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Resonance Raman: a powerful tool to interrogate carotenoids in biological matrices

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Contents

Abstract.....	2
Carotenoids: Electronic Properties.....	2
Electronic Structure of Carotenoids.....	2
Vibrational spectroscopy: tools to describe molecules precisely.....	4
Resonance Raman of carotenoid molecules.....	5
Origin of the bands observed in carotenoid resonance Raman spectra.....	6
Probing the Molecular Structure of Carotenoids using their Vibrational Properties.....	8
Resonance Raman of Carotenoid Molecules: Practical Aspects.....	9
Applications of resonance Raman to Carotenoids in Biological Systems.....	10
Linear Carotenoids in Light-Harvesting Proteins from Purple Bacteria.....	10
Identifying Carotenoid Absorption Using Resonance Raman.....	11
Playing with the Configuration: Cyclic Carotenoids in Photosynthetic Proteins.....	11
Playing with the cis/trans Configuration : Carotenoids in Photosynthesis.....	13
Carotenoid Photochemistry in flowers and fruits.....	14
Conclusions.....	15
Acknowledgements.....	15
References.....	15

Abstract

Resonance Raman spectroscopy is one of the most powerful techniques in analytical science due to its molecular selectivity, high sensitivity, and the fact that, in contrast to IR absorption spectroscopy, the presence of water does not hamper or mask the results. Originating in physics and chemistry, the use of Raman spectroscopy has spread and now includes a variety of applications in different disciplines, including biology. In this chapter, we introduce the basic principles of Raman and resonance Raman scattering, and show resonance Raman can be applied to study carotenoid molecules, in complex biological or chemical matrices. We describe the type of information that can be extracted from resonance Raman spectra, illustrating the power of this method by a series of example applications.

Carotenoids: Electronic Properties

Electronic Structure of Carotenoids

Carotenoids are tetraterpenoid derivatives initially formed of 8 isoprene molecules. Carotenoids exhibit significant structural diversity, since their carbon skeletons may vary from purely linear, including cycles and/or functional groups such as carbonyls, alkynes or allenes (Britton, Liaaen-Jensen et al. 2008)(Britton 2022). Figure 1a shows the structure of a few representative carotenoids that are among the better characterized. Carotenoids can be described as having a C_{2n} point symmetry, which allows a classification of the electronic states as rotationally symmetric (A) or asymmetric (B). Hence, the electronic transitions are limited to those that change state inversion symmetry ($g \leftrightarrow u$) and Pariser alternancy symmetry (pseudoparity) ($+ \leftrightarrow -$) (Hudson and Kohler 1974, Christensen 1999). The absorption properties of carotenoids can be described by a simplified system which includes three main electronic states, the S_0 ($1^1A^-_g$), S_1 ($2^1A^-_g$) and S_2 ($1^1B^+_u$) electronic levels. The strongly-allowed $S_0 \rightarrow S_2$ transition of carotenoids usually exhibits a characteristic three-peak structure, corresponding to the lowest three vibronic bands of the electronic transition, termed 0-0, 0-1 and 0-2 (Figure 1b). Note that the $S_0 \rightarrow S_1$ transition is one-photon silent (forbidden) for symmetry reasons (Tavan and Schulten 1987). For the purposes of this review, when addressing the energy of the $S_0 \rightarrow S_2$ electronic transition, we will refer specifically to the energy of the (0-0) band.

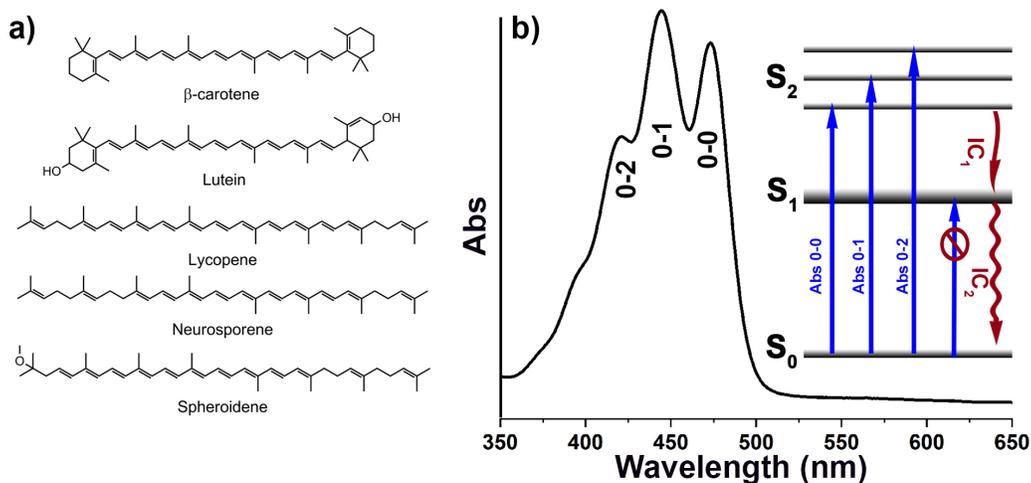


Figure 1 | a) Structures of several simple carotenoids (β -carotene, lutein, lycopene, neurosporene and spheroidene). b) Representative carotenoid absorption spectrum (lutein in *n*-hexane at room temperature), exhibiting 0-0, 0-1 and 0-2 vibronic bands. **Inset:** Simplified energy diagram of carotenoids: blue arrows - absorption (Abs), forbidden for $S_0 \rightarrow S_1$; red arrows - internal conversion (IC) by non-radiative decay, for $S_2 \rightarrow S_1$ (IC_1) and $S_1 \rightarrow S_0$ (IC_2), respectively.

