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# Raman spectroscopy analysis of molecular configuration forms of the macular xanthophylls

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**Abstract**

Macula lutea, the yellow spot of the retina of the human eye, comprises three xanthophyll pigments: lutein, zeaxanthin, and *meso*-zeaxanthin, playing numerous important biological functions. Macular xanthophylls are exposed to relatively strong illumination in the eye, owing to the fact that the yellow spot is localized on the optical axis, in the frontal layer with respect to photoreceptors. In the present work, we address the problem of photostability and possible photoisomerization of macular xanthophylls with the application of resonance Raman spectroscopy. The results show photostability of the two major isomers of the macular xanthophylls, all-*trans* and 9-*cis*, and efficient photoconversion of the 13-*cis* isomer to the 9-*cis* and mostly to the all-*trans* form. We report the Raman spectra of the main molecular configuration forms of the macular xanthophylls, opening an avenue for the examination of their possible presence and photoconversion in natural systems.

**KEYWORDS**

carotenoids, cis-xanthophylls, macula, retina, xanthophylls

## 1 | INTRODUCTION

Carotenoid pigments are ubiquitous in the biosphere and play numerous important physiological roles, including protection against oxidative damage and filtering harmful, short-wavelength radiation.[1–3] These functions are particularly important from the standpoint of photoprotection and integrity of the eye operating under conditions characterized by exposure to light and the presence of dissolved molecular oxygen in highly vascularized tissues.[4] An anatomical structure in the retina of the human eye particularly rich in carotenoids is referred to as the *macula lutea* or the yellow spot, due to its intense colouration.[5] Three polar carotenoids, called xanthophylls, have been identified in the macular pigment pool, namely, lutein (Lut), zeaxanthin (Zea) and *meso*-zeaxanthin (*m*-Zea) (see Figure 1).[4,6] Resonance Raman spectroscopy of macular xanthophylls can be effectively applied to examine their properties in the natural system[7] or even for imaging the retina,[8] owing to the relatively strong Raman scattering signal of polyene dyes.[9] On the other hand, a laboratory experience with carotenoids shows that molecules from these particular groups can undergo light-induced molecular configuration changes.[10,11] In the present work, we address the problem of possible light-induced molecular reconfiguration of macular xanthophylls, with the application of resonance Raman spectroscopy.

  

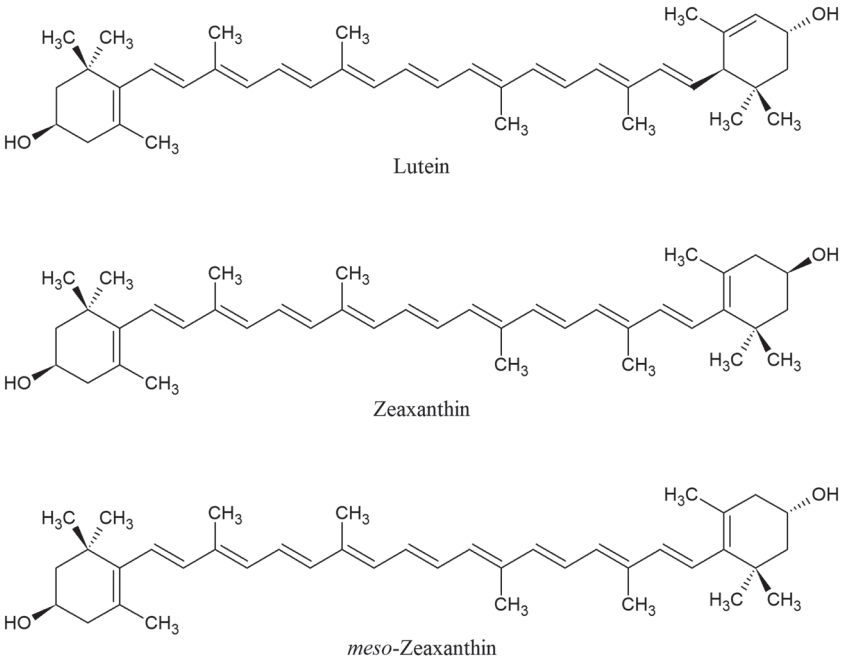


**FIGURE 1** Chemical structures of the macular xanthophylls in the molecular configuration all-*trans*. The structures of the xanthophylls in the molecular configurations *cis* are shown in Figures S1–S4

