Optical detection methods for carotenoids in human skin

Igor V. Ermakov *, Werner Gellermann

Department of Physics and Astronomy, University of Utah, Salt Lake City, UT 84112, USA

Article info

Article history:
Received 26 November 2014
and in revised form 16 January 2015
Available online 28 January 2015

Keywords:
Carotenoids
Antioxidants
Human skin
Raman spectroscopy
Reflection spectroscopy

Abstract

Resonance Raman spectroscopy and reflection spectroscopy are non-invasive optical quantitative methods for the measurement of carotenoid antioxidant levels in human skin in vivo. Since all tissue carotenoids are derived from the diet, optical monitoring in skin may serve as an objective indicator for fruit and vegetable intake, and more broadly also as an effective biomarker for integrative health. The two optical methods have already found enthusiastic application in the Nutritional Supplement Industry where they are used with large populations to measure skin carotenoid uptake upon consumption of carotenoid-containing dietary supplements. Applications in medical fields such as nutrition science and epidemiology have been awaiting rigorous correlation studies between the optical carotenoid detection methods and the established gold standard detection method of high-performance liquid chromatography, which requires excised tissue samples. In this article we review the principles of the methods along with the current status of validations so the reader can assess the merits of the optical methods in their respective fields of interest.

Introduction

Carotenoids play an important protective role in healthy human tissues through their optical filtering and/or antioxidant functions [1]. They cannot be synthesized by the human body; rather they need to be taken up from the diet, where they exist in high concentrations in a wide range of fruits and vegetables [2]. The most common carotenoids in the diet are alpha-carotene, beta-carotene, beta-cryptoxanthin, lycopene, lutein, zeaxanthin, phytoene and phytofluene [3]. Usually all these carotenoids are absorbed from the diet by the intestine and transported by lipoproteins through the bloodstream to various target tissues [4]. Carotenoid levels in the blood stream are correlated with dietary intake, typically increasing significantly in response to fruit and vegetable consumption in intervention settings [5]. In the retina, where the carotenoid uptake is mediated by binding proteins, only lutein and zeaxanthin are selectively taken up from the blood stream. They are deposited in high concentrations in the macular region of the retina, where they help prevent or delay the onset of age-related macular degeneration [6]. In skin, all carotenoids are taken up that are present in the blood stream. Here, the carotenoids may help prevent premature skin aging [7] and certain skin cancers [8]. Skin is a relatively stable storage medium for carotenoids and non-invasive optical measurements in this tissue may serve as a novel non-invasive biomarker for fruit and vegetable intake. Since adequate fruit and vegetable consumption is closely linked to reductions in chronic diseases such as various cancers [9], cardiovascular disease [10], age-related degenerative diseases [11] and obesity [12], rapid painless optical screening methods for large populations could have tremendous utility. It would be possible to identify populations at particular risk for inadequate intake of fruits and vegetables with these screening methods, and to evaluate the success of interventions aimed at increasing fruit and vegetable intake.

In this manuscript, we review the principles of the two spectroscopy methods, validation studies carried out to date, and their feasibility for applications in field and clinical settings.

Resonance Raman spectroscopy, RRS

RRS is a laser spectroscopy method that detects the characteristic vibrational/rotational energy levels of a molecule. Carotenoid molecules are particularly well suited for RRS-based detection since their chain-like carbon backbone is conjugated. It consists of a sequence of alternating carbon double – and single bonds (C=C and C–C bonds, respectively), with the outer electron of the molecule free to move along the chain. This leads to strong absorption bands in the blue region of the optical spectrum as shown in Fig. 1(a) and the possibility to obtain resonantly enhanced Raman scattering responses when exciting the carotenoids with suitable
blue laser lines or other spectrally narrowed light sources. The RRS responses occur as spectrally sharp spectral lines (Stokes lines) at discrete frequencies that are downshifted from the excitation laser frequency by the respective vibrational frequencies of the carotenoid backbone constituents, i.e. at shifts that are due, respectively, to the C=C and C–C stretch frequencies, and the rocking frequency of the CH3 molecules attached on the side of the carbon backbone [13]. As an example, we show in Fig. 1(b) a typical RRS spectrum of carotenoids for a solution in methanol, featuring three strong and clearly resolved carotenoid Stokes lines at ~1525 cm⁻¹ (C=C stretch), 1159 cm⁻¹ (C–C stretch), and 1008 cm⁻¹ (C–CH3 rocking motions). A relatively weak line exists also at ~960 cm⁻¹. It is due to out-of-plane wagging motions of the chain hydrogens (C–H groups), coupled with C=C torsional modes [14,15], but its intensity is too weak for quantitation purposes.

In homogenous, optically thin solvent systems, the Raman scattering intensity of any of the Stokes lines, \( I_s \), is linearly correlated with the carotenoid concentration, \( N \), according to the relation

\[
I_s = N \cdot \sigma \cdot I \cdot l
\]

where \( \sigma \) and \( l \) represent the Raman scattering cross section and laser intensity, respectively, and \( I \) is the sample path length monitored by the detection system. The RRS line intensity can therefore be used as quantitative optical measure for the carotenoid concentration.

