

Noninvasive selective detection of lycopene and β -carotene in human skin using Raman spectroscopy

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Abstract. The predominant long-chain carotenoids found in human skin are lycopene and β -carotene. They are powerful antioxidants and thought to act as scavengers for free radicals and singlet oxygen formed by normal metabolism as well as excessive exposure of skin to sunlight. The specific importance of the particular representatives of the carotenoid antioxidants regarding skin defense mechanisms is of strong current interest. We demonstrate fast and noninvasive detection of β -carotene and lycopene concentrations in living human skin using Raman detection of the molecules' carbon-carbon double bond stretch vibrations. Employing excitation with suitable blue and green laser lines, and taking advantage of differing Raman cross sectional profiles for β -carotene and lycopene, we determine the relative concentration of each carotenoid species. This novel technique permits the quantitative assessment of individual long-chain carotenoid species rather than their composite level in human skin. The obtained results reveal significant differences in the carotenoid composition of the subjects' skin and show that the ratio between β -carotene and lycopene concentration can vary from 0.5 to 1.6. The technique holds promise as a method for rapid screening of carotenoid compositions in human skin in large populations and should be suitable for clinical studies correlating carotenoid status with risk for cutaneous diseases.

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1 Introduction

Carotenoid molecules play an important role in the skin's antioxidant defense system.¹ The five most concentrated carotenoid antioxidants in human skin are lycopene, α -carotene, β -carotene, phytoene, and phytofluene, with lycopene and the carotenes accounting for about 60%–70% of total carotenoid content (see Table 1 and Ref. 2). They are thought to act as scavengers for free radicals,³ singlet oxygen,^{4,5} and other harmful reactive oxygen species⁶ which are all formed, e.g., by metabolic processes or by excessive exposure of skin to the ultraviolet components of sunlight. If unbalanced by antioxidants, the destructive effects of reactive oxygen species and free radicals can lead to skin malignancies and disease. In animal models carotenoids have been shown to inhibit carcinoma formation in the skin.⁷ It has been shown that skin carotenoid levels are strongly and significantly correlated with carotenoid levels in plasma.⁸ As is found in plasma, skin carotenoid levels are lower in smokers than in nonsmokers. β -carotene levels in skin are known to increase with supplementation,⁹ and supplemental β -carotene is used to treat patients with erythropoietic protoporphyria, a photosensitive disorder.¹⁰ Supplemental carotenoids have also been shown to delay erythema in normal healthy subjects exposed

to UV light.^{11–13} There is limited evidence that they may be protective against skin malignancies,² but more research is needed to confirm these findings.

For many decades, the standard technique for measuring carotenoids has been high-pressure liquid chromatography (HPLC). This time consuming and expensive chemical method works well for the measurement of carotenoids in serum, but it is difficult to perform in skin tissue since it requires biopsies of relatively large tissue volumes. Additionally, serum antioxidant measurements are more indicative of short-term dietary intakes of antioxidants rather than steady-state accumulations in body tissues exposed to external oxidative stress factors such as smoking and UV-light exposure. Nevertheless, the scientific basis of carotenoid function in the human body has been extensively studied for over 30 years using the HPLC methodology.

Recently we developed resonance Raman spectroscopy as a novel noninvasive optical alternative to HPLC for the measurement of carotenoids in living human tissues, including the human retina, skin, and oral mucosal tissue.^{14–16} In skin, the absolute levels of carotenoids are much lower than in the human retina, but the laser power can be much higher and acquisition times can be much longer to compensate for this. Background fluorescence of the tissue can be quite high, but base line correction algorithms are still adequate to yield caro-

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Table 1 Carotenoid composition of human skin in ng per g tissue (adopted from Ref. 2).