## 2 | MATERIALS AND METHODS

### 2.1 | Xanthophylls and solvents

Crystalline xanthophylls (all-*trans*)-lutein [(3R,3′R, 6′R)-β, ε-carotene-3,3′-diol)] and (all-t*rans*)-Zeaxanthin [(3R,3′R)-β,β-carotene-3,3′-diol)] were obtained from Extrasynthese. (All-*trans*) *meso*-Zeaxanthin [(3R,3′S)-β, β-carotene-3,3′-diol)] was obtained from U.S. Pharmacopeia. In order to remove possible degradation products, xanthophylls were repurified directly before use by means of high-performance liquid chromatography (HPLC) technique according to the previous report[10] (more details of purification are presented below). All the solvents for chromatographic xanthophyll purification were purchased from POCH (Poland) and were of the chromatographic quality: methanol (99.8%), methyl tert-butyl ether (99.8%), acetonitrile (99.9%), and dichloromethane (99.8%). Ultrapure water was obtained from a Milli-Q system (Millipore, France). The specific resistivity of water was 18 MΩ cm. Tetrahydrofuran (THF) for spectroscopic measurements (99.8%) was purchased from POCH (Poland).

### 2.2 | Xanthophyll isomerization

Isomerization of Zea and *m*-Zea was performed according to the procedure described previously.[10] All-*trans m*-Zea and all-*trans* Zea were dissolved in dichloromethane. Iodine catalyst in hexane was added at a concentration of 2% (w/w). The mixture of iodine and pigment was illuminated for 1 hr using a halogen lamp illuminator (150 W) equipped with a bandpass blue filter (B-390, Shimadzu, Japan). In order to remove iodine through reduction reaction, an aqueous solution of 5% sodium thiosulfate was added. After phase separation, the upper phase was collected and sodium thiosulfate was removed by washing with distilled water. The obtained isomer mixture was dried and dissolved in an HPLC mobile phase and subjected to chromatographic separation. In the case of Lut, two separate protocols were applied, preferentially yielding isomerization to 9-*cis* and 9′-*cis* forms and to 13-*cis* and 13′-*cis* forms. In order to obtain a higher concentration of 9-*cis* and 9′-*cis* forms, a solution of all-*trans* lutein in hexane was supplemented with an $I_2$ solution in the same solvent (the final concentration of the catalyst 2% w/w) and incubated for 20 min at 30°C. Iodine was removed from pigment solutions as in the case of isomerization of Zea and m-Zea. In order to obtain higher concentrations of 13-*cis* and 13′-*cis* forms of lutein, a solution of all-*trans* lutein in ethanol was incubated for 2.5 hr at 80°C.

Chemical formulas of the main molecular configuration forms of Lut, Zea and *m*-Zea are shown in Figures S1–S4.

### 2.3 | Xanthophyll separation and purification

Isomers of Zea, *m*-Zea, and Lut were separated and purified chromatographically with the application of a Shimadzu LC-20AD system equipped with an SPD-

M20A diode array detector and with a C-30 coated, phase-reversed column (YMC GmbH, Germany), internal diameter 4.6 mm, length 250 mm, and particle size 5 μm. In the case of Zea and *m*-Zea, a mixture of methanol and methyl tert-butyl ether (95: 5, v/v) was used as a mobile phase and elution rate was 1 ml/min. In the case of lutein, two chromatographic phases (acetonitrile: dichloromethane: methanol, 54: 28: 18, v:v:v) and (acetonitrile: methanol: water, 72: 8: 3, v:v:v) were mixed in the proportion 7:3 and applied isocratically with the elution rate 1.5 ml/min. Chromatographic fractions of xanthophylls were identified according to the literature data.[10,12] Typical HPLC elution profiles along with the absorption spectra of the main xanthophyll fractions are presented in Figures S5–S7.