The unique Raman scattering properties of carotenoids led us to explore RRS for the non-invasive quantitative optical measurement of carotenoids and their spatial distributions in living human tissue environments. Challenges could be anticipated in this complex tissue matrix from other tissue chromophores existing in the measured tissue volume simultaneously with the carotenoids of interest, and from laser intensity restrictions due to safety constraints. However quantitative RRS detection could be readily demonstrated in the human retina (macula) [16–18], in human skin [13,19–22] and in oral mucosal tissue [13].

A prototype RRS instrument used for skin carotenoid measurements is shown in Fig. 2(a). It consists of a 488 nm solid-state laser, a spectrograph/CCD array combination for detection of the backscattered light, and a computer for exposure control, data acquisition, processing, and display. Excitation and backscattered light is routed to and from the skin tissue site of interest with the help of fiber optics and a light delivery and collection module that is placed in direct contact with the skin tissue site of interest. Light backscattered from the skin is filtered to reject the laser excitation light, spectrally dispersed by a compact, small focal length spectrograph, and recorded with an interfaced CCD array. Using custom developed software algorithms, the recorded spectra are analyzed for the intensity of the strongest RRS line at the carbon double bond (C=C) frequency at 1525 cm⁻¹, which under 488 nm excitation occurs at 527 nm. RRS spectra with high signal to noise ratios (approximately 50:1 in best cases) can be obtained with ~9 mW exposure for 30 s at a laser excitation disk at the skin surface of ~2.5 mm diameter [13,22].

Fig. 2(b) shows the computer display after a single in vivo measurement. The measured raw spectrum, shown in the left panel, features a strong spectrally broad “autofluorescence” background, along with the three weak tissue Raman carotenoid lines, which are superimposed on this background. Even though the autofluorescence intensity is about 100 times stronger compared to the carotenoid RRS lines, it is possible to quantify the latter with high accuracy by employing a detector with high dynamic range. After fitting of the fluorescence background with a higher order polynomial and subsequent subtraction from the raw spectrum, an isolated skin carotenoid RRS spectrum is obtained (right panel) that is virtually undistinguishable from the RRS spectrum of a pure \( \beta \)-carotene solution (Ref. Fig. 1(b)).

The skin carotenoid RRS response originates from contributions of all carotenoid species present in the tissue volume that absorb in the blue wavelength region [23]. Since all individual C=C Raman line positions are identical at the instrument’s spectral resolution, the intensity of the C=C RRS line at 1525 cm⁻¹ can be used as a measure for the combined concentrations of all carotenoids existing in the measured skin tissue volume (with the exception of the “color-less” phytoene and phytolfluene, which absorb in the UV range).

The RRS intensity varies with excitation light intensity and of course depends also on the sensitivity of the instrumentation (light throughput, optical alignment, specifications of CCD detector array, etc.). It is therefore susceptible to intensity fluctuations and requires an external calibration standard for routine sensitivity checks and cross-calibration purposes between instruments. A suitable external standard is sodium nitrate, featuring a Raman line which is in close proximity to the carotenoid RRS lines of interest. The RRS intensities of the standard are measured prior to a skin carotenoid measurement session and used to correct any potential deviations of the instrument response from its original sensitivity. A comprehensive description of the RRS methods for quantitative skin carotenoid measurements is published elsewhere [13,22,23].

**Selective RRS detection of carotenes and lycopene in human skin**

Skin carotenoid RRS measurements under blue excitation, as described above, provide a measure for the total concentration of all carotenoid species absorbing in that wavelength region since
all are excited equally efficient. However, lycopene has an increased conjugation length compared to the other blue absorbing carotenoids in skin, causing a small (~10 nm) red shift of its absorption band [20]. This shift can be exploited to selectively measure skin lycopene levels in a sequential two-wavelength excitation scheme with 488 and 514.5 nm lasers [20], leading to RRS lines at 527 and 558 nm, respectively. The longer excitation wavelength leads to 6-fold higher lycopene RRS intensities relative to the carotene RRS intensities, and lycopene levels can be derived from the measurements along with total carotene levels using a simple carotene/lycopene absorption scenario [20,22].

In seven volunteer subjects measured with this two-wavelength RRS methodology [20], a strong, almost threefold variation in carotene-to-lycopene ratios could be observed between subjects, ranging from 0.54 to 1.55. This demonstrates that substantially different carotenoid compositions can exist between subjects, with some subjects exhibiting almost twice the skin concentration of lycopene versus carotene, and other subjects exhibiting the opposite. The differences are due to different dietary patterns in the consumption of lycopene or lycopene-containing vegetables, or due to individual differences in uptake/depletion processes for the respective carotenoid species.