Carotenoid	Skin source			Mean
	1	2	3	
Lycopene+Z-isomers	105	9	93	69
Carotenes α , β , γ , ξ	96	8	55	53
Lutein+Zeaxanthin	26	ND ^a	ND ^a	9
Phytoene+Phytofluene	113	51	74	79
Total	340	68	222	210

^a Nondetectable.

tenoid resonance Raman spectra with excellent signal-to-noise ratios. The Raman method exhibits excellent sensitivity and specificity² and is an appealing alternative to reflectance methods¹⁷ since it does not require complex correction models. Also, this method allows one to measure absolute carotenoid levels in these tissues employing suitable calibration procedures, so the method does not have to rely on induced concentration changes. Deep melanin pigmentation likely interferes with penetration of the laser beam, so measurements are routinely performed on the palm of the hand where pigmentation is usually quite light even in darkly pigmented individuals. As with reflectometry,¹⁷ relatively high levels of skin carotenoids are measured by the Raman method on the forehead and on the palm of the hand, while other body areas are lower.²

Measurements of large populations with the Raman device have demonstrated a bell-shaped distribution of carotenoid levels in the palm of the hand, as shown in Fig. 1 for a group of 57 healthy volunteers (both genders, 25–75 years of age). Clearly, the histogram of Raman carotenoid response demonstrates a strong between-subject variability. Approximately

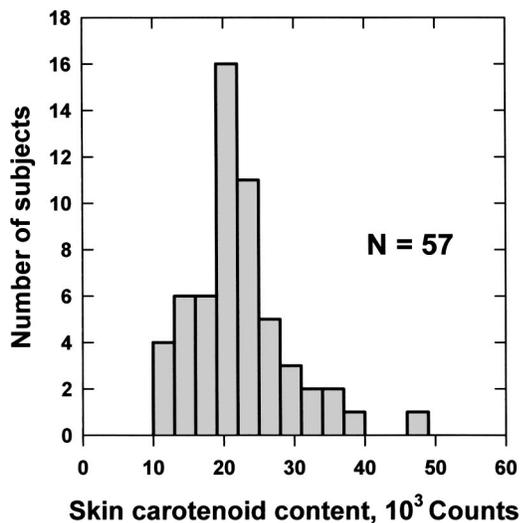


Fig. 1 Histogram of Raman measurements for a group of 57 healthy subjects, showing a wide distribution of carotenoid levels in the large subject base. Measured skin site is the inner palm.

50% of all subjects form the center (average) of the distribution. Another 50% contribute to the wings of the distribution, causing a fivefold variation of carotenoid content (Raman signals varying from 10 000 to 49 000 counts). In previous measurements² we established the short-term and day-to-day-repeatability of the Raman response of a particular subject to be within 10% for the same skin region. Therefore we interpret the between-subject variation in Fig. 1 as substantial evidence for significant variations in tissue carotenoid content in different subjects.

Field studies have recently been carried out where a population of 1375 healthy subjects could be screened within a period of several weeks.¹⁸ Preliminary analysis of the data confirmed that smokers had dramatically lower levels of skin carotenoids as compared to nonsmokers. Furthermore, the study showed that people with habitual high sunlight exposure have significantly lower skin carotenoid levels than people with little sunlight exposure, independent of their carotenoid intake or dietary habits.

In all previous Raman measurements of dermal carotenoids we measured the composite level of the long-chain carotenoid species since they all are simultaneously excited under the used visible excitation conditions and all contribute to the overall Raman response. Short-chain carotenoids like phytoene and phytofluene absorb in the near-UV wavelength range and are not resonantly excited with visible laser excitation. In this paper we demonstrate a technique for the selective measurement of two important representatives of the carotenoids in human skin, i.e., carotenes and lycopene. According to a HPLC analysis of excised skin samples, these carotenoid species are the most dominant ones in this particular tissue (see Table 1), as they are in human blood,⁸ and they are thought to play special roles in the human body. For example, there is considerable interest in a specific role for lycopene in prevention of prostate cancer and other diseases,^{13,19} and a noninvasive biomarker for lycopene consumption would be of tremendous utility.

