2-1 (h) and the raphides in Figure 2-2 (b) are likely different crystal forms of calcium oxalate. Ranking the different species according to their estimated degree of mineralization (Figure 2-3) immediately revealed the two extremes; *B. mori*, with no calcium oxalate present in the cocoon shell and *G. postica*, with calcium oxalate covering the entire cocoon shell with no visible silk. There appeared by eye to be a close relationship between the estimated degree of mineralization and the SEM appearance of the outermost part of the cocoon wall. In this connection, it is interesting to note that the wild silks that are commercially exploited at present, like *A. pernyi* and *A. mylitta*, are fairly low in their degree of mineralization (ratio: ~1, Figure 2-3). This granular mineral coating filled the interstices between fibers. In the species studied, this resulted in a compact and hard cocoon shell impossible to wet reel in all species except *B. mori* (Figure 2-3).

Figure 2-1 (on next page). High magnification pairs of SEM pictures (500x, scale bar 50µm) of the same untreated (mineralized; left-hand side) and treated (demineralized; right-hand side) cocoon shell for some selected silkmoth species: *Antheraea frithi* (a), *Antheraea mylitta* (b), *Antheraea pernyi* (c), *Attacus atlas* (d), *Bunaea alcinoe* (e), *Hyalophora cecropia* (f), *Philosamia canningii* (g), *Saturnia pyri* (h). All other wild silk species studied exhibited similar results.



After demineralizing, large quantities of granular material were no longer seen on the surfaces and interstices between the fibers in all species, exposing the underlying network of silk fibers in the cocoon shell (Figure 2-1 (right-hand side)). EDX and FTIR showed that calcium oxalate was removed after demineralizing the cocoon shells (spectra were similar to Figure 2-4 (c, 5)). All demineralized cocoon shells were still intact and similar in appearance to Figure 2-2 (c).

Table 2-1. The weight and mass density of the *G. postica* cocoon discs of the untreated cocoons (A) versus the effect of demineralization (B) and combined demineralization and degumming (C). Each box shows the mean, standard deviation and two tailed probability (ANOVA).

			Weight (mg)	mass density (g/cm ³)	Samples (n)
A	Natural		6.15	0.541	50
	st.dev.	p-value (A-B)	1.04 <0.0001	0.028 <0.0001	
B	Demineralised		4.67	0.474	50
	st.dev.	p-value (B-C)	1.12 <0.0001	0.043 <0.0001	
C	Demineralised and degummed		1.70	0.276	25
	st.dev.	p-value (C-A)	0.83 <0.0001	0.082 <0.0001	
R ² -value			0.89	0.82	

In more detail, all *G. postica* cocoon discs lost about 24% of their original weight after the demineralizing treatment ($p < 0.001$; $n = 125$) (Table 2-1). Also, the measured mean relative mass density significantly changed (from 0.541 g/cm³ to 0.474mg/cm³; $p < 0.0005$ after demineralizing. Figure 2-2 (a and b) shows that the outer surfaces of the natural cocoons of *G. postica* were heavily mineralized with a complete covering of narrow elongated monoclinic crystals similar in size and shape to the calcium oxalate crystals observed in Saturnid cocoons described elsewhere^{91, 100, 101, 106}. The cocoon fibers were difficult to distinguish (Figure 2-2, b (arrows)) and the cocoon appeared very compact.

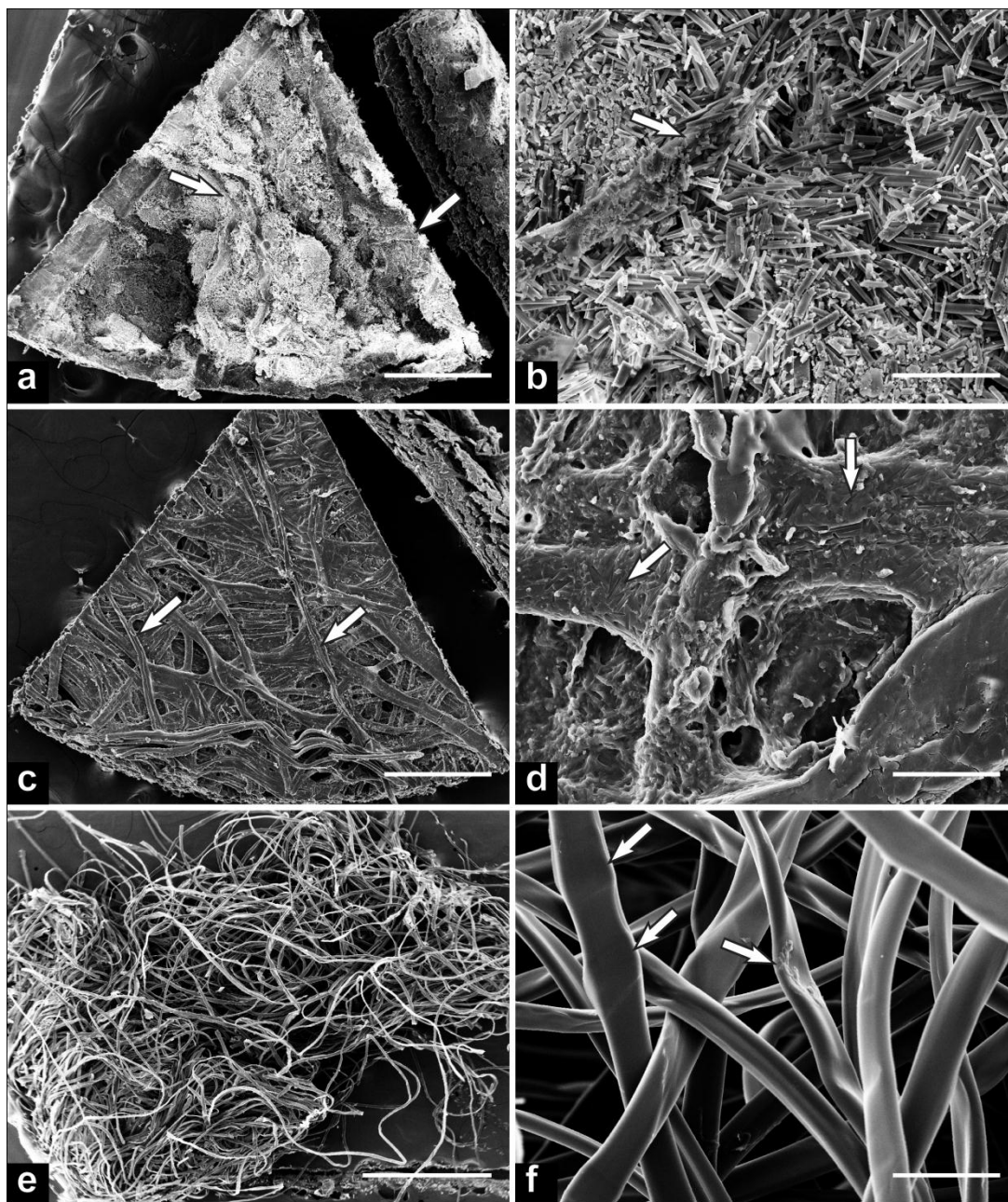


Figure 2-2. Low and high magnification SEM pictures of quadrants of cocoon discs of *G. postica*. Untreated (natural) cocoon disc, (a) low magnification (scale bar 500 µm) and (b) detail of covering mineral crystals (scale bar: 50 µm) with arrows indicating embedded fibers. Demineralized cocoon disc (c) with arrows indicating visible fibers in the cocoon shell network (scale bar: 500 µm) and (d) detail of cocoon disc surface showing fibers and gum, with arrows indicating crystal imprints (scale bar: 50 µm). A degummed cocoon disc (e) low magnification (scale bar: 1mm) showing the unravelling of the disc and (f) a detail of the fibers with arrows indicating imprints showing that brins were deformed where they crossed one another in forming the network (scale bar: 50 µm).

After demineralizing the cocoon discs, mineral crystals had disappeared and the underlying fibrous structure became visible (Figure 2-2, c and d). A network of cocoon fibers was now visible and the cocoon appeared less compact with many cavities and holes left by removing the mineral. In more detail it was clear that the surrounding gum was still intact connecting all cocoon fibers together in a bonded but open network, comparable to that of *B. mori*. The continuing presence of the gum after demineralization was further confirmed by the observation that the places where the crystals had been incorporated in the gum were still visible as imprints in the gum coating (Figure 2-2, d: arrows). When degumming the cocoons, directly from the natural cocoon or after first demineralizing, the compact network and binding between cocoon fibers broke-down completely (Figure 2-2, e and f) and the cocoon disc drastically expanded in volume, as shown by a decrease in mass density, to give a tangled loose mesh of fibers. In Figure 2-2 (f, arrows) the fibers after degumming appeared very smooth and no crystal imprints were found confirming the removal of gum. On the surface of some smooth fibers, although the original bonded network had broken down, the points at which the fiber had been in contact with other fibers was still recognizable as faint deformations in the surface of the fibroin of the brins (Figure 2-2, f: arrows).

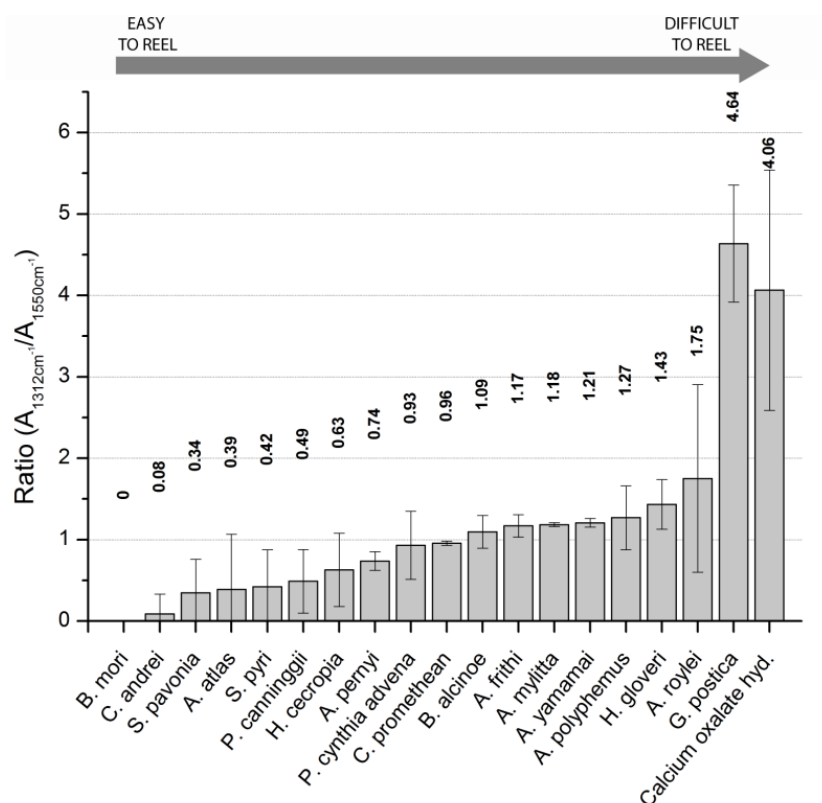


Figure 2-3. Shows the ranking of the species according to the estimated approximated degree of mineralization (see method section) and the ease of reeling these cocoons according to this ranking.

2.3.2. Spectrographic evidence for identification of the mineral and its removal

Figure 2-4 shows spectrographic evidence from EDX, XRD and ATR-FTIR for the identification of the mineral on/in the cocoon and for the effectiveness of its removal by demineralization with EDTA. The extracted powder from the cocoons of *G. postica* clearly showed the presence of calcium, oxygen and carbon in the EDX (Figure 2-4 a, blue trace). The EDX of the cocoon discs also indicated that after demineralizing, the calcium and oxygen peaks that were initially present in the untreated sample (Figure 2-4 a, black trace) were no longer present (Figure 2-4 a, red trace).

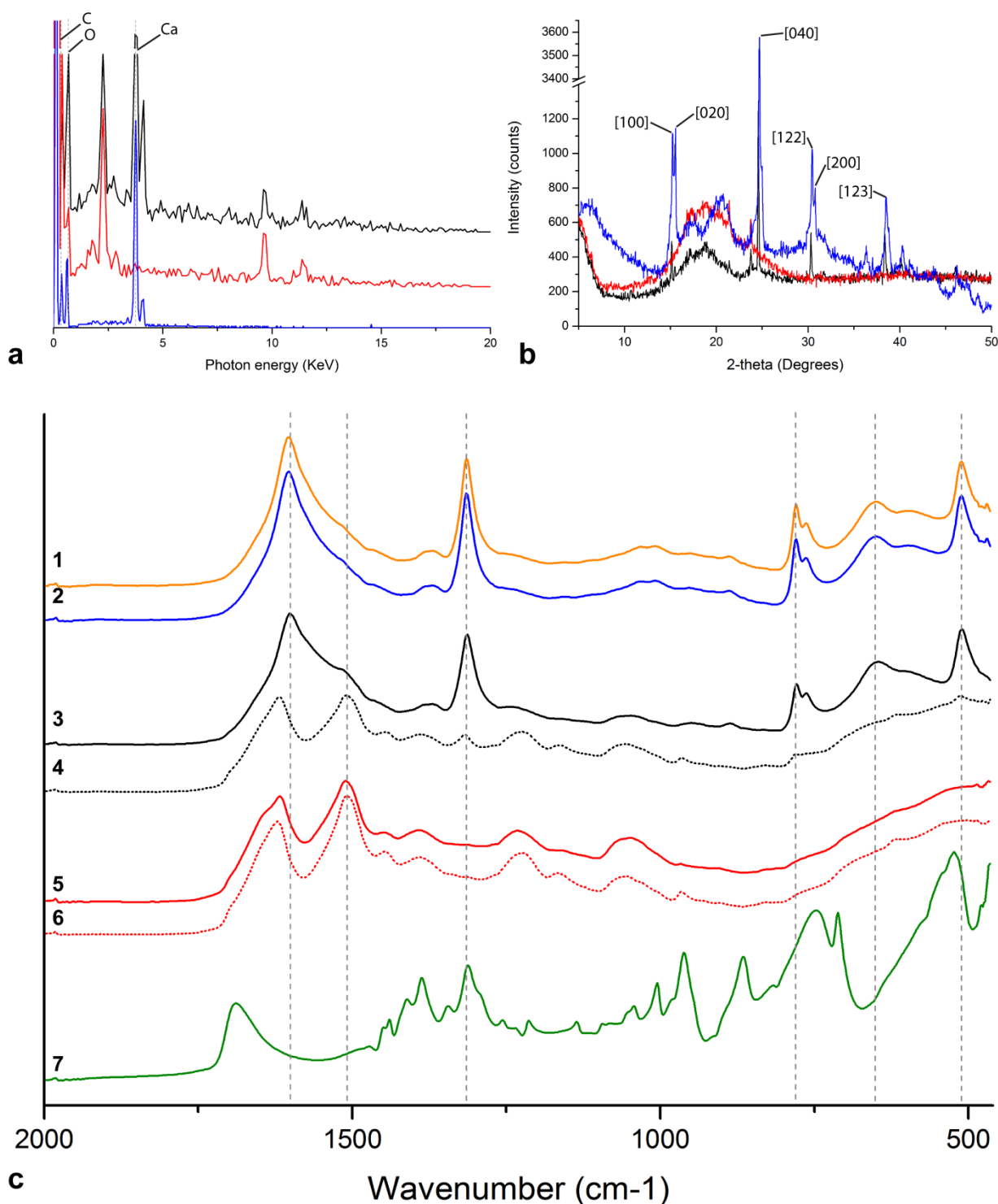


Figure 2-4. Spectra of the extracted mineral powder (blue trace), its presence in the natural cocoon shell (black trace) and its removal (red trace) from the cocoon shell by the demineralizing treatment. The EDX shows that the mineral powder consists of calcium, oxygen and carbon (a, blue trace), while the XRD spectra of the extracted powder (b, blue trace) of the *G. postica* cocoon was identified as, and indexed against whewellite (JCPDS card 01-077-1160).

The EDX (a) and XRD (b) spectra both show that the peaks characteristic for calcium oxalate disappeared after the demineralization treatment (black line, natural cocoon disc; red line, demineralized cocoon disc). The FTIR spectra (c) showed that the extracted powder from the cocoons (2, blue line) matches the calcium oxalate hydrate spectra (1, orange line) bought from Sigma. The untreated (black line) cocoon discs (3) matched the calcium oxalate spectra (1, orange line) and that almost no mineral was present on the inside of the untreated disc (4). After demineralizing (red line) the calcium oxalate was completely removed on the outside (5) as well as on the inside (6) of the cocoon with no traces of EDTA (7, green line).

The XRD spectra (Figure 2-4 (b, blue)) of the powder showed major reflections at 2-theta [100], [020], [040], [122], [200] and [123] that could be indexed to calcium oxalate hydrate (JCPDS card of whewellite, pattern 01-077-1160). Further, XRD demonstrated that the six peaks characteristic for calcium oxalate hydrate were lost after demineralizing the cocoon discs. The FTIR-spectra of the extracted powder and the outside of the mineralized cocoon discs (Figure 2-4 (c, 2 and 3)) matched the spectrum of pure calcium oxalate hydrate (Sigma) (Figure 2-4 (c, 1)) and confirmed that there was a large quantity of calcium oxalate present on the outside of the cocoon discs. On the inside of the natural cocoon discs some calcium oxalate peaks could be seen superimposed on the silk spectrum but at much reduced height (Figure 2-4 (c, 4)). After demineralizing the cocoon discs the peaks characteristic for calcium oxalate (main peaks at 1599.8cm^{-1} and 1311.8cm^{-1}) disappeared completely on the outside as well as on the inside of the cocoon (Figure 2-4 (c, 5 and 6)). No evidence of remnants of the EDTA used for demineralization was found in the spectra of the demineralized cocoon discs (Figure 2-4 (c, 7)).

We did not determine whether calcium oxalate was the only component removed by demineralization, which is likely, not the case.

2.3.3. Reeling

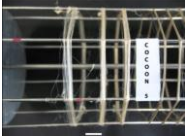
Prolonged degumming in a pineapple extract or in boiling sodium carbonate solution was effective in removing both the mineral and the gum, however the cocoons became impossible to reel because the fibers together with enclosed remains of the pupa produced tangles and finding the beginning of the fiber or reeling became impossible. The breakdown of the gum connections, separation of the fibroin brins and their entanglement was seen in SEM images (Figure 2-2, e and f) helping to explain why reeling was not possible after these treatments. In contrast, the five cocoons that were first demineralized were easy to reel (Table 2-2). This is probably because the gum connections holding the fibers together were still intact preventing tangling yet sufficiently swollen and weakened to allow them to be broken by the forces generated by wet reeling. Additionally, removal of the mineral made it easy to find the beginning of the fibers. The total length of reeled silk varied from a minimum of 45 meter to maximum of 572 meter (Figure 2-5, a). Although this somewhat less than that which can be obtained from *B. mori* (300 to 1300m; unpublished data) it still represents a commercially useful quantity. Continuous lengths up to 350 meter (Figure 2-5 (a): cocoon 1 and 4) could be reeled from *G. postica* cocoons which represents a very great improvement over the short staple fibers produced by carding after degumming with pineapple extract. The much longer fibers should allow stronger and more even singles and twists to be made for the first time from this silk.

Almost all silk cocoons were reeled to completion and reeling stopped when only a thin transparent layer was left surrounding the pupa, as with *B. mori* reeled cocoons. The number of breaks varied but was comparable to the number in industrially reeled *B. mori* cocoons (Table 2-2). The number of breaks in cocoon 5 was significantly higher than the other cocoons because the trap door of this cocoon was rectangular, snagging the fibers and causing them to break.

To yield a large quantity of reeled silk, the *G. postica* cocoon needed a volume > 10ml or more specifically, a width of >20mm and length <22mm (unpublished data).

The demineralizing method enabled *G. postica* cocoons to be reeled at a speed of 6.2m/min which is less than that used industrially for multi-end reeling of *B. mori* silk, 50 to 80m/min. However, higher reeling speeds were not tried.

Table 2-2. Reeling data of five different *G. postica* cocoons. Measures of the cocoon (width, height and volume), number of breaks during reeling, total length of silk reeled and a visual estimate of the completeness of the reeling are tabulated for each cocoon. The pictures show the cocoon before and after reeling and the amount of reeled silk (white scale bar = 1cm).

Cocoon	Cocoon before reeling	Cocoon height (mm)	Cocoon width (mm)	Calculated cocoon volume (ml)	Reeled silk	Fibre breaks (n)	Reeled length (m)	Cocoon after reeling	Reeling completion
1		50.08	21.36	12.0		9	572.0		almost complete
2		48.28	19.99	10.1		7	434.2		almost complete
3		43.15	18.62	7.8		4	45.1		complete
4		49.79	19.96	10.4		3	332.2		complete
5		48.12	22.34	12.6		23	179.0		partial

Cocoons from *A. mylitta*, *A. pernyi*, *C. Andrei*, *S. canningii* and *G. postica* could be readily wet reeled after demineralizing as described above. Other species were not tried. Most cocoons available for this study had emergence-holes where the larva broke through the cocoon shell. This led to breaking of the fibers at these spots, which in turn prevented the reeling of long continuous lengths of fiber. Accordingly we have with a few exceptions no reliable data on maximal reelable length for most species. However, we do have reliable mechanical data on the effect of the treatments on fiber properties. Moreover, we also have reliable data on reeling length for one selected species, namely *G. postica*.

2.3.4. Tensile properties

Figure 2-5 (b) compares the averaged stress strain curves (n: 25) for *G. postica* demineralized brins of four different cocoons (black curves), demineralized/sodium carbonate degummed brins of those same four cocoons (red) and brins from carded and spun commercial *G. postica* yarns consisting of multiple different cocoon filaments degummed with pineapple juice (blue).

The first two treatments gave a much more consistent pattern of stress strain curves probably as a result of the fibres originated from the same cocoon compared with pineapple degummed and carded where fibres originated from many different cocoons. Alternatively the more consistent pattern could be a result of less fiber damage produced by wet reeling in contrast to the pineapple degumming and carding.. Silk fibers reeled from demineralized cocoons were significantly ($p < 0.001$) and markedly stronger than the silk from commercial *G. postica* yarn as judged by their mean breaking load, 0.084N against 0.056N respectively (Table 2-3). However, the mean breaking stress of the demineralized silk filaments was significantly less ($p < 0.05$) than that of the commercial yarn due to the large and highly significant ($p < 0.001$)

reduction in the cross sectional area of the brins produced by the vigorous action of the pineapple extract.

It is not clear whether the smaller mean cross sectional area of pineapple degummed material resulted solely from more efficient removal of gum or whether it additionally removed some weakly stabilized fibroin from the surface of the brin.

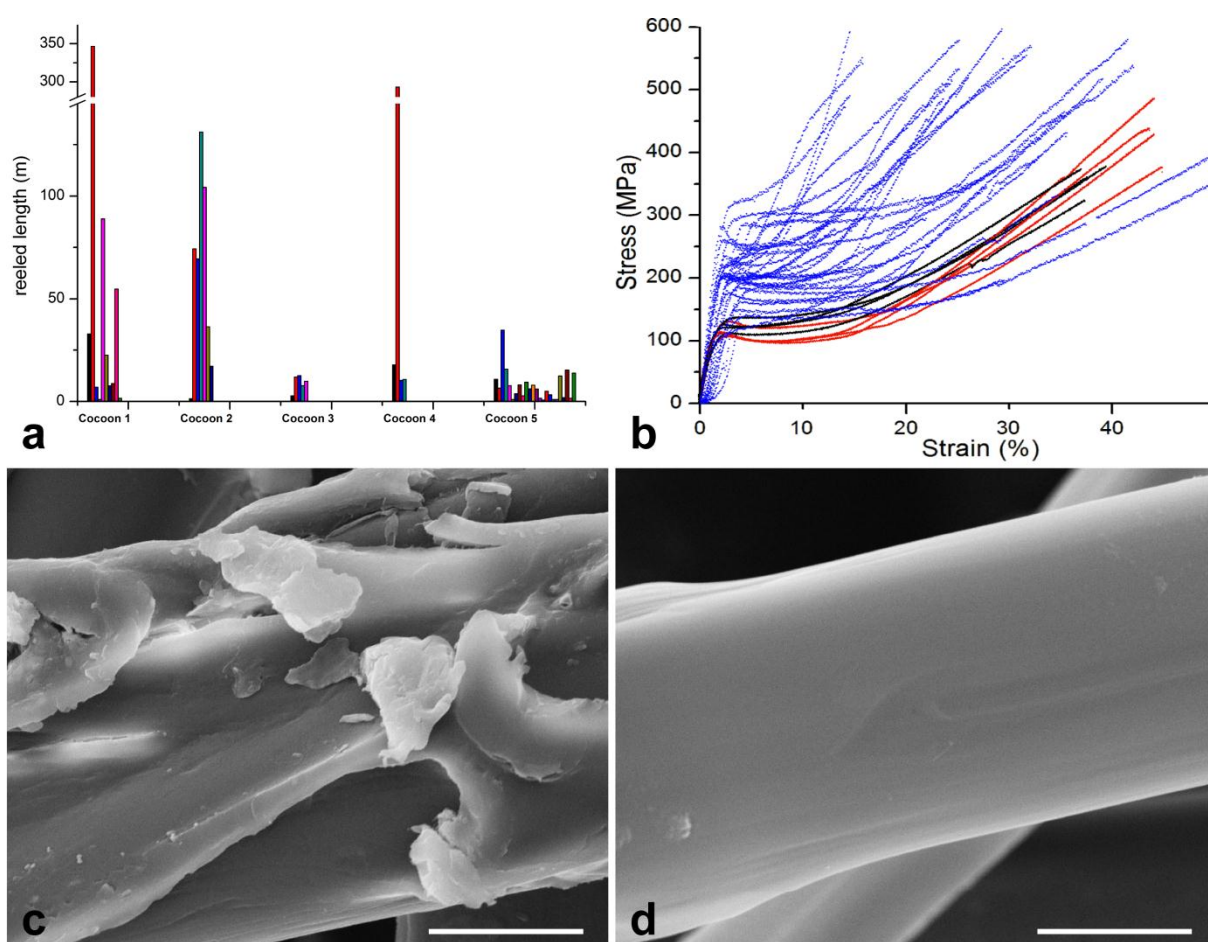


Figure 2-5. (a) The maximum continuous length of reeled silk fiber per cocoon, each column representing a new try after a fiber breakage. (b) Stress-strain graphs are shown from *G. postica* silk with different treatments: Treated with pineapple juice, carded and unravelled from hand spun yarn (blue); Silk fibers extracted by reeling after demineralizing the cocoon before (black); and after sodium carbonate degumming (red) (n = 4 cocoons, averaged representative curves). Figure 2-5 (c-d) SEM pictures of demineralized (c) and sodium carbonate degummed (d) fibers after reeling (scale bar: 10μm).

Sodium carbonate degumming of previously demineralized fibers reduced the cross sectional area of the fiber resulting in an increased breaking stress (Figure 2-5, c and d). However, the break load did not significantly change (Table 2-3 (A, B), $p>0.05$) after sodium carbonate degumming indicating that the gum did not contribute to the strength of the fiber.

Although the mean breaking energy of the demineralized/degummed filament was not significantly greater ($p>0.05$) than the pineapple degummed ones they did show a significantly larger ($p<0.001$) mean elongation to break (Table 2-3). Thus the *G. postica* fibers produced by wet reeling after demineralizing and sodium carbonate degumming are probably more consistent in mechanical properties and significantly better in strength measured by breaking load and elongation to break compared with the commercial fibers from the same species produced by pineapple degumming, carding and hand spinning. Using a milder degumming method, comparable to the pineapple degumming in effectiveness of gum removal, in place of sodium carbonate degumming may result in a much smaller cross section of the demineralized fibers and improved breaking stress.

Table 2-3. The effect of various treatments on the tensile properties of *G. postica* fibers: (A) demineralized and reeled; (B) demineralized, reeled and sodium carbonate degummed; (C) fibres produced by degumming with pineapple juice, carded, hand spun and unravelled. Also showing, a recalculation of the tensile properties demineralized, reeled and sodium carbonate degummed *G. postica* fibres based on the cross section of pineapple degummed fibres (see text). Each box shows the mean (top left), standard deviation (bottom left) and two tailed probability (bottom right, ANOVA).

		Cross section (μm^2)	Break Load (N)	Break Stress (MPa)	Break Strain (%)	Breaking energy (J/cm ³)	Initial modulus (MPa)	# fibres (n)
A	Demineralised	254	0.084	326	28.4	53.6	8398	101
	st.dev. p-value (A-B)	53 <0.001	0.015 0.24	72 <0.001	9.5 <0.001	18.0 <0.001	1541 0.889	
B	Demineralised + degummed	198	0.082	401	37.9	77.9	8363	100
	st.dev. p-value (B-C)	36 <0.001	0.016 <0.001	98 0.031	7.5 <0.001	26.2 0.615	1385 <0.001	
C	Pineapple juice	125	0.058	443	27.7	75.3	10960	30
	st.dev. p-value (C-A)	26 <0.001	0.017 <0.001	129 <0.001	11.7 0.728	35.7 <0.001	3188 <0.001	
	R ² -value	0.40	0.24	0.23	0.22	0.21	0.22	
D	Re-calculated <i>G. postica</i>	125	0.080	638	37.9	-	-	100
	st.dev.	26	0.019	155	7.5			
E	Commercially degummed <i>B. mori</i>	280¹	0.08¹	500²	15²	65.1¹	8114¹	10
	st.dev.	50	0.014	3	3.4	19.3	750	

¹ Juan Guan personal communication

² Shao, Z. Z. and F. Vollrath (2002). "Surprising strength of silkworm silk." *Nature* **418**(6899): 741-741

Because the gum of the fiber does not contribute to the breaking load of the fiber (Table 2-3, A and B) it is possible to re-calculate the breaking stress of a demineralized fiber based on the cross section of the pineapple degummed fibers (Table 2-3, C). This means that our demineralized/degummed *G. postica* fibers compared favorably with a typical commercial degummed *B. mori* silk as follows: mean breaking stress 0.638 GPa, 0.500 GPa; Mean breaking strain 37.9%, 15.2%; initial modulus 8.3 GPa, 8.2 GPa; breaking energy 77.9 Jcm⁻³, 60 Jcm⁻³ (Table 2-3, D and E).

2.4. Discussion

It emerged from our studies that the cocoons of wild Silkmoths belonging to a range of taxa can indeed be wet reeled and produce high-quality fibers using the demineralizing method novel for silkworm cocoons.

Using *Gonometa postica* cocoons as an example, we present strong evidence derived from SEM, EDX, FTIR and XRD demonstrating that calcium oxalate monohydrate (whewellite) forms a major structural component of the outer layer of the cocoon of *G. postica* and is present in smaller quantities throughout the rest of the cocoon shell. Our evidence also strongly suggests that the extensive infiltration of whewellite between the silk fibers accounts for the difficulty of reeling in this and many other Wild Silkworm species. We suggest that the currently used methods for softening *Gonometa* cocoons, namely prolonged retting or digestion with pineapple extract are effective in removing the mineral but simultaneously remove the gum that holds the fibers together resulting in a collapse of the cocoon's structure and entanglement of the silk fibers making reeling impossible.

In contrast, removal of the mineral with EDTA left the gum intact preventing collapse and entanglement of the cocoon fibers but evidently softened them sufficiently to enable wet reeling for the first time for this species. This method that clearly differs from degumming can be referred to as “demineralizing”. We show that substantially all of the silk in *G. postica* cocoons can be reeled in this way using standard commercial reeling equipment to produce a total length of silk of up to 570 m per cocoon in unbroken lengths of up to 350 m. Crucially we show that the mechanical properties of the silk reeled in this way and subsequently degummed are markedly better than commercial silk fibers produced from the same batch of cocoons using pineapple juice degumming, carding and hand-spinning.

Equally crucially, when suitably degummed, the mechanical properties are also comparable to those of conventionally degummed commercial *B. mori* silk.

Importantly, other species of wild silkmoths with more established commercial potential such as *Antheraea mylitta*, *Antheraea pernyi*, *Cricula andrei* and *Samia canninggii* responded to the EDTA treatment in a comparable fashion suggesting that the treatment crosses not only species boundaries but also those of higher taxa (families) and thus has more generic implications.

2.5. Conclusion

Wild Silkmoth cocoons are difficult or impossible to reel under conditions that work well for cocoons of the Mulberry silkmoth, *Bombyx mori*. Here we report evidence that this is caused by mineral reinforcement of wild silkmoth cocoons and that washing these minerals out allows for the reeling of commercial lengths of good quality fibres with implications for the development of the ‘wild silk’ industry which will be discussed in Chapter 3. We show that in the Lasiocampid silkmoth *Gonometa postica*, the mineral is whewellite (calcium oxalate monohydrate). Evidence is presented that its selective removal by ethylenediaminetetraacetic acid (EDTA) leaves the gum substantially intact preventing collapse and entanglement of the network of fibroin brins, enabling wet reeling. Therefore this method clearly differs from the standard “degumming” and should be referred to as “demineralizing”. Mechanical testing shows that such preparation results in reeled silks with markedly improved breaking load and extension to break by avoiding the damage produced by the rather harsh degumming, carding or dry reeling methods currently in use.

This suggests that our novel degumming method should increase the range of wild silkmoth species that can be wet reeled and may be important for the development of the silk industries not only in Asia but also in Africa and South America.

Chapter 3

Practical implications of demineralizing Wild Silkmoth cocoons

Abstract and motivation

In Chapter 2 we have shown that the inability of reeling Wild Silkmoth cocoons was mainly due to a mineral coating present in most of these cocoons. Furthermore, a method was described for the selective removal of this mineral enabling reeling of these cocoons, in most cases for the very first time. Because many Wild Silkmoth species exist and are distributed worldwide in contrast to *B. mori*, the impact of this finding has huge commercial significance. Therefore, this chapter will deal with the intellectual property protection of this method in the form of a filed patent application (GB 1018269.9).

The composition of writing a patent is quite different from that of a general paper outline, thus here we will first give a brief background and description of the invention, followed by a detailed protocol of the demineralizing method and to conclude the claims that give the scope of protection for this patent.

This Chapter can be linked back to Chapter 1.4.1 in that this technique will eliminate a lot of inter- and intra-specific variability in silkmoth silks and in this way will lead to a broadening of the range of available silks for (biomedical) applications as it will increase the range of available silk types, decrease the variation in silk properties (mainly tensile properties), decrease or abolish cytotoxicity and increase solubility of these silks. In Chapter 4 the cocoon spinning behaviour between *B. mori* and Wild Silkmoth silks is studied revealing that the main reason why *B. mori* is reelable in contrast to all others is probably because it has been selected for over 3000 years for the cocoons that can be reeled best. This means there are two main ways of ensuring cocoons are reelable; by “human” selection which can easily take several thousands of years or by the technique described in this chapter that will take a few days.

3.1. Technical field

The present invention relates to a method for demineralising wild silk cocoons before the silk is further processed and to materials with improved properties produced according to the method. This method makes it possible both to reel cocoons from species which cannot be reeled with prior arts and to wet reel cocoons from other which can only be dry reeled or semi-dry reeled with the prior arts. Compared with the prior arts the present invention gives silk fibres with improved quality and reduced or abolished toxicity and allergenicity.

3.2. Background of the invention

The term Wild Silk refers to silks that are derived from wild silkmoth cocoons rather than silk derived from the domesticated silkmoth, *Bombyx mori*. Wild Silkmoth cocoons for the manufacture of silk are usually gathered directly from the wild though partial domestication has been achieved for several wild species. There are three families of wild silkmoths: Saturniidae, Lasiocampidae, and Thaumetopoeidae. In addition the Bombycid silkmoth *Bombyx mandarina* is found in the wild and has not been domesticated and can therefore be considered to be a wild silkmoth. In India, China and Japan, the cocoons of several species of moths are currently exploited commercially for the production of wild silks and many other species have potential for exploitation in other parts of the world. In addition, there is growing interest particularly in Africa in the potential of wild silkmoth species for the production of commercial wild silks though the industries for these are at an early stage of development¹⁰⁷. Many species of wild silkmoth are indigenous and adapted to less developed parts of the World where some are regularly found at high population density and are sometimes present as pests of economic importance.

The cocoons of wild silkmoths are generally difficult to soften and this makes reeling from the cocoon difficult or impossible and in consequence most of the wild silk produced commercially is not reeled but carded and spun into staple silk^{98, 108}. Compared with reeling, particularly wet reeling, carding and spinning is disadvantageous as silks prepared in this way have reduced strength; toughness and resistance to wear caused by the shortness of the staple fibre and by damage to the fibres resulting from the carding process and the harsh treatments required to enable the silks to be carded. Several factors contribute to the difficulty of softening wild silk cocoons to enable them to be reeled, of which the most important is that the sericin gum which cements the fibroin brins together in the cocoon is relatively insoluble and resistant to swelling compared to that of the domesticated *Bombyx mori* silk worm. This resistance is thought to result from chemical interaction between silk sericin and inorganic minor components or tannins contained in the wild silk¹⁰⁹. This makes the cocoon impossible or difficult to reel with existing methods. Where reeling is possible, it is usually achieved by dry or semi-dry reeling and the additional mechanical force required for this compared with conventional wet reeling is likely to damage the silk. In some wild silks it is necessary to resort to prolonged boiling in alkaline solutions and/or treatment with aggressive enzymes or prolonged retting to soften the sericin before the silk can be carded or reeled directly. These treatments are likely to cause significant degradation to the fibroin brins leading to reduced tensile strength, toughness and resistance to wear.

The Lasiocampid silks are particularly difficult to reel as the sericin appears to be particularly hard and resistant to swelling. The sericin of the Lasiocampid silkworm *Gonometa postica* is difficult to remove¹¹⁰. The sericin of *Gonometa rufobrunnea* could only be removed by the harsh treatment of heating for 1 hour to 95°C in a strongly alkaline soap solution¹¹¹. Brins of *Gonometa rufobrunnea* and *Gonometa postica* degummed by heating to 95°C in a strongly alkaline solution showed low ultimate strength (290 and 178

MPa respectively) and low mean moduli (1.2 and 1.4 GPa)¹¹². This may be partly attributable to damage during degumming and carding.

The cocoons of *Gonometa* spp cannot be softened and reeled with existing methods. Even after prolonged boiling, the silk of *Gonometa postica* cannot be reeled and so must be carded and spun. Alternatively retting for 2-3 weeks buried in moist and warm soil is required to remove the sericin before the silk can be carded and spun.

Saturniid cocoons are also difficult to soften and reel. For example *Antheraea mylitta* cocoons require prolonged cooking or cooking in soda followed by dry reeling or prolonged boiling in soap and hydrogen peroxide¹¹³. *Philosamia ricini* cocoons are usually carded and spun as a staple because they cannot be wet reeled using existing methods¹¹⁴. For example, *Antheraea proylei* cocoons could not be reeled by soaking in distilled water for 20 hrs at 26-31° C or for 4 hrs at 60 °C after an initial boiling for 30 minutes at 60 °C and could only be reeled by prolonged treatment with a pineapple extract containing bromelain¹⁰⁴. These authors disclose that treatment with bromelain is disadvantageous in that it reduces the tenacity of silk fibers obtained from *Antheraea proylei* cocoons.

Thaumetopoeid cocoons are also difficult to degum. Those of *Anaphe* sp. could only be carded and spun after prolonged boiling in concentrated sodium carbonate solution¹¹⁵.

Calcium oxalate has been shown to be present in the cocoon of *Antheraea assama*¹⁰⁰, while the hardness of *Antheraea mylitta* cocoons has been attributed to the presence of these crystals¹⁰⁶. Calcium oxalate has also been identified in the cocoon of the Lasiocampid *Malacosoma neustria testacea*¹⁰¹, the authors commented that it is difficult to remove but do not suggest a function for it in the cocoon, considering it only as an excretion from the silk worm's Malpighian tubules. Akai et al⁹¹ also report the presence of calcium containing

crystals in the cocoon of the Lasiocampid *Gonometa* sp. although the authors suggested the packing of these crystals between the silk fibers might account for the difficulty of reeling the hard outer layer of the cocoon, they did not suggest that agents to remove calcium minerals or tannins might facilitate reeling.

While it is widely understood that sericin, calcium oxalate and tannins and other substances present in wild silk cocoons may have toxic, irritant or allergenic effects the literature does not suggest how these may be removed from cocoons under mild conditions.

Furthermore, it is clear that existing methods of reeling silk from wild silkmoth cocoons from the Saturniid family damage the silk resulting in frequent breaking of the silk baves during reeling. This increases the time required for finding fresh ends and results in considerable wastage of unreelable or partially reelable cocoons. Damage to the baves during the dry or semi-dry reeling of wild silks also reduces the strength and toughness of the baves and fibres formed from them with a consequent reduction in resistance to abrasion and wear in wild silk fabrics. The damage also results in longitudinal splitting or partial transverse fracture of the fibre and the production of several forms of imperfection in the yarn including short regional thickenings known as slubs. All these imperfections can reduce the hand, lustre and value of the yarn and the woven fabric. They can also reduce the ease with which twists, yarns and braids can be formed.

Bombyx mori cocoons can be easily reeled by softening the cocoons in warm water. In contrast this simple method of softening cocoons does not generally permit reeling of wild silks necessitating the use of prolonged cooking or boiling in alkaline solutions with or without soaps or the use of aggressive enzymatic treatments or retting to enable sufficient removal or softening of the sericin to enable the fibres to be separated. These treatments are

likely to damage the silk fibres. The difficulties of reeling wild silkworm cocoons usually makes it necessary to card the silk and spin it into yarns with very short staples. These yarns have very uneven thickness and greatly reduced strength, toughness resistance to wear and lustre compared to the very long fibres in wet reeled Mulberry silk. Where conventional treatment of silks does permit reeling the softening or removal of the sericin is often inadequate necessitating the use of the greater force involved in dry or semi-dry reeling. This in turn is likely to damage the silk fibres.

Thus obtaining undamaged, long silk fibres by reeling of wild Silk moth cocoons represents an unsolved problem. It is clear that an efficient method for doing this would greatly encourage the development of a sustainable wild silk industry in less-developed parts of the world with significant social and economic benefit enabling it to compete with other silk industries.

3.3. Brief description of the invention

It is therefore the object of the present invention to provide a method for the efficient reeling of undamaged, long silk fibres from Wild Silkmoth cocoons by first removing all or substantially all of the mineral matter from them under mild conditions and in so doing, to produce wild silk singles, twists, yarns and cloths with reduced numbers of imperfections, and improved lustre, tensile properties, length, resistance to abrasion and wear, and improved biocompatibility. For some Wild Silks including by way of example only those of the Lasiocampidae this method makes it possible for the first time to reel the fibres as baves or singles thereby obviating the necessity for damaging degumming and carding treatments needed which yield short staples for spinning into yarns. For other wild silkmoth cocoons

including by way of example only those of certain Saturniids the method of demineralising cocoons is highly advantageous in that it enables wild silk cocoons to be wet reeled efficiently with existing equipment used for reeling Mulberry silk thereby reducing damage to fibres resulting from dry or semi-dry reeling or carding. The method of demineralising cocoons therefore produces silks with improved mechanical properties, resistance to wear and abrasion, and improved lustre and hand. It is also highly advantageous in that the process removes the toxic calcium oxalate, tannins, polyphenols and other toxic agents thus greatly increasing the biocompatibility of wild silks and thereby making them suitable for medical uses and akin the silk fibres less irritant when woven into apparel. The method of demineralising of cocoons is also advantageous in that it reduces the labour and cost required for producing fibres from wild Silk and reduces wastage by enabling all or most of the silk to be reeled from cocoons. The method is further advantageous in that it enables the dissolution and regeneration of certain wild silks which cannot be regenerated under mild conditions with the prior arts enabling the formation of regenerated silk materials with improved properties and reduced cost.

Thus the method and materials produced by it overcome the disadvantages and limitations inherent in existing processes and in the silks obtained from them.

3.4. Detailed description of the invention

In the first aspect of the invention, wild silk cocoons are treated by immersing them in a demineralising agent preferably an aqueous solution containing 0.2–1M ethylenediamine-tetraacetic acid (EDTA) or one of its salts or a combination of EDTA with one of its salts or with another chelating agent capable of removing divalent cations or with an aqueous

solutions containing citric acid or an aqueous solution containing a fruit juice containing citric acid at a pH between 5 and 10, preferably pH 7. Such solutions containing citric acid could by way of example only be lemon juice, orange juice or mausammi juice. Less preferably by way of example other demineralising agents can be used instead including by way of example only deionised water, formic acid or solutions containing aluminium nitrate, phosphate ions or magnesium ions. It is to be understood that a combination of one or more demineralising agents can be used to remove the calcium oxalate and other minerals from the cocoon and partially remove or soften the matrix between the silk fibres.

In each case, treatment with the demineralising agent is carried out at temperatures between 10 and 100°C and preferably between 15 and 60°C. Treatment under mild conditions with the demineralising agent disclosed here advantageously avoids the degradation to the silk fibres caused by the use of alkaline solutions, soaps, enzymes, retting or prolonged or high temperature treatments.

To facilitate wet reeling it is advantageous for the demineralising agent to leave at least some of the gum intact while softening it. Failure to leave some of the gum intact allows collapse of the cocoon fibres into a compact and readily tangled mass from which it is difficult to reel or find fresh ends. Failure to soften the gum is also disadvantageous in raising the force required for reeling leading to a greater risk of damaging the fibres. It will be clear from this that demineralisation as described above is distinguished from degumming in that it leaves some or substantially all of the gum intact (see Chapter 2).

It is further advantageous if part or all of the treatment of the cocoons by immersion in one or more of the demineralising agents described above is carried out under reduced pressure

to facilitate the removal of air from the cocoon shell and thereby the penetration of the demineralizing agent.

Highly advantageously, demineralising the wild silk cocoons as disclosed in this invention makes it possible to reel most or substantially all of the silk from the cocoon using standard equipment used for the wet reeling of *Bombyx mori* silk. Demineralizing in this way permits the cocoons of some species of wild silkmoth to be reeled for the first time and for other wild silk species to be reeled without recourse to damaging reagents or retting procedures. It also allows the damaging dry reeling or semi-dry reeling to be replaced by gentler wet reeling. Moreover it avoids the use of other damaging treatments required in some species to enable the silk to be carded. It is to be understood that the method of demineralizing the cocoons facilitates the subsequent degumming of the silk in skeins or as silk fabrics both prepared from either reeled or carded and spun silk. Thus demineralizing the cocoons enables the fibres, yarns, threads, tops, noils or silk fabrics prepared from them to be more completely, more rapidly, more fully degummed or degummed under mild conditions using sodium carbonate with or without soap solutions, enzymes or other reagents used in the mulberry silk industry and known to a person skilled in the art. The method is further highly advantageous in that it enables the dissolution and regeneration of wild silks which cannot be regenerated under mild conditions with prior arts thereby greatly improving the properties of the regenerated silk materials obtained.

In a second aspect of the invention the silk fibres obtained by reeling wild silk cocoons after demineralising as disclosed here with or without subsequent degumming in solutions of sodium carbonate or solutions of sodium carbonate with or without added soap solutions or solutions containing one or more enzymes have advantageous properties compared with fibres obtained using the conventional methods for the preparation of wild silks according

to the previous arts. By way of example only the average ultimate tensile strength of *Gonometa postica* silk fibres prepared by demineralising with EDTA and subsequently degumming with sodium carbonate according to the current invention was 400 MPa (n=100) compared to a value of 178 MPa reported by Colomban et al. (op. cit.) obtained for fibres degummed by heating for 1 hour to 95°C in a strongly alkaline soap solution and carding. Similarly the average Young's modulus of fibres prepared according to the present invention was 8.3 GPA (n = 100) compared with 1.4 GPA for fibres prepared with the prior art. Thus the current invention produces fibres of superior tensile strength and Young's modulus compared with the prior art.

As shown in Table 3-2, load to break and elongation to break of *Gonometa postica* silk obtained using the protocol described below is significantly higher (both $p < 0.0001$) compared to fibres obtained by carding after treating cocoons with fresh pineapple extract containing bromelain according to the previous art.

A further advantageous property of the silk produced by demineralising and degumming according to the present invention as described below in section 3.5 is that all mineral matter including the toxic calcium oxalate is entirely removed from wild silk. To illustrate this by way of example only, Figure 3-1 shows that after demineralisation no calcium oxalate could be detected in *Gonometa postica* silk using a Bruker D8 powder X-ray spectrometer. As calcium oxalate is highly toxic, its removal greatly improves the biocompatibility of wild silks prepared in this way.

In a further aspect of the invention demineralised and degummed fibres prepared according to the present invention can be dissolved in a chaotropic agent under milder conditions than those required in the prior arts. These milder conditions comprise a reduction in the

temperature of the chaotropic agent and/or a reduction in the concentration of the chaotropic agent and in some cases the use of an aqueous solution of lithium bromide in place of the more damaging lithium thiocyanate solution.

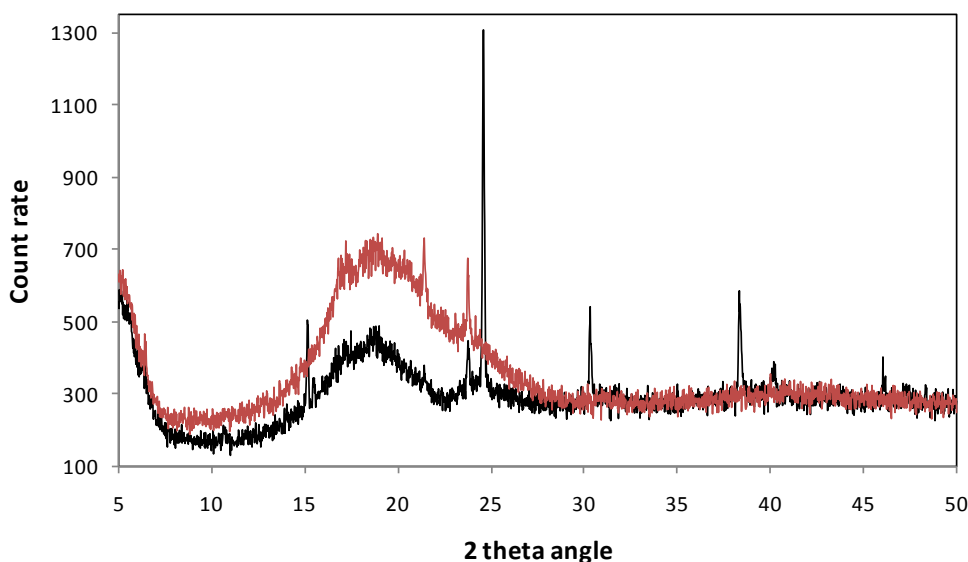


Figure 3-1: A comparison of the X-ray scattering spectra of the same *Gonometa postica* cocoon outer shell section before (shown in black)) and after demineralisation (shown in red). The peaks due to the crystalline mineral are entirely absent in the demineralised material.

Thus the milder conditions required for dissolving demineralised silks compared with those of the prior arts enables the formation of regenerated silk materials with improved properties and reduced cost. Silk solutions prepared from demineralised silk prepared according to the invention can be dialysed against deionised water and concentrated as will be understood by a person skilled in the art. Such a person will also know a plurality of ways for using resulting concentrated silk solution to form materials.

In a further aspect of the material prepared according to any aspect of the invention can be used in the manufacture of medical devices or for non-medical uses.

3.5. Protocol 1: demineralizing and degumming Wild Silk cocoons.

For a description of protocol 1 please refer to previous section 2.2.2 and for a more detailed description see Appendix A.2.

