A spectroscopic investigation of polyacetylenic molecules in the molluskan biogenic matrix

by

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“And in like manner we see the race of shells painting
the lap of earth, where with gentle waves the sea beats
on the thirsty sand of the winding shore.
And so I must insist these things must be:
The atoms are by nature made, not all alike,
not turned out by factory’s mass production.
Differ then they must.”

Lucretius, II, 374 – 380
Abstract:
A spectroscopic investigation of polyacetylenic molecules in the molluskan biogenic matrix

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Molluscan shells show great diversity in colour, which is due to many different classes of chemical compounds. The identification of the pigments found in the molluscan shells of a diverse group of marine species was done in situ using Resonance Raman spectroscopy, and found to be unsubstituted polyacetylenes.

Two South African species (D. serra and J. janthina) were chosen for extraction of the pigment-containing organic component, and it was found that the vibrational bands in the Raman spectra of the extracted component shifted relative to that obtained in the molluscan matrix, indicating structural changes in the pigmentary molecules upon extraction.

Published correlations were used to evaluate the possible length of the conjugated chain, and a new correlation was established using previous reported data. The conjugated chain length predicted from the new correlation of the studied pigmentary molecules ranges between ten and twelve double bonds.
Samevattting:
A spectroscopic investigation of polyacetylenic molecules
in the molluskan biogenic matrix
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Werner Barnard
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Weekdier skulpe toon 'n groot verskeidenheid in kleur as gevolg van verskillende chemiese verbindinge. Die identifikasie van die pigmentte wat in 'n verskeidenheid weekdierspesies se skulpe gevind word, is in situ gedoen deur middel van Resonans Raman spektroskopie. Die pigmentte is geïdentifiseer as ongesubstitueerde poliasetileen molekules.

Twee Suid-Afrikaanse spesies (D. serra en J. janthina) is gekies om pigmenthoudende organiese deel te ekstraer. Die vibrasie bande in die Raman spektra van die geëkstraerde deel het geskuif relatief tot die vibrasie band posisies verkry in die skulp matriks, wat aanduidend is van structurele veranderinge in die pigment molekules as gevolg van die ekstraksie.

Gepubliseerde verwantskappe was gebruik om die moontlike lengte van die gekonjugeerde ketting te bepaal, en 'n nuwe verwantskap is verkry deur gebruik te maak van voorheen gepubliseerde data. Die lengte van konjugasie wat voorspel is deur die nuwe verwantskap vir die pigment molekules wat bestudeer is, is tussen tien en twaalf dubbelbindings.
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List of Related Achievements

Poster Presentations


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1. “Raman Investigation of Molluscan Carotenoid Pigments” SASS Young Spectroscopists Symposium, Rand Afrikaans University, 12 August 2003, awarded 1$^{st}$ place.

2. “Raman Investigation of Molluscan Carotenoid Pigments” SACI/RSC Young Chemist Symposium, University of the Witwatersrand, 24 October 2003, awarded 2$^{nd}$ place.


Chapter 1

Introduction

1.1 Molluscan Pigments

Nature has never disappointed in the use of pigmentary molecules for colour in the fauna and flora kingdoms. For centuries mankind has harvested and used these colouring agents to adorn themselves or beautify their clothing. One of the most prized of these colouring agents was Tyrian purple (6,6'-dibromoindigo), and was obtained from molluscs belonging to either the genus Muricidae or Thaisidae.

The pigmentary molecules responsible for the colouration of specifically the molluscan shell was particularly difficult to determine relative to that of the soft parts of a mollusc – the reason being the difficulty in extraction of the pigment. Some early methods report the use of 6N HCl solutions at 70ºC, compared to a simple ether or acetone extraction for the soft tissue parts. Granted, some chemical compounds, might be stable under these conditions, but many may not be. ‘Softer’ methods entailed the digestion of the mainly CaCO₃ molluscan shell by means of Na₂EDTA, or 5% HCl in acetone. These methods, first used on corals, were later employed, and proved successful in the extraction of the pigment from the molluscan shell.

Different types of pigmentary molecules in different parts of the molluscan body have been identified using various techniques such as UV-Visible spectroscopy, column chromatography and HPLC. The most important of these pigments include the carotenoids, indole pigments (indigoids and melanins), tetrapyrroles (porphyrins and bilichromes) and hemocyanins. Of these, carotenoids offer the strongest link to terrestrial life as they are found in...
many plants and land animals, not part of the phylum Mollusca, as colouring agents. The majority of pigments so far identified in molluscan shells seems to be of carotenoid nature\textsuperscript{4,5,6}.

Carotenoids can be described as relatively short linear polyene chains with substituents. The common chemical denominator for all classical carotenoids is the tetramethyl substituted polyene backbone shown in Figure 1-1. The structures of some of the more common carotenoids are shown in Figure 1-2.

![Figure 1-1: The classical carotenoid polyene backbone showing the positions of the four methyl groups.](image1)

![Figure 1-2: Structures of some common carotenoids showing different terminal group functionalities.](image2)
Carotenoids are of great industrial importance in the food industry for both their nutritional and aesthetical value. Two industries that use it as an important additive in feedstock is the poultry and salmon fishing industry, for both the aforementioned reasons, and have therefore done extensive research to find a more economically viable source of the required carotenoids.\textsuperscript{11,12,13}

1.2 The Molluscan Shell

The molluscan shell is an excellent example of what is known as a biogenic matrix that consists of both an organic and inorganic component. The organic component is largely made up of the conchoilin-proteins that hold the inorganic part of the matrix together.\textsuperscript{14} Also part of the organic component is the all-important pigment of the shell. It is usually only found deposited on the surface of the shell, be it internally or externally.

The major component of the molluscan shell is the inorganic part that is largely made up of CaCO\textsubscript{3}, but Mg\textsuperscript{2+}, Sr\textsuperscript{2+} can also be found in significant quantities.\textsuperscript{15} Other minor trace elements such as iron and magnesium have also been reported to be present.\textsuperscript{14,15} CaCO\textsubscript{3} itself can be found in three different phases, which are calcite, aragonite and vaterite.\textsuperscript{16} The two dominant phases seem to be calcite and aragonite, and can either occur separately or in combination with one another in the molluscan shell.\textsuperscript{14,15} The carbonate phase of the shell seems to be dependent on many factors,\textsuperscript{14} but the most important seems to be the conchoilin composition to initialise the biomineralisation process.\textsuperscript{17}

1.3 The use of Raman Spectroscopy

Raman spectroscopy has been developed as a powerful, non-destructive tool for the \textit{in situ} observation or identification of chemical compounds. Raman spectroscopy also becomes the preferred method of investigation when it comes to very complex, inhomogeneous samples as it can be utilised as a
micro probing technique. The sample is simply placed under the microscope and the laser focused on the desired area for which a spectrum needs to be obtained. Very little sample preparation is usually required for Raman spectroscopy, and it has therefore become the desired technique when working with valuable or microscopic samples that need to be investigated in situ or in vivo.

A good example of this is the study or verification of archaeological samples and historical artworks\(^\text{18}\). Raman spectroscopy has also found wide application in the biological field for in situ or in vivo studies and diagnostic purposes\(^\text{19,20}\). Carotenoids have favourably lent themselves as the ideal molecule for such studies, as they show a dramatic Resonance Raman (RR) effect with blue to green laser wavelengths. This enables scientists to detect carotenoids at very low concentrations in complex biological mixtures if the incident laser wavelength is selected correctly. One such successful example is the use of this technique for the probing of carotenoids in vivo in living human subjects for the early detection of certain skin and retinal problems\(^\text{21,22,23}\).