2 Optical Properties and Raman Scattering of β -Carotene and Lycopene

Lycopene and β -carotene are π -electron conjugated carbon-chain molecules. Their chemical structures are shown in the inset of Fig. 2. The “backbone” of these molecules consists of alternating carbon single and double bonds. The β -carotene molecule has nine conjugated carbon double bonds along its backbone and four methyl groups attached as side groups. Furthermore, the molecule is terminated on each end by an ionone ring, each having an additional internal carbon bond, and each adding to the effective conjugation length of the molecule. Lycopene has 11 conjugated carbon double bonds along its backbone, two un-conjugated double bonds, and no end groups.

The stretching vibrations of the carbon double and single bonds of the carotenoids, as well as the rocking motion of the methyl side groups, can be detected with Raman spectroscopy.²⁰ In Fig. 2 we show the absorption spectra for a solution of β -carotene and lycopene dissolved in methanol. The electronic absorptions are strong in each case, occur in broad bands (~ 100 nm width), and are centered at ~ 450 and 460 nm, respectively. Both show a clearly resolved substructure.

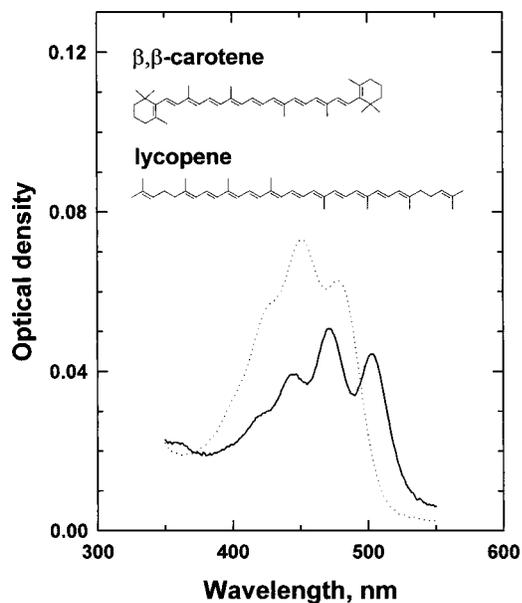


Fig. 2 Absorption spectra of solutions of lycopene (solid line) and β -carotene (dotted line) in methanol. At top, the structures of both molecules are shown. Note the vibronic substructure of the absorption bands and the noticeable spectral shift originating from the difference in effective conjugation length of the two molecules.

ture with a spacing of $\sim 1400\text{ cm}^{-1}$. The spectral shift between both bands is a result of the different effective conjugation lengths of the two carotenoids. This absorption behavior is due to electric-dipole allowed vibronic transitions of the molecule's conjugated π -electron from the 1^1A_g singlet ground state to the 1^1B_u singlet excited state.

Optical excitation within the absorption band leads to very weak, Stokes-shifted luminescence bands (width $\sim 70\text{ nm}$) centered at $\sim 530\text{ nm}$ in both cases (not shown). The extremely low quantum efficiency of the luminescence is caused by the existence of a second excited singlet state, a 2^1A_g state, which lies below the 1^1B_u state. Following excitation of the 1^1B_u state, the carotenoid molecule relaxes very rapidly, within $\sim 200\text{--}250\text{ fs}$,²¹ via nonradiative transitions, to this lower 2^1A_g state from which electronic emission to the ground state is parity forbidden. The low $1^1B_u \rightarrow 1^1A_g$ luminescence efficiency ($10^{-5}\text{--}10^{-4}$) and the absence of $2^1A_g \rightarrow 1^1A_g$ fluorescence of the molecules allow us to explore the resonant Raman scattering response of the molecular vibrations while avoiding potentially masking fluorescence signals.

