

Non-Invasive Raman Spectroscopic Detection of Carotenoids in Human Skin

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Carotenoids are thought to play a significant part in the skin's anti-oxidant defense system, and may help prevent malignancy. Inability to measure skin carotenoid content readily has, however, made it difficult to establish the relationship between carotenoid concentration and the occurrence of cutaneous malignancy. We have measured *in vivo* carotenoid concentration using a noninvasive optical method, Raman spectroscopy. To validate our instrumentation, abdominoplasty skin was evaluated by both Raman spectroscopy and high-performance liquid chromatography determination for carotenoid content. Evaluation of the Raman signal in specific carotenoid solutions was also performed. Precision of Raman measurements within skin sites, within subjects, and between subjects was measured. Sensitivity of the method was evaluated as a function of anatomical region and the distribution of carotenoids within the stratum corneum. Lastly, we evaluated

the Raman signal in actinic keratosis and basal cell carcinoma lesions and perilesional skin and compared this with region-matched sites in healthy subjects. Our results indicate that the Raman scattering method reflects the presence of carotenoids in human skin and is highly reproducible. Evaluation of five anatomical regions demonstrated significant differences in carotenoid concentration by body region with the highest carotenoid concentration noted in the palm. Comparison of carotenoid concentrations in basal cell carcinomas, actinic keratosis, and their perilesional skin demonstrate a significantly lower carotenoid concentration than in region-matched skin of healthy subjects. These results represent the first evidence that carotenoid concentration in the skin correlate with the presence or absence of skin cancer and precancerous lesions. **Key words:** carotenoids/human/Raman spectroscopy/skin. *J Invest Dermatol* 115:441-448, 2000

Carotenoids are thought to play a part in the anti-oxidant system of the skin; however, due to the difficulty of their identification in human skin, specific evidence has been lacking. In animal models carotenoids have been shown to inhibit carcinoma formation in the skin (Chen *et al*, 1993) as well as other tissues such as mammary gland (Nagasawa *et al*, 1995), lung (Murakoshi *et al*, 1992), liver (Murakoshi *et al*, 1992), and colon (Narisawa *et al*, 1996). Carotenoids role in cancer prevention is thought to be secondary to their photoprotective (Krinsky, 1991), radical quenching (Bohm *et al*, 1995), and anti-oxidant properties (Burton and Ingold, 1984); with lycopene identified as the most potent singlet oxygen quencher of the carotenoids (Conn *et al*, 1991). Because the carotenoids are lipophilic molecules, they are well placed in the skin to act as chain-breaking anti-oxidants protecting epidermal polyunsaturated fatty acids from oxygen radical peroxidation (Krinsky and Deneke, 1982).

Previous epidemiologic studies have attempted to assess the role of carotenoids in humans in the development of skin cancer (Breslow

et al, 1995; Karagas *et al*, 1997); however, quantitation of carotenoids in skin was not performed as the standard measurement of carotenoids using high pressure liquid chromatography (HPLC), is often tedious and requires prohibitively large skin samples. Therefore, analysis of carotenoid concentration was performed in serum rather than skin. In addition, only β -carotene in the serum was examined, ignoring the contribution of several other carotenoids that have been identified in human skin (Biesalski *et al*, 1996; Wingerath *et al*, 1998).

In this study we have measured total carotenoid concentration in human skin using a noninvasive method, Raman spectroscopy. Raman spectroscopy is a powerful laser spectroscopic technique that detects the characteristic vibrational/rotational energy levels of a molecule. When light irradiates an ensemble of molecules, most of it is scattered elastically. This elastically scattered light, which has the same frequency as the incident light, is termed Rayleigh scattering. A small amount of light, however, is scattered inelastically. The inelastically scattered light, termed Raman scattered light, exhibits a frequency shift with respect to the incident light, and the frequency shift corresponds exactly to the vibrational/rotational energy transitions of the molecules. Raman scattering is a process in which energy is exchanged between the incident light and the scattering molecules. The Raman spectrum gives a spectral fingerprint of the molecules, and the intensity of the Raman peak is directly proportional to the concentration of molecules (Koningstein, 1971).

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Carotenoids are a family of polyene molecules that share a common $C_{40}H_{56}O_2$ formula. Because of the molecular structure of polyene molecules, they elicit very strong Raman intensities, especially when resonantly excited in their $\pi-\pi^*$ electronic absorption transition in the visible (violet/green) wavelength range. Because carotenoids are ideal for Raman analysis, they have been examined by Raman spectroscopy in human retinal tissue (Bernstein *et al*, 1998; Gellermann *et al*, 1998), isolated erythrocyte membrane (Verma and Wallach, 1975a), thymocyte endoplasmic reticulum (Verma and Wallach, 1975b), and in the lymphocytes of healthy and lung cancer patients (Bakker Schut *et al*, 1997). To date, carotenoid concentration in normal skin and malignant skin lesions by Raman spectroscopy has not been investigated.

To validate the instrumentation, abdominoplasty skin was evaluated for carotenoid concentration by both Raman spectroscopy and HPLC analysis. Evaluation of the Raman signal in specific carotenoid solutions was also performed. Precision of Raman measurements within skin sites, within subjects, and between subjects was measured. Sensitivity of the method was evaluated as a function of anatomical region as well as relative carotenoid disposition in the stratum corneum *versus* other skin layers. Lastly, the Raman signal within actinic keratosis and basal cell carcinoma lesions as well as healthy perilesional skin was compared with region-matched sites in healthy subjects. We believe the Raman spectroscopy method allows total cutaneous carotenoid concentration to be ascertained noninvasively.

MATERIALS AND METHODS

Materials The reference samples of carotenoids were either synthesized or isolated from natural sources according to previously published procedures (Khachik *et al*, 1992a, b, c). Zeaxanthin and ethyl β -apo β '-carotenoate was donated by Hoffmann-LaRoche (Basel, Switzerland); the latter was employed as an external standard for the HPLC separation of reference carotenoids. Lutein was isolated from marigold flowers (Khachik, 1995). Retinol and γ - and α -tocopherol were obtained from Eastman Kodak (Rochester, NY). Butylated hydroxytoluene and N,N-diisopropylethylamine were purchased from Aldrich (Milwaukee, WI). Ascorbic acid (99% catalog no. A5960) and synthetic phytol (catalog no. T3251) were obtained from Sigma (St Louis, MO). Extraction and HPLC grade solvents, tetrahydrofuran, acetonitrile, dichloromethane, hexane, methanol, and ethanol (Baxter Scientific Division, McGaw Park, IL) were used without further purification. Adhesive tape discs (D-Squame) used for the skin stripping experiments were used as purchased from CuDerm (Dallas, TX).

Abdominal female human skin evaluated in the HPLC analysis was obtained from local hospitals under a tissue collection program in the Department of Dermatology within 4 h of surgery. Human skin was dermatomed to a thickness of ≈ 0.5 mm using Pagett's electrokeratome in the laboratory and stored at -70°C until shipped on dry ice to Dr Khachik for HPLC analysis.

