Conjugated polyenes as chemical probes of life signature: use of Raman spectroscopy to differentiate polyenic pigments

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Polyenes, which are represented by carotenoids, carotenoids and conjugated polyenals, are some of the most important targets for astrobiology, because they can provide strong evidence of the presence of organic compounds in the most extreme environments, such as on Mars. Raman spectroscopy has been used as the main analytical tool in the identification of such compounds, for the greatest variety of living species, from microorganisms to animals and plants. However, using only the position of the characteristic Raman bands can lead to errors in tentatively identifying chemicals. In this work, we present a series of observations that can provide a more complete and robust way to analyse the Raman spectrum of a polyenal, in which the position, the intensity, the use of various laser lines for excitation, and the combination of more than one pigment can be considered in the complete analysis.

1. Introduction

It is well known that our neighbouring planet Mars is a possible exobiological habitat, and many efforts have been performed to search for similar examples of life in extreme conditions (extremophiles) here on the Earth to be compared with the situation that can be seen on Mars.
As an example, there is a report published by the European Space Agency, entitled *ROME: Response of Organisms to the Martian Environment*, in which more than a dozen investigations tackled the subject [1–3]. One of the most elegant studies presented in that report was one that involved simulation experiments with complex microbial soil communities, in which the authors report their contributions to answer the main question: is there life on Mars? [4]. In another important paper, Edwards *et al.* [5] suggested the use of Raman spectroscopy as a tool for studying complex systems, because many of the main organic substances can be observed in the spectrum obtained, based on the analysis of the specific band markers of each of the organic constituents in the sample.

There are several target substances that it is very important to observe in natural samples; living organisms produce them to survive or at least to preserve certain characteristics that would be impossible without such molecules. Chlorophylls [6], conjugated polyenes [7], scytonemin [8], mycosporines [9] and melanins [10] are examples of biomolecules that occur in numerous organisms, from bacteria to animals and plants from the present far into history. These compounds act as pigments that are especially important in protecting organisms against photoinhibition and UV photodamage. The importance of identifying such molecules is crucial in understanding the strategies adopted by organisms from the early evolutionary process to the current time.

Conjugated polyenes are one type of such molecules that include tetraterpenes (carotenoids) [11], linear polyunsaturated aldehydes (polyenals) [12] and laetiporic acids [13] (figure 1).

**Figure 1.** Chemical structures of conjugated polyenes: (1) peridinin, (2) astaxanthin, (3) lutein, (4) zeaxanthin, (5) β-carotene, (6) lycopene, (7) polyenal and (8) laetiporic acid.
Typical carotenoids present a C$_{40}$ carbon skeleton containing from seven to 11 double bonds. Carotenoids function as accessory pigments, protecting against photo-oxidation and harmful oxygen species [7,14,15]. They are biomarker molecules that have been used as diagnostics of the past environment [16]; they are produced by bacteria, archaea, fungi, algae, lichens and plants but can be found in animals due to dietary intake or from association with microorganisms [7,17–19]. Non-carotenoid linear conjugated polyenes, such as polyenals (or unsubstituted polyacetylenes) and laetiporic acids, are not as diverse as carotenoids, and they are restricted to a few taxonomic groups. For example, polyenals containing from six to nine conjugated double bonds, named psittacofulvins, were identified from parrots [12]; polyenals containing from six to 14 double bonds were described for pearls [20,21], mollusc shells [22–24] and octocorals [25–30]; and laetiporic acids [13] containing 10–12 conjugated double bonds were found in fungi. Synthetic polyenals present antioxidant, antitumoural and anti-inflammatory activity [31], and laetiporic acids are potential food colorants [13].

The literature has described several different analytical techniques to identify qualitatively and quantitatively the presence of conjugated polyenes; however, such techniques are laborious and require time and reagent consumption. Raman spectroscopy has in recent years become a preferred technique to analyse the presence of conjugated polyenes in several different types of living organisms, because the existence of conjugated carbon–carbon double bonds gives the chemical system a very characteristic vibrational movement, based on the stretching of the single and the double bonds, which are very intense in the Raman spectrum; even in very low concentrations, both bands can be observed in the spectra [32]. The presence of such bands in a Raman spectrum can be used as a fingerprint to confirm the presence of conjugated polyenes in the analysed samples; however, determining the type of conjugated polyene, for instance a carotene or a polenal, is a more difficult task. Therefore, the objective of this work is to present several tricks and tips for using Raman spectroscopy as an analytical technique to analyse the presence of conjugated polyenes and to conclude what type of compound is present in the analysed sample. Thus, we present the Raman spectra of several different conjugated polyenes, from pure samples to living organisms such as corals and sponges, and we present a discussion to understand the subtle differences in each spectrum obtained with various laser excitations.