Indeed, measuring the resonance Raman spectrum of β -carotene and lycopene in acetone, we observe strong and clearly resolved Raman signals superimposed on a weak fluorescence background, as shown in Fig. 3, where we used 488 nm as well as 514.5 nm argon laser excitation. The Raman response in both cases is characterized by two prominent Stokes lines at ~ 1159 and 1524 cm^{-1} , which have nearly identical relative intensities. These lines originate, respectively, from carbon-carbon single-bond and double-bond stretch vibrations of the conjugated backbone; the 1008 cm^{-1} line is attributed to rocking motions of the molecule's methyl components.²⁰ We found that other carotenoids found in human skin such as zeaxanthin, lutein, canthaxanthin, and astax-

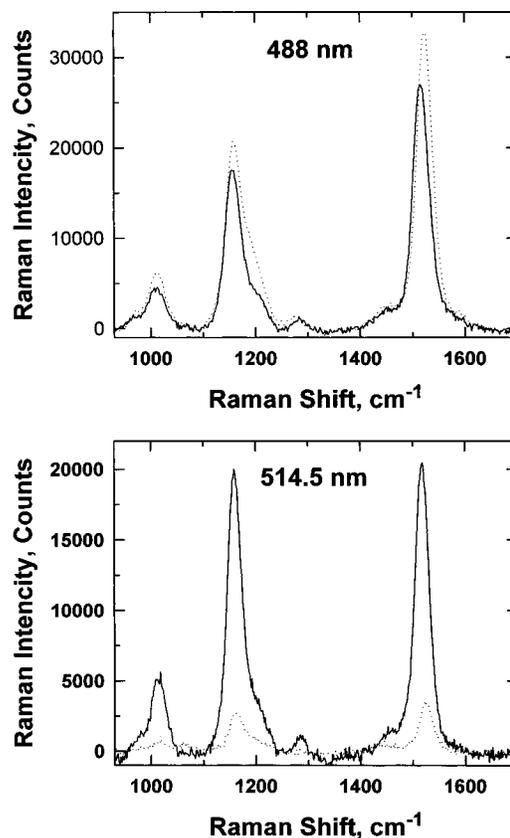


Fig. 3 Resonance Raman spectrum of an acetone solution of lycopene (solid lines) and β -carotene (dotted lines), measured under argon laser excitation at 488 nm (upper panel) and 514.5 nm (bottom panel). Both solutions had identical carotenoid concentrations. Raman spectra were recorded using identical excitation power and sensitivity-matched instruments. Strongest Raman peaks correspond to the stretch vibrations of the carbon single and double bonds of the molecule (at ~ 1159 and $\sim 1525\text{ cm}^{-1}$, respectively).

anthin had virtually identical resonance Raman spectra at room temperature within the resolution limit ($\sim 10\text{ cm}^{-1}$) of our spectrometer.

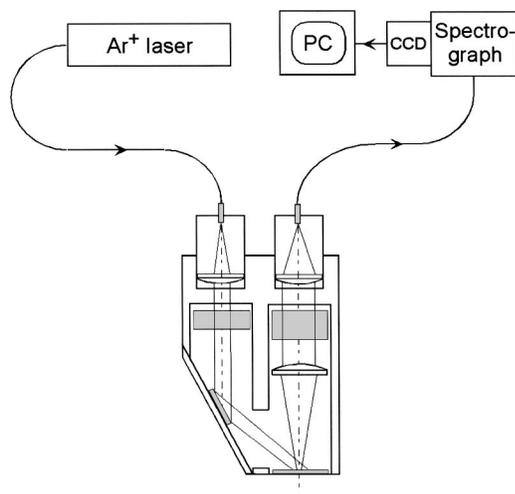
As seen in Fig. 3 and Table 2, the Raman signal excitation efficiency for lycopene and β -carotene under 488 nm radiation is approximately the same. However, under irradiation with 514.5 nm wavelength, the Raman excitation efficiency for lycopene is a factor of about 6 higher than for β -carotene. This is easily understood since the *resonance* Raman scattering cross section usually follows the absorption spectral profile²² and since the lycopene absorption band is shifted by $\sim 10\text{ nm}$ toward longer wavelengths compared to β -carotene as a consequence of the increased conjugation length. This spectral difference in absorption then makes it possible to measure either the composite response of these carotenoids or to measure their individual contributions. The composite response is obtained by excitation with 488 nm, where β -carotene and lycopene contribute almost equally toward the overall Raman response. The individual responses are obtained from measuring, in addition, the Raman response under excitation with a green laser line, which is chosen to excite mostly the long-wavelength shoulder of lycopene.