3.6. Protocol 2: tensile testing of Wild Silks

For a description of protocol 2 please refer to previous section 2.2.2 or for a more detailed description see Appendix A.2.

3.7. Efficiency of reeling

Demineralised *Gonometa postica* cocoons could be completely reeled as shown in Table 3-1.

Table 3-1: Reeling data of four different *G. postica* cocoons is shown. Both the total length of silk reeled from each cocoon and a visual estimate of the completeness of the reeling are tabulated.

Cocoon	Reeling completion	Total length reeled (m)
1	Almost complete	572
2	Complete	434
3	Complete	332
4	Partial	179

3.8. Results of tensile testing

The effect of combined demineralization and degumming using Protocol 1 (section 3.5) on the stress strain curve of *Gonometa postica* silk filaments is shown in Figure 3-2. The shape of the stress strain curve is much more consistent in the demineralised and degummed fibres compared to those degummed according to the prior art with pineapple juice. This probably results from highly variable damage during the carding used to separate the filaments.

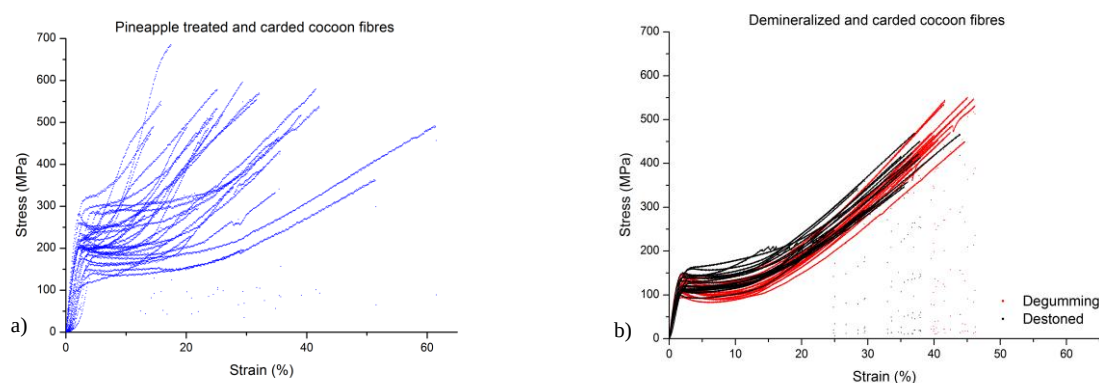


Figure 3-2: Stress-strain graphs from *G. postica* fibres extracted according to the prior art which involves treating with pineapple juice and carding (a), and fibres extracted by reeling after demineralising the cocoon (b) without degumming (black) and with degumming (red).

The effect of demineralization on its own, combined demineralization and degumming using sodium carbonates solution, and degumming without demineralization using the pineapple juice treatment of the prior art on the tensile properties of *Gonometa postica* silk filaments is shown in Table 3-2 and Figure 3-2.

Table 3-2: The effect of demineralisation, combined demineralisation and degumming according to the present method and treatment with pineapple juice according to the prior art on the tensile properties. Each box shows the mean, standard deviation and one tailed probability (ANOVA).

		Cross section (μm^2)	Break Load (N)	Break Stress (MPa)	Break Strain (%)	Breaking energy (J/cm ³)	Initial modulus (MPa)	# fibres (n)
A	Demineralised	254	0.080	310	28.4	51.8	7960	101
	st.dev. p-value (A-B)	53 <0.001	0.019 0.819	81 <0.001	9.5 <0.001	19.5 <0.001	1610 0.115	
B	Demineralised + degummed	198	0.080	401	37.9	77.9	8363	100
	st.dev. p-value (B-C)	36 <0.001	0.019 <0.001	98 0.037	7.5 <0.001	26.2 0.622	1385 <0.001	
C	Pineapple juice	125	0.056	443	27.7	75.3	10960	30
	st.dev. p-value (C-A)	26 <0.001	0.016 <0.001	129 <0.001	11.7 0.728	35.7 <0.001	3188 <0.001	
R ² -value		0.40	0.16	0.23	0.22	0.21	0.22	

Compared with the prior art, *Gonometa postica* silk filaments prepared by demineralising the cocoon are highly significantly stronger ($p < 0.001$), 0.056N against 0.080N (Table 3-2). However, the breaking stress of the demineralised silk filaments is significantly less ($p < 0.05$) due to the greatly reduced cross sectional area of the filaments prepared according to the prior art.

Figure 3-3(c) shows that pineapple juice produces a highly significantly ($p < 0.001$) reduction in the cross sectional area of the fibres ($125 \mu\text{m}^2$) in comparison to the two other treatments. This may result from the high efficiency of pineapple juice for removing the sericin gum, most probably because of the active bromelain in this extract. The enzyme might also contribute to the greater variability of the mechanical properties of the silk treated with pineapple juice compared with the demineralised/degummed filaments. Although the breaking energy (Figure 3-3(f)) is not significantly greater ($p > 0.05$) compared with the pineapple degummed fibres the demineralised/degummed filaments do have a significantly larger ($p < 0.001$) elongation to break (Figure 3-3(e)).

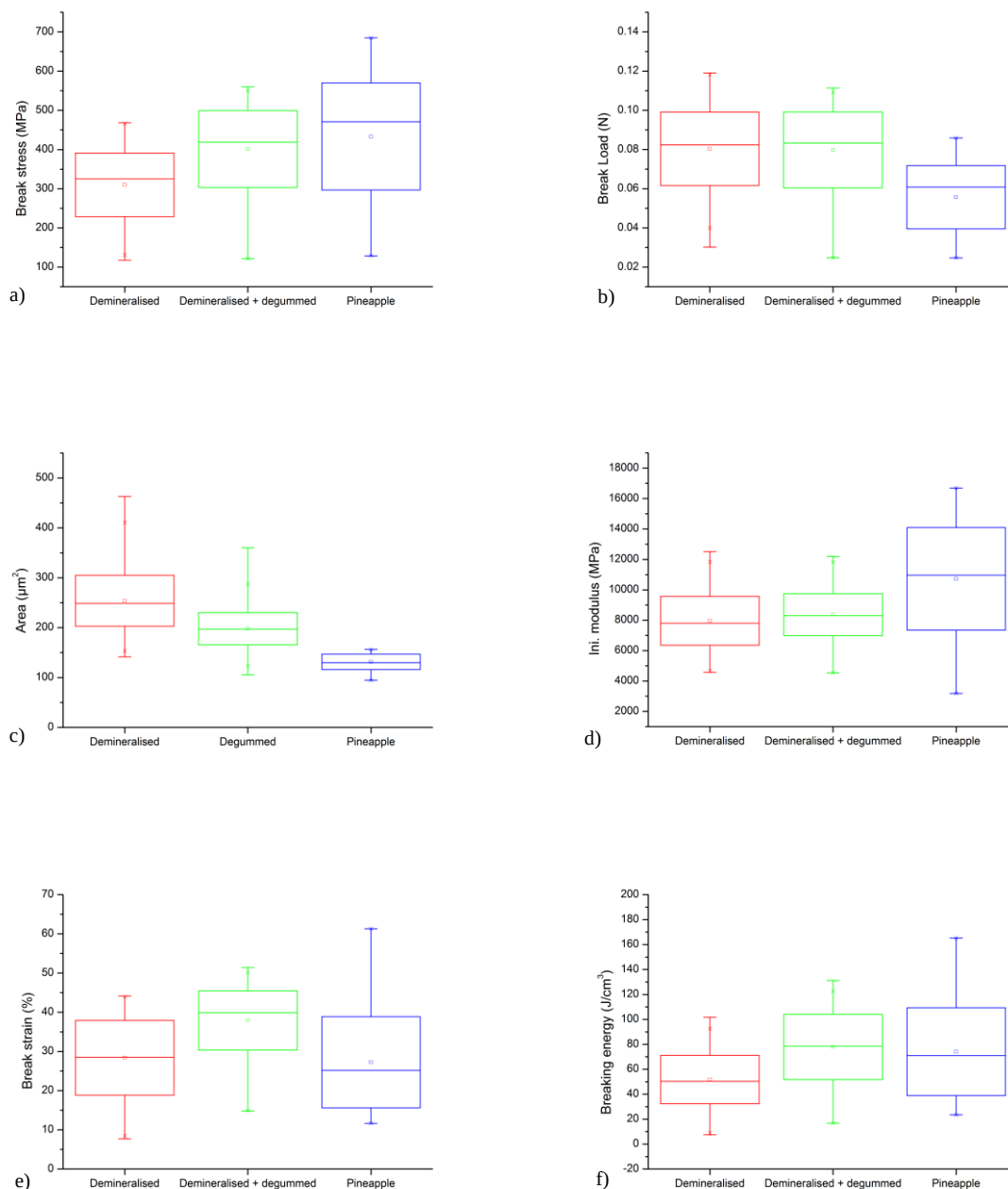


Figure 3-3: Box plots comparing the effect of demineralisation (red), demineralisation/sodium carbonate degumming (green) and pineapple degumming (blue) on the tensile properties of the silk filaments: break stress (a); the break load (b); the cross sectional area (c); the Young's modulus (d; break strain (e); and breaking energy (f). The top and bottom of the boxes represent $\pm$ the standard deviations while the vertical bars represent the minimum and maximum values. The mean value is represented by a dot in the box while the line in the box represents the median; n = 150 in each case.

3.9. Claims

- 1) A method to facilitate the reeling or carding of the cocoons of wild silkworms by first treating them with an aqueous solution of a demineralising agent comprising one or more of the following:
 - ethylenediaminetetraacetic acid
 - ethylenediaminetetraacetic acid salt
 - ethyleneglycoltetraacetic acid
 - ethyleneglycoltetraacetic acid salt
 - citric acid
 - fruit juice containing citric acid
 - deionized water
 - aluminium nitrate
 - phosphate ions
 - magnesium ions
- 2) A method for according to claim 1 wherein the cocoon is pre-treated with a dilute aqueous solution of ammonia or ammonium ions before treatment with the demineralising agent.
- 3) A method according to either of claims 1 to 2 wherein the demineralised silk cocoons are subsequently reeled.
- 4) A method according to claims 1 to 3 wherein the substantially all of the silk in the cocoon can be reeled.

- 5) A method according to any of claims 1 to 3 wherein the demineralised cocoons or reeled silk derived from them are subjected to the additional steps of :
- Removing the sericin coating by a degumming method;
 - Dissolution in a chaotropic agent;
 - Dialysis against deionised water or an aqueous solution;
 - Concentration of the silk protein;
 - Forming of the silk protein into an object.
- 6) A material prepared from wild silk cocoons treated with a demineralizing agent comprising one or more of the following aqueous solutions:
- ethylenediaminetetraacetic acid
 - ethylenediaminetetraacetic acid salt
 - ethyleneglycoltetracetic acid
 - ethyleneglycoltetracetic acid salt
 - citric acid
 - fruit juice containing citric acid
 - deionized water
 - aluminium nitrate
 - phosphate ions
 - magnesium ions
- 7) A material prepared by reeling or carding wild Silk cocoons from wild silk cocoons treated with a demineralizing agent to claim 6.
- 8) A silk material according to any of claims 6 to 7 wherein the silk material is a brin, bave, single, twist, thread, yarn , or cloth in which the filaments or staples have a mean staple length between 35mm and 1 m.

- 9) A silk material according to any of claims 6 to 7 wherein the silk material is a brin, bave, single, twist, thread, yarn , or cloth in which the filaments or staples have a mean staple length between 1 m and 350 m.
- 10) A silk material prepared from demineralised cocoons according to any of claims 6-9 and comprised of regenerated wild silk.
- 11) A silk material according to any of claims 6 to 10 wherein the calcium oxalate or other mineral initially present in the cocoon has been completely removed leaving much or substantially all of the sericin coat intact.
- 12) A silk material according to any of claims 5 to 11 wherein the material is wholly or substantially biocompatible.
- 13) A silk according the claims 5 to 11 wherein one or more of the following list of properties are significantly increased by prior treatment of the cocoons with demineralising agent relative to materials prepared without demineralisation:
 - Breaking load,
 - Extension to break,
 - Young's modulus,
 - Breaking energy,
 - Wear resistance,
 - Lustre,
 - Hand,
 - Obtained yarn
- 14) A silk material according to claims 5 to 13 wherein the material is formed into a medical device.

3.10. Conclusion

A method for demineralising wild silk cocoons under mild conditions is described, making it possible both to reel cocoons from species which can only be carded with the prior arts and to wet reel cocoons from species which can only be dry reeled or semi-dry reeled with the prior arts. This, in both cases reduces damage caused in processing and results in single brins and baves with markedly improved consistency in mechanical properties and increased strength and elongation to break. The method also allows substantially all of the silk to be wet reeled from cocoons. For species where the current invention enables silk to be reeled where the prior arts only enabled the carding and spinning of silk, the invention makes it possible to form singles, twists and yarns with very long staple lengths and hence greatly superior properties including greater strength, toughness, consistency of diameter, and lustre compared with the short staple fibres prepared according to the prior arts. Demineralisation according to the invention also enables regenerated silk solutions to be prepared under milder conditions compared with the prior arts reducing costs and improving the properties of materials formed from the regenerated silk. The method of demineralising wild silks according to the present invention also produces materials with reduced or abolished toxicity and allergenicity.

Chapter 4

Comparison of the behavioural aspects of mineralisation in *B. mori* and *G. postica*

Abstract and motivation

In previous chapters, Chapter 2 and Chapter 3, a close relationship was found between the mineralisation of Wild Silkmoth cocoons and the ease of reeling these cocoons. This led to the formulation of the hypothesis that the ease of reeling of *B. mori* cocoons might result from the lack of mineralisation of the cocoon shell. The aim of the work for the present chapter was to test this hypothesis and compare the spinning behaviour of this species with that of *G. postica* to discover whether this could explain the difference in mineralisation of the cocoons. The latter species was selected as it has the most heavily mineralised cocoon of all the species we have studied.

We show that a slight change in the timing of the release of calcium oxalate from the Malpighian tubules has dramatic consequences on the calcium oxalate content of the cocoon shell and consequently on the ease with which it can be reeled.

4.1. Introduction

Worldwide there is a vast range of silkworm species (Bombycidae, Saturniidae, Lasiocampidae, Thaumetopoeidae) that produce cocoons while only a few species have been reeled commercially of which the mulberry silkworm, *Bombyx mori*, was by far the most important. The ease of reeling *B. mori* cocoons undoubtedly contributes to this fact. In contrast, only a few species of Wild Silkworms' cocoons can be softened and reeled, including *Antheraea mylitta* (Tussah silk), *Antheraea pernyi* (Tussah silk), *Antheraea assama* (Muga silk) and *Phylisomia ricini* (Eri silk).

The range of silks that could be wet reeled has recently been extended by selectively removing the mineral phase present in Wild Silkworm cocoons prior to reeling, which we have

termed “demineralising” (seen in Chapter 2 and Chapter 3). This investigation demonstrated the significance of the mineral phase, calcium oxalate, in preventing cocoons from being reeled (Chapter 2).

This now raised the question how and where calcium oxalate was formed in silkworm cocoons and why *B. mori* cocoons were easily softened. The main aim of the present chapter is to investigate the timing of the release of calcium oxalate from the Malpighian tubules in *B. mori* and *Gonometa postica*.

We demonstrate that the timing of the release of the contents of the Malpighian tubules before the commencement of spinning in *B. mori* resulted in the absence of a mineral phase in these cocoons. We show that in contrast in Wild Silkworms the contents of the Malpighian tubules are released during the construction of the outermost layer (shell) of the cocoon. We suggest that for four millennia the common silkworm *B. mori* may have been selected for this behaviour in order to obtain the most easily reelable cocoons.

4.2. Material and methods

4.2.1. Material

For this study, it was decided to compare the spinning behaviour and cocoon composition of *B. mori* with a representative species of the Wild Silkworms, namely *Gonometa postica*, as this species (a) had been the subject of an excellent study of its ecology¹⁰⁵, yet the cocoon spinning behaviour had never been reported (b), had one of the most heavily mineralised cocoons (Figure 2-3) and (c) had a cocoon structure with some additional defensive features in contrast to other Wild Silkworms. All together, these features made *G. postica* the most interesting of the Wild Silks to investigate because any additional information about the

cocoon composition and formation would result in a better understanding of how to obtain and prepare cocoons for reeling, ultimately to increase further their commercial potential.

For the study of the cocoon spinning behaviour, eggs of *Gonometa postica* were obtained from ICIPE, Kenya. The emerging hatchlings were fed on leaves of *Acacia* sp. at room temperature and a relative humidity of 65%. After 13 to 16 weeks the final instar spun a cocoon whereof three were filmed while spinning a cocoon.

For *Bombyx mori*, hatchlings were fed on White Mulberry leaves (*Morus alba* L.), at ICIPE in Kenya and sent over in their final instar (5th). On arrival, they were fed a few more days on White Mulberry leaves at room temperature and a relative humidity of 60% where after cocoons were being spun. Five silkmoths were recorded spinning a cocoon. The naturally excreted Malpighian fluid was collected on Whatman filter paper (Nr.4) from final instar *B. mori*, after defecation and before spinning, and was, eluted from the paper with demineralised water (type I, MilliQ) and freeze dried to give a white powder.

Cocoons of *G. postica* and *B. mori* were obtained from ICIPE in Kenya. To analyze the mineral content of the cocoon shell, several samples were used per cocoon (n: 5) per species, typically after the emergence of the moth and the pupal remains were removed through the emergence hole using forceps. From each cocoon, 10 discs with a diameter of 4.86 mm were pressed out of the cocoon at random locations with a sharp hole-punch. Of these discs, four were used for making cross sections of the cocoon shell by means of a sharp razor blade, whereby one half of each disc was used as a control and the other half was demineralised.

4.2.2. Methods

Demineralising method: the same standard protocol for demineralisation was used as described in previous chapter 2.2. In short, the samples (10 discs per 600ml) were immersed in a warm (40°C), 1M solution of ethylenediamine-tetraacetic acid (EDTA) at pH 10 for 72

hours and gently stirred. Samples were then thoroughly washed with running tap water (three times three hours) and air dried overnight at room temperature.

Cocoon spinning: Silkworms were put in a plastic enclosure (30cm x 60cm x 40cm) with branches of the host plant at room temperature and a relative humidity of 60%. The silkworms were gradually time shifted over a week to an inverse 12L/12D period and the worms started spinning at 7pm shifted time. During darkness a red filter was used for filming with a JVC camera and recorded in real-time to DVD.

Amino acid analysis (AAA): One small sample per cocoon (n: 3) of *G. postica* before and after demineralising was cut off the cocoon shell and send to the Oxford University Biochemistry Department for amino acid analysis. The resulting glycine values may be overloaded but the narrowness of the standard deviation suggested that this effect may be constant or small. Sadly the sample of *B. mori* was not analyzed as a result of closure of this facility.

The methods and settings used for SEM, EDX, XRD and FTIR were identical to those described in Chapter 2. For these instruments, five discs and five cross sections per cocoon (n: 5) per species were used before and after demineralising. Representative spectra for each instrument and treatment are given below. For FTIR, five discs of each cocoon (n: 5) were sampled three times on each side (inside and outside of cocoon shell) before and after demineralising. All spectra per treatment, disc side and species were averaged.

To make the false colour image presented in Figure 4-1 (b, top half), raw images of the mapping for calcium and oxygen of both species were loaded in Adobe Photoshop CS3 (version 10.0.1) and a threshold level was set to 100. The remaining pixels were then coloured red for calcium and yellow for oxygen. The resulting images were then halved and superimposed over the original SEM image.

4.3. Results and discussion

4.3.1. Cocoon spinning behaviour

A. *Gonometa postica*

The Lasiocampid, *Gonometa postica*, constructed its cocoon in the same four successive phases that were recognized in other wild silkmoth species, like *Antheraea pernyi*^{116, 117} and *Platysamia cecropia*^{118, 119}. In our study, the start of cocoon spinning was taken from the gut purging moment of the silkmoth, adding one extra phase referred to as site location prior to these four previously recognised phases as we show that this phase is important in the difference between *G. postica* and *B. mori* cocoon forming behaviour.

This resulted in the following five successive phases in cocoon spinning (Figure 4-1):

- 1) site location
- 2) cocoon scaffolding
- 3) formation of the outer cocoon
- 4) cocoon impregnation
- 5) formation of the inner cocoon

Before spinning, *G. postica* fed on a large quantity of leaves followed by gut evacuation (known as gut purge (GP)). The gut purge produced a sticky mass of faeces with the absence of a watery secretion (see further). A period of wandering followed (2 ± 1 h) in which the silkworm located a suitable spinning place (site location, Figure 4-1, a (1)). Once the location was determined the silkworms started spinning the scaffold in a head down position. The scaffolding consisted of fibres attached to leaves and twigs, parallel to the stem of the twig. In this phase, much time was spent on the claw-like, scaffold attachment of the silk (cocoon) to the stem. Once the scaffolding finished (1.5 ± 0.5 h, Figure 4-1, a (2)) the silkworm started spinning in the figure-of-eight movement, also seen in *B. mori*, marking the start of the outer layer of the cocoon (Figure 4-1, a (3)), making a bag like structure from the bottom up. Once the initial bag was finished, the silkworm settled in a doubled up posture, continuing spinning

while closing the top of the initially open bag. After spinning part of the outer layer (± 3 h), the silkworm suddenly started secreting a white fluid on this layer, marking the commencement of the impregnation phase (Figure 4-1, a (4)) which lasted approximately thirty minutes. This liquid was distributed on the inner wall of the bag by the anus and the silkworm continued spinning in this secretion. Upon wetting with this fluid, the cocoon was seen to shrink. The silkworm appeared to respond to this by pushing the cocoon outward with its head capsule. This behaviour was very similar to the cocoon expansion behaviour (CEB) seen in *B. mori*¹²⁰. Directly after the impregnation phase and before this secretion was dry (± 24 h), specially adapted hairs originating from the silkmoths' back were pushed through the cocoon shell. These were cemented into the outer layer by the secretion upon drying, probably forming an additional defence against predators (Figure 4-1, a (4)). Once the secretion was dry, it became impossible to see the silkworm spinning making it difficult to determine the exact time spend on the outer and inner layer. However, when the secretion was dry (± 24 h), we could hear a slight rustling sound revealing that the silkworm spun for a further 24h.

As seen in other Wild silkmoths (Figure 4-1, a), the end of the impregnation marked the beginning of the spinning of the inner layer. This seemed unlikely for *G. postica* because the silkworm was only spinning for about three hours on the outside layer until the impregnation phase and was very short in comparison to the other wild silkmoths, such as *A. pernyi* (10-20h) and *Cecropia* (24h) (Figure 4-1, a (3)). Therefore we suggest that the outer layer continued to be spun after the impregnation phase until this secretion was dry. After the impregnation fluid was dry, the silkworm might have taken a break while the secretion dried or continued spinning the inner layer for another 24h while the outer layer dried. Both possibilities could account for the very distinct separation seen between the two layers in the cocoon shell shown in Figure 4-1 (b) and the observation that the inner layer was as thick as or in some cases thicker than the outer layer.

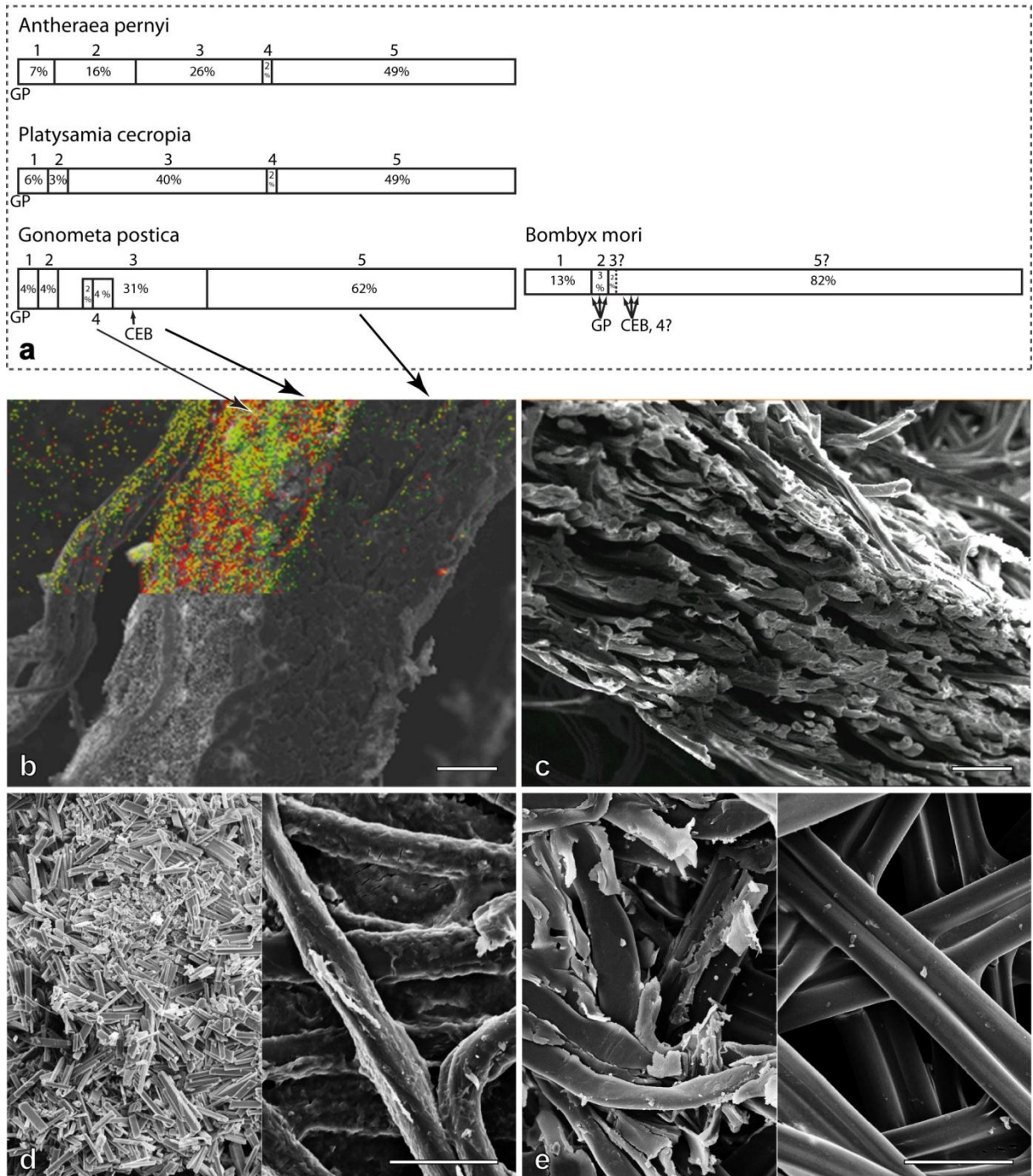


Figure 4-1. a) Cocoon construction time lines for *A. pernyi*, *S. cecropia*, *G. postica* and *B. mori* showing the percentage time (%) spend on each spinning phase (total time: ± 48 h) mentioned in section 4.3.1.A. (CEB: cocoon expansion behaviour). b-c) High magnification SEM pictures (scale bar: 100 μ m) of the cross-section of the cocoon shell of *G. postica* (b) and *B. mori* (c), split in an upper and lower half, showing the natural and demineralised cocoon respectively with superimposed the EDX mapping for oxygen (yellow) and calcium (red). d-e) High magnification pairs of SEM pictures (500x, scale bar 50 μ m) of the same untreated (natural; left-hand side) and treated (demineralized; right-hand side) cocoon shell of *G. postica* (d) and *B. mori* (e).

B. *Bombyx mori*

The cocoon spinning behaviour of *Bombyx mori* was studied before by Yanagisawa¹²¹⁻¹²³ and more recently by Kiyosawa^{124, 125} who observed that *B. mori* had five successive phases in cocoon spinning, namely: finding an appropriate cocooning place (1), construction of a scaffold (2), formation of the large rough shape of the cocoon (3), reducing the shape into the final size (4) and spinning the cocoon shell (5). These phases generally resemble the phases seen in Wild Silkmoths however there are some significant differences.

It was clear that the spinning of *B. mori* differed significantly from that of *G. postica* and other Wild Silkmoths. Instead of the usual gut purge seen in Wild Silkmoths indicating the start of cocoon spinning, the silkworms of *B. mori* first searched for a suitable cocooning place (site location, 8h) after feeding (Figure 4-1, a (1)). Once a suitable location was found, an initial scaffold was spun (Figure 4-1, a (2)). In the first half of the scaffolding phase, the gut was purged (GP) forming a white/pinkish stool. Right after or simultaneously with the gut purge, a white, watery secretion was secreted (Figure 4-1, a (4)). Following the gut purge, they either left their scaffold and started spinning elsewhere (n: 3) or continued spinning in the old scaffold (n: 2, 3 hours). After a few hours, the silkworm started spinning a large rough cocoon shape (Figure 4-1, a (n= 3)). Later this shape was reduced to its final size. During this phase the previously observed cocoon expansion behaviour (CEB) was noticed in which the larva stretched its neck region and pressed the head outward for a moment, expanding the cocoon¹²⁴. Afterwards, the silkworm spun the cocoon shell, taking about 48h to three days for completion (Figure 4-1, a (5?)).

C. Comparison

Whereas the cocoon spinning behaviour of *G. postica* generally followed the same successive stages seen in other Wild Silkmoths, *B. mori* differed significantly from these (Figure 4-1, a). In *B. mori* many of the cocoon spinning phases seemed to have been shifted compared to Wild Silkmoths, making it difficult to correctly identify the impregnation phase and the inner/outer layer spinning phase. However, the characteristic cocoon expansion behaviour was found in both species (Figure 4-1, a (CEB)). In *G. postica* this behaviour was seen in association with the shrinking of the cocoon upon wetting during the impregnation phase. The same expansion behaviour was seen in *B. mori*, implying that the silkworm was spinning the outer layer of the cocoon and that the GP or peeing during the scaffolding in *B. mori* could be a vestige of, or analogous to, the impregnation phase. Furthermore, this assumption suggested that the outer layer was very much reduced and that the main thickness of the cocoon shell of *B. mori* was actually the inner layer (Figure 4-1, a (5)).

Additionally, the gut purge was delayed to before, during or after the scaffolding phase in *B. mori*, in complete contrast with the Wild Silkmoth species where the gut purge happens even before the site location phase (Figure 4-1, a). Furthermore, the gut purge was followed by a white, watery secretion in *B. mori*. This timing is not seen in Wild Silkmoth species. This secretion in *B. mori* resembled the one seen in the impregnation phase in Wild Silkmoths, containing the mineral phase and thus suggested that this secretion actually was the impregnation liquid. Although the FTIR-spectra of the gut purge and watery secretion (Figure 4-2, c (9-10)) indicated that both shared some similar compounds, the presence or absence of calcium oxalate, oxalate ions or calcium carbonate could neither be confirmed nor excluded. Either way, this secretion took place outside the cocoon resulting in no mineralisation and no initial tanning of the cocoon.

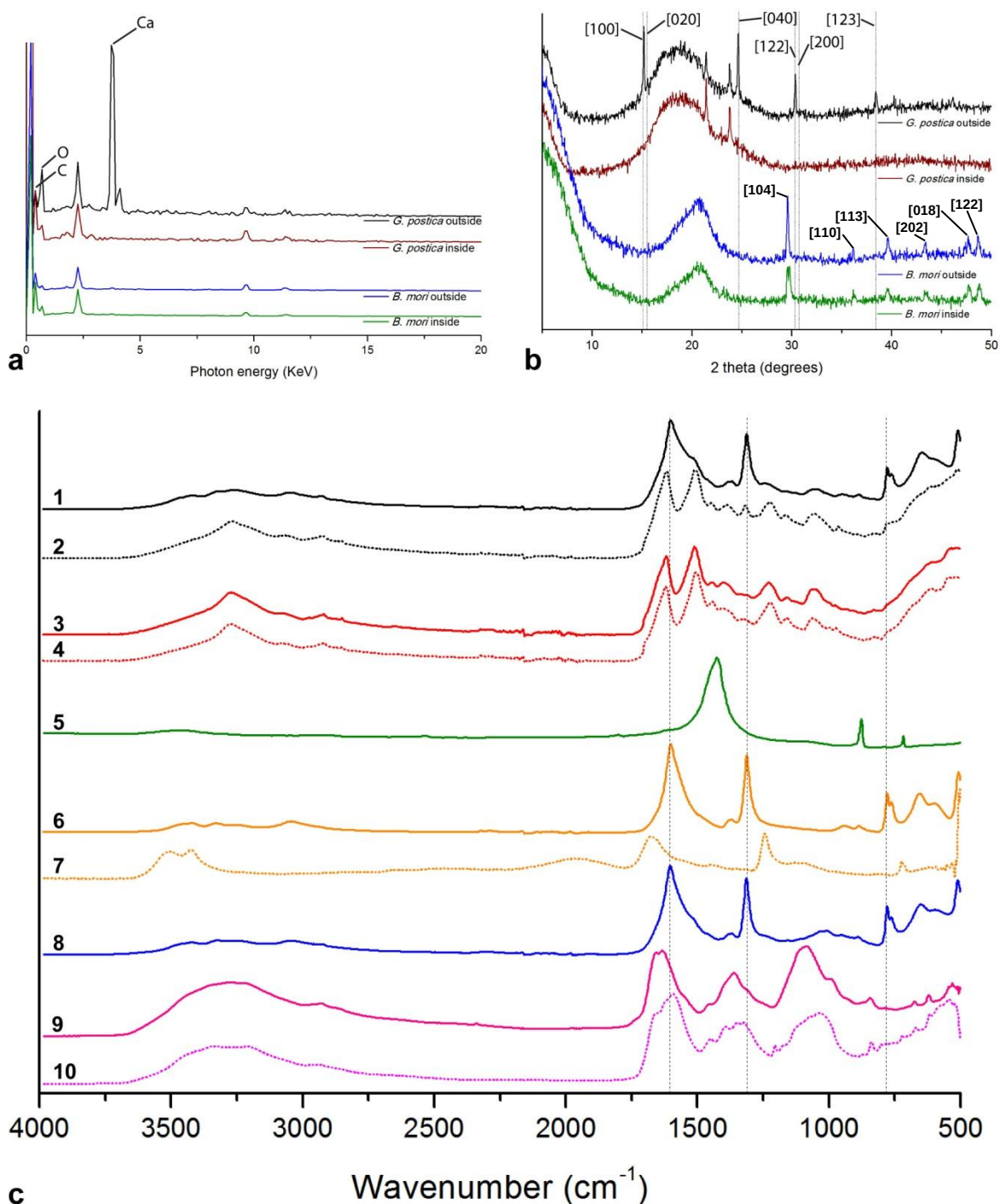


Figure 4-2. a-b) The EDX (a) and XRD (b) of the outer (black) and inner (red) layer of the cocoon in *G. postica* in comparison with the outer (blue) and inner (green) layer of the cocoon in *B. mori* indexed to calcium oxalate (JCPDS card 01-077-1160). Some traces of calcium carbonate were seen in the XRD-spectra of *B. mori* but were absent in the EDX-spectra. The FTIR-spectra (c) indicated that calcium oxalate was present in the outer (1) and inner (2) layer of *G. postica* and not in the outer (3) and inner (4) layer of *B. mori*. Also the impregnation fluid of *G. postica* (8) and the secretion (9) and poo (10) of *B. mori* during spinning were compared with pure calcium carbonate (5), calcium oxalate (6) and oxalate ion (7).

4.3.2. Cocoon shell analysis

In Figure 4-1 (d and e, left side) it is shown that the outer layer of the cocoon of *B. mori* did not appear to have the mineralized outer cocoon shell of *G. postica* and other Wild Silkmoth species (see chapter 2.3.1). The XRD-spectra (Figure 4-2, b) indicated the presence of calcium oxalate hydrate in the outer layer of *G. postica* (black) and the absence in the cocoon of *B. mori* (outer (blue) and inner (green) layer) and inner layer of *G. postica* (red). However in *B. mori*, the XRD-spectrum of the cocoon shell showed major reflections at 2-theta [104], [110], [113], [202], [018] and [122] that could be indexed to calcite (JCPDS card of calcite, pattern 01-077-1160) (Figure 4-2, (b), green and blue). Nevertheless, this mineral presence could not be confirmed by FTIR or EDX (Figure 4-2; a (blue and green) and c (red, 3 and 4)) and was therefore considered to be present in minute quantities in the cocoon shell of *B. mori*. Later, we found out that the encountered calcium carbonate was probably an artefact introduced by our suppliers who added powdered calcium carbonate during rearing of the silkmoths to prevent diseases. Overall it was concluded that any form of mineral coating was missing in the cocoon shell of *B. mori* in contrast to most Wild Silkmoths whose outer cocoon shell was coated with a mineral layer of calcium oxalate.

This was further confirmed by the clear division seen between the outer and inner layer of the cocoon shell of *G. postica*, with the outer layer having a high electron emissivity appearing white in scanning electron micrographs and the inner layer appearing dark grey (Figure 4-1, b (bottom half)). Thus, the previously observed white secretion during the impregnation phase was only located in this outer layer. When mapping this cross section with EDX for calcium (red) and oxygen (yellow) (Figure 4-1, b (upper half)), it was confirmed that only this white outer layer contained an abundance of calcium and oxygen, and consequently consisted of calcium oxalate. In *B. mori* the cocoon shell was extremely uniform lacking a clear separation between outer and inner layer as seen in Wild Silkmoth cocoons (Figure 4-1, c (bottom half)).

Furthermore, EDX mapping did confirm the absence of a mineral phase (no calcium or oxygen) in the cocoon shell cross section of *B. mori* (Figure 4-1, c (upper half)).

When the outer layer of *G. postica* and *B. mori* before and after demineralizing were compared, the mineral coating in *G. postica* was clearly removed whereas in *B. mori*, without any mineral phase, the fibres looked much cleaner implying some material was removed from these fibres by demineralizing. The observation that the cocoon was still compact after demineralizing suggested that the sericin was still present holding the fibres together and that an outer component or the outer most sericin layer of the fibre was removed by this treatment. The small quantity of material removed by the demineralising treatment may represent soluble salts or insoluble mineral or other small molecular weight compounds, originating from the inorganic components of the sericin or perhaps the fibroin, which precipitate or crystallise on the surface of the silk baves as they dry slowly after passing through the spigot. Amino acid analysis of the cocoon shell of *G. postica* before and after demineralizing indicated that this coating may contain peptides rich in aspartic acid, glutamic acid, serine and threonine (Figure 4-3). In *B. mori*, the same amino acids were found in abundance in the outer most sericin coating of the silk fibres^{109, 126}. These amino acids are known to play a role in controlling the calcium oxalate formation¹²⁷⁻¹²⁹ and therefore this outer sericin layer may be an interaction layer for the binding of the silk fibre/cocoon with calcium oxalate.

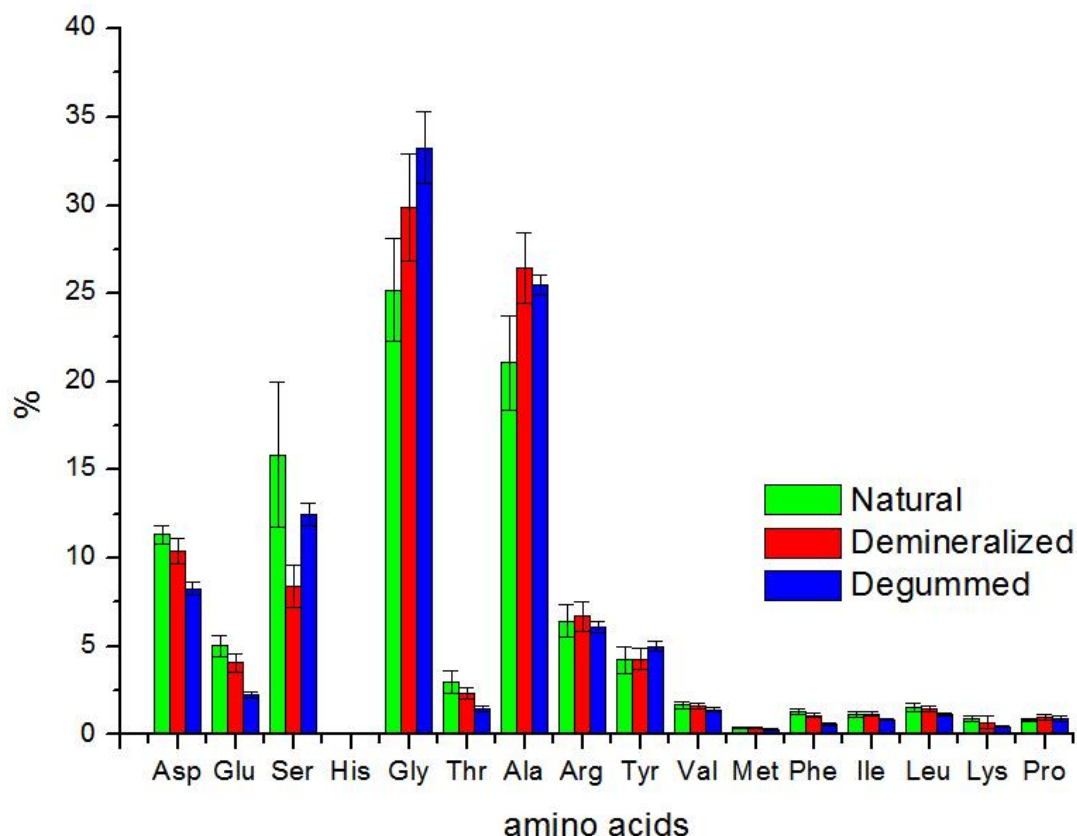


Figure 4-3. Column graph of the mean percentage amino acid composition of the natural cocoon shell of *G. postica* (green, n: 5), after demineralizing (red, n: 5) and after demineralizing/degumming (blue, n: 5) with the standard deviation indicated by lines.

4.4. Conclusion

The spinning behaviour of *G. postica* followed the main successive cocoon spinning stages seen in other Wild Silkmoth species. We found that the impregnation liquid consisted of calcium oxalate monohydrate and that it was selectively deposited in the outer shell of the cocoon. Demineralizing the cocoon shell of *G. postica* did seem to remove some peptides rich in aspartic acid, threonine, glutamic acid and serine. These amino acids are known to play a

role in the initiation, inhibition and formation of calcium oxalate crystals¹²⁷⁻¹³² and may play an important role in the binding and formation of the calcium oxalate shell.

B. mori differed from the Wild Silkmoths studied in that no detectable endogenous mineral material was present in the cocoon shell and no impregnation phase was seen during spinning inside the cocoon. However, during the scaffolding phase a whitish watery fluid was secreted together with or after the gut purge (GP). This early timing of discharge of fluid is thought to prevent mineralisation and tanning in *B. mori*. Although no proof was presented that this liquid did contain calcium oxalate, the presence of calcium oxalate in the leaves of the White Mulberry tree¹³³ and the abundant calcium oxalate crystals found in the Malpighian tubules of *B. mori*^{134, 135} strongly suggested that the watery Malpighian secretion of this species did contain calcium oxalate but was voided before it could be incorporated into the cocoon shell.

These observations together with the observed cocoon expansion behaviour and the outer sericin, lead us to suggest that the successive phases seen in Wild Silkmoths also occur in *B. mori* but that these somehow have been shifted backward in time resulting in the impregnation phase taking place before the wall of the cocoon is constructed. Because this leads to a mineral free cocoon and therefore a reelable cocoon, we suggest that *B. mori* has been selected for more than 4000 years on this behaviour for obtaining the best reelable cocoon for use in textiles.

Chapter 5

Identification of the yellow pigment in *Nephila edulis*

5.1. Introduction and motivation

In this chapter, the focus is on small molecules secreted together with the silk fibroin during spinning (see Figure 1-4). As discussed in chapter 1.4, polyphenols could either reversibly bind to the silk peptide by ionic or hydrogen bonding, or irreversibly through covalent bonding. Small molecules in the form of phenolics were detected in wild silkmoth silks and have shown to be involved in the cross-linking of these silks⁴⁷. In silkmoths, the cocoon is only used for pupating and therefore the silk is not recycled. By cross-linking the silk, the cocoon shell stiffens incredibly forming a strong protection against possible predators. Furthermore, this cocoon shell can be additionally reinforced by a mineral coating and defensive hairs as seen in Chapter 2.

In spiders however, neither mineralisation nor irreversible cross-linking occurs as the resources for making the silk fibres are scarce and therefore spiders recycle their silks, except for the egg sac silk. Because phenols and polyphenols were also found in the dragline silk of *Nephila clavipes*⁸⁶, we formulated the hypothesis that these small molecules were reversibly binding to the fibroin and in this way may contribute to the tensile properties of the resulting fibre. This was the subject of my PRS, showing that by washing these compounds out of the dragline fibres (*Nephila edulis*) the tensile properties decreased and these properties restored or even improved by washing the compounds back into the fibre (this however was not included in the thesis). However, the problem with this experiment was that the composition of the extraction as well as the concentration of the compounds present was unknown.

In the dragline silk of *Nephila clavipes* three compounds were found, namely xanthurenic acid, a hydroxylated benzoquinone or naphthoquinone and an unknown compound⁸⁶. In *Nephila edulis* mainly one compound was found responsible for the yellow coloration of the

fibre. This compound displayed the same characteristics as the unknown compound described for *Nephila clavipes*⁸⁶ and in this way needed identification.

The aim of this chapter is first to identify the unknown yellow pigment molecule in the dragline silk of *Nephila edulis*. Understanding the structure of this molecule will enable us to understand how it interacts with the protein and how it could be synthesized in large quantities for further experiments. Secondly, this yellow pigment is compared with that of egg sac silk to establish if they are identical. This would not only provide us with a second means of obtaining this pigment in large quantities but also show that this same molecule is present in different types of silk.

Therefore, the pigment is identified by using NMR, MS, FTIR and UV-spectroscopy. First we will discuss the chromatographic separation and purification by HPLC of the dragline and egg sac extractions. Subsequently these purified fractions were subjected to NMR to elucidate the main structure of the molecule enabling us to find a reference molecule to verify our assignments. These observations are then further confirmed by mass spectrometry. The obtained structure for the yellow pigment molecule from NMR and MS is validated by FTIR and UV spectroscopy.

We conclude that the yellow pigment present in dragline and egg sac silk was identical and is indeed a polyphenol, being xanthurenic acid with an additional substituted nitro group (7-nitro xanthurenic acid). This molecule has enormous hydrogen bonding capabilities and may play a role in the tensile properties of the fibre as discussed in chapter 1.4 and will be discussed in more detail in the general discussion (Chapter 7). However, we are unable to investigate this effect in the scope of this thesis because of the inability to obtain this molecule in large enough quantities. Therefore our focus is on synthesizing this molecule and a possible route for that is given in the next chapter (Chapter 6).

5.2. Material and methods

5.2.1. Samples

Spiders of the genus *Nephila edulis* were reared in the greenhouse at the University of Oxford (UK). The humidity in the greenhouse was kept constant with two rain cycles a day (2 x 5 min). The spiders were fed with house flies and fruit flies. The egg sacs were collected directly from the greenhouse and the silk was extracted after removal of the eggs. All dragline silk samples were obtained by forcible reeling from immobilized but awake spiders at room temperature ($25 \pm 2^\circ\text{C}$) and humidity ($60 \pm 5\%$). The samples were collected on glass slides (50 x 150mm) that were cleaned with ethanol to avoid contamination. In total 250 spiders were reeled for their dragline silk from the major ampullate glands at a reeling speed of 7.5mm/s. The egg sac and dragline silk was then washed with a 28% ammonia solution on a shaker at 330rpm (30min). In both cases, the washing solution extracted an orange/yellow pigmentation leaving the silk white to the naked eye. The wash solution was then evaporated in a vacuum oven at 60°C until a small volume of the extract was left and then diluted again with pure grade I MilliQ (Millipore) water. The resulting solution was filtered through a $0.2\mu\text{m}$ Acrodisc Minispine filter (Sigma-Aldrich) and the filtrate was freeze dried. A small fraction (0.5mg) of both resulting powders was then dissolved in 0.3ml water and used for analytical HPLC. The remaining powder of both extracts ($\pm 10\text{mg}$) was dissolved in 1ml water each and run on a semi-preparative column as described below. The yellow pigment peak (eluting at $\pm 14, 5\text{min}$) was collected manually from both extracts. These fractions were then evaporated to a smaller volume, repeatedly washed with Millipore water and freeze dried through several cycles to remove trifluoroacetic acid (TFA) derived from the HPLC mobile phase. The resulting purified powder (3.5mg) was then used for NMR, MS and FTIR.

5.2.2. Reagents

Standards of kynurenic acid, xanthurenic acid, L-tyrosine, 3-nitro-L-tyrosine, sodium hydroxide and hydrochloric acid were obtained from Sigma-Aldrich (Dorset, UK). For liquid chromatography and mass spectrometry (MS), HPLC grade solvents were obtained from Fisher Scientific: grad I water, methanol (UV cut-off: 200nm), acetonitrile and trifluoroacetic acid (TFA). For NMR, D₂O and formic acid were used (Sigma). Where not mentioned, all water used was ultrapure grade I MilliQ (Millipore) quality.

5.2.3. Methods

High Pressure Liquid Chromatography (HPLC): Separations of the dragline pigments were carried out on an HPLC-system from Varian, which consisted of three stand-alone modular units: a 9012Q solvent delivery system (Varian), an AI-200 Dynamax autosampler (Midas) and a Prostar 330 Photo Diode Array detector (PDA, Varian). Data was obtained on the Star Chromatographic Workstation (Star 6.41, LC Control Software) and spectral data was analyzed with the Polyview 2000 software. For the analytical separation, an X-Bridge C18 column (5µm, 4.6 x 250mm; Waters) with an X-Bridge C18 guard column (5µm, 4.6x20mm; Waters) was used. Extracted dragline samples and standard samples were injected (50µl) with a 100µl loop. Each sample was injected five times and the spectra and retention times were averaged.

For the preparative separation of the pigment for purification, a Discovery HS F5 C18 semi-preparative column (5µm, 10 x 250mm; Supelco) was used. Samples were injected with a 1000µl loop and the fraction containing the yellow pigment was collected manually.

For both, the analytical and preparative separation, the mobile phase consisted of 0.1% (v/v) trifluoroacetic acid (TFA, phase A) and methanol (MeOH) with 0.1% (v/v) TFA (phase B). Gradient elution was carried out at 1ml/min in a linear gradient from 5 to 95% (v/v) B/A over

a time span of 20 minutes followed by a 10 minutes equilibration of the column at 5% (v/v) phase B/phase A. The sample tray was cooled at 4°C, the column was kept at 40°C in the column oven of the autosampler, injections were performed with a 250 µl syringe and washes (30µl, 100% acetonitrile (ACN)) were programmed between injections to rinse the injection needle. The UV spectra (200-800nm) were obtained with a sampling interval of 200ms and a slit width of 1nm. The eluate was monitored continuously at 270nm and 250nm. Prior to each injection the noise of the PDA detector was measured (1 min) for an accurate noise setting for peak detection.

Fourier Transform Infra-red Spectroscopy (FTIR): The machine and settings used for this experiment were exactly the same as described in Chapter 2. Samples were prepared by dissolving a few small grains (50ug) of the purified pigment powder in 100 µl water (grade I, MilliQ, Millipore). Reference samples of pure xanthurenic acid (Sigma-Aldrich) were prepared in the same way. Under vacuum, the samples were dried to coat the ATR-crystal.