2. Experimental set-up

(a) Collection

Samples of _Phyllogorgia dilatata_ Esper, 1806 (Alcyonacea, Gorgoniidae) were collected by scuba divers at a depth of 6–9 m at Saco dos Cardeiros, Arraial do Cabo, RJ (23°44′ S–42°02′ W). _Leptogorgia punicea_ samples were collected in Enseada dos Ingleses at a depth of 12–20 m, Arraial do Cabo, RJ (23°30′ S–42°26′ W). The octocorals _Leptogorgia setacea_ collected at Praia do Forte, Cabo Frio, RJ (MNRJ 2779), _Renilla muelleri_ collected at Cabo Frio-RJ (MNRJ 3982) and _Muricea flamma_ (MNRJ 4738) in Bahia State (19°45′36″ S–39°31′36″ W) were donated by Dr Clovis B. Castro (UFRJ-MNRJ). Samples of _Hymeniacidon heliophila_ were collected in Niteroi, RJ (22°52′ S–43°0′ W).

(b) Raman measurements

Fourier transform Raman spectra were measured using a Bruker RFS 100 instrument and a Nd:YAG laser operating at 1064 nm, equipped with a Ge detector cooled with liquid nitrogen. The spectral resolution was 4 cm$^{-1}$, and good signal-to-noise ratios were obtained with 516 scans, using a range of laser powers at the sample of between 100 and 150 mW. A SENTERRA dispersive Raman microscope instrument using laser lines at 532, 632.8 and 785 nm was also used, equipped with a CCD detector; good spectra were obtained using a range of laser powers between 0.2 and 2.0 mW, and three or five accumulations for 3 s, with a spectral resolution of 3 cm$^{-1}$. 

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3. Results and discussion

Conjugated polyenes, such as polyenals and carotenoids, have been characterized by the analysis of vibrational bands attributed to $\nu(C=C)$, $\nu(C-C)$ and $\delta(C-CH_3)$ in polyenes or polyenals and carotenoids. As mentioned above, Raman spectroscopy has been suggested to be one of most suitable tools to investigate conjugated systems, and it is extensively used in the characterization of carotenoids and polyenals or unsubstituted polyacetylenes [33–36]. Polyenic molecules are strong Raman scatterers due to the strong electronic/vibrational coupling, and their vibrational modes can be selectively enhanced by resonance excitation near the $\pi-\pi^*$ electronic transition [26]. The most intense lines in the Raman spectra of polyenals and carotenoids are due to the modes coupled to the conjugated system, with a great contribution from the C=C bond stretching.

The wavenumber position of C=C stretching vibrations may be influenced by the length of the conjugated carbon–carbon chain, by the substitution pattern in the chain, and by the interaction with other chemical constituents present in the biological matrix [37–39].

The main differences between polyenals and carotenoids observed from Raman spectroscopic analysis are the red-shifted wavenumber position of the $\nu(C-C)$ stretching mode of polyenals by ca 30 cm$^{-1}$ when compared with that of carotenoids, as well as the absence in polyenals of the deformation mode related to the $\delta(C-CH_3)$ group at ca 1000 cm$^{-1}$ and the presence of $\delta(C-CH_3)$ at ca 1020 cm$^{-1}$ [33]. The differences and similarities of carotenoids and polyenals in Raman spectra are shown in figures 2–4, represented by the in situ analysis of biological and standard samples.

Raman spectroscopic analysis of polyenals or unsubstituted polyacetylenes has revealed that the position of the $\nu(C=C)$ and $\nu(C-C)$ bands are chain-size and matrix-effect dependent (figures 2 and 3) [27,29,40]. The wavenumber position of $\delta(C-CH_3)$ at ca 1020 cm$^{-1}$ presents different behaviour from that of methyl groups arranged as in terpene units; in carotenoids, there is a significant contribution of the $\delta(C-CH_3)$ to the $\nu(C-C)$ mode [41]. The shift of the
wavenumber positions of the ν(C−C) stretching mode by ca 30 cm$^{-1}$ may be an indication that polyenals do not contain the same methylation pattern as occurs in carotenoids; the ν(C−C) mode downshifts as the number of methyl groups decreases in the polyenic chain [40]. Subtle differences in the wavenumber position of δ(C−CH$_3$) account for the identification of totally or partially unmethylated polyenes [40]. Brambilla et al. [40] proposed that pigments from the octocoral Corallium rubrum are not completely demethylated, as are psittacofulvins. Analysis of the overtones and combination bands indicated that the polyconjugated chain may contain −CH$_3$ groups; however, no combination band involving C−CH$_3$ was observed in the psittacofulvin spectra. Summarizing, the position of the δ(C−CH$_3$) band matches neither the position observed in carotenoids nor in psittacofulvins.