Photon absorption thus promotes an electron from S_0 to S_2 , the latter having an ultrashort lifetime (hundreds of femtosecond). The S_2 state relaxes vibrationally to the S_1 state, which decays non-radiatively to the ground state in several picoseconds (Polívka and Sundström 2004). Although the lifetimes of these excited states are extremely short, carotenoids efficiently harvest solar photons in the blue-green range during the first steps of photosynthesis. These short lifetimes are otherwise ideal for providing photoprotection to photosynthetic organisms, whereby lutein accepts the excitation energy from chlorophyll and rapidly turns this energy into heat (Ruban, Berera et al. 2007).

A major role of carotenoids in biology is protection against oxidative stress, as carotenoids also possess a triplet state, whose energy is lower than that of singlet oxygen, and this triplet state actually deactivates this harmful singlet oxygen chemical species efficiently in biological tissues. In addition, the spurious chlorophyll triplet states formed during the early steps of photosynthesis are efficiently deactivated by triplet transfer to the neighbouring carotenoid molecules. A number of additional electronic states have been suggested for carotenoids by time-resolved spectroscopy studies, although these elusive states have not yet been fully characterized. The energy of carotenoid excited states and their excited state lifetimes are generally inversely proportional to the number of C=C double bonds present in the conjugated chain. This effect is straightforward for carotenoids such as neurosporene ($N = 9$), spheroidene ($N = 10$), and lycopene ($N = 11$), which contain a linear chain of π -electron-conjugated C=C bonds. However, the concept of *effective conjugation length* N_{eff} had to be introduced to account for the electronic properties of carotenoids containing chemical groups, such as conjugated end-rings, ketones and allene groups. Effective conjugation length includes the effect of distortions of the conjugated chain in its equilibrium state, which influence the electronic structure of these molecules. For instance, the conjugated β -rings in lutein or β -carotene

contribute by the equivalent of only 0.3 of a C=C bond, as their configuration is partly out of the plane of the C=C chain. It should be noted that even the N_{eff} parameter is not sufficient to describe carotenoids containing conjugated oxygen atoms, as this electron-rich atom has additional effects on the electronic structure (Mendes-Pinto, Sansiaume et al. 2013, Llansola-Portoles, Pascal et al. 2017).

As fluorescence and phosphorescence yields of carotenoids are extremely low, steady-state and time-resolved electronic absorption are the main electronic spectroscopies used to characterize the electronic properties and photophysics of carotenoids. However, these absorption methods do not yield details about the electronic and structural configuration of these molecules. In the absence of such information, it remains difficult to understand the functional properties of carotenoids, or to perform a complete modelling of them.

Vibrational spectroscopy: tools to describe molecules precisely

Vibrational spectroscopy gives access to the energy of vibrational levels of polyatomic molecules, which in turn are extremely sensitive to their structural and electronic configuration. Such methods thus complement electronic absorption, in particular for carotenoid studies. There are two main vibrational approaches, infrared absorption and Raman spectroscopy. Infrared absorption requires the presence of a transition dipole between the ground state and the vibrational excited state. As carotenoids are highly symmetric, and mainly composed of carbon atoms, the information content of their infrared absorption spectra are relatively poor. The presence of oxygen atoms in carotenoid structures induces the appearance of very intense absorption bands, which may be used to control the intactness of the carotenoid structures during purification, but this technique has found little application to investigate carotenoids in biological systems.

Raman scattering is the phenomenon of a change in the frequency of light when it is scattered by polyatomic molecules, reflecting energy exchange between the incoming photon and the scattering molecule. Classically, the Raman effect can be described as an interaction between the oscillating electric field of the incident light, which disturbs the electronic charge distribution in the molecule, and the intrinsic oscillations (normal modes) of the illuminated molecule. The product of these two oscillating functions can be approximated by a cosine product, which can be written in the form of three cosine functions possessing i) the frequency of the incident light, ii) the sum of the frequency of the incident light and that of each vibrational mode which influences the molecular polarizability, and iii) the difference between these frequencies (Long 2002). This simplified view predicts the appearance in the scattered light of new frequencies, shifted from the energy of the incident light by the energy of the vibrational modes. When reasoning in terms of quantified energy levels, the Raman effect corresponds to an ultrafast interaction, leading the molecule to a virtual state, during which it may either gain or lose the energy level of a vibrational state (Stokes or anti-Stokes scattering, respectively; figure 2a). Raman spectroscopy can thus be used as an analytical method for determining the chemical structure of molecules, as well as their conformation and/or the intermolecular interactions in which they are involved. The units used for describing Raman spectra correspond to the shift between the energy of the photons provided by the exciting laser beam and those emitted by the scattering molecule, expressed in wavenumbers for convenience (or cm^{-1}). The Raman effect is a very low probability process so that the signal measured is

usually very weak, and thus can be easily contaminated by spurious fluorescence. However, if the energy of the incident light matches that of an electronic transition of the irradiated molecule, an enhancement of a subset of Raman-active modes is observed, which may reach many orders of magnitude (figure 2b). This is the so-called *resonance* phenomenon, which becomes apparent only when a full quantum mechanical description of the effect is performed (Long 2002). The resonance effect allows for the selective observation of a pigmented molecule of interest (*e.g.* carotenoids) in complex media, such as in proteins, membranes and even whole cells. The phenomenon not only dramatically increases the intensity of the signal, but also reduces the complexity of the spectra. The only vibrational modes contributing to the spectra under resonance conditions are those involving nuclear motions which correspond to distortions experienced by the molecule during transition between the ground and excited states used for inducing the resonance (Albrecht and H. 1961). This intra-mode selection is far from being a limitation. Indeed, thanks to this process, resonance Raman yield selective information on those modes which are coupled with the electronic transition of the molecule – this transition being generally at the origin of its biological activity. Resonance Raman thus reveals the ‘biologically relevant’ vibrational part of these molecules almost exclusively.