Nuclear Magnetic Resonance (NMR): The purified pigment powder ($\pm$ 3mg) was dissolved in D₂O in a quartz NMR tube (5 mm diameter bulb) titrated with aqueous 0.1M HCl or NaOH solution as required. The samples were then placed in a Bruker AVII 500 spectrometer equipped with a dual ¹³C-¹H cryogenic probe, operating at 500 MHz for ¹H and 125 MHz for ¹³C. The probe was regulated at 298K. A Bruker AVIII 700 equipped with a triple ¹H/¹³C/¹⁵N cryogenic probe, operating at 700 MHz for ¹H and 175 MHz for ¹³C also at 298K.

Mass spectrometry (MS): 50µg of the purified pigment powder was dissolved in Millipore water (500µl) and 10µL was injected into an LC/MS system. This comprised of a Waters Acquity UPLC-system coupled to a Waters SYNAPT HDMS-system. Accurate mass analysis was also performed using a Bruker uTOF and a Bruker 9.4T Fourier Transform-Ion

Cyclotron Resonance-MS (High resolution). The general LC-method used was a reversed phase linear gradient over 5 minutes using water in mobile phase A and acetonitrile (2.5% TFA) in mobile phase B. The mass spectrometers were all externally calibrated using a directly infused sodium formate solution.

5.3. Results

5.3.1. High Pressure Liquid Chromatography (HPLC)

A. Dragline pigment

Analytical separation of the extracted dragline pigment resulted in a chromatogram (270nm trace) with a peak at 14.56 minutes (± 0.40 min, n: 252, Figure 5-1, a (orange trace)). With the void peak at 4.5min this meant the pigment eluted at about 50/50, A/B mobile phase. The UV-absorbance spectrum (200 to 800nm) taken from the apex of that peak is given in Figure 5-1 (b, orange trace). The peak had a very characteristic and broad UV-absorbance spectrum reaching from 200 to 540nm with peaks at 226, 277, 337 and 396nm.

B. Egg sac pigment

Injection of the egg sac extract resulted in one common peak (yellow pigment) with a retention time of 14.59min (± 0.26 min, n: 64; Figure 5-1, a (red trace)). A two-tailed student's t-test indicated that this retention time was not significantly different from the retention time of the yellow pigment peak in the dragline extraction ($p > 0.05$). Furthermore, the UV-spectra of the yellow pigment peak of the egg sac extract (see Figure 5-1, b (red trace)) was an exact match (similarity index: 0.999) with that of the dragline extract (see Figure 5-1, b (orange trace)).

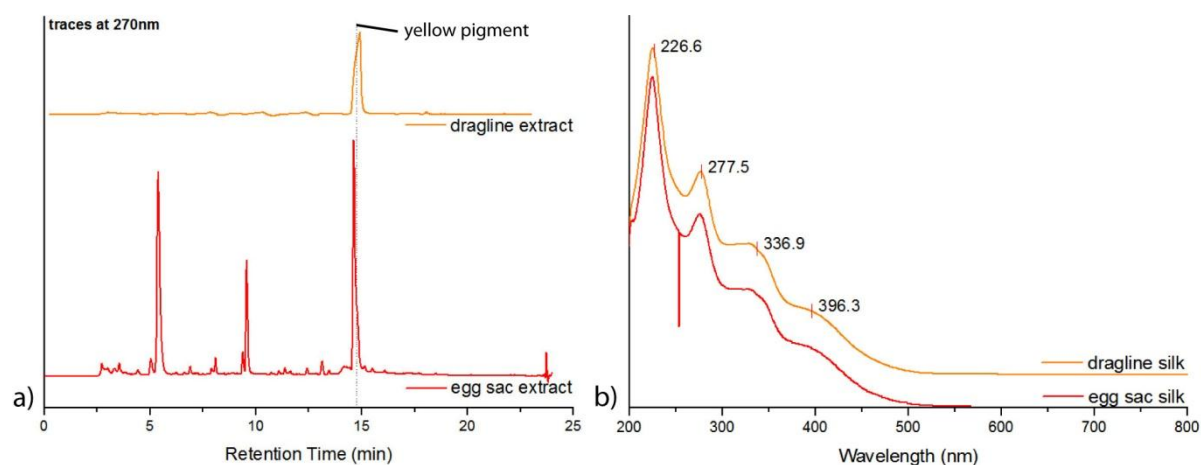


Figure 5-1. Chromatographic data of the dragline extract (orange trace) and egg sac extract (red trace) with (a) the chromatograms resulting in a common peak with apex at 14.5 minutes (analytical column see methods section) and UV-absorption spectra (200 to 800nm) of the apex of both chromatograms (b).

C. Summary

The broad UV-spectra and late retention time of the yellow pigment molecule indicated the molecule was possibly a ring structure with many conjugated double bonds.

Finding the same UV-spectra and retention time in both extractions, dragline and egg sac silk, were a strong indication that the yellow pigment was identical, however further confirmation was needed from other instruments like NMR, MS and FTIR (see next chapters).

5.3.2. Nuclear Magnetic Resonance (NMR)

A. General

Nuclear Magnetic Resonance (NMR) is a powerful technique for elucidating the structure of both small and large molecules^{136, 137}. Here ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC) was used for the study of the short range couplings whereas ^1H - ^{13}C Heteronuclear Multiple Bond Correlation (HMBC) was used for the longer range couplings.

In this subchapter, the dragline pigment is analyzed first which resulted in a partial structure of the molecule. Subsequently the egg sac pigment is compared with the dragline pigment as it was shown to be identical in the HPLC. Finally, to confirm the structure of the dragline pigment molecule the NMR-spectra are compared with those of xanthurenic acid as a reference molecule with a very similar structure. In this way it is not only possible to confirm the structure but also to elucidate the location and identity of any additionally substituted groups.

B. Dragline pigment

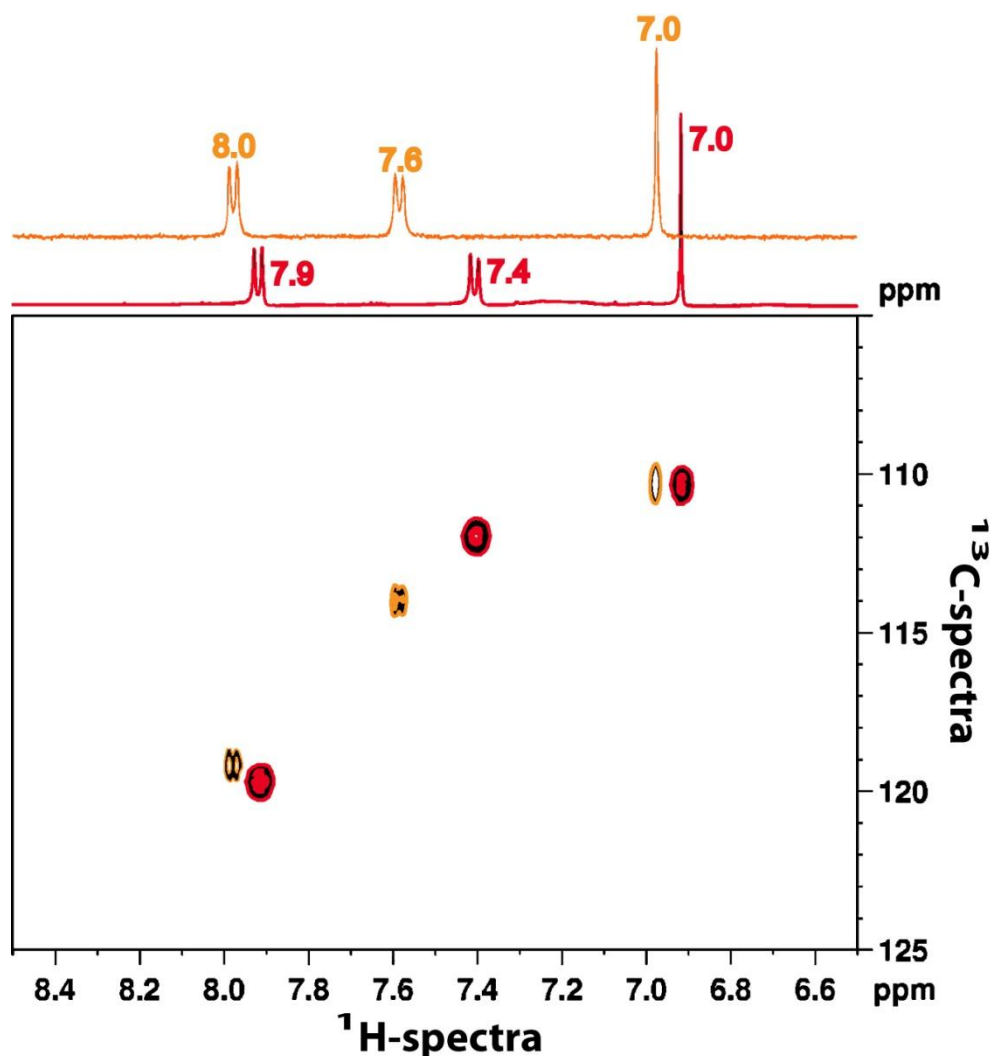


Figure 5-2. HSQC of the purified extracted dragline pigment (orange) and egg sac pigment (red) in acidified D_2O .

Integrating the peaks from the HSQC ^1H spectra revealed three single classes of hydrogen atoms (Figure 5-2, orange). In the HSQC (Figure 5-2, orange) as well as in the HMBC (Figure 5-3, orange), the hydrogens had chemical shifts in the range of 7 to 8 ppm indicating that these were aromatic hydrogen atoms (6 to 8.5 ppm)^{136, 137}. From the HSQC direct couplings between the hydrogen and carbon, it can be inferred that the hydrogen at 7.0 ppm was linked to the carbon atom 110ppm, the hydrogen at 7.6ppm was linked to the carbon at

114ppm and the hydrogen at 8.0ppm was linked to the carbon atom at 119ppm (see Figure 5-2 and Table 5-1).

The ^{13}C spectra of the HMBC revealed ten carbon atoms. Most of these carbons fell in the ppm range of aromatic carbons (arenes: 115-145ppm) indicating the molecules' main structure was possibly a double ring structure^{136, 137}.

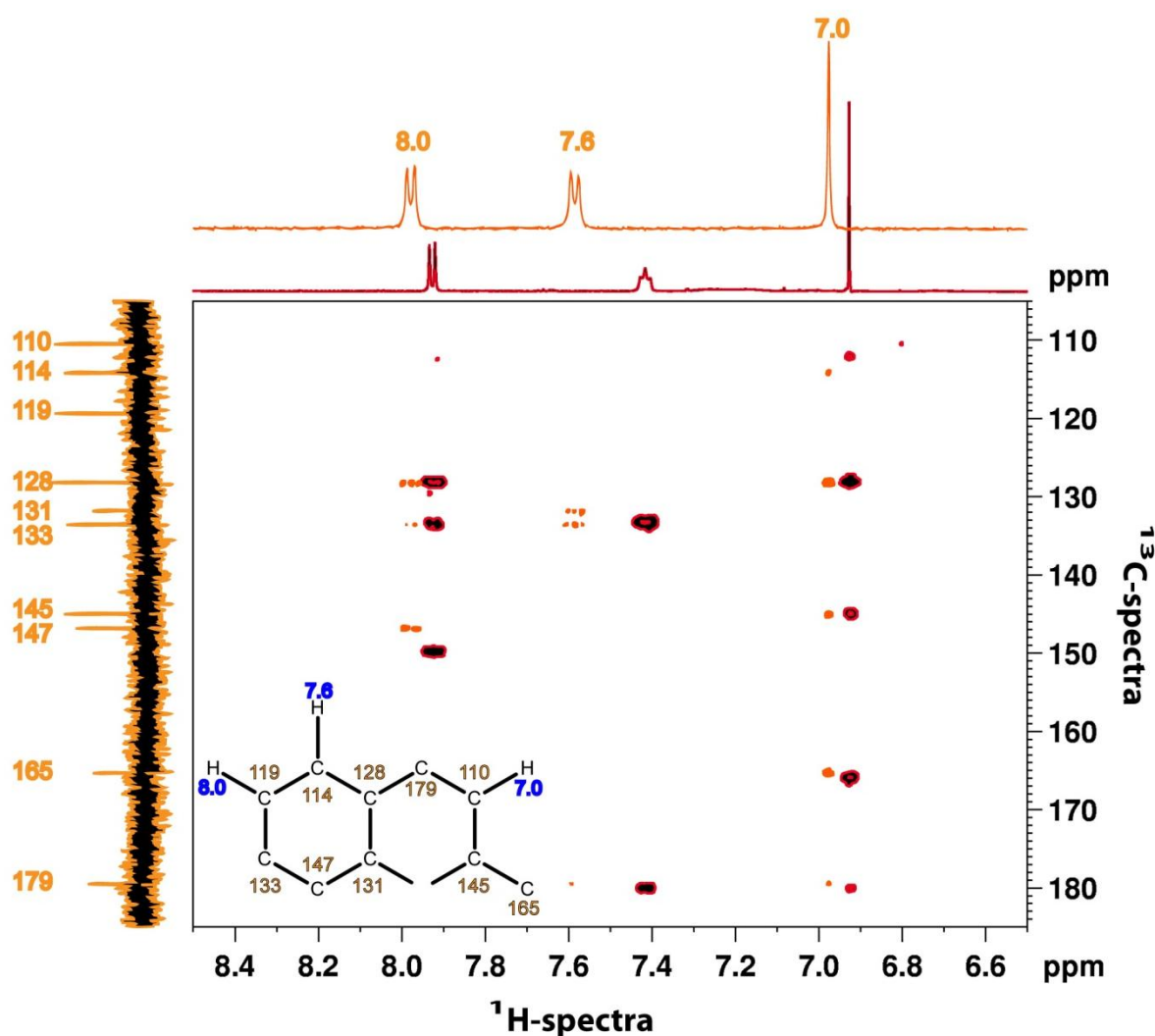


Figure 5-3. HMBC of the purified extracted dragline pigment (orange) and egg sac pigment (red) in acidified D_2O and the resulting solved structure from the ^{13}C -assignments.

Table 5-1. The direct coupling of the ^1H to the ^{13}C was listed from the HSQC together with the long range coupling of the ^1H with the ^{13}C -carbons from the HMBC for the purified extracted dragline pigment.

HSQC	^{13}C	HMBC									
		^1H									
		110	114	119	128	131	133	145	147	165	179
110	7.0		4		3			2		3	2
114	7.6					3	3				3
119	8.0				3		2		3		

Table 5-1 shows the short range coupling (HSQC) as well as the long range coupling correlations (HMBC) between the ^1H and ^{13}C . From these couplings the structure shown in Figure 5-3 was solved. Neither hydrogen nor carbon could be a substitute on the carbon at 179 ppm; however oxygen and nitrogen were possible candidates. The deshielding indicated the substituent was a strong electron acceptor group, making an oxygen atom the most likely candidate in the form of a carbonyl¹³³ which was later confirmed by MS (chapter 5.3.3.A). The carbon shift of 165ppm indicated that this carbon was part of a carboxylic acid (160 to 185 ppm range) group¹³³ and this was confirmed by MS (chapter 5.3.3.A). The atom to complete the double ring structure was most likely a nitrogen atom as this was within the shift range of the two neighbouring carbon atoms¹³⁷ and fitted the structural formula predicted by MS (5.3.3.A). The carbon at 147ppm had a hydroxyl group as the shift was induced by an electron acceptor group but not as strong a one as a carbonyl^{136, 137}. The carbon at 133ppm was more difficult as the shift was too low to be induced by an oxygen atom. Predictions showed that a nitrogen atom could cause a similar shift. A nitro substituent was a likely candidate and also fitted the MS data (chapter 5.3.3.A).

The predicted structure of the dragline pigment from the NMR data is given in Figure 5-4.

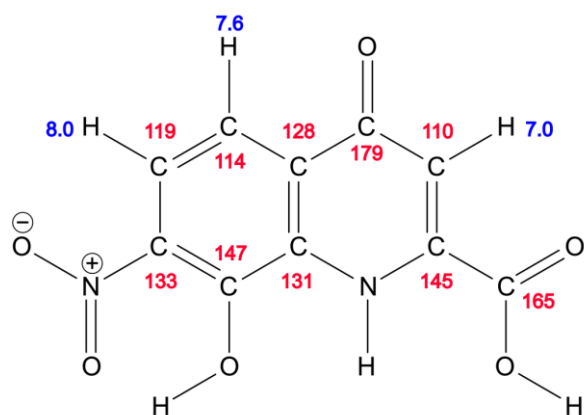


Figure 5-4. The predicted structure from NMR data of the extracted purified dragline pigment. The ¹³C chemical shifts are shown in red and the ¹H shifts in blue (ppm).

C. Egg sac pigment

Integrating the peaks in the HSQC ¹H spectra revealed three single classes of hydrogen atoms (Figure 5-2, red) with similar direct couplings between the ¹H and ¹³C shifts seen in dragline pigment (Figure 5-2, orange); the hydrogen at 7.0ppm was linked to the carbon atom at 110ppm, the hydrogen at 7.4ppm to the carbon at 112ppm and the hydrogen at 7.9ppm to the carbon atom at 120ppm. This indicated that the egg sac pigment and dragline pigment had the same amount of hydrogen atoms and coupled to carbons with similar shifts, indicating both compounds have a very similar structure. Moreover the upshift (7.9ppm) of the proton at carbon 120ppm (Figure 5-2, red) is very similar to the one seen in the dragline pigment (proton 8.0ppm at carbon 119ppm, Figure 5-2, orange) suggesting the same substituent was present in the same place in the molecule. The ¹H spectra of the HMBC showed a slightly shifted proton at 7.4 but this proton had been seen to move about with pH conditions (Appendix A.4) meaning the egg sac pigment was in a slightly more alkaline environment when measuring the HMBC.

Again, ten carbon atoms were found in the ^{13}C spectra of the HMBC of the egg sac pigment (Figure 5-3, red) and the ^{13}C shifts matched the shifts of the carbon atoms in the dragline pigment (Figure 5-3 (orange) and Table 5-2). The similarity in ^{13}C shifts ($\pm 3\text{ppm}$) strongly indicated the structure of both, the egg sac and dragline pigment molecule, were the same (double ring structure) and that the molecule had the same substituted groups present (Table 5-2 and Figure 5-3 (red and orange)).

The most striking similarities between both spectra were the exact same ^{13}C shift of the carbon atom at position 2 (for atom positions see Figure 5-7, a) in egg sac pigment at 134ppm and dragline pigment at 133ppm, confirming the same unknown substituent was present at the same carbon atom (Table 5-2). Also the ^{13}C shift of 180 and 166ppm indicated the same carbonyl and carboxylic acid were present in the same location. The resulting solved structure was exactly the same as the one found for the dragline pigment (Figure 5-4).

		dragline pigment (ppm)	egg sac pigment (ppm)	difference (ppm)
Carbon shifts	C1	119	120	-1
	C2	133	134	-1
	C3	147	150	-3
	C4	131	133	-2
	C5	128	128	0
	C6	114	112	2
	C8	145	145	0
	C9	110	110	0
	C10	179	180	-1
	C12	165	166	-1
Hydrogen shifts	nitro/H18	nitro?	nitro?	
	H21/H19	8	7.9	0.1
	H22/H20	7.6	7.4	0.2
	H23/H21	7	7	0

Table 5-2. Comparison and difference between the carbon and proton shifts of the extracted purified dragline pigment and egg sac pigment. The most important and characteristic shifts for the dragline pigment are indicated in yellow.

D. Comparison with xanthurenic acid

To confirm the predicted structure for the yellow pigment (from the dragline and egg sac extraction), NMR spectra from xanthurenic acid were obtained under the same conditions, because it had an identical structure but lacked the nitro group.

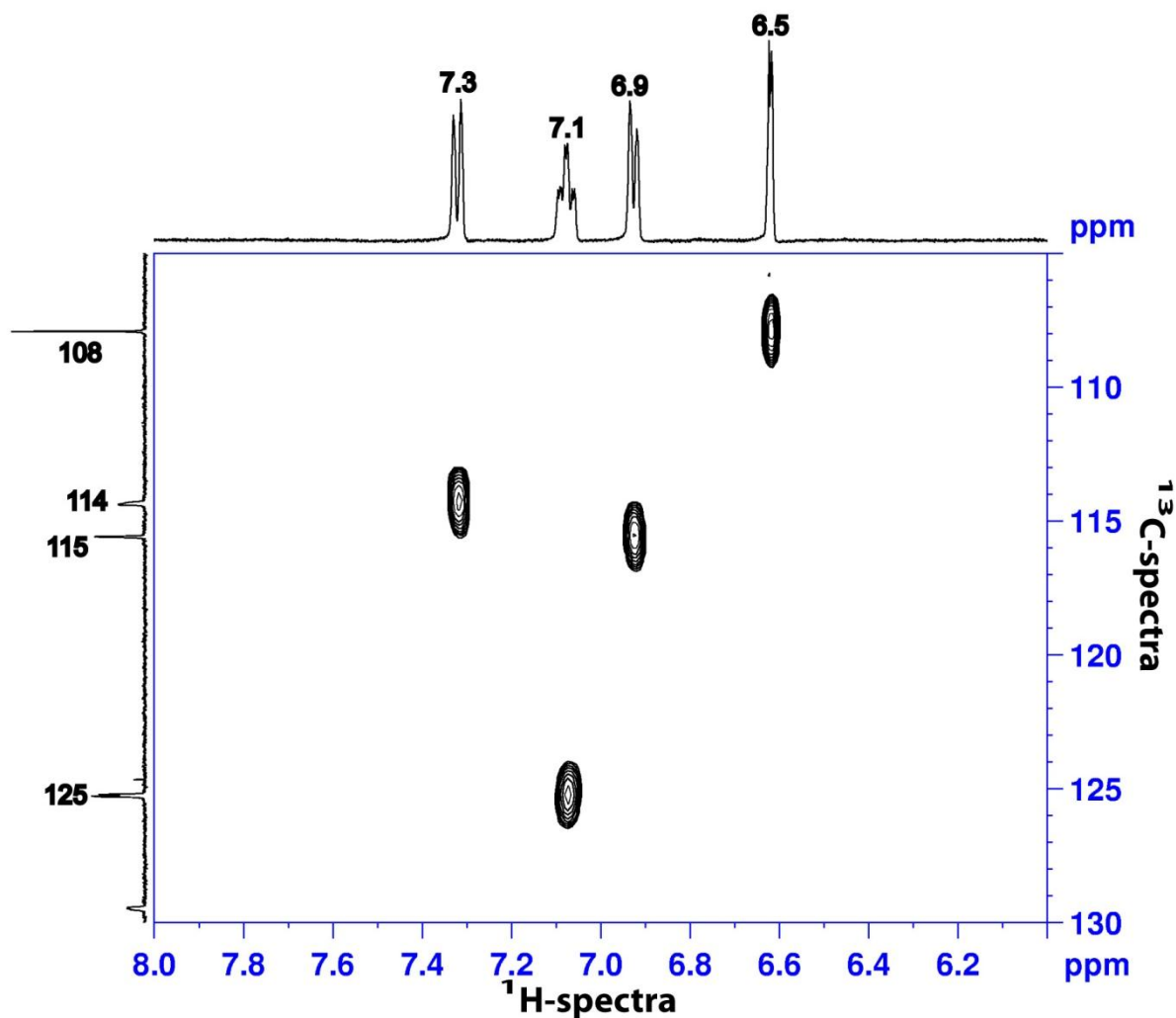


Figure 5-5. HSQC of xanthurenic acid (Sigma) in acidified D_2O .

For xanthurenic acid, four aromatic hydrogen atoms (from 6.6ppm to 7.4ppm)^{136, 137} were found in accordance with its known structure (Figure 5-5). The existence of four chemical shifts for xanthurenic acid's aromatic protons instead of the three for the dragline pigment (see above) indicated that one of the hydrogen atoms of xanthurenic acid had been substituted in xanthurenic acid to result in the pigment structure.

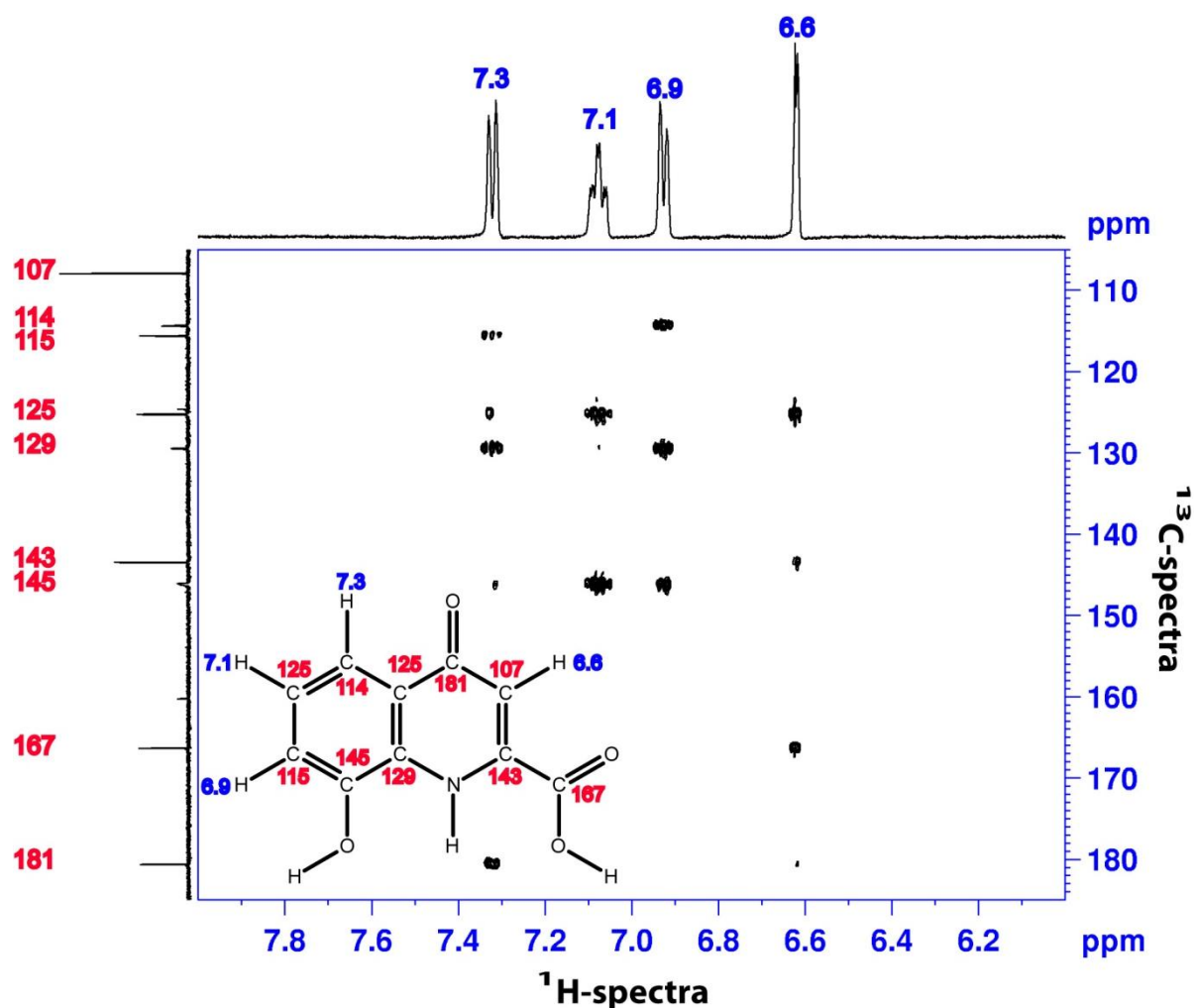


Figure 5-6. HMBC of xanthurenic acid (Sigma) in acidified D₂O and the resulting structure and ¹³C assignments from the HMBC spectra.

The direct correlation between the hydrogen and carbon atom was given in the HSQC (Figure 5-5 and Table 5-3). From the HMBC, ten carbon atoms were found in the range of 110 to 185ppm confirming the double ring structure of xanthurenic acid (Figure 5-6) and identical to the yellow pigment structure. The long and short range coupling correlations from the HSQC and HMBC were summarized in the table below (Table 5-3).

Table 5-3. The direct coupling of the ^1H proton to the ^{13}C carbons were listed from the HSQC together with the long range coupling of the ^1H proton with the ^{13}C carbons from the HMBC for xanthurenic acid (Sigma).

^{13}C	^1H	HMBC									
		^{13}C									
		108	114	115	125	125	129	143	145	167	181
108	6.5				3			2		3	2
114	7.3			3	2		3		4		3
115	6.9		3				3		2		
125	7.1				3				3		

Because the structure of xanthurenic acid was known it was easy to assign the ^{13}C -shifts to the appropriate carbon atoms in the molecule (Figure 5-6). In this way it became possible to compare the predicted structure for the dragline pigment with the known structure of xanthurenic acid. In the figure below the structure was given for the dragline pigment (Figure 5-7, a) and xanthurenic acid (Figure 5-7, b) with their respective atom labelling.

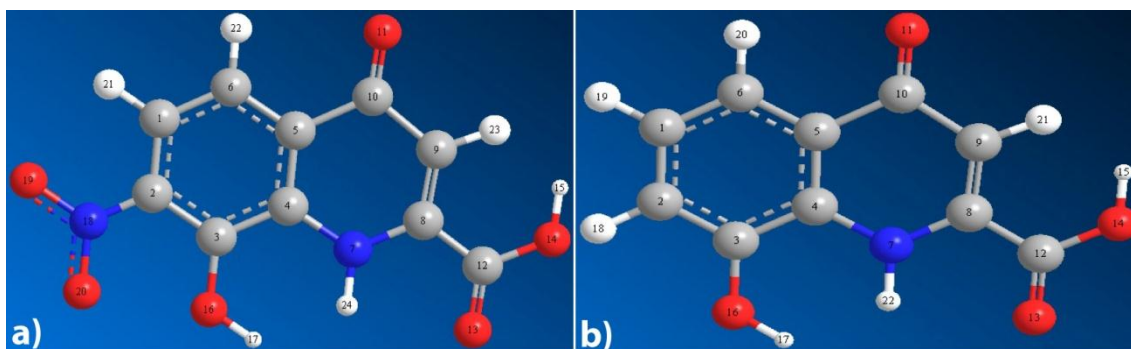


Figure 5-7. Chemical structures of (a) the dragline pigment molecule and (b) its most closely structurally related molecule, xanthurenic acid. Carbons and hydrogens are numbered for comparison in the Table 5-2 and Table 5-4.

The ^{13}C -shifts of both spectra confirmed the presence of ten carbon atoms and that the majority of the carbon shifts matched ($\pm 2\text{ppm}$), confirming the main structure of the yellow pigment molecule was like xanthurenic acid (Figure 5-6 and Table 5-4). The general difference of 2ppm between the shifts of the two spectra was most likely due to slight changes in condition between the two experimental runs.

Table 5-4. Comparison and difference between the carbon and proton shifts of the extracted purified dragline pigment and xanthurenic acid. The major differences between the two compounds are highlighted in yellow.

		dragline pigment (ppm)	xanthurenic acid (ppm)	difference (ppm)
Carbon shifts	C1	119	125	-6
	C2	133	115	18
	C3	147	145	2
	C4	131	129	2
	C5	128	125	3
	C6	114	114	0
	C8	145	143	2
	C9	110	107	3
	C10	179	181	-2
	C12	165	167	-2
Hydrogen shifts	nitro/H18	nitro	6.9	
	H21/H19	8	7.1	0.9
	H22/H20	7.6	7.3	0.3
	H23/H21	7	6.6	0.4

The most striking difference between both spectra was the presence of an extra hydrogen atom for xanthurenic acid confirming the presence of a substituted group on the ring for the dragline pigment molecule (Figure 5-6). The ^{13}C -shift of the carbon atom at position 2 of the dragline pigment (133ppm) differed greatly from that same carbon atom in xanthurenic acid (115ppm, Table 5-4 (highlighted)). This kind of shift can only be induced by a strong substituent on this carbon and a nitro group was very likely.

The ^{13}C -shift value of the carbon at position 2 in the dragline pigment molecule (133ppm) was in agreement with the predicted shift for a substituted nitro group. Also the rather large upfield proton shift of 0.9ppm of the neighbouring proton (H21) was characteristic for the presence of a nitro or carboxylic acid group on a neighbouring carbon atom (Table 5-4). Because there was no evidence for an extra carboxylic acid (^{13}C -shift) group on the molecule, the substituted group was most likely a nitro-group. This was later confirmed by MS (chapter 5.3.3.A).

E. Summary

Comparing the NMR data of the yellow pigment in the dragline and egg sac pigment revealed that both components were identical.

The structure of the yellow pigment molecule was partly solved and resembled the structure of xanthurenic acid with the difference that the dragline pigment had one more substituted group, most likely a nitro-group. Because side groups on the molecule could only be indirectly seen by the ^{13}C -shift value, these needed confirmation from mass spectrometry.

5.3.3. Mass spectrometry (MS)

A. Dragline pigment

A.1. Exact mass and structural formula determination

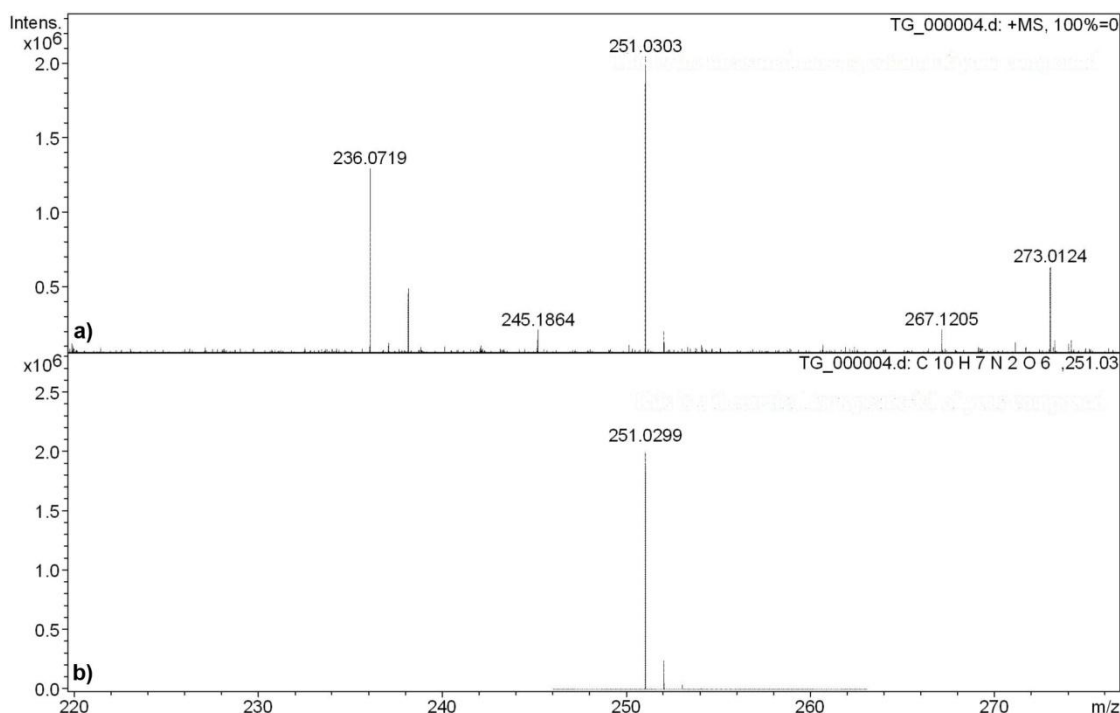


Figure 5-8. a) Mass spectra obtained from purified dragline pigment on the SYNAPT with positive ionisation electrospray and at a current of 1.24×10^4 eV. The mass spectrogram of the relative abundance of the ions is plotted against their mass (m/z). b) Theoretical spectrum of the yellow pigment.

In the purified yellow dragline pigment, the peak at 251 (representing M+H) was the most abundant (Figure 5-8, a) and was seen in all mass spectrometers used (section 5.2.3). The mass given by the accurate mass measurement (Bruker uTOF) was determined at 251.0303 with an error of 1.59ppm predicting an even number of nitrogen atoms¹³⁷. The obtained accurate mass resulted in the following possible calculated structural formulas (Table 5-5).

Table 5-5. Predicted structural formulas for the measured accurate mass of the yellow pigment (251.0303)

Calculated mass + one positive charge	Difference between measured and calculated mass	Calculated structural formula
251.0438	0.0038	C12 H5 N5 O2
251.0451	0.0051	C14 H7 N2 O3
251.0339	0.0061	C15 H7 O4
251.0326	0.0075	C13 H5 N3 O3
251.0312	0.0088	C11 H3 N6 O2
251.0492	0.0091	C19 H7 O
251.0497	0.0096	C5 H9 N5 O7
251.0299	0.0101	C10 H7 N2 O6
251.0510	0.0110	C6 H5 N9 O3
251.0510	0.0110	C7 H11 N2 O8

Many of the above listed structural formulae can be discarded on chemical grounds. The isotope mass clearly indicated the presence of carbon atoms and together with the NMR-data which showed that there were ten carbon atoms present in the molecule (see 5.3.2.B) made it possible to narrow the results down to one unique structural formula for the molecular ion $M+H$, namely $C_{10}H_7N_2O_6$ (calculated mass: 251.0299, measured mass: 251.0303) (Figure 5-8, b). Due to the additional proton coupled to the molecule by electrospray ionisation for mass spectrometry the accurate structural formula for the molecule was given by $C_{10}H_6N_2O_6$ (predicted accurate mass: 250.0226).

The structural formula obtained from the accurate mass already confirmed that the unknown group found in the yellow pigment with NMR was indeed a nitro-group (chapter 5.3.2.D). For further confirmation the fragmentation pathway of the yellow pigment was compared with that of xanthurenic acid.

A.2. Fragmentation pathway: structure determination

The dragline pigment molecular ion (251 m/z) appeared to break up initially in the same way as in xanthurenic acid (Appendix A.7) resulting in the mass fragments 233, 223 and 205 (Figure 5-9). These masses fitted with the molecular ion losing water ($M+H-OH$: 233 (Table 5-6, B)), the carboxyl group ($M+H-COOH$: 205 (Table 5-6, D)) and carbon monoxide ($M+H-C=O$: 223 (Table 5-6, C))^{137, 138}. The loss of a nitro group was also plausible, however a mass loss of 30 (NO) was not found and the breakdown pathway of the mass at 206 was completely different from that observed for xanthurenic acid (Appendix A.7) indicating the nitro group was not lost in the initial fragmentation of the yellow pigment molecule.

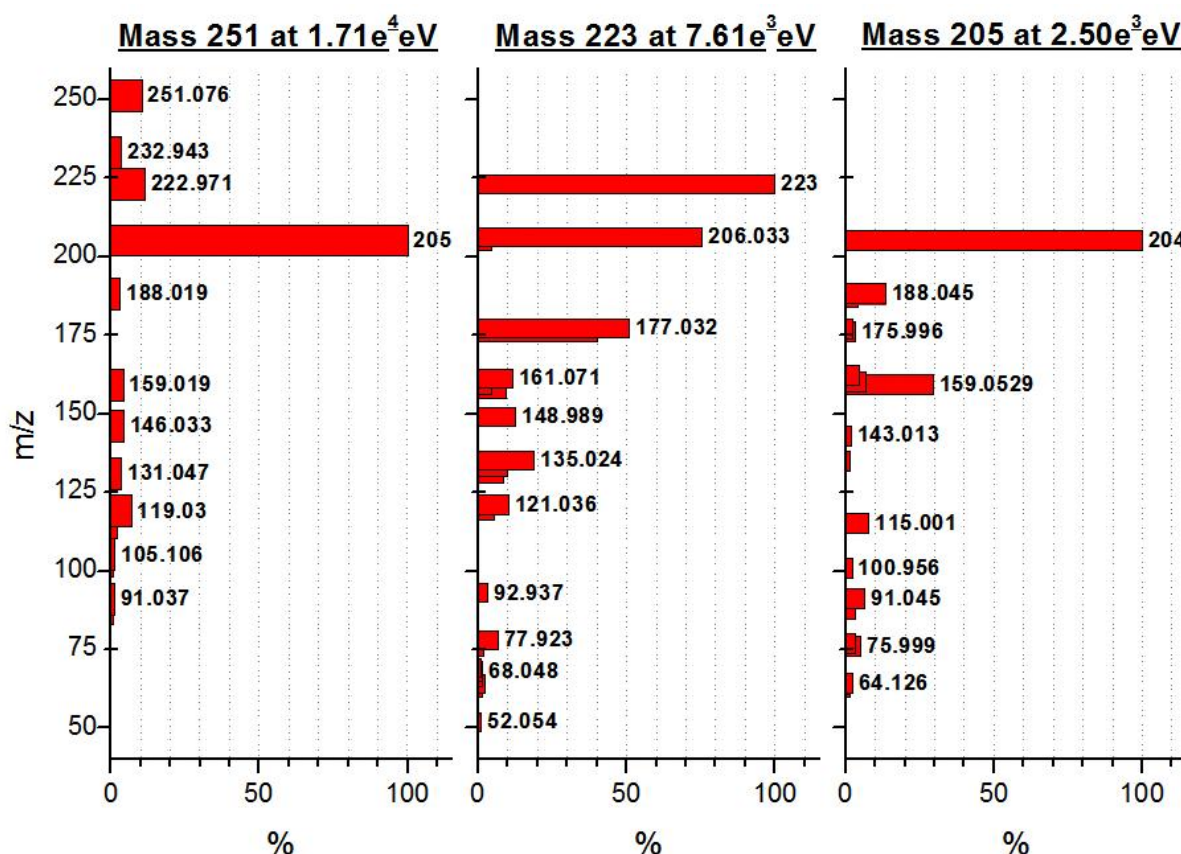


Figure 5-9. The fragmentation of the yellow pigment on the SYNAPT with positive ionisation spray of the mass at 251 (at 1.71e⁴) and the fragments of mass 223 (at 7.61e³) and 205 (at 2.50e³). The raw data can be found in Appendix A.7.

The mass of 205 derived from the dragline pigment appeared to fragment like that seen in xanthurenic acid (Appendix A.7), although many additional fragments were found in different relative abundances (Figure 5-9). This suggests the molecular ion resembled xanthurenic acid but was not exactly the same confirming our prediction that the nitro group was more stable than the carboxyl group and was still present after the initial fragmentation (loss of carboxyl group). Fragmentation of 205 mass resulted in a main mass of 119 which was unique for the dragline pigment molecule. This meant the 205 mass lost a fragment with a total mass of 86.9956, corresponding to C_2HNO_3 and the main molecular ion fragment would be C_7H_5NO (119 mass) indicating that a nitro group was present on the molecule (Figure 5-10). In smaller quantities, similar molecular ions were found to those derived from xanthurenic acid and this would indicate the fragmentation was as follows: the loss of water ($M+H-OH$: 188 (Table 5-6, B)), the nitro group ($M+H-NO_2$: 159 (Table 5-6, D)) and nitrogen monoxide ($M+H-N=O$: 178 (Table 5-6 (C)) (Figure 5-10). The other smaller fragments found (masses 76, 103, 131 and 146) were most likely all produced by the further breakdown (Figure 5-10)) of the double ring structure (159 mass) which were also observed in xanthurenic acid (Appendix A.7)^{137, 138}.

The most likely fragmentation pathway was given in Figure 5-10 and has some similar breakdown pathways to those found in xanthurenic acid (Appendix A.7).

Table 5-6. All mass fractions obtained from the fragmentation of the dragline pigment in MS were listed and indexed (A till G) together with the mass difference from the ion fractions with the yellow pigment. For all ions the possible structural formulas were calculated and the most likely ion was tabulated next to it.

	MS	Allowed structural formulas from mass fraction	Most likely structural formula	MS difference of fragment A	Allowed structural formula for the mass difference of A	Most likely mass fraction of A
A	251.0517	C10H7N2O6	C10H7N2O6		dragline pigment	
B	233.0407	C10 H5 N2 O5 (-0.021402251)	C10 H5 N2 O5	18.011	H2O (-4.366999E-4)	H2O
C	223.0544	C9 H7 N2 O5 (-0.01945218)	C9 H7 N2 O5	27.9973	CO (-0.002385)	CO
D	205.0448	C9H5N2O4 (-0.019874)	C9H5N2O4	46.0069	CH2O2 (-0.001422) ; H2N2O (+0.0098096) ; NO2 (-0.013998) ;	CH2O2
E	188.0407	C9H4N2O3 (-0.018512) ; C7H12N2O4 (+0.0389980) ; C9H2NO4 (-0.042321) ; C8H14NO4 (+0.0515740) ; C8N2O4 (-0.054897) ; C9H16O4 (+0.0641500)	C9H4N2O3	63.011	C4HN (-1.022E-4) ; CH3O3 (-0.002783) ; H3N2O2 (+0.0084483) ; C5H3 (+0.0124737) ; HNO3 (-0.015359) ; CH5NO2 (+0.0210243)	CH3O3
F	177.0655	C9H9N2O2 (+8.960000000000001E-4) ; C6H13N2O4 (+0.0220226) ; C9H7NO3 (-0.022912) ; C7H15NO4 (+0.0345986) ; C8H5N2O3 (-0.035488) ; C9H5O4 (-0.046720)	C9H7NO3	73.9862	CNO3 (+0.0016155) ; C2H2O3 (+0.0141915) ; C5N (+0.0168732) ; CH2N2O2 (+0.0254238) ; C6H2 (+0.0294492) ; C2H4NO2 (+0.0379998)	CNO3
G	159.0498	C6H9NO4 (+0.0033510) ; C9H7N2O (+0.0060326) ; C5H7N2O4 (-0.009224) ; C7H11O4 (+0.0159270) ; C9H5NO2 (-0.017775) ; C6H11N2O3 (+0.0271592)	C9H5NO2	92.0019	C4N2O (-8.394999E-4) ; CH2NO4 (-0.003521) ; C2H4O4 (+0.0090548) ; C5H2NO (+0.0117364) ; C5O2 (-0.012071) ; N2O4 (-0.016097)	CH2NO4
H	146.0403	C9H6O2 (-0.003524) ; C5H8NO4 (+0.0050263) ; C4H6N2O4 (-0.007549) ; C8H6N2O (+0.0077080) ; C8H4NO2 (-0.016100) ; C6H10O4 (+0.0176024)	C8H4NO2	105.0114	C5HN2O (-0.002514) ; C2H3NO4 (-0.005196) ; C3H5O4 (+0.0073794) ; C6H3NO (+0.0100611) ; C6HO2 (-0.013747) ; CHN2O4 (-0.017772)	CH3NO5
I	131.05	C9H7O (-3.1370000000000004E-4) ; C4H7N2O3 (-0.004339) ; C5H9NO3 (+0.0082368) ; C8H7N2 (+0.0109185) ; C8H5NO (-0.012889) ; C5H7O4 (-0.015571)	C9H6N?	120.0017	C5N2O2 (-0.005725) ; C6H2NO2 (+0.0068506) ; C2H4N2O4 (+0.0154012) ; C6O3 (-0.016957) ; C7H4O2 (+0.0194266) ; C3H6NO4 (+0.0279772)	CH3NO6?
J	119.0292	C6H3N2O (-0.004665) ; C4H7O4 (+0.0052286) ; C3H5NO4 (-0.007347) ; C7H5NO (+0.0079103) ; C7H3O2 (-0.015897) ; C3H7N2O3 (+0.0164608)	C7H5NO	132.0225	C8H4O2 (-0.001373) ; C3H4N2O4 (-0.005398) ; C4H6NO4 (+0.0071772) ; C7H4N2O (+0.0098588) ; C7H2NO2 (-0.013949) ; C5H8O4 (+0.0197531)	C3H2NO5
K	115.0413	C8H5N (+8.9619999E-4) ; C5H7O3 (-0.001785) ; C4H7N2O2 (+0.0094468) ; C7H3N2 (-0.011679) ; C9H7 (+0.0134721) ; C4H5NO3 (-0.014361)	?	136.0104	C9N2 (-0.004253) ; C7H4O3 (+0.0056406) ; C6H2NO3 (-0.006935) ; C6H4N2O2 (+0.0168730) ; C5N2O3 (-0.019511) ; C7H6NO2 (+0.0294489)	?
L	103.0546	C8H7 (+1.722E-4) ; C3H7N2O2 (-0.003853) ; C4H9NO2 (+0.0087228) ; C7H5N (-0.012403) ; C4H7O3 (-0.015085) ; H11N2O4 (+0.0172734)	C7H5N?	147.9971	C6N2O3 (-0.006211) ; C7H2NO3 (+0.0063647) ; C7O4 (-0.017443) ; C8H4O3 (+0.0189407) ; C7H4N2O2 (+0.0301730) ; C8H6NO2 (+0.0427490)	?
M	91.0504	C2H7N2O2 (+3.468E-4) ; C7H7 (+0.0043722) ; C6H5N (-0.008203) ; C3H7O3 (-0.010885) ; C3H9NO2 (+0.0129228) ; C5H3N2 (-0.020779)	?	160.0013	C8H2NO3 (+0.0021647) ; C7N2O3 (-0.010411) ; C9H4O3 (+0.0147407) ; C8O4 (-0.021643) ; C8H4N2O2 (+0.0259730) ; C9H6NO2 (+0.0385490)	?
N	76.0411	C2H6NO2 (-0.001250) ; C6H4 (-0.009801) ; C3H8O2 (+0.0113249) ; CH4N2O2 (-0.013826) ; C5H2N (-0.022377) ; C2H8N2O (+0.0225573)	C5H2N?	175.0106	C8H3N2O3 (+0.0037624) ; C9H3O4 (-0.007469) ; C9H5NO3 (+0.0163384) ; C8HNO4 (-0.020045) ; C9H7N2O2 (+0.0401468) ; C6H11N2O4 (+0.0612734)	?
O	63.0338	CH5NO2 (-0.001775) ; C5H3 (-0.010326) ; C2H7O2 (+0.0108004) ; H3N2O2 (-0.014351) ; CH7N2O (+0.0220327) ; C4HN (-0.022902)	?	188.0179	C9H4N2O3 (+0.0042870) ; C9H2NO4 (-0.019521) ; C8N2O4 (-0.032097) ; C7H12N2O4 (+0.0617980) ; C8H14NO4 (+0.0743740) ; C9H16O4 (+0.0869500)	C9H4N2O3

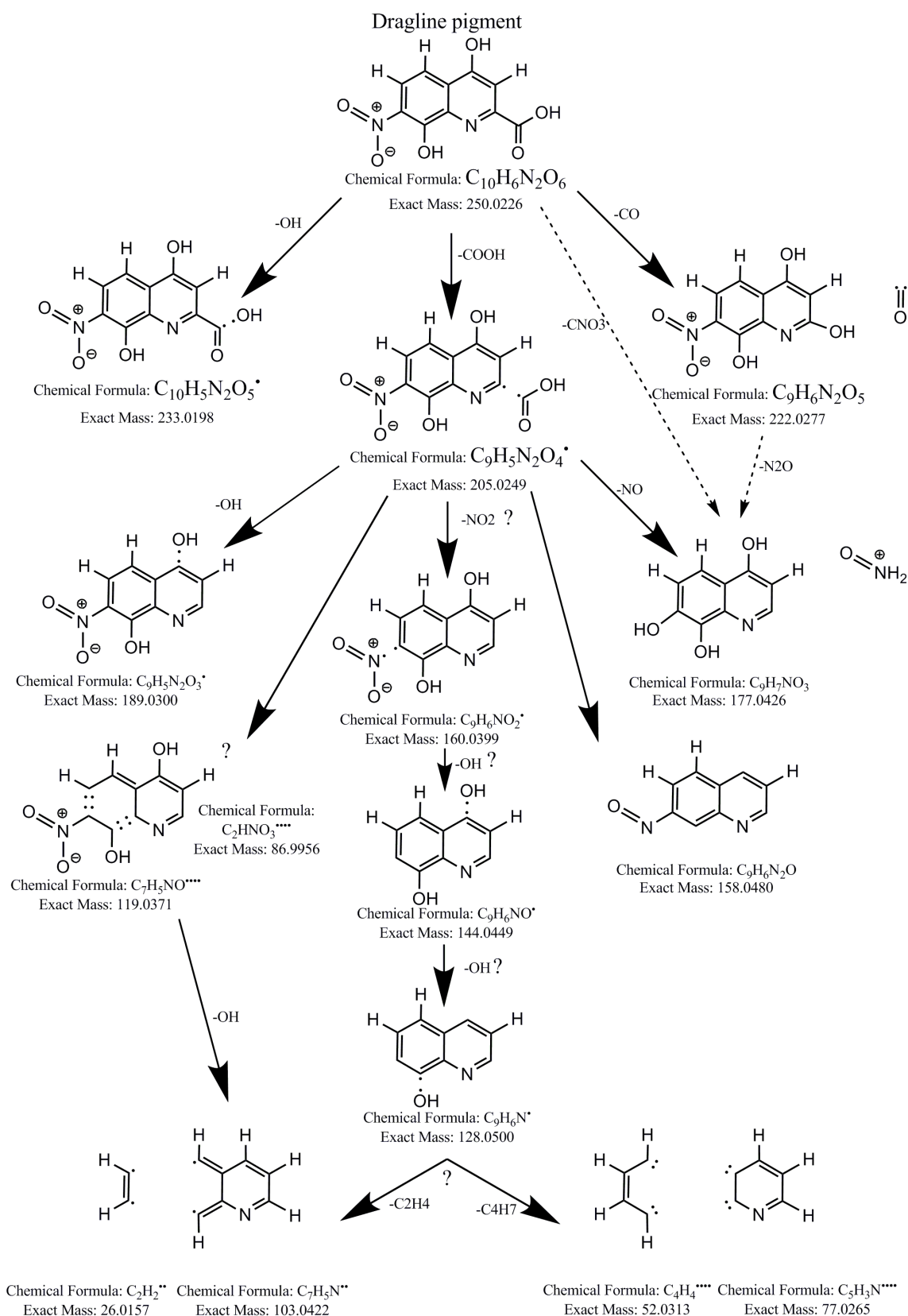


Figure 5-10. A hypothetical fragmentation pathway of the dragline pigment based on the experimental data. The fragmentation suggested the presence of a nitro-group.