## 2.4 | Raman spectroscopy

Raman spectroscopy measurements were carried out using an inVia confocal Raman microscope (Renishaw, UK) with an argon laser (Stellar-REN, Modu-Laser™, USA) operating at 488 nm (or at 514.5 nm, when indicated), equipped with 20x long working distance objective (Olympus SLM Plan, NA = 0.25). All spectra were recorded in the spectral region between 445–1885 $cm^{-1}$ at 1-s exposure time, 120 accumulations, with EMCCD Newton 970 camera (Andor Technology, UK) cooled to 223 K. Spectral resolution was 1 $cm^{-1}$ (2,400 lines/mm grating). Measurements were performed with varying output power of the laser, in the range between 1.1 μW (0.05% of the nominal power) and 240.8 μW (10%), as measured in the sample compartment. Samples were scanned at selected temperatures, 298, 196, and 118 K, stabilized with the application of the Linkam THMS600 temperature-controlled stage from Linkam Scientific (UK) equipped with LNP96 and T95-PE controller units. An exact temperature at which spectra were recorded was monitored by a temperature probe placed within the sample compartment. Directly before measurements, xanthophyll samples were dissolved in THF, placed in a 0.1-mm quartz cuvette and incubated for 10 min at the appropriate temperature. Xanthophyll concentration in the samples subjected to Raman spectroscopy measurements was in the range between 0.85 and 1.15 × $10^{-5}$ M. Spectroscopic measurements were also conducted on diluted samples (information provided in appropriate figure legends). All spectra were preprocessed by cosmic ray removing and baseline correction using WiRE 4.2 software from Renishaw, UK. The procedure of subtraction of spectra was performed with the application of the same software. Absorption spectra of carotenoid samples, in the UV-Vis region, were recorded with Cary 60 UV-Vis Spectrophotometer from Agilent.

## 3 | RESULTS AND DISCUSSION

Figures 2–4 present the resonance Raman spectra of Lut, Zea, and *m*-Zea in the molecular configuration 13-*cis*, recorded at 196 K. The spectra recorded at different temperatures, also from the all-*trans* and 9-*cis* isomers of the macular xanthophylls are presented in Figures S8–S16. For each the pigment sample, spectra were recorded several times, with increasing light intensity, in the laser power range between 0.05% and 10%. Four main bands can be resolved in the Raman spectra recorded, typical for carotenoids.[9] The principal $\nu_1$ band in the spectral region between 1,500 and 1,600 $cm^{-1}$, representing the C═C stretching vibrations in the conjugated double bond systems, the $\nu_2$ band in the spectral region between 1,100 and 1,250 $cm^{-1}$, representing the C—C stretching vibrations in the conjugated double bond systems coupled either to the C—H in-plane bending or to the C—$CH_3$ stretching modes, the $\nu_3$ band centering at ~1,000 $cm^{-1}$, representing the $CH_3$ in-plane rocking vibrations and the $\nu_4$ band at ~950 $cm^{-1}$, representing the out-of-plane wagging modes of the ═C—H groups. As can be seen, the exposure to strong light influences the shape of resonance Raman spectra of individual

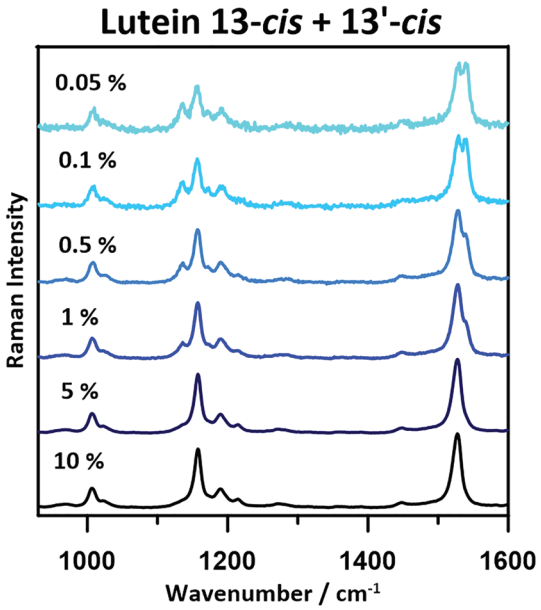


**FIGURE 2** Resonance Raman spectra of lutein in the molecular configuration 13-*cis* and 13′-*cis* recorded with increasing the light intensity of the laser, in the power range between 0.05% and 10% (marked). Spectra were recorded at 196 K, with a 488-nm laser, from the xanthophyll sample in tetrahydrofuran

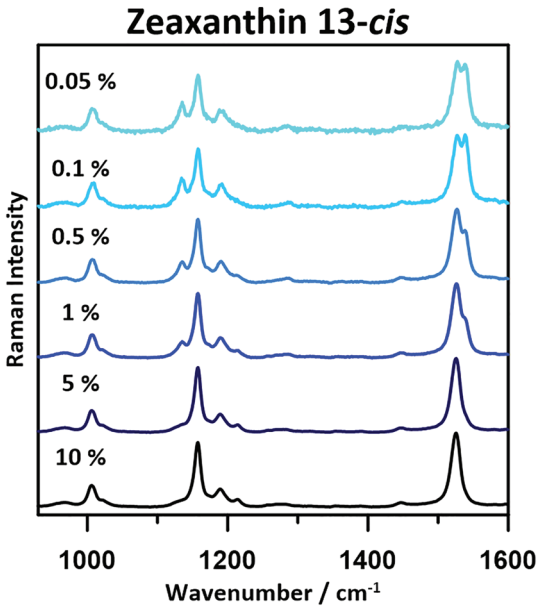


**FIGURE 3** Resonance Raman spectra of zeaxanthin in the molecular configuration 13-*cis* recorded with increasing the light intensity of the laser, in the power range between 0.05% and 10% (marked). Spectra were recorded at 196 K, with a 488-nm laser, from the xanthophyll sample in tetrahydrofuran