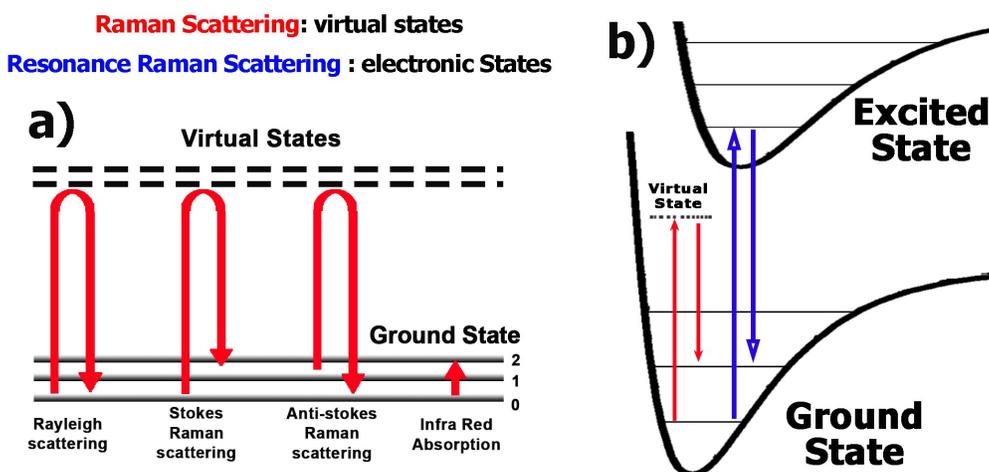


Figure 2 | Principle of Raman Spectroscopy: **a)** Energy Levels involved in the Raman scattering phenomenon. From left to right: Rayleigh scattering, Stokes and anti-Stokes Raman scattering, and infra-red absorption (for comparison). **b)** Principle of resonance Raman spectroscopy: virtual and electronic energy levels involved in the Raman and resonance Raman scattering phenomena (red, blue respectively).

Resonance Raman of carotenoid molecules

Carotenoid molecules are ideally suited for resonance Raman studies, as they display a very high resonance intensity, actually the highest among natural biomolecules. The first resonance Raman spectra of an *all-trans* carotenoid were measured in 1970, and the four main groups of bands observed were termed ν_1 to ν_4 (Gill, Kilponen et al. 1970). The origin of these modes was assessed by comparison with the spectra of polyenes (Rimai, Heyde et al. 1973). However, the first normal coordinates calculation of carotenoids was performed only in the late 1980's (Saito and Tasumi 1983). Precise prediction of these spectra is now

possible, although intensive calculations by Density-functional theory (DFT) are required, combined with quantum mechanics/molecular mechanics (QM/MM) calculations or studies using *ab initio* molecular dynamics (QMD)(Troullier and Martins 1991, Martyna and Klein 1992, Perdew, Burke et al. 1996, Wong 1996, Rudberg, Sałek et al. 2005, Dreuw, Harbach et al. 2010, Cohen, Mori-Sánchez et al. 2012, Hutter 2012, Liu, Wang et al. 2012, Kühne 2014, Chung, Sameera et al. 2015, Mardirossian and Head-Gordon 2017). The fact that the resonance Raman spectra of specific, phenyl-containing carotenoid derivatives could be predicted before they were actually measured, is a striking illustration of the success of such calculations (Macernis, Galzerano et al. 2015). Figure 3 shows the characteristic resonance Raman spectra of *all-trans* and *cis* carotenoids, with the ν_1 to ν_4 regions indicated.

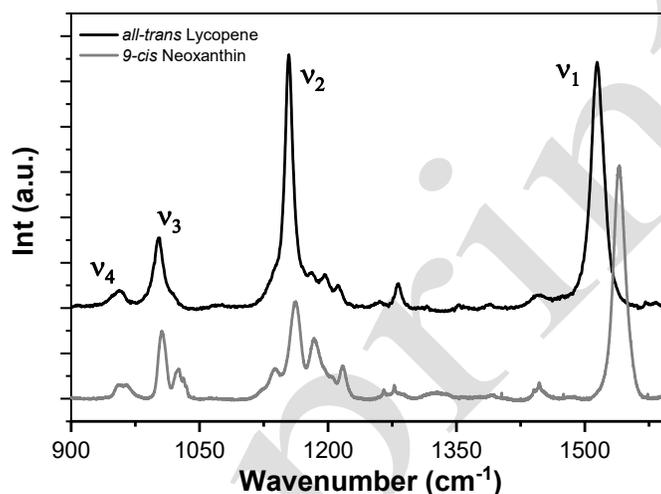


Figure 3 | Resonance Raman spectra of *all-trans*-lycopene and *9-cis* neoxanthin in *n*-hexane, upon excitation at 488.0 & 476.5 nm, respectively (black, grey lines).