In another pilot study, we demonstrated the capability of the two-wavelength method to detect and track the selective accumulation of lycopene in skin upon dietary supplementation. Three volunteer subjects consumed a tomato extract at a dose of 20 mg/day.
for a period of 50 days [24], in addition to their normal diet. As shown in Fig. 3, the dermal RRS lycopene levels increased in all cases, doubling for the subject with lowest starting level, and increasing by ~75% and ~50%, respectively, for the others. In Fig. 4, both lycopene and carotene kinetics are shown for the subject with lowest starting level. In agreement with the dietary pattern, the RRS lycopene and carotene kinetics are shown for the subject with lowest starting level. In Fig. 4, both lycopene and carotene kinetics are shown for the subject with lowest starting level.

The obtained results show clearly that lycopene and carotene skin levels can be measured independently with the RRS method in a suitable two-wavelength instrument configuration.

**Validation studies**

The carbon stretch vibrations have relatively high frequencies and originate from internal molecule vibrations, i.e. from their carbon backbone. Since the molecule frequencies are not appreciably shifted by their own end groups, the tissue environment can be expected to have only a minor effect, a shift of only a few wavenumbers at most. Carotenoids RRS responses should therefore be virtually identical for the isolated molecule, the molecule in solution, or the molecule in a cell environment. However, the applicability of the method may heavily depend on potentially confounding tissue properties such as a saturation of the carotenoid RRS response at high concentrations, and/or the combined effects of absorption, fluorescence and scattering caused by other molecules simultaneously present in the measured tissue volume. A crucial task for the acceptance of the skin carotenoid RRS detection method therefore is its validation for the particular tissue environment.

For portable skin carotenoid RRS instrumentation, 488 nm and 473.2 nm solid state lasers or 488 nm air-cooled argon ion lasers were initially available as excitation light sources. In spite of their differing spectral positions and bandwidths, we showed that the resulting carotenoid RRS responses are essentially comparable in all cases and that there is no need to validate the RRS method separately for each excitation laser [23]. The linearity of the carotenoid RRS response with exposure duration and excitation power, as expected from Eq.(1), was verified for in vivo skin measurements [23]. Also, the intrinsic test–retest reproducibility of the RRS intensity could be shown to be extremely high for a fixed tissue location (standard deviation of about 1%), while the RRS intensities varied, between 1% and 15%, in accordance with the degree of tissue inhomogeneities [23]. In each case, the repeatability figures could be kept at a minimum in sequential measurements by tightly controlling the position of the optical contact module with respect to the tissue location of interest.

The gold standard for tissue carotenoid measurements is based on high performance liquid chromatography, HPLC, methods, which require highly invasive chemical extraction of the sample of interest, but as an advantage are able to detect individual carotenoid concentrations. In first validation experiments, we compared skin carotenoid RRS measurements with HPLC levels of fasting serum for 104 healthy male and female volunteer subjects [25]. As tissue site for the measurements, the inner palm of the hand was chosen since it contains minimal concentrations of melanin, which as a strongly absorbing and scattering chromophore can potentially attenuate the skin carotenoid RRS response [13]. The correlation result is shown in Fig. 5(a), demonstrating a statistically significant correlation with p < 0.001 and a high coefficient of 0.78.

In a recent study, RRS/HPLC serum correlations were further investigated for 29 subjects participating in controlled dietary intervention phases over a time period of 28 weeks [26]. The phases included two diet phases low in carotenoids/provided fruit and vegetables, a phase with a diet rich in carotenoids, and a subsequent normal diet phase. Blood was collected at 9 time points at the starting and mid points of the respective intervention phases. Fig. 5(b) shows the correlation between skin RRS and plasma carotenoids obtained at the time points when blood samples were collected. At baseline, skin RRS and plasma HPCL total carotenoid values correlated well (r = 0.61, p < 0.001). Also, per maximum likelihood for all phases, the overall within-individual correlation between skin RRS and plasma carotenoids was as high as 0.7, and the between-individual correlation was 0.72 (p < 0.001 for both).

Further high correlations between skin RRS carotenoid and plasma HPLC carotenoids were seen in studies involving adults: N = 28, R = 0.62, p = 0.006 [27], post-delivery woman: N = 30, R = 0.63, p < 0.001 [28], school children N = 38, R = 0.62, p < 0.0001 [29], N = 45, R = 0.7–0.73, p < 0.001 [30], and 51 infants and children (up to 7 years old): R = 0.78, p < 0.0001 [31]. The HPLC
analyses in all these studies had been limited to subset of serum carotenoids involving only the 4–5 most common species (\(\beta\)-carotene, lycopene, lutein, and \(\beta\)-cryptoxanthin). Since all RRS carotenoid results are generated as composite scores for all carotenoid species present in the measured tissue (typically about a dozen), the obtained correlations with the incomplete HPLC data are therefore artificially lowered.