Carotenoids also have the favourable property that they do not show an appreciable fluorescence with blue to green laser wavelengths, thus reducing the necessity of spectral corrections. Furthermore, many low intensity vibrational bands can be enhanced, making it easier to be distinguished. The major reason for the lack of fluorescence for carotenoids is the preferred pathways of relaxation that exists after excitation by the incident wavelength\(^\text{23}\). From Figure 1-3 it can be seen that after optical excitation from the \(1^1\text{A}_g\) ground state to the \(1^1\text{B}_u\) excited state, three pathways of relaxation exist for the molecule: The first being the Raman effect itself, and the other two pathways is either the fluorescent relaxation, or the nonradiative relaxation to another excited state, the \(2^1\text{A}_g\) state, found below the \(1^1\text{B}_u\) state. Once relaxation has occurred using the latter pathway, an electronic transition from the \(2^1\text{A}_g\) state to the \(1^1\text{A}_g\) ground state is parity forbidden\(^\text{23}\). Unfortunately,
many of the biological matrices in which the carotenoids are found, fluoresce which can become a problem.

Figure 1-3: Diagram$^{23}$ showing the different pathways of relaxation to the ground state that occurs in most carotenoids after excitation to an excited state.

As CaCO$_3$ is the major component of the molluscan shell, the problem of matrix fluorescence is eliminated in the Raman spectrum of the molluscan shell pigment. The positions of the Raman bands of CaCO$_3$ do not interfere with those of the carotenoid, and no Resonance Raman effect is observed for it in the blue to green wavelength region. The strong symmetrical stretching mode of the CO$_{3}^{2-}$ ion acts as a good internal reference in the obtained spectra. The stability of the pigment in the matrix is also favourable, allowing for acceptable spectra to be obtained without any visible degradation$^{24}$. 
Taking into account all these aspects, Raman spectroscopy seems to be the most favourable technique with which to investigate the pigments of molluscan shells *in situ*.

### 1.4 References


Chapter 2

Theoretical Aspects

2.1 The Theory of Vibrational Spectroscopy

The energy of any polyatomic system can be separated in four main quantised components. These are the energies associated with the translational motion, the electrons, the vibrations and the rotations of the polyatomic unit:

\[ E_T = E_{\text{trans}} + E_e + E_{\text{vib}} + E_{\text{rot}} \]  \hspace{1cm} (2.1)

Of these, only \( E_e \), \( E_{\text{vib}} \) and \( E_{\text{rot}} \) are usually of interest to the molecular spectroscopist. The relationship between these three energies for a diatomic unit is illustrated in Figure 2-1. Here only the vibrational energy will be discussed. The two complementary techniques that are most often employed in obtaining vibrational information are Infrared and Raman spectroscopy, but the origins of these two underlying phenomena are very different.

2.1.1 Infrared (IR) Spectroscopy

The electric dipole moment \( \mu \) of a chemical bond can be described as

\[ \mu = qd \]  \hspace{1cm} (2.2)

where \( q \) is the charge product at one pole and \( d \) the distance between the poles. As a molecular bond vibrates, the rate of change in the dipole moment is the same as the frequency of the bond vibration. For a polyatomic molecule
having a permanent dipole, \( \mu \neq 0 \) at any given moment during the vibration. Because quantum mechanics show that molecular vibrational energy levels are quantised, the dipole moment will only couple with the electric field of the infrared radiation that matches the correct frequency of vibration of the molecule, allowing for an absorption and emission of infrared photons between two vibrational energy levels in the molecule’s electronic ground state. Infrared spectroscopy is therefore a measurement of the frequency during the change of the permanent dipole moment as the molecule vibrates.

**Figure 2-1:** Schematic showing the relationship of the electronic, vibrational and rotational energy levels for a diatomic unit\(^1\). Not drawn to scale.
2.1.2 Raman Spectroscopy

In Raman spectroscopy, we observe electromagnetic radiation, usually visible light, scattered by molecules when it is irradiated with a monochromatic light source. The source of the monochromatic radiation is usually a laser set to a specific frequency. When the light is scattered by the molecule, two possible scattering processes can occur: elastic and inelastic scattering of the light. The elastic scattering process is when the frequency of the light scattered by the molecule is equal to the frequency $\nu$ of the incident light, and is called Rayleigh scattering. This is by far the major effect observed. When light is scattered inelastically by a molecule, the frequency of the light scattered by the molecule changes to a frequency $\nu_i$. If $\nu_i < \nu$ the scattered radiation is called Stokes radiation, and if $\nu_i > \nu$ it is called anti-Stokes radiation. The difference $\nu_0 = |\nu - \nu_i|$, corresponds to frequencies or energies in the infrared region. In both the inelastic and elastic scattering process the molecule is excited to a virtual state. These virtual states can be seen as being a sum over all excited states, when we look at Raman as a two photon process. This entails the virtual absorption of the first photon to numerous excited vibrational states, whereafter the second photon is virtually emitted to return to the ground state$^{2,3}$. These virtual states do not correspond to true vibrational or electronic levels, as molecules have a continuum of virtual states dependent only on the frequency of the exciting laser.

Molecules that are centrosymmetric or those with high symmetry such that the vector sum of the individual bond dipoles gives $\mu = 0$, will have no permanent dipole, even though each bond might have a nonzero dipole moment associated with it. When a molecule is placed in an electric field $E$ a dipole moment $\mu_i$ is induced, with

$$\mu_i = \alpha E$$ (2.3)
where the proportionality constant $\alpha$ is called the polarizability. The electric field $E$ has an associated fluctuation frequency $\nu$, and therefore it can be expressed as

$$E = E_0 \cos 2\pi vt$$

(2.4)

with amplitude $E_0$ over time $t$. The induced dipole in the electric field can now be expressed as

$$\mu_i = \alpha E_0 \cos 2\pi vt.$$  

(2.5)

The displacement $x$ of the atoms from the equilibrium position, vibrating at a frequency $v_0$ can be expressed as

$$x = x_0 \cos 2\pi v_0 t,$$

(2.6)

and because polarizability is a molecular property, it is also dependent on the displacement from the equilibrium position and will vary according to the vibrational frequency of the molecule giving:

$$\alpha = \alpha_0 + \left( \frac{\partial \alpha}{\partial x} \right)_0 x$$

$$= \alpha_0 + \left( \frac{\partial \alpha}{\partial x} \right)_0 x_0 \cos 2\pi v_0 t$$

(2.7)

where $\alpha_0$ is the polarizability at equilibrium, and $\left( \frac{\partial \alpha}{\partial x} \right)_0$ is the rate of change of $\alpha$ during the change of $x$ at the equilibrium position. Substituting Eq. 2.7 into Eq. 2.5 yields

$$\mu_i = \left[ \alpha_0 + \left( \frac{\partial \alpha}{\partial x} \right)_0 x_0 \cos 2\pi v_0 t \right] E_0 \cos 2\pi vt.$$  

(2.8)
Use of the identity \( \cos A \cos B = \frac{1}{2} [\cos(A+B) + \cos(A-B)] \), after the rearrangement of Eq. 2.8, finally gives

\[
\mu = \alpha E_0 \cos 2\pi \nu t + \frac{1}{2} \left( \frac{d\alpha}{d\tau} \right) x_0 E_0 \{ \cos [2\pi (\nu + \nu_0) t] + \cos [2\pi (\nu - \nu_0) t] \}. \tag{2.9}
\]

The first term in Eq. 2.9 refers to the elastic Rayleigh \((\nu)\) scattering while the last term refers to the two inelastic processes of anti-Stokes \((\nu + \nu_0)\) and Stokes \((\nu - \nu_0)\) scattering. In Figure 2-2 the relationships between the different vibrational processes are clearly indicated. Because Raman spectroscopy measures a difference between the incident and scattered light, the positions of the vibrational bands are not dependent on the wavelength of laser excitation.