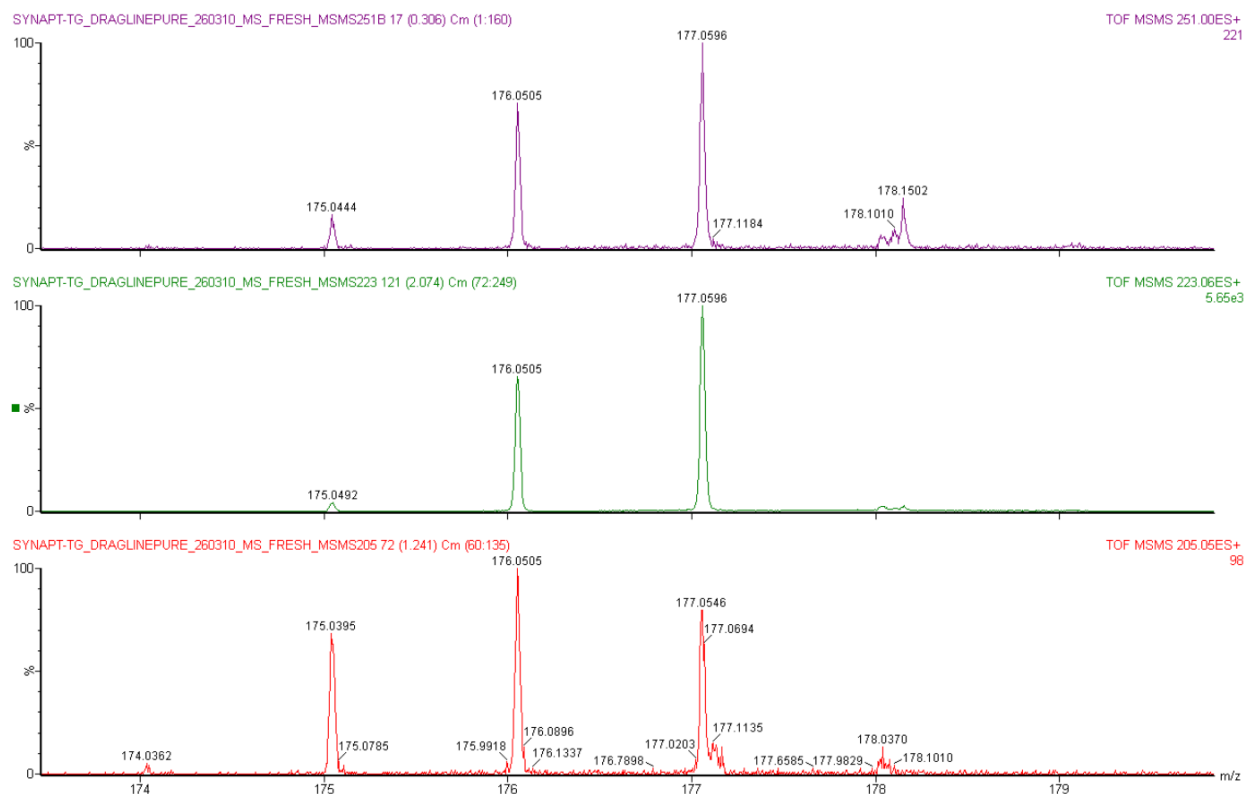


Figure 5-11. A detail of the mass region 173 to 180 of the mass spectra obtained after fragmenting masses 251 at 221 eV (a), 223 at 5.65e^3 eV (b) and 205 at 98 eV (c) indicating the loss of CNO_3 , NO_2 and NO respectively.

The molecular ion with mass ± 177 in the fragmentation pattern of the dragline pigment was of interest because this ion also confirmed the loss of a nitro group. Fractioning the masses at 251 (221 eV), 223 (5.65e^3 eV) and 205 (98 eV) consistently displayed a mass at 177 (Figure 5-11). In the case of the mass at 251 this indicated that CNO_3 had been lost, for the mass of 223 the NO_2 had been lost and from the mass at 205, NO had been lost (see Figure 5-10 and Table 5-7).

Table 5-7. Mass loss obtained from the difference between the ions at 177, 176 and 175 with masses 251, 223 and 205 respectively, indicating the fragmentation of the nitro-group.

	MS difference	Allowed structural formulas from mass fraction	Most likely structural formula
MSMS 251			
251-177.0596	73.9404	CNO ₃ (+0.0474155); C ₂ H ₂ O ₃ (+0.0599915); C ₅ N (+0.0626732); CH ₂ N ₂ O ₂ (+0.0712238); C ₆ H ₂ (+0.0752492); C ₂ H ₄ N ₂ O ₂ (+0.0837998)	CNO ₃
251-177.0596	74.9495	CHNO ₃ (+0.0461401); C ₂ H ₃ O ₃ (+0.0587160); C ₅ HN (+0.0613977); CH ₃ N ₂ O ₂ (+0.0699484); C ₆ H ₃ (+0.0739738); C ₂ H ₅ N ₂ O ₂ (+0.0825243)	CHNO ₃
251-177.0546	75.9556	CO ₄ (+0.0240564); N ₂ O ₃ (+0.0352886); C ₅ O (+0.0393140); CH ₂ NO ₃ (+0.0478646); C ₄ N ₂ (+0.0505464); C ₂ H ₄ O ₃ (+0.0604407)	CH ₂ NO ₃
MSMS 223.06			
223.06-176.0505	46.0004	CH ₂ O ₂ (+0.0050774); NO ₂ (-0.007498); H ₂ N ₂ O (+0.0163097); CH ₄ NO (+0.0288857); C ₂ H ₆ O (+0.0414616); CH ₆ N ₂ (+0.0526940)	NO ₂
223.06-176.0505	47.0095	CH ₃ O ₂ (+0.0038020); HNO ₂ (-0.008774); H ₃ N ₂ O (+0.0150343); CH ₅ NO (+0.0276102); C ₂ H ₇ O (+0.0401862); CH ₇ N ₂ (+0.0514185)	HNO ₂
223.06-176.0505	48.0108	H ₂ NO ₂ (-0.002249); CH ₄ O ₂ (+0.0103265); C ₄ (-0.010800); H ₄ N ₂ O (+0.0215589); O ₃ (-0.026057); CH ₆ NO (+0.0341348)	H ₂ NO ₂
MSMS 205.05			
205.05-175.0444	27.9954	CO (-4.858999E-4); N ₂ (+0.0107464); CH ₂ N (+0.0233224); C ₂ H ₄ (+0.0358984); H ₁₂ O (+0.0934093); H ₁₄ N (+0.1172176)	NO
205.05-175.0444	28.9995	CHO (+0.0032387); HN ₂ (+0.0144710); CH ₃ N (+0.0270469); C ₂ H ₅ (+0.0396229); H ₁₃ O (+0.0971338); H ₁₅ N (+0.1209421)	NO
205.05-175.0395	30.0105	CH ₂ O (+6.33E-5); H ₂ N ₂ (+0.0112956); NO (-0.012512); CH ₄ N (+0.0238715); C ₂ H ₆ (+0.0364475); H ₁₄ O (+0.0939584)	NO

B. Egg sac pigment

Mass spectrometry indicated a mass of 251.0308 m/z which was identical to the mass found for the dragline pigment molecule (251.0303). This resulted in the same predicted structural formula as the one found for the dragline pigment, namely C₁₀H₆N₂O₆. Also the mass fragments were similar to those found in the dragline pigment (same as Figure 5-9) meaning that the structure was identical.

C. Summary

The mass of the dragline pigment molecule was determined at 251.0303 with mass spectrometry and resulted in the following structural formula: $C_{10}H_6N_2O_6$ (predicted monoisotopic mass: 250.0226). This confirmed the NMR data that the additional unknown group on the yellow pigment molecule (in contrast to xanthurenic acid) was indeed a nitro-group. Mass fragmentation showed that the structure of the dragline pigment was similar to that of xanthurenic acid except for the fact that the nitro group was more stable than the carboxylic acid group. This resulted in a similar loss of the carboxylic acid group at first but then deviated as the presence of the nitro group resulted in different fragments. However, once the nitro group was lost and the basic double ring conformation was obtained, the mass fragments became similar again.

The mass spectra data from the dragline and egg sac pigment were identical meaning the compound was identical in both extractions, confirming the HPLC and NMR data.

5.3.4. Fourier Transform Infra-Red Spectroscopy (FTIR)

A. General

The FTIR spectra obtained for the dragline pigment (Figure 5-12) were compared with spectra in the databases of the NIST, Organic Compound database, AIST, etc. but did not match any compound. Also the interpretation software of OMNIC (interpreter+) did not produce a match with any compounds.

From NMR and MS (chapter 5.3.2.D and 5.3.3.A) it was shown that the dragline pigment was structurally close to xanthurenic acid with the exception that it had an additional substituted group, most likely a nitro group. To further confirm the presence of that group a detailed analysis of the FTIR spectrum of the dragline pigment was compared to the spectrum of xanthurenic acid. For a more detailed analysis of the spectrum of xanthurenic acid (XA) please refer to Appendix A.5.

B. Dragline pigment

In general, the FTIR spectrum obtained for the dragline pigment (Figure 5-12) was similar to that of xanthurenic acid (Appendix A.5). Like xanthurenic acid, the dragline pigment had a broad peak that stretched from 2400 to 3700 cm^{-1} , however markedly shifted to high wavenumbers. Even fewer peaks were seen in this region than in xanthurenic acid, indicating a lack of some functional groups (hydroxyl). The broad stretch of this peak suggested the presence of a carboxylic acid. This assignment was reinforced by the presence of four peaks (1294, 1231, 1146 and 1051 cm^{-1}) in the 1300-1000 cm^{-1} range, characteristic for the C-O stretching^{137, 139}.

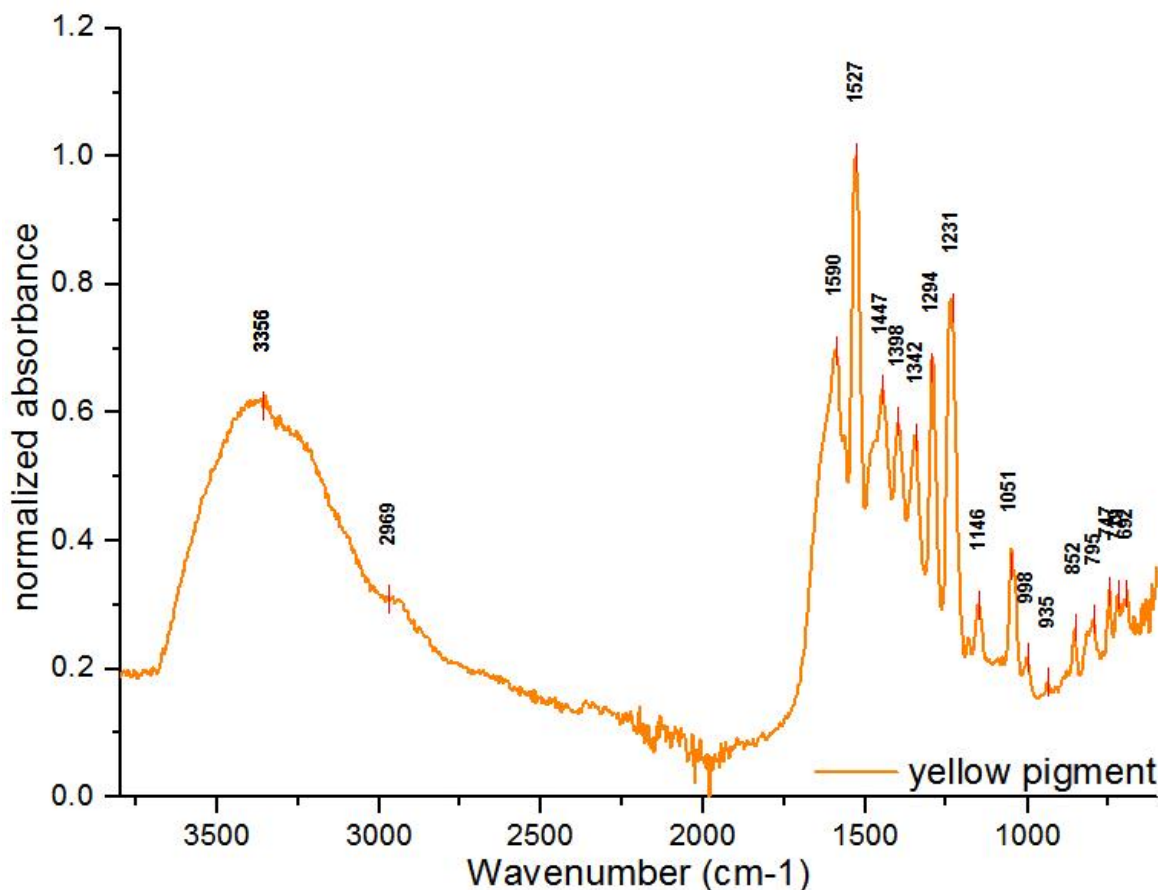


Figure 5-12. FTIR-spectra of purified dragline pigment with peaks indicated.

The aromatic C-H stretches were usually very weak but can be noticed at $3100\text{--}3000\text{cm}^{-1}$ indicating an aromatic ring. The clear N-H stretch that was noticeable in the xanthurenic acid spectrum was much less marked in the dragline pigment spectrum. The peak at 3356 cm^{-1} might indicate an N-H stretch in a molecule like XA^{136, 137}.

Like XA, the main C=O stretching was observed at 1590cm^{-1} peak; much lower than its usual 1700 cm^{-1} value^{140, 141}. The difference could be explained by the electron density delocalisation due to the conjugation^{137, 140}. The electron withdrawing capabilities of a substituted nitro group on the double ring would be able to cause an additional shift like this. This assignment was confirmed by the presence of the peak at 1398cm^{-1} which was characteristic for a carbonyl group ($1405\text{--}1395\text{cm}^{-1}$)^{137, 141} and the peaks in the region 1000--

1300cm^{-1} . The aromatic C=C bonds are usually in the region of $1600\text{-}1500\text{cm}^{-1}$ with two or three bands¹⁴⁰. Here, three peaks were found at 1590 , 1560 and 1527cm^{-1} which clearly indicated an aromatic compound and was confirmed by the presence of four peaks in the $850\text{-}730\text{cm}^{-1}$ region (852 , 795 , 747 and 719cm^{-1}) indicating aromatic C-H stretches^{137, 139}. The strong and main peak of the spectra at 1527cm^{-1} indicated an N=O ($1600\text{-}1500\text{cm}^{-1}$ region) or N-O stretch ($1550\text{-}1475\text{cm}^{-1}$ region)^{137, 139-141}. This, together with the peaks at 1342cm^{-1} and 1231cm^{-1} indicated that there was an aromatic nitro group present^{137, 139-141}.

C. Egg sac pigment

The FTIR spectrum from the purified egg sac extraction was entirely different from that of the purified dragline pigment or xanthurenic acid (see Appendix A.6). The broad peaks observed in this spectrum suggested the presence of a mixture of compounds that was confirmed by NMR, showing an additional small peptide present in the purified fraction. Therefore the interpretation of the FTIR-spectrum would be incorrect (Appendix A.6).

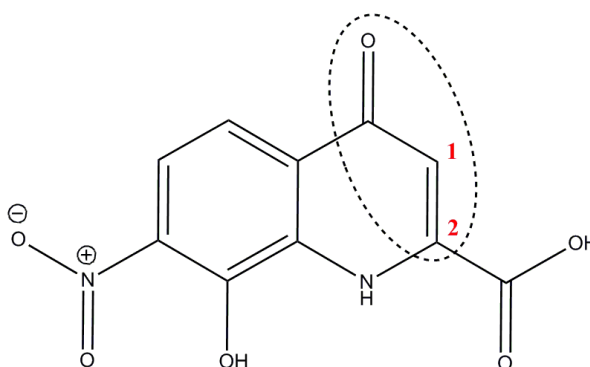
D. Summary

The FTIR-spectrum of the extracted dragline pigment (Figure 5-12) resembled that of xanthurenic acid (Appendix A.5) however one additional group was found in the yellow pigment that was absent in xanthurenic acid. The spectrum confirmed the compound was aromatic and indicated the presence of a hydroxyl, carboxyl and nitro group, confirming the HPLC, NMR and MS data.

5.3.5. Ultra-violet spectroscopy (UV-spectroscopy)

A. Prediction of the UV-spectrum of the yellow pigment: Woodward-Fieser rules.

Detailed predictions of UV-spectra are difficult and not routinely undertaken. However, the empirical rules of Woodward-Fieser assign a $\lambda_{\max}$ value to the $\pi \rightarrow \pi^*$ transition of the parent chromophore and tabulated increments for the effect of added substituents could give very useful structural information^{136, 137} (see below). The reason for using these rules was to confirm the structure of the yellow pigment molecule by predicting the lowest $\pi \rightarrow \pi^*$ transition and comparing it with the $\lambda_{\max}$ obtained from the measured UV-spectrum (peak at 396nm) (Figure 5-1).



Woodward-Fieser calculations of the predicted structure			
Parent six-ring 1,2-unsaturated ketone			215nm
1-substituents:			none
2-substituents:	alkyl group	1 x 12	12nm
	Ring residue	1 x 12	12nm
Exocyclic double bonds		3 x 5	15nm
Double bond extending conjugation		5 x 30	150nm
Solvent correction (50% water + 50% methanol)		-8/2	4nm
predicted $\lambda_{\max}$			400nm

The calculated value (400nm) of the predicted structure was very close to the observed λ_{max} value of 396nm in the yellow pigment molecule, agreeing with the predicted structure. Additionally it can be concluded that the substituted group must have a double bond present and that it has to be conjugated to a neighbouring one. This was shown by the lack of a nitro group in xanthurenic acid, resulting in one less extended conjugated bond (-30nm) and exocyclic bond (-5nm). This means in turn that the calculated λ_{max} of xanthurenic acid should be 365nm which fits the observed value of 360nm closely (Appendix A.3). The 4nm error in both cases could be explained by the added stabilization of the molecule by the presence of TFA (0.1%).

B. Summary

Using the Woodward-Frieser rules on the predicted molecular structure of the yellow pigment by NMR, MS and FTIR, resulted in a calculated λ_{max} that was extremely close to the measured λ_{max} of the yellow pigment molecule. This data again confirmed that the predicted structure for the yellow pigment was correct and that the substituted group must contain a conjugated double bond confirming the presence of a nitro-group.

Chromatographically the addition of a nitro-group could increase the retention time of a molecule and also induce a red shift in the UV-spectra (Appendix A.4) which was exactly the difference seen between xanthurenic acid and the dragline pigment (Appendix A.3).

5.4. Conclusion

The structure of the yellow pigment was determined by nuclear magnetic resonance and mass spectrometry. Both these methods provided strong evidence for the structure of the yellow pigment molecule (Figure 5-13). The NMR showed that with the exception of one additional substituted group on carbon atom two (carbon shift: 133 ppm) the molecular structure was identical to that of xanthurenic acid (Figure 5-7, b). This shift was characteristic for a substituted nitro-group and this substitution was confirmed by the structural formula for the molecule obtained by mass spectrometry ($C_{10}H_6N_2O_6$) and by the mass fragmentation pathway. Peaks at 1527 cm^{-1} and 1343 cm^{-1} in the FTIR spectra further confirmed the presence of an aromatic nitro compound in the yellow pigment molecule. Lastly, the UV-spectra and relative retention time were consistent with the molecule having an additionally substituted nitro-group in comparison to the xanthurenic acid molecule.

The purified egg sac pigment had exactly the same mass like the dragline pigment resulting in the same structural formula: $C_{10}H_7N_2O_6$. The NMR-data further confirmed that they both had exactly the same structure and the same substituted side groups, including the nitro- group. Also the retention time and UV-spectra matched, further confirming both pigments were identical compounds.

To conclude, the yellow pigment in dragline silk was 7-nitro xanthurenic acid or more fully 4,8-dihydroxy-7-nitroquinoline-2-carboxylic acid with structural formula: $C_{10}H_6N_2O_6$ (Figure 5-13).

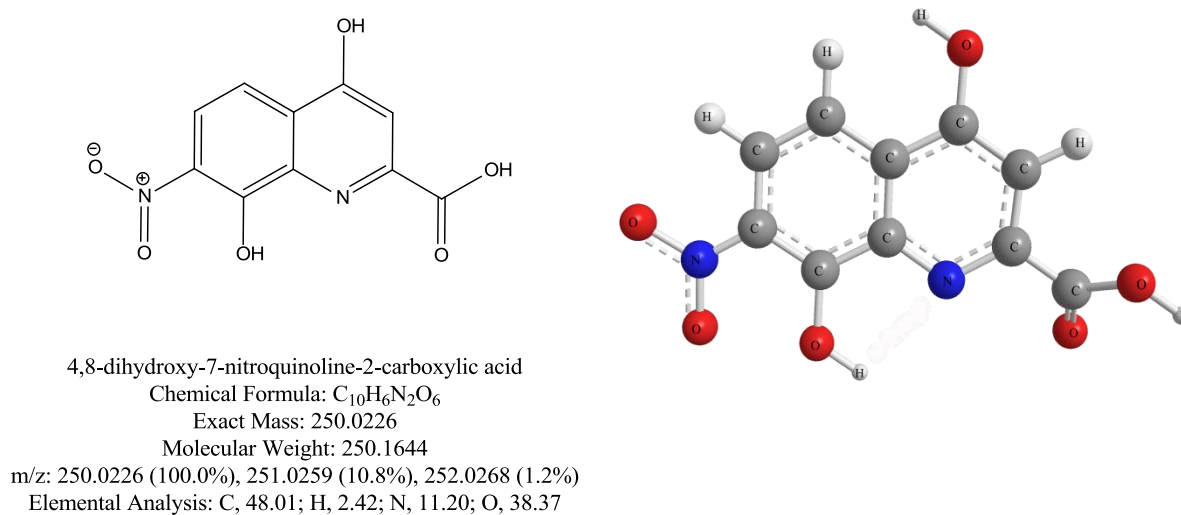


Figure 5-13. The predicted structure for the dragline pigment with its chemical properties (a) and a graphical representation (balls and sticks model) of the dragline pigment (b).

Chapter 6

In vitro synthesis of the yellow pigment

6.1. Introduction

In the previous chapter (Chapter 5) we have identified the molecule responsible for the yellow pigmentation in the dragline and egg sac silk of *N. edulis*, namely 7-nitro xanthurenic acid. Whereas xanthurenic acid was a known product of the tryptophan metabolism, the 7-nitro form of xanthurenic acid had never been observed and therefore was a newly discovered molecule. Because xanthurenic acid was an end metabolite, it was assumed that this molecule was nitrated to form 7-nitro xanthurenic acid found in the silk of *N. edulis*. *In vivo*, there are two ways nitration of molecules happen, namely enzymatic or chemically. The best known chemical nitration of compounds is via the nitronium ion (NO_2^+) which is typically produced by a “mixed-acid” such as nitric acid and sulphuric acid. *In vivo* however, this reaction would most likely occur via free-radical formation. The free radical chemistry however is very complex and there are still many uncertainties in how this works *in vivo*. Moreover, free radicals are less site-specific and were likely to produce more side products. In contrast, enzymatic nitration of tyrosine residues in peptides is known to occur via a peroxidase (e.g. myeloperoxidase, eosinophil, etc.)^{142, 143}. More recently it was found that even tryptophan derivatives could be nitrated via a peroxidase, more specifically Horseradish peroxidase (HRP) and Lactoperoxidase (LPO)¹⁴⁴.

The aim of this chapter was to synthesize 7-nitro xanthurenic acid *in vitro*. This was necessary because; large quantities were needed for future experiments, to make a ^{15}N isotope of the yellow pigment making the nitro group visible for NMR to further confirm its presence and study the possible interactions, and to get an idea of how 7-nitro xanthurenic acid was synthesized in the spider *in vivo*.

In this chapter, the most energetically favoured positions for substituting a nitro-group on xanthurenic acid were discussed first to give us an idea of the most likely yield. Next, we will

discuss the chemical nitration of xanthurenic acid via the most typically known process, the “mixed-acid” nitration which involves the use of nitric and sulphuric acid. Although this was unlikely to happen *in vivo*, this method was still preferred above the free-radical formation method as it was straight forward, more controllable and was expected to produce less side products.

For the enzymatic nitration of xanthurenic acid we chose to use Horseradish peroxidase as this peroxidase was known to nitrate tryptophan derivatives¹⁴⁴ and general ring structures¹⁴⁵. Because this enzymatic nitration depended on sodium nitrite and horseradish peroxidase¹⁴⁴, a range of concentrations of both reagents was tested. Whereas the highest sodium nitrite and hydrogen peroxide concentration resulted in a product that matched the retention time and UV-spectrum of 7-nitro xanthurenic acid, the yield of this product was very low. The unexpected low yield was thought to originate from multiple nitrations of the product over time and xanthurenic acid being in the wrong resonant form (Appendix A.4). Therefore this same reaction was run over different time intervals within 15 minutes and at two pH-values, pH 7.5 and 6.0, to explore the difference between the two main resonant forms of xanthurenic acid. This resulted in the synthesis of 7-nitro xanthurenic acid in a good yield at pH 6.0.

For identification and confirmation that this synthesized product was indeed 7-nitro xanthurenic acid, this method was up scaled and the putative 7-nitro xanthurenic acid was purified and manually fraction collected from preparative HPLC.

UV-spectroscopy, FTIR and NMR indicated this collected fraction was impure and consequently we were not able to confirm the identity of the compound. Therefore this impure fraction was separated again on a UPLC-MS system resulting in two compounds with one being identical to 7-nitro xanthurenic acid.

This showed that the nitration of xanthurenic acid via the enzyme HRP was able to form 7-nitro xanthurenic acid that was found in the dragline and egg sac silk of *N. edulis* in Chapter 5. In Chapter 7 we will further discuss the implications of this newly identified compound and its synthesis.

6.2. Material and method

6.2.1. Reagents

Xanthurenic acid, sodium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide, hydrochloric acid, sodium nitrite, horseradish peroxidase, hydrogen peroxide, sulphuric acid and nitric acid were obtained from Sigma-Aldrich (Dorset, UK). For liquid chromatography and mass spectrometry (MS), HPLC-grade solvents were obtained from Fisher Scientific: grade I water, methanol (UV cut-off: 200nm), acetonitrile (UV cut-off: 200nm) and trifluoroacetic acid (TFA). For NMR, D₂O and formic acid were used (Sigma). Where not mentioned, all water used was ultrapure grade I MilliQ (Millipore) quality.

6.2.2. Samples

The yellow pigment was extracted from the dragline silk of *Nephila edulis* (n: 60) as described in chapter 5.2.1. Subsequently, the resulting powder (± 1 mg) was dissolved in one millilitre of pure grade I MilliQ (Millipore) water and used as a reference for the synthesized product in high pressure liquid chromatography (HPLC), mass spectrometry (MS) and FTIR. For NMR, the same purified dragline sample was used as in previous chapter (chapter 5.2.1) because the weight obtained from the last extraction (± 1 mg) was too little for NMR.

6.2.3. Preparations

A. Chemical nitration of xanthurenic acid

Xanthurenic acid (500mg) was exothermically nitrated using a nitrating mixture consisting of 55% sulphuric acid, 35% nitric acid and 10% water, left to react at a temperature of 50°C for 3 hours followed by an elevation of the temperature to 90°C for one hour on a Bunsen burner. This solution was then dried on a rotary evaporator and the resulting powder was prepared in a concentration of 1mg/ml for analytical and preparative HPLC as described below.

B. Enzymatic nitration of xanthurenic acid

In a first attempt the effects of different concentrations of sodium nitrite and hydrogen peroxide were assessed on the enzymatic production of 7-nitro xanthurenic acid (Figure 6-1). Therefore, a sodium phosphate buffer was prepared (50ml 0.1M, pH 7.5 at 21°C; ionic strength: 0.268M) to which 22mg of horseradish peroxidase (HRP) was added to give a 1µM solution. To this solution, xanthurenic acid (130mg) was added to get a 0.01M concentration. To aliquots of this enzyme/substrate solution (3ml per sample, 4 samples per concentration) sodium nitrite was added to give final concentrations of 0.1M, 0.02M and 0.005M. To each of these samples, hydrogen peroxide solution (40%, Sigma-Aldrich) was added to give final concentrations of 10, 1, 0.1 and 0.01mM. This resulted in twelve samples (Figure 6-1).

In a second attempt, the effect of a lower pH and reaction time was studied (Figure 6-1). Therefore, a sodium phosphate buffer was prepared (0.1M, 50ml, pH 7.5 at 21°C; ionic strength: 0.268M) to which 22mg of horseradish peroxidase (HRP) was added to give a 1µM solution. To this solution, xanthurenic acid (130mg) was added to get a 0.01M concentration. Further, hydrogen peroxide and sodium nitrite were added to this solution to give a respectively 10mM and 0.1M concentration. Half of this solution (25ml) was kept at the initial pH 7.4 and the other half of the solution (25ml) was adjusted with concentrated

hydrochloric acid to pH 6.0. The samples at different pH were left to react for different time intervals (10sec, 1min, 5min, 10min and 15min) where after the reaction was stopped by submersing the vials in boiling water when sonicated.

For both experiments, all solutions were purged with dry nitrogen gas for at least 20 minutes prior to the addition of hydrogen peroxide. Each sample was injected 5 times, the chromatographic data was averaged and representative chromatograms were used in this chapter.

Controls were run in the absence of enzyme to rule out the possibility of a non-catalytic reaction and no products were formed indicating the enzyme was necessary for the synthesis.

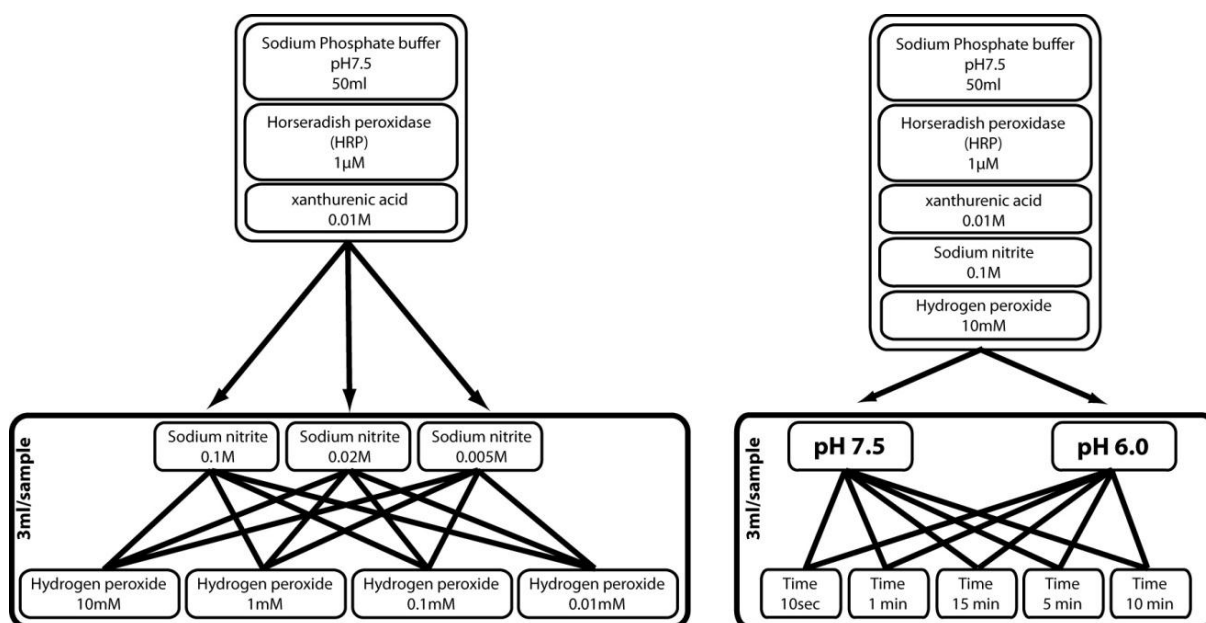


Figure 6-1. The sample preparation of the two experiments for different concentrations (on the left) and at different pH (on the right).

C. Large scale preparation of nitrated xanthurenic acid

Xanthurenic acid (130mg) was added to 20ml of the same phosphate buffer (as described above). Horseradish peroxidase (HRP) was added to give a 1µM solution and the solution was adjusted to pH 6.0. To this enzyme/substrate solution, hydrogen peroxide (10mM) and sodium nitrite (0.1M) were added. All solutions were purged with dry nitrogen gas for at least 30 minutes prior to the addition of hydrogen peroxide. The mixture was left to react for 15 minutes where after it was dialysed (MWCO: 14 kDa) four times against 500ml of ultrapure grade I MilliQ water (Millipore) overnight to separate the product (7-nitro xanthurenic acid, MWCO < 14 kDa) from the reaction mixture containing the HRP (MW: 44 kDa). This dialysand was then boiled down to a smaller volume and freeze dried. Finally, the 7-nitro xanthurenic acid was purified from the resulting powder with preparative HPLC. The peak at 14.5 min was manually collected for FTIR, MS and NMR.

6.2.4. Methods

Lowest substitution energy predictions: These predictions were made with chemical software, namely Chemdraw (v. 12.0, Scienomics as part of the MAPS suite). The density functional theory method was used to calculate the energy state of the proposed xanthurenic acid molecule and the difficulty of substituting each carbon atom in the ring.

The techniques (HPLC, NMR, MS and FTIR) and methods used for the identification of the synthesized pigment were identical to the ones described in Chapter 5.2.3.

6.3. Results

6.3.1. Synthesis

A. Preferred position of a substituted nitro group on xanthurenic acid

Density function method (Turbomole) simulations were used to calculate the energy of the proposed dragline pigment molecule using different substitution positions of the nitro group on xanthurenic acid. The calculations did not take into account the actual state of the acid group structure (ionisation) due to pH conditions used to obtain the protonated state of the other groups.

The two proposed resonance isomers (Appendix A.4) for the xanthurenic acid were given below; together with their calculated substitution energies (Figure 6-2).

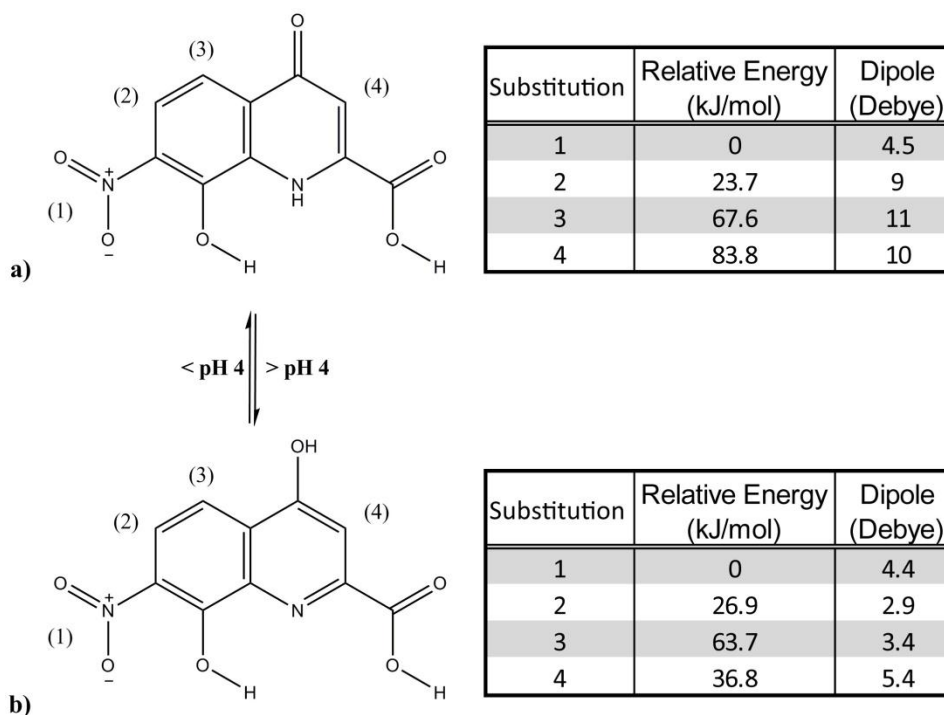


Figure 6-2. Two resonant forms of xanthurenic acid: (a) the carbonyl form at pH<4 and (b) the hydroxyl form at pH > 4, with numbers indicating the possible nitration sites in the structure (1 to 4) and tabulated next to it the energy required to nitrate this position and the dipole to overcome.

From the predictions, nitration at position 1 gave the most energetically favoured structure and this was also the nitration position of the yellow pigment (Chapter 5). This position was likely to be preferred for non-enzymatic nitration of xanthurenic acid in vitro.

B. Chemical synthesis

B.1. Analytical separation

The most straight forward way of synthesizing 7-nitro xanthurenic acid was by chemically nitrating xanthurenic acid with a mixture of nitric acid and sulphuric acid. Because the predicted preferred site for nitration in xanthurenic acid was exactly the position where the substitution occurred in the yellow pigment (7-nitro xanthurenic acid), a large yield of the pigment molecule was expected by chemical nitration.

The isogram of the chemical nitration product did show a peak at 14.5 minutes (Figure 6-3). The spectrum from the apex at 14.5minutes of the chemical nitrated xanthurenic acid (with PuP-value of 255.6 and relative retention time of 14.5) matched that of 7-nitro xanthurenic acid saved in the library (average of 25 spectra with PuP-value of 255.658 and relative retention time of 14.463) with a similarity index of 0.99641 and a dissimilarity of 0.08461 (Figure 6-3). A similar retention time and UV-spectrum was a strong indication that this product was indeed 7-nitro xanthurenic acid.

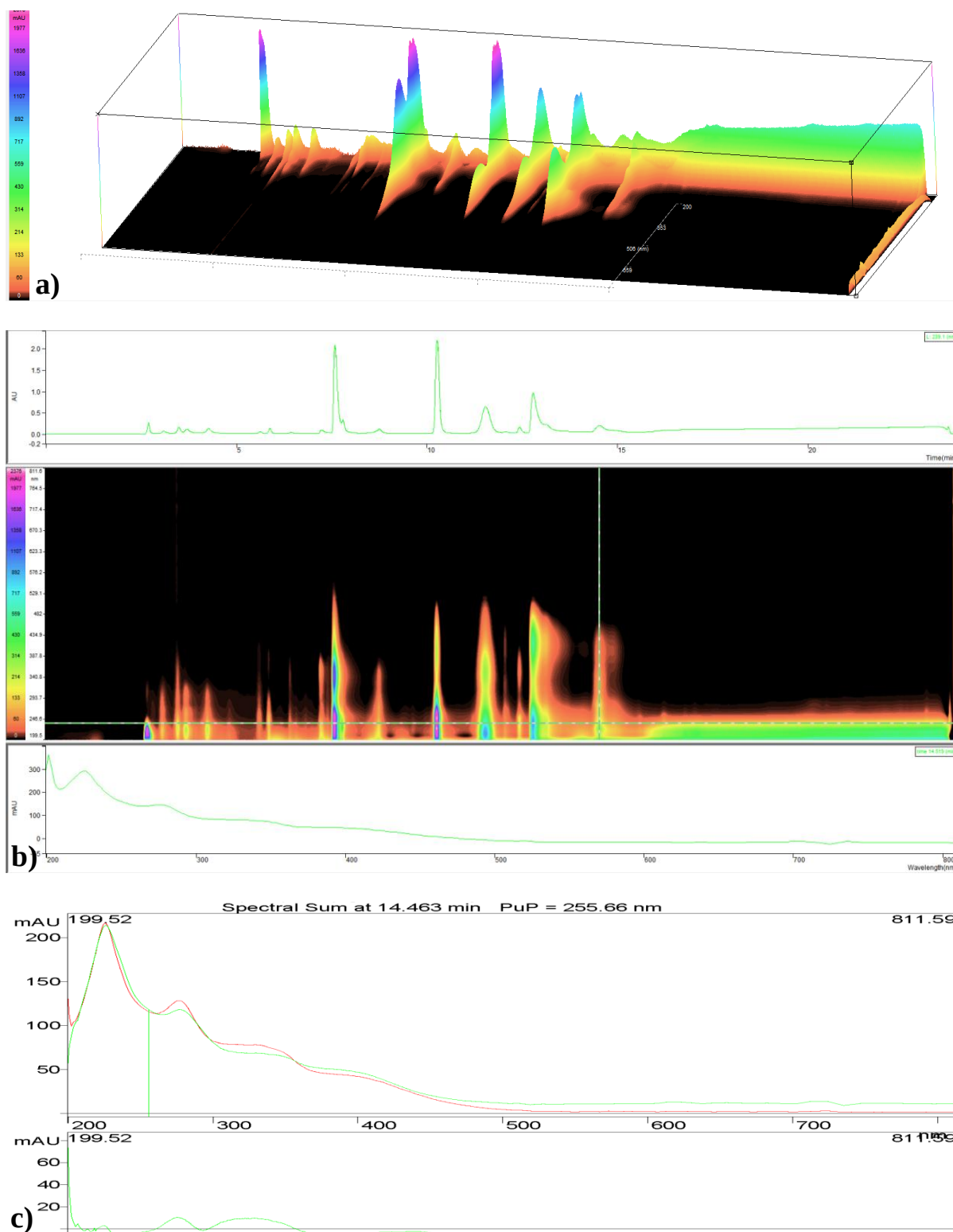


Figure 6-3. Chromatographic data of injected chemically nitrated xanthurenic acid showing (a) a 3D-representation of the isogram of the run with the X-axis as retention time (0 to 25min), Y-axis as absorbance (mAU) and Z-axis as wavelength (from 200-800nm), (b) the 2D-isogram with the peak selected for the dragline pigment showing the measured UV-spectra and chromatogram (270nm) at this point (green), and (c) The spectral match of the measured UV-spectrum of the apex at 14.4 minutes (green) with the spectrum from 7-nitro xanthurenic acid (red, n:25) and the difference spectrum between both spectra (below, green).

Figure 6-4 shows the chromatogram of the nitrated xanthurenic acid measured at 270nm indicating the common peak (7-nitro xanthurenic acid) between the dragline extract and the chemical nitration products. The chemical nitration product with a small peak at 14.5 minutes matched the retention time and UV-spectra of 7-nitro xanthurenic acid. Another fit between the chromatogram of dragline extract and the chemical nitration product was the presence of a peak at 2.6 minutes with identical UV-spectra and is most likely sodium nitrite (Figure 6-4). However as this was an ion and was eluting in the void volume of the column we can only suggest its presence. The peak at 12.7 minutes in the chemical nitration product was found to be xanthurenic acid which fitted the UV-spectrum and retention time of standard injected xanthurenic acid (Sigma) and was not completely used in the chemical nitration. In contrast to what was expected, chemical nitration of xanthurenic acid resulted in many unknown side products (main peaks at 5.8 min, 7.5 min, 8.7 min, 10.2 min and 11.5 min).

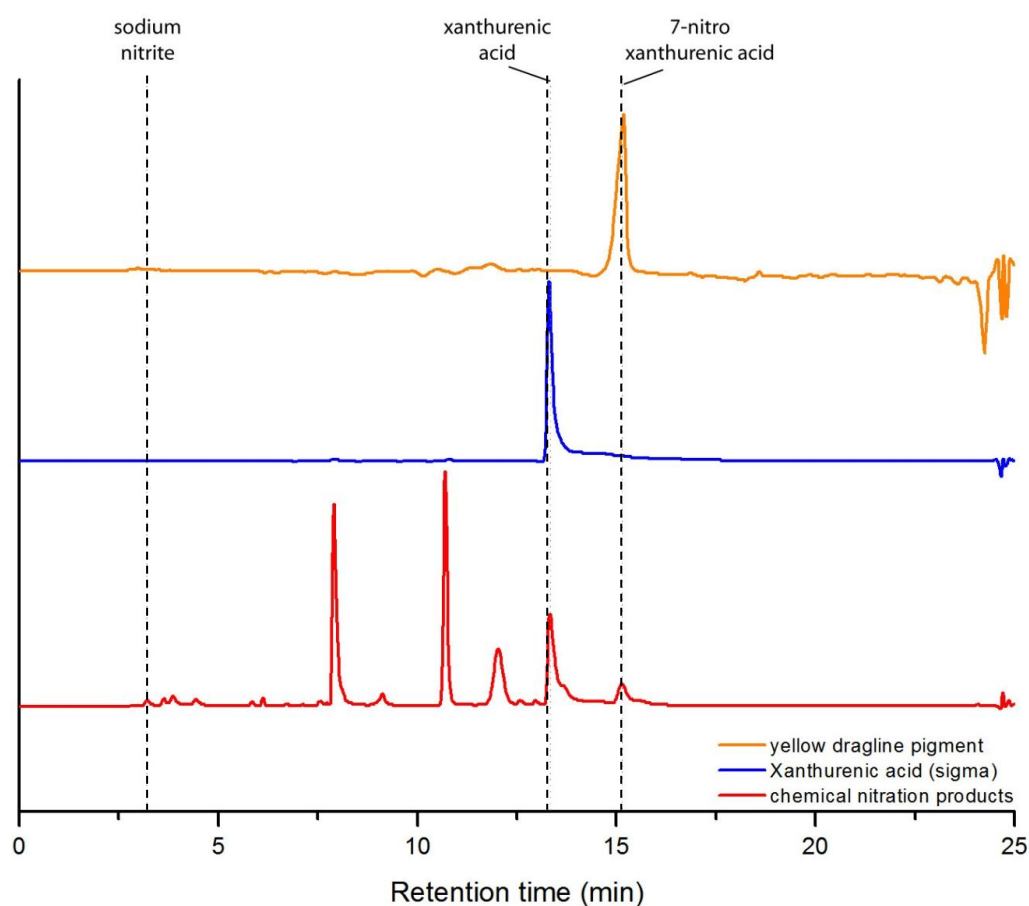


Figure 6-4. Chromatographic traces taken at 270nm of extracted dragline pigment (yellow trace), xanthurenic acid (blue trace) and chemically nitrated xanthurenic acid (red trace) with some characteristic peaks indicated with dotted lines (sodium nitrite, xanthurenic acid and 7-nitro xanthurenic acid (yellow pigment)).

B.2. Preparative separation

Preparative separation was attempted despite the low yield, but the separation of the components was not as good as that obtained with the analytical column. Despite many adjustments including changes to the gradient, running time and different mobile phases, no satisfactory separation of the mixture was obtained (see Figure 6-5) and co-elutants were detected in the main peak at 19.1 minutes. Therefore, it was impossible to obtain the purified fraction of the peak at 14.5min from the chemical nitrated product (Figure 6-4) for further identification with NMR and MS.

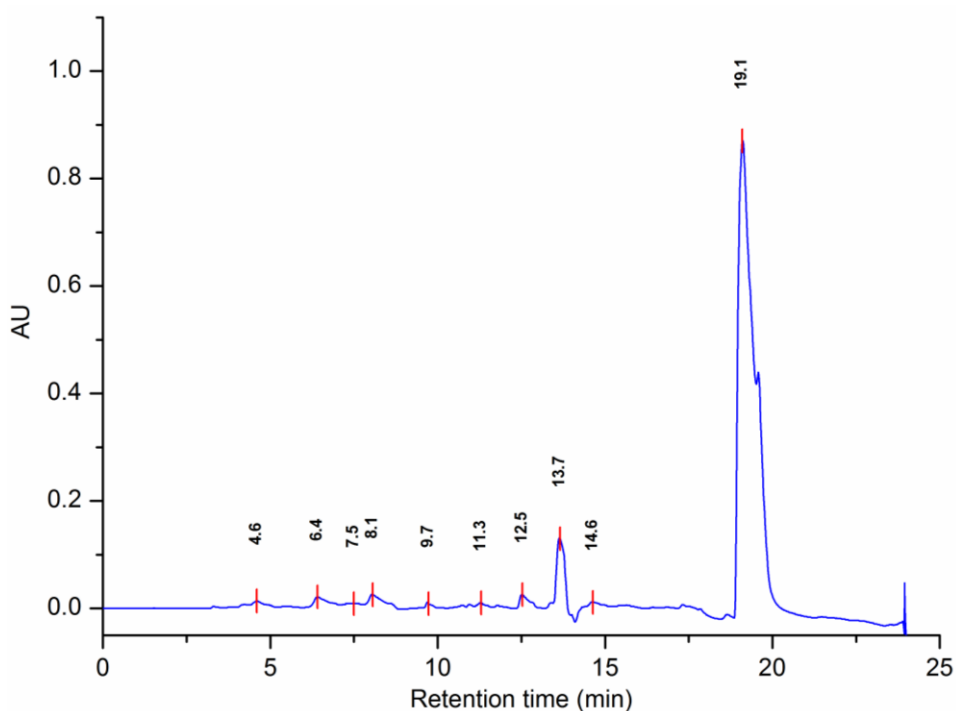


Figure 6-5. Chromatogram at 270nm of attempt at chemical synthesis of nitrated xanthurenic acid run on the semi-preparative column resulting in poor separation and a massive impure peak at 19.1 minutes.

B.3. Summary

The chemical nitration of xanthurenic acid resulted in a nitration product that had exactly the same retention time and UV-spectrum as 7-nitro xanthurenic acid found in the dragline silk extracts. However due to the fact that the yield was so low, many side-products were produced and no satisfactory separation was obtained by semi-preparative chromatography, it was impossible to purify this compound for further identification with NMR and MS.