Raman spectra of polyenals identified from certain octocorals, such as Leptogorgia setacea, M. flamma, R. muelleri, L. punicea (figure 2), Chromonephthea braziliensis and Phyllogorgia dilatata (not shown) [27–29], presented spectral features resembling those of Corallium spp. [21,25]. Previous works from Maia et al. [27,29] and comparison with the literature data [13] have demonstrated that the red octocoral L. punicea may contain 11 conjugated double bonds due to the presence of ν(C=C) modes at ca 1509 cm$^{-1}$. However, deconvolution of the in situ Raman spectra using the 1064 nm laser line for vibrational modes ranging from 1550 to 1480 cm$^{-1}$ and from 1040 to 980 cm$^{-1}$ showed two distinct bands. Based on these data along with Raman analyses previously

Figure 3. Fourier transform Raman spectra of carotenoids: (a) pumpkin rind, (b) zeaxanthin, (c) β-carotene, (d) peridinin and (e) lycopene. (Online version in colour.)
Figure 4. Raman spectra of the carotenoid astaxanthin with addition of calcium carbonate for various excitation lines: (a) 532, (b) 632.8 and (c) 785 nm. The marked band refers to the carbonate ion vibration, used as an internal standard for comparison.

reported for the yellow *L. setacea*, pink-orange *Chromonephthea braziliensis* [29] and coloured pearls [36], it can be suggested that the pigmentation of such organisms is composed of a mixture of polyenals with various numbers of conjugated double bonds. Moreover, in figure 2a,b, the matrix effect can also be seen, which promoted a shift of the Raman bands when *L. punicea* tissues were demineralized. In an important investigation, de Oliveira *et al.* have demonstrated the matrix effect by studying the inclusion of certain characteristic carotenoids, such as β-carotene and astaxanthin, in β-cyclodextrin, for which it is clearly possible to see even a shift of more than 10 cm$^{-1}$ for the $\nu$(C=C) mode for the carotenoids included in the matrix [39].

Despite the growing number of organisms that produce polyenals, there is no clear evidence of the biosynthetic route; in parrots, it has been hypothesized that they are endogenously synthesized [42], and in corals, these pigments are embedded in calcium carbonate sclerites produced by skeleton-forming cells [43]. This is not the case for carotenoids, which are widespread in nature and well studied in many different aspects. In addition to their vast biological activities [1,7,8], carotenoids have been considered a target compound for astrobiological investigations because they have been identified from Bacteria [44], Archaea [18] and Eukarya (Protista, Fungi, Plantae and Animalia) [45,46]. A massive chemical study of carotenoids has been reported from higher plants, followed by algae, lichens and microorganisms.

Raman spectroscopy has been unique in the detection and characterization of carotenoids in many different samples [19,47]. Examples from vegetables, corals and standard carotenoids are shown in figures 2 and 3 which also demonstrate the influence of the matrix effect and variations in the wavenumber position due to the lengths of the conjugated double bonds.

Figure 3 shows the *in situ* Raman analysis of pumpkins [38], known to produce carotenoids with nine conjugated double bonds, such as β-carotene, lutein and zeaxanthin [48], which present bands at 1526, 1157 and 1006 cm$^{-1}$. For comparison, the Raman spectrum from standard β-carotene (Sigma-Aldrich Co.) is provided, with characteristic bands at 1515, 1157 and 1008 cm$^{-1}$, and that from standard zeaxanthin (Sigma-Aldrich Co.) with bands at 1519, 1158 and 1006 cm$^{-1}$; the lutein data were obtained from the literature, with bands at 1522, 1157 and 1008 cm$^{-1}$ [37]. As an attempt to investigate the composition of the carotenoids in
pumpkin samples by Raman spectroscopy, a band decomposition analysis was performed. In the deconvoluted spectrum using the 1064 nm excitation line for pumpkin rind, two distinct bands at 1515 and 1525 cm$^{-1}$ (figure 3) were observed. The occurrence of $\beta$-carotene could be suggested by the presence of the band at 1515 cm$^{-1}$; however, the differences between the Raman signals of the pure standards lutein and zeaxanthin are very small to distinguish between both pigments in the natural sample. Other examples shown in figure 3 are carotenoids with 11 conjugated double bonds, such as lycopene, and the C37 skeletal nor-carotenoid peridinin, containing eight all-trans double bonds, an allene functional group and a lactonic carbonyl group, demonstrating that either the length of the conjugated double bond in the chain or the molecular substituent may influence the wavenumber position of the $\nu$(C=C) and $\nu$(C−C) vibrational modes.