![Figure 2-2: Schematic showing the relationships between the different vibrational processes.](image-url)
The Stokes scattering intensities are more intense than those of the anti-Stokes scattering. The explanation for this can be found in the populations of the different vibrational energy levels. From the Maxwell-Boltzmann distribution law, the relative populations of two energy levels in a harmonic oscillator with associated energies $\varepsilon_A$ and $\varepsilon_B$ ($\varepsilon_A < \varepsilon_B$), having degeneracies $g_A$ and $g_B$ is

$$\frac{n_B}{n_A} = \frac{g_B}{g_A} e^{-\Delta \varepsilon / kT} \tag{2.10}$$

where $n_A$ and $n_B$ are the molecule populations in each state, $k$ is Boltzmann’s constant and $T$ is the temperature in Kelvin. Because vibrational levels are nondegenerate $g_A = g_B = 1$, and between any two consecutive vibrational levels $\Delta \varepsilon = h\nu_0$, giving

$$\frac{n_{\nu+1}}{n_{\nu}} = e^{-h\nu_0 / kT} . \tag{2.11}$$

Stokes scattering originates from the $\nu = 0$ vibrational level whereas anti-Stokes scattering originates from the $\nu = 1$ vibrational level. Evaluation of real experimental data with Eq. 2.11 will show that the vibrational levels are rarely populated above the $\nu = 0$ level at room temperature, thus explaining the difference in intensities observed. Spectroscopists therefore usually obtain the Stokes spectrum.

### 2.1.2.1 Overtone and Combination Bands

The quantum mechanical treatment of the harmonic oscillator has lead to the conclusion that vibrational energy levels are quantised and that the selection rule allows for transitions of only the kind $\Delta \nu = \pm 1$. The transition from $\nu = 0$ to $\nu = 1$ is called the fundamental transition. Overtones are defined as vibrational bands that are integer multiples of the fundamental transition, and combination bands are either the difference or sum of two fundamental bands.
Both are forbidden by the selection rule, but because of the anharmonicity of true molecules that deviate from the harmonic oscillator model, they are sometimes observed. For a harmonic oscillator the potential curve is given by

\[ V = \frac{1}{2} k x^2 \]  

(2.12)

where \( k \) is the force constant. For systems that deviate from the harmonic model, a better approximation can be made using

\[ V = \frac{1}{2} k x^2 - gx^3 \quad (k \gg g) \]  

(2.13)

with corresponding eigenvalues

\[ E_v = h c \omega_v (v + \frac{1}{2}) - h c x_v \omega_v (v + \frac{1}{2})^2 + \ldots \]  

(2.14)

We can approximate the anharmonicity corrected wavenumber \( \omega_v \) and the anharmonicity magnitude \( \omega_v x_v \) by rearranging Eq. 2.14 to give

\[ (E_v - E_0) / hc = v \omega_v - x_v \omega_v (v^2 + v) + \ldots \]  

(2.15)

with

- Fundamental : \( v_1 = \omega_v - 2 x_v \omega_v \)
- 1\(^{st}\) overtone : \( v_2 = 2 \omega_v - 6 x_v \omega_v \)
- 2\(^{nd}\) overtone : \( v_3 = 3 \omega_v - 12 x_v \omega_v \)

Knowing \( \omega_v \) we can more accurately approximate the force constant \( k \) using

\[ k = 4\pi^2 c^2 \omega_v^2 \mu \]  

(2.16)

where \( c \) is the speed of light, and \( \mu \) is the reduced mass of the two atoms involved in the bond.
2.1.2.2 Resonance Raman (RR) Spectroscopy

Figure 2-3: The processes of Normal and Resonance Raman as well as fluorescence which can occur during irradiation by laser light.

When the energy of the laser wavelength used for excitation, is such that it is coincides with the energy of an electronic transition of the molecule, the RR effect can occur. In other words, the virtual state energies of the Raman effect approaches that of the electronic excited states. When this occurs, the polarizability of the electron cloud of the molecule increases, creating a higher probability for the Raman transition to occur. Returning to the sum-over-states approximation, the resonant electronic state terms are seen to dominate the sum-over-states expression for the virtual states, making the
nonresonant terms negligible\(^2\). This results in an increase of the intensity of certain Raman bands by as much as \(10^6\) times\(^{2,3}\). At the same time the less desired effect of fluorescence can also occur. Figure 2-3 illustrates the difference between normal and resonance Raman scattering, and fluorescence. If combination and overtone bands that originate from the resonance enhanced fundamentals are observed, their intensities are also enhanced by the resonance effect.

### 2.2 The Effective Conjugation Coordinate (ECC) Theory for Polyenes\(^5\)

The use of ECC theory has become the preferred method of modeling polyconjugated systems, as it has been successfully applied to chemically different conjugated systems such as polyacetylene\(^5,6,7\), dithienothiophene derivatives\(^8\), polyaromatic polymers\(^5,9\) and conjugated polymers consisting of heteroatomic units\(^5,9\). Polyacetylene has been the most studied of these systems, and the different chemical variations of the finite and infinite polyconjugated chain for both the doped and undoped forms correlate well with the theory\(^5,6,7\). For polyacetylene, the simplest system is neutral, undoped all-\textit{trans} polyacetylene.

ECC theory is a generalized reformulation of Amplitude Mode (AM) Theory\(^9\). Originally AM Theory gave a good simplified model of polyacetylene, but could not be applied to doped polyacetylene systems\(^9\). ECC theory defines coordinates that describe the electron-phonon coupling that occurs between the conjugated \(\pi\)-electrons and the molecular geometry\(^7,10\). We can define the symmetry coordinates for polyacetylene using the internal coordinates as defined in Fig 2-4 relative to the point group \(C_{2h}\)\(^5\).
For the $a_g$ mode the most used coordinate is the ‘effective conjugation coordinate’ $\mathcal{Y}$. For a finite chain with $N$ C=C bonds and $M$ C-C bonds it is defined as:

$$\mathcal{Y} = \left( N_{\text{C=C}} + M_{\text{C-C}} \right)^{-1} \left( \sum_{n=1}^{N_{\text{C=C}}} R^{(n)}_{\text{C=C}} - \sum_{n=1}^{M_{\text{C-C}}} r^{(n)}_{\text{C-C}} \right)$$

(2.17)

Where $R^{(n)}_{\text{C=C}}$ and $r^{(n)}_{\text{C-C}}$ are the lengths of the $n$th carbon double and single bonds respectively. Extending to the infinite chain $\mathcal{Y}$ becomes

$$\mathcal{Y} = \sqrt{2} \left( R_{\text{C=C}} - r_{\text{C-C}} \right).$$

(2.16)

A full description for the symmetry coordinates of the four $a_g$ modes of trans-polyacetylene can be obtained upon defining the following three coordinates together with $\mathcal{Y}$:

$$R^+ = \sqrt{2} \left( R_{\text{C=C}} + r_{\text{C-C}} \right)$$

(2.17)

$$d^+ = \sqrt{2} (d_1 + d_2)$$

(2.18)
\[ W^+ = \frac{1}{\sqrt{2}} (\beta_1 + \beta_2 - \delta_1 - \delta_2) \]  