All raw materials used in the Raman scattering spectroscopic studies were measured on a Sartorius ultramicroscale (serial no. 47MP8) with a sensitivity of $0.1 \mu\text{g}$. All chemicals were completely solubilized in the solvents utilized.

HPLC analysis of skin samples were conducted on a Hewlett-Packard (HP) system equipped with a rapid scanning ultraviolet (UV)/visible photodiode array detector, and an HP-1050 autosampler. The data were stored and processed by means of Compaq DeskPro 590 computing system using the HP Chem-Station program (version A.05.02) on Windows-97, in combination with a high resolution color display monitor, Model MaxTech MPRII, and an HP-Laserjet 4 Plus printer. Dietary carotenoids, α -tocopherol, γ -tocopherol, and retinol in extracts from human abdominal skin were separated and quantitated on a Microsorb (25 cm length \times 4.6 mm i.d.) C_{18} (5 μm spherical particles) reversed phase column (Rainin Instrument, Woburn, MA) according to a published procedure (Khachik *et al*, 1997). The column was protected with a Brownlee guard cartridge (3 cm length \times 4.6 mm i.d.) packed with spheri-5- C_{18} (5 μm particle size). Lutein and zeaxanthin and their oxidative metabolites were separated on a silica-based nitrile bonded (25 cm length \times 4.6 mm i.d.; 5 μm spherical particle) column (Regis, Morton Grove, IL), which was protected with a Brownlee nitrile bonded guard cartridge (3 cm length \times 4.6 mm i.d.; 5 μm particle size) (Khachik *et al*, 1992c, 1997).

Methods

Skin extraction procedure All tissue extractions were performed under gold fluorescent light (Grainger, Baltimore, MD). Dermatomed abdominal skin samples previously stored at -70°C were immediately extracted after thawing at ambient temperature. Tissues (6.50 ± 0.34 g, n mean \pm SD) were homogenized with tetrahydrofuran (75 ml; containing 0.1% butylated hydroxytoluene) by sonication in an ice bath each time for 30 min. The solvent was decanted off into a 500 ml round bottom flask and the extraction was repeated twice, as described above. The combined tetrahydrofuran extract was concentrated on a rotary evaporator at 40°C . The residue was microfiltered into a graduated 5 ml centrifuge vial using dichloromethane and the solvent was evaporated under nitrogen. The skin extract was submitted to two independent HPLC assays (i) reversed phase HPLC assay which separated the carotenoids, vitamin A and α - and γ -tocopherol, and (ii) normal phase HPLC assay, which separated lutein, zeaxanthin, and their oxidative metabolites (Khachik *et al*, 1992c, 1997).

HPLC assay Carotenoids were identified by comparison of their retention times and HPLC/UV visible spectra obtained by a photodiode array detector with those of authentic standards. UV absorption was simultaneously monitored at 446, 400, 350, and 290 nm in the reverse phase HPLC assay and at 446 and 325 nm in the normal phase HPLC assay.

Because the use of an internal standard could possibly interfere with the presence of unknown carotenoids in the skin extracts, no internal standard in the extraction of the current skin tissue samples was employed; however, to monitor the accuracy and reproducibility of the HPLC analysis of carotenoids, a solution containing known concentrations of ethyl β -apo β '-carotenoate (external standard), lycopene, lutein, α -cryptoxanthin, β -cryptoxanthin, α -carotene, β -carotene, retinol, γ -tocopherol, and α -tocopherol was routinely analyzed. The recovery and reproducibility of the HPLC analysis for carotenoids, vitamin A, α -tocopherol, and γ -tocopherol was shown to be greater than 95%. Limit of detection of the carotenoids in the assay was approximately 2 ng per ml.

Raman scattering spectroscopy The Raman scattering spectroscopy method for the noninvasive measure of carotenoids in human skin *in vivo* was validated for precision, accuracy, specificity, and sensitivity. Twenty subjects (five females and 15 males), ranging in age from 28 to 79 y, volunteered for the studies after signing an informed consent form of an Institutional Review Board approved protocol at the University of Utah.

The Raman scattering instrument used in combination with a hand-held light beam delivery and collection module ("Raman probe"), is schematically shown in **Fig 1**. The Raman excitation light, originating from a small air-cooled argon laser, is routed via fiber, beam expanding lens (L_3), laser bandpass filter (F_2), dichroic beamsplitter (BS), and focusing lens (L_2) to the surface of the skin of a subject. In combination with a slightly diverging incident beam, lens L_2 , having a focal length of 25 mm, focuses the laser to a 2 mm circular disk in its focal plane. The focal plane is positioned inside a thin window (not shown) covering an opening of the Raman probe below lens L_2 . For all measurements, the window is placed against the tissue site of interest. This arrangement provides a fixed distance from the lens L_2 to the tissue and guarantees identical laser spot sizes and corresponding identical illumination conditions for all measurements. The Raman scattered light is collected in 180° back scattering geometry with the same focusing lens L_2 , and routed through beam splitter (BS), holographic notch filter (F_1), fiber input coupling lens L_1 , and a fiber bundle to a commercial spectrograph (Jarrell Ash F/3.8). The Raman spectra were recorded with a thermoelectrically cooled CCD camera (Santa Barbara, Inc. Model ST-6) and processed with Windows-based software ("KestrelSpec", Catalina Scientific/Rhea Corporation). All Raman measurements were obtained with a laser power of 10 mW and an exposure time of 20 s. Accounting for the 2 mm laser spot size, this results in an intensity of 0.2 W per cm^2 at the tissue surface, which is considered safe by ANSI Z136.1 standards.

A typical Raman spectrum of human skin on the ventral forearm, obtained *in vivo* with laser excitation at 488 nm laser wavelength, 10 mW laser power, 20 s exposure time, and a 2 mm spot size, is shown in **Fig 2**. The spectrum in **Fig 2(A)** was obtained directly after laser exposure, and reveals a broad, featureless, and strong fluorescence background of skin with three superimposed sharp Raman peaks characteristic for the carotenoid molecules at 1015, 1159, and 1524 cm^{-1} . **Figure 2(B)** illustrates the Raman spectrum obtained after fitting the fluorescence background with a fifth-order polynomial and subtracting it from **Fig 2(A)**, thus revealing the frequency region of the two strongest carotenoid peaks, at 1159 and 1524 cm^{-1} at an expanded scale.