The wavenumber shifts could also be a consequence of excitation at various laser lines, as exemplified by the system composed of astaxanthin and calcium carbonate in figure 4. The Raman spectra obtained with excitations at 532, 632.8 and 785 nm showed differences in the signal intensities and band positions. The spectra obtained with the 632.8 and 785 nm laser lines showed a signal enhancement of the $\nu$(C=C) and $\nu$(C−C) modes when compared with the spectrum obtained with the 532 nm laser line. In this way, under resonance or pre-resonance conditions ($\lambda_0 = 632.8$ nm), there is clearly a preferred signal enhancement for the bands at 1504 and 1150 cm$^{-1}$. This effect, known as the resonance Raman effect, can also be used for the study of several conjugated polyenes present in the most different samples, such as corals [25,49], the shells of molluscs [24], pearls [21,50], plants [41], etc. It is also clear that the presence of the band at 1008 cm$^{-1}$, related to the $\delta$(C−CH$_3$) mode, occurs as one of the enhanced modes in the Raman spectra of carotenoids, according to figure 4, in which it can be seen in the spectrum of pure astaxanthin. This characteristic has been shown in other investigations based on the resonance Raman profile of carotenoids, such as bixin, the main constituent of Bixa orellana, and used as a colorant in the food industry; the results indicate that the $\delta$(C−CH$_3$) mode is present in the chromophoric group and is part of the Raman modes that are enhanced under resonant conditions [41].

On the other hand, the Raman spectra of the conjugated polyenes known as polyenals do not present such enhancement for the band at 1020 cm$^{-1}$ [40] for the same vibrational mode. This can be explained by the presence of the methyl groups in the middle of the conjugated chain of the carotenoids and the complete absence of such groups at the same positions in the polyenal structure. As an important conclusion, this can be used as the main differentiation between the two families of compounds: under resonant conditions, the band at ca 1010 cm$^{-1}$ is enhanced for carotenoids, whereas the band at ca 1020 cm$^{-1}$, present in the polyenal spectra, is not.

Focusing on the use of the Raman technique in the identification of carotenoids relevant to astrobiology, we present a new target of study from the eukaryote domain. Sponges (phylum Porifera) are evolutionarily ancient metazoans, well represented in oceans and in freshwater; owing to their simplicity and adaptation ability, they are considered living fossils [51,52]. Carotenoids are well represented in the phylum Porifera [53], members of which are known to live in association with microorganisms such as cyanobacteria, bacteria, archaea and fungi [54,55]. It has been hypothesized that sponge microbial association is composed of a mixture of evolutionarily ancient bacteria and bacteria acquired from the water column [54]; they are reservoirs of diverse microorganisms. Therefore, the use Raman spectroscopy to characterize carotenoids from sponges is promising, as can be seen from the spectrum of Demospongiae Hymeniacidon heliophila (figure 5), a species associated with ammonia-oxidizing archaea, proteobacteria and cyanobacteria [56]. In addition, clones of bacteria closely related to the ones from an Indian Ocean hydrothermal vent were also identified [44]. The in situ analysis of the orange-coloured tissue showed major bands at 1525, 1159 and 1006 cm$^{-1}$. These fingerprint bands pointed to the presence of carotenoids in the sponge tissues. It is straightforward to note that this is the initial investigation on this subject, and many more data will be provided in the near future for the chemical and spectroscopic characterization of marine sponges.
In sum, Raman spectroscopy is suitable for the investigation of various pigments in most living organisms, and it can also be used to study marine sponges, which are reservoirs of microbes and can provide valuable chemical information about the strategies for survival, adaptation and radiation in microorganisms from the early terrestrial environment.

4. Conclusion

The main differences between polyenals and carotenoids observed in Raman spectroscopic analyses are the red-shifted wavenumber position of the $\nu$($C\equiv C$) stretching mode of polyenals by $ca$ 30 cm$^{-1}$ when compared with that of carotenoids, as well as the absence in polyenals of the deformation mode related to the $\delta(C\equiv CH_3)$ group at $ca$ 1000 cm$^{-1}$ and the presence of the same $\delta(C\equiv CH_3)$ mode at $ca$ 1020 cm$^{-1}$. Within polyenals, subtle differences have been observed in the identification of completely or partially unmethylated polyenes, based on the wavenumber position of $\delta(C\equiv CH_3)$ at $ca$ 1020 cm$^{-1}$. The positions of the Raman bands from carotenoids and polyenals are equally susceptible to the matrix effect, size of the conjugated double bond system and laser excitation line. Polyenals have been reported in the feathers of parrots and the mineralized structures of shells, pearls and octocorals, which may also co-occur with carotenoids. The unequivocal characterization of each compound in the analysed samples is very important for the investigation of the polyenic nature in many other living organisms.

As Raman spectroscopy is one of the most suitable techniques to identify conjugated polyenes, it has been applied in the study of the carotenoids present in marine sponges, the most ancient metazoans, which live in association with several microorganisms. Sponges, as reservoirs of microbes, may provide valuable chemical information about the strategies of survival, adaptation and radiation in microorganisms from the early terrestrial environment. In other words, Raman spectroscopy can provide a complete assignment of the chemical composition for living species containing polyenes, taking into account the points addressed here.

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