(2.19)

d, \beta \text{ and } \delta \text{ are defined in Fig 2-4. Both } \mathcal{R} \text{ and } \mathcal{R}^+ \text{ contribute to the C=C, C-C and HC=CH vibrational modes in different degrees}^{5}, \text{ but modes that are dominant in the Raman spectrum are those skeletal modes that have the largest electron-phonon coupling}^{11}, \text{ and in the case of polyacetylene these are the C=C and C-C stretching modes}^{5,11}. \text{ If any change were to occur in the frequencies of these normal modes involved in the carbon/carbon stretching modes, i.e. a change in the conjugation length, this would indicate that a change has occurred in the force constants associated with their vibrations namely } F_{\mathcal{R}} \text{ and } F_{\mathcal{R}^+}. \text{ The greatest variation seems to be in } F_{\mathcal{R}}. \text{ The reason can be found in the expressions of } F_{\mathcal{R}} \text{ and } F_{\mathcal{R}^+}, \text{ made up from entries in the reduced force constant matrix for the } a_g \text{ coordinates}^{5}:

\[ F_{\mathcal{R}} = \frac{1}{2} (k_k + k_r) - \sum_n \left( -f^n_R - f^n_r + 2f^n_{RR} \right) \]  

(2.20)  

\[ F_{\mathcal{R}^+} = \frac{1}{2} (k_k + k_r) + \sum_n \left( + f^n_R + f^n_r + 2f^n_{RR} \right) \]  

(2.21)

Application of ‘the rule of alternance’ will show that the sum in } F_{\mathcal{R}} \text{ is always positive, and the sum in } F_{\mathcal{R}^+} \text{ remains relatively constant. This rule states that the matrix entries for all } f_k \text{ and } f_r \text{ are always negative, and for all } f_{RR} \text{ they are positive}^{5}. \text{ The crux of the whole matter is that spectroscopically the } \mathcal{R} \text{ coordinate, representing the antisymmetric carbon/carbon stretching mode, is the best descriptor of the state of conjugation, as the dependency of the wavelength is well described by } F_{\mathcal{R}}. \text{ Thus, after the spectrum has been obtained, the wavenumber can be related to the force constant } F_{\mathcal{R}} \text{ from a masterplot of } \nu/F_{\mathcal{R}}. \text{ This masterplot can be obtained by the diagonalisation of the reduced force constant matrix defined by } F^{a_g} (0)^5. \text{ The application of ECC theory has been successfully applied to naturally occurring carotenoid systems}^{11}, \text{ showing the generality of the theory. For an in-depth review of ECC theory see the chapter of Gussoni et al. in Ref [5].}
2.3 References


Chapter 3

Article submitted to the
Journal of Raman Spectroscopy

Raman Investigation of
Pigmentary Molecules in the
Molluscan Biogenic Matrix

The pigments of some molluscan shells was identified as carotenoid derivatives. A new relationship was used together with previously reported ones to evaluate the length of conjugation present in the molluscan pigments.

W. Barnard, D. de Waal

Raman investigation of pigmentary molecules in the molluscan biogenic matrix
Raman investigation of pigmentary molecules in the molluscan biogenic matrix

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Department of Chemistry, University of Pretoria, Pretoria 0002, South Africa

3.1 Abstract

Raman spectra of the pigments of several marine mollusc shells have been obtained using the 514.5 nm laser line of an Ar⁺ laser. Shell species were chosen to obtain a variety of colour. The spectra obtained for the molluscan pigments indicated that they are polyacetylenic in nature, and from the spectral features it was deduced that the pigments were carotenoids, with unmethylated polyacetylenic backbones of various conjugation lengths. A logarithmic relationship was established between the number of conjugated double bonds (N) and the C=C vibration (ν₁) of previous reported data for finite polyacetylenic systems, and this relationship was used to evaluate the data obtained in this study. The aragonite and calcite phases of CaCO₃ were also identified to be present in different species of molluscs.

Keywords: A. Mollusc, B. Raman spectroscopy, C. Polyene, D. Carotenoid, E. Marine Pigment

3.2 Introduction

Carotenoids are the dominant source of colour in nature¹,² and have been identified in many different body parts of marine invertebrates¹,³,⁴. The
phylum Mollusca make up the second largest phyla in the animal kingdom, and the shells of the species found under this phylum vary greatly in size, ornamentation and colour. Different colouration scenarios can also occur: One could have a single specie showing a great variation in colour (eg. *Chlamys australis*), or a single shell containing distinct, separate coloured areas (eg. *Conus virgo* & *Drupa morum*). Molluscs are not able to synthesise carotenoids themselves, as these are synthesised afresh only in the plant kingdom\(^1\). The exact chemical nature of the marine carotenoids of molluscan origin is not known, but they are believed to be carotenoid derivates\(^5,6,7\). Extraction and limited characterisation has been done on other marine invertebrates such as sponges\(^8\) and the ‘By the wind sailor’ (*Velella velella*)\(^9,10\). Carotenoids can chemically be described as conjugated polyacetylenic molecules of finite length, having varied substitution on the terminal ends of the polyene chain, as well as the characteristic four methyl groups substituted on the polyenic backbone.

Raman spectral features have been used extensively for probing the structural nature of carotenoids\(^5-7,11-13\) and of the carotenoids’ industrial counterparts, the polyacetylene derivatives\(^14-19\). Also, the 514.5 nm laser wavelength falls within or close to the energy required for the electronic transition of most carotenoids or polyacetylenic systems, resulting in the Resonance Raman effect that occurs for carotenoids. This feature makes Raman spectroscopy an ideal tool for *in situ* investigation of these pigments in the molluscan matrix.

### 3.3 Experimental

#### 3.3.1 Molluscan Shells

The molluscan shells in Figure 3-1 were obtained from private collections (*Chlamys australis* – yellow & purple forms, *Conus chaldeus*, *Conus virgo*, *Coralliophila neritiodea*), collected from the South African coastline (*Conus*...
tinianus – pink & brown forms), or bought from shell dealers (Chlamys australis – orange form, Drupa morum, Pecten raveneli). Two other molluscan species (Donax serra, Janthina janthina), shown in Figure 3-2, were chosen for extraction of the organic matrix, by means of decalcification, for further investigation. These two species were freshly collected from the Southern Cape coast of South Africa.

Figure 3-1: Shells of the molluscan species investigated; from the top, left to right: Chlamys australis (purple, yellow and orange forms), Conus tinianus (pink), Corallophila neritoidea (purple), Drupa morum (purple), Conus tinianus (brown), Conus chaldeus (brown), Pecten raveneli (maroon), Conus virgo (purple).

Figure 3-2: Shells of the two purple coloured molluscan species that were selected for decalcification. Donax serra (left) and Janthina janthina.
3.3.2 Raman Spectroscopy

The micro-Raman spectra were obtained using a Dilor XY multichannel Raman spectrometer equipped with a liquid nitrogen-cooled CCD detector, and the laser beam focussed on the sample using a confocal Olympus microscope with a 50X objective. All the samples were excited using the 514.5 nm line of a Coherent Innova 300 Ar⁺ laser, with a laser output at the source of 100 mW. Approximately 10% of the laser output reached the sample under the microscope. The spectral resolution was 3 cm⁻¹.

3.3.3 Decalcification

A modified procedure from a previously reported method was used⁵,⁶,⁷. In this study the shells were not treated with NaOH at any stage of the extraction. The molluscan shells were ground as finely as possible, and stirred overnight in a saturated solution of Na₂EDTA in distilled water. The suspended brown leaflets that were present after decalcification were filtered off and washed with distilled water until no more of the EDTA containing solution was present. The filtrate was then washed with methanol and dried overnight in a desiccator.