To validate the skin carotenoid RRS detection method more directly with excised tissue samples, a study was carried out in which 28 healthy adults had their total RRS carotenoid levels measured for a posterior hip tissue location and compared with HPLC results or subsequently biopsied tissue samples (3 mm punch). Total carotenoid levels by RRS were significantly correlated with total carotenoid levels by HPLC (\(R = 0.66, p = 0.0001\)) [27]. Lycopene skin levels as measured with selective two-wavelength RRS correlated with corresponding HPLC biopsy lycopene levels even higher (\(R = 0.74, p < 0.0001\)), as shown in Fig. 6(a). This is explainable due to the HPLC analysis of exactly the same species. Errors in the HPLC analysis can be assumed to occur due to the relatively small tissue volumes of the punch biopsies and due to processing of tissue depths exceeding the light penetration, i.e. the two methods compare different tissue volumes.

To eliminate these error sources, heel skin RRS carotenoid levels were measured in another in vivo study for eight volunteer subjects, and compared with HPLC results for subsequently excised thin skin slivers. While thin (~0.6 mm), the chosen tissue samples had a higher mass (10–50 mg) relative to the skin biopsies (~3 mg). Also, the HPLC analysis was extended to a larger subset of species, including lutein, zeaxanthin, cis-lutein/zeaxanthin, \(\alpha\)-cryptoxanthin, \(\beta\)-cryptoxanthin, trans-lycopene, cis-lycopene, \(\alpha\)-carotene, trans-\(\beta\)-carotene, cis-\(\beta\)-carotene, and canthaxanthin. The correlation is plotted in Fig. 6(b). It now shows a near perfect coefficient \(R = 0.95, p < 0.001\), with the regression line fit passing through the origin and the RRS carotenoid levels increasing linearly over a wide range of physiological skin carotenoid concentrations. The obtained correlation results allow us to directly calibrate the used RRS instrument in tissue carotenoid concentration units, where the total skin carotenoid content, \(C\), measured in \(\mu g\) per \(g\) of skin tissue is linked to the height of the \(C\)-carotenoid RRS intensity, \(I\), in the instrument by the relation

\[
\frac{C}{\mu g/g} = 4.3 \cdot 10^{-5} \cdot I/\text{counts}.
\]

Applications

Nutritional Supplement Industry

Following proof-of-concept developments, the RRS method was enthusiastically embraced in the Nutritional Supplement Industry, where it is used to identify customers with low skin carotenoid levels, to track uptake levels upon consumption of carotenoid containing dietary supplements and in this way to prove the efficacies of the supplements [25,32]. Initially, the laboratory RRS instrumentation was miniaturized into a commercial portable field usable platform based on a 473 nm solid state frequency up-conversion laser and a compact 65 mm spectrograph/CCD detector combina-
A second generation instrument followed soon after in which the laser was replaced with a more rugged, spectrally narrowed LED light source [33]. Known as the “Biophotonic Scanner” (NuSkin/Pharmanex Inc., Provo, Utah), about ten thousand portable skin carotenoid RRS scanners are presently in use in this industry, with the total number of measured subjects exceeding 10 million. An early histogram of RRS skin carotenoid levels measured for 32,948 subjects is shown in Fig. 7(a). It features a bell shaped distribution with a slight skew toward higher RRS levels ("skin carotenoid score"). The halfwidth of the distribution is large (about 75% of the peak score), showing that individual skin carotenoid levels can vary widely between subjects. Similar distributions were observed in a screening study involving 1375 healthy volunteer subjects [25,27], and in other smaller-scale studies [13,27,29].

The high population numbers easily achievable with the RRS methodology provide insight, with high statistical significance, into the effects of diets, external stress factors such as smoking, and demographic differences. Analysis of the data confirmed a pronounced positive relationship between self-reported fruit and vegetable intake and skin Raman response [25,27–29,31,34,35]. Subjects with habitual high sunlight exposure had significantly lower skin carotenoid levels than people with little sunlight exposure, independent of their carotenoid intake or dietary habits, and smokers had dramatically lower levels of skin carotenoids as compared to nonsmokers [22].

In a study involving 22 subjects, Meinke et al. [36] evaluated a commercial oil extract of kale (Lutrex™), which was rich in carotenoids, versus a placebo oil to assess bioavailability. Measuring RRS skin carotenoid levels in each group of 11 subjects, they found an increase in both skin and blood carotenoids with Lutrex™ supplementation. The blood values increased faster in the first 14 days as compared to the subsequent 14 days, suggesting saturation. In contrast, the skin levels did not appear to saturate. When Lutrex™ supplementation ceased, carotenoid decreases were much more rapid in serum than in skin. This is consistent with a much longer half-life of carotenoids in skin.

**Nutrition science**

RRS based skin carotenoid measurements are particularly attractive for assessment of fruit and vegetable intake in infants and children. Accurate measurement of fruit and vegetable consumption by questionnaire-based parent or self-report is challenging and of questionable validity. Plasma HPLC carotenoid concentrations is rarely conducted in school settings in children, mostly due to parental and child concerns regarding the invasive nature of blood draws.