The three characteristic carotenoid Raman peaks shown in **Fig 2** originate, respectively, from rocking motions of the molecule's methyl components (1015 cm^{-1}), from carbon-carbon single-bond stretch vibra-

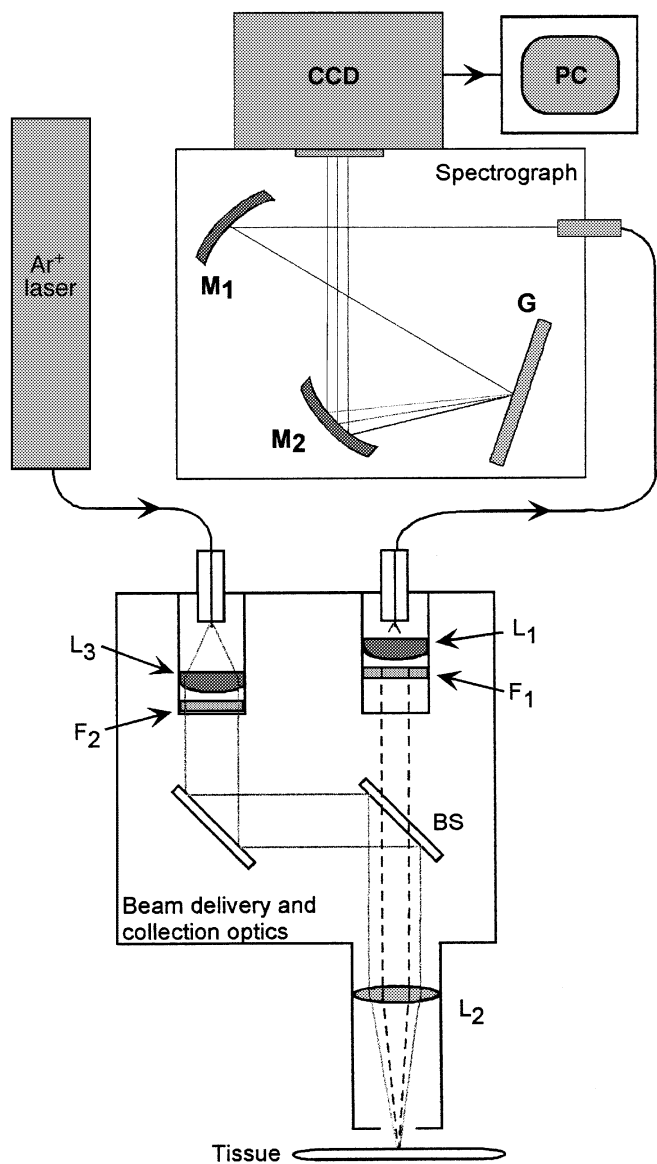


Figure 1. Schematics of basic Raman scattering instrumentation used for detection of carotenoid pigments in human tissue.

Excitation light from an argon laser is routed via optical fiber, beam expanding lens L_3 , laser bandpass filter F_2 , dichroic mirror BS, and lens L_2 to the tissue. The Raman shifted backscattered light is collimated by lens L_2 , directed through BS, filtered by holographic rejection filter F_1 , focused by lens L_1 on to a fiber, and sent to a spectrograph. The wavelength dispersed signals are detected by a charge-coupled array detector CCD, and displayed on a computer monitor PC.

tions (1159 cm^{-1}), and from carbon-carbon double-bond stretch vibrations (1524 cm^{-1}) of the molecule's backbone (Koyama *et al.*, 1988). The 1524 cm^{-1} Raman peak produces the strongest signal, and therefore was used throughout this study, as the method for quantitating the carotenoid concentration present in the tissue investigated.

Based on the literature (Anderson and Parrish, 1981; Jacques, 1989; Van Gemert *et al.*, 1989) we estimate that light in the green wavelength range penetrates human skin to a depth of about 0.5 mm. The back-scattered Raman scattered light would then originate from a maximum sampling depth of about half this value, i.e., $\approx 250\text{ }\mu\text{m}$. The absolute depth of penetration is a result of the combined action of absorption (by chromophores) as well as scattering.

Variability Six healthy nonsmoking subjects, ranging in age from 28 to 46 y (three males and three females), with no concomitant medications, except nutritional vitamins, were evaluated. Three skin sites, $\approx 1.5\text{ cm}^2$ surface area, and spaced 2–3 cm center-to-center apart, were demarcated on the left volar forearm of each subject in an area that was a minimum of 3 cm above

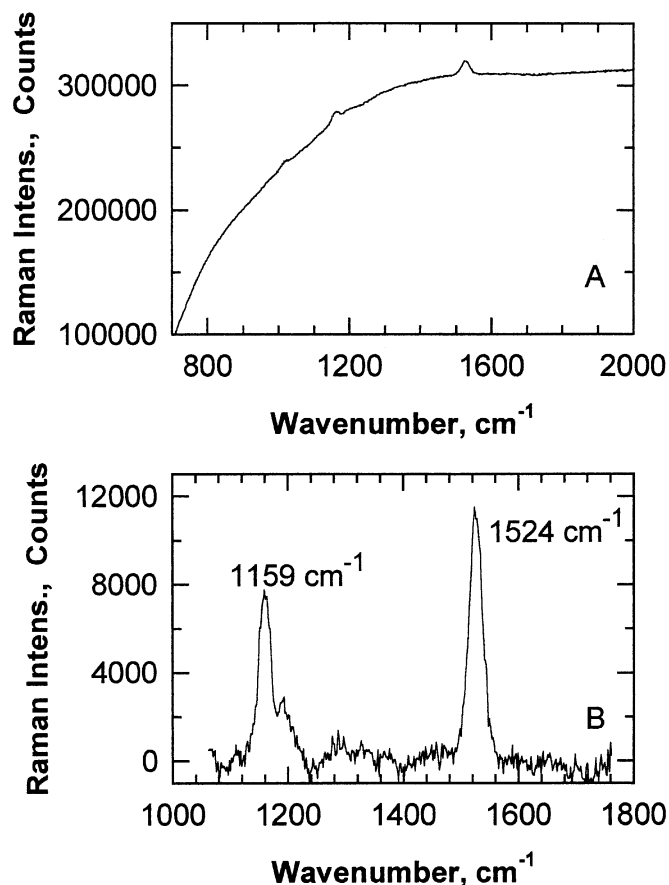


Figure 2. Typical Raman spectra for human ventral forearm skin, measured *in vivo*. Illumination conditions: 488 nm laser wavelength, 10 mW laser power, 20 s exposure time, 2 mm spot size. Spectrum shown at top is spectrum obtained directly after exposure, and reveals broad, featureless, and strong fluorescence background of skin with superimposed sharp Raman peaks characteristic for carotenoid molecules. Spectrum at bottom is difference spectrum obtained after fitting fluorescence background with fifth-order polynomial and subtracting it from top spectrum. The main characteristic carotenoid peaks are clearly resolved with good signal-to-noise ratio, at 1159 and 1524 cm^{-1} .

the wrist and 3 cm below the antecubital fossa. Triplicate measurements of each of skin site were collected. All measurements from a single subject were obtained within a 1 h time period.

Within-site precision was evaluated from the replicate measurements at each skin site of the six subjects. Within-individual precision was evaluated from the mean 1524 cm^{-1} Raman intensity (counts) of the individual skin sites on the volar forearm of each subject. Interindividual precision of the method was evaluated with the mean 1524 cm^{-1} Raman intensity (counts) of all subjects evaluated. Mean, SD, and coefficient of variation of the 1524 cm^{-1} Raman intensity (counts) were calculated in MS Excel (MS office 98, MacIntosh) spreadsheet.