The KBr disk was prepared using 100 mg of spectroscopic grade KBr and approximately 2 mg of the organic extract of the *D. serra* specimen. The two components were ground together, and the pellet pressed at 10KPa for 20 minutes.

3.4 Results and Discussion

Figures 3-3, 3-4 and 3-5 show the recorded Raman spectra in the region 600 – 1700 cm⁻¹ of the pigmentary molecules of all the molluscan species investigated. All the obtained values for the Raman bands of all species studied are summarised in Table 3-1. Figure 3-6 is the recorded Raman spectrum for the pigment found in *J. janthina* for the region 500 – 4000 cm⁻¹,
clearly showing the many combination and overtone bands observed at higher wavenumbers. Similar combination and overtone bands were observed for the other samples studied, and are also included in Table 3-1. Figure 3-7 shows the spectrum obtained for the region 600 – 1700 cm\(^{-1}\) of the extracted organic part of the two species *J. janthina* and *D. serra*. These values are also summarised in Table 3-1.

![Image of Raman spectra](image)

**Figure 3-3:** Raman spectra of the carotenoid pigments as recorded on the shell for *J. janthina* (top) and *D. serra* (bottom), showing the four fundamental vibrational modes \(\nu_1 \text{–} \nu_4\) of the pigments as described in the text. The CaCO\(_3\) phase for the two species is aragonite (bands at 702 and 706 cm\(^{-1}\)). The Raman band at 1086 cm\(^{-1}\) is the symmetric carbonate stretching mode. The two enlargements are of the two low intensity fundamental bands \(\nu_3\) (1298 cm\(^{-1}\)) and \(\nu_4\) (~1017 cm\(^{-1}\)).
Figure 3-4: Raman spectra of the shell species that were found to consist of the aragonite phase of CaCO$_3$ (bands at 702 and 706 cm$^{-1}$). From top to bottom the species are: *C. chaldeus* (brown), *C. virgo* (purple siphonal canal), *C. tinianus* (brown), *C. tinianus* (pink), *C. neritoidea* (purple), *D. morum* (purple mouth). The band at 1086 cm$^{-1}$ is typical for the carbonate ion, while the four bands ($v_1$, $v_2$, $v_3$, $v_4$) can be attributed to the carotenoidal pigment in the molluscan shells as assigned in Table 3-1.

Figure 3-5: Raman spectra of the shell species that were found to consist of the calcite phase of CaCO$_3$ (band at 714 cm$^{-1}$). From top to bottom the species are: *P. raveneli* (maroon), *C. australis* (purple, orange & yellow). The characteristic carbonate band at 1086 cm$^{-1}$ is observed again, as well as the four fundamental vibrations of the molluscan shell pigment listed in Table 3-1.
Figure 3-6: The Raman spectrum of the pigment in the carbonate shell matrix of *J. janthina* for the region 500 – 4000 cm\(^{-1}\), showing many combination and overtone bands. The highest intensity bands are indicated in the spectrum, but all bands are reported and assigned in Table 3-1. Similar overtone and combination bands were observed for all the pigments in the molluscan shells and are listed in Table 3-1.

Figure 3-7: The spectrum of the extracted organic part of the shell matrix of *J. janthina* (top) and *D. serra* (bottom). A shift of the bands occur relative to the spectra of the pigments (Figure 3-3) obtained in the carbonate shell matrix (See Table 3-1).
### Table 3-1: Raman wavenumbers (cm⁻¹) of the fundamental, combination and overtone bands of the pigmentary molecules in the various molluscan species compared to that of selected synthetic carotenoids.

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<th>v₂</th>
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Values and assignments obtained from:

a Ref. [20]; b Ref. [21]; c Ref. [22]
3.4.1 The Molluscan Shell

The inorganic component of molluscan shells primarily consists of CaCO$_3^{23}$. CaCO$_3$ can exist as three polymorphs: calcite, aragonite and vaterite. It is possible to distinguish between the three phases by Raman spectroscopy, as each of them has unique, non-overlapping peaks. Vaterite has bands at 738 cm$^{-1}$ and 750 cm$^{-1}$, aragonite a doublet at 700 cm$^{-1}$ and 705 cm$^{-1}$, and calcite a band at 711 cm$^{-1}$. The most intense band for all three phases occurs at ~1086 cm$^{-1}$, and is assigned to the symmetric CO$_3^{2-}$ stretching mode$^{24,25}$. Only aragonite and calcite were observed in the molluscan species studied. The Raman spectra of the aragonite shells ($J. janthina$, $D. serra$, $C. chaldeus$, $C. virgo$, $C. tinianus$, $C. neritoidea$ and $D. morum$) are shown in Figures 3-3 and 3-4, while Figure 3-5 contains the Raman spectra of the calcite species ($P. raveneli$, $C. australis$).

3.4.2 Polyacetylene

Four main bands are observed in the Raman spectrum of unsubstituted, all trans-polyacetylenic molecules$^{26,27,28}$. The two bands of highest intensity are the C=C ($\nu_1$) and C-C ($\nu_2$) stretching modes, which occur between 1450 – 1680 cm$^{-1}$ and 1070 – 1210 cm$^{-1}$ $^{21,26,29}$, respectively. These two bands owe their high intensities to resonance enhancement with the 514.5 nm laser line. The $\nu_3$ band is usually observed between 1285 – 1315 cm$^{-1}$ $^{26,29}$, and is assigned to the CH=CH in-plane rocking mode of the olefinic hydrogens. The $\nu_4$ band can be found between 1000 – 1015 cm$^{-1}$ $^{21,27}$, and is assigned to the CH out-of-plane wagging mode$^{14,16,27}$. The assignment of the $\nu_4$ band was substantiated by Raman studies done on trans-acetylene/acetylene-$d_2$ copolymers, and recently verified by a DFT study of finite, CH$_2$ terminated polyacetylenes by Fujimori and co-workers, which showed that the Raman inactive in-phase mode of the out-of-plane wagging mode becomes Raman active due to a distortion of the polyenic backbone$^{27}$. 
3.4.3 Carotenoids

Classical Carotenoids have methyl groups substituted on the polyacetylenic backbone. The Raman spectra of carotenoids contain the four vibrational bands previously mentioned, as well as many other small bands specific to the carotenoid. The regions in which the $v_1$, $v_2$ and $v_3$ bands for carotenoids have been reported are $1480 - 1580 \text{ cm}^{-1}$, $1100 - 1200 \text{ cm}^{-1}$ and $1260 - 1290 \text{ cm}^{-1}$ respectively\textsuperscript{11-13,21,25}. The $v_4$ band has been reported to be present between $990 - 1015 \text{ cm}^{-1}$\textsuperscript{13,21,25}, but is assigned to the rocking mode of the backbone CH$_3$ groups\textsuperscript{13,21}. The spectrum of $\beta$-carotene shows two medium-to-low intensity bands at $1008 \text{ cm}^{-1}$ and $1018 \text{ cm}^{-1}$. These have been assigned to the out-of-phase and in-phase combinations of the backbone CH$_3$ rocking modes, respectively, by means of a normal-coordinate analysis of $\beta$-carotene\textsuperscript{30}. A comparison of this assignment with the spectrum of the unmethylated analogue of $\beta$-carotene, tetradesmethyl-$\beta$-carotene, found that the absence of the band at $1008 \text{ cm}^{-1}$ conclusively supports the assignment\textsuperscript{20}. No mention was made of the band at $1018 \text{ cm}^{-1}$, and on inspection of the reported spectrum of tetradesmethyl-$\beta$-carotene\textsuperscript{20}, a weak band is observed at $\sim 1000 \text{ cm}^{-1}$.