The dirtiness of the chemical nitration procedure may result from lack of specificity of the site of nitration and the formation of breakdown products resulting from the use of the nitric and sulphuric acid for the nitration. This suggested that the nitration of xanthurenic acid probably was not chemically *in vivo*, as free-radical nitration would result in the same amount of side products not seen in the natural extract. An attempt was therefore made to use a bio-inspired enzymatic synthesis in hope that this would provide a cleaner method.

C. Enzymatic synthesis

Chemical nitration resulted in many side products that were absent in the natural extraction of dragline silk suggesting that nitration of xanthurenic acid was probably enzymatically catalyzed *in vivo*. From the literature we found that tryptophan derivatives could be nitrated by horseradish peroxidase and lactoperoxidase¹⁴⁴ and that horseradish peroxidase could easily nitrate benzene rings as well¹⁴⁶. Furthermore a peroxidase was found in the silk gland of *N. edulis* that was suggested to be involved in the yellow pigment synthesis^{147, 148}. Therefore we tested if an enzymatic nitration of xanthurenic acid via a peroxidase was a possible synthesis route.

C.1. Effect of sodium nitrite and hydrogen peroxide concentration

After an initial run proved successful, a range of different conditions were tried systematically in an attempt to optimise the method (Table 6-1 and Figure 6-6).

a) General

In Figure 6-6, representative chromatograms were listed of all tested samples with different concentrations of sodium nitrite and hydrogen peroxide. The chromatographic profile of sodium nitrite was very characteristic in that it had two peaks (start and end of the sodium nitrite “region”, indicated in shaded grey) with a very broad peak in between. The size of the last two peaks increased with increasing sodium nitrite concentration (see Figure 6-6). Peaks in this region were likely to have been obscured by this effect, however the products of interest elute later (7-nitro xanthurenic acid: 14.5min). The main peak in the chromatogram was identified by its UV-spectrum and elution time as xanthurenic acid and thus represents the substrate that was not nitrated.

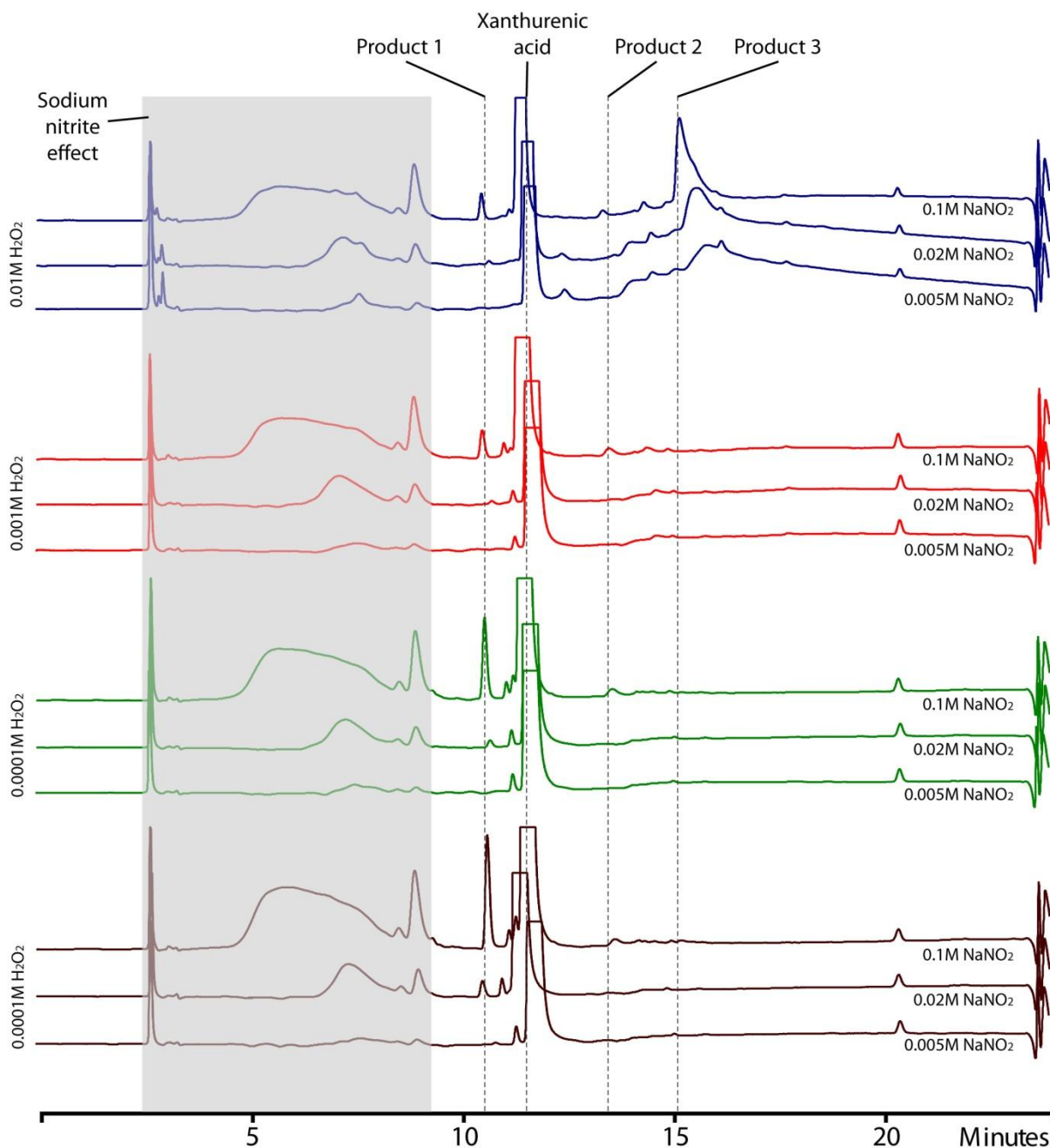


Figure 6-6. The chromatograms (at 270nm with normalized Y-axis) for the three different concentrations of sodium nitrite (0.1M, 0.02M and 0.005M) grouped together for the four different concentrations of hydrogen peroxide used (0.01M, 0.001M, 0.0001M and 0.00001M). The region that was affected by sodium nitrite is indicated. The four main peaks that varied outside this region are labelled (xanthurenic acid and unknown products 1, 2 and 3).

Three peaks in the chromatogram were changing with varying concentrations of sodium nitrite and hydrogen peroxide and were labelled: product 1, product 2 and product 3 (Figure 6-6). The UV spectrum of the apex of each product peak was given in Figure 6-7. Integration of the main four peaks resulted in a relative amount of each peak which could be compared between samples (Table 6-1).

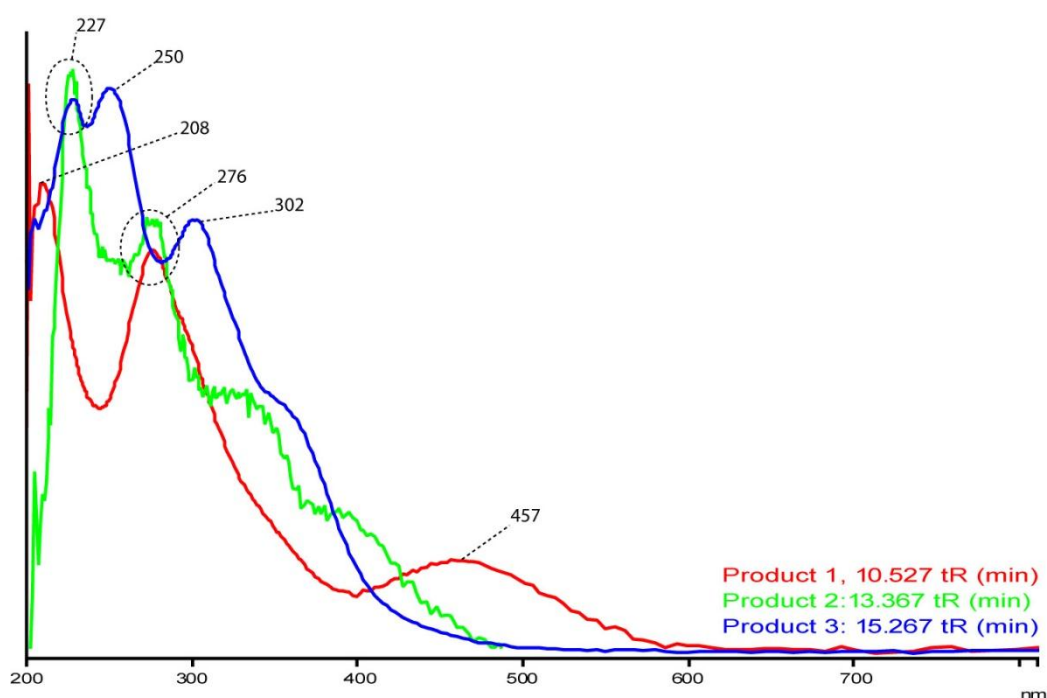


Figure 6-7. UV spectrum of the three product peaks (1, 2 and 3) in the enzymatic nitration of xanthurenic acid. Product 2 had a similar UV spectrum as 7-nitro xanthurenic acid (yellow pigment) and whereas both other spectra were unidentified spectra. Peak maxima were labelled.

b) Products

Product 2 (the putative yellow pigment: 7-nitro xanthurenic acid): This peak had a retention time of 13.3 minutes (Figure 6-6) and therefore eluted about one minute earlier than 7-nitro xanthurenic acid (yellow pigment). However, the UV-spectrum of product 2 matched that of 7-nitro xanthurenic acid with a similarity index of 0.93 and dissimilarity of 0.37 (Figure 6-7). This was a poor match but could be due to the low concentration of this product.

It was remarkable that product 2 was only produced at the highest sodium nitrite concentration (0.1M) (Table 6-1). At this concentration the yield produced at the different hydrogen peroxide concentrations tested was not significantly different ($p > 0.05$). Furthermore, product 2 was only synthesized in the beginning of the synthesis (within the first 15 minutes) and gradually decreased in amount over time until it was completely absent after 24 hours (Figure 6-8). This is possibly the result of multiple nitration or peroxidation of product 2 over time.

Table 6-1. The averaged peak areas (n: 3, at 270nm) of the four main peaks (xanthurenic acid and three products (1, 2 and 3)) were tabulated for each sample. Peaks were listed in order of elution time.

NaNO ₂	H ₂ O ₂	Tested after 15 minutes				Tested after 24 hours			
		Product 1	Xanthurenic acid	Product 2	Product 3	Product 1	Xanthurenic acid	Product 2	Product 3
0.1M	0.01M	1.2 E+06	1.1 E+08	3.9 E+05	1.6 E+07	4.0 E+06	1.8 E+07	0	7.6 E+06
	0.001M	1.5 E+06	1.5 E+08	6.5 E+05	2.1 E+05	1.5 E+07	2.0 E+08	0	1.3 E+06
	0.0001M	4.2 E+06	1.6 E+08	7.7 E+05	1.6 E+05	2.3 E+07	1.9 E+08	0	2.5 E+06
	0.00001M	5.8 E+06	1.6 E+08	4.4 E+05	9.1 E+04	2.6 E+07	1.9 E+08	0	2.0 E+06
0.02M	0.01M	1.4 E+05	1.1 E+08	0	1.2 E+07	2.8 E+05	1.4 E+08	0	1.7 E+07
	0.001M	1.4 E+05	1.5 E+08	0	1.4 E+05	3.3 E+05	1.9 E+08	0	2.1 E+06
	0.0001M	3.5 E+05	1.6 E+08	0	7.6 E+04	2.7 E+06	1.4 E+08	0	1.9 E+05
	0.00001M	8.4 E+05	1.6 E+08	0	0	3.8 E+06	2.0 E+08	0	1.6 E+05
0.005M	0.01M	1.1 E+05	1.1 E+08	0	3.7 E+06	6.7 E+05	1.4 E+08	0	7.4 E+06
	0.001M	0	9.6 E+07	0	1.6 E+05	0	7.7 E+07	0	4.5 E+06
	0.0001M	0	1.6 E+08	0	7.8 E+04	0	5.7 E+07	0	1.7 E+05
	0.00001M	0	1.6 E+08	0	0	7.5 E+05	9.1 E+07	0	1.4 E+05

Xanthurenic acid: this was the substrate molecule and had a retention time of 11.3 minutes which was about one minute earlier than when it was run without sodium nitrite (12.4min). The amount of xanthurenic acid did not change significantly with sodium nitrite concentration ($p>0.05$) and neither with hydrogen peroxide concentration ($p>0.05$), meaning that the enzymatic synthesis did not synthesize many products in large quantities.

Product 1: this peak had a retention time of 10.4 minutes (Figure 6-6) and the UV-spectrum did not match any compound stored in my personal spectral library (Figure 6-7). The synthesized amount of this compound was positively correlated with the concentration of sodium nitrite, meaning more sodium nitrite resulted in more Product 1 (see Figure 6-6). There was no significant difference ($p>0.05$) in synthesized amount between the hydrogen peroxide concentrations. However a trend was noticed that less hydrogen peroxide resulted in more Product 1 production (see Table 6-1). The amount of Product 1 produced after 24 hours was significantly higher than the amount synthesized after 15 minutes ($p<0.05$) (see Figure 6-8 and Table 6-1). The increasing amount of Product 1 over time and the disappearance of Product 2 after time (see further) together with the UV-spectra of this compound, all indicate that this product was multiple nitrated (we suggest three nitro groups).

Product 3: this peak had a retention time of 15.1 minutes (Figure 6-6) and the UV-spectrum of the apex did not match any compound stored in my personal spectral library (Figure 6-7). The yield of this product did not significantly change over time ($p>0.05$), neither did it significantly change with sodium nitrite concentrations ($p>0.05$) (Figure 6-8 and Table 6-1). However, the synthesis of this product was very dependent on the hydrogen peroxide, where the highest concentration (0.01M) produced significantly more Product 3 than the other concentrations ($p<0.001$) (see Table 6-1). This was most likely a side product as the free radicals in the mixture would increase with increasing hydrogen peroxidase concentration.

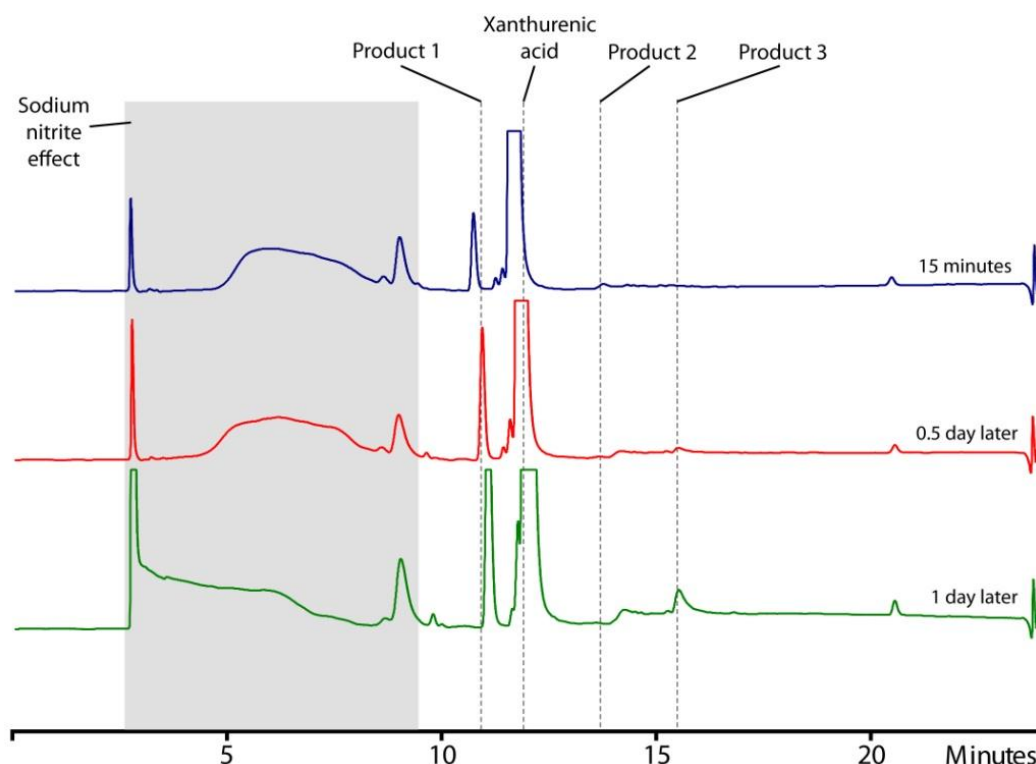


Figure 6-8. Chromatographic traces (270nm) of one sample (0.1M NaNO₂ and 0.00001M H₂O₂) after increasing time intervals during synthesis. Xanthurenic acid and the enzymatic synthesis products are labelled.

c) Summary

In general, via enzymatic nitration of xanthurenic acid it was possible to get a product (Product 2) that had exactly the same UV-spectrum as the yellow pigment (7-nitro xanthurenic acid). However this compound eluted one minute earlier than the yellow pigment. This could be due to the altered chromatographic conditions in the column produced by sodium nitrite and was also seen in the earlier elution time of xanthurenic acid. Alternatively, the compound (Product 2) may have a nitro group in a different position (which would result in the same λ_{max}). Furthermore the compound was obtained in a low yield comparable to the chemical nitration (both a peak height of 150mAU) complicating the large scale production and purification. In addition, the synthesis of the compound was time dependent and was only produced in the first 15 minutes where after the product was probably nitrated further.

C.2. Effect of pH

a) *General*

In the previous section it was shown that a peak (Product 2) matched that of 7-nitro xanthurenic acid (yellow pigment) and that it could be synthesised from xanthurenic acid using horseradish peroxidase in the presence of hydrogen peroxide and sodium nitrite. However the yield was very low and thus not favourable for large scale preparation and purification. Xanthurenic acid was known to have two pH-dependent isomeric structures and therefore it was possible that xanthurenic acid was in the wrong isomeric form at pH 7.5 (Appendix A.4). The transition of xanthurenic acid to its two different resonant structures was easily noticeable as a colour change from yellow in alkaline solutions to colourless in acidic conditions. With pure xanthurenic acid solution this colour change happened at pH 6 on acidification. Therefore it was decided to test the enzymatic synthesis of 7-nitro xanthurenic acid from xanthurenic acid at two pH conditions, namely pH 7.5 and pH 6.0, with the aim of cleaning up the synthesis and obtaining an increased yield. The synthesis was stopped after 15sec, 1min, 5min, 10min and 15min because the previous experiment had shown that the quantity and cleanness of the synthesis of the putative pigment (Product 2) was time dependent, the longer the reaction lasted the fewer products was left possibly by multiple nitrations.

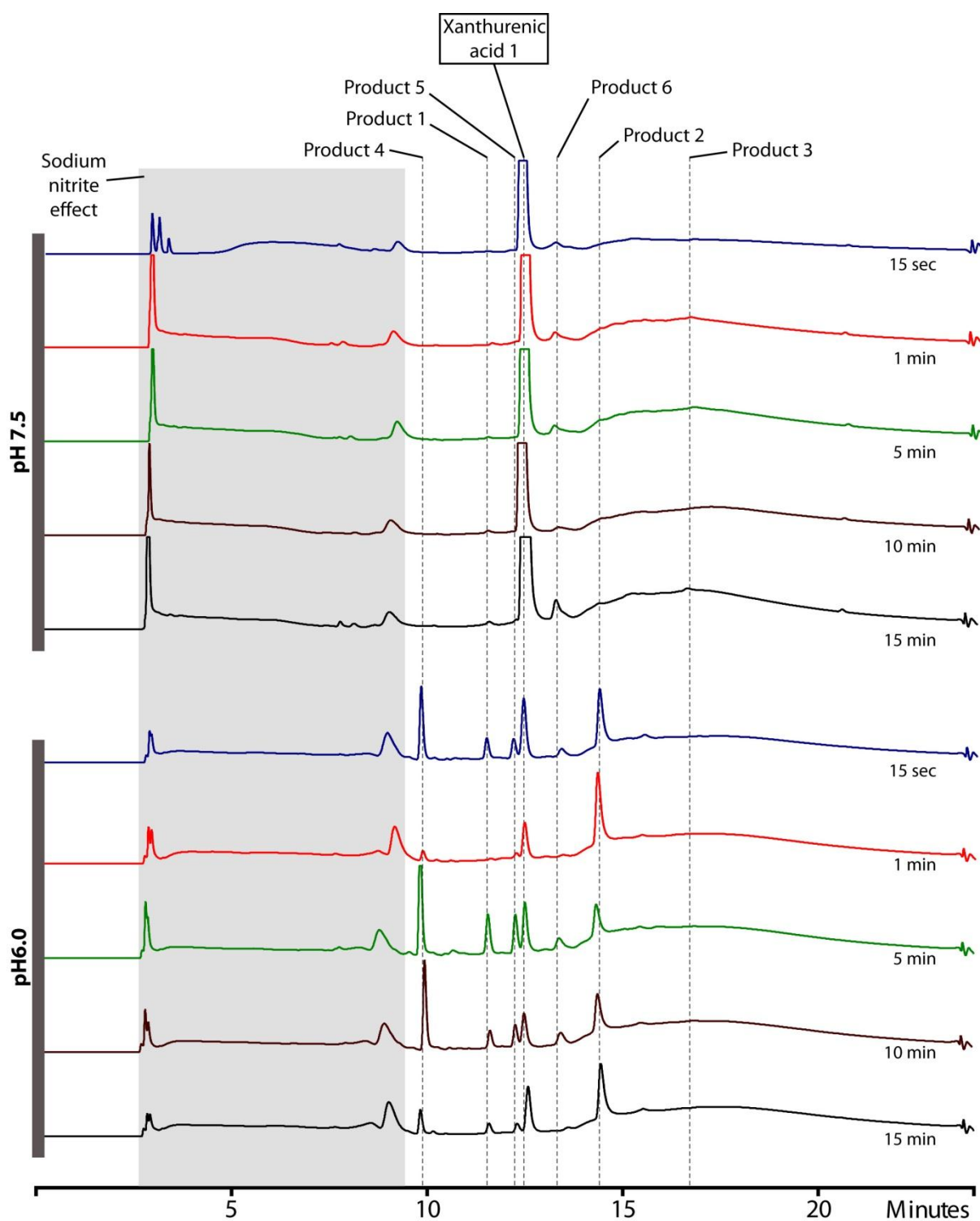


Figure 6-9 shows the averaged chromatograms (n: 5, trace at 270nm, Y-axis: 0 to 1AU) for both pH conditions (pH 6.0 and pH 7.5) at the different time intervals (15sec, 1min, 5min, 10min and 15min). The effect of sodium nitrite was shaded and labelled. The six main peaks that varied outside this region (xanthurenic acid and six product peaks) were marked.

Figure 6-9 shows the effect of the enzymatic synthesis at pH 6.0 and 7.5 and at varying time intervals. As described in previous chapter, the sodium nitrite effect (shown in grey) was variable and not considered. The chromatograms of the synthesised products at pH 6.0 were dramatically different from those at pH 7.5 in that much more Product 2 was produced (the putative 7-nitro xanthurenic acid (yellow pigment)) from the three commonly produced products (1, 2 and 3) from previous experiment and three additional products were synthesized (products 4, 5 and 6) at this pH.

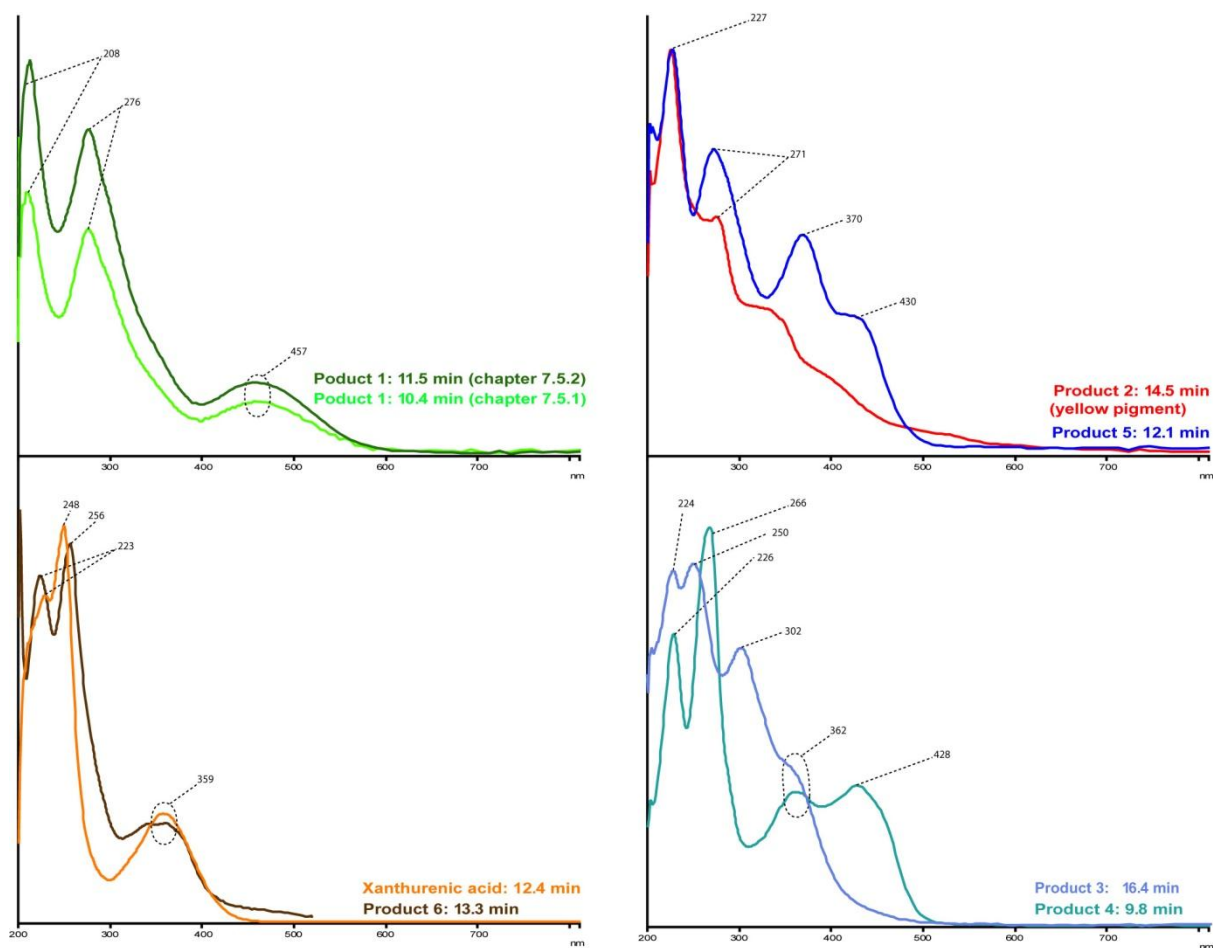


Figure 6-10. UV-spectra at the apex of the different product peaks in the chromatograms shown in Figure 6-9.

b) Products

Note that all peaks eluted about one minute later than the previous set of experiments. When looking at the peak of Xanthurenic acid, Product 1 and Product 3, these exact same compounds eluted now about 1 minute later confirming the malfunction of the machine in the first experiment.

Table 6-2. The averaged peak areas (n:3, at 270nm) of the seven main peaks (xanthurenic acid and six products (1 to 6)) were tabulated for both pH conditions, pH6.0 and 7.5, for the different time intervals (15sec, 1min, 5min, 10min and 15min). Peaks were listed in order of elution time.

Substrate solution	Adjusted to	Reaction time (min)	Product 4	Product 1	Product 5	Xanthurenic acid	Product 6	product 2	Product 3
Xanthurenic acid (0.01M) HRP (1μM) NaNO ₂ 0.1M H ₂ O ₂ 0.01M in phosphate buffer (0.1M, pH7.5)	pH 7.5	0.16	0.0 E+00	2.6 E+05	0	1.3 E+08	7.8 E+05	0	6.0 E+05
		1	1.8 E+05	7.2 E+05	0	1.4 E+08	6.2 E+06	1.3 E+06	6.2 E+04
		5	1.2 E+05	6.3 E+05	0	1.4 E+08	6.1 E+06	3.7 E+06	1.8 E+06
		10	9.4 E+04	1.3 E+06	0	1.3 E+08	1.9 E+06	0	0
		15	2.1 E+05	1.9 E+06	0	1.6 E+08	1.0 E+07	1.1 E+06	1.9 E+06
	pH 6.0	0.16	2.1 E+07	7.3 E+06	6.4 E+06	2.3 E+07	3.8 E+06	2.5 E+07	4.6 E+05
		1	2.8 E+06	5.8 E+05	3.2 E+06	1.4 E+07	6.2 E+05	3.3 E+07	1.4 E+06
		5	3.1 E+07	1.4 E+07	1.2 E+07	1.8 E+07	5.7 E+06	1.5 E+07	9.8 E+05
		10	2.5 E+07	6.2 E+06	7.5 E+06	1.4 E+07	5.7 E+06	1.9 E+07	1.2 E+06
		15	7.0 E+06	3.6 E+06	3.4 E+06	1.9 E+07	4.8 E+05	2.7 E+07	6.7 E+05

Product 2 (putative yellow pigment (7-nitro xanthurenic acid)): This peak had a retention time of 14.4 minutes (Figure 6-9) and a UV spectrum (Figure 6-10) similar to that of the yellow pigment (7-nitro xanthurenic acid). This UV spectrum matched that of 7-nitro xanthurenic acid (stored in the spectral library) with a similarity index of 0.9993 and a dissimilarity index of 0.036, indicating probably identical compounds.

The synthesized amount of Product 2 at pH 6.0 was significantly different from that produced at pH 7.5 ($p < 0.05$) and the synthesis increased by 90% at pH 6.0. The synthesis was not time dependent in the time frame up to 15 minutes ($p > 0.05$) (Table 6-2).

Xanthurenic acid: The main peak at about 12.4 minutes (Figure 6-9) with its UV-spectrum (Figure 6-10) matched that of xanthurenic acid and was the substrate compound in the enzymatic synthesis.

The amount of xanthurenic acid left after synthesis was significantly less ($p < 0.001$) at pH 6.0 than at pH 7.5 (Table 6-2), indicating that the synthesis was more successful (in contrast to the first experiment) and more xanthurenic acid was used as a substrate for nitration during synthesis.

Product 6: This peak did not significantly change with pH or with reaction time ($p < 0.05$). This compound eluted one minute later (13.3min, Figure 6-9) than xanthurenic acid but has exactly the same UV-spectrum (Figure 6-10) and was possibly a different conformation of xanthurenic acid.

Product 1: this product had a retention time of 11.5 minutes (Figure 6-9) and the UV-spectrum matched that of “Product 1” from the previous experiment indicating it probably was the same compound (Figure 6-10). The synthesized amount was independent of pH conditions ($p > 0.05$). In contrast to the first experiment, the synthesis did not significantly increase over this short time period ($p > 0.05$) (Table 6-2).

Product 3: This peak eluted at 16.4 minutes (Figure 6-9) and matched the UV-spectrum of “Product 3” from previous experiment indicating it was probably the same compound (Figure 6-10). The synthesized amount of this product was not pH dependent ($p > 0.05$) and the amount did not significantly change within a 15 minute time period ($p > 0.05$) (Table 6-2). This was still in agreement that it was a side product of radical formation due to hydrogen peroxide.

Product 4: This product eluted at 9.8 minutes (Figure 6-9) and was the most polar product (Figure 6-9). The λ_{max} (500nm) of the UV spectrum was similar to that of 7-nitro xanthurenic acid but displayed a different shape (Figure 6-10).

The synthesis of “Product 4” was significantly higher ($p < 0.05$) at pH 6.0 than it was at pH 7.5. The amount increases by a hundred fold and thus is highly favoured at this pH. The reaction however did not seem to be time dependent ($p > 0.05$) (Table 6-2).

Product 5: This product eluted at 12.1 minutes (Figure 6-9) and the λ_{max} (500nm) of the UV spectrum was similar to that of 7-nitro xanthurenic acid but displayed a different shape (Figure 6-10). The synthesis of this product was not time dependent ($p > 0.05$) and this compound was only produced at pH 6.0 (Table 6-2). Therefore it could be suggested that this compound would only be synthesized when xanthurenic acid was in the right conformation.

c) Summary

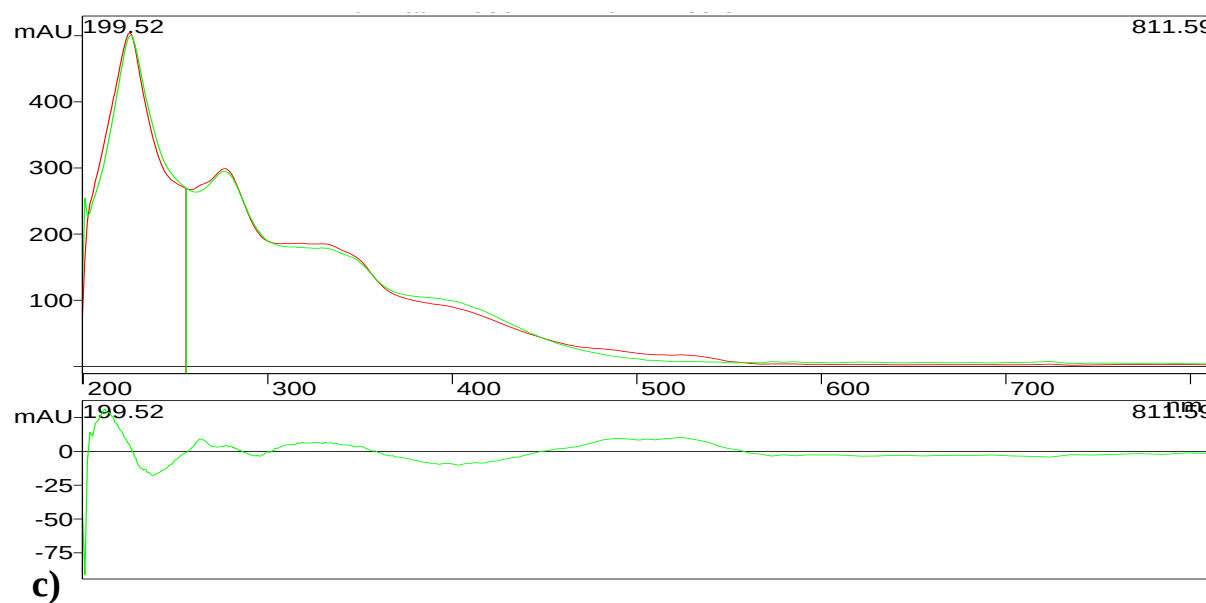
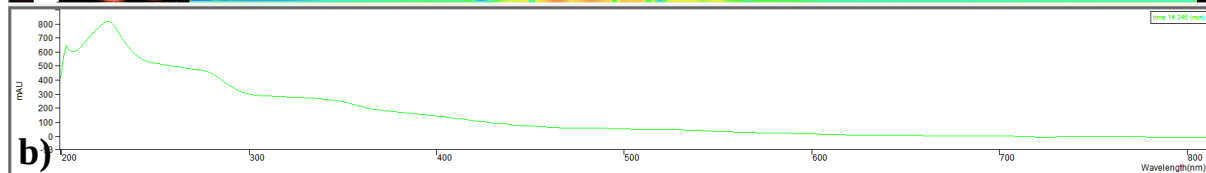
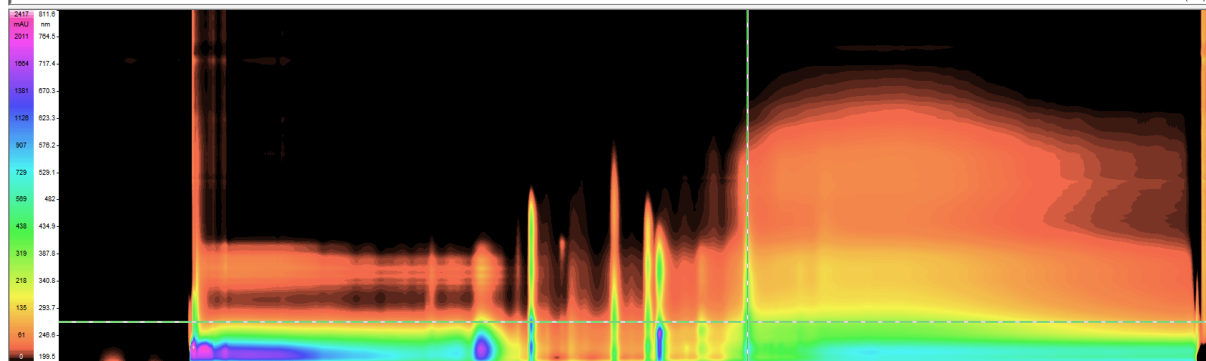
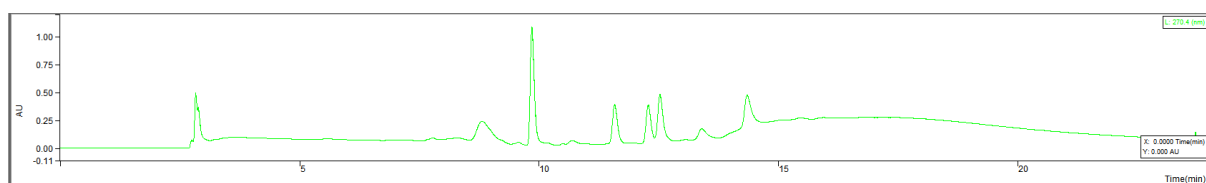
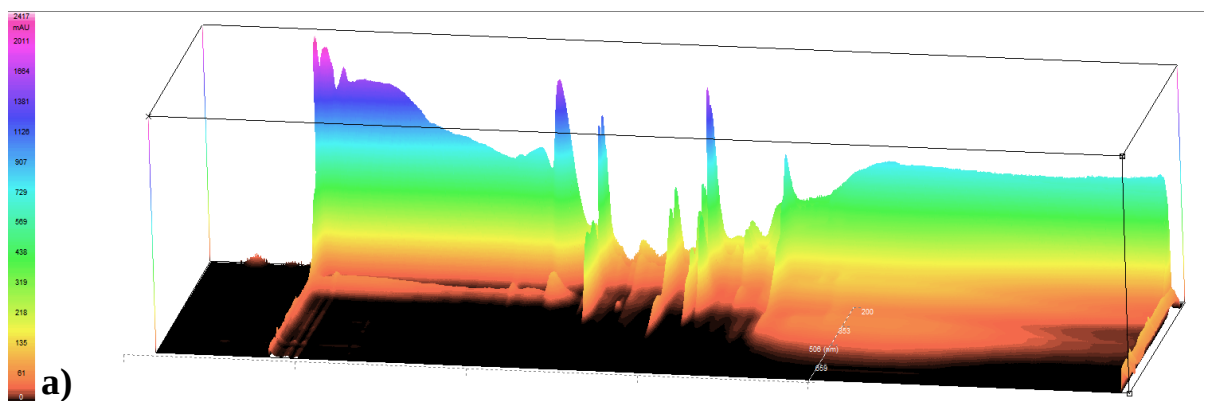
In general, enzymatic nitration of xanthurenic acid at pH 6.0 resulted in a product (Product 2) which matched the retention time and UV-spectrum of 7-nitro xanthurenic acid. Lowering the pH to 6.0 increased the yield of “Product 2” by 90% in contrast to the amount synthesized at pH 7.5 (Figure 6-11). Whereas, HRP was optimal at a pH in the range of 6.0-6.5, the activity of the enzyme at pH 7.5 would still be 84% of its maximum. Thus, if this increase was solely due to a more optimal working of the HRP at pH 6.0 only an increase of 6% in the production should be seen. Here an increase of 90% was seen what strongly suggested this increased production of “Product 2” was due to the isomeric structure of xanthurenic acid in a more acidic environment allowing the enzyme to nitrate the molecule in the right position.

D. Conclusion

Here we present preliminary evidence from the close similarity between the elution time and the UV-spectrum that a product (Product 2) identical to that of the yellow pigment, 7-nitro xanthurenic acid, had been successfully synthesised by chemical and enzymatic nitration. However, the chemical and enzymatic nitration (at pH 7.5) resulted in a very low yield and proved unusable. However the enzymatic synthesis of this product (Product 2) increased by 90% when the pH was lowered to 6.0. The synthesis at this pH was extremely fast and was detectable after 15 seconds of incubation. However, besides “Product 2” a few more side products were produced via this enzymatic synthesis indicating the enzyme was not entirely product specific (Figure 6-11).

In section C.1 above, the concentration of “Product 2” decreased with increasing reaction time (longer than 15minutes) and therefore a reaction time of 15 minutes was favoured for the large scale preparation (see further). Because the best synthesis of “Product 2” was found at pH 6.0 at the highest sodium nitrite and hydrogen peroxide concentrations, these conditions were used for the large scale preparation of the pigment for further verification and identification with NMR and MS.

Figure 6-11 (on next page). Chromatographic data of injected xanthurenic acid/HRP reaction mixture showing (a) a 3D-representation of the isogram with the X-axis as retention time (min), Y-axis as absorbance (AU) and Z-axis as wavelength (200-800nm), (b) the 2D-isogram with “Product 2” (the putative yellow pigment) showing the UV-spectrum and Chromatogram (270nm) at this point, and (c) The spectral match of the UV-spectrum of the apex at 14.4 minutes (green) with the spectrum from 7-nitro xanthurenic acid (n:25, red) and the difference spectrum.



6.3.2. Identification of the enzymatic produced dragline pigment

A. HPLC: large scale preparation

Having improved the yield of “Product 2” produced by the enzymatic nitration with HRP/H₂O₂/NaNO₂, a large batch was prepared for further identification and verification if this product was indeed 7-nitro xanthurenic acid. After a reaction time of 15 minutes, the reaction mixture was dialysed and the dialysand was saved and concentrated. The dialysis step was introduced for practical reasons (enzyme not blocking the column) and as an efficient way of stopping the reaction (pH of dialysis water was ± 7.0 slowing the reaction down and sodium nitrite and hydrogen peroxide were dialysed out of the reaction mixture) without the introduction of inhibition products for disabling the enzymes that were also likely to bind and interfere with our synthesised products.

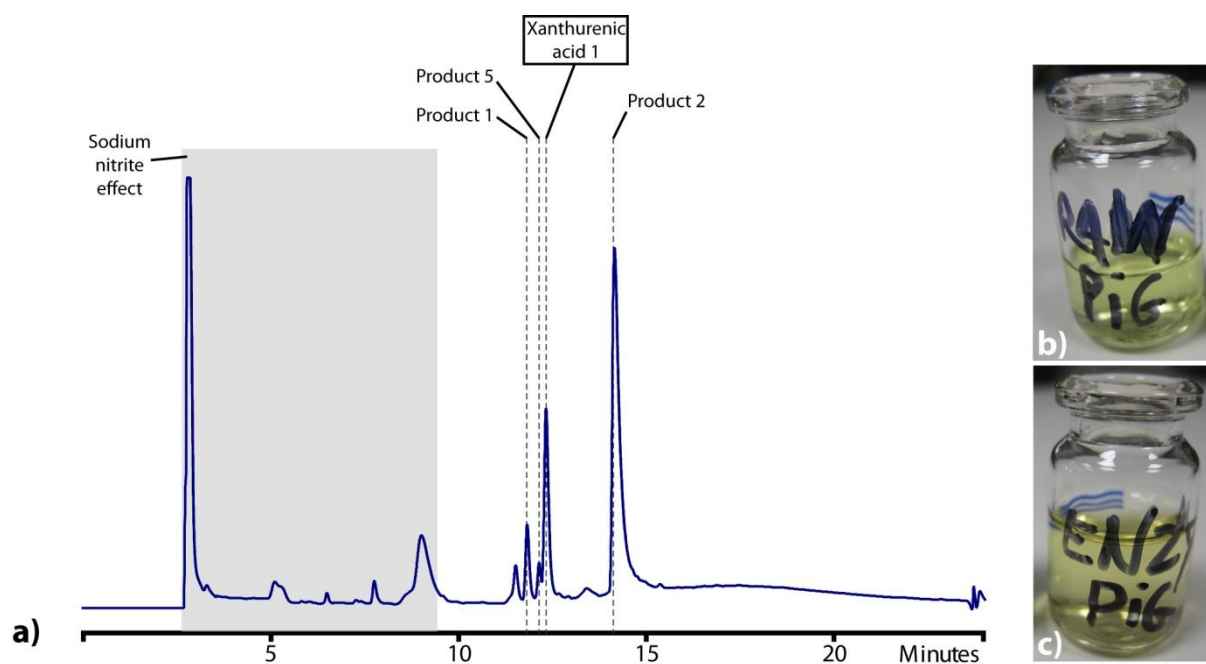


Figure 6-12. a) The preparative chromatogram (270nm, Y-axis: 0 to 2 AU) for the enzymatic synthesized dragline pigment. The effect of sodium nitrite was shaded and labelled. The products that were found back from the analytical separation were labelled, b) purified extracted yellow pigment of dragline silk (7-nitro xanthurenic acid), and c) the purified enzymatic synthesized Product 2 (the putative 7-nitro xanthurenic acid).

A typical chromatogram of the synthesis product is given in Figure 6-12 (a), showing a good yield of “Product 2”, the putative 7-nitro xanthurenic acid (1.7AU at the apex of the peak). Three more side products were co-synthesized of which two were identical to the synthesized Products 1 and 5 from previous experiment; however their abundance was low in comparison to “Product 2”. The fact that despite identical synthesis conditions as previous experiment, three products (3, 4 and 6) were absent in the preparative run suggested that some peaks might co-elute on the preparative column as a result of differences in its contents or packing. A purity calculation of the “Product 2” peak indicated that the front of the peak was impure (purity parameter: 219.465nm) in comparison to the apex and rest of the peak (purity parameter: 262.697nm). This means another compound was possible co-eluting with the product of interest, “Product 2” (this will be confirmed later with UPLC-MS). Adjusting the HPLC-method did not result in a better separation and therefore the entire peak, “Product 2” at 14.5 minutes, was selected for purification (Figure 6-12).

The purified “Product 2” was then freeze dried, resulting in a very clear difference between both extractions, being an orange/red powder for the purified dragline pigment and a black powder for the purified synthesized “Product 2”. However, when dissolving both powders back into water, both resulted back into a similar yellow solution (Figure 6-11, b and c).

B. FTIR of the purified synthesized product

From the FTIR spectra, it was difficult to confirm the identity of synthesized “Product 2” as being 7-nitro xanthurenic acid (the yellow pigment). This was most likely caused by the fact that “Product 2” was not entirely pure (as indicated above), meaning the resulting spectra were a sum of a mixture rather than from one synthesized compound. Also the spectra were dominated by the presence of TFA, even after repeated extensive freeze-drying. However, in two spectra (Appendix A.10, 4 and 5) a peak was noticed at 1533 cm^{-1} which did indicate the presence of an aromatic nitro-group also present in 7-nitro xanthurenic acid (yellow pigment) (Chapter 5.3.4.B).

C. Nuclear magnetic resonance (NMR)

Integrating the peaks of the proton spectra of the synthesized “Product 2” revealed three single hydrogen atoms (Figure 6-13) just like 7-nitro xanthurenic acid (Figure 6-13, red trace). In the HSQC and the HMBC (Figure 6-13 and Figure 6-14, purple trace), the hydrogens’ were in the ppm-range of aromatic hydrogen atoms (6 to 8.5ppm)^{136, 137} similar to those in 7-nitro xanthurenic acid. In general the proton spectra looked similar to 7-nitro xanthurenic acid, however some unusually large shifts were seen (Figure 6-13). The peak at 6.4ppm was rather low in contrast to those seen in 7-nitro xanthurenic acid (6.9 to 7.6 ppm range) and more generally, the three protons in “Product 2” were more shielded. The direct coupling of the protons with the carbons was as follows: the proton at 7.8ppm with the carbon at 124ppm, the proton at 6.9ppm with the carbon at 111ppm and the proton at 6.4ppm with the carbon at 110ppm (Figure 6-13).

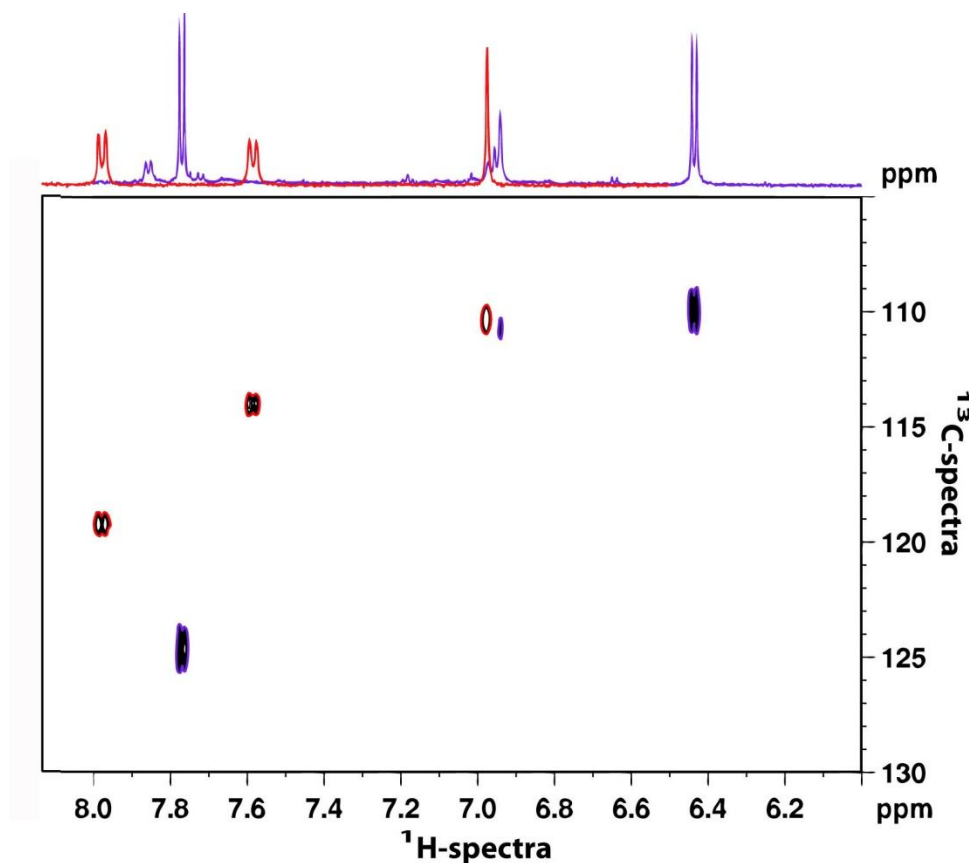


Figure 6-13. HSQC of the purified synthesized “Product 2” fraction (shown in purple) compared to that of 7-nitro xanthurenic acid (shown in red).

The HMBC of the synthesized “Product 2” only revealed 9 carbon atoms but this was because of the broad ^1H -peak at 6.9ppm. The carbons fell in the ppm-range of the aromatic carbons (arenes: 115-145ppm)^{136, 137} similar to 7-nitro xanthurenic acid which suggests that the main structure was the same (Figure 6-14). The coupling in the HMBC did show some similarity with 7-nitro xanthurenic acid but also showed an unusual correlation between the proton at 7.8ppm with the carbon at 161ppm which was not seen in 7-nitro xanthurenic acid. This peak was difficult to assign as the only peak present in this region was usually the acid at 165ppm (Figure 6-14). Because of these difficulties, the structure could not be resolved from this spectrum.