Table 2 Relative excitation efficiency of the C=C double bond Raman line for β -carotene and lycopene dissolved in acetone.

Carotenoid	Excitation wavelength	
	488.0 nm	514.5 nm
β -carotene	1.00	0.11
lycopene	0.82	0.62

3 Instrumentation

The prototype Raman instrument used in combination with a hand-held light delivery and collection module is schematically shown in Fig. 4. It has been described in detail elsewhere.¹⁴ The instrument is suitable for Raman measurements on human skin *in vivo*. It consists of a small air-cooled 488 nm argon laser, a small light delivery and collection module, "Raman probe," and a high throughput spectrograph that is coupled to a charge coupled device (CCD) camera. For a typical measurement, the Raman probe window is placed in contact with the skin. The excitation laser light is routed through an optical fiber, collimated with a lens, filtered with a narrow-band dielectric filter, and directed onto the skin where it illuminates a 2-mm-diam spot. The Raman scattered light is collected in an off-180° backscattering geometry with a second lens, passed through a holographic notch filter and routed via fiber bundle to the spectrograph. The spectrograph camera is interfaced to a personal computer. In all measurements on living human skin we used a laser power of 5 mW and an exposure time of 20 s. Taking into account the 2 mm laser light spot size on the skin, this results in an intensity of 0.16 W/cm² at the skin surface, which is considered safe by ANSI Z136.1-2000 standards.²³ In fact, for the used laser intensity on the skin, the exposure time required for a measurement is about a factor of 1000 below the maximum allowed as set under this safety standard. The probe window material, fused silica, was seen to have no confounding effects on the measurements since its optical response was negligible.

**Fig. 4** Schematics of the carotenoid Raman detector, showing beam delivery and collection optics (not to scale).

For selective lycopene and β -carotene measurements we constructed a second Raman instrument with identical mechanical and optical design, but using a 514.5 nm argon laser for excitation and using suitable laser line and holographic notch filters. To calibrate the instruments we used Raman active KNO₃ powder as a standard. We measured the relative intensities of the strongest KNO₃ Raman line, originating from the A_g vibrational mode of the (NO₃)⁻ ion under visible, nonresonant excitation with 488 and 514.5 nm, and compared them with the Raman intensities expected according to their λ^{-4} wavelength dependence. Then the sensitivity of the 514.5 nm instrument was adjusted to the sensitivity of the 488 nm device. Since the Raman response of the calibration material is spectrally close to that of the carotenoids, the correction coefficient established in this way compensates for any differences in the spectral response of the CCD detectors and other optical components such as grating and holographic notch filters of the spectrograph.

4 Selective Detection of β -Carotene and Lycopene: Experiments with Living Human Skin

Based on the spectral absorption shifts of lycopene and β -carotene, we investigated their Raman response in human skin using two-wavelength excitation with 488 and 514.5 nm argon laser lines. A skin site suited particularly well for carotenoid Raman measurements is the inner palm, which, besides accessibility, has several other advantages: (a) carotenoid concentrations are among the highest of all sites² (since carotenoids are lipophilic, and since the skin of the palm has a high lipid-to-protein ratio), (b) the differences in pigmentation among various skin types are minimal in the palm, and (c) the *stratum corneum* thickness of the palm ($\sim 400 \mu\text{m}$) is high compared with other skin sites. The small laser light penetration depth in this highly scattering skin layer will confine our measurement to the *stratum corneum*. Assuming that the morphology of the *stratum corneum* does not change appreciably from person to person, a consistent intra-subject sampling will then be realized.

Following the calibration procedures outlined above, we measured the inner palm of seven healthy volunteers using the 488 and 514 nm Raman probes in succession. Care was taken to measure the same tissue spot with each probe; a potential mismatch was estimated to be smaller than 0.5 mm. Since the skin Raman response changes only very gradually throughout the palm (it is essentially the same within a 1-cm-diam area), this minor sampling mismatch would not cause any errors caused by measuring tissue sites with different concentrations. A typical skin Raman carotenoid spectrum obtained from a subject with the "488 nm instrument" is shown in Fig. 5. Similar spectra were measured with the "514.5 nm instrument."