Specificity Specificity of the Raman scattering spectroscopy method was evaluated using various carotenoids and other known anti-oxidants found in human skin. Stock concentrations of the carotenoids; β -carotene, lycopene, and the other anti-oxidants, ascorbic acid and α -tocopherol, were prepared at $2\text{ }\mu\text{g}$ per ml in tetrahydrofuran and stored at -70°C . For the determination of Raman scattering efficiencies of the various carotenoids, the stock standards of lycopene, β -carotene were diluted to various concentrations: 0, 20, 64, 125, 235, and 421 ng per ml in ethanol immediately prior analysis.

In addition, additivity of the 1524 cm^{-1} Raman intensity (counts) for individual and combined carotenoids was evaluated comparing freshly prepared 64 ng per ml solutions of lycopene and β -carotene with and without other anti-oxidants, ascorbic acid and α -tocopherol in ethanol from the stock standards. Raman scattering spectra were obtained for each freshly prepared individual and combined solution(s).

Sensitivity The ability of the instrument to measure changes in concentration was assessed by evaluating the 1524 cm^{-1} Raman intensity (counts) at five different anatomical regions in healthy subjects. In addition, changes in Raman signal strength was measured with successive removal of stratum corneum using a skin stripping procedure (Pershing *et al*, 1992).

Single measurements at five anatomical sites, including palm, forehead, inner arm, lateral arm, and dorsal hand were evaluated using the 1524 cm^{-1} Raman intensity (counts) on six healthy nonsmoking subjects (three males and three females) ranging in age from 28 to 46 y, with no concomitant medications, except nutritional vitamins.

To evaluate the relative disposition of the carotenoids in the stratified epidermis *versus* the viable epidermis-dermis, the 1524 cm^{-1} Raman intensity (counts) within various stratum corneum layers was evaluated at three skin sites, 1.33 cm^2 surface area, on the volar forearm of each of six healthy subjects (three males and three females) ranging in age from 28 to 46 y, after sequential removal of the stratum corneum using a skin stripping technique (Pershing *et al*, 1992). Raman scattering spectra were measured before skin stripping (baseline), and after skin stripping 10 \times , 20 \times , 30 \times , and 40 \times , thus producing five separate 1524 cm^{-1} Raman intensities (counts) at each skin site.

Correlation with HPLC carotenoids Total detectable lutein and zeaxanthin, lycopene and its Z-isomers, and α -, β -, γ -, and ζ -carotene concentrations quantitated by HPLC analysis were compared with the 1524 cm^{-1} Raman intensity (counts) in the same three skin sources. The plant carotenoids, phytoene and phytofluene, were excluded from the comparison due to the inability of the instrument to detect these plant carotenoids with the current laser excitation wavelength (488 nm).

Skin cancer evaluation Subjects with biopsy-confirmed basal cell skin cancer or clinically verifiable actinic keratosis (12 males and two females ranging in age from 46 to 79 y) and site-matched otherwise normal-appearing uninvolved skin in healthy subjects (three males and three females, age ranging from 28 to 46 y) were evaluated and compared using the 1524 cm^{-1} Raman intensity (counts). Raman spectra were obtained by placing the probe on the intact cancerous or precancerous lesion, and the perilesional skin 1–2 cm away from the lesion margin in the cancer subjects, or on region-matched sites in the healthy subject volunteers. The healthy-appearing perilesional site in the cancer subjects was chosen so as not to involve scaly skin, which might affect the optical perception of the instrument.

Statistical methods Collected data included subject ID, age, gender, anatomical region, UV radiation status, and type of lesion as assessed by the investigator. Data was entered into a spreadsheet (Excel, version MS Office 98, Microsoft, Redmond WA) and evaluated for mean, standard deviation and coefficient of variation. Statview (version 4.1, Abacus Concepts, Calabasas, CA) was utilized to evaluate the statistical significance between groups using an $\alpha = 0.05$ in ANOVA; Fishers protected least significant difference method and the Mann-Whitney test.

RESULTS

HPLC carotenoid analysis of human skin Eighteen dietary carotenoids including five metabolites were identified by HPLC in human abdominal skin extracts. Among these, α -cryptoxanthin, β -cryptoxanthin, lycopene, 5Z-lycopene, 5Z,5'Z-lycopene, γ -carotene, ζ -carotene, α -carotene, β -carotene, all-E + Z-phytofluene and phytoene were separated and quantitated by reverse phase HPLC. In addition, lutein, zeaxanthin, and several oxidative metabolites of these compounds, namely 3'-epilutein, ϵ,ϵ -carotene-3,3'-dione, 3'-hydroxy- ϵ,ϵ -caroten-3-one, 3-hydroxy- β,ϵ -caroten-3'-one, and one metabolite of lycopene, 2,6-cyclolycopene-1,5-diol were separated and quantitated by normal phase HPLC. Foremost among the mean detectable carotenoids in the human abdominal skin extracts was lycopene and its cis (Z)-isomers ($69 \pm 52\text{ ng per g}$, mean \pm SD), a carotenoid found in tomatoes and tomato products (Table I). Phytoene ($65 \pm 24\text{ ng per g}$), the combined γ -, β -, α -, and ζ -carotenes ($53 \pm 44\text{ ng per g}$), lutein and zeaxanthin ($\approx 26\text{ ng per g}$), and phytofluene (15 ± 8) were also quantitated in the skin extracts in descending order of concentration. β -carotene represented $\approx 50\%$ of the combined ($\alpha + \beta + \gamma + \zeta$) carotenes.

The oxidative metabolites of lutein and zeaxanthin: 3'-epilutein (6 ng per g), ϵ,ϵ -carotene-3,3'-dione (10 ng per g), 3'-

Table I. Carotenoid concentrations in human skin (ng per g tissue)

Carotenoid	Skin source				
	1	2	3	Mean	SD
Lycopene+ Z-isomers	105	9	93	69	52
Carotenes					
γ	20	ND ^a	ND	20	NA ^a
β	38	2	37	26	20
α	13	2	8	8	6
ζ	25	4	10	13	11
Total	96	8	55	53	44
Lutein + Zeaxanthin	26	ND	ND	26	NA
Total	227	17	148	131	106
Phytoene	92	45	57	65	24
Phytofluene	21	6	17	15	8
TOTAL	340	68	222	210	136

^aNA, not available; ND, nondetectable.

hydroxy- ϵ,ϵ -caroten-3-one (19 ng per g), 3-hydroxy- β,ϵ -caroten-3'-one (14 ng per g), and lycopene, 2,6-cyclolycopene-1,5-diol (36 ng per g), respectively, present in the skin extracts have also been previously identified and isolated from human serum (Khachik *et al*, 1992a, 1997). Whether the presence of these carotenoids in human skin is the result of *in vivo* oxidation as a result of the previous exposure to UV radiation or simply reflective of human serum is unknown at this time.