Origin of the bands observed in carotenoid resonance Raman spectra

Appearing around 1500 cm^{-1} , the most intense contribution to these spectra is termed ν_1 . This band arises from the ensemble of stretching modes of the conjugated C=C double bonds. Its frequency depends on the length of the π -electron conjugated chain, which in turn is sensitive to the molecular configuration of the carotenoid (Rimai, Heyde et al. 1973, Koyama, Kito et al. 1982, Koyama, Takii et al. 1983, Koyama, Takatsuka et al. 1988, Koyama and Fujii 1999). The frequency of this band was shown to be temperature dependent, shifting by 3-6 cm^{-1} between 293 and 77 K, probably because of temperature-induced changes in both the vibronic coupling and the extent of π -electron delocalization in the carotenoid molecule (Andreeva, Apostolova et al. 2011, Mendes-Pinto, Galzerano et al. 2013). Around 1160 cm^{-1} a complex cluster of bands, termed ν_2 , can be observed. These contributions arise from the ensemble of stretching vibrations of C-C single bonds, coupled with in-plane C-H bending modes. Systematic studies of β -carotene (Koyama, Takii et al. 1983) and spheroidene (Koyama, Kito et al. 1982) isomers showed that this region can be used as a fingerprint for most carotenoid *cis*-isomers. For instance, *9-cis* configuration of the C=C chain is accompanied by the appearance in this region of a fingerprint band at 1132 cm^{-1} (Yan, Liu et al. 2001), while *13-cis* carotenoids exhibit a contribution at 1138 cm^{-1} (Koyama, Kito et al. 1982, Koyama,

Takatsuka et al. 1988). At about 1000 cm^{-1} , the so-called ν_3 band contributes, which arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent C-H groups (Koyama, Takii et al. 1983). It was recently shown that the ν_3 band can be used as a fingerprint of the configuration of conjugated end-rings (Mendes-Pinto, Galzerano et al. 2013). Finally, the group of bands termed ν_4 , located around 960 cm^{-1} , arises from C-H out-of-plane wagging motions coupled with C=C torsional modes (out-of-plane twists of the carbon backbone) (Saito and Tasumi 1983). When the carotenoid conjugated system is fully planar, these out-of-plane modes are not coupled with the electronic transition (which occurs in-plane), and so they exhibit little resonance enhancement. Upon rotations around C-C single bonds, the electronic transition loses its purely in-plane character, and these modes (or some of them) become more coupled with it, and consequently gain intensity. This region can be used as an indicator of the distortions experienced by the carotenoid backbone - see *e.g.* (Lutz, Szponarski et al. 1987). Finally, the presence of additional conjugated groups in the carotenoid structure may produce additional bands. For example, the fucoxanthin spectrum shows a vibrational mode around 1660 cm^{-1} arising from the stretching mode of its conjugated keto group, while diadinoxanthin exhibits a mode around 980 cm^{-1} which is seemingly unrelated to the presence of twists around C-C bonds (Premvardhan, Bordes et al. 2009). It must be noted that the participation of these additional conjugated groups to the resonance Raman spectra of carotenoids does not depend only on their conjugation. For these modes to be resonance-Raman-active, they should experience a change in coordinates during the corresponding electronic transition. For instance, spheroidenone possess a clearly conjugated C=O group, but the corresponding C=O stretching mode is not observed in spheroidenone resonance Raman spectra.

Resonance Raman relies on an ultrafast transition, and thus allows the conception of time-resolved experiments. Pump-probe experiments in resonance Raman are limited in time resolution to a few picoseconds, as the frequency broadening of shorter laser pulses blurs the Raman signal. Faster time resolution (down to 50 femtoseconds) can be achieved by a non-linear approach, namely stimulated Raman, where a combination of three laser pulses is used to produce the signal (McCamant, Kim et al. 2002, McCamant, Kukura et al. 2003, Kukura, McCamant et al. 2004). Resonance Raman of isolated carotenoid molecules in their S_1 , S_2 , T_1 triplet and charged states have been reported (Hashimoto and Koyama 1988, Hashimoto and Koyama 1989, Hashimoto and Koyama 1990, Jeevarajan, Kispert et al. 1996, McCamant, Kukura et al. 2003, Kloz, Weißenborn et al. 2016). Resonance Raman of excited (and charged) states of carotenoid display the same groups of active vibrational modes, with shifted frequencies due to the changes of the electronic structure induced by the promotion of an electron into the singlet (triplet) excited state. Up to now, only a few studies showing Raman signatures of carotenoid excited states have been reported in *biological* systems (Vrettos, Stewart et al. 1999, Telfer, Frolov et al. 2003, Gall, Berera et al. 2011, Hontani, Kloz et al. 2018, Artes Vivancos, van Stokkum et al. 2020). Carotenoid molecules can also be oxidized, although the redox potential necessary to do so is quite high ($0.8/1.0\text{ V}$ vs SCE). Resonance Raman of a pure carotenoid cation has not yet been observed *in vitro* - the second oxidation potential, leading to the carotenoid dication, is close to that of Car/Car^+ , which usually results in production of a mixture of mono- and di-cations. However, carotenoid cations were observed in photosystem II reaction centers, in conditions where electron transfer from the Manganese cluster to the primary electron donor is impaired (*e.g.* low temperature). These experiments, together with EPR measurements, revealed that a β -carotene was the first

molecule to reduce the primary electron donor to protect photosystem II when the manganese cluster is damaged (Hanley, Deligiannakis et al. 1999, Telfer, Frolov et al. 2003).