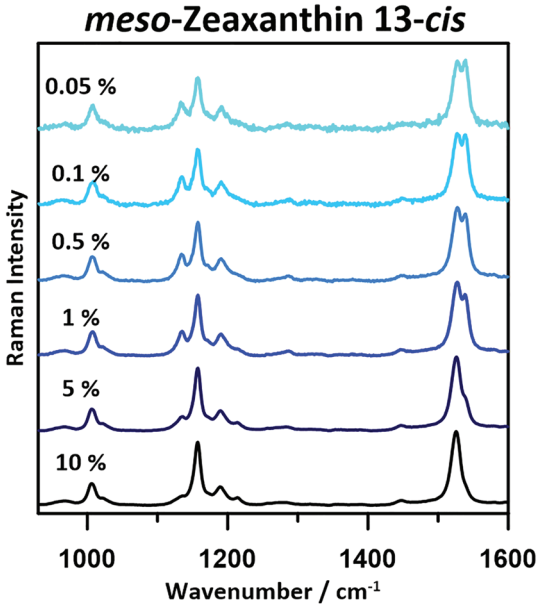


**FIGURE 4** Resonance Raman spectra of *meso*-zeaxanthin in the molecular configuration 13-*cis* recorded with increasing the light intensity of the laser, in the power range between 0.05% and 10% (marked). Spectra were recorded at 196 K, with a 488-nm laser, from the xanthophyll sample in tetrahydrofuran. The *m*-Zea sample contained a mixture of the 13-*cis* and 13′-*cis* isoforms that are indistinguishable both chromatographically and on the basis of ultraviolet–visible absorption spectroscopy

xanthophylls in molecular configuration 13-*cis*, thus pointing possible phototransitions between the molecular configuration forms (Figures 2–4). The splitting of the $\nu_1$ band, visible very clearly in the spectra, is a manifestation of this light-induced process. In contrast, photostability in the same light intensity range can be observed for the all-*trans* (Figures S9, S12, and S15) and 9-*cis* (Figures S10, S13, and S16) molecular configurations. A detailed analysis of the Raman spectra of the pigments in molecular configuration 13-*cis*, recorded with the application of higher laser powers, shows that among the photoconversion products dominate the isoform 9-*cis* (or 9′-*cis*) with some contribution from the form all-*trans* (see Figures S17–S22). It is intriguing why just one molecular configuration (namely 13-*cis*), in the case of all the xanthophylls examined, undergoes the light-induced isomerization. The solvent was chromatographically pure, and the same solvent was used in the case of the other xanthophyll isomers, which were found to be photostable. This minimizes the possibility of a photoisomerization sensitized by solvent impurities. On the other hand, there is a certain probability that possible impurities may possibly interact differently with different carotenoid isomers. A very likely mechanism of the photo-isomerization observed would be a reaction driven by the triplet excited state originating from the singlet-singlet fission in carotenoid molecular aggregated structures.[13] In order to check the possibility of formation of molecular ensembles of xanthophylls in a concentrated solution subjected to Raman spectroscopic analysis, absorption spectra were recorded in the UV-Vis region of the samples both concentrated and diluted. The spectra recorded for Zea in the molecular configuration 13-*cis* and all-*trans* are presented in Figures 5 and 6, respectively. As can be seen from the difference spectrum presented in the lower panel of Figure 5, the concentrated solution of 13-*cis* Zea contains a certain fraction of molecules involved in the excitonic interactions that give rise to the hypsochromic spectral shifts of both the main S0→S2 absorption band originally located between 400 and 500 nm ($1^1A_g^- \rightarrow 1^1B_u^+$) and the so-called "*cis* band" with the maximum at 344 nm ($1^1A_g^- \rightarrow 1^1A_g^+$). This fraction of molecules can potentially sensitize light-driven isomerization observed in the experiments. As can be seen from the absorption spectra presented in Figure 6, the concentrated solution of Zea in the molecular configuration all-*trans* does not show spectral shifts diagnostic for formation of molecular aggregates in this system. The fact that xanthophyll molecules in such a system are photostable, in contrast to Zea 13-*cis* involved in the formation of molecular structures and undergoing the light-induced isomerization, leads to the conclusion that this process can be