Resonance Raman scattering spectra of carotenoids in human skin Figure 2 illustrates a typical Raman spectrum of human skin measured under excitation with the 488 nm line of an argon laser for 20 s at a laser power of 10 mW, and illuminated spot size diameter of 2 mm. The spectrum in Fig 2(A) is obtained directly after exposure. It reveals a broad, featureless, and strong fluorescence background of skin (originating from chromophores and collagen), and the weaker, superimposed, sharp Raman peaks, characteristic for carotenoid molecules. The spectrum in Fig 2(B) is the difference spectrum obtained after fitting the fluorescence background with a fifth-order polynomial and subtracting it from the spectrum in Fig 2(A). The main characteristic carotenoid peaks, associated with the stretching vibrations, are clearly resolved with good signal-to-noise ratio, at 1159 and 1524 cm^{-1} . As Raman spectroscopy is a linear method with respect to the scattering molecule concentration, the Raman spectrum intensity can be correlated with the carotenoid concentration in the skin, provided that the measurement conditions remain exactly the same.

Raman scattering spectroscopy method correlates with HPLC analysis Previous work (Gellermann *et al*, 1998) demonstrated that carotenoid concentration in the human macula densa of the retina can be detected and quantitated by Raman scattering spectroscopy and was linearly correlated with total carotenoid concentration in that tissue as determined by HPLC. Cutaneous carotenoid concentration measured by HPLC analysis also demonstrates a positive correlation with 1524 cm^{-1} Raman intensity (counts). Skin sources 1 and 3 with similar total carotenoid concentrations also have similar 1524 cm^{-1} Raman intensity (counts). Skin source 2, which had minimal total carotenoid concentration (17 ng per g tissue), demonstrated nondetectable 1524 cm^{-1} Raman intensity (counts). (Table II). The greater Raman scattering intensity in the skin source 3 (10,000 counts) compared with skin source 1 (7255 counts) despite less total

Table II. Total cutaneous carotenoid concentration versus laser-Raman scattering spectrophotometer values

Skin source	Total carotenoid ^a (ng per g tissue)	Laser-Raman scattering value
1	227	7255
2	17	ND
3	148	10000

^aIncludes lycopene and its Z-isomers, lutein and zeaxanthin, and γ , β , α , ζ carotenes.

carotenoid (148 vs 200 ng per g tissue) may reflect differences in the amount of tissue analyzed by HPLC versus Raman spectroscopy. With HPLC analysis, the entire 500 μm depth of dermatomed $\approx 10\text{ cm}^2$ skin samples was extracted and analyzed for carotenoids, whereas the Raman spectroscopy method analyzed skin to the laser depth of 250 μm . Therefore, carotenoid concentrations in the deeper skin layers below 250 μm depth were likely not included in the Raman analysis, but were included in the extraction of the whole tissue by the HPLC method. In addition, the stability of the carotenoids to the -70°C storage and transport conditions is unknown and may have differentially affected the Raman scattering versus HPLC analysis of these tissues.

Raman scattering spectroscopy method is specific, sensitive, reproducible and dose responsive Specificity of the Raman scattering spectroscopy method was investigated with freshly prepared ethanolic solutions of β -carotene, lycopene, and the non-Raman scattering anti-oxidants, ascorbic acid and α -tocopherol. β -carotene and lycopene produced dose responsive 1524 cm^{-1} Raman intensities (counts), as a function of increasing concentrations from 20 to 421 ng per ml. β -carotene, however, produced a 4-fold increase in 1524 cm^{-1} Raman intensity (counts) than lycopene at the same concentration. Thus, β -carotene has a 4-fold higher Raman scattering cross-section than lycopene with laser excitation at 488 nm. The latter can be attributed to the increased absorption of β -carotene at 488 nm compared with lycopene. As expected, the other known anti-oxidants of the skin, ascorbic acid and α -tocopherol did not exhibit a 1524 cm^{-1} Raman intensity (counts) at similar concentrations (Fig 3). Whereas the Raman response to lycopene is linear up to a concentration of 400 ng per ml, the β -carotene Raman response is only linear to ≈ 140 ng per ml, and demonstrates a saturation behavior at higher concentrations. This behavior reflects the increased absorption coefficient of β -carotene with respect to lycopene, which at higher concentrations results in nonuniform excitation of the solution, as well as self-absorption of the Raman scattered light in the long-wavelength shoulder of the β -carotene absorption. It should be noted, however, that β -carotene concentrations are sufficiently low in the skin, such that the area of nonlinearity of β -carotene would not be attained with the current Raman method. Carotenoid detection by Raman scattering spectroscopy is additive for each carotenoid species present. Despite differences in absorption coefficients between β -carotene and lycopene, a combined ethanolic solution containing 64 ng per ml of each carotenoid (total carotenoid concentration = 128 ng per ml) produces a 1524 cm^{-1} Raman intensity (counts) equal to the sum of the signals of each individual carotenoid solution (data not shown).

Limits of sensitivity of the current method for individual carotenoids is approximately 20 ng per ml, which is equal to ≈ 20 ng per g skin. The lack of a 1524 cm^{-1} Raman intensity (count) in skin source 2 (Table II) is consistent with the low total carotenoid concentration of ≈ 17 ng per g in that tissue sample. These data confirm that the Raman scattering spectroscopy method is accurate, dose-responsive, specific, sensitive, and additive for carotenoids that are excited with the 488 nm laser light.

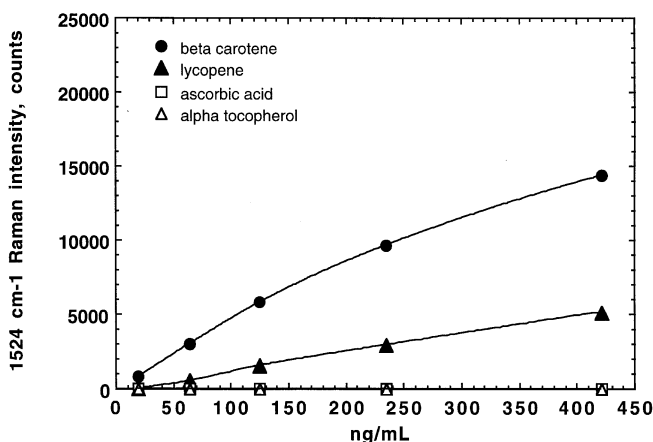


Figure 3. Raman scattering activity in solutions of various anti-oxidants as a function of concentration in ethanol. ●, β -carotene; ▲, lycopene; □, ascorbic acid; △, α -tocopherol.