The strongest feature of the optical response is a spectrally broad background response originating from skin autofluorescence (which presumably is caused by collagen, porphyrins, elastin, etc.), and not by carotenoids.² Superimposed on the large fluorescence background response are the characteristic Raman peaks of the carotenoids, which appear as small bumps in the top trace of Fig. 5. In the course of the measurements we discovered that the fluorescence background bleached partially over a time period of several minutes.¹⁴

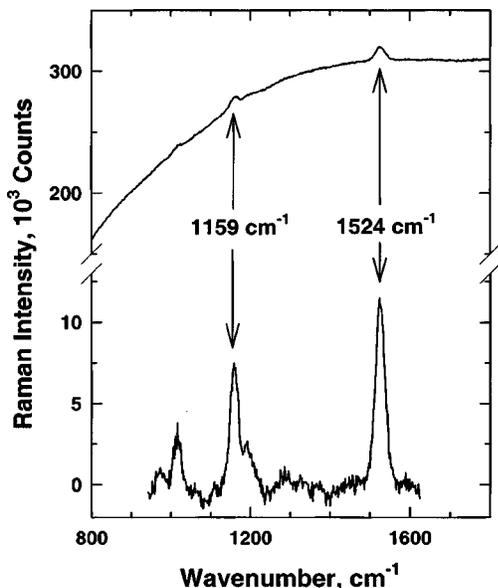


Fig. 5 Typical Raman spectra for human skin, measured *in vivo* under 488 nm laser excitation. Top trace—spectrum obtained before subtraction of autofluorescence background; bottom trace—after subtraction.

While the fluorescence signal is seen to significantly decrease due to bleaching, the Raman signal levels remain unchanged.¹⁴ Raman peaks can be retrieved with excellent resolution and high signal-to-noise ratio. This is demonstrated in the lower trace of Fig. 5, where the fluorescence background has been fitted with a fourth-order polynomial and subtracted from the original spectrum. The two strong Raman peaks correspond to the 1159 and 1524 cm⁻¹ carbon–carbon single- and double-bond stretching vibrations, respectively, of the carotenoid molecules, and their peak heights correlate with the existing carotenoid concentrations in the skin.

5 Selective Detection of β -Carotene and Lycopene: Data Analysis

The amplitudes of the C=C Raman peaks obtained under resonant 488 nm excitation, $I^{488}(\text{C}=\text{C})$, and under 514.5 nm excitation, $I^{514}(\text{C}=\text{C})$, were measured for the inner palm of seven healthy subjects. They are shown in the second and third column of Table 3. Values for the Raman signal obtained from the skin under 514.5 nm excitation were corrected as described in Sec. 3.

We consider a very simple model for the skin Raman response, assuming that only two carotenoids, β -carotene and lycopene, contribute to the Raman signal of human skin, and are excited either with 488 or 514.5 nm laser light. In this case, the measured amplitude of the C=C Raman peak under 488 nm excitation, I^{488} , can be described by

$$I^{488} = k^{488}(\sigma_B^{488}N_B + \sigma_L^{488}N_L), \quad (1)$$

where N_B and N_L are the concentrations of β -carotene and lycopene in the particular skin sample, and σ_B^{488} and σ_L^{488} are the resonance Raman scattering cross sections for β -carotene

Table 3 Experimental intensities, I , of the C=C double bond Raman line measured for the palms of seven volunteer subjects under blue and green laser excitation. The ratio N_B/N_L , determined with Eq. (3), indicates the ratio of β -carotene to lycopene in the subject's skin.