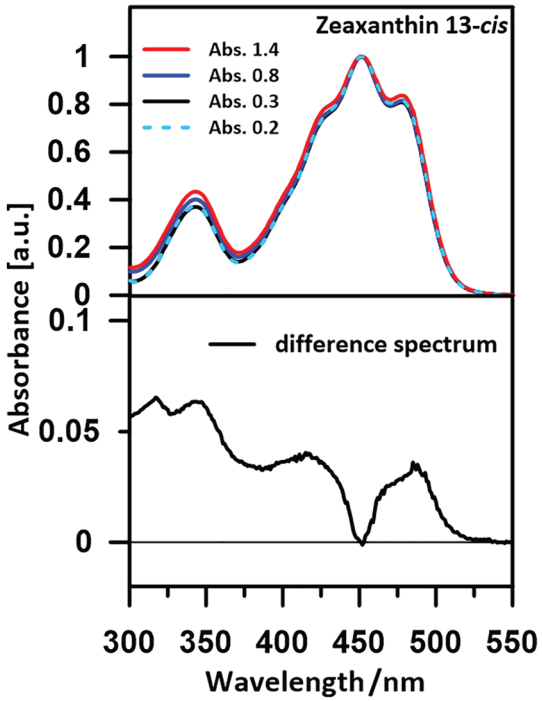


**FIGURE 5** Absorption spectra of zeaxanthin in the molecular configuration 13-*cis* in tetrahydrofuran solution used for resonance Raman analysis (absorbance level at the maximum 1.4 in a 1-cm cuvette) and diluted to the absorbance levels 0.8, 0.3, and 0.2, indicated. The pure solvent was recorded as a reference. The spectra were normalized at the maximum. The lower panel presents the difference spectrum calculated from the spectra presented in the upper panel: concentrated (absorbance 1.4) minus diluted (absorbance 0.3)

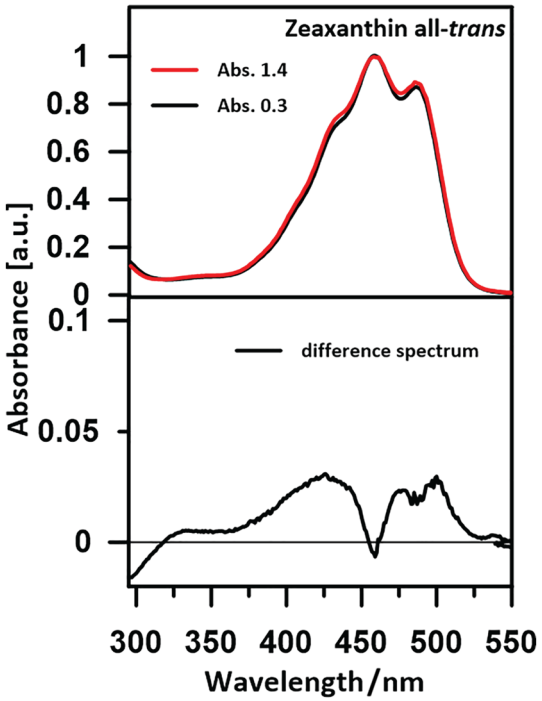


**FIGURE 6** Absorption spectra of zeaxanthin in the molecular configuration all-*trans* in tetrahydrofuran solution used for resonance Raman analysis (absorbance level at the maximum 1.4 in a 1-cm cuvette) and diluted to the absorbance level 0.3, indicated. The pure solvent was recorded as a reference. The spectra were normalized at the maximum. The lower panel presents the difference spectrum calculated from the spectra presented in the upper panel (concentrated minus diluted)