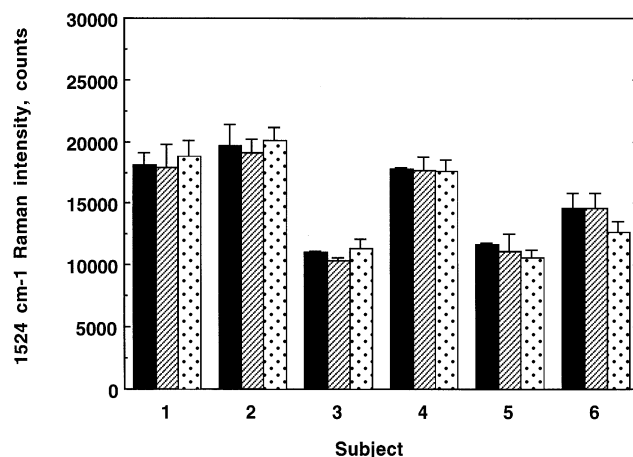


Figure 4. Within site and between-site precision of Raman intensity at 1524 cm^{-1} (counts) at skin sites on the ventral forearm. Mean \pm SD of triplicate readings. ■, Proximal site; ▨, medial site; ▤, distal site.

Between subject variability in the six healthy subjects evaluated is relatively high, $\approx 50\%$ (Fig 4), as expected and consistent with other documented variability in other intact skin parameters (Pershing *et al*, 1993). Triplicate readings of a single skin site (within-site variability), however, were not significantly different within a subject ($p > 0.05$; ANOVA: Fisher's PLSD). Further, between site carotenoid values (distal versus medial versus proximal) were not significantly different in the same subject ($p > 0.05$, ANOVA: Fisher's PLSD). The coefficient of variation at the most distal site, mid site, and proximal site on the forearm varied from 0.9% to 7.4%, 2.4% to 10.5%, and 1.7% to 8.8%, respectively, in the six individuals evaluated. These data demonstrate excellent reproducibility in cutaneous carotenoid quantitation with Raman scattering spectroscopy.

Carotenoid concentrations vary as a function of anatomical region Evaluation of carotenoid concentration at various anatomical regions; palm, forehead, inner arm, volar arm, and dorsal hand, illustrates that carotenoid deposition in skin is not equal throughout the body (Fig 5). Data illustrate that the palm region has the highest mean carotenoid concentration of the regions evaluated. The forehead and inner arm carotenoid values, however, were not significantly different in the small population of subjects evaluated. Noteworthy, is that both the volar arm and dorsal hand were significantly different ($p < 0.05$) from the palm region. The 1524 cm^{-1} Raman intensity (counts) did not appear to be primarily

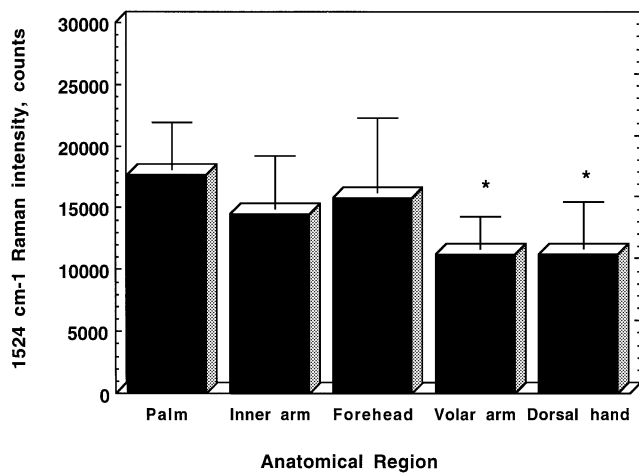


Figure 5. Sensitivity of Raman intensity at 1524 cm⁻¹ (counts) as a function of anatomical region. Mean \pm SD, n = 6 subjects. *p < 0.05 from palm region, by ANOVA, Fisher's PLSD.

influenced by sun-exposed body regions, as the sun-protected sites of the inner arm and volar forearm were not significantly different than the sun-exposed site of the dorsal hand. These data suggest that the carotenoid signal detected with the Raman scattering spectroscopy method is influenced by a combination of factors; total carotenoid concentration, stratum corneum thickness, laser light penetration depth, and likely UV exposure history. The fact that β -carotene has a 4-fold higher Raman scattering intensity than lycopene and the possibility of different ratios of β -carotene to lycopene in skin may also account for measured regional differences.

Carotenoid concentrations in the stratum corneum are high Carotenoids are lipophilic, and as such will tend to partition to those areas of the body where there is a high lipid content. Stratum corneum has a high lipid/protein concentration ratio, which is the basis of its barrier role in maintaining internal body environment. The contribution of the stratum corneum carotenoids to the cutaneous Raman spectra was evaluated by sequentially applying and removing tape adhesive strips (also called skin stripping) at a designated skin site on the ventral forearms of six healthy subjects so as to remove this layer of skin. The stripping method used has been previously validated by Pershing (Pershing *et al*, 1992, 1993) and confirmed to remove approximately 250 μ g stratum corneum per cm² surface area with 10 skin strippings and 113 μ g per cm² after 20 skin strippings. Forty skin strippings removes >90% of the stratum corneum, as previously confirmed by light microscopic histology (data not shown). Baseline measurements, which represent fully intact stratum corneum, had mean 1524 cm⁻¹ Raman intensities (counts) of \approx 15500 (Fig 6). After 10 and 20 skin strippings, the mean 1524 cm⁻¹ Raman intensities (counts) were not different from baseline (p > 0.05, ANOVA, Fisher's PLSD); however, after 30 and 40 skin strippings, the mean 1524 cm⁻¹ intensity (counts) were significantly reduced 21% from baseline (p < 0.05). That 21% of the total 1524 cm⁻¹ Raman intensity (counts) in intact skin is represented by only \approx 6% of the total skin thickness evaluated by the instrument (250 μ m) documents that carotenoids are in a relatively high concentration in the stratum corneum and further support their hypothesized role in the initial protective barrier to UV insult and skin cancer. The remaining 80% of the Raman intensity (counts) after removal of the stratum corneum, confirms that carotenoids are also present in the deeper skin layers.

Precancerous lesions and skin neoplasms have lower carotenoid concentrations than healthy skin The noninvasive differentiation of normal skin from clinically verifiable actinic keratosis and biopsy proven basal cell carcinomas was investigated using the noninvasive Raman scattering spectroscopic method. Preliminary evaluation of carotenoid levels adjacent to as

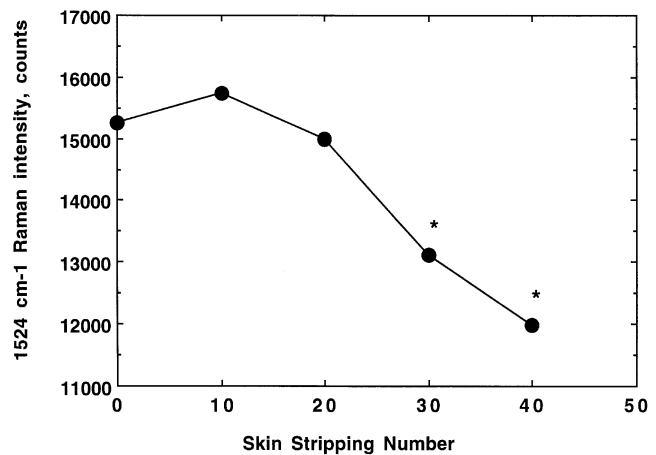


Figure 6. Raman scattering spectroscopic detection of carotenoid disposition in human ventral forearm stratum corneum as a function of stratum corneum removal by skin stripping. Mean values presented, *p < 0.05 from baseline (0) by ANOVA Fisher's PLSD.