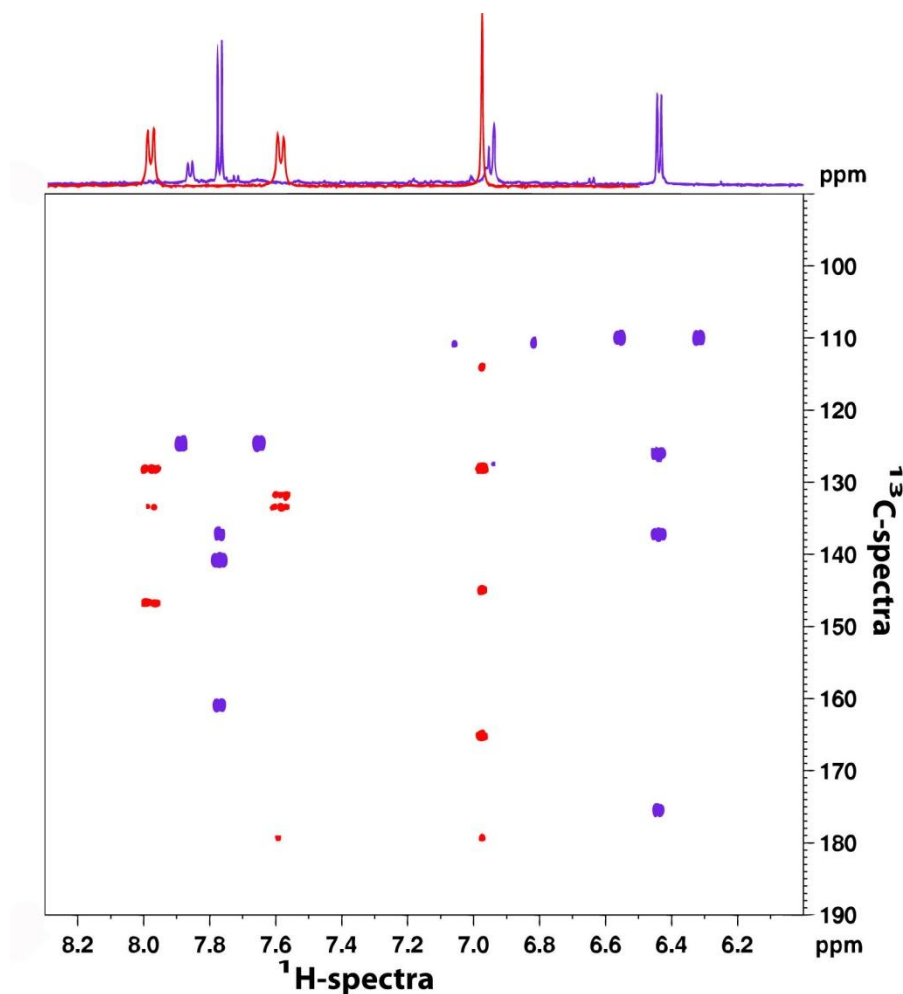


Figure 6-14. HMBC comparison between the purified synthesized “Product 2” (shown in purple) compared to that of 7-nitro xanthurenic acid (shown in red).

To conclude, the NMR data was unable to confirm the synthesized “Product2” was in fact 7-nitro xanthurenic acid present in the extraction of dragline silk. The shifts seen in the proton and carbon-spectra could be due to a mixture of compounds or a nitro-group that has been substituted in a different position on the xanthurenic acid ring, e.g. opposite the hydroxyl group. Furthermore, the unusual correlation seen from the proton at 7.8ppm with the carbon at 161ppm together with the low proton at 6.4ppm suggested a possible different substitution of the nitro group on xanthurenic acid, but the data were incomplete due to the broad proton-peak at 6.9ppm. Do note that the ^1H -spectra did show some smaller peaks that could indicate the presence of another compound and in this way would suggest a mixture of compounds.

D. Mass spectrometry (MS)

Because UV-spectroscopy, FTIR and NMR indicated that the purified “Product 2” from the large scale preparation (section A) was still impure, aliquots of this mixture were run on two UPLC/MS-systems, a TOF- and uTOF-system, in an attempt to separate the mixture as these systems have higher resolution. The nano-chromatograms (Figure 6-15, a and b) of both systems were the same in that two peaks were found for the purified synthesized “Product 2” (purple) in contrast to the purified dragline pigment, where only one peak was found, namely 7-nitro xanthurenic acid (red). This meant the purified synthesized “Product 2” was a mixture of two compounds here named “Peak 1” and “Peak 2”.

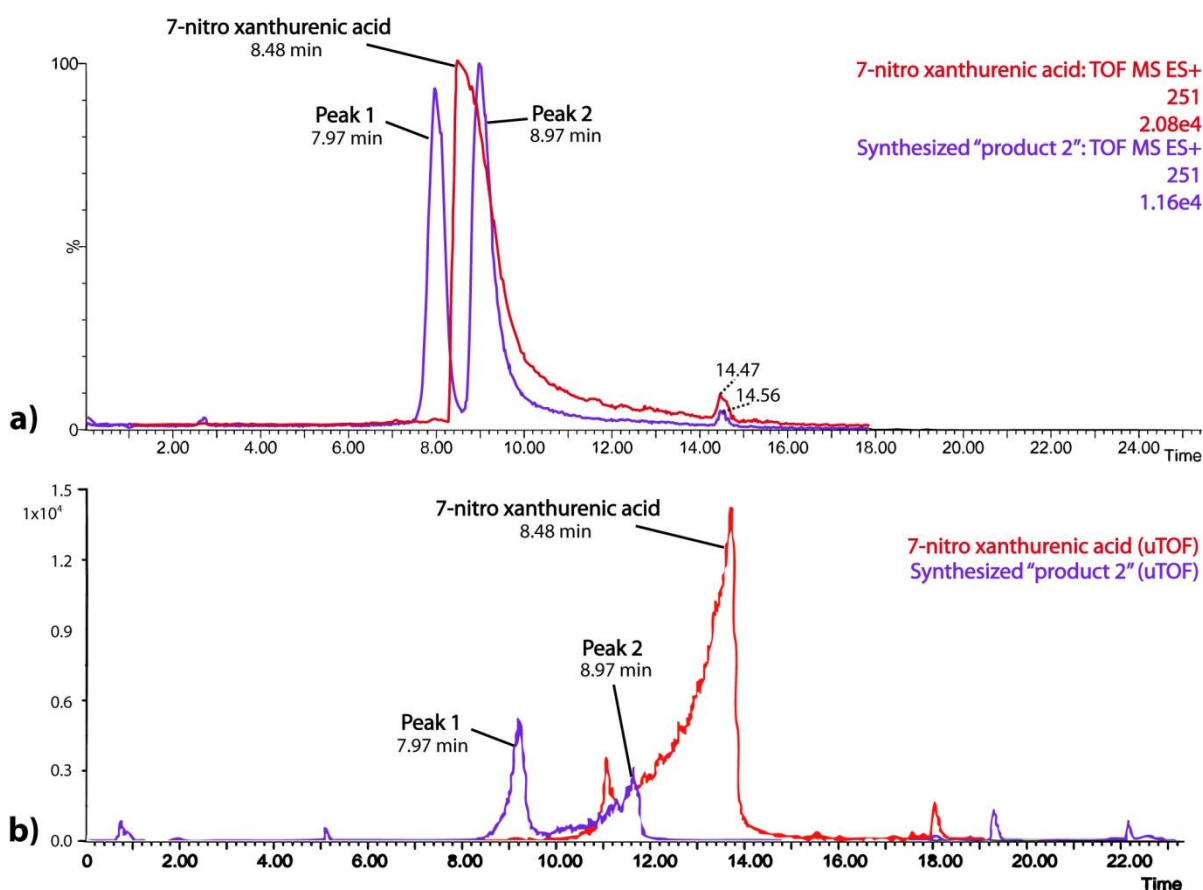


Figure 6-15. Nano-chromatograms obtained from the TOF- (a) and uTOF-system (b) showing the trace for 7-nitro xanthurenic acid (red trace) and the purified synthetic “Product 2” (purple trace). Note that the nano-chromatogram (b) of 7-nitro xanthurenic acid was run with a different method and therefore the retention time was not reliable in these chromatograms.

For the TOF-system (Figure 6-15, a), the mass analysis of 7-nitro xanthurenic acid was determined at 251.0910 (red trace), whereas the mass of both peaks of the purified synthetic “Product 2” (purple trace), namely peak 1 (7.97min) and peak 2 (8.97min), had an identical mass of 251.1027 (Appendix A.11). This was confirmed by the second instrument, the uTOF-system, which determined the mass of 7-nitro xanthurenic acid at 251.0303 whereas the mass of the first and second of the synthesized “Product 2” were respectively 251.0306 and 251.0308 (Figure 6-16). In Figure 6-16 it was shown that the measured mass spectrum of 7-nitro xanthurenic acid (1) and both synthetic products, Product 1 (2) and Product 2 (3), matched the simulated mass spectrum (4) based on the predicted formula $C_{10}H_7N_2O_6$, meaning that both synthesized compounds were nitrated xanthurenic acid compounds with exactly the same structural formula $C_{10}H_7N_2O_6$. However, in the mass spectrum of Product 1, one additional mass was found at ± 250 (2, red circled) which was not present in 7-nitro xanthurenic acid, Product 2 and the predicted mass spectrum (see Figure 6-16), indicating that this compound was possibly different from these compounds.

This indicated that the enzymatic nitration of xanthurenic acid resulted in two isomers from the structural formula: $C_{10}H_7N_2O_6$. However, four different substitution places were possible in xanthurenic acid meaning there are four possible isomers with an identical mass and the same structural formula. Therefore, confirmation was needed that one of both synthesized isomers was 7-nitro xanthurenic acid which was obtained by analyzing the mass fragmentation pattern.

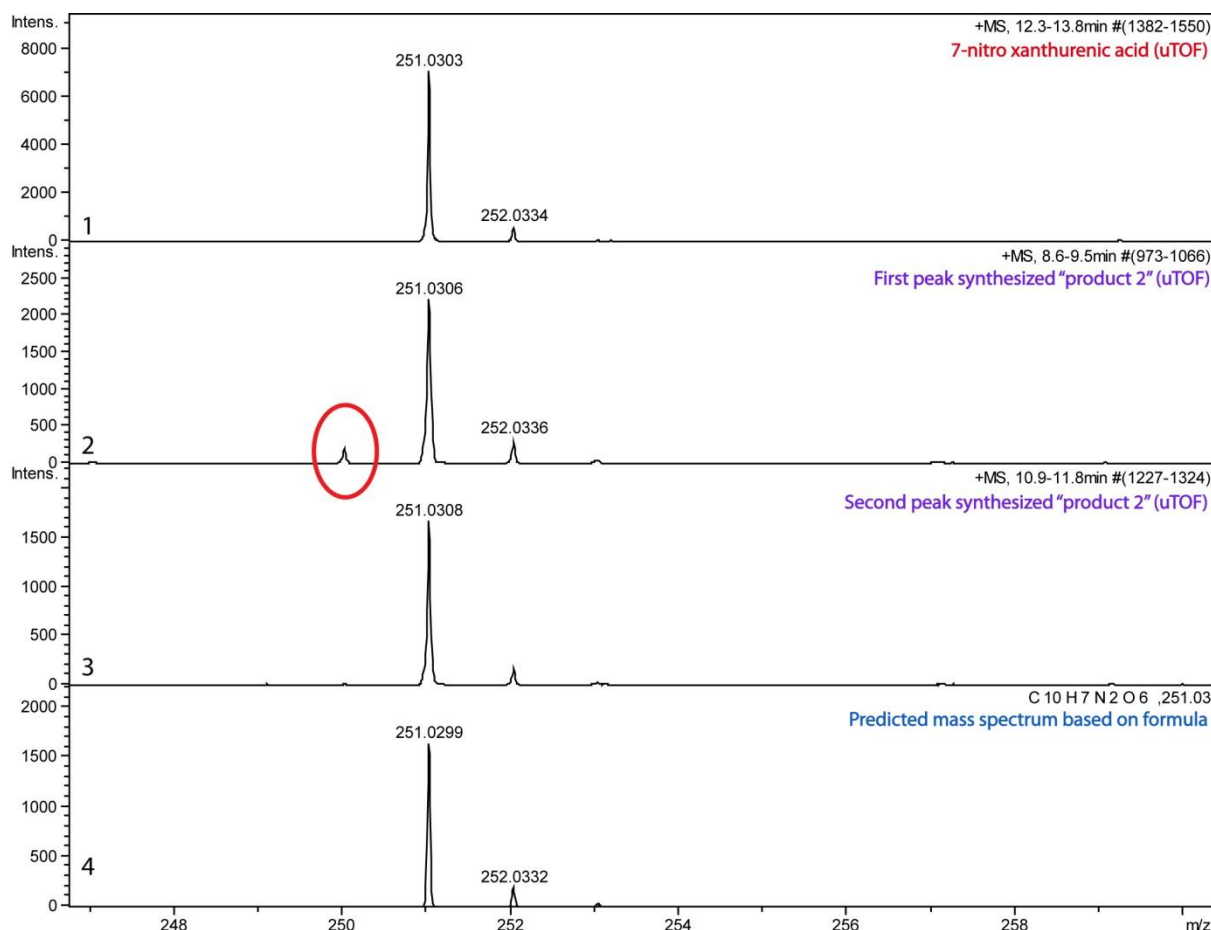


Figure 6-16. Showing the enlarged mass spectrum from the main mass at ± 251 from 7-nitro xanthurenic acid (1), Product 1 (2), Product 2 (3) and the predicted mass spectrum based on the structural formula, $C_{10}H_7N_2O_6$ (4).

Figure 6-17 (a and b) shows the fragmentation of the molecular ion ($M+H$, 251) at low current resulted in similar mass fragments for the three compounds (7-nitro xanthurenic acid, Peak 1 and 2), namely ± 233 (loss of H_2O (18.0011)), ± 223 (loss of CO (27.9837)) and ± 205 (loss of CH_2O_2 (46.0014)) indicating the initial loss of a carboxylic acid group. Furthermore, the fragmentation patterns of both products were different from that of xanthurenic acid confirming the presence of the nitro group. This meant that the nitro-group in both synthetic products, Peak 1 and 2, was more stable than the carboxylic acid. Note that the abundance of

smaller fragments was larger in Peak 1 compared to that of 7-nitro xanthurenic acid and Peak 2 indicating this compound was possibly different.

Remarkably, the mass fragmentation pattern of the molecular ion ($M+H$) at higher current of Peak 2 (blue) was identical to that of 7-nitro xanthurenic acid (Figure 6-17, d (red)). All mass fragments were present in the same relative amounts resulting in a symmetrical pattern (Figure 6-17, d), indicating the two compounds had identical structures. This strongly confirmed that the synthesized product Peak 2 was identical to 7-nitro xanthurenic acid.

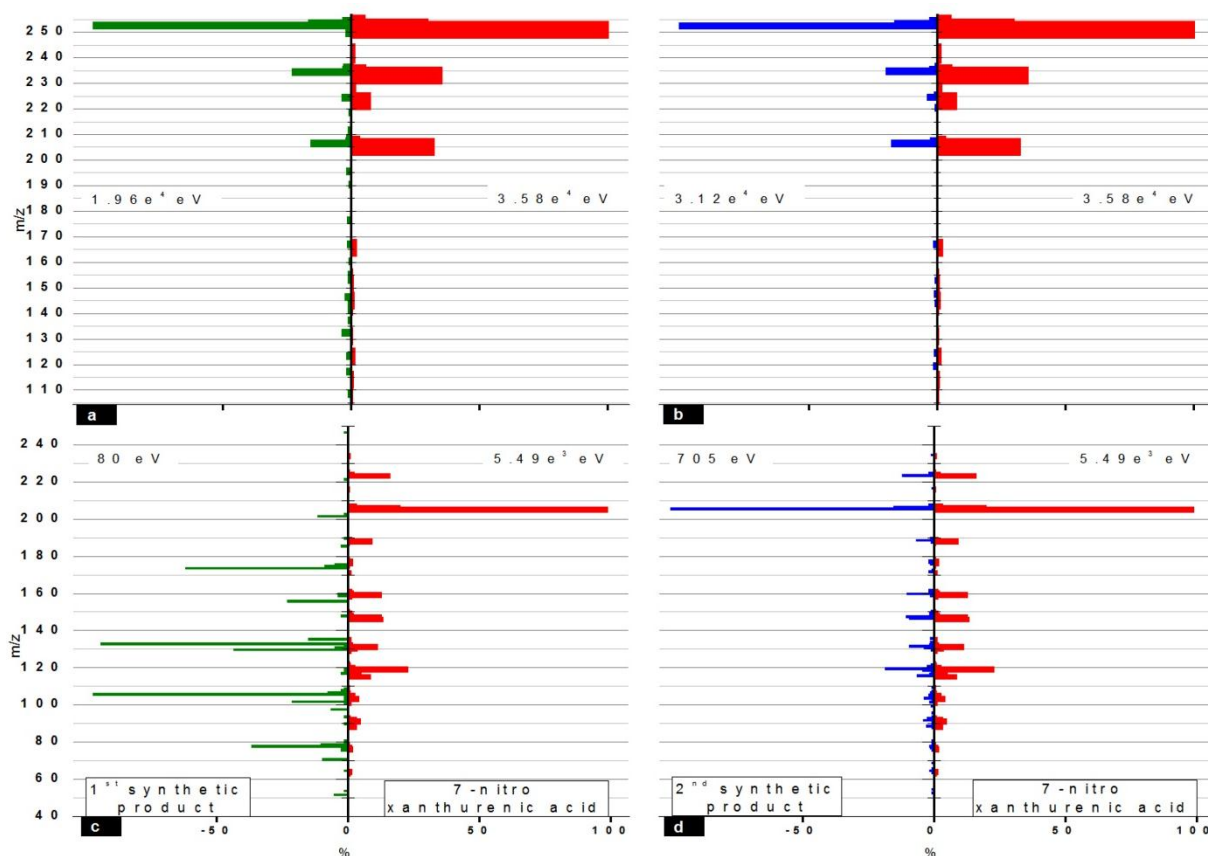


Figure 6-17. The mass fragmentation patterns at low current (a and b) and at a higher current (c and d) of 7-nitro xanthurenic acid from the extracted dragline (red), and Peak 1 (green) and Peak 2 (blue) from the purified synthesized “Product 2”. Raw data in Appendix A.11.

In contrast, the mass fragmentation pattern of Peak 1 was quite different from that of both other compounds (Figure 6-17, c (green)). Although many similar mass fragments were present, like ± 205 , ± 175 , ± 159 , etc., some additional mass fragments, like 109, 108, 107, 99, 80, 79, 66 and 51 were found in this product that were not present in 7-nitro xanthurenic acid nor in the Peak 2 product. Also, the relative abundance of the mass fragments was entirely different from that of 7-nitro xanthurenic acid which strongly indicated a different nitration site (resulting in an asymmetrical pattern in Figure 6-17, c). This was further confirmed by the low abundance of the 205 mass (12%) together with a massive relative abundance of lower mass fragments, suggesting some structural instability of the left-over molecular fragment. The more abundant mass fractions 175.0871 (65%) and at 159.0899 (25%) did indicate the loss of NO (29.9972) and NO₂ (45.9944) respectively, confirming that the molecular mass fragment 205 was nitrated.

E. Summary

Mass spectrometry did confirm that the purified “Product 2” from the large scale preparation was indeed a mixture of two compounds, as suggested by HPLC, NMR and FTIR. The mass of both compounds was identical to that of 7-nitro xanthurenic acid and thereby resulted in the same structural formula: C₁₀H₇N₂O₆, confirming that both compounds were isomeric nitrated xanthurenic acid molecules. The mass fragmentation pattern of one of these isomers (Peak 2) was identical to that of 7-nitro xanthurenic acid. This meant that the yellow pigment (7-nitro xanthurenic acid) could be produced in large quantities by horseradish peroxidase, HRP.

6.4. Conclusion

Whereas chemical nitration of xanthurenic acid resulted in a product that probably was 7-nitro xanthurenic acid (yellow pigment, not confirmed), the yield was limited and the synthesis resulted in many side products.

In contrast, the enzymatic nitration of xanthurenic acid by horseradish peroxidase did result in the production of 7-nitro xanthurenic acid in a large yield and was confirmed by an identical mass and mass fragmentation pattern.

This confirmed that a peroxidase was able to substitute nitro groups on xanthurenic acid and was thereby the most likely enzyme to synthesize 7-nitro xanthurenic acid (the yellow pigment) *in vivo* in dragline silk. However, a few side products (e.g. an isomer of 7-nitro xanthurenic acid) were co-synthesized indicating the enzyme used was not entirely site specific.

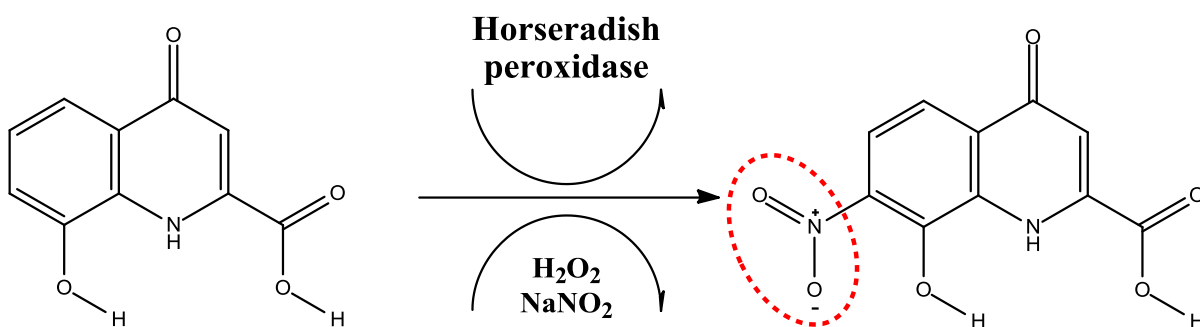


Figure 6-18. Enzymatic synthesis of 7-nitro xanthurenic acid via sodium nitrite and hydrogen peroxide in vitro and probably also in vivo.

Chapter 7

Summary and future work

Abstract and motivation

The purpose of this chapter is to provide the reader with a coherent view of the presence and effects of small molecules in silks. Therefore we give a general discussion first exploring the origin, mechanism and function of the two small molecules studied in this thesis. This will pull together Chapter 2, Chapter 3 and Chapter 4 for the discussion of calcium oxalate, and Chapter 5 and Chapter 6 for the discussion of the yellow pigment molecule.

Furthermore we discuss the practical applications of our findings on the properties and variability in silk previously discussed in Chapter 1. Thereafter we discuss taking this research further.

7.1. General discussion

7.1.1. Calcium oxalate

In this thesis we: identify the main mineral found in Wild Silkmoth cocoons as calcium oxalate hydrate, in the form of Whewellite (Chapter 2); provide evidence about how this is integrated in the cocoon by the spinning behaviour (Chapter 4); relate the mineralisation to the inability to reel these cocoons (Chapter 2) and develop a method for removing this mineral coating that enabled reeling of these cocoons (Chapter 3).

A. Origin

The most common minerals made by living organisms are hydroxyapatite (calcium phosphate), which make up the bones and teeth in many animals^{149, 150} and, calcite and aragonite (both forms of calcium carbonate) found in the shell in many marine organisms^{149,}

¹⁵¹.

Although calcium oxalate forms kidney stones in man and other mammals, there do not appear to be other cases apart from the cocoons of certain Wild Silkmoths of its use as component of a functional structural material in the animal kingdom although it is frequently found in vascular plants. Its lack of use as a structural material in animals is somewhat surprising in that oxalate ions can be readily synthesized by many organisms and the elongated monoclinic crystals of whewellite, a form of hydrated calcium oxalate, would make good filler particles¹⁴⁹.

However in silkmooths, the metabolic synthesis of calcium oxalate from citrate and glucose is minimal¹⁵² and the large quantity of calcium oxalate found in the Malpighian tubules of silkworms originate from free or bound calcium oxalate present in their food^{134, 152, 135}. Because silkmooths feed on leaves of their food plant, the calcium oxalate found in their Malpighian tubules and consequently in their cocoons, originated from these plants¹³⁵.

In plants, calcium oxalate is produced in all major groups of photosynthetic organisms including algae, lower vascular plants, gymnosperms and angiosperm¹⁵³. However, not all plants do produce calcium oxalate and the produced amount can vary on an inter- or intra-specific level. As a direct consequence, the amount of calcium oxalate found in silkmooth cocoons and the ease of reeling these cocoons (Chapter 2), is likely to directly reflect the relative calcium oxalate concentration found in their food plant and would follow the same trend as that seen in Figure 2-3. For example, because *G. postica* had the most heavily mineralised cocoon of the Wild Silkmooths studied suggested that its food plant, *Acacia* sp, would contain the largest abundance of calcium oxalate in their leaves of the food plants of silkmooths.

B. Mechanism

In plants, production of calcium oxalate functions as a high-capacity calcium regulation system and as a protection mechanism against herbivores with calcium oxalate acting as an anti-feedant¹³⁵. In silkmoths, secretion of calcium oxalate was unlikely to regulate the amount of calcium in the silkmoth but more likely acted as a protection mechanism, in that the absorbed harmful calcium oxalate via their food was eliminated by storing it in the Malpighian tubules^{134, 152}. The Malpighian tubules were known to be involved in the cyclical development of calcium oxalate necessary in the moulting cycle of silkmoths, whereby during ecdysis the calcium oxalate crystals leave the Malpighian tubules via the anus to coat the new cuticle¹³⁴. Whereas these secretions with each moult were relatively small, the amount of calcium oxalate in the Malpighian tubules increased rapidly during the 3th and 5th day of the last instar and was correlated to the excessive food consumption of the silkmoth for its silk synthesis¹⁵². This final calcium oxalate accumulation has been shown to be voided by the Malpighian tubules as it is during ecdysis, but instead of coating the new cuticle, the silkworm coated the cocoon shell instead. This resulted in a very specific behaviour seen during the cocoon construction in silkmoths, known as the impregnation phase¹¹⁷. In this thesis, this impregnation behaviour was observed in detail for *G. postica* (Chapter 4) and the excreted fluid was confirmed as calcium oxalate hydrate (Chapter 2 and Chapter 4). During the impregnation phase, the cocoon was seen to shrink upon which the silkmoth appeared to react by pushing the cocoon outward with its head. This behaviour was found to be closely similar to the previously observed “expansion behaviour” in *B. mori* and other studied Wild Silkmoths, and was the first time that an explanation is given for this particular behaviour. In general, the cocoon construction of *G. postica* was very similar to that seen in other Wild Silkmoth species^{53, 116-119}.

C. Function

Although the initial function of calcium oxalate secretion may have been the elimination of this harmful substance from their food, its function could have evolved in relation to its cocoon construction. Because the impregnation phase resembled the coating of the new cuticle in the ecdysis process, the silk cocoon could be seen as its final “external cuticle” to protect the pupa during its most vulnerable phase, the metamorphosis. Therefore the main function of the cocoon would be protection and therefore mineralization would help in stiffening, hardening and toughening these cocoons¹⁴⁹. This assumption was further confirmed by the observation of an additional, specialized behaviour seen in *G. postica*, by which the silkmoth pushed specialized sharp hairs from its skin through the cocoon shell during the impregnation phase (Chapter 4). Upon drying these sharp hairs were cemented in the cocoon shell by the calcium oxalate and probably forming a dual action defence layer against possible predators.

Furthermore the use of calcium oxalate in the cocoon shell could have an additional advantage in that calcium oxalate was toxic and could help prevent predation and perhaps fungal or bacterial attack. The construction of the cocoon shell whereby calcium oxalate was very precisely administered on the outside layer of the cocoon and coated with an oxalate free inner layer could indicate that calcium oxalate was probably also toxic for the silkmoth itself.

An additional function of calcium oxalate could be the control of the humidity inside the cocoon, however this needs further investigation.

D. Behavioural and evolutionary aspects: comparison with *B. mori*.

In Wild Silkmoths the mineralisation of cocoons seemed very important and probably co-evolved with the very specialized cocoon spinning behaviour. The additional behaviour found in the cocoon spinning of *G. postica*, namely the hair implantation phase, did heavily depend on the mineralisation of the cocoon shell and would fail without it (Chapter 4). Therefore I think the mineralisation of the cocoon shell became so vitally important that the silkmoth became dependent on that source of calcium oxalate, the food plant, which could explain why silkmoths became so extremely food specific. This would correlate with the observation that the calcium oxalate in Mulberry leaves did not show a negative effect on feeding in *B. mori*¹³³.

Because mineralisation with calcium oxalate appears to be a common characteristic in Wild Silkmoth species, the behavioural mechanism by which this is achieved during cocoon formation is likely to occur in all Wild silkmoths. When cocoons of *B. mori* were compared with those of Wild Silkmoths, it was obvious they differed in that these cocoons were purely white and easily softened. In Chapter 4 we determined that the cocoon shells of *B. mori* cocoons did not contain calcium oxalate in measurable quantities and that this probably contributed to the ease of reeling these cocoons. However, as in Wild Silkmoths, *B. mori* is known to be extremely food specific and only eats White mulberry leaves (*Morus alba* L.), that contain a lot of calcium oxalate¹³⁴. Calcium oxalate was also seen to accumulate in the *B. mori* Malpighian tubules¹³⁵, however no crystals have been found in their cocoon¹⁵² (Chapter 4). A comparative study was therefore undertaken to discover whether differences in cocoon forming behaviour in *B. mori* from that in Wild Silkmoths could account for this.

In Chapter 4 we proposed that the absence of mineralisation in *B. mori* resulted from the release of secretion from the Malpighian tubules before, rather than after the onset of spinning as seen in Wild Silkmoths.

However, demineralising the cocoon shell of *B. mori* gave the cocoon fibres a much cleaner appearance in the SEM suggesting that some material was removed from the surface of the silk fibres by this treatment. The observation that the cocoon structure did not fall apart indicates that although much of the sericin must remain intact to prevent this happening, the demineralizing process could have selectively removed the outer layer of the sericin coating or additional material coating this. This coating clearly did not originate in *B. mori* from the calcium oxalate in the Malpighian tubules as this is released before spinning and is not applied to the surface of the cocoon. This implied the coating was administered during spinning and may be derived from, the drying and consequent crystallisation or precipitation of components of the solution of mineral salts which passes out with the silk bave as it is extruded from the spigot.

Amino acid analysis of the cocoon shell of *G. postica* before and after demineralizing indicated a removal of protein(s) containing a preponderance of aspartic acid, glutamic acid, threonine and serine, also found in abundance in the outermost sericin layer of *B. mori*^{109, 126}. Peptides rich in these amino acids are known to control mineralization^{127, 128, 154, 155} and in this way the sericin coating on the fibres could serve as a coating for interacting with calcium oxalate and favouring mineralisation. The tight bonding of the sericin matrix to the mineral phase via salt linkage to carboxyl side chains of the glutamic and aspartic acid could help to toughen the resulting sericin/calcium oxalate composite.

In *B. mori*, it is possible that the early release of the Malpighian tubule contents before spinning and the storage of calcium oxalate storage in the Malpighian tubules represents the

retention of vestiges of cocoon forming behaviour and physiology derived from a common ancestor to Mulberry and Wild Silkmoth species. The change in behaviour could have arisen in *B. mori* as a result of artificial selection for the best reelable cocoon and in this way the selection of behaviour that did not incorporate the calcium oxalate would eliminate the difficulty in reeling seen in Wild Silkmoth cocoons as has been shown in Chapter 2, Chapter 3 and Chapter 4.

7.1.2. Yellow pigment (7-nitro xanthurenic acid)

In this thesis we identified the molecule responsible for the yellow pigmentation in dragline and egg sac silk of the spider *N. edulis* (Chapter 5) and demonstrated an enzymatic synthesis method for making this molecule from xanthurenic acid in vitro (Chapter 6) which very likely occurs *in vivo*.

A. Origin

In Chapter 5 we identified the molecule responsible for the yellow pigmentation in dragline and egg sac silk as being 7-nitro xanthurenic acid (yellow pigment). This molecule has never been reported before in nature and its occurrence in silk was therefore novel. The closest structurally related molecule was xanthurenic acid and differed from 7-nitro xanthurenic acid by lacking the nitro group (Chapter 0).

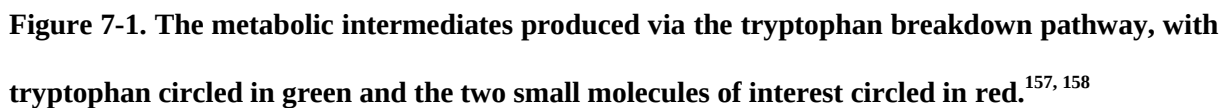
Xanthurenic acid was a known metabolic intermediate in the abnormal tryptophan breakdown pathway and has been associated with some diseases including diabetes mellitus. It has been found to be readily produced from tryptophan by vitamin B₆ (pyridoxine) deficient animals¹⁵⁶.

In the extractions of the yellow dragline and egg sac silk, xanthurenic acid was sporadically found together with 7-nitro xanthurenic acid suggesting xanthurenic acid could be a precursor

to 7-nitro xanthurenic acid. In Chapter 6 further proof was found that this was the case as 7-nitro xanthurenic acid could be readily synthesized from xanthurenic acid via a peroxidase, a peroxidase was also present in silk glands¹⁴⁷.

There are two possible routes by which the spider may obtain the xanthurenic acid from which the 7-nitroxanthurenic acid is synthesised. In the first xanthurenic acid is derived from tryptophan in the absence of pyridoxine. In the second the precursor xanthurenic acid may be derived directly from the spider's prey. The observation that the amount of xanthurenic from insect prey would be very small and that the content of yellow pigment was quite high (approximately 4% by weight of the dried silk, data not shown) both argued against the possibility spiders obtained xanthurenic acid from their food. It is therefore probable that spiders synthesise the precursor xanthurenic acid themselves via the tryptophan breakdown pathway^{157, 158} given in Figure 7-1. This assumption was further reinforced by the observation that in silkmoth silks another small related molecule 3-hydroxyanthranilic acid has been shown to be present⁴⁷, and this molecule was also known as a metabolic intermediate of the tryptophan breakdown (Figure 7-1). Whereas the production of tough silks are thought to have evolved independently in spiders and silkmoths the small molecules found in their silks are probably both derived from tryptophan metabolism. If that is the case it could point to a much more fundamental function of this pathway and its metabolic intermediates in relation to silk production.

Furthermore, because 7-nitro xanthurenic acid was probably produced via the tryptophan breakdown pathway, the production and abundance of this compound would still be food related because tryptophan is an essential amino acid.



B. Mechanism

In Chapter 5 we confirmed that the same yellow pigment, namely 7-nitro xanthurenic acid, was found in two different silks, dragline and egg sac silk originating from different silk glands, the major-ampullate and cylindriform glands. It had been observed previously that *N. edulis* dragline silks were not always yellow, but could sometimes colour white without any apparent reason^{86, 159}. During the many forced reeling sessions undertaken we further observed that:

- Extensively reeling a major ampullate silk gland until depletion, showed a gradual decrease in the yellow colouration of the reeled dragline silk.
- The dragline silk was white just before oviposition and the resulting egg sac silk was coloured bright yellow.
- When the reeled major-ampullate silk was yellow just before oviposition, the cocoon produced by the cylindriform glands was partly white.

In a pilot study we observed that the white reeled dragline silk did not contain any 7-nitro xanthurenic acid (data not shown) showing that the lack of colour was not due to the presence of this compound in a colourless state and that the secretion of yellow pigment in the silk could be “switched off”. Furthermore, the observations above suggest that the secretion of the pigment and incorporation of the yellow pigment in their silk might be “switched off” in the major-ampullate gland for incorporation in the egg sac silk. This “switch” could be a transport or enzyme that was enabled or disabled. As histochemical evidence suggests that the peroxidase activity necessary for the nitration of xanthurenic acid can be enabled or disabled (Chapter 6), it is possible that this provides the mechanism for the switching.

The third observation listed above suggests that there may be a common store of pigment or pigment precursor which can be moved from the major-ampullate to the cylindriform glands.

The observation that the non-nitrated xanthurenic has been sporadically detected in extracts of dragline silk (data not shown and literature¹⁶⁰) suggests this compound may be the precursor. This might be stored in the hemolymph or in other tissues. .

The nitration of xanthurenic acid to 7-nitro xanthurenic acid via peroxidase and sodium nitrite was illustrated in Chapter 6. Peroxidase activity has been observed previously in the major-ampullate glands of *N. edulis* in the distal part of the B-zone, however no definite function was assigned to it^{83, 147}. This presence of peroxidase in the silk gland and the ability of a peroxidase to nitrate xanthurenic acid strongly suggested that *in vivo* the yellow pigment (7-nitro xanthurenic acid) was synthesized from xanthurenic acid by a peroxidase as described in Chapter 6. The many side products generated by horseradish peroxidase in the yellow pigment synthesis in contrast to the very clean product of yellow pigment found in silk could be explained if the horseradish peroxidase was less specific than the peroxidase found in the silk gland.

C. Function

C.1. Tryptophan metabolism and metabolites

The literature on the relation between an abnormal tryptophan metabolism and diseases is vast and it was not in the scope of this thesis to give a full review on this matter. However, it was interesting to note that in vitamin B₆ (pyridoxine)-deficiency a change in tryptophan metabolism resulted in an increased production of xanthurenic acid and 3-hydroxy anthranilic acid¹⁵⁶ (Figure 7-1) and that these two metabolic intermediates were indeed found respectively in spider (Chapter 5) and Wild Silkmoth silk⁴⁷. Both molecules were related to aging and diseases including bladder cancer, Hodgkin's disease, and depression^{161, 162}. Xanthurenic acid itself was found to trigger mitosis, apoptosis, mitochondrial migration, cytochrome C release and the destruction of mitochondria and nuclei^{162, 163}. The switch to

increased production of xanthurenic acid and 3-hydroxy anthranilic acid in pathological conditions and in normal silk production may be mediated by changes in the concentration of quinolinic acid (Figure 7.1) feeding back on one or more enzymes in the tryptophan metabolism pathway or at the gene level.

C.2. 7-nitro xanthurenic acid

The function of 7-nitro xanthurenic acid produced in the gland and secreted in the silk is still a mystery. However, the active addition of a nitro-group to xanthurenic acid by the spider allows us to postulate that the nitro-group may have a quite important function in silk. The fact that the 7 position in the pigment is substituted with a nitro-group rather than a carboxylic group led us to suspect that the nitro-group had some additional advantages over a carboxylic group. Nevertheless, the yellow pigment could still be a simple side-product of peroxidase activity in the silk gland for the synthesis of something else. Yet the fact that spiders appear to be able to actively control the pigment secretion and apparently save it for the yellow pigment for the egg sac silk does imply that this was not the case.

An additional nitro-group on xanthurenic acid would result in additional hydrogen bonding capabilities of the molecule, as the nitro-group could act as a proton acceptor¹⁶⁴. This would result in the yellow pigment having extensive hydrogen bonding capabilities similar to those of polyphenols which are known to interact strongly with proteins⁴⁶. These hydrogen bonding capabilities could help the large silk peptides fold in the right conformation and thereby help remove the water in the spinning of the fibre, or contrarily could prevent the peptide from folding prematurely in the silk gland by preventing peptide chains from interacting with each other. The latter function was more probable, as the pigment content appeared larger as the gland content increased and the protein was possibly stored in a safe conformation³⁶.

Polyphenols have been shown to inhibit amyloid fibril formation¹⁶⁵ and accordingly the yellow pigment may act as an inhibitor and prevent premature folding of the fibroins in the silk gland. In contrast to these observations, a pilot experiment indicated that washing the yellow pigment out of the dragline fibre was associated with a decrease in tensile properties whereas placing the silk in an impure solution containing yellow pigment back restored or improved the tensile properties of the fibre. However, the yellow pigment was not the sole compound washed out and back into the fibre and there was the additional effect to consider from the washing solution (aqueous 30% w/v ammonium hydroxide).

Nonetheless, it may well be possible the pigment fulfilled both functions listed in the previous paragraph as it has two different resonant structures at acidic and alkaline pH (appendix A.4). At an alkaline pH of 7.2 as seen in the spider gland, the molecule may prevent folding of the fibroin for storage whereas at acidic pH it may help folding the protein in its beta-sheet rich structure resulting in a fibre.

The yellow pigment, 7-nitro xanthurenic acid, could also function as an anti-feedant or anti-bacterial compound as suggested for xanthurenic acid and naphthoquinone by Bernays⁹⁶ and Estable⁹⁷. The heterocyclic part consisting of the quinone and carboxylic acid in the structure of 7-nitro xanthurenic acid is widely found in many pesticides. Therefore the pigment may well function as a pesticide and that would also agree with the observation that the pigment was saved for the egg sac.

From the UV-spectrum of the molecule one more function is possible where the molecule could serve as a UV-protectant in the fibre. The UV-spectra of 7-nitro xanthurenic acid was extremely broad with potentially a wide spectrum absorption of UV-light resulting in reduced UV-damage of the silk proteins and eggs/embryos contained within the spider's silk cocoon.

7.1.3. Overall significance

This thesis clearly demonstrated that small molecules perform important functions in silks and may contribute to the vast variability in properties seen in silks. The small molecules in silks are thought to be either obtained directly from the food like calcium oxalate and gentisic acid (silkmoth silks⁴⁷), or indirectly from the food in the form of tryptophan, an essential amino acid. Tryptophan could then be metabolised via the tryptophan breakdown pathway to xanthurenic acid in spiders (the precursor for 7-nitro xanthurenic acid) and 3-hydroxy anthranilic acid in silkmoths. Either way, both of these mechanisms point to the importance of food in silkmoths and spiders, and could even have contributed to the extreme food selectiveness of silkmoths (Figure 7-2).

7.2. Practical implications

A. Calcium oxalate

Brunet has shown that cross-linking of certain Wild silks happened via small molecules taken up directly (gentisic acid) or synthesised (3-hydroxy anthranilic acid) from food components and that this tanning was possibly initiated by actively wetting the cocoon with a watery secretion⁴⁷. This tanning resulted in the relative insolubility of silk, the inability to degum these and increased tensile properties⁴⁷ (Figure 7-2 and see also Chapter 1). In this thesis we showed that this watery secretion used for tanning the silk also contained a large concentration of mineral crystals obtained from the food, identified as calcium oxalate monohydrate, and that these were used for reinforcing the cocoon shell. This resulted in a heavily mineralized cocoon in Wild Silkmoths and proved to be the major obstacle of reeling these cocoons. Furthermore calcium oxalate is a known toxic compound and therefore increases the cytotoxicity and may lower the biodegradability of the silk (Figure 7-2).

In Chapter 2 and Chapter 3 a technique was proposed for removing this mineral layer (demineralising) in Wild Silkmoth cocoons, resulting in:

- A vast range of Wild silkmoth cocoons that could be reeled which were previously impossible to reel.
- Fibres with improved and more consistent mechanical properties as a consequence of less damage resulting from milder treatment and the use of wet reeling rather than carding.
- A probable reduction or possible elimination of toxicity improving the biocompatibility for most Wild silk types resulting in a wider range of different silk types that can be used for medical applications or for sensitive skins.
- A more energetically optimal industrial method for treating cocoons at 40°C in contrast to currently used methods involving prolonged boiling.

This in turn could lead to:

- The emergence of new textiles, textile industries and biomedical applications based on Wild Silks
- A larger supply of different silk types for further research and making textiles, reconstituted silks, etc.

B. 7-nitro xanthurenic acid (yellow pigment)

The practical implications of the identification of the yellow pigment and its synthesis are still unclear. Whereas Brunet showed in silkmoth silks that small molecules were involved in tanning the silk⁴⁷, the identified yellow pigment did not seem to perform the same function in dragline silk of *N. edulis* as this pigment could be readily washed out (Figure 7-2). However, we do note that this possibility was not excluded in egg sac silk as not all of the pigmentation could be washed out of this fibre type (Figure 7-2).

Furthermore, even if the yellow pigment is not involved in cross-linking, the molecule is likely to have enormous potential to reversibly bind and interact with the proteins and could be important in the induction or prevention of protein folding. If the former, it could be a missing constituent necessary for making “native” reconstituted silk solution and therefore lead to more useful properties in materials derived from these. If the latter, it could mean that the molecule had been evolved and optimized to prevent peptides from aggregating prematurely, making it an optimized candidate drug for use in preventing and treating amyloid diseases.

In the case that the yellow pigment functions as a pesticide, it would be likely to be cytotoxic with implications for biomedical uses of silks. It is also possible that the compound which can be relatively easily synthesized might have direct applications as a fungicide, bactericide, vermicide or insecticide. If the yellow pigment functions largely as a UV-filter, it could be a useful molecule for protecting skin, biological materials and polymers from UV-radiation.

7.3. Future work

In general this thesis showed that implications of small molecules for the properties of silks and silk composites, deserves more consideration. Spiders and silkmooths are both ideal systems to study biomineralization with calcium oxalate and the effect of small molecules on proteins as both can be studied outside the body.

A. Calcium oxalate monohydrate (Whewellite)

The study of calcium oxalate formation in silkmooth silks looks promising in that it provides researchers a unique system for studying this process on the cocoon shell and in the Malpighian tubules as both are likely to provide an organic matrix for the control of crystal growth.

Furthermore, the relation between the calcium oxalate content in plant leaves, the silkmooths eating behaviour, the mineral present in the cocoon and the ability to reel the cocoons needs further investigation because these might lead to artificial diets for Wild Silkmooths which yield cocoons that are easier to reel.

B. Yellow pigment (7-nitro xanthurenic acid)

Further research needs to be done on the yellow pigment to elucidate its function, synthesis and the control of its secretion. Furthermore the possible link between the tryptophan metabolism pathway in silks and various diseases states needs to be clarified.

To conclude, future research on small molecules in relation to silk looks extremely exciting as there are many similarities between the assembly of silks and amyloidogenic fibrils and this could help in understanding the mechanism of the latter in an important class of diseases.

Appendix A

Supplementary information

A.1. Media coverage

a) Oxford university website

http://www.ox.ac.uk/media/news_stories/2011/reeling_in_a_wild.html



20
MAY

Reeling in a wild silk harvest

Science

20 May 11



Gonometa Postica cocoon

A new way of treating wild silkmoth cocoons could see new silk industries springing up wherever wild silk is found in Africa and South America, as well as silk's Asian heartland.

A team led by Oxford University scientists found that the surfaces of wild cocoons are coated with a layer of the mineral calcium oxalate, which makes them difficult to unravel. When the researchers removed this layer using an acidic solution they discovered that it was possible to reel cocoons into long strands of silk comparable to those derived from the domesticated Mulberry silk moth (*Bombyx mori*).

The team publish a report of their research this week in the American Chemical Society's journal *Biomacromolecules*.

'Unlike traditional ways of treating wild silk cocoons – such as degumming using pineapple juice, carding and hand-spinning – our new method softens the cocoon so that it can be reeled but does not damage the strands which give silk its sought-after properties,' said Tom Gheysens of Oxford University's Department of Zoology, who led the work with Oxford supervisor Professor Fritz Vollrath. 'The demineralising process makes it possible, for the first time, to create long continuous strands of silk from wild cocoons producing a high quality wild silk which is, potentially, a match for the 'farmed' variety.'

The researchers tested their new technique on the cocoons of the wild silkworm *Gonometa postica*. They found that, unlike traditional methods, their technique, using a warm solution of EDTA (Ethylenediaminetetraacetic acid), softens the cocoons enough they can be reeled like farmed silk without collapsing the cocoon fibres that make them strong and durable.

'Whilst these experiments were with the wild silkmoth *Gonometa postica* our findings suggest that the cocoons of other wild silkmoth species could be treated and reeled using this method,' said Professor Vollrath of Oxford University's Department of Zoology. 'This has the potential to bring a 'wild silk revolution' to places such as Africa and South America where wild silk is abundant but which are unsuited to domestic silk farming.'

Other wild silkmoth species with commercial silk-producing potential include *Antheraea mylitta*, *Antheraea pernyi*, *Cricula andrei*, and *Samia canningii*.

A report of the research, entitled 'Demineralization enables reeling of Wild Silkmoth cocoons', is published in this week in the American Chemical Society's journal *Biomacromolecules*.

b) European commission

http://ec.europa.eu/research/headlines/news/article_11_05_31_en.html



European Commission
CORDIS

European Commission > CORDIS > News > Researchers develop novel method for silk thread unravelling

Home News Funding Results Themes Go local Look it up Interact Help

CORDISNews

Search all CORDIS

Search news database:

Detailed search

Latest on...

FP7

FP6

EU Presidency

Lisbon Strategy

Calls

Forthcoming events

Interviews

EU R&D Newsroom

research.eu

CORDIS Express

CORDIS Wire

Press Corner

E-mail notification

Send news

What is RSS?

FP7 News

Researchers develop novel method for silk thread unravelling

[Date: 2011-05-31]

Scientists led by Oxford University in the United Kingdom have discovered that a layer of mineral calcium oxalate covers the surfaces of wild cocoons, making unravelling a complex process. Removal of this layer facilitates the unreeling of cocoons into long strands of silk comparable to those derived from the domesticated mulberry silkworm (*Bombyx mori*, B. mori). The research was funded in part by the SABIP ('Silks as biomimetic ideals for polymers') project, which has clinched a European Research Council (ERC) grant worth almost EUR 2.3 million under the EU's Seventh Framework Programme (FP7). Presented in the journal *Biomacromolecules*, the findings could lead to the development of new silk industries in Asia, Africa and South America, areas where wild silkworm numbers are in abundance.



People have long recognised how crucial a role silkmoths play in the silk industry. Silk fabric first materialised in ancient China, as far back as 3500 BC. Made by the unravelling of fine, soft thread from the silkworm's cocoons, the majority of silk found in today's markets comes from cocoons of *B. mori*. Unravelling (unreeling) the threads from this silkworm is easy, allowing long continuous strands to emerge.

For those working in the silk industry, unravelling the cocoon threads of 'wild' species is not as easy. In order to get the product they want, workers use harsher methods on the cocoons. The drawback, however, is that the strands are usually damaged, resulting in poorer quality silk. Enter the Oxford team, which successfully developed and tested a method to unravel the strands of silk of wild cocoons without damaging them.

They tested their new method on the wild silkworm *Gonometa postica*. Using a warm solution of ethylenediaminetetraacetic acid (EDTA), the team softened the cocoons enough that unravelling the famed silk without damaging the threads went without a hitch.

By 'demineralising' the surfaces of wild cocoons - i.e. removing the mineral calcium oxalate layer - the researchers were able to unreel the cocoons into long continuous strands with commercial reeling equipment. These strands could easily compete against those of the Mulberry silkworm cocoons.

'Unlike traditional ways of treating wild silk cocoons - such as degumming using pineapple juice, carding and hand-spinning - our new method softens the cocoon so that it can be reeled but does not damage the strands which give silk its sought-after properties,' explains Tom Gheysens from the Department of Zoology at Oxford University, who led the work with Oxford supervisor Professor Fritz Vollrath. 'The demineralising process makes it possible, for the first time, to create long continuous strands of silk from wild cocoons producing a high quality wild silk which is, potentially, a match for the 'famed' variety.'

Writing in the paper, the researchers conclude: 'The fibres of *G. postica*, which previously had not been examined in any great detail, when obtained this way showed competitive properties to fibres produced by the commercial mulberry silkworm *B. mori*. This suggests that our degumming method should increase the range of wild silkworm species that can be wet reeled and may be important for the development of the silk industries not only in Asia but also in Africa and South America.'

Scientists from the University of Bristol and the Commercial Insects Program, International Center of Insect Physiology and Ecology in Kenya contributed to this study.

