

Resonance Raman detection of carotenoid antioxidants in living human tissues

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Received March 5, 2001

We have used resonance Raman scattering as a novel noninvasive optical technology to measure carotenoid antioxidants in living human tissues of healthy volunteers. By use of blue-green laser excitation, clearly distinguishable carotenoid Raman spectra superimposed on a fluorescence background are obtained. The Raman spectra are obtained within less than a minute, and the required laser light exposure levels are well within safety standards. Our technique can be used for rapid screening of carotenoid levels in large populations and may have applications for assessing antioxidant status and the risk for diseases related to oxidative stress. © 2001 Optical Society of America

OCIS codes: 170.3890, 170.6930, 160.4890, 170.5660.

Carotenoid molecules are powerful antioxidants that play important roles throughout the human body. Studies have shown an inverse correlation between high dietary intake of carotenoids and the risk of various cancers, cardiovascular disease, and degenerative diseases.¹ We have developed a noninvasive optical technique based on resonance Raman scattering to measure carotenoid levels in living human tissues, including the skin, retina, oral cavity, and other tissues.

In human skin, the five most-concentrated carotenoid antioxidants are lycopene, α -carotene, β -carotene, phytoene, and phytofluene, with lycopene and the carotenes accounting for 60–70% of total carotenoid content.² These molecules play an important role in the skin's antioxidant defense system. They are thought to act as scavengers for free radicals,³ singlet oxygen,^{4,5} and other harmful reactive oxygen species⁶ that are formed, e.g., by excessive exposure of skin to sunlight. If they are unbalanced by carotenoids and other antioxidants, the effects of reactive oxygen species can lead to premature skin aging, oxidative cell damage, and even the formation of skin cancers such as basal cell carcinoma, squamous cell carcinoma, and malignant melanoma. In animal models, carotenoids have been shown to inhibit carcinoma formation in the skin.⁷ Carotenoids have also been studied as inhibitors of a variety of other cancers and precancers, including sustained remissions in oral leukoplakia patients.⁸ In the retina, studies have shown an inverse correlation between carotenoid levels and age-related macular degeneration, a leading cause of blindness. Based on previous work in the human retina,⁹ it was found that the Raman technique works well for detection of the macular carotenoid pigments. However, the concentration of the macular carotenoids is ~ 100 times higher than the concentration in other tissues, so it is not clear whether Raman detection would also work in these cases.

The standard technique for measuring carotenoids is high-pressure liquid chromatography. This chemical method works well for the measurement of carotenoids in serum but is difficult to perform in tissue, since it requires biopsies and processing of relatively large tissue

volumes. We explored resonance Raman scattering as a fast and noninvasive optical method to measure tissue carotenoid levels throughout the body.

Carotenoids are accumulated in the body through fruit and vegetable consumption. They are π -electron conjugated carbon-chain molecules ($C_{40}H_{56}$) and are similar to polyenes with regard to their structure and optical properties. Their optical absorptions are strong and occur in broad bands (~ 100 -nm width) centered at ~ 450 and ~ 460 nm, respectively, for β -carotene and lycopene. Optical excitation leads to very weak, Stokes-shifted luminescence bands (width ~ 70 nm) centered at ~ 530 nm in both cases. The extremely low quantum efficiency of the luminescence is a striking feature of carotenoids and is caused by the existence of a second excited energy level that lies below the energy level reached in absorption and that has the same parity as the ground state. Following excitation, the molecule relaxes very rapidly, within ~ 200 – 250 fs,¹⁰ via nonradiative transitions to this lower excited state, from which electronic emission to the ground state is parity forbidden. The absence of intrinsic carotenoid luminescence allows us to explore the resonant Raman-scattering response to these molecules for their optical detection in tissues, provided that there is no overwhelming luminescence from other tissue emitters that might be optically excited along with carotenoid molecules.

To test the Raman response of thin, filmlike, carotenoid samples with physiologically relevant carotenoid concentrations, we spotted β -carotene-methanol solutions onto Teflon substrates and measured resonance Raman spectra in a backscattering geometry. Using a spectrograph with a spectral resolution of 1.2 cm^{-1} in these initial experiments, we observed strong and clearly resolved Raman signals superimposed on a weak fluorescence background under 488-nm laser excitation, as shown in Fig. 1. The Raman response is characterized by two prominent Stokes lines at 1159 and 1524 cm^{-1} . These lines originate, respectively, from carbon-carbon single- and double-bond stretch vibrations of the conjugated backbone; the 1008-cm^{-1} line is attributed to rocking