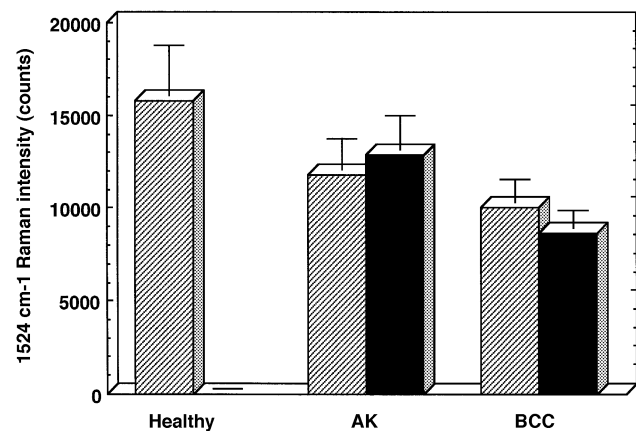


Figure 7. Raman intensity of healthy and lesional skin. Comparison of Raman intensity at 1524 cm⁻¹ (counts) in region-site matched healthy (hatched bars) versus perilesional (hatched bars), cancerous (solid bars) and precancerous (solid bars) skin in humans. Mean \pm SEM of healthy (n = 6); AK, actinic keratosis (n = 14); BCC, basal cell carcinoma (n = 14)

well as within basal cell carcinomas and actinic keratosis demonstrate a significantly lower carotenoid concentration in these patients than in site-matched skin of healthy subjects by the Raman scattering spectroscopy method (Fig 7). These data document that the carotenoid concentration in the skin surrounding, as well as within these precancerous and neoplastic tissues are indeed altered from healthy skin and suggest an important part for carotenoids in skin cancer and the skin defense to UV insult.

DISCUSSION

Carotenoids are thought to play a part in the skin's anti-oxidant defense system. Also included in this defense system are superoxide dismutase, glutathione and glutathione peroxidase, α -tocopherol, ascorbic acid, melanins, and other metals and free radical mediators (Steenvoorden and Beijerbergen van Henegouwen, 1997). Previous studies have shown that members of this anti-oxidant system work in conjunction with one another, and indicate that a balance among the levels of these anti-oxidants must be maintained to achieve greatest efficacy and protection against cellular oxidation (Shindo *et al*, 1993). Raman scattering spectroscopy has the ability to discern carotenoids from among these other anti-oxidant moieties in the skin, due to its unique ability to "fingerprint" molecules with resonant double-bond structures. When these molecules are stimulated by laser

light, carbon backbone vibrations generate a Raman shift, which may then be measured by the Raman spectrometer. This method differs significantly from fluorescence energy, which is emitted from many different kinds of molecules as a complex signal (Kollias *et al*, 1998). Previous investigators have evaluated the use of noninvasive fluorescence spectra to distinguish between healthy and diseased states; however, this technology did not enable specific quantitation of carotenoids in skin (Lohmann *et al*, 1989; Hung *et al*, 1991; Cordeiro *et al*, 1995).

In this study, we have demonstrated that the Raman scattering spectroscopy method correlates with HPLC analysis of carotenoids in human abdominoplasty skin, with a limit of quantitation of 17 ng per g (Table II). Precision of the method demonstrates an average coefficient of variation of less than 10% across all subjects, exhibiting excellent reproducibility. Sensitivity of this method was also confirmed by its dose responsiveness of increasing concentrations of known individual and combined carotenoid ethanolic solution *in vitro*, and differentiation among various regions of the body *in vivo*.

HPLC analysis utilizing reversed-phase HPLC separations allowed for the separation of dietary carotenoids as well as vitamin A and the two forms of vitamin E, α tocopherol and γ -tocopherol. The normal phase HPLC separation allowed the separation of lutein, zeaxanthin, and their oxidative metabolites. This enabled the identification of lycopene as the most prevalent carotenoid, followed by phytoene, the combined carotenes, lutein and zeaxanthin, and phytofluene as the most prevalent in human skin. Because Raman scattering spectra was collected under excitation at 488 nm, the carotenoids that do not absorb at this wavelength, i.e., phytoene and phytofluene, are not detected with this method. The most prevalent carotenoids, however, lycopene and the combined carotenes are easily detected, as expected, by their absorption spectra. The predominance of lycopene in the skin is in agreement with its presence in other tissues, such as the prostate (Rao *et al*, 1999) and the ovary (Stahl *et al*, 1992).

The significant change in Raman carotenoid values associated with sequential removal of the stratum corneum (skin stripping) demonstrates that carotenoids are well represented within the stratum corneum. Thus, the stratum corneum may serve as first-line protection from UV and oxidative insult.

The lower carotenoid concentrations within perilesional, actinic keratosis, and basal cell carcinoma sites compared with region-matched skin in healthy individuals are significant and suggest that decreased carotenoid concentrations may predispose an individual to the development of skin cancer. Although preliminary, these data represent the first evidence that carotenoid levels in the skin correlate with the presence or absence of skin cancer.

In conclusion, quantitation of carotenoid content in human skin using Raman scattering spectroscopy is precise, accurate, specific, and sensitive. The noninvasive method shows promise in assessing skin cancer risk. Further work is needed to understand the relative contributions of dietary and supplemental carotenoids on skin carotenoid concentration, as well as the possible correlation with human cutaneous pathology.