As an example, Fig. 7(b) illustrates a distribution for a group of 256 school children (ages 10–12 years) who had their skin carotenoid levels measured over two consecutive school days with research-grade RRS instrument. Remarkably, the shape of this distribution mirrors the shape for adults, with the slight differences in maxima likely attributable to a different demographic.

A different study investigated RRS measured skin carotenoid levels in relation to parent-supported fruit and vegetable intake in preschool children (N = 381). As a result, a near-normal inter-subject distribution was found. Also, parent-reported fruit and vegetable consumption could be established as the major biomarker in this population [35]. In a similar study by Aguilar et al. involving children ages 10–17 (N = 45) and a commercial RRS device, skin carotenoids were measured at three different time points, with reported correlations between skin and serum carotenoids of R = 0.7, R = 0.71, and R = 0.73, respectively [30]. They also reported a correlation between skin carotenoids and reported food intake (R = 0.57). Latest cross-sectional school-based studies [29] found associations between self-reported carotenoid intake and RRS measurements with correlations as high as R = 0.40 (p < 0.0001, N = 128) while the total plasma carotenoid concentrations correlated with RRS skin carotenoid levels strongly (R = 0.62, p < 0.0001, N = 38). In conclusion of the studies [35,30,29] it can be stated that children accept skin carotenoid RRS measurements very easily, and skin carotenoid status as measured by RRS can be a strong predictor of plasma carotenoid status and dietary intake of carotenoids in children.

To investigate whether the RRS method is capable of tracking carotenoid uptake in adults upon regular dietary intervention instead of upon carotenoid supplementation, a comprehensive multi-phase study was carried over a 28-week (196 days) time span in which 29 subjects consumed a diet varying drastically in carotenoid content among phases [26]. Specifically, the study design included a depletion phase with a diet low in carotenoids (days 1–42; 1.4 mg/day), a “feeding” phase with a diet high in carotenoids (days 43–98; 62 mg/day), a phase with a diet again low in carotenoids (days 99–140; 1.5 mg/day), and a final repletion phase with a normal diet (days 141–196; 9 mg/day). Carotenoid status in skin was assessed each day with RRS during the feeding phase and 2–5 times a week during other phases. As tissue site, the inner palm was chosen for the RRS carotenoid measurements. Fig. 8 shows an example of typical skin carotenoid kinetics observed for one of the volunteers. Following the depletion phase, a twofold increase is observed during the feeding phase, followed by a significant decrease during the subsequent depletion phase, and a slow increase again during the natural repletion phase. Interestingly,

Fig. 7. Carotenoid levels measured in the palm of the hand for (a) 32,948 adults and (b) 256 elementary school children. Shown as histograms, the levels have near bell-shaped distributions with large widths that are slightly skewed toward higher levels.
the individual dermal carotenoid level increases observed for most participants during the feeding phase occurred with comparable slopes. This effect would be in agreement with the standardized dose of carotenoids each subject ingests with the regular diet.

The kinetics of RRS skin carotenoid levels and HPLC plasma concentrations, averaged for all 29 subjects, are shown in Fig. 9(a) and (b), respectively. Both skin and plasma carotenoid values decreased significantly ($p < 0.001$) between baseline and the end of the first depletion phase and increased significantly during the feeding phase. Plasma carotenoids returned to baseline by the middle of the second depletion phase, whereas skin levels returned to baseline only by the middle of the natural repletion phase (phase 4), i.e. they were delayed by more than a month compared to plasma levels. Clearly, the kinetics show [26] that plasma concentrations decrease more rapidly than skin levels, which is consistent with blood serving as a transport medium whereas skin serves as a storage medium.

Considering skin and blood as tissues with different uptake kinetics, correlations between carotenoid skin levels and plasma concentrations were examined in more detail. The horizontal dashed line in Fig. 9(c) separates all data points to two groups of relatively high correlation coefficients ($R = 0.56–0.75$) and relatively low coefficients ($R = 0.3–0.5$), respectively. Low blood/skin correlations exist for the dietary intervention phases; highest correlations occur for baseline, first depletion, and (middle and end) of the repletion phases, i.e. whenever a steady-state conditions were present in which a constant dietary carotenoid intake over a sufficiently long time had resulted in stationary carotenoid levels in both blood and skin. The condition for a high correlation between skin carotenoid RRS levels and blood HPLC levels therefore depends on the carotenoid lifetimes in blood and in the skin, as well as on a higher carotenoid intake rate compared to the lower blood to the skin transfer rate. In view of these results it is understandable why blood carotenoid HPLC measurements are not optimal for validation of the results by skin RRS since they may not have been carried out in steady-state conditions.

In conclusion, the RRS method is capable of accurately measuring changes in carotenoid levels in response to an intervention involving a diet enriched in fruits and vegetables.