3.4.4 The Molluscan Pigments

The two vibrational bands of highest intensity that were recorded between $1501 - 1526 \text{ cm}^{-1}$ and $1120 - 1135 \text{ cm}^{-1}$ are assigned to the C=C stretching mode ($v_1$) and C-C stretching mode ($v_2$), respectively\textsuperscript{6,7,21}. The band between $1295 - 1300 \text{ cm}^{-1}$ is assigned to the CH=CH in-plane rocking mode ($v_3$), whereas the $v_4$ band between $1015 - 1021 \text{ cm}^{-1}$ is assigned to the deformation-activated CH=CH wagging mode\textsuperscript{14,27} rather than the methyl rocking mode\textsuperscript{6,7}. This assignment is based on the average value of $1017 \text{ cm}^{-1}$ observed for the molluscan carotenoids, and corresponds well with the DFT calculated value for CH$_2$ terminated linear polyacetylenes of $1018 \text{ cm}^{-1}$\textsuperscript{27} and the structural deductions which follow.
3.4.5 Analysis of the Four Fundamental Modes

Several structure-related factors can influence the band position of the four fundamentals of the carotenoid pigments in various degrees. The highest intensity bands are mainly associated with the polyene backbone, and the factors determining these band positions are thus also dependent on the nature of the backbone. The most important factors that influence the fundamental mode band positions, mentioned in the literature are: the chemical factors of substitution and the chemical matrix/environment in which the carotenoid is found; conformation, isomerism and electronic factors such as length of conjugation of the polyenic backbone, which affect the extent of the \( \pi \)-delocalisation.

3.4.6 Chemical Factors

3.4.6.1 Substitution

Carotenoids have two main types of substitution, namely terminal and polyene backbone substitution. The type of substitution on the terminal ends of a finite polyene chain seems to have little or no effect on the position of the four fundamental vibrations on comparison of carotenoids that have the same polyene backbone length\(^{13,21}\), and seems to become even less significant as the polyenic chain increases in length\(^{15}\). Terminal substitution and chain conformation are said to give rise to low intensity bands in the 1200 – 1400 cm\(^{-1}\) region, but these could be overshadowed by the resonance enhancement of the \( v_1 \) and \( v_2 \) bands\(^{13}\). Both the \( v_1 \) and \( v_2 \) bands are sensitive towards substitution of the polyenic backbone. As the \( \pi \)-conjugation decreases due to the deformation of the backbone as a result of substitution, the C=C \( v_1 \) band shifts to a higher wavenumber, and therefore the unsubstituted analogues will have \( v_1 \) at lower wavenumbers\(^{21}\). At the same time, if methyl groups are absent from the polyenic backbone, the C-C stretching mode \( v_2 \) wavenumber will be \( \sim 20 \text{ cm}^{-1} \) lower than that of the...
methylated analogue of the same chain length\textsuperscript{13}. The \( v_1 \) and \( v_2 \) values for dodecapreno-\( \beta \)-carotene (a methyl substituted, extended polyenic backbone carotenoid) are 1492 cm\(^{-1}\) and 1146 cm\(^{-1}\) respectively\textsuperscript{21}. This example shows that \( v_2 \) is a more reliable indicator for the presence of methyl groups, as \( v_2 \) is not as sensitive towards the extent of the \( \pi \)-conjugation as \( v_1 \). In the present work, the average wavenumber value of the \( v_2 \) band of the molluscan shell carotenoids is 1126 cm\(^{-1}\), which is \(~31\) cm\(^{-1}\) downshifted with respect to the average value of the \( v_2 \) band (\(~1156\) cm\(^{-1}\)) of the methylated carotenoids. This shift indicates that an unsubstituted, all-\textit{trans} polyacetylenic molecule is more than likely present in the molluscan shell\textsuperscript{6,7,21}.

The position of the \( v_3 \) band has also been used as an indicator for polyenic backbone substitution\textsuperscript{13}. In the Raman spectrum of conjugated polyenes, the position of the \( v_3 \) band occurs at a lower wavenumber for the unsubstituted chain than for the methyl-substituted chain of the same length\textsuperscript{13}. There seems to be no specific wavenumber value that can help to distinguish between methyl substituted or unsubstituted chains of variable lengths. For short-chain polyacetylenes (\( N = \sim 4 \)) it seems that \(~1300\) cm\(^{-1}\) could be used as a reference value\textsuperscript{29,31,32}. The \( v_3 \) band of carotenoids is found at approximately 1273 cm\(^{-1}\)\textsuperscript{21}. Corresponding unmethylated analogues should then, according to reference [13], have their \( v_3 \) bands at even lower wavenumbers. Specifically, \( \beta \)-carotene’s \( v_3 \) band is at 1273 cm\(^{-1}\)\textsuperscript{21}. On closer inspection of the spectrum of tetradesmethyl-\( \beta \)-carotene, a possible low intensity \( v_3 \) band is observed at 1280 – 1300 cm\(^{-1}\). This observation contradicts the above statement. Interestingly, it must be said that the possible \( v_3 \) band at 1280 – 1300 cm\(^{-1}\) for tetradesmethyl-\( \beta \)-carotene compares well with the \( v_3 \) wavenumber values of 1294 cm\(^{-1}\) for infinite (C=C > 100) unsubstituted polyacetylene\textsuperscript{16}, or \(~1292\) cm\(^{-1}\) for finite polyacetylene systems\textsuperscript{15}. Thus, the above statement from reference [13] seems to hold only for short polyene systems and not for those that have carotenoidal nature and lengths (C=C > \(~8\)). The average value of \( v_3 \) for the molluscan carotenoids in this work is 1298 cm\(^{-1}\), which compares well with that reported for the
aforementioned infinite and finite unsubstituted polyacetylene systems, substantiating the earlier deduction that the molluscan carotenoid backbones are unsubstituted.

### 3.4.6.2 The Chemical Matrix

Carotenoids are not usually found in the free form in nature but rather as complexed with some form of protein\(^4,9-12\). These complexes, or carotenoproteins, commonly differ greatly in colour relative to that of the uncomplexed carotenoid. A prime example is the carotenoprotein \(\alpha\)-crustacyanin that contains the carotenoid astaxanthin. It has a bathochromic shift in its absorption spectrum of \(~150\) nm from the free to complexed carotenoid\(^9,33\). The crystal structure of \(\alpha\)-crustacyanin has been reported\(^9\), showing the strained nature of the backbone and the terminal rings of the astaxanthin molecule relative to the uncomplexed structure. This strain is also observed in the Raman spectrum of \(\alpha\)-crustacyanin\(^11\). Upon complexation of the carotenoid with a protein, the most important change in the spectrum is the appearance of many more vibrational bands\(^11-13,33\), and the downshift of the \(v_1\) band, which is rationalised by the increase in \(\pi\)-delocalisation\(^11\). None of these extra bands were observed in the obtained spectra of the molluscan carotenoids, thus making the possibility of interaction between the carotenoid and the conchoilin protein of the shell highly unlikely. A CaCO\(_3\)-carotenoid complex has been proposed to exist in the calcareous skeletons of corals\(^34,35\), and some shells\(^7\). This would also be a more plausible explanation for the currently reported molluscs.