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REFERENCES

- Anderson RR, Parrish JA: The optics of human skin. *J Invest Dermatol* 77:13-19, 1981
- Bakker Schut TC, Puppels GJ, Kraan YM, Greve J, Van Der Maas LLJ, Figdor CG: Intracellular carotenoid levels measured by Raman microspectroscopy: Comparison of lymphocytes from lung cancer patients and healthy individuals. *Int J Cancer* 74:20-25, 1997
- Bernstein PS, Yoshida MD, Katz WB, McClane RW, Gellermann W: Raman detection of macular carotenoid pigments in intact human retina. *Invest Ophthalmol Vis Sci* 39:2003-2011, 1998
- Biesalski HK, Hemmes C, Hopfenmuller W, Schmid C, Gollnick HPM: Effects of controlled exposure of sunlight on plasma and skin levels of β -carotene. *Free Rad Res* 24:215-224, 1996
- Bohm F, Tinkler JH, Truscott TG: Carotenoids protect against cell membrane damage by the nitrogen dioxide radical. *Nat Med* 1(2):98-99, 1995
- Breslow RA, Alberg AJ, Helzlsouer KJ, *et al*: Serological precursors of cancer: Malignant melanoma, basal and squamous cell skin cancer, and prediagnostic levels of retinol, β -carotene, lycopene, α -tocopherol and selenium. *Cancer Epid* 4:837-842, 1995
- Burton GW, Ingold UK: β -carotene: an unusual type of lipid anti-oxidant. *Science* 224:569-573, 1984
- Chen LC, Sly L, Jones CS, De Tarone R, Luca LM: Differential effects of dietary beta-carotene on papilloma and carcinoma formation induced by an initiation-promotion protocol in SENCAR mouse skin. *Carcinogenesis* 14(4):713-717, 1993
- Conn PF, Schaleh W, Truscott TG: The singlet oxygen and carotenoid interaction. *J Photochem Photobiol* 11:41-47, 1991
- Cordeiro PG, Kirschner RE, Hu QY, *et al*: Ultraviolet excitation fluorescence spectroscopy: a noninvasive method for the measurement of redox changes in ischemic myocutaneous flaps. *Plastic Reconstr Surg* 96:673-680, 1995
- Gellermann W, Yoshida MD, McClane RW, Balashov N, Bernstein PS: Raman detection of pigments in the human retina. *Proceedings of Optical Biopsy II* January 25-26, 1998, San Jose, California, SPIE
- Hung J, Lam S, Le Riche JC, Palcic B: Autofluorescence of normal and malignant bronchial tissue. *Lasers Surg Med* 11:99-105, 1991
- Jacques SL: Simple theory, measurements, and rule of thumb for dosimetry during photodynamic therapy. *SPIE Photodynamic Ther: Mechanisms* 1065:100-108, 1989
- Karagas MR, Grenbert R, Nierenberg D, Stukel TA, Morris JS, Stevens MM, Baron JA: Risk of squamous cell carcinoma of the skin in relation to plasma selenium, α -tocopherol β -carotene, and retinol: a nested case-control study. *Cancer Epidemiol* 6:25-29, 1997
- Khachik F: Process for isolation, purification, and recrystallization of lutein from saponified marigolds oleoresin and uses thereof. US Patent, 5,382714, January 17, 1995
- Khachik F, Beecher GR, Goli MB, Lusby WR, Smith JC: Separation and identification of carotenoids and their oxidation products in extracts of human plasma. *Anal Chem* 64:2111-2122, 1992a
- Khachik F, Beecher GR, Goli MB, Lusby WR: Separation and quantification of carotenoids in foods. In: Packer L (ed.). *Methods in Enzymology*, Vol. 213a. New York: Academic Press, 1992b, pp 347-359
- Khachik F, Englert G, Daitch CE, Beecher GR, Lusby WR, Tonucci LH: Isolation and structural elucidation of the geometrical isomers of lutein and zeaxanthin in extracts from human plasma. *J Chromatogr Biomed Application* 582:153-166, 1992c
- Khachik F, Spangler CJ, Smith JC, Jr, Canfield LM, Pfander H, Steck A: Identification, quantification, and relative concentrations of carotenoids, their metabolites in human milk and serum. *Anal Chem* 69:1873-1881, 1997
- Kollias N, Gillies R, Moran M, Kochevar IE, Anderson RR: Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging. *J Invest Dermatol* 111(5):776-783, 1998
- Koningsstein JA: *Introduction of the Theory of the Raman Effect*. Dordrecht: D. Reidel Publishing, 1971
- Koyama Y, Takasuka I, Nakata M, Tasumi M: Raman and infrared spectra of the all-trans, 7-cis, 9-cis, 13 cis, and 15 cis isomers of β -carotene: Key bonds distinguishing stretched or terminal-bent configurations from central-bent configurations. *J Raman Spectroscopy* 19:37-49, 1988
- Krinsky NI: Effects of carotenoids in cellular and animal systems. *Am J Clin Nutr* 53:238S-248S, 1991
- Krinsky HI, Deneke SM: The interaction of oxygen and oxyradicals with carotenoids. *J Nat Cancer Inst* 69:205-210, 1982
- Lohmann W, Mussmann J, Lohmann C, Kunzel W: Native fluorescence of the cervix uteri as a marker for dysplasia and invasive carcinoma. *Eur J Obstet Gynecol Reprod Biol* 31:249-253, 1989
- Murakoshi M, Nishino H, Satomi Y, *et al*: Potent action of α -carotene against carcinogenesis: spontaneous liver carcinogenesis and promoting stage of lung and skin carcinogenesis in mice are suppressed more effectively by α -carotene than by β -carotene. *Cancer Res* 52:6583-6587, 1992
- Nagasawa H, Mitamura T, Sakamoto S, Yamamoto K: Effects of lycopene on spontaneous mammary tumor development in SHM virgin mice. *Anticancer Res* 15:1173-1178, 1995
- Narisawa T, Fukaura Y, Haseve M, *et al*: Inhibitory effects of natural carotenoids, α -carotene, β -carotene, lycopene, and lutein, on colonic aberrant crypt formation in rats. *Cancer Lett* 107:137-149, 1996
- Pershing LK, Silver BS, Krueger GG, Shah VP, Skelley JP: Feasibility of measuring the bioavailability of topical betamethasone dipropionate in commercial formulations using drug content in skin and a skin blanching bioassay. *Pharmaceut Res* 9:45-51, 1992
- Pershing LK, Lambert LD, Krueger GG, Shah VP: Variability and correlation of chromameter and tape stripping methods with the visual skin blanching assay in the quantitative assessment of topical 0.05% betamethasone dipropionate bioavailability. *Int J Pharmaceutics* 86:201-210, 1993
- Rao AV, Fleshner N, Agarwal S: Serum and tissue lycopene and biomarkers of

- oxidation in prostate cancer patients: a case control study. *Nutr Cancer* 33(2):159-164, 1999
- Shindo Y, Witt E, Packer L: Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light. *J Invest Dermatol* 100(3):260-265, 1993
- Stahl W, Schwarz W, Sundquist AR, Sies H: Cis-trans isomers of lycopene and beta-carotene in human serum and tissues. *Arch Biochem Biophys* 294:173-177, 1992
- Steenvoorden DPT, Beijerbergen van Henegouwen GMJ: The use of endogenous antioxidants to improve photoprotection. *Photochem, Photobiol* 41:1-10, 1997
- Van Gemert MJC, Jacques SL, Sterenborg HJCM, Star WM: Skin optics. *IEEE Trans Biomed Eng* 36(12):1146-1154, 1989
- Verma SP, Wallach DFH: Carotenoids as Raman-active probes of erythrocyte membrane structure. *Biochim Biophys Acta* 401:168-176, 1975a
- Verma SP, Wallach DFH, Schmidt-Ullrich R: The structure and thermotropism of thymocyte plasma membranes as revealed by laser-Raman spectroscopy. *Biochim Biophys Acta* 394:633-645, 1975b
- Wingerath T, Sies H, Stahl W: Xanthophyll esters in human skin. *Arch Bio Biophys* 355:271-274, 1998