Medical research

Skin carotenoid RRS measurements have found use as rapid, objective, carotenoid/antioxidant biomarkers in otolaryngology [37], ophthalmology [28,31], neonatology [28,38,39], epidemiology [40], and orthopedic medicine [41].

In otolaryngology, carotenoids measured non-invasively by catheter-coupled RRS are investigated in their capacity as cancer preventing agents [37]. In neonatology, carotenoid levels measured by RRS can serve as valuable biomarkers for serum carotenoid levels which are difficult to obtain since blood volume in newborn infants is small [38]. Systemic carotenoid levels were observed to dramatically drop after birth and to be barely detectable in premature infants in the newborn ICU as a result of oxidative stress. Skin carotenoid levels by RRS are a predictor also for macular carotenoid levels in the developing retina, unlike in adults [31]. The method therefore holds potential as a non-invasive tool in intervention strategies aimed at preventing risk for retinopathy of prematurity. Skin carotenoid levels by RRS are further useful to investigate interrelationships between maternal carotenoid status
and newborn infant systemic as well as macular pigment levels [28], which can be influenced by maternal diet composition and factors such as intrauterine growth restriction (IUGR). The value of breast milk diet in supporting the systemic carotenoid status of preterm infants was demonstrated in [39]. One group of infants was fed mother’s milk, whereas other was fed a preterm infant formula that was not enriched with carotenoids. The infants fed human milk had a higher serum total carotenoid concentration and skin RRS counts than formula-fed infants. The human milk-fed infants’ serum total carotenoid concentrations and skin RRS values did not change during the study period; however, the formula-fed group’s total serum and skin carotenoid decreased significantly during the study, suggesting that formula-fed infants may benefit from carotenoid supplementation.

In epidemiology, skin carotenoid levels measured by RRS that deviate from “normal” levels may serve as an indicator for potential diseases. For example, in study [40] involving 155 patients referred for dietary assessment, with 44% suffering from metabolic syndrome, average skin carotenoid RRS levels in the “healthy” subpopulation were 32.011 ± 5.514 counts, and RRS levels in the metabolic syndrome group were 23.058 ± 9.812 counts, i.e. they were reduced by about 30% on average.

In orthopedic medicine, skin carotenoid levels by RRS may serve as a biomarker for carotenoid levels in bone [41]. The optimal health and homeostasis of bone involves a dynamic balance between bone formation and resorption as well as a multitude of biochemical reactions necessary for life. This balance requires an intimate relationship with the environment, particularly as it relates to food intake. Epidemiological studies have suggested that high intakes of vegetables and fruits reduce the risk of osteoporosis, that carotenoid antioxidants are beneficial micronutrients for the maintenance of normal bone metabolism, that serum levels are associated with bone mineral density in post-menopausal women [42] and that total carotenoid and lycopene intake has a protective effect on hip fracture [43]. Measurements of biopsied tissue samples from total knee arthroplasty with molecule-specific RRS and high performance liquid chromatography revealed that all carotenoids known to exist in human skin are also present in human bone. This includes all carotenes, lycopene, beta-cryptoxanthin, lutein and zeaxanthin [41]. This will allow one to establish potential correlations between internal bone and surrounding tissue carotenoid levels with skin, and to potentially use already existing optical skin carotenoid tests as surrogate marker for bone carotenoid status.

### Reflection spectroscopy, RS

Reflection spectroscopy (RS) had been investigated as a potential optical method for the non-invasive and quantitative detection of skin carotenoids more than a decade ago [44,45]. Taking into account the inhomogeneity of chromophore distributions in the living tissue, a complex spectral de-convolution algorithm was used in a first-principles approach, involving multi-compartment modeling for skin chromophores. These researchers found a significant correlation between baseline skin and serum carotenoid levels in a 12-week β-carotene supplementation study, and they were able to document an apparent rise in response to supplementation in a small group of volunteer subjects [44]. However, the interpretation of reflection spectra within the diffusive light transport model in turbid media was recognized to be problematic for the assessment of the relatively weakly absorbing carotenoid chromophores [46], and the particular methodology has not found widespread application.

A further reflection-based variant explored skin color saturation measurements [47]. In this approach, one of the color tri-stimulus values, the b’-value, was measured and compared to the chromaticity diagram of a white reflection standard. Since the b’-value measures the color saturation from the yellow to the blue region, it can be expected to be influenced by the absorption of skin carotenoids occurring in this spectral range. Also, the measurements are influenced not only by the carotenoid absorption but also by the superimposed absorption and scattering effects of blood and melanin, thus leading to rather unspecific results with relatively large errors.