Probing the Molecular Structure of Carotenoids using their Vibrational Properties

It can be predicted that both the position of the electronic absorption of carotenoid molecules (expressed in energy) and the frequency of the ν_1 band linearly depend on the inverse of the length of the carotenoid C=C conjugated chain (Tavan and Schulten 1986). While this relationship was observed for the simplest carotenoid molecules, the properties of carotenoids containing conjugated end-rings were clearly deviating from it. For instance, the formal conjugation length of 11 for β -carotene includes two conjugated β -rings, and its absorption peak at 478 nm in n-hexane contrasts with that of the equivalent linear N=11 carotenoid, lycopene, at 502 nm (see structures in Figure 1a). On the other hand, the strict linear relationship between the frequency of the ν_1 band and the absorption position is nevertheless conserved for β -carotene and all measured carotenoids that possess conjugated end-rings. In order to account for this observation, the concept of *effective* conjugation length (N_{eff}) was introduced, which reflects the actual influence of the conjugated bonds to the carotenoid structure. For β -carotene (figure 4a), the measured ν_1 frequency (1525 cm^{-1}) yields an effective conjugation length of 9.6, indicating that the C=C bonds present in the end-rings only contribute an equivalent of 0.3 C=C to its conjugated chain. Indeed this is fully consistent with the three-dimensional structure of β -carotene, where steric hindrance forces the β -rings out of the plane by about 45° , reducing the extent of their conjugation (Sterling 1964). This concept could be extended to all carotenoids with conjugated end-rings, as well as to aryl carotenoid molecules.

The effect of the refractive index of the surrounding environment on the position of the absorption transition of carotenoid molecules has been studied extensively (Araki and Murai 1952, LeRosen and Reid 1952, Hirayama 1955, Suzuki and Mizuhashi 1964, Andersson, Gillbro et al. 1991, Kuici, Nagae et al. 1994, Frank, Bautista et al. 2000, Chen, Lee et al. 2006, Renge and Sild 2011). The position of the $S_0 \rightarrow S_2$ electronic transition in solution linearly depends on the solvent polarizability, defined as $R(n) = (n^2 - 1)/(n^2 + 2)$, where n is the solvent refractive index. For linear carotenoids, the $S_0 \rightarrow S_2$ transition shifts to longer wavelength as the refractive index increases (Renge and Sild 2011), due to dispersive interactions between the solvent environment and the large transition dipole moment of the molecule (Renge and Sild 2011). A significant linear correlation was found between the frequency of the ν_1 Raman band and the polarizability of the solvent for different linear carotenoids (including those with conjugated end-rings), proving an influence of the solvent polarizability on the carotenoid ground state (Mendes-Pinto, Sansiaume et al. 2013). Figure 4b displays a practical example of the polarizability effect on the absorption spectra and ν_1 Raman band of β -carotene. Again, this linear relationship extends to a large variety of carotenoids (including those with conjugated end-rings), with quite similar slopes (Mendes-Pinto, Sansiaume et al. 2013, Llansola-Portoles, Pascal et al. 2017). The ν_1 Raman band reflects polarizability-induced changes in the ground-state only, while the absorption shift results from the combined effects on both S_0 and S_2 . As a result, the linear relationship between the absorption position and ν_1 frequency according to solvent polarizability (for a given carotenoid in different solvents) possesses a much steeper slope than the equivalent relationship plotted according to effective conjugation length (different carotenoids in the same solvent) (Mendes-Pinto, Sansiaume et al. 2013, Llansola-Portoles, Pascal et al. 2017).

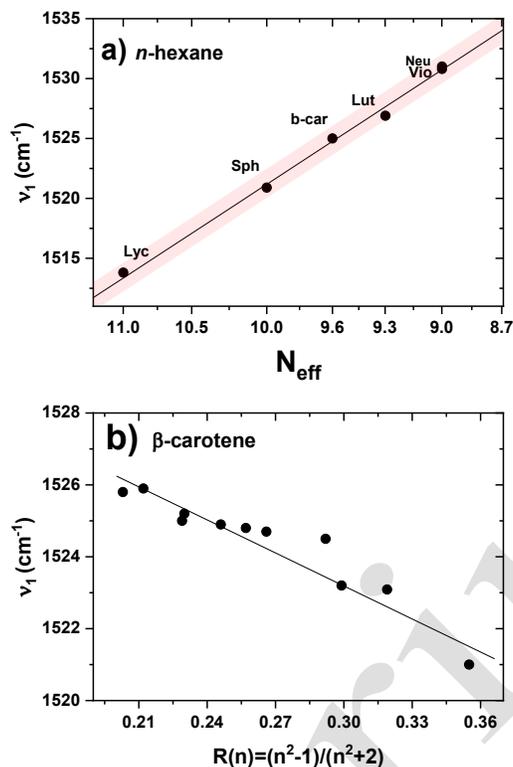


Figure 4| a) Correlation between the frequency of the ν_1 Raman band in *n*-hexane and effective conjugation length (N_{eff}) (reciprocal scale). **b)** Correlation between the frequency of the ν_1 Raman band of β -car and solvent polarizability.