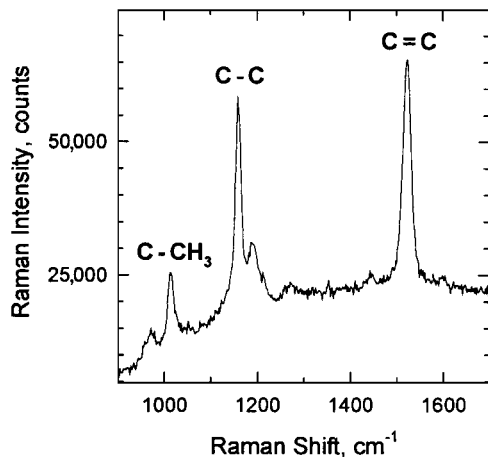


Fig. 1. Resonance Raman spectrum of a dried β -carotene-methanol solution spotted on a thin Teflon substrate.

motions of the molecule's methyl components.¹¹ We found the Raman-signal excitation efficiency for lycopene and β -carotene under 488-nm radiation to be approximately the same. However, under irradiation with the 514.5-nm wavelength, the Raman excitation efficiency for lycopene is at least a factor of 10 higher than for β -carotene. This behavior is understandable in terms of the absorption of these carotenoids, since the resonance Raman scattering cross section usually follows the absorption spectrum,¹² and the lycopene absorption is shifted toward longer wavelengths compared with β -carotene. This feature offers one the opportunity to measure either the mixture of these carotenoids (using 488-nm excitation) or just the lycopene portion (using 514.5-nm laser excitation). We found the intensity of the Raman peaks to scale linearly with the known carotenoid concentration in these measurements; therefore the strength of a Raman line is a measure of the concentration of the carotenoids.

Following the experiments with pure carotenoids, we constructed a prototype instrument suitable for Raman measurements on human tissue *in vivo*. The instrument, described in more detail elsewhere,⁹ consists of a small air-cooled 488-nm argon laser, a small home-made light delivery, collection, and filtering module, a Raman probe, and a home-made spectrograph coupled to a CCD camera (SBIG Model ST-6). The spectrograph design favors high light throughput at the expense of (unnecessary) spectral resolution (35 cm^{-1}). For a typical measurement in skin, a window in the Raman probe is placed in contact with the skin, ensuring a reproducible measuring condition, and the Raman-scattered light from a 2-mm-diameter illuminated spot is measured through the glass window in a 180° backscattering geometry. In our measurements on living human skin we used a laser power of 5 mW and an exposure time of 20 s, resulting in an intensity of 0.16 W/cm^2 at the skin surface, which is ~ 1000 times less than the exposure limit set by ANSI Z136.1-2000 standards.¹³

Typical carotenoid Raman spectra obtained from the skin and oral cavity tissues of a healthy human

volunteer are shown in Fig. 2. The strongest signal component of the spectra (top traces) is a spectrally broad background originating from native tissue fluorescence¹⁴ and not from carotenoids. Superimposed upon the fluorescence background are the characteristic Raman peaks of the carotenoids, appearing as small bumps on the traces. By use of the high dynamic range of our CCD detector, these Raman peaks can be resolved with a high signal-to-noise ratio. This is demonstrated in the bottom traces of Fig. 2, showing skin and oral cavity tissues after the fluorescence background has been fitted with a fourth-order polynomial and subtracted from the original spectra.

In the course of our Raman measurements we discovered that the fluorescence background was bleached partially over a period of several minutes. This effect is shown in Fig. 3(a), in which the top curve corresponds to the fluorescence background immediately after exposure of a fresh skin spot and the bottom curve corresponds to the fluorescence after 7-min exposure with 488-nm light (using a safe power density of $\sim 200\text{ mW/cm}^2$). Although the shape of the fluorescence remains unchanged, the intensity has dropped to $\sim 70\%$ of its initial value. We further investigated the kinetics of this bleaching effect, using lower (20-mW/cm^2) and higher (200-mW/cm^2) light-exposure levels. The result for the fluorescence signal at 530 nm is shown in Fig. 3(b), together with the 1524-cm^{-1} peak of the Raman response of the carotenoids. Although the fluorescence signal can be seen to decrease significantly due to bleaching, the Raman signal remains unchanged.