Recently, a pressure-mediated RS variant was developed that derives skin carotenoid absorptions via reflectivity measurements under application of topical pressure [48,49]. The method holds promise as a particularly simple and inexpensive method since it does not require any narrow-band light sources for excitation or sensitive detectors for intrinsically weak Raman responses. Sketched in Fig. 10(a), it uses a spectrally broad “white light” source provided by a tungsten halogen lamp or “white” LED. A light delivery/collection module projects the white light onto the skin as a 3 mm diameter excitation disk. A plano-convex lens serves as the

<table>
<thead>
<tr>
<th>Wavelength, nm</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>0.55</td>
</tr>
<tr>
<td>480</td>
<td>0.60</td>
</tr>
<tr>
<td>610</td>
<td>0.65</td>
</tr>
<tr>
<td>800</td>
<td>0.70</td>
</tr>
</tbody>
</table>

\[ A(\lambda) = - \lg(R(\lambda)/100) \]

\[ Acar = A_1 - A_2 \]

\[ P = 3 \times 10^5 \text{ Pa} \]

\[ P = 0.0 \text{ Pa} \]

Fig. 10. (a) Computer controlled, portable, pressure-mediated reflection spectroscopy instrument for skin carotenoid measurements; (b) typical reflection derived absorption spectra obtained from the thumb tissue of a healthy adult volunteer subject. A characteristic carotenoid absorption band can be clearly resolved and quantified at ~480 nm after temporal removal of blood chromophores from the measured tissue. The skin carotenoid optical density in the shown example is 0.07, which for an approximately 0.7 mm effective light path corresponds to tissue concentration of ~3 µg per gram of skin. The plots were adapted from [49].

---

1 Abbreviations used: IUGR, intrauterine growth restriction; RS, reflection spectroscopy; HbO₂, oxyhemoglobin.
window of the module and is in direct contact with the skin. Light diffusively reflected at 45° from the skin surface is collimated and focused into a spectrograph that is interfaced to a laptop computer for data acquisition, processing, and display. The apparent absorption, A, at a given spectral location, $\lambda$, is related to the measured reflectivity, $R(\lambda)$, according to the relation

$$A(\lambda) = -\log[R(\lambda)/100]$$

(3)

where lg is the logarithm to base ten.

Upon contact with the measured skin surface, the window applies a topical pressure of about 1 atm. and squeezes the skin’s blood away from the vertex location of the window into radial tissue directions. In this process, the blood circulation in the squeezed tissue volume is temporarily blocked, oxyhemoglobin (HbO2) absorptions are strongly reduced, and any residual HbO2 is converted into deoxyhemoglobin, Hb, with the latter having about 2.5 times lower absorption strength relative to HbO2 (see Fig. 10(b)). To monitor the topical pressure, a piezo-resistive load cell is mounted onto the lens assembly, providing visual feedback for the measured subject, who is asked to keep the pressure constant within a pre-selected optimal pressure range of $\sim 10^3$ Pa (1 atm) ± 10%. As alternative, mechanical spring – clamp combination can be used to provide constant pressure during the RS measurements, as implemented in our other instruments and shown in Fig. 10(a).

To derive tissue carotenoid levels from a measured apparent absorption spectrum, a simple algorithm is used that calculates the absorbance difference between the absorbance at 480 nm and the absorbance at 610 nm in optical density units, as sketched in Fig. 10(b), thus disregarding the background tissue absorbance in the carotenoid region of interest. This approach assumes that under the applied topical pressure the scattering background at 480 nm is comparable to the background at 610 nm, and that any scattering contributions from the skin matrix will be very similar between subjects. The light excitation disk is relatively large (~3 mm dia.) and thus helps to average out the small tissue inhomogeneities due to ridges, sweat glands, etc. during the measurements. As tissue sites, sites with minimal melanin content are preferred, such as the tips of fingers, the palm of the hand, or the heel of a foot. In its current implementation, the RS methodology quantifies all carotenoid species absorbing in the 480 nm range, including lycopene. As illustrated in Fig. 10(b), the measured value includes contributions from all carotenoids, who are all absorbing with approximately equal weight, with the exception of the UV absorbing short-chain carotenoids phytoene and phytofluene. The feasibility of the method for selective detection of skin phytoene and phytofluene levels is still an open question due to high scattering and absorption backgrounds in the UV range.

RS validation experiments

For the validation of the RS methodology, excised heel skin samples were used, which like the palm of the hand are bloodless, free of melanin, and contain a relatively thick stratum corneum layer [23]. RS-derived absorbance levels of the samples were roughly the same as those derived via direct transmission measurements of the excised slivers [49]. Also, the obtained background corrected absorption spectra were very similar to the absorption spectrum of β-carotene.

The viability of the method for the detection of skin carotenoid uptake upon dietary supplementation was explored in a recent pilot experiment involving three volunteer subjects consuming two three-ounce servings of carrot juice per day with their regular diet. Measuring the skin of a thumb as tissue site, the RS derived carotenoid levels stayed constant (within ~10%) during a 10-day baseline phase prior to juice consumption. Upon juice consumption, the levels increased quickly for two of the subjects with relatively low starting levels, doubling within about 3 weeks, and saturating shortly after. The subject with high starting levels revealed only a modest increase over the supplementation phase [49].