For more information, please visit:

Oxford University:
<http://www.ox.ac.uk>

Biomacromolecules:
<http://pubs.acs.org/journal/bomaf6>

European Research Council:
<http://erc.europa.eu/>

Category: Project results
Data Source Provider: Oxford University
Document Reference: Gheysens, T., et al. (2011) Demineralization enables reeling of Wild Silkworm cocoons. *Biomacromolecules*. DOI: 10.1021/bm2003362.
Subject Index: Coordination, Cooperation; Life Sciences; Scientific Research; Social Aspects

RCN: 33467

[Previous article](#) | [Next article](#)

Top | Sitemap | A-Z Index | Glossary | FAQ | Help Desk | ©

CORDIS is managed by the Publications Office

c) New York Times

<http://www.nytimes.com/2011/05/24/science/24obsilk.html>

OBSERVATORY

Silk Production Takes a Walk on the Wild Side

By SINDYAN BHANOO

Published: May 20, 2011

Most commercial silk today comes from the cocoons of domesticated silkworms, bred over thousands of years to produce cocoons that are easy to unravel into silk that is easy to dye. The use of silkworms is thought to have originated in China nearly 6,000 years ago. There are many species of wild silkworms as well, but their cocoons are more difficult to unravel.

[Enlarge This Image](#)



C. Holland/OxfordSilkGroup

A *Gonometa* silkmoth cocoon.

[A newly discovered technique may make the unraveling process easier](#), and help the silk industry expand to new regions beyond Asia, according to a study in the journal *Biomacromolecules*.

Researchers from England and Kenya have discovered a way to remove the mineral layer that coats the cocoons of wild silkworms using an acidic solution. Removing this layer makes it easy to unfurl long strands of silk from the cocoons, the study's authors report.

They tested out a variety of solutions, with the goal of finding one that removed the mineral layer without harming the silk.

"You want to use chemicals to demineralize, but you don't want to use nasty ones, and you don't want to use ones that will damage the silk quality," said Fritz Vollrath, a zoologist at the University of Oxford and one of the study's authors.

Because the textures and colors of wild silks differ from those of silk produced by domesticated worms, wild silk could be highly valued by the fashion industry, he said.

The method could also lead to greater silk production in Africa and South America, where the climate is well suited for wild worms. Presently, the silk industry is concentrated in China and India.

"Silk uses very little space, and it's a very high-value product," Dr. Vollrath said. "As an agricultural product it really, seriously offers something to regions of poverty."

He and his colleagues are seeking a patent for their technique.

RECOMMEND

TWITTER

SIGN IN TO E-MAIL

PRINT

REPRINTS

SHARE

SNOW FLOWER
and the SECRET FAN
WATCH THE TRAILER

Related

Observatory: Fossil Sheds Light on the Lizard-Snake Divide (May 24, 2011)

[More Observatory Columns](#)

RSS Feed



Get Science News From The New York Times »

A.2. Detailed protocol 1 and 2 of Chapter 3

a) Protocol 1: demineralizing and degumming Wild Silk cocoons.

- 1) Freshly spun cocoons of *Gonometa postica* were obtained before or after emergence of the moth. To maintain the continuity of the fibre, cocoons were harvested with secouters while still attached to a small piece of branch. For the same reason, cocoons with holes produced by eclosion of parasitic wasps were not used. Cocoons from which the moths had not emerged were stored at -25°C to kill the pupae but other methods of doing this can be used instead as would be understood by a person skilled in the art. For hatched cocoons, pupal shells were removed through the emergence hole using forceps.
- 2) A solution of ethylenediaminetetraacetic acid (1M, EDTA) was adjusted to pH 10 with a concentrated sodium hydroxide solution. The cocoons were immersed in this for 48 hour at 40°C on shaker/stirrer and under vacuum as in step 1 above.
- 3) The cocoons were then thoroughly washed with tap water containing calcium ions at room temperature. The use of tap water instead of deionized water improves the ease of subsequent reeling possibly by replacing calcium ions removed by the EDTA within the fibre. A better characterised solution containing calcium can be used instead of tap water.
- 4) The cocoons were kept wet and were then ready to be reeled or carded, all at room temperature and using standard methods as used by a person skilled in the art. All or substantially all of the silk in un-emerged cocoons could be reeled into a yarn after demineralising, sometimes without the necessity of finding a new end.
- 5) Degumming was carried out for one hour with 0.5% sodium carbonate solution at 80° C. For preparation of regenerated silk cocoons demineralised as in step 2 were degummed directly, omitting step 3.

b) Protocol 2: tensile testing of Wild Silks

- 1) Single brins or baves of *Gonometa postica* silk were obtained respectively with and without degumming using Protocol 1 shown above. For comparison with the industrial method for degumming *Gonometa postica* silk we used an industrial yarn. The yarn was prepared by degumming cocoons in pineapple extract according to a standard protocol. The silk was then hand carded and hand spun into a yarn. Brins for mechanical testing were gently teased out of the yarn.
- 2) Hatched cocoons of *Antheraea mylitta* and *A. pernyi* were demineralised as in protocol 1. For comparison, industrially reeled *A. pernyi* and *A. mylitta* silk skeins were used directly as obtained from commercial suppliers.
- 3) Samples were mounted without preloading onto cardboard frames to give an initial gauge length of 15 mm. The fibres were equilibrated at 40% RH and 26°C for five minutes prior to tensile testing under the same environmental conditions on an Instron Universal Materials Testing Instrument at a strain rate 50% per minute using a 5 N load cell.
- 4) Cross sectional areas were obtained by clamping orientated silk threads in a holder and cutting them across with a new razor blade before sputtering them with gold and mounting them in a scanning electron microscope (SEM) whose magnification had been calibrated with a standard TEM grid. As it was impractical to obtain cross sectional areas for each sample adjacent to each test sample, normalized cross-sectional areas were obtained by averaging 150 brins for each sample.
- 5) Engineering ultimate tensile strengths are reported here. Stress strain curves were plotted using Excel. The J-integral method was used to determine the energy to break. Exelstat 7.5.2 was used for statistical analysis of the data with ANOVA (two tailed).

A.3. Comparison xanthurenic acid and yellow pigment: HPLC and UV

The NMR and MS data showed that the extracted yellow pigment structure was identical to xanthurenic acid but with one additional substituted group, most likely a nitro-group. Because an additional group would alter the retention time and the UV spectra in liquid chromatography, xanthurenic acid and the yellow pigment were co-injected (Figure A-1). The xanthurenic acid eluted about 1.5 minutes before the yellow pigment (Figure A-1, a). The later elution time of the yellow pigment confirmed that it was larger (more substituted groups) and/or more hydrophobic than the xanthurenic acid. In both cases an additional nitro group could cause such a delayed retention time (see appendix A.4).

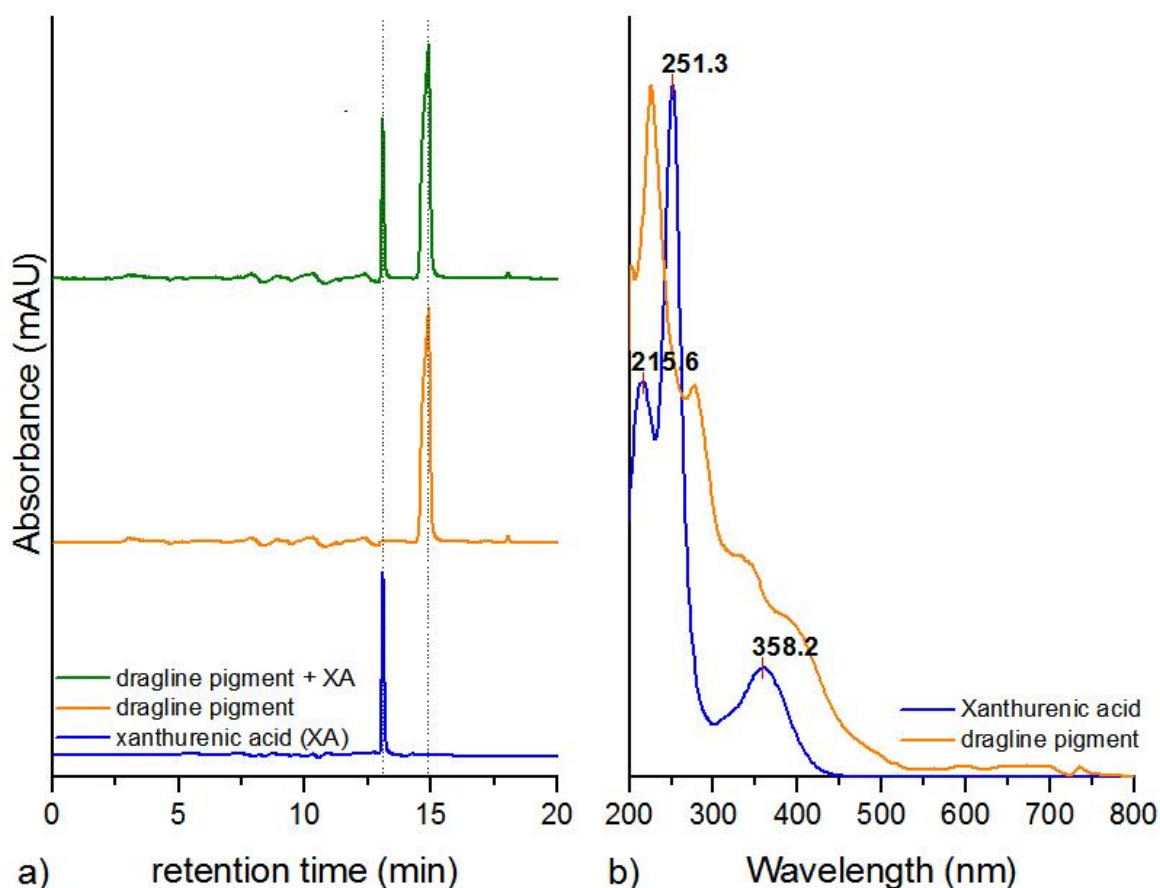
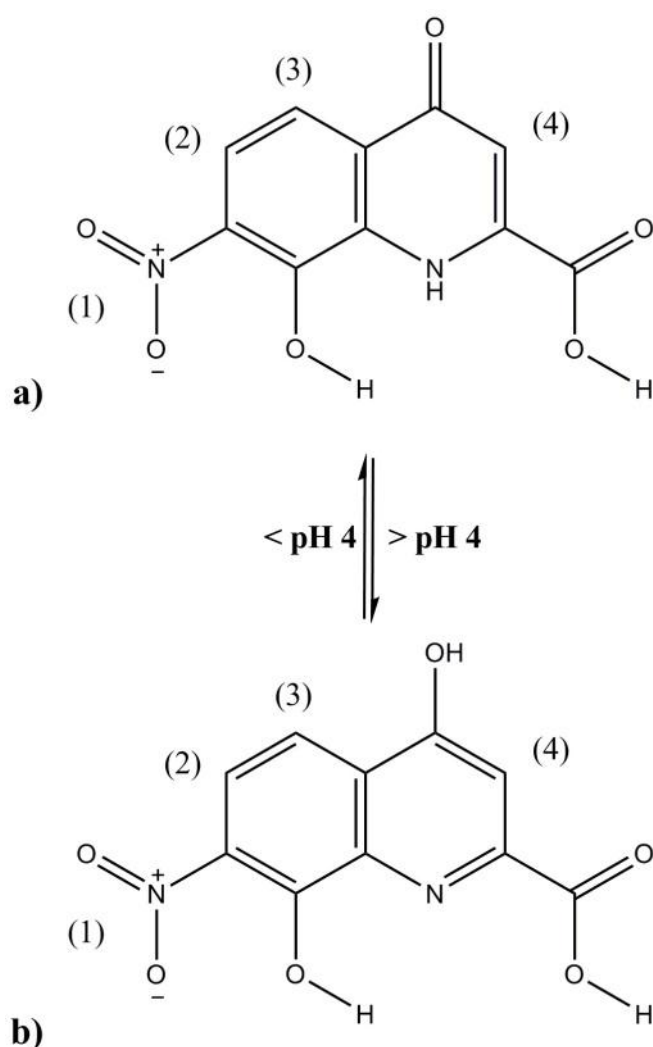


Figure A-1. a) Chromatographic trace of xanthurenic acid (black trace) with apex at 13.1 minutes, yellow pigment (orange trace) with apex at 14.9 minutes and co-injected xanthurenic acid and yellow pigment resulting in the two separate peaks illustrating the difference between both compounds (trace at 270nm). B) The UV absorption spectrum of xanthurenic acid with maxima at 213, 251 and 359nm compared to the UV absorption spectrum of extracted dragline pigment, the yellow pigment molecule.

The UV spectrum of xanthurenic acid was markedly different from the dragline pigment with peaks at 213, 251 and 360nm (Figure A-1, b). This marked difference in the spectra indicated that the electronic state of the pigment was quite different from that of xanthurenic acid and this agreed with the existence of an additional substituent. Compared with the UV spectrum of xanthurenic acid, the main peak of the yellow pigment showed a blue shift (to a shorter wavelength). This shift indicates that the pigment was either dissolved in a more polar solvent and/or the pigment itself was inherently more polar as a result of the additional group. Because the xanthurenic acid eluted first and so had a more polar solvent than the dragline pigment it was impossible to decide whether the blue shift of the pigment was due to an additional polar group or to the greater polarity of the mobile phase at the point in the elution gradient that it eluted. However it was possible that the additional group retained the molecule for longer in the mobile phase giving a slower elution for the pigment compared with the xanthurenic acid. Furthermore the addition of the substituted group seemed to broaden the peaks with λ_{max} greater than 250nm and shifted them to the red (higher wavelengths). A nitro group (see appendix A.4) was able to induce both effects at the same time.

A.4. The effect of pH on the NMR ^1H spectrum of 7-nitro xanthurenic acid

In general, for 4-hydroxyquinolines, two tautomeric resonance forms can be written making the compound a phenol or a ketone¹⁶⁶. The ketone form is most predominantly observed in acidic environments whereas the phenolic form is observed in alkaline environments¹⁶⁶. In our case the ketone form was present at $\text{pH} < 4$ indicated by no coloration and in the form of a phenol at $\text{pH} > 4$ indicated by a yellow coloration.



The two resonant forms of the yellow pigment: (a) the carbonyl form at $\text{pH} < 4$ and (b) the hydroxyl form at $\text{pH} > 4$, according to Reitsema¹⁶⁶.

In NMR, adjusting the dragline pigment in D₂O to a nominal pH of 3.0 as determined by a glass electrode from an acidic pH makes the hydrogens shift upfield in general, meaning they become more shielded in a basic pH (Figure A- 2). The hydrogen at 7.6ppm was most strongly affected by the pH, the more basic (pH 8.0) the more shielded it became. It could shift from 7.6 to 7 ppm with increasing pH.

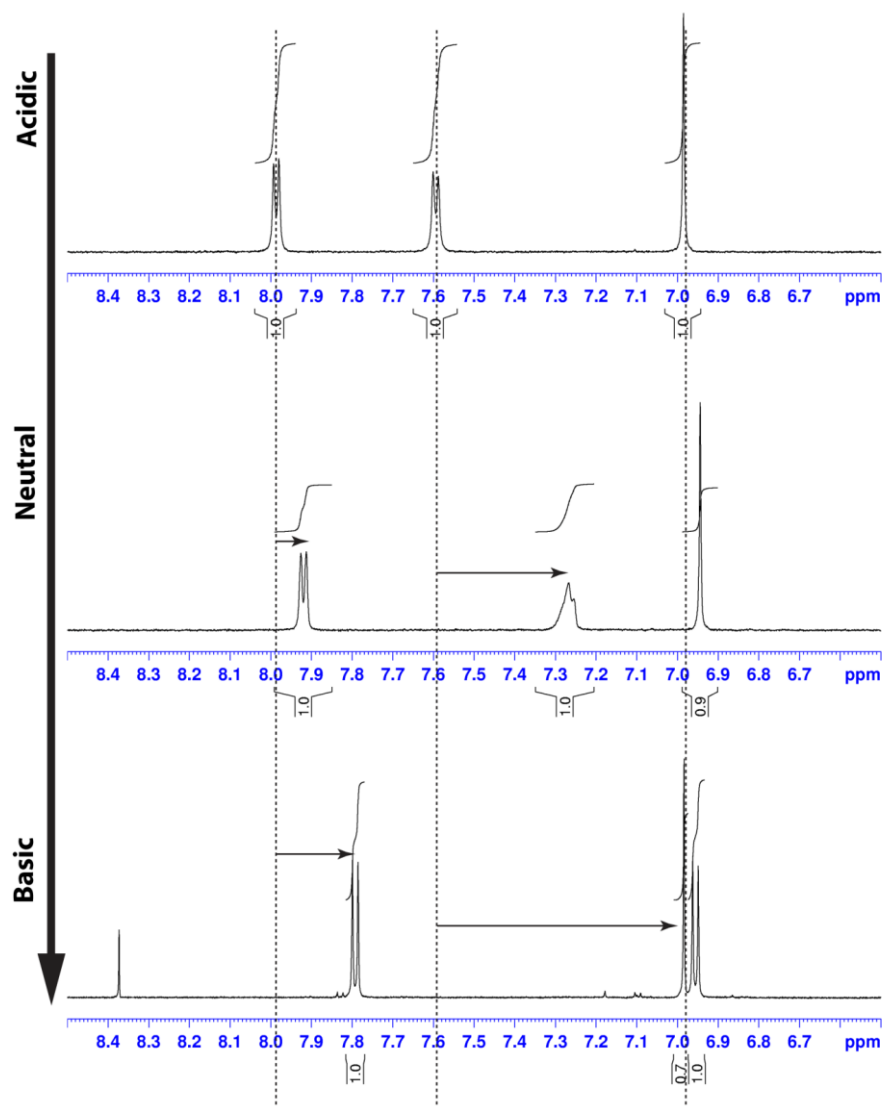


Figure A- 2. The proton spectra for the same extracted purified dragline pigment in D₂O at different pH values. The proton at 7.6ppm had the largest shift to the right when made basic.

A.5. FTIR spectrum and interpretation of Xanthurenic acid

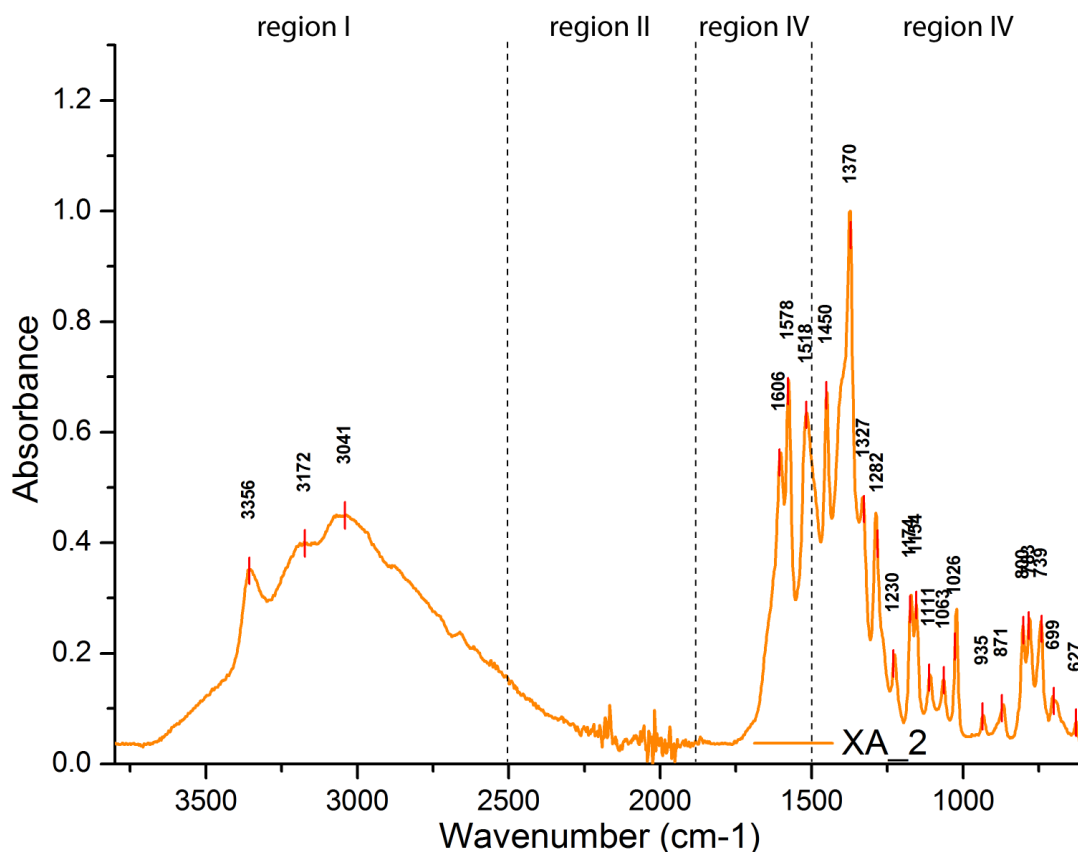


Figure A-3. FTIR-spectra of xanthurenic acid with peaks analyzed.

A broad peak was seen that stretches from 2400 to 3600 cm^{-1} and the lack of very sharp peaks in this region suggested the molecule had extended hydrogen bonding capabilities which is characteristic for alcohols and phenols^{137, 139, 140}. Although the OH-absorption band is always broad in solid state, the fact that the broad peak covered the range from 3400 to about 2400 cm^{-1} with the maximum at around 3000 cm^{-1} also indicated the presence of a carboxylic acid^{137, 139-141}.

This was further confirmed by the presence of several peaks in the $1300\text{-}1000\text{cm}^{-1}$ range characteristic for the C=O stretch. However the main peak of the C=O stretch was not seen in the 1700cm^{-1} region which could suggest a carboxylate in which the region was shifted to lower wavenumbers. The two peaks 3351 and 3200 superimposed on the broad peak together with the peak at 1601 could indicate the N-H stretching. Aromatic C-H stretches were weak at 3041cm^{-1} .

The region $1900\text{-}1500\text{cm}^{-1}$ is where the double bonds absorb. Normally there should be a strong band at 1700 for the carbonyl group due to the OH broad peak we see in the $2400\text{-}3400\text{cm}^{-1}$ region. Here, a strong band at 1601cm^{-1} was seen which was still in the carbonyl region $1800\text{-}1600\text{cm}^{-1}$. The reason for the lower value could result from the loss of the hydrogen on the carbonyl (lowers frequency), the carbonyl was conjugated to a C=C bond ($-50\text{cm}^{-1}\text{cm}^{-1}$) or/and hydrogen bonding to the carbonyl oxygen ($-50\text{cm}^{-1}\text{cm}^{-1}$).

The main peak at 1370cm^{-1} was difficult to assign but it had been identified elsewhere as the COOH stretch when ligand bound. The aromatic C=C bonds were usually in the region of $1600\text{-}1500\text{cm}^{-1}\text{cm}^{-1}$ with two or three bands. Here, two peaks were found at 1575cm^{-1} and 1515cm^{-1} which clearly indicated an aromatic compound and was confirmed by the presence of three peaks in the $850\text{-}730\text{cm}^{-1}\text{cm}^{-1}$ region (800, 778 and 740).

The FTIR-spectrum of xanthurenic acid (Figure A-3) nicely illustrated the structure and the substituents of the molecule. The spectrum confirmed the hydroxyl and carboxyl group and indicated the molecule was aromatic. It also hinted that the molecule was heterocyclic.

A.6. The FTIR-spectrum of the purified egg sac pigment fraction

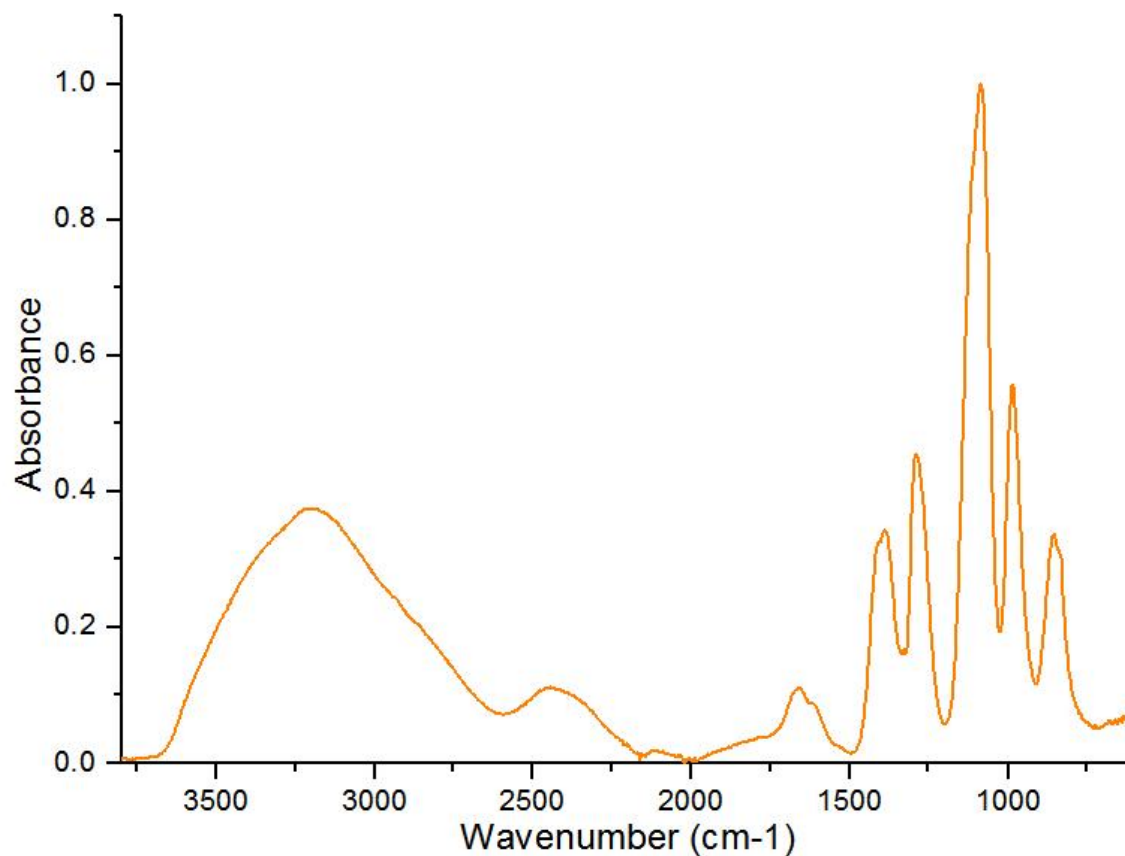
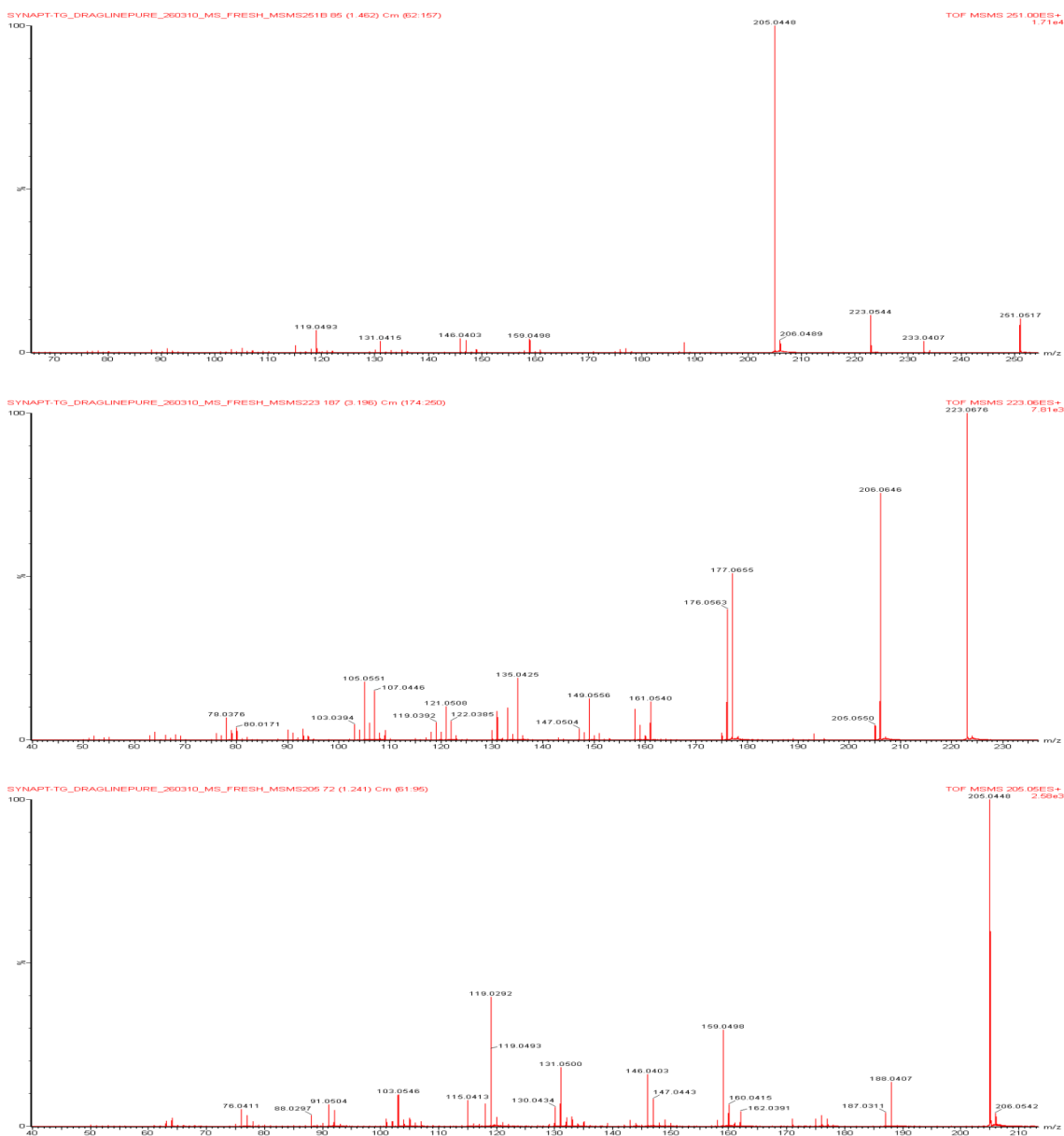


Figure A-4. FTIR-spectra of the purified egg sac pigment, showing broad band's indicating a mixture of compounds.

A.7. Raw data mass spectrograms of the yellow pigment



The fragmentation of the yellow pigment on the SYNAPT with positive ionisation spray. The main fraction pattern of the mass at 251 was illustrated in (a), where after the mass at 223 was fractioned at a current of 7.61e3 (b) and the mass at 205 at a current of 2.50e3 (c).

A.8. Mass spectroscopic data of Xanthurenic acid

To provide a framework for identifying the mass fragments generated from the purified dragline pigment molecule by mass spectrometry, the break-down pathway of a known similar molecule, xanthurenic acid, was investigated. This molecule was used because NMR spectroscopy indicated that the yellow pigment molecule only differed from xanthurenic acid by the addition of a substituted nitro group (Chapter 5.4). As most of its structure was identical it should result in a very similar breakdown pattern of the main molecule and identical mass fragments should occur.

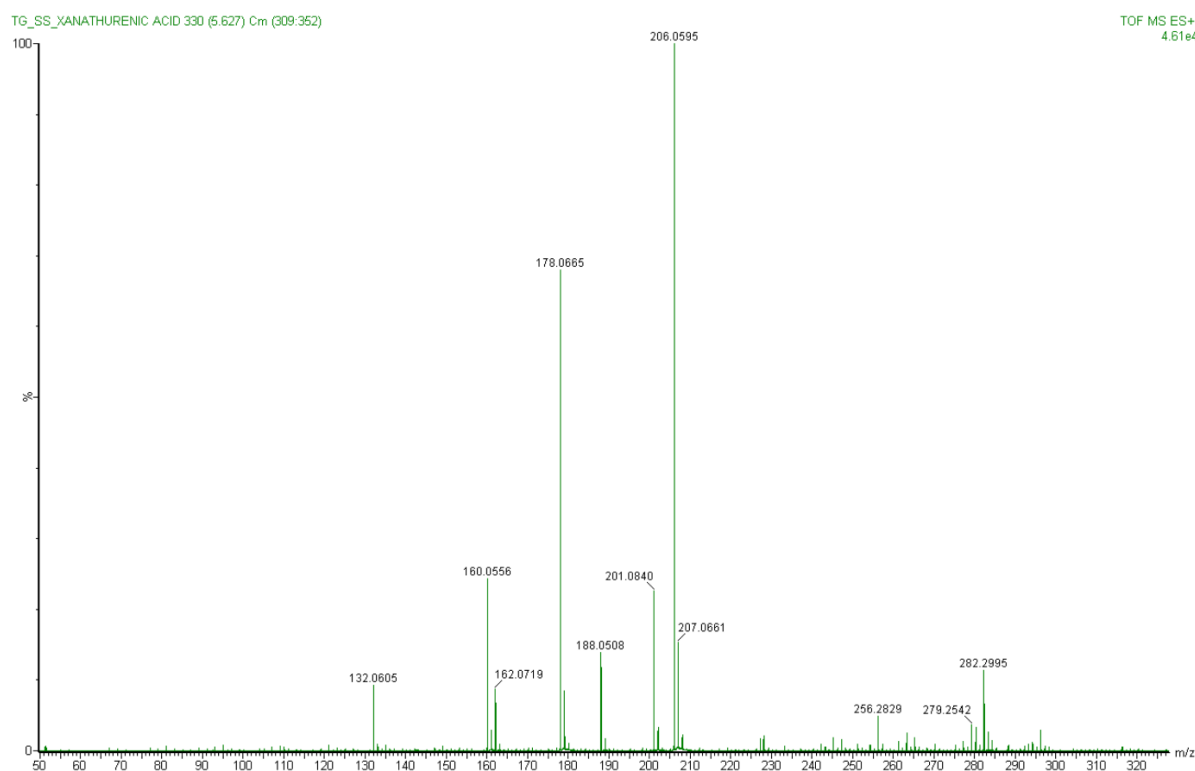


Figure A-5. Mass spectra obtained from xanthurenic acid on the SYNAPT with positive ionisation spray and at a current of 4.61e^4 eV. A bar plot of the relative abundance of the ions was plotted against their mass.

The main mass of xanthurenic acid was found at 206.0 and gave the following structural formula for the molecular ion $M+H$: $C_{10}H_8N_2O_6$ (Figure A-5). After removing the additional proton, the accurate structural formula for the molecule was $C_{10}H_7N_2O_6$ which was in agreement with structural formula of the reference, xanthurenic acid (Sigma).

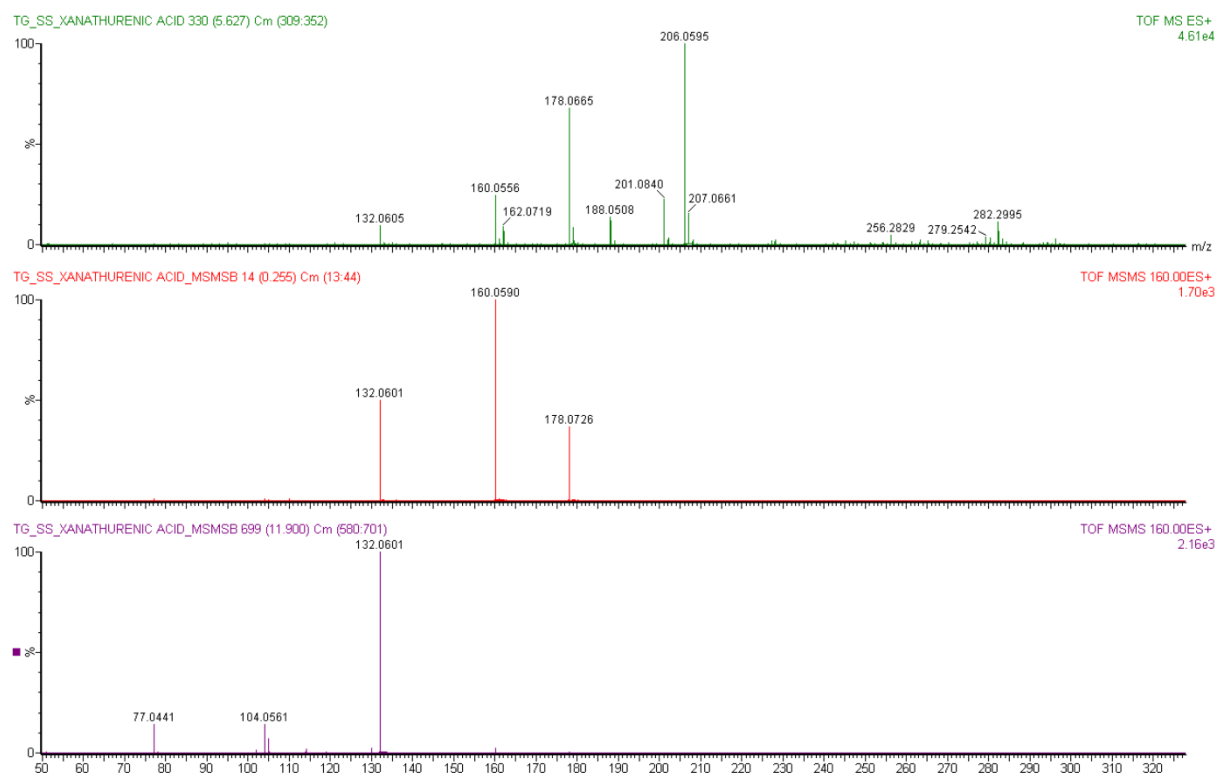


Figure A-6. The fragmentation of xanthurenic acid on the SYNAPT with positive ionisation spray. The main fraction pattern of the mass at 206 is illustrated in (a) where after the mass at 160 was fractioned at higher currents of $1.70e^3$ eV (b) and $2.16e^3$ eV (c) resulting in smaller fragments.

In general, substituted groups were the most easily lost leaving a more stable molecule. In the case of xanthurenic acid, the double ring structure (mass: 132.06) was most stable whereas the substitutes were most easily fragmented. In Figure A-6 (green) it was noticeable that the main molecular ion had a mass of 206, but it had many fragmented masses with a lower relative

abundance at 178, 160 and 188. These masses fit with the molecular ion losing water (M^+ -OH: 178 (Table A- 1, B), the carboxyl group (M^+ -COOH: 160 (Table A- 1, D)) and carbon monoxide (M^+ -C=O: 188 (Table A- 1, C)) (Figure A-6 (green)).

Table A- 1. All mass fractions obtained from the fragmentation of xanthurenic acid in MS were listed together with the mass difference from the ion fractions. For all ions the possible structural formulas were calculated and the most likely ion was tabulated next to it.

	MS	Allowed structural formulas from mass fraction	Most likely structural formula	MS difference of fragment A	Allowed structural formula for the mass difference of A	Most likely mass fraction of A
A	206.0595	C10H7NO4	C10H7NO4	Xanthurenic acid (Sigma)		
B	188.0508	C10H6NO3 (-0.016036)	C10H6NO3	18.0087	H2O (+0.0018633)	H2O
C	178.0665	C10H10O3 (-0.003511); C9H8NO3 (-0.016087)	C9H8NO3	27.993	CO (+0.0019141); CH2N (+0.0257224); C2H4 (+0.0382984);	CO
D	160.0556	C6H10NO4 (+0.0053756); C9H6NO2 (-0.015750); C7H12O4 (+0.0179516)	C9H6NO2	46.0039	CH2O2 (+0.0015774); NO2 (-0.010998)	CH2O2
E	132.0601	C9H8O (-0.002589); C5H10NO3 (+0.0059615); C8H6NO (-0.015165); C5H8O4 (-0.017846); C9H10N (0.020675773)	C9H10N	73.9994	C2H2O3 (+0.0013915); C5N (+0.0040732); CNO3 (-0.011184); C6H2 (+0.0166492);	CH4O4
F	104.0561	C7H6N (-0.006079); C8H8 (+0.0064967); C4H8O3 (-0.008760); C4H10NO2 (+0.0150474)	C7H6N	102.0034	C6NO (-0.001712); C3H2O4 (-0.004394); C7H2O (+0.0108632); C2NO4 (-0.016970);	C3H2O4
G	77.0441	C2H7NO2 (+0.0035735); C6H5 (-0.004976); C3H9O2 (+0.0161496); C5H3N (-0.017553); C2H5O3 (-0.020234)	C5H3N	129.0154	C5H5O4 (+0.0033794); C8H3NO (+0.0060611); C4H3NO4 (-0.009196); C8HO2 (-0.017747)	C5H5O4

The mass peak at 160 (M^+ -COOH: 160 (Table A- 1, D)) was seen at a higher current, to fragment yielding two mass peaks, 132 and 178 mass units (Figure A-6, red). This again fitted with the 160 ion losing the two hydroxyl groups (M^+ -CO₄H₃: 132 (Table A- 1, E) while the ion at 178 is most likely M^+ -COOH. This was thought to arise from 160 fragment (Table A- 1, D) reacting with carbon dioxide. To break up the mass at 132 an even higher current was required as this structure has a stable double ring structure (Figure A-6, purple). When breaking up the mass at 160 with an even higher current three mass fragments were found whereas the mass at 132 was the double ring structure and two additional fragments at 77 and 104. Because these two masses cannot be accounted for as two parts of the mass 160 they must result from a breakdown of this mass fragment in two different ways.

The mass at 104 should be derived from the double ring containing the nitrogen by the breaking off of an ion with mass 26, but the range of the instrument did not permit us to check this; however this ion was still very likely as C_2H_2^+ is a well known MS-ion. This implied that the other ion at 104 was the hetero-cyclic ring $\text{C}_7\text{H}_5\text{N}^+$: 104 (Table A- 1, F)). The molecular ion with mass 77 was identified as $\text{C}_5\text{H}_3\text{N}^+$ (Table A- 1, G) with the other fragment of the parent molecule at mass 52 (C_4H_4^+) visible in the MS plot as a trace ion.

The resulting breakdown pathway for xanthurenic acid was given in Figure A- 7 and was in agreement with the breakdown pattern for xanthurenic acid published elsewhere¹⁶⁷.

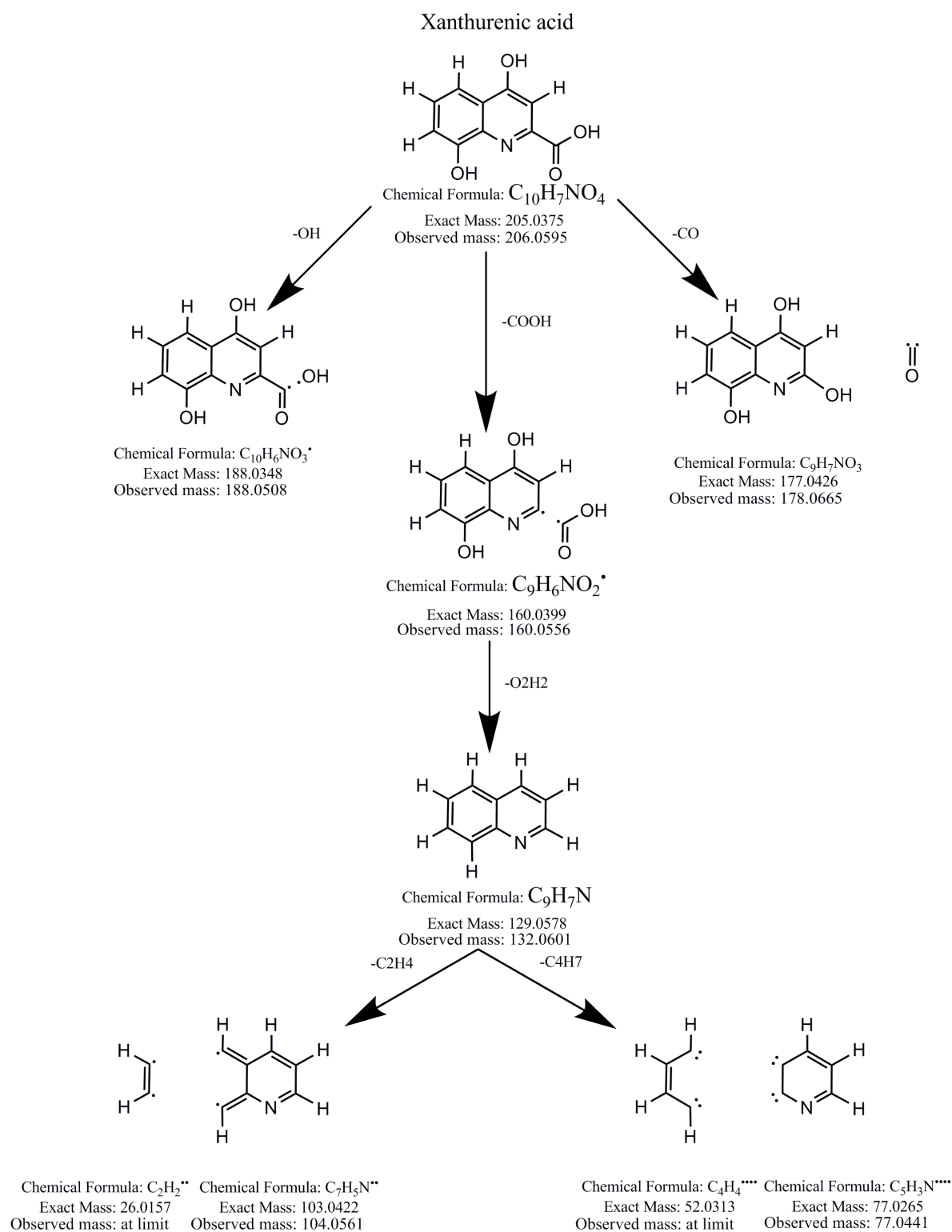


Figure A- 7. The predicted fragmentation pathway of xanthurenic acid based on the experimental data. This fragmentation pathway fits well with the fragmentation pathway described for xanthurenic acid elsewhere¹⁶⁷.

A.9. The effect of substituted groups on Liquid Chromatography (HPLC)

a) A hydroxyl group

The effect of one additional OH-group on the double ring of kynurenic acid (Figure A-8, a) to form xanthurenic acid (Figure A-8, b) made the component elute a little earlier (by 0.2min), because it became more hydrophilic (Figure A-8, c). There was also a red shift visible in the UV-spectra but the main shape was still identical (Figure A-8, d). This shift was caused by the electron donor capability of the hydroxyl group.

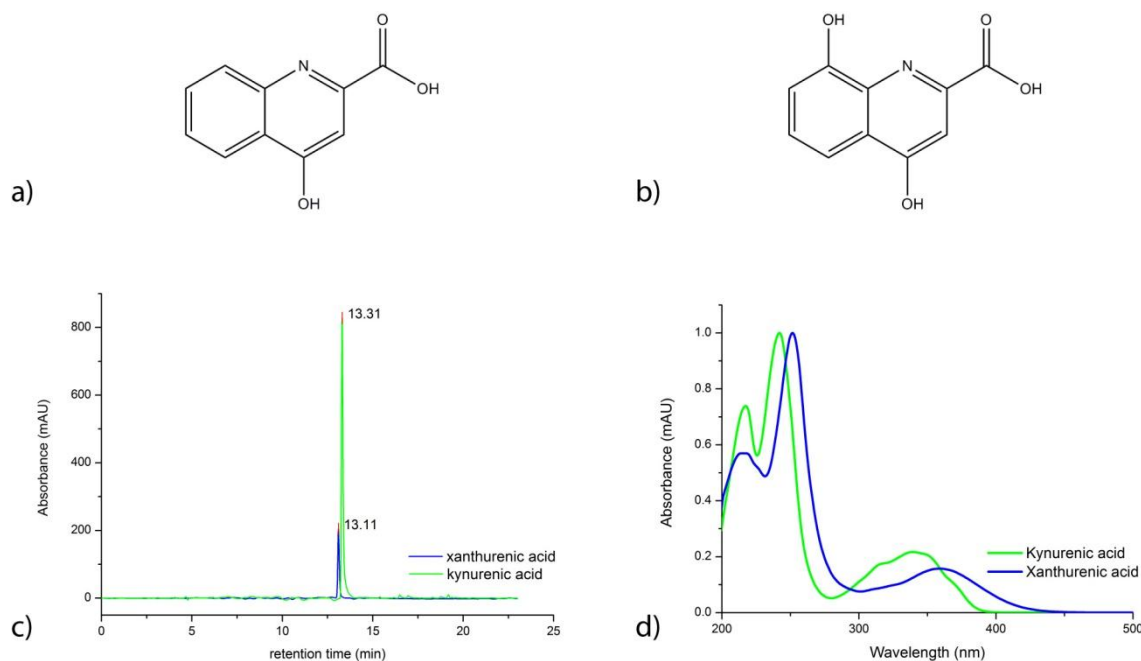


Figure A-8. Comparison of kynurenic acid and xanthurenic acid. The chemical structure of (a) kynurenic acid and (b) xanthurenic acid, (c) Xanthurenic acid (blue trace) eluted sooner from the analytical column than kynurenic acid (green trace). (d) The differences in UV-absorption spectra of kynurenic acid (green trace) and xanthurenic acid (blue trace) in which the hydroxyl-group induced a small red shift.

The later retention time of the dragline pigment and the differences in its UV spectra suggested that it was unlikely that there was an additional hydroxyl group in the dragline pigment molecule compared to xanthurenic acid. Also it was unlikely that there was an additional carboxyl group in the pigment as this would induce a blue shift and a reduced retention time resulting from a more hydrophilic molecule.

b) A nitro-group

The effect of an additional nitro-group (Figure A-9, b) on L-tyrosine (Figure A-9, a) was investigated to shed light on the effect of nitration of xanthurenic acid. This produced a large increase in the retention time (about 2 minutes; Figure A-9 (c)), most likely due to the larger molecule or/and the more hydrophobic group. A nitro-group also had a large effect on the UV spectra in that it induced a large red shift (Figure A-9, d).

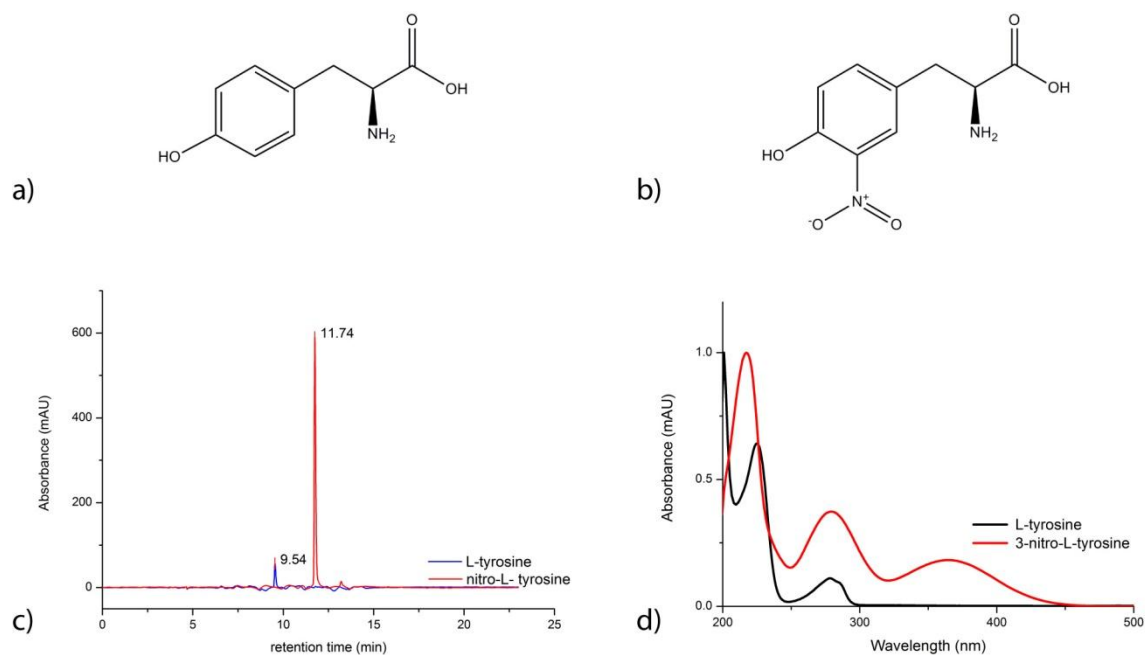


Figure A-9. Comparison of L-tyrosine and 3-nitro-L-tyrosine. The chemical structure of (a) L-tyrosine, (b) 3-nitro-L-tyrosine, (c) the chromatogram of 3-nitro-L-tyrosine (red trace) eluting two minutes later than L-tyrosine (blue trace) and (d) the UV absorption spectra of L-tyrosine (black trace) and 3-nitro-L-tyrosine (red trace) showing that the nitro-group induced a large red shift in the spectrum.

c) Conclusion

These two effects were also visible between xanthurenic acid and the dragline pigment molecule. Consequently, a nitro group could explain the longer retention time and the important difference in UV spectrum in comparison to xanthurenic acid.