Upon extraction of the organic component from the shells of \(J.\) \(janthina\) and \(D.\) \(serra\), the colour changed from purple to golden brown. On comparison of Figure 3-3 with Figure 3-7 slight shifts were observed in the Raman spectra for all four of the fundamental modes. The \(v_1\) and \(v_2\) bands shifted to a higher wavenumber, and the \(v_3\) and \(v_4\) showed a downward shift. These changes in the band positions could possibly be explained by the orientation of the
carotenoids in the biogenic CaCO$_3$ matrix, as the structure of the aragonite biogenic matrix is mostly crossed lamellar$^{36}$ and the partial orientation of a carotenoid has been observed in the feather of the bird species *Cyanoramphus novalandiae*.$^{21}$ It is therefore likely that the carotenoid might orientate itself in a lamellar packed fashion as well in the CaCO$_3$ matrix. Also, the position of the $v_1$ band for carotenoids has been observed to differ in solution from the solid form.$^{21}$ The result when the organic extract of *D. serra* used in this study was compressed in a KBr disk was that the $v_1$ band position shifted 4 cm$^{-1}$ towards a lower wavenumber value (towards the $v_1$ band position obtained for the pigment on the shell) relative to the position of the $v_1$ band of the extract – a positive indication for preferential orientation of the carotenoid in the biogenic CaCO$_3$ matrix.

### 3.4.7 Electronic Factors

The extent of the $\pi$-delocalisation, i.e. the effective length of conjugation, mainly depends on a few factors. The $v_1$ wavenumber is affected most by the extent of the $\pi$-delocalisation$^{6,7,21,26}$, whereas no appreciable change is observed for the $v_3$ and $v_4$ vibrational bands$^{11,21,30}$.

#### 3.4.7.1 Conjugation Length

Schaffer et al. showed that on going from a 3-ene to 12-ene tert-butyl capped all-trans conjugated system, the $v_1$ band downshifted by $\sim$133 cm$^{-1}$ while the $v_2$ and $v_3$ bands only varied by approximately 15 cm$^{-1}$ and 3 cm$^{-1}$ each.$^{15}$ Similar observations have been made on shorter polyene chains$^{29,31,37}$, and confirmed with DFT calculations on all-trans polyene chains of the kind $\text{H(\(CH=CH\))}_n\text{H}$ with $n = 1 - 12$.$^{38}$
3.4.7.2 Isomerism

The bulk of carotenoids have an all-trans configuration of the polyenic backbone, but some cis-carotenoids have been reported\(^\text{39}\). A normal-coordinate analysis has also been done on 15-cis-\(\beta\)-carotene\(^\text{30}\), and on comparison with the all-trans isomer, the \(v_1\) band position for the cis isomer is at a higher value. This indicates the loss of some \(\pi\)-delocalisation of the polyenic backbone i.e. the length of the trans-conjugation segment in the molecule will be shorter, resulting in the \(v_1\) band position to shift to a higher wavenumber\(^\text{13,30}\).

3.4.7.3 Conformation

If the conformation of the polyenic backbone was to deviate from planarity some of the \(\pi\)-delocalisation is lost, resulting in the \(v_1\) band to shift to a higher wavenumber. On comparison of the \(v_1\) band position of some carotenoids in the solid state and in solution\(^\text{21}\), one sees that the solid-state value is lower than that in solution, and is again indicative of some loss of the \(\pi\)-delocalisation in solution\(^\text{21}\). For \(\beta\)-carotene this shift is \(\sim 9\ \text{cm}^{-1}\)\(^\text{30}\).

Taking all three these factors into account (conjugation length, isomerism and chain conformation), it then appears that the band position of \(v_1\) is a good indicator of the ‘effective conjugation length’, rather than a rigid predictor of the number of conjugated double bonds\(^\text{21,25,26}\).

3.4.8 Conjugation Length of the Molluscan Carotenoids

Because of the dependence of the \(v_1\) on the ‘effective conjugation length’ (maximum number of conjugated double bonds) and the ground electronic state\(^\text{7,40}\), empirical correlations for the possible number of conjugated double bonds \((N)\), the C=C vibrational mode \((v_1)\), the C-C vibrational mode \((v_2)\) and the absorption wavelength in solution have been obtained\(^\text{6,7,14,40}\). These
correlations have been used to make predictions about carotenoids of which the spectra were obtained in situ\textsuperscript{25} or extracted\textsuperscript{6,7}. The first of these relationships was published for polyacetylene, and relates the value of the $\nu_1$ band with the number of conjugated double bonds, $N$\textsuperscript{14}.

$$\nu_1 = 1459 + \frac{720}{N + 1} \text{ (cm}^{-1}\text{)} \quad (3.1)$$

Evaluation of reported experimental Raman data of carotenoids with CH$_3$ groups present on their polyene backbones using Equation 3.1, shows that $N$ will be overestimated\textsuperscript{6,7}. Similar mathematical relationships, for which the equations are given here, were obtained for the series of tert-butyl capped polyacetylenes for both the $\nu_1$ and $\nu_2$ bands\textsuperscript{15}:

$$\nu_1 = 1438 + \frac{830}{N} \text{ (cm}^{-1}\text{)} \quad (3.2)$$

and

$$\nu_2 = 1082 + \frac{476}{N} \text{ (cm}^{-1}\text{)} \quad (3.3)$$

Equations 3.2 and 3.3 were obtained using only the data reported for tert-butyl capped polyacetylenes having $7 \leq N \leq 12$ and $8 \leq N \leq 12$ respectively\textsuperscript{15}. As previously stated, the value of the $\nu_1$ vibration would be an indication of the ‘effective conjugation length’ rather than of a fixed number of double bonds. This concept of ‘effective conjugation length’ is best described by the ECC (effective conjugation coordinate) theory as reviewed by Zerbi et al\textsuperscript{15}. ECC theory states that the $\gamma$ mode (which is a combination of $a_g$ symmetry coordinates) is the most enhanced of the totally symmetric modes, and the wavenumber of the $a_g$ modes is dependent on the force constant $F_{\gamma}$\textsuperscript{25,26}. $\gamma$ (the effective conjugation coordinate) is pivotal in expressing the state of conjugation\textsuperscript{26,41}. Thus, a much better description of the conjugation state can
be obtained by comparing the force constant $F_\text{ag}$ and the wavenumber of the $a_g$ modes (eg. $\nu_1$ or $\nu_2$)\textsuperscript{21,26}. To obtain such a master curve for comparison, a diagonalisation of the force constant matrix for the $a_g$ coordinates, $F^{ag}(0)$, needs to be done\textsuperscript{26}.

The values obtained from Equations 3.1 – 3.3 for $N$ for the molluscan carotenoids in this study are listed in Table 3-2. The plot of $\ln(1/N)$ vs $\nu_1$ for the data reported in reference [15], resulted in a linear relation with a least square regression analysis ($R^2$) value of 0.9987 for $3 \leq N \leq 12$. Figure 3-8 shows the graph and the linear relationship,

$$\nu_1 = 97.07\ln(1/N) + 1745 \quad (\text{cm}^{-1}) \quad (3.4).$$

![Graph of ln(1/N) vs the \nu_1 wavenumber](image)

**Figure 3-8:** A plot of $\ln(1/N)$ vs the $\nu_1$ wavenumber, where $N$ is the number of conjugated double bonds, for the series of tert-butyl capped polyacetylenes with $3 \leq N \leq 12$ obtained from the data of Reference [15]. This correlation should be more accurate, as it contains more data points as previously reported correlations obtained from the same set of data\textsuperscript{15}. This relationship is used in the text as Equation 3-4.
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Table 3-2: Comparison of the different mathematical relations previously reported (Eq’s. 3.1 – 3.3) with the presently reported Eq. 3.4 compared to the possible prediction of the number of double bonds (N) obtained from Fig. 3-9 for the pigments in the molluscan samples.