potentially sensitized by the triplet states generated via the singlet-singlet fission. To test whether some other mechanisms could lead to light-induced isomerization, observed specifically for the 13-*cis* xanthophylls, Raman spectra were recorded from diluted samples in which the absorption spectra showed no signs of pigment aggregation (see Figure 5). Moreover, Raman spectra from such samples were additionally recorded with application of the laser line 514.5 nm that is out of resonance with the aggregated forms of the 13-*cis* isomers of Zea owing to the hypsochromic spectral shift (see the difference spectrum in Figure 5). The results of the experiments (Figures S23 and S24) show that 13-*cis* Zea in a monomeric form also undergoes the light-induced isomerization. The photoisomerization of the 13-*cis* isomers can be explained in terms of different excited state dynamics that enables effective photoisomerization to other molecular configurations. It is possible that the intramolecular charge transfer state, observed for the 13-*cis* isomer of another polar carotenoid fucoxanthin, distinctively different than in the case of the all-*trans* form,[14] could be involved in a photo-conversion of the 13-*cis* isomers to other molecular configurations. On the other hand, the operation of both mechanisms, the singlet fission and intramolecular charge transfer, and/or other mechanisms may not be excluded. An interesting and important issue is the light-induced isomerization of xanthophylls in the environment of THF that is observed even at 118 K (see Figure S14). Taking into consideration the fact that the freezing point of THF is at 164.8 K, one can presume that the photoisomerization observed at 118 K involves a local melting of the pigment-environment, induced by a thermal deexcitation. It is noteworthy that light-driven isomerization of the 13-*cis* forms of the macular xanthophylls was observed to be substantially more effective at higher temperatures (196 K and 298 K; see Figures 2–4 andS8). In addition to the obvious kinetic reasons of such an effect one can additionally consider a structure-stabilizing effect of THF that is frozen at 118 K. The subtraction of the spectra of the 9-*cis* and all-*trans* forms from the original Raman spectra recorded from the chromatographically pure samples of the 13-*cis* isomers yields the "pure" spectra that can be attributed to the molecular

configuration 13-*cis* (Figures S17–S22). As can be seen from the comparison of the spectra derived for the 13-*cis* forms of *m*-Zea, Zea and Lut, the procedure applied yields very similar final spectra, independently of actual laser power and differences in scaling factors selected to subtract the spectra representing the contribution of the 9-*cis* and all-*trans* forms. As mentioned above, the light-induced conversions of the 13-*cis* forms of all the macular xanthophylls are more efficient at the room temperature (Figure S8) as compared with lower temperatures (196 K, Figure S11 and 118 K, Figure S14). This can be judged based on a comparison of the spectral splitting in the $\nu_1$ region at the same laser powers. On the other hand, the other molecular configurations of all the macular xanthophylls (all-*trans* and 9-*cis*) were found to be photostable even at the room temperature, in the same power range of the laser (see Figures S9 and S10). The Raman spectra of the main molecular configuration forms of Lut, Zea, and *m*-Zea, recorded at 196 K, are presented in Figures 7–9 (and recorded at other temperatures in Figures S25–S30). As can be expected,

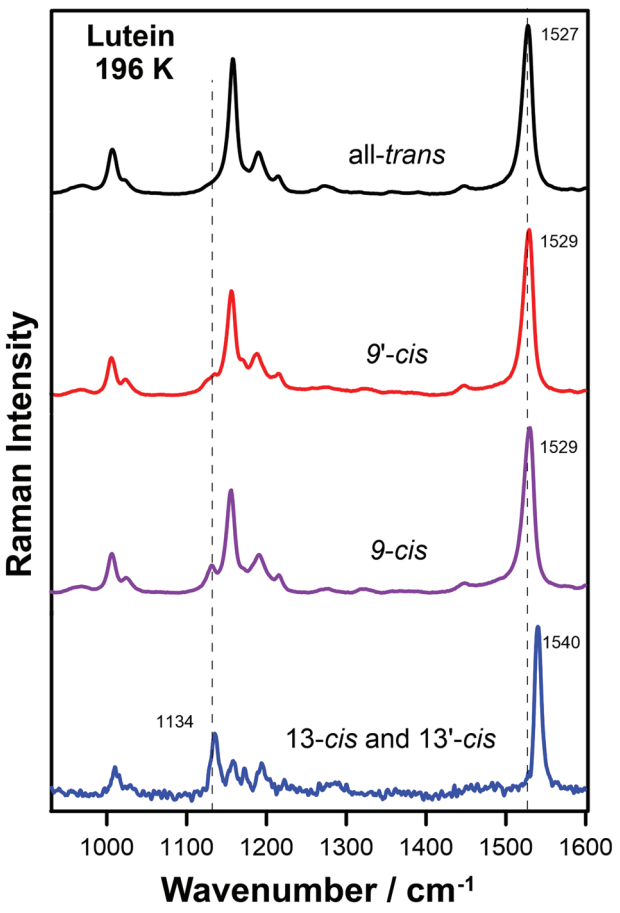


**FIGURE 7** Comparison of the resonance Raman spectra of lutein in the molecular configurations all-*trans*, 9-*cis*, 9′-*cis* and the mixture of 13-*cis* and 13′-*cis*, indicated. Recorded at 196 K, with a 488-nm laser, from the samples in tetrahydrofuran