A.10. Detailed analysis of the FTIR-spectrum of the enzymatically synthesized compound

Not surprisingly, the FTIR spectra of the freeze dried powder (4 times) from the synthesized dragline pigment were dramatically different from the extracted dragline pigment (Figure A- 10, 2 and 8). When comparing the spectrum of the synthesized pigment with TFA it was clear that TFA was still present and heavily bonded to the product in contrast to the extracted dragline pigment. Even after ten freeze drying cycles, it was not possible to say that the TFA was entirely removed from the synthesized product. The two main characteristic peaks for TFA at 1200 cm^{-1} and 1151 cm^{-1} are the C-F stretches (Figure A- 10, c and d) and were visible in all acquired spectra of the synthesized pigment spectra (Figure A- 10,(2, 3, 4 and 5)) even after extensive freeze drying. One possibility for this is that the synthesized product is different from the dragline pigment or there was more than one product that together with TFA made a tightly bonded network. The latter explanation was more likely because another component was co-eluting with the dragline pigment product in the purification step (see chapter 6.3.2.A). In two spectra (Figure A- 10, 4 and 5) a peak was noticed at 1533 cm^{-1} which matched the dragline pigment and confirmed the presence of an aromatic nitro-group (Figure A- 10, b).

In more detail, another difference was the much broader peaks in the synthesized pigment spectra. The very broad peak in the $3700\text{-}2500\text{ cm}^{-1}$ suggested much hydrogen bonding, whereas the “broad” peaks in the $1700\text{-}1000\text{ cm}^{-1}$ suggested a mixture of compounds. This again agreed with the compound being impure.

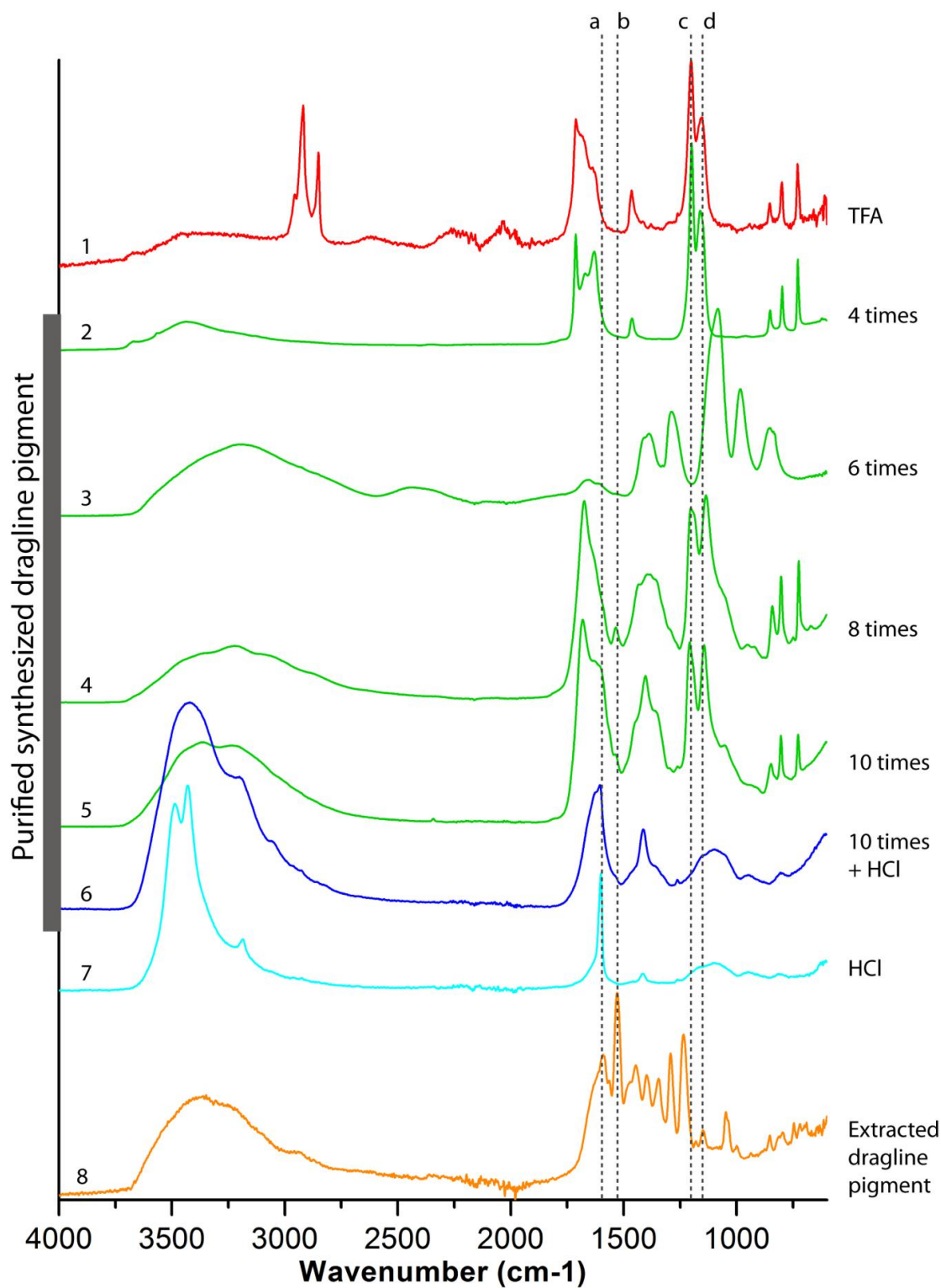


Figure A- 10. FTIR-spectra of synthesized dragline pigment after different cycles of freeze drying: 4 times (2), 6 times (3), 8 times (4) and 10 times (6) and 10 times with addition of HCL (7) in comparison to TFA 0.1% (1), HCl (7) and extracted dragline pigment (8).

A.11. Raw data of the mass spectrometric fragmentation pattern of the enzymatic synthesised product

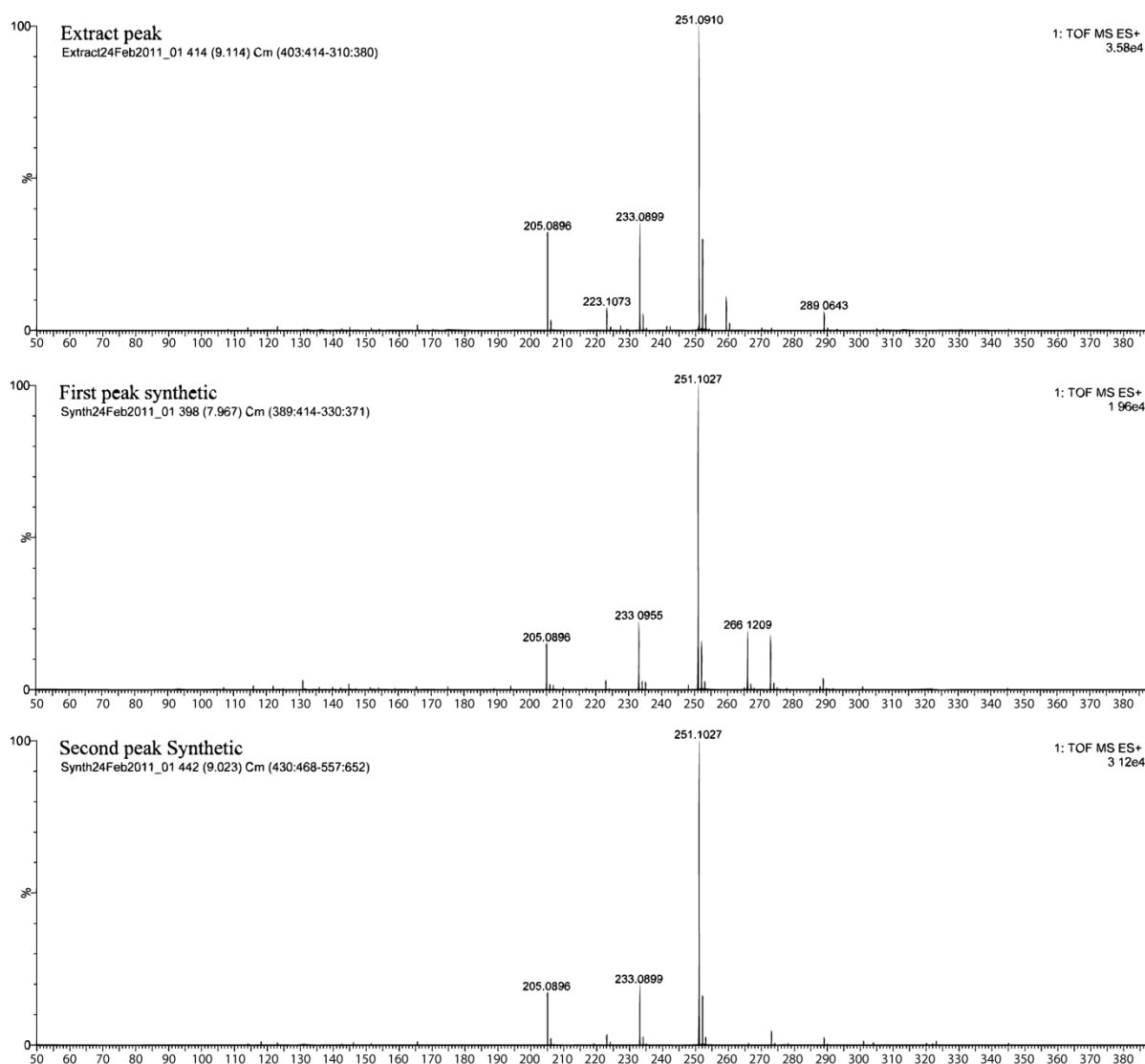


Figure A- 11. Mass spectrometry on the SYNAPT with positive ionisation spray of the extracted yellow pigment (top, $3.58e^4$), the first synthetic purified product (middle, $1.96e^4$) and the second synthesized purified product (bottom, $3.12e^4$) with main mass each time at 251.

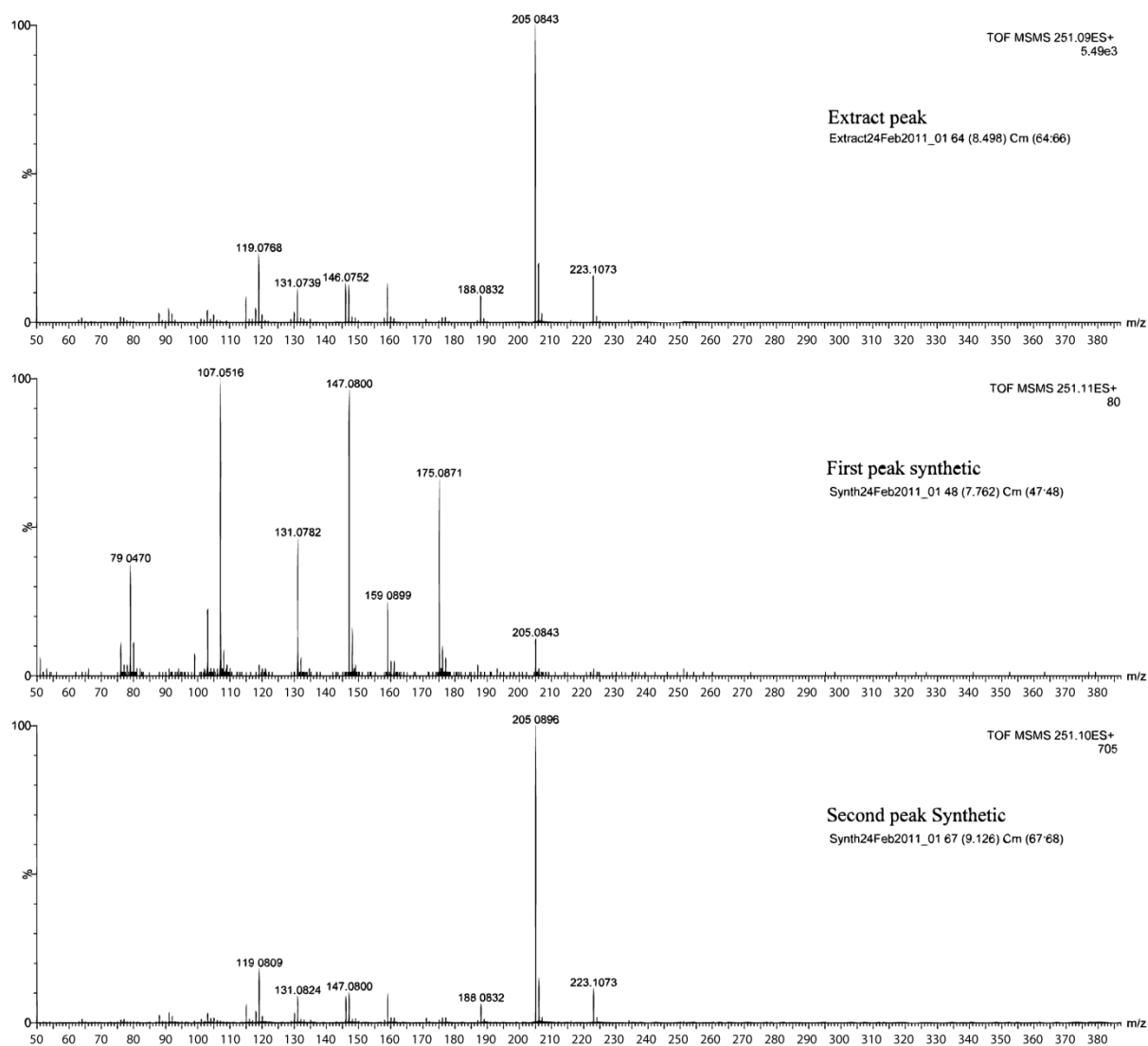


Figure A- 12. Mass fragmentation pattern of the main mass 251 on the SYNAPT with positive ionisation spray of the extracted yellow pigment (top, $5.49e^3$), the first synthetic purified product (middle, 80) and the second synthesized purified product (bottom, 705).

Appendix B

References

1. Vollrath, F.; Knight, D. P., Liquid crystalline spinning of spider silk. *Nature* **2001**, 410, (6828), 541-548.
2. Craig, C. L., *Spidersweb and Silk: Tracing evolution from Molecules to Genes to Phenotypes*. Oxford University Press: Oxford, N.Y, 2003.
3. Craig, C., Evolution of arthropod silks. *Ann. Rev. Entomol.* **1997**, 42, 231-267.
4. Vollrath, F., Biology of spider silk. *Int. J. Biol. Macromol.* **1999**, 24, (2-3), 81-88.
5. Vollrath, F., Spider Silk: Evolution and 400 Million Years of Spinning, Waiting, Snagging, and Mating. *Nature* 466, (7304), 319-319.
6. Dale, S. F., "Silk Road, Cotton Road or Indo-Chinese Trade in Pre-European Times". *Modern Asian Studies* **2009**, 43, 79-88.
7. Reddy, N.; Yang, Y. Q., Morphology and tensile properties of silk fibers produced by uncommon Saturniidae. *International Journal of Biological Macromolecules* **2010**, 46, (4), 419-424.
8. Inoue, S. I.; Tanaka, N.; Magoshi, Y.; Magoshi, J., Structure of filament spun by silkworm in different stages: Bombyx mori and wild silkworm. *Abstr. Pap. Am. Chem. Soc.* **2000**, 219, 277-PHYS.
9. Lucas, F., Spiders and their silks. *Discovery* **1964**, 25, 20-26.
10. Xu, M.; Lewis, R. V., Structure of a protein superfiber: spider dragline silk. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, 87, (18), 7120-4.
11. Kovoov, J., Comparative Structure and Histochemistry of silk-producing organs in Arachnids. In *Ecophysiology of Spiders*, Nentwig, W., Ed. Springer: Berlin-Heidelberg-New York, 1987; pp 160-186.
12. Haupt, J.; Kovoov, J., Silk-gland system and silk production in mesothelae (araneae). *Ann. Sci. Nat. Bot. Zool.* **1993**, 14, (2), 35-48.
13. Chris, H.; Vollrath, F. t. a.; Holland, C. Silk rheology. Thesis (D.Phil.)--University of Oxford, 2008., 2008.
14. Eisoldt, L.; Smith, A.; Scheibel, T., Decoding the secrets of spider silk. *Materials Today* **2011**, 14, (3), 80-86.
15. Kluge, J. A.; Rabotyagova, O.; Leisk, G. G.; Kaplan, D. L., Spider silks and their applications. *Trends Biotechnol.* **2008**, 26, (5), 244-251.
16. Hardy, J. G.; Romer, L. M.; Scheibel, T. R. k., Polymeric materials based on silk proteins. *Polymer* **2008**, 49, (20), 4309-4327.
17. Porter, D.; Vollrath, F., Nanoscale toughness of spider silk. *Nano Today* **2007**, 2, (3), 6-6.
18. van Beek, J. D.; Hess, S.; Vollrath, F.; Meier, B. H., The molecular structure of spider dragline silk: Folding and orientation of the protein backbone. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, 99, (16), 10266-10271.
19. Porter, D.; Vollrath, F.; Shao, Z., Predicting the mechanical properties of spider silk as a model nanostructured polymer. *The European Physical Journal E* **2005**, 16, (2), 199-206.
20. Gatesy, J.; Hayashi, C.; Motriuk, D.; Woods, J.; Lewis, R., Extreme Diversity, Conservation, and Convergence of Spider Silk Fibroin Sequences. *Science* **2001**, 291, (5513), 2603-2605.
21. Bini, E.; Knight, D. P.; Kaplan, D. L., Mapping domain structures in silks from insects and spiders related to protein assembly. *J. Mol. Biol.* **2004**, 335, (1), 27-40.
22. Sponner, A.; Unger, E.; Grosse, F.; Weisschart, K., Conserved C-termini of spidroins are secreted by the major ampullate glands and retained in the silk thread. *Biomacromolecules* **2004**, 5, (3), 840-5.

23. Sponner, A.; Vater, W.; Rommerskirch, W.; Vollrath, F.; Unger, E.; Grosse, F.; Weisshart, K., The conserved C-termini contribute to the properties of spider silk fibroins. *Biochem. Biophys. Res. Commun.* **2005**, 338, (2), 897-902.
24. Huemmerich, D.; Helsen, C. W.; Quedzuweit, S.; Oschmann, J.; Rudolph, R.; Scheibel, T., Primary structure elements of spider dragline silks and their contribution to protweil solubility. *Biochemistry* **2004**, 43, 13604-13612.
25. Jin, H. J.; Kaplan, D. L., Mechanism of silk processing in insects and spiders. *Nature* **2003**, 424, (6952), 1057-1061.
26. Hardy, J. G.; Römer, L. M.; Scheibel, T. R., Polymeric materials based on silk proteins. *Polymer* **2008**, 49, (20), 4309-4327.
27. Pauling, L., THE NATURE OF THE CHEMICAL-BOND - 1992. *J. Chem. Educ.* **1992**, 69, (7), 519-521.
28. Lewis, G. N., THE ATOM AND THE MOLECULE. *J. Am. Chem. Soc.* **1916**, 38, (4), 762-785.
29. Brown, C. H. k., A REVIEW OF THE METHODS AVAILABLE FOR THE DETERMINATION OF THE TYPES OF FORCES STABILIZING STRUCTURAL PROTEINS IN ANIMALS. *Quarterly Journal of Microscopical Science* **1950**, 91, (3), 331-339.
30. Parsegian, V.; Parsegian, V. A., *Van der Waals forces : a handbook for biologists, chemists, engineers, and physicists*. New York : Cambridge University Press: New York, 2006.
31. Perez-Rigueiro, J.; Viney, C.; Llorca, J.; Elices, M., Mechanical properties of single-brin silkworm silk. *J. Appl. Polym. Sci.* **2000**, 75, (10), 1270-1277.
32. Marsh, R. E.; Corey, R. B.; Pauling, L., An Investigation of the Structure of Silk Fibroin. *Biochim. Biophys. Acta* **1955**, 16, (1), 1-34.
33. Knight, D. P.; Vollrath, F., Biological liquid crystal elastomers. *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* **2002**, 357, (1418), 155-163.
34. Carmichael, S.; Barghout, J. Y. J.; Viney, C., The effect of post-spin drawing on spider silk microstructure: a birefringence model. *Int. J. Biol. Macromol.* **1999**, 24, (2-3), 219-226.
35. Geurts, P.; Zhao, L.; Hsia, Y.; Gnesa, E.; Tang, S.; Jeffery, F.; Mattina, C. L.; Franz, A.; Larkin, L.; Vierra, C., Synthetic Spider Silk Fibers Spun from Pyriform Spidroin 2, A Glue Silk Protein Discovered in Orb-Weaving Spider Attachment Discs†. *Biomacromolecules* **2010**, 11, (12), 3495-3503.
36. Dicko, C.; Kenney, J. M.; Knight, D.; Vollrath, F. k., Transition to a beta-sheet-rich structure in spidroin in vitro: The effects of pH and cations. *Biochemistry* **2004**, 43, (44), 14080-14087.
37. Arunan, E.; Desiraju, G. R.; Klein, R. A.; Sadlej, J.; Scheiner, S.; Alkorta, I.; Clary, D. C.; Crabtree, R. H.; Dannenberg, J. J.; Hobza, P.; Kjaergaard, H. G.; Legon, A. C.; Mennucci, B.; Nesbitt, D. J., Definition of the hydrogen bond (IUPAC Recommendations 2011). *Pure Appl. Chem.* **2011**, 83, (8), 1637-1641.
38. Knight, D. P.; Knight, M. M.; Vollrath, F., Beta transition and stress-induced phase separation in the spinning of spider dragline silk. *Int. J. Biol. Macromol.* **2000**, 27, (3), 205-210.
39. Porter, D.; Vollrath, F., The role of kinetics of water and amide bonding in protein stability. *Soft Matter* **2008**, 4, (2), 328-336.
40. Head-Gordon, T., Is water structure around hydrophobic groups clathrate-like? *Proceedings of the National Academy of Sciences* **1995**, 92, (18), 8308-8312.
41. Tanaka, K.; Kajiyama, N.; Ishikura, K.; Waga, S.; Kikuchi, A.; Ohtomo, K.; Takagi, T.; Mizuno, S., Determination of the site of the disulfide linkage between heavy and light

- chains of silk fibroin produced by *Bombyx mori*. *Biochim. Biophys. Acta* **1999**, 1432, 92–103.
42. Brunet, P. C. J. k., SCLEROTINS. *Endeavour* **1967**, 26, (98), 68-&.
 43. Ittah, S.; Michaeli, A.; Goldblum, A.; Gat, U., A Model for the Structure of the C-Terminal Domain of Dragline Spider Silk and the Role of Its Conserved Cysteine. *Biomacromolecules* **2007**, 8, (9), 2768-2773.
 44. Raven, D. J.; Earland, C.; Little, M., Occurrence of dityrosine in Tussah silk fibroin and keratin. *Biochimica et Biophysica Acta (BBA) - Protein Structure* **1971**, 251, (1), 96-99.
 45. Haslam, E. k., POLYPHENOL-PROTEIN INTERACTIONS. *Biochemical Journal* **1974**, 139, (1), 285-288.
 46. Haslam, E., *Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action*. Cambridge University Press: Cambridge, 1998; p 440.
 47. Brunet, P. C. J.; Coles, B. C., Tanned silks. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* **1974**, 187, (1087), 133 - 170.
 48. Brunet, P. C. J.; Kent, P. W. k., MECHANISM OF SCLEROTIN FORMATION - THE PARTICIPATION OF A BETA-GLUCOSIDE. *Nature* **1955**, 175, (4462), 819-820.
 49. Brunet, P. C. J.; Coles, B. C. k., Tanned silks. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* **1974**, 187, (1087), 133-170.
 50. Lin, L. H.; Edmonds, D. T.; Vollrath, F., Structural engineering of an orb-spider's web. *Nature* **1995**, 373, (6510), 146-148.
 51. Chen, F.; Porter, D.; Vollrath, F., Silkworm cocoons inspire models for random fiber and particulate composites. *Phys. Rev. E* **2010**, 82, (4).
 52. Lounibos, L. P., The cocoon spinning behaviour of the chinese oak silkworm, *Antheraea pernyi*. *Anim. Behav.* **1975**, 23, (Part 4), 843-853.
 53. Yagi, N., The cocooning behavior of a Saturnian caterpillar (*Dictyoploca japonica*); a problem in analysis of insect conduct. *Jour Exp Zool* **1966**, 46, ((2)), 245-261.
 54. Lounibos, L. P., Initiation and maintenance of cocoon spinning behaviour by saturniid silkworms. *Physiol. Entomol.* **1976**, 1, (3), 195.
 55. Montes-G, G. M.; Draughn, R. A., Slow crack propagation in composite restorative materials. *Journal of Biomedical Materials Research* **1987**, 21, (5), 629-642.
 56. Zhao, H. P.; Feng, X. Q.; Yu, S. W.; Cui, W. Z.; Zou, F. Z., Mechanical properties of silkworm cocoons. *Polymer* **2005**, 46, (21), 9192-9201.
 57. Meinel, L.; Hofmann, S.; Karageorgiou, V.; Kirker-Head, C.; McCool, J.; Gronowicz, G.; Zichner, L.; Langer, R.; Vunjak-Novakovic, G.; Kaplan, D. L., The inflammatory responses to silk films in vitro and in vivo. *Biomaterials* **2005**, 26, (2), 147-155.
 58. Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J. S.; Lu, H.; Richmond, J.; Kaplan, D. L., Silk-based biomaterials. *Biomaterials* **2003**, 24, (3), 401-416.
 59. Gosline, J. M.; Guerette, P. A.; Ortlepp, C. S.; Savage, K. N., Mechanical design of spider silk: from fibroin sequence to mechanical function. *J. Exp. Biol.* **1999**, 202, 3295-3303.
 60. Vepari, C.; Kaplan, D. L. k., Silk as a biomaterial. *Prog. Polym. Sci.* **2007**, 32, (8-9), 991-1007.
 61. Holland, C.; Terry, A. E.; Porter, D.; Vollrath, F., Comparing the rheology of native spider and silkworm spinning dope. *Nat. Mater.* **2006**, 5, (11), 870-874.
 62. Fahnestock, S. R.; Bedzyk, L. A., Production of synthetic spider dragline silk protein in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **1997**, 47, (1), 33-39.
 63. Xia, X. X.; Qian, Z. G.; Ki, C. S.; Park, Y. H.; Kaplan, D. L.; Lee, S. Y., Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, 107, (32), 14059-14063.

64. Huemmerich, D.; Scheibel, T.; Vollrath, F.; Cohen, S.; Gat, U.; Ittah, S., Novel assembly properties of recombinant spider dragline silk proteins. *Curr. Biol.* **2004**, *14*, (22), 2070-2074.
65. Scheller, J.; Guhrs, K. H.; Grosse, F.; Conrad, U., Production of spider silk proteins in tobacco and potato. *Nat. Biotechnol.* **2001**, *19*, (6), 573-577.
66. Miao, Y. G.; Zhao, A. C.; Zhang, Y. S.; Nakagaki, K.; Meng, Y.; Zhao, T. F.; Nakagaki, M., Silkworm, *Bombyx mori* larvae expressed the spider silk protein through a novel Bac-to-Bac/BmNPV baculovirus. *Journal of Applied Entomology* **2006**, *130*, (5), 297-301.
67. Holland, C.; Terry, A. E.; Porter, D.; Vollrath, F., Natural and unnatural Silks. *Polymer* **2007**, *48*, 3388-3392.
68. Xie, F.; Zhang, H.; Shao, H.; Hu, X., Effect of shearing on formation of silk fibers from regenerated *Bombyx mori* silk fibroin aqueous solution. *International Journal of Biological Macromolecules* **2006**, *38*, (3-5), 284-288.
69. Collins, A. M.; Skaer, N. J. V.; Cheysens, T.; Knight, D.; Bertram, C.; Roach, H. I.; Oreffo, R. O. C.; Von-Aulock, S.; Baris, T.; Skinner, J.; Mann, S., Bone-like Resorbable Silk-based Scaffolds for Load-bearing Osteoregenerative Applications. *Adv. Mater.* **2009**, *21*, (1), 75-78.
70. Gellynck, K.; Verdonk, P. C. M.; Van Nimmen, E.; Almqvist, K. F.; Gheysens, T.; Schoukens, G.; Van Langenhove, L.; Kiekens, P.; Mertens, J.; Verbruggen, G., Silkworm and spider silk scaffolds for chondrocyte support. *Journal of Materials Science-Materials in Medicine* **2008**, *19*, (11), 3399-3409.
71. Yang, Y.; Ding, F.; Wu, H.; Hu, W.; Liu, W.; Liu, H.; Gu, X., Development and evaluation of silk fibroin-based nerve grafts used for peripheral nerve regeneration. *Biomaterials* **2007**, *28*, (36), 5526-5535.
72. Bunning, T. J.; Jiang, H.; Adams, W. W.; Crane, R. L.; Farmer, B.; Kaplan, D., Applications of Silk. *Silk Polymers* **1994**, *544*, 353-358.
73. Omenetto, F. G.; Kaplan, D. L., New opportunities for an ancient material. *Science* **2010**, *329*, (5991), 528-31.
74. Dandu, R.; Ghandehari, H., Delivery of bioactive agents from recombinant polymers. *Prog Polym Sci* **2007**, *32*, (8-9), 1008-1030.
75. Colgin, M. A.; Lewis, R. V., Spider silk: A biomaterial for the future. *Chem. Ind.* **1995**, (24), 1009-1012.
76. Madsen, B.; Shao, Z. Z.; Vollrath, F., Variability in the mechanical properties of spider silks on three levels: interspecific, intraspecific and intraindividual. *Int. J. Biol. Macromol.* **1999**, *24*, (2-3), 301-306.
77. Madsen, B.; Shao, Z. Z.; Vollrath, F. In *Variability in the mechanical properties of spider silks on three levels: interspecific, intraspecific and intraindividual*, 2nd International Symposium on Silk, Charlottesville, Virginia, Jun, 1998; Charlottesville, Virginia, 1998; pp 301-306.
78. Vollrath, F.; Madsen, B.; Shao, Z. Z., The effect of spinning conditions on the mechanics of a spider's dragline silk. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* **2001**, *268*, (1483), 2339-2346.
79. Shao, Z. Z.; Vollrath, F., Surprising strength of silkworm silk. *Nature* **2002**, *418*, (6899), 741-741.
80. Vollrath, F.; Knight, D. P.; Hu, X. W., Silk production in a spider involves acid bath treatment. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* **1998**, *265*, (1398), 817-820.
81. Dicko, C.; Kenney, J. M.; Knight, D.; Vollrath, F., Transition to a beta-sheet-rich structure in spidroin in vitro: The effects of pH and cations. *Biochemistry* **2004**, *43*, (44), 14080-14087.

82. Zhou, P.; Xie, X.; Knight, D. P.; Zong, X.-H.; Deng, F.; Yao, W.-H., Effects of pH and calcium ions on the conformational transitions in silk fibroin using 2D Raman correlation spectroscopy and ¹³C solid-state NMR. *Biochemistry-Us* **2004**, 43, (35), 11302-11.
83. Vollrath, F.; Knight, D. P., Structure and function of the silk production pathway in the Spider *nephila edulis*. *International Journal of Biological Macromolecules* **1999**, 24, (2-3), 243-249.
84. Madsen, B.; Vollrath, F. k., Mechanics and morphology of silk drawn from anesthetized spiders. *Naturwissenschaften* **2000**, 87, (3), 148-153.
85. Liu, Y.; Shao, Z. Z.; Vollrath, F., Relationships between supercontraction and mechanical properties of spider silk. *Nat. Mater.* **2005**, 4, (12), 901-905.
86. Holl, A.; Henze, M. In *Pigmentary constituents of yellow threads of Nephila webs*, European Society of Arachnology: 11th european colloquium, 1988; 1988; p 350.
87. Kurioka, A.; Yamazaki, M., Purification and identification of flavonoids from the yellow green cocoon shell (Sasamayu) of the silkworm, *Bombyx mori*. *Biosci. Biotechnol. Biochem.* **2002**, 66, (6), 1396-1399.
88. Prommuak, C.; De-Eknamkul, W.; Shotipruk, A. k., Extraction of flavonoids and carotenoids from Thai silk waste and antioxidant activity of extracts. *Separation and Purification Technology* **2008**, 62, 444-448.
89. Yamada, H.; Kato, Y., Green colouration of cocoons in *Antheraea yamamai* (Lepidoptera: Saturniidae): light-induced production of blue bilin in the larval haemolymph. *J. Insect. Physiol.* **2004**, 50, (5), 393-401.
90. Veldtman, R.; McGeoch, M. A.; Scholtz, C. H., Fine-scale abundance and distribution of wild silk moth pupae. *B. Entomol. Res.* **2007**, 97, (1), 15-27.
91. Akai, H.; Nagashima, T., Calcium Crystals of Cocoon Shell from African Gonometa Silkworm (Lasiocampidae). *Int. J. Wild Silkmoth & Silk* **2003**, 8, 1-5.
92. Opell, B. D., Economics of spider orb-webs: the benefits of producing adhesive capture thread and of recycling silk. *Funct. Ecol.* **1998**, 12, (4), 613-624.
93. Vollrath, F.; Fairbrother, W. J.; Williams, R. J. P.; Tillinghast, E. K.; Bernstein, D. T.; Gallagher, K. S.; Townley, M. A., Compounds in the Droplets of the Orb Spiders Viscid Spiral. *Nature* **1990**, 345, (6275), 526-528.
94. Townley, M. A.; Tillinghast, E. K., Orb Web Recycling in *Araneus-Cavaticus* (Araneae, Araneidae) with an Emphasis on the Adhesive Spiral Component, Gabamide. *J. Arachnol.* **1988**, 16, (3), 303-319.
95. Fischer, E., Über Spinnenseide. *H-S. Z. Physiol. Chem.* **1907**, 53, 126-139.
96. Bernays, E.; Lupi, A.; Bettolo, R. M.; Mastrofrancesco, C.; Tagliatesta, P., ANTIFEEDANT NATURE OF THE QUINONE PRIMIN AND ITS QUINOL MICONIDIN FROM MICONIA SPP. *Experientia* **1984**, 40, (9), 1010-1011.
97. Estable, C.; Ardao, M. I.; Fieser, L. F., GONYLEPTIDINE. *J. Am. Chem. Soc.* **1955**, 77, (18), 4942-4942.
98. Franck, R. R., *Silk, mohair, cashmere and other luxury fibres*. Woodhead publishing limited: 2001.
99. Das, S.; Chowdhury, S. K.; Das, N. K. k., An Improved Method of Tussah Silk Reeling. *Journal of the Textile Institute* **1992**, 83, (2), 279 - 281.
100. Freddi, G.; Gotoh, Y.; Mori, T.; Tsutsui, I.; Tsukada, M., Chemical structure and physical properties of *antheraea assama* silk. *J. Appl. Polym. Sci.* **1994**, 52, (6), 775-781.
101. Ohnishi, E.; Takahashi, S. Y.; Sonobe, H.; Hayashi, T. k., Crystals from Cocoons of *Malacosoma neustria testacea*. *Science* **1968**, 160, (3829), 783-784.

102. Das, S.; Chowdhury, S. K., A modified approach to the reeling of tropical tasar silk with adhesives. *Journal of the Textile Institute* **1991**, 82, (4), 505-507.
103. Yolande, O. Die evaluerende van peroksiedbleiking van *Gonometa postica*. master, Universiteit van die Vrystaat, Bloemfontein, Suid Afrika, 2007.
104. Singh, L. R.; Devi, Y. R.; Devi, S. K., Enzymological characterization of pineapple extract for potential application in oak tasar (*Antheraea proylei* J.) silk cocoon cooking and reeling. *Electronic Journal of Biotechnology* **2003**, 6, (3), 198-207.
105. Veldtman, R. The ecology of southern African wild silk moths (*Gonometa* species, Lepidoptera: Lasiocampidae): consequences for their sustainable use. University of Pretoria, 2005.
106. Shamitha, G., Studies on the filament of tasar silkworm, *Antheraea mylitta* D(Andhra local ecorace). *Current science* **2006**, 90, (12), 1667.
107. Paterson, B., Prospects for wild-silk production in South Africa. **2002**, 161, (3696), 67.
108. Kumar, A.; Choudhury, R., *Textile preparation and dyeing* Science Publishers: Enfield, (NH), 2006.
109. Mondal, M.; Trivedy, K.; Nirmal Kumar, S., The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* Linn., - a review. *Caspian J. Env. Sci.* **2007**, 5, (2), 63-76.
110. Hess, K. P., *Textile fibres and their use*. 5th ed.; Lippincott: 1954; p 547.
111. Freddi, G.; Svilokos, A.; Ishikawa, H.; Tsukada, M., Chemical-composition and physical-properties of *gonometa- rufobrunnae* silk. *J. Appl. Polym. Sci.* **1993**, 48, (1), 99-106.
112. Colomban, P.; Dinh, H. M.; Riand, J.; Prinsloo, L. C.; Mauchamp, B., Nanomechanics of single silkworm and spider fibres: a Raman and micro-mechanical in situ study of the conformation change with stress. *J. Raman Spectrosc.* **2008**, 39, (12), 1749-1764.
113. Moor, M.; Mitra, G., Indian silk. **2007**, 46, (1), 20-22.
114. Tuskes, P. M. P.; Paul, P. M., *The wild silk moths of North America : a natural history of the Saturniidae of the United States and Canada*. Cornell University Press: 1996.
115. Kato, H.; Hata, T.; Yasuta, K.; Kanda, C. k., Degumming, dyeing and structural properties of wild silkworm cocoon spinning yarns from *Anaphe*, *Cricula trifenestrata* and *Attacus atlas* Linnaeus. *J. Seric. Sci. Jpn.* **1999**, 68, (5), 405-415.
116. Lounibos, L. P., Initiation and maintenance of cocoon spinning behaviour by saturniid silkworms. *Physiological Entomology* **1976**, 1, (3), 195-206.
117. Lounibos, L. P., The cocoon spinning behaviour of the chinese oak silkworm, *Antheraea pernyi*. *Anlm. Behav.* **1975**, 23, 843-853.
118. Vanderkloot, W. G.; Williams, C. M., Cocoon construction by the cecropia silkworm. 1.The role of the external environment. *Behaviour* **1953**, 5, (2), 141-156.
119. Vanderkloot, W. G.; Williams, C. M., Cocoon construction by the cecropia silkworm. 2. The role of the internal environment. *Behaviour* **1953**, 5, (3), 157-174.
120. Miura, M.; Morikawa, H.; Kato, H.; Iwasa, M., Analysis of the construction process of cocoon shape by *Bombyx mori*. *Journal of Sericultural Science of Japan* **1997**, 66, (1), 23-30.
121. Yanagisawa, N., On the mathematical expression of the forms of the cocoons and the motion of the silkworms during the cocooning. (I). *The journal of sericultural science of Japan* **1941**, 13, 105-134.
122. Yanagisawa, N., On the mathematical expression of the forms of the cocoons and the motion of the silkworms during the cocooning. (II). *The journal of sericultural science of Japan* **1942**, 13, (a), 152-167.

123. Yanagisawa, N., On the mathematical expression of the forms of the cocoons and the motion of the silkworms during the cocooning. (III). *The journal of sericultural science of Japan* **1942**, 14, (b), 105-134.
124. Kiyosawa, M.; Ito, E.; Shirai, K.; Kanekatsu, R.; Miura, M.; Kiguchi, K., Cocoon spinning behavior in the silkworm, *Bombyx mori* : Comparison of three strains constructing different cocoons in shape. *Zool. Sci.* **1999**, 16, (2), 215-223.
125. Miura, M.; Kuwako, N.; Morikawa, H. k. In *Statistical analysis of silkworm spinning behaviour under abnormal circumstances*, 6th China International Silk Conference/2nd International Textile Forum, Suzhou, PEOPLES R CHINA, Sep 13-14, 2007; Bai, L., Ed. Chemical Industry Press: Suzhou, PEOPLES R CHINA, 2007; pp 151-153.
126. Takasu, Y.; Yamada, H.; Tsubouchi, K. k., Isolation of Three Main Sericin Components from the Cocoon of the Silkworm, *Bombyx mori*. *Bioscience, Biotechnology, and Biochemistry* **2002**, 66, (12), 2715-2718.
127. Brecevic, L.; Kralj, D., THE INFLUENCE OF SOME AMINO-ACIDS ON CALCIUM-OXALATE DIHYDRATE TRANSFORMATION. *J. Cryst. Growth* **1986**, 79, (1-3), 178-184.
128. Sayan, P.; Sargut, S. T.; Kiran, B., Calcium oxalate crystallization in the presence of amino acids, proteins and carboxylic acids. *Crystal Research and Technology* **2009**, 44, (8), 807-817.
129. Sargut, S. T.; Sayan, P.; Kiran, B., Influence of essential and non-essential amino acids on calcium oxalate crystallization. *Crystal Research and Technology* **2010**, 45, (1), 31-38.
130. Shen, Y. H.; Li, S. K.; Xie, A. J.; Xu, W. H.; Qiu, L. G.; Yao, H.; Yu, X. R.; Chen, Z. X., Controlled growth of calcium oxalate crystal in bicontinuous microemulsions containing amino acids. *Colloids and Surfaces B-Biointerfaces* **2007**, 58, (2), 298-304.
131. Orme, C. A.; Noy, A.; Wierzbicki, A.; McBride, M. T.; Grantham, M.; Teng, H. H.; Dove, P. M.; DeYoreo, J. J., Formation of chiral morphologies through selective binding of amino acids to calcite surface steps. *Nature* **2001**, 411, (6839), 775-779.
132. Shiraga, H.; Min, W.; Vandusen, W. J.; Clayman, M. D.; Miner, D.; Terrell, C. H.; Sherbotie, J. R.; Foreman, J. W.; Przysiecki, C.; Neilson, E. G.; Hoyer, J. R., INHIBITION OF CALCIUM-OXALATE CRYSTAL-GROWTH INVITRO BY UROPONTIN - ANOTHER MEMBER OF THE ASPARTIC ACID-RICH PROTEIN SUPERFAMILY. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, 89, (1), 426-430.
133. Nagaoka, S.; Katayama, H.; Fujibayashi, Y.; Sugimura, Y., Calcium oxalate crystals in mulberry leaves: No negative effect on feeding the silkworm, *Bombyx mori*. *Journal of Insect Biotechnology and Sericology* **2010**, 79, (2), 71-74.
134. Teigler, D. J.; Arnott, H. J., CRYSTAL DEVELOPMENT IN MALPIGHIAN TUBULES OF BOMBYX-MORI (L). *Tissue Cell* **1972**, 4, (1), 173-&.
135. Wyman, A. J.; Webb, M. A., Calcium oxalate accumulation in malpighian tubules of silkworm (*Bombyx mori*). In *Renal Stone Disease*, Evan, A. P.; Lingeman, J. E.; Williams, J. C., Eds. Amer Inst Physics: Melville, 2007; Vol. 900, pp 407-411.
136. Harwood, L.; Claridge, T. D. W., *Introduction to Organic Spectroscopy*. Oxford, 1996.
137. Harwood, L. M.; Moody, C. J.; Percy, J. M., *Experimental organic chemistry - standard and microscale*. 2nd ed.; 1999.
138. Cole, R. B., *Electrospray ionization mass spectrometry*. New York, 1997.
139. Silverstein, R. M.; Bassler, G. c.; Morrill, T. e., *Spectrometric Identification of Organic Compounds*. 5th edn ed.; New York, 1991.
140. Bellamy, L., *The Infrared Spectra of Complex Molecules*. 2nd edn ed.; london, 1981.
141. Field, L.; Sternhell, S.; Kalman, J., *Organic Structures from Spectra*. 2nd edn ed.; New York, 1995.

142. Ulrich, M.; Petre, A.; Youhnovski, N.; Promm, F.; Schirle, M.; Schumm, M.; Pero, R. S.; Doyle, A.; Checkel, J.; Kita, H.; Thiyagarajan, N.; Acharya, K. R.; Schmid-Grendelmeier, P.; Simon, H. U.; Schwarz, H.; Tsutsui, M.; Shimokawa, H.; Bellon, G.; Lee, J. J.; Przybylski, M.; Doring, G., Post-translational tyrosine nitration of eosinophil granule toxins mediated by eosinophil peroxidase. *Journal of Biological Chemistry* **2008**, 283, (42), 28629-28640.
143. Wu, W. J.; Chen, Y. H.; Hazen, S. L., Eosinophil peroxidase nitrates protein tyrosyl residues - Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *Journal of Biological Chemistry* **1999**, 274, (36), 25933-25944.
144. Sala, A.; Nicolis, S.; Roncone, R.; Casella, L.; Monzani, E., Peroxidase catalyzed nitration of tryptophan derivatives - Mechanism, products and comparison with chemical nitrating agents. *European Journal of Biochemistry* **2004**, 271, (13), 2841-2852.
145. Ricoux, R.; Boucher, J.-L.; Mansuy, D.; Mahy, J.-P., Microperoxidase 8 catalyzed nitration of phenol by nitrogen dioxide radicals. *European Journal of Biochemistry* **2001**, 268, (13), 3783-3788.
146. Dai, R.-J.; Huang, H.; Chen, J.; Deng, Y.-L.; Xiao, S.-Y., Nitration Reaction Catalyzed by Horseradish Peroxidase in the Presence of H₂O₂ and NaNO₂. *Chinese Journal of Chemistry* **2007**, 25, (11), 1690-1694.
147. Pouchkina, N. N.; Stanchev, B. S.; McQueen-Mason, S. J., From EST sequence to spider silk spinning: identification and molecular characterisation of *Nephila senegalensis* major ampullate gland peroxidase NsPox. *Insect Biochem. Mol. Biol.* **2003**, 33, (2), 229-238.
148. Vollrath, F.; Knight, D. P. k. In *Structure and function of the silk production pathway in the Spider nephila edulis*, 2nd International Symposium on Silk, Charlottesville, Virginia, Jun, 1998; Elsevier Science Bv: Charlottesville, Virginia, 1998; pp 243-249.
149. Weiner, S., Biomineralization: A structural perspective. *Journal of Structural Biology* **2008**, 163, (3), 229-234.
150. Elliott, J. C., Calcium Phosphate Biominerals. *Reviews in Mineralogy and Geochemistry* **2002**, 48, (1), 427-453.
151. Rahman, M. A.; Oomori, T.; Wörheide, G., Calcite Formation in Soft Coral Sclerites Is Determined by a Single Reactive Extracellular Protein. *Journal of Biological Chemistry* **2011**, 286, (36), 31638-31649.
152. Takahashi, S. Y.; Suzuki, G.; Ohnishi, E., Origin of oxalic acid in Ca oxalate crystals in the Malpighian tubes of the tent caterpillar, *Malacosoma neustria* testacea. *J. Insect Physiol.* **1969**, 15, (3), 403-407.
153. Franceschi, V. R.; Nakata, P. A. k., Calcium oxalate in plants: Formation and function. *Annu. Rev. Plant Biol.* **2005**, 56, 41-71.
154. Hauschka, P. V.; Lian, J. B.; Gallop, P. M., DIRECT IDENTIFICATION OF CALCIUM-BINDING AMINO-ACID, GAMMA-CARBOXYGLUTAMATE, IN MINERALIZED TISSUE. *Proc. Natl. Acad. Sci. U. S. A.* **1975**, 72, (10), 3925-3929.
155. Li, X. X.; Zhang, D. Z.; Lynch-Holm, V. J.; Okita, T. W.; Franceschi, V. R., Isolation of a crystal matrix protein associated with calcium oxalate precipitation in vacuoles of specialized cells. *Plant Physiology* **2003**, 133, (2), 549-559.
156. Lepkovsky, S.; Roboz, E.; Haagen-Smit, A. J., Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine- deficient rats. *Journal of Biological Chemistry* **1943**, 149, (1), 195-201.
157. Nelson, D. L.; Cox, M. M., *Lehninger Principles of Biochemistry*. Fourth Edition edition ed.; Freeman, W. H.: 2005.

158. Salway, J., *Metabolism at a Glance*. 3rd Edition edition ed.; Wiley-Blackwell: Oxford, 2004.
159. Putthanarat, S.; Tapadia, P.; Zarkoob, S.; Miller, L. D.; Eby, R. K.; Adams, W. W., The color of dragline silk produced in captivity by the spider *Nephila clavipes*. *Polymer* **2004**, 45, (6), 1933-1937.
160. Holl, A.; Henze, M., Pigmentary constituents of yellow threads of *Nephila* webs. In *Europaisches Arachnologisches Colloquium: comptes rendus du XI. Colloque d'Arachnologie (colloque international europeen)*. J. Hout: Technische Universität Berlin (Institut für Biologie FB-14), Berlin., 1988; Vol. XI, p 350.
161. Rose, D. P., ASPECTS OF TRYPTOPHAN METABOLISM IN HEALTH AND DISEASE - REVIEW. *Journal of Clinical Pathology* **1972**, 25, (1), 17-&.
162. Malina, H.; Richter, C.; Mehl, M.; Hess, O., Pathological apoptosis by xanthurenic acid, a tryptophan metabolite: activation of cell caspases but not cytoskeleton breakdown. *BMC Physiology* **2001**, 1, (1), 7.
163. Li, J.; Li, G., Transamination of 3-Hydroxykynurenine to Produce Xanthurenic Acid: A Major Branch Pathway of Tryptophan Metabolism in the Mosquito, *Aedes aegypti*, During Larval Development. *Insect Biochem. Mol. Biol.* **1997**, 27, (10), 859-867.
164. Baitinger, W. F.; Schleyer, P. v. R.; Murty, T. S. S. R.; Robinson, L., Nitro groups as proton acceptors in hydrogen bonding. *Tetrahedron* **1964**, 20, (7), 1635-1647.
165. Porat, Y.; Abramowitz, A.; Gazit, E. k., Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. *Chem. Biol. Drug Des.* **2006**, 67, (1), 27-37.
166. Reitsema, R. H., THE CHEMISTRY OF 4-HYDROXYQUINOLINES. *Chem. Rev.* **1948**, 43, (1), 43-68.
167. Otter, D.; Cao, M.; Lin, H.-M.; Fraser, K.; Edmunds, S.; Lane, G.; Rowan, D., Identification of Urinary Biomarkers of Colon Inflammation in IL10(-/-) Mice Using Short-Column LCMS Metabolomics. *J. Biomed. Biotechnol.* **2011**.