This relationship should be better than Equations 3.2 and 3.3 as it derives from a larger data set. The number of double bonds predicted by Equation 3.4 is listed in comparison with Equations 3.1 – 3.3 in Table 3-2. Figure 3-9 shows a plot similar to the one published by Veronelli et al. to compare the tert-butyl conjugated systems to the studied molluscan carotenoids. Comparing the values predicted by Equations 3.1 – 3.4 with the values estimated for the molluscan carotenoids in Figure 3-9, shows that Equation 3.1 overestimates the number of double bonds. Comparison of the values obtained for N from Equations 3.2, 3.3 and 3.4 with the molluscan values in Figure 3-9, it would seem that Equation 3.4 gives a more accurate simulation of the obtained data. Possible reasons could be that even though Equation 3.2 is based on the position of the $v_1$ (C=C) band, it was fitted only for a region that seems linear, which could account for the few incorrect values, whereas Equation 3.3 is based on the position of the $v_2$ (C-C) band position, which is not as sensitive towards substitution as the C=C stretching vibration (a total shift of ~15 cm$^{-1}$ going from the 3-ene to the 12-ene$^{15}$), thus accounting for the less accurate prediction.
Figure 3-9: Plot of the C=C wavenumber ($v_1$) versus C-C wavenumber ($v_2$) similar to that obtained by Veronelli et al., for bird feathers, showing the relation of the data obtained for the molluscan carotenoids (◊) to that of known conjugation length tert-butyl capped polyacetylenes$^{15}$ (●). The number next to each ● indicates the number of conjugated double bonds present in that specific tert-butyl capped polyacetylene.

3.5 Conclusions

The Raman spectra indicated that all the bivalves except for $D. \ serra$ were found to be calcite, and all the gastropods were aragonite. From the spectral features it was found that the molluscan pigments in the present study were unsubstituted polyacetylenes, possibly of the carotenoid class of compounds, and are probably not associated with the conchoiin protein in the shell. The pigments are probably found in a preferred orientation in the shell matrix, and upon evaluation of a new correlation, Equation 3.4, between the C=C wavenumber ($v_1$) and the number of conjugated double bonds, it was found that the pigments probably contain between 10 – 12 conjugated double bonds. No plausible explanation can be given for the great diversity in colour, even though the number of double bonds only differed by a maximum of two.

As a result of the structural deductions, the assignment of the band at $\sim$1015
An interesting observation that was made is the difference in the reported spectrum of *V. velella*\(^{10}\) and that obtained for *J. janthina*. *J. janthina* lives in the neuston (top 10 – 20 cm of the ocean’s surface), drifting intercontinentally on the ocean’s currents. Many other invertebrates of the genusses *Physalia*, *Glauxa*, *Porpita*, and specifically the species *V. velella*, live in symbiosis with the purple mollusc in the neuston. Most are either transparent or coloured blue or purple. *J. janthina* is a carnivorous mollusc feeding on these species, and it can therefore be postulated that it sources its own colour from these invertebrates. On comparison of the Raman spectrum of the pigment in *V. velella* with that obtained for *J. janthina* it was seen that they differ in band positions, and there are extra bands observed in the spectrum of *V. velella*. The spectrum of *V. velella* was reported as being that of an astaxanthin-protein complex\(^{10}\). This could therefore be a possible indication that the mollusc is chemically altering the carotenoid, for reasons unknown.

### 3.6 Acknowledgements

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### 3.7 References

Chapter 4

Conclusions

4.1 Structure of the Pigment

From the position of the $v_1-v_4$ vibrational bands in the obtained Raman spectra of the molluscan shells, it was deduced that the pigmentary molecules are of a polyacetylenic nature, most probably of the carotenoid class of compounds. Most important, is that the spectral data indicates that the polyacetylenic chain is unsubstituted, therefore lacking the four methyl groups that are usually associated with the backbone of a carotenoid (See Figure 1-1). This has only been reported in four previously published papers\(^1\)-\(^4\). No evidence could be found in the spectra to help in determining the chemical nature of the terminal groups of the polyacetylenes.

Association with the conchoilin protein seems highly unlikely, as there was no spectral evidence to support this. Also, comparison of the Raman spectra of the extracted organic part of *D. serra* and *J. janthina* with that of the pigment *in situ* in the shell, shows that some conformational changes of the backbone could have occurred, even though the colour changed dramatically from purple to golden brown. No dramatic spectral shifts occurred similar to those usually observed for a carotenoid relative to its protein-complexed form\(^5\).

For the extracted organic component it was found that the length of conjugation was shortened, but this is not sufficient enough to explain the dramatic colour change of the organic extract (golden brown) relative to the pigment in the molluscan matrix (purple). Possible reasons for this colour change could rather be interaction with the matrix or other pigmentary
molecules, but not interaction with the conchoilin protein as the Raman spectra did not indicate any such interaction.

4.2 Length of Conjugation of the Pigments

Using a new correlation (Equation 3-4) obtained from previously reported data, the number of conjugated double bonds in the molluscan pigments were predicted to range from ten to twelve double bonds. This correlation (Equation 3-4) has a $R^2 = 0.9987$, which makes it more reliable than the previously reported correlations. The ten to twelve double bond range predicted for the pigments investigated here, is a relatively small structural change for such a diversity of colour, and therefore conjugation cannot be the only contributor to the colour of the molluscan pigment. Most probably other factors could include: the nature of the terminal groups, interaction with the shell matrix and concentration of the pigment in the biogenic matrix. A more detailed discussion of the conclusions pertaining to the length of conjugation and the correlation can be found in Chapter 3 under Sections 3.4 and 3.5.

4.3 Conjugation Length and Colour

Empirically comparing the colour of the shell with the prediction of the conjugation length, it is seen that the observations fit well with what one would classically expect. The shells range from purple through yellow, and therefore the shells that are coloured purple are absorbing the longer wavelength colours (yellows, reds and oranges), thus indicating that they should have the longer length of conjugation, similarly this should hold for the yellow and orange coloured shells, and one expects them to have the shorter conjugation length to absorb the shorter light wavelengths. To easily see this correlation it is represented in Figure 4-1. This is a very simplistic view, but always reassuring to see that what one intuitively expects does occur.
Figure 4-1: Schematic showing the empirical relation of the shell colour relative to the C=C ($\nu_1$) predicted length of conjugation from Figure 3-9. Notice that the yellow shell (*C. australis*) seems not to fit with the trend, but that its number of double bonds are very similar to the two orange species.

### 4.4 The CaCO$_3$ of the Biogenic Matrix

Of the three phases of CaCO$_3$ only aragonite and calcite was identified. On classifying the species into their phases it was found that all the gastropods (*J. janthina*, *C. chaldeus*, *C. virgo*, *C. tinianus*, *C. neritoidea* and *D. morum*) were aragonite, and all the bivalves (*P. raveneli*, *C. australis*) were calcite, except for the bivalve *D. serra* which was aragonite. Aragonite is known to be thermodynamically less stable, and more soluble than calcite under pressure. The locales of the aragonite species are mostly cold-water current regions. The calcite species have their main locations in warm, tropical regions, lending itself as a possible factor for the choice of carbonate phase. If one of the contributing factors for the choice of phase is the temperature, it could also explain the exception of the bivalve *D. serra* which is a cold-water bivalve found mainly of the Southern coast of South Africa.
4.5 References