Subject ID	$I^{488}(\text{C}=\text{C})$	$I^{514}(\text{C}=\text{C})$	$r = I^{488}/I^{514}$	N_B/N_L
I	26 622	12 393	2.1	0.7
M	17 174	6445	2.7	1.2
W	28 830	11 743	2.5	1.0
P	23 051	7709	3.0	1.55
R	23 088	10 483	2.2	0.76
J	28 125	13 968	2.0	0.54
U	35 000	11 619	3.0	1.55

and lycopene under excitation with 488 nm, respectively. The signal measured under 514.5 nm laser excitation, I^{514} , is described with the similar expression

$$I^{514} = k^{514}(\sigma_B^{514}N_B + \sigma_L^{514}N_L). \quad (2)$$

Here, σ_B^{514} and σ_L^{514} are resonance Raman scattering cross sections for β -carotene and lycopene under 514.5 nm excitation.

Since the optical properties of healthy human skin (absorption and scattering) do not change drastically over the narrow spectral range from 488 to 514.5 nm, as can be seen from Fig. 6, we can assume that they are essentially the same for either excitation wavelength. A similar assumption can be made for the Raman responses (at 527 and 558 nm), and therefore it can be assumed that the sampling volume for either excitation wavelength will be the same. Furthermore, the constants k^{488} and k^{514} in formulas (1) and (2) then represent the respective

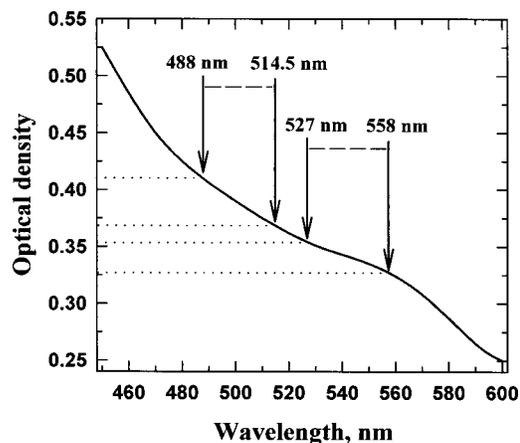


Fig. 6 Absorption spectrum of normally pigmented human skin in the visible spectral range. The spectrum, adapted from Ref. 24, shows a slowly changing monotonically declining absorption behavior. Note the small difference in absorption for two pairs of wavelengths corresponding to excitation (488 and 514.5 nm) and Raman (527 and 558 nm) wavelengths.

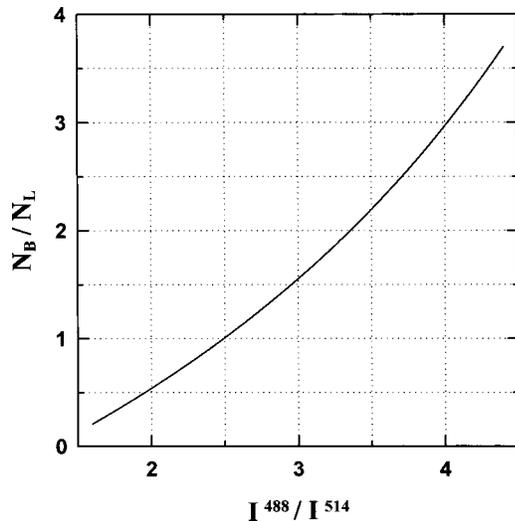


Fig. 7 Plot used to evaluate the carotenoid composition of human skin if the Raman response of the skin under 488 nm and under 514.5 nm is known. The ratio of β -carotene to lycopene concentration in human skin, N_B/N_L , scales according to expression (4).

sensitivities of the 488 and 514.5 nm instruments, provided that the 488 and 514.5 nm Raman probes deliver an equal intensity to the skin.