In a separate uptake study, 90 volunteer participants were separated into four groups, with three groups consuming two-ounce servings of β-carote-containing juices twice daily over a period of 7 weeks (total daily carotenoid amount of 9 mg), and a control group continuing with an un-supplemented regular diet. A 200% increase of skin RS carotenoid levels was observed for the subject with the highest response while the average level increase of the whole group was approximately 80% increase. Unchanged carotenoid levels were observed for the control group [49].

It is interesting to compare the pressure mediated RS method also with the RRS method. Measurements for 10 volunteer subjects with both methods are plotted in Fig. 11(a). For each subject the RS derived carotenoid absorbance is shown along with the corresponding RRS C=C intensity. A linear correlation intersecting the origin is obtained for the two methods with a correlation coefficient of $R = 0.92$ in this cross sectional study [49].

Comparing both methods also in uptake studies, similar increases in skin carotenoid levels are observable with both methods over time. An example is shown for a subject in Fig. 11(b), where in a 12 days baseline phase with regular diet no skin carotenoid level changes were observed, and where skin carotenoid levels per the two methods increased with similar kinetics in a supplementation

![Fig. 11. (a) Correlation between reflection- and RRS derived skin carotenoid levels for measurements of ten volunteer subjects, demonstrating a high correlation coefficient between the two methods ($R = 0.92$). (b) Tracking of skin carotenoid levels with both methods for a volunteer subject participating in a supplementation study with a 20 mg/day dose of Lyc-O-Mato carotenoid extract [24]. Both methods track carotenoid increases similarly over time.](image-url)
phase in which 20 mg of a lycopene extract [24] per day was consumed with the regular diet.

Application in Nutritional Supplement Industry

Similarly to RRS, the pressure-mediated RS method is readily finding applications in the nutritional supplements industry for screening of skin carotenoid levels in large populations in field settings and tracking of skin carotenoid levels upon supplementation. The commercial RS instrumentation has an advantage over RRS in its relative simplicity. Based on a white light emitting diode, photometric two-wavelength detection scheme, microprocessor interfacing for signal acquisition, processing and display, RS derived skin carotenoid levels can be generated in a small portable platform with short measurement times of about 15 s. For instrument control and data display, wireless communication of the devices with smart phones or tablet computers can be employed.

Comparing pressure-mediated RS to RRS (see Table 1), the RS method is less molecule specific and less precise due to overlapping chromophores, but the reflected light levels are relatively strong. The method can be implemented with inexpensive broadband light sources for excitation and simple filters for detection. The insensitivity of the RS method to the particular composition of dermal carotenoids present in the measured tissue volume can be regarded as an advantage over RRS [49]. Also, RS is an absolute detection technique, i.e. the signal strength changes with excitation light intensity and detection sensitivity. It is therefore susceptible to intensity fluctuations and requires an external calibration standard for accurate measurements (see section on RRS). In contrast, the reflection method is a relative method. It derives the optical density of dermal carotenoid levels via measurements against a reference spectrum or reference wavelength. Intensity variations therefore cancel out during the measurements and data processing routines.

RS has high specificity and high precision. However it requires excitation with a narrow-bandwidth laser or tightly filtered LEDs, as well as the detection of narrow-bandwidth spectral regions centered on the carotenoid Stokes lines. To compensate for the small Raman scattering cross section, excitation with a relatively high-power light source and detection with a sensitive, high-dynamic range CCD array or photomultiplier detector must be used. As a result the instrumentation is relatively complex and expensive.

Both RRS and RS may be influenced by excessive dermal melanin; in the case of RRS, excessive melanin levels will lead to an underestimation of carotenoid levels due to attenuation of both excitation and Raman light. In contrast, in the RS method, melanin leads to an overestimation of dermal carotenoids since undercompensated absorption levels would count toward carotenoid apparent absorption. Refinements of the method such as combination with independently measured melanin indices, can be expected to remove these limitations.

As optical methods, both RRS and RS sample a comparable skin tissue volume. Both methods have found already widespread application in the Nutritional Supplement Industry and they are holding potential as objective biomarkers for fruit and vegetable intake also in Nutrition Science and Medical research.

Acknowledgments

This work was supported by Image Technologies Inc., Salt Lake City, UT.

Table 1

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>High molecule specificity</td>
<td>Weak signal levels</td>
<td>High melanin content</td>
</tr>
<tr>
<td>Fast</td>
<td>Complex instrumentation</td>
<td></td>
</tr>
<tr>
<td>High sensitivity</td>
<td>Needs external sensitivity calibration</td>
<td></td>
</tr>
<tr>
<td>Allows selective detection of carotenes and lycopene</td>
<td>Lower molecule specificity</td>
<td></td>
</tr>
<tr>
<td>Reflectivity</td>
<td>High melanin content</td>
<td></td>
</tr>
<tr>
<td>High signal levels</td>
<td>Lower sensitivity</td>
<td></td>
</tr>
<tr>
<td>Simple instrumentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References