Resonance Raman of Carotenoid Molecules: Practical Aspects

Raman-active molecular vibrations are those involving changes in molecular polarizability. As a result, the Raman signal of water is very low, and thus seldom interferes with that of the biological molecules being studied. This feature constitutes an important advantage for the Raman technique over infrared absorption spectroscopy. However the Raman signal is usually very weak, and is thus easily contaminated by spurious fluorescence from the biological/chemical matrix. In resonance conditions, the large gain in the Raman signal (up to 10^6) allows easier measurements on biological samples. Carotenoids have the highest resonance Raman cross-section among natural pigments, and these measurements can thus be achieved at very low carotenoid concentrations (less than micromolar) in media as complex as whole organisms. The volume of the sample needed for a Raman experiment is determined by the size of the focused laser beam, *i.e.* a few microliters. Even nanoliter-sized samples can be used, by coupling a microscope (which may be confocal) with the Raman spectrometer. Note that in some measurement conditions, such as those at room temperature and/or when using a highly-focussed beam in a microscope, anaerobic conditions may be required to avoid photo-bleaching of the sample.

Applications of Resonance Raman to Carotenoids in Biological Systems

Linear Carotenoids in Light-Harvesting Proteins from Purple Bacteria

Light-harvesting (LH) pigment–protein complexes from purple photosynthetic bacteria can bind many different carotenoids, with significant variations observed between bacterial species but also for the same species in different habitats (Takaichi 1999) – *e.g.* spheroidene, neurosporene (in *Rhodobacter sphaeroides*), or spirilloxanthin (in *Rhodospirillum rubrum*). These carotenoids can have different functions depending on their configuration and environment, either as auxiliary light-harvesting molecules (Desamero, Chynwat et al. 1998, Feng, Tseng et al. 2017) or as photoprotective species quenching chlorophyll excited states (Angerhofer, Bornhäuser et al. 1995, Takaichi 1999, Dilbeck, Tang et al. 2016). A room-temperature MP-graph, correlating the ν_1 Raman band, the position of the $S_0 \rightarrow S_2$ transition, the polarizability of the environment, and the effective conjugation length, was constructed for a range of such protein-bound carotenoids, in comparison with the same carotenoids *in vitro* (figure 5). This representation allows us to determine the environment polarizability and the N_{eff} of a given carotenoid through measurement of its ν_1 Raman band and $S_0 \rightarrow S_2$ transition (Mendes-Pinto, Sansiaume et al. 2013, Llansola-Portoles, Pascal et al. 2017). In figure 5, the relationship between the pairs of values (ν_1 , $S_0 \rightarrow S_2$) for isolated carotenoids in *n*-hexane ($R = 0.229$; blue circles) and when bound to LH proteins (red circles) lies along the slope drawn for *in vitro* changes in polarizability (black arrow). These results suggest that the average polarizability of the LH protein binding pocket is the dominant factor for tuning the position of the $S_0 \rightarrow S_2$ transition upon binding to their LH protein host. This average polarizability, which is nearly identical for the three homologous proteins, corresponds to a value, $R(n)$, of about 0.334, slightly lower than that found in carbon disulfide ($R = 0.355$). This value is very high, and can be explained by the fact that LH-bound carotenoids are in close contact with bacteriochlorophyll molecules, which may provide them a highly polarizable environment (McDermott, Prince et al. 1995). Similar results were found by Dilbeck et al. (Dilbeck, Tang et al. 2016) for six LH2 proteins from genetically-modified strains of the purple photosynthetic bacterium *Rhodobacter (Rb.) sphaeroides*. It was again found that the shift in the ν_1 Raman band and the absorption spectrum for the studied LH-bound carotenoids (neurosporene, spheroidene, lycopene, spirilloxanthin, ketospirilloxanthin or diketospirilloxanthin) could be explained by the polarizability of the environment alone.

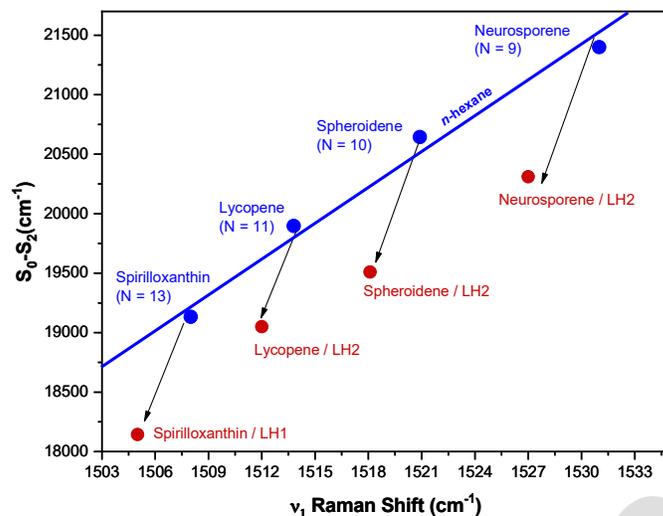


Figure 5 | Correlation between the position of the $S_0 \rightarrow S_2$ electronic transition and the ν_1 Raman band for spirilloxanthin, lycopene, spheroidene, and neurosporene, in *n*-hexane (blue circles) and bound to LH proteins (red circles) (Mendes-Pinto, Sansiaume *et al.* 2013, Dilbeck, Tang *et al.* 2016).