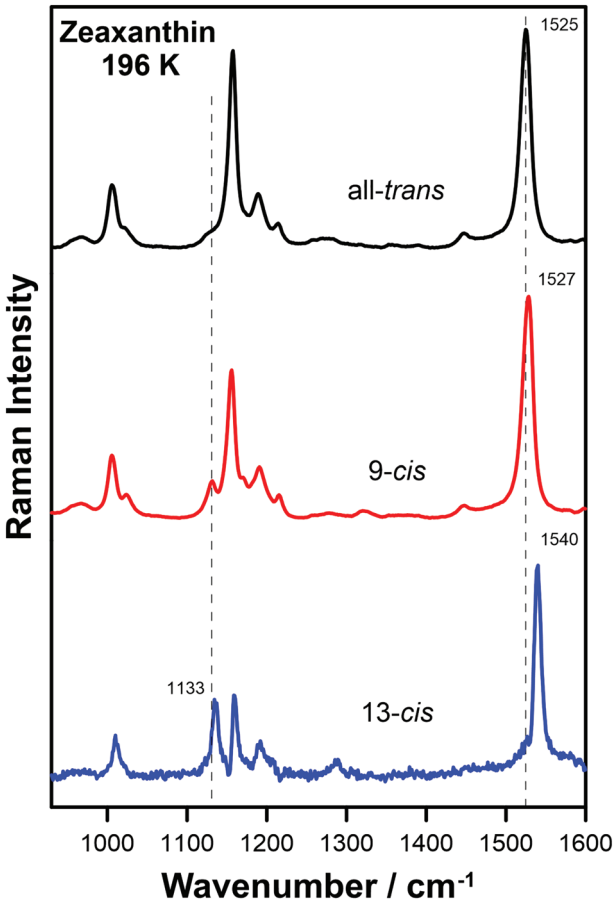


**FIGURE 8** Comparison of the resonance Raman spectra of zeaxanthin in the molecular configurations all-*trans*, 9-*cis*, and 13-*cis*, indicated. Recorded at 196 K, with a 488-nm laser, from the samples in tetrahydrofuran

pigments in the configuration *cis* present $\nu_1$ bands shifted towards higher wavenumbers with respect to molecular configuration all-*trans*, owing to slightly shortened conjugation length.[15,16] Importantly, the specific features of the Raman spectra of macular xanthophylls in the $\nu_2$ region correspond very well to the same isoforms of β-carotene.[15,16] It can be concluded that intensity of the peripheral band in the $\nu_2$ spectral region, centering at ~1133 $cm^{-1}$, relative to the intensity of the principal $\nu_2$ band, centering at ~1157 $cm^{-1}$, is diagnostic and critical for an assignment of appearance of *trans-cis* molecular configuration forms of carotenoids, including the macular xanthophylls Lut, Zea, and *m*-Zea. Knowledge of Raman spectra of molecular configuration forms of the xanthophylls opens an avenue to study their photostability and possible photoconversion in complex natural systems, including the retina of the eye. The resonance Raman spectra recorded at room temperature with the 488-nm laser line from the macula lutea of the human retina, prepared post mortem[7,17] and in vivo[17]