A skin site that is particularly well suited for assessing the antioxidant level in living human skin is the inner palm. This site is convenient not only for accessibility but also for the following reasons: (a) the carotenoid concentrations in the palm are

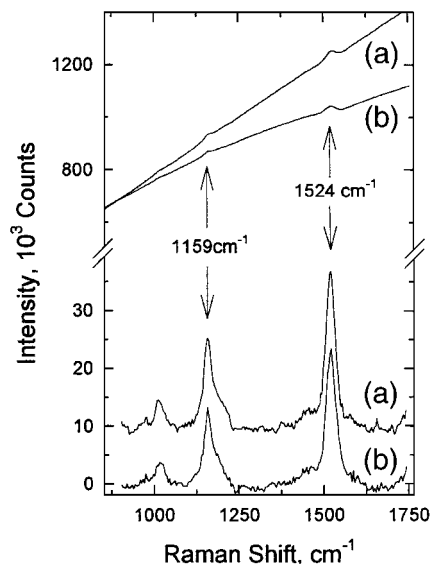


Fig. 2. Typical Raman spectra for (a) human forearm skin and (b) oral cavity tissue, measured *in vivo*. Top traces, spectra obtained before subtraction of native fluorescence background; bottom traces, after subtraction.

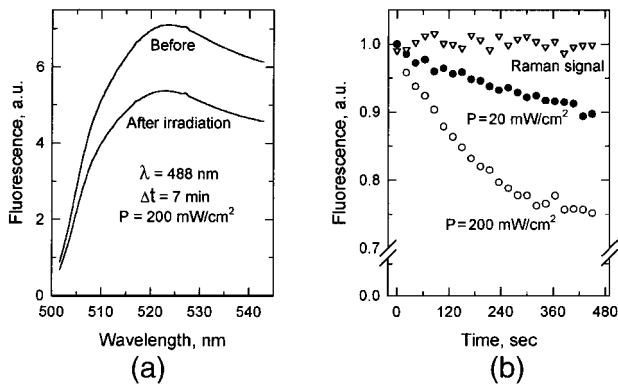


Fig. 3. Bleaching behavior of human skin fluorescence. (a) Fluorescence spectra before and after 7-min illumination at 200-mW/cm² intensity. (b) Fluorescence intensities (at 530 nm) and Raman response versus time during irradiation with 488-nm light at 20 and 200-mW/cm². Note that the Raman response stays constant, whereas the fluorescence bleaches with time.

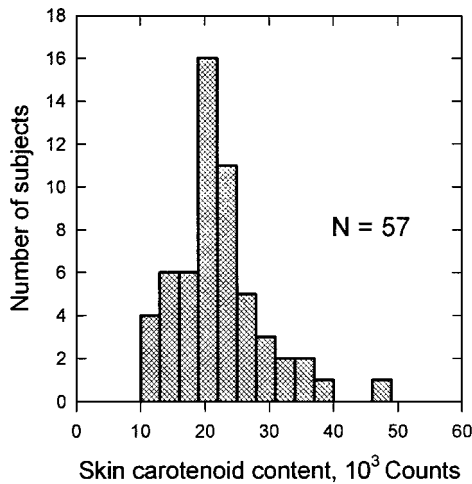


Fig. 4. Histogram of Raman measurements for a group of 57 healthy subjects, showing a wide distribution of carotenoid levels among them.

among the highest found in skin² (because carotenoids are lipophilic and palm skin has a high lipid/protein ratio), (b) differences in pigmentation among various skin types are minimal in the palm, and (c) the *stratum corneum* thickness of the palm (~400 μm) is high compared with other skin sites. The laser 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 intrasubject sampling volume should be realized.

Shown as a histogram in Fig. 4 is the Raman carotenoid response (carbon double-bond peak) measured in 57 healthy volunteers (both sexes, 25–75 years of age). The data show that approximately 50% of all subjects form the center (average) of the distribution. Another 50% contribute to the wings of the distribution, showing a fivefold variation of carotenoid content in this group. In previous measurements² we established the short-term and day-to-day repeatabil-

ity 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. 4 as substantial evidence for significant variations in tissue carotenoid content in different subjects.

In conclusion, resonance Raman scattering appears to be a feasible optical technique for the measurement of carotenoid antioxidants in living human tissue. It is precise, accurate, specific, and sensitive. Most importantly, it is noninvasive and suitable for clinical studies. Pending a careful calibration of our measurement with that determined by high-pressure liquid chromatography, it should be possible to replace the highly invasive chemical method with a completely noninvasive and quantitative optical alternative. The laser excitation power used on our experiments is well below safety standards and can be easily increased to permit nearly real-time data collection. Our Raman technique may become a useful method to evaluate the correlation between tissue carotenoid levels and risk for malignancies or other diseases associated with oxidative stress.

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