Identifying Carotenoid Absorption Using Resonance Raman

As explained above, the Raman scattering intensity of a given carotenoid is highly dependent on the precise position of the Raman excitation. In complex proteins containing more than one carotenoid, this dependence can be used to attribute each absorption transition to a given carotenoid species. This identification method was first used by Ruban *et al.*, in the major light-harvesting protein LHCII from higher plants. This trimeric protein binds two luteins and a 9-*cis* neoxanthin per monomer, carotenoids which exhibit quite different resonance Raman spectra - in particular because the neoxanthin is in a 9-*cis* configuration. By scanning the Raman excitation across the carotenoid absorption range, and identifying at which positions the contribution of one or other carotenoid were dominant, these authors could conclude that the two luteins display different electronic transitions, one absorbing at 495 nm and the other redshifted to 510 nm, while the 9-*cis* neoxanthin absorption peaks at 486 nm (Ruban, Pascal *et al.* 2000). This strategy has been widely used in algal light-harvesting proteins, and led to the conclusion that, in all complexes studied thus far, the pair of carotenoids equivalent to the central luteins in higher plants possess shifted absorption transitions relative to each other (Llansola-Portoles, Uragami *et al.* 2016, Llansola-Portoles, Litvin *et al.* 2017, Streckaite, Llansola-Portoles *et al.* 2021). In addition, this strategy was essential for determining the stoichiometry of pigments bound to the fucoxanthin-chlorophyll proteins in diatoms (Premvardhan, Bordes *et al.* 2009).

Playing with the Configuration: Cyclic Carotenoids in Photosynthetic Proteins

As mentioned above, the LHCII protein assembles into a trimer in the photosynthetic membrane, with each monomer containing two lutein molecules. In LHCII monomers, both luteins absorb at 495 nm, while in LHCII trimers, one lutein (lut_1) absorbs at 495 nm whereas the absorption of the second one (lut_2) is

shifted to 510 nm (Ruban, Pascal et al. 2000, Caffarri, Croce et al. 2001). Plotting the lut_1 pair of (ν_1 , $S_0 \rightarrow S_2$) values on a MP-plot shows that the position of its electronic transition is mainly governed by the polarizability of its protein binding site (as is the case for both luteins in LHCII monomers). However, considering the (ν_1 , $S_0 \rightarrow S_2$) pair leads to the conclusion that it is not the case for lut_2 . Instead, the lut_2 values suggest that the conjugated chain of the carotenoid is increased by nearly one C=C double bond at constant polarizability. The apparent length of the conjugated chain for lutein in solvent (and for lut_1 in LHCII) is 9.3, with its β -ring being only partially conjugated because of its out-of-plane conformation. N_{eff} for the red-absorbing lut_2 in LHCII is ~ 10 , and it was proposed that the β -ring in the latter case is forced back into the conjugated plane, resulting in a gain in conjugation length (Mendes-Pinto, Galzerano et al. 2013). A similar effect was observed with the two β -carotene molecules in the photosystem II reaction centre, which also display shifted absorption (van Dorssen, Breton et al. 1987, Kwa, Newell et al. 1992, Tomo, Mimuro et al. 1997). It was similarly concluded that the red-absorbing β -carotene in this protein has a longer apparent conjugation length (calculated as ~ 10.2), and that steric hindrance forces one of its β -rings into an in-plane configuration (Mendes-Pinto, Galzerano et al. 2013).

High-light inducible proteins (HLIPs) constitute a family of photoprotective proteins in cyanobacteria, which contain carotenoids able to quench the excited states of chlorophyll molecules. Among this family of LH-related proteins, the recently-purified HliD and HliC contain two bound β -carotenes with even more distinct electronic absorption transitions (Staleva, Komenda et al. 2015, Llansola-Portoles, Sobotka et al. 2017, Shuklaa, Llansola-Portoles et al. 2017). Figure 6 shows the absorption spectra at 4.5 K of HliC, with one β -carotene peaking at 492 nm and the other at 527 nm, with corresponding ν_1 frequencies at 1525 and 1516 cm^{-1} , respectively (Shuklaa, Llansola-Portoles et al. 2017). Plotting (ν_1 , $S_0 \rightarrow S_2$) pairs on an MP-graph for these two carotenes again led to the conclusion that, while the electronic properties of the blue carotene are tuned by the polarizability of the protein binding pocket only, the red-absorbing species displays a longer effective conjugation length. The effective length of this carotene lies between 10.5 and 10.6 C=C, which can be explained by both β -rings being forced back into an in-plane configuration (the expected N_{eff} for a fully-conjugated β -carotene is 11).

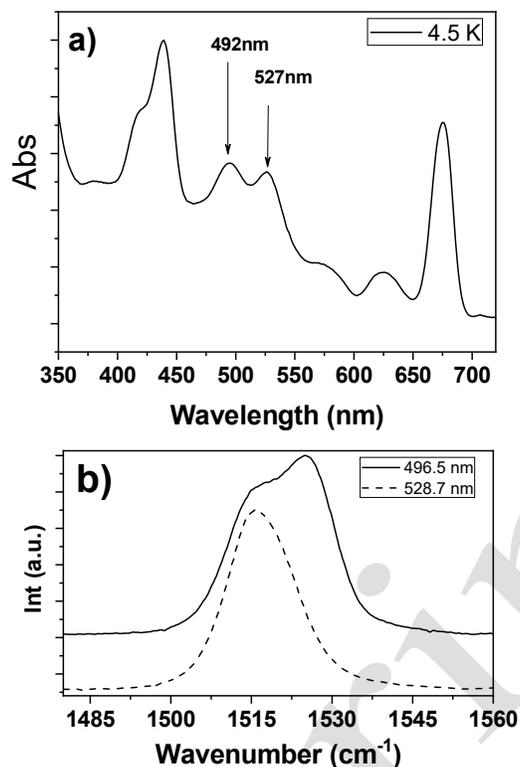


Figure 6 | **a)** 4.5 K absorption spectra of His-HliC. **b)** 77 K resonance Raman spectra of His-HliC at 496.5 and 528.7 nm excitation.