Combining Eqs. (1) and (2), we can obtain an analytical expression (3), linking the ratio N_B/N_L , i.e., β -carotene concentration over lycopene concentration, to the ratio of measured Raman signals, $r = I^{488}/I^{514}$, and to the optical parameters of the carotenoid molecules

$$\frac{N_B}{N_L} = \frac{\alpha\sigma_L^{488} - \sigma_L^{514}r}{\sigma_B^{514}r - \alpha\sigma_B^{488}} \quad (3)$$

In this expression $\alpha = k^{488}/k^{514}$ and equals 1 if the 488 nm instrument has the same sensitivity as the 514.5 nm instrument and if both probes deliver the same intensity at the skin. For practical applications, expression (3) can be rewritten as

$$\frac{N_B}{N_L} = \frac{0.82 - 0.62r}{0.11r - 1} \quad (4)$$

where we use a relationship between the resonant Raman cross sections of the carotenoids as reported in Table 2. A plot of expression (4), shown in Fig. 7 and giving N_B/N_L as a function of r , can be used to find the ratio of β -carotene to lycopene, N_B/N_L . The following expressions (5) and (6), obtained from Eq. (1), can be used to find the relative concentration of lycopene, N_L , and β -carotene, N_B

$$N_L = \frac{1}{k^{488}\sigma_B^{488}} \cdot I^{488} \cdot \left(\frac{N_B}{N_L} + 0.82\right)^{-1} \sim I^{488} \cdot \left(\frac{N_B}{N_L} + 0.82\right)^{-1} \quad (5)$$

$$N_B = N_L \cdot \left(\frac{N_B}{N_L}\right) \sim I^{488} \left(\frac{N_B}{N_L}\right) \cdot \left(\frac{N_B}{N_L} + 0.82\right)^{-1} \quad (6)$$

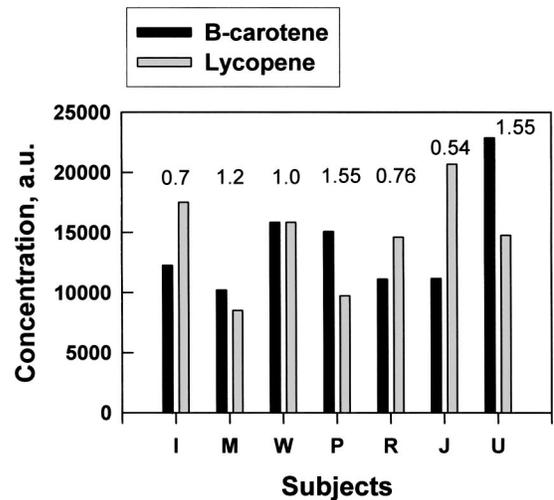


Fig. 8 Carotenoid composition of human inner palm skin obtained for seven healthy volunteers using selective resonant Raman spectroscopy.

The results are tabulated in Table 3 and are also shown as a bar graph in Fig. 8 along with the relative concentrations of each carotenoid species found in the skin of the subjects.

As seen from Table 3, there is a strong, almost threefold, variation in carotene-to-lycopene ratio even in a relatively small group of human subjects, ranging from 0.54 to 1.55. That means that substantially different carotenoid compositions exist in normal human skin. Some subjects exhibit almost twice the concentration of lycopene compared to carotene, and other subjects show just the opposite effect. This behavior could reflect different dietary patterns regarding the intake of lycopene or lycopene-containing vegetables, or it could point towards differing abilities between subjects to accumulate these carotenoids in the skin.

6 Conclusion

Resonance Raman scattering is a viable optical technique for the measurement of carotenoid antioxidants in living human skin. It is precise, specific, and sensitive. Most importantly, it is also noninvasive, fast, and suitable for clinical and population studies. It permits the assessment of antioxidant status in a large subject base and it may become a useful method to evaluate the correlation between skin carotenoid levels and risk for malignancies caused by oxidative stress.

Using two-wavelength excitation, it is possible to selectively measure the two most prominent skin carotenoids, lycopene and β -carotene. According to the medical literature, these compounds may play different roles in the human body and be part of different tissue defense mechanisms. First measurements of the carotenoid skin composition in a group of seven healthy volunteers reveals a wide inter-subject variation in the β -carotene-to-lycopene concentration ratio, showing either lycopene or β -carotene as the predominant carotenoid in the particular subject. This finding may reflect a different dietary pattern for different subjects participating in the study. Alternatively, we can hypothesize that opposite carotenoid composition patterns of human skin may be a consequence of

differing individual abilities to uptake carotenes and lycopenes, or consequences of differing external stress factors.

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