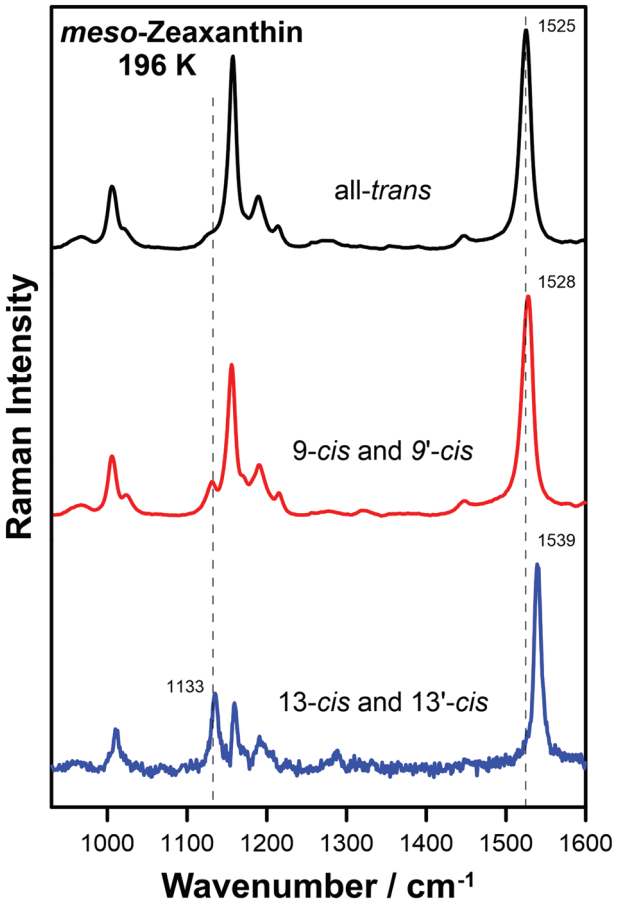


**FIGURE 9** Comparison of the resonance Raman spectra of *meso*-zeaxanthin in the molecular configurations all-*trans*, the mixture of 9-*cis* and 9′-*cis*, and the mixture of 13-*cis* and 13′-*cis*, indicated. Recorded at 196 K, with a 488-nm laser, from the samples in tetrahydrofuran

are very close to the all-*trans* lutein, recorded in the present work.

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## REFERENCES

[1] A. Kaczor, M. Baranska, *Carotenoids. Nutrition, Analysis and Technology*, Wiley, Oxford **2016**.
[2] T. Bohn, *Int. J. Vitam. Nutr. Res.* **2018**, *87*, 1.
[3] J. Fiedor, K. Burda, *Nutrients* **2014**, *6*, 466.
[4] P. S. Bernstein, B. X. Li, P. P. Vachali, A. Gorusupudi, R. Shyam, B. S. Henriksen, J. M. Nolan, *Prog. Retin. Eye Res.* **2016**, *50*, 34.
[5] R. A. Bone, J. T. Landrum, L. M. Friedes, C. M. Gomez, M. D. Kilburn, E. Menendez, I. Vidal, W. Wang, *Exp. Eye Res.* **1997**, *64*, 211.
[6] R. Arunkumar, C. M. Calvo, C. D. Conrady, P. S. Bernstein, *Eye* **2018**, *32*, 992.
[7] A. A. Arteni, M. Fradot, D. Galzerano, M. M. Mendes-Pinto, J. A. Sahel, S. Picaud, B. Robert, A. A. Pascal, *Plos One* **2015**, *10*, e0135779.
[8] P. S. Bernstein, D. Y. Zhao, M. Sharifzadeh, I. V. Ermakov, W. Gellermann, *Arch. Biochem. Biophys.* **2004**, *430*, 163.
[9] B. Robert, *Photosynth. Res.* **2009**, *101*, 147.
[10] J. Milanowska, W. I. Gruszecki, *J. Photochem. Photobiol. B: Biol.* **2005**, *80*, 178.
[11] W. Grudzinski, E. Janik, J. Bednarska, R. Welc, M. Zubik, K. Sowinski, R. Luchowski, W. I. Gruszecki, *J. Phys. Chem. B* **2016**, *120*, 4373.
[12] D. J. Li, Y. D. Xiao, Z. Y. Zhang, C. Q. Liu, *J. Pharm. Biomed. Anal.* **2014**, *100*, 33.
[13] C. Wang, C. J. Berg, C. C. Hsu, B. A. Merrill, M. J. Tauber, *J. Phys. Chem. B* **2012**, *116*, 10617.
[14] V. Kuznetsova, P. Chabera, R. Litvin, T. G. Polivka, M. Fuciman, *J. Phys. Chem. B* **2017**, *121*, 4438.
[15] Y. Koyama, in *Carotenoids*, (Eds: G. Britton, S. Liaaen-Jensen, H. Pfander), Birkhauser, Dordrecht **1995**; Vol. Volume 1B: Spectroscopy 135.
[16] Y. Koyama, R. Fujii, in *The Photochemistry of Carotenoids*, (Eds: H. A. Frank, A. J. Young, G. Britton, R. J. Cogdell), Kluwer Academic Publ, Dordrecht **1999** 161.
[17] W. Gellermann, I. V. Ermakov, M. R. Ermakova, R. W. McClane, D. Y. Zhao, P. S. Bernstein, *J. Opt. Soc. Am. A Opt. Image Sci. Vis.* **2002**, *19*, 1172.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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