Playing with the *cis/trans* Configuration : Carotenoids in Photosynthesis

As early as 1976, it was reported by the group of Lutz that the spheroidene bound to the bacterial reaction centre displays a very unusual resonance Raman spectrum. In this spectrum, the ν_2 region was much more complex, exhibiting many more bands, while the intensity in the ν_4 region was as high as the very intense ν_1 band (Lutz, Kleo et al. 1976). Comparing with the spectra of a series of *cis* isomers, it was concluded that this spectrum probably arises from a 15-15' *cis* isomer, although this conclusion was not able to explain the anomalous intensity of the ν_4 band (Koyama, Kito et al. 1982). One acute problem of extracting carotenoids from such chlorophyll-containing proteins is that photon absorption by the chlorophyll molecules sensitises the formation of carotenoid triplets, which may induce *cis-trans* isomerisations (as the energy barriers for isomerisation are lower in the carotenoid triplet state). When the spheroidene was extracted in total darkness, it could be shown that the purified carotenoid displayed the typical spectrum of a 15-*cis* carotenoid, while removal of the carotenoid from the binding site resulted in disappearance of the anomalously intense ν_4 . It was thus concluded that the reaction centre-bound spheroidene was a 15-*cis* carotenoid, which was highly distorted by its protein binding site (Lutz, Szponarski et al. 1987). In other photosynthetic proteins, resonance Raman experiments showed that carotenoids are generally in the *all-trans* conformation, with a few notable exceptions (*e.g.* 9-*cis* β -carotene in cytochrome b_6/f ; 9-*cis* neoxanthin in LHCII; photosystem I may also contain some 9-*cis* β -carotene). Why

the carotenoids bound to bacterial reaction centers display such an unusual conformation is still unclear, but it should be noted that the isomerization is apparently induced by the binding of the carotenoid to the protein - there is no specific biosynthetic pathway for this isomer (Mathies, van Hemert et al. 2011).

Carotenoid Photochemistry in flowers and fruits

As mentioned above, resonance Raman spectra of the excited states of monomeric carotenoids have been measured in solvents, and occasional applications on carotenoid excited states in biology have been reported. Carotenoid molecules can associate into self-assemblies, which may modify their electronic states significantly. Resonance Raman was used to characterize carotenoid aggregates as early as 1977 by Salares et.al. (Salares, Young et al. 1977), and the first observation of triplet states was performed by picosecond resonance Raman spectroscopy by Wang et.al. (Wang and Tauber 2010). In biology, the bright colours of many flowers and fruits originate from carotenoid assemblies in specialized plastids in the plant cell, termed chromoplasts (Wijk and Kessler 2017). The yellow chromoplasts in daffodil flowers (*Narcissus pseudonarcissus L.*) mainly contain violaxanthin and lutein carotenoids, associated in membrane structures. On such samples, the high selectivity of resonance Raman confirmed the presence of carotenoid assemblies and helped in determining the different aggregated species, as well as localizing the absorption of each of these species. By scanning across the absorption range in *Narcissus* chromoplasts, it was found that excitations at 488.0, 501.7 & 406.7 nm produced different vibrational signatures, with ν_1 frequencies at 1530.4, 1526.7 & 1528.0 cm^{-1} , respectively. This revealed the presence of three different species of carotenoid aggregates (Quaranta, Krieger-Liszkay et al. 2021).

Tomato chromoplasts contain crystalloids containing mainly lycopene. In these crystalloids, interactions between lycopene molecules red-shift the lycopene absorption to *ca* 586 nm. Time-resolved absorption, performed on whole tomato chromoplasts, revealed a more complex photochemistry than expected, with the formation of a long-lived excited state (in the microsecond range). Resonance Raman experiments were used to determine the nature of this long-lived excited species. For such long-lived species, power-dependent resonance Raman spectroscopy allows the dynamic accumulation of transient excited states in the sample, by playing on the power of the laser used to produce the Raman signal (Gall, Berera et al. 2011, Ho, Kish et al. 2017). Experimentally, it is necessary to set the laser excitation in a region where both ground and excited state have absorption. Low-intensity resonance Raman yields the vibrational modes of the ground state, while at higher intensity the vibrational modes of the dynamically-accumulated excited state(s) appear. Using this technique in lycopene crystalloids, a new contribution was observed at 1135 cm^{-1} in the C–C stretching region, with a shoulder at 1128 cm^{-1} , while the ν_1 C=C stretching mode was downshifted. These vibrational features are fingerprints of carotenoid triplet excited states. Such resonance Raman measurements formally demonstrated the presence of significant levels of carotenoid triplet states in illuminated chromoplasts, generated by singlet fission. This is the first example of a single fission mechanism in biology (Llansola-Portoles, Redeckas et al. 2018). This phenomenon has also been observed in *Narcissus* (Quaranta, Krieger-Liszkay et al. 2021), suggesting that singlet fission is a general photochemical mechanism in carotenoid-containing fruits and flowers.

Conclusions

This review gives several examples of resonance Raman applications to study carotenoids in complex chemical and biological matrices. It shows how the high sensitivity and selectivity of resonance Raman spectroscopy makes it an excellent tool for studying the vibrational properties of carotenoids in their ground and excited states. With the on-going development of Raman imaging techniques and non-linear Raman methodologies (steady-state and femtosecond time-resolved), it should in the near future extend its current range of applications.

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