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Optical methods for noninvasive determination of carotenoids in human and animal skin

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Abstract. Carotenoids are important substances for human skin due to their powerful antioxidant properties in reaction of neutralization of free radicals and especially reactive oxygen species, including singlet oxygen. Concentration of carotenoids in the skin could mirror the current redox status of the skin and should be investigated *in vivo*. Optical methods are ideally suited for determination of carotenoids in mammalian skin *in vivo* as they are both noninvasive and quick. Four different optical methods could be used for *in vivo* measurement of carotenoids in the human or animal skin: (1) resonance Raman spectroscopy; (2) Raman microscopy; (3) reflection spectroscopy; (4) skin color measurements. The advantages, shortcomings, and limitations of the above-mentioned optical methods are discussed. © *2013 Society of Photo-Optical Instrumentation Engineers (SPIE)* [DOI: 10.1117/1.JBO.18.6.061230]

Keywords: resonance Raman spectroscopy; Raman microscopy; reflection spectroscopy; skin color measurements; antioxidants.

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1 Introduction

Free radicals and especially the reactive oxygen species, including one of the most powerful singlet oxygen, are known to be strong oxidizers in human skin. They could be a main reason for the development of different cutaneous diseases including skin cancer.[1] Antioxidants neutralize free radicals before they react with the skin compartments, thus protecting the skin against oxidation. Vitamins, enzymes, and carotenoids are well-known cutaneous antioxidants,[2–6] acting synergistically in order to protect each other against the direct destruction.[7–10] Most antioxidants, including carotenoids, cannot be synthesized by the organism and must be taken up with food, which is rich in these substances. The digestion and metabolism of dietary carotenoids in humans is a complex process.[11,12] After digestion, the carotenoids accumulate mostly in the adipose tissue, liver, and blood.[13] The epidermis, dermis, and subcutaneous fat of the skin also contain carotenoids. The two main pathways for the accumulation of carotenoids in the epidermis of human skin have been clarified: diffusion from the subcutaneous fat, blood, and lymph flows to the epidermis and the secretion of carotenoids onto the skin surface via the sweat glands and/or sebaceous glands and their subsequent penetration into the epidermis.[14] Carotenoids also appear to contribute to human skin color.[15]

The group of carotenoids in human skin includes alpha-, beta-, gamma-, sigma-carotene, lutein, zeaxanthin, lycopene, lutein, zeaxanthin, and their isomers,[16] the most prominent of them being beta-carotene and lycopene.[17,18] The ability of carotenoids to quench singlet oxygen is related to the conjugated carbon double-bond system, and maximum protection is provided by those having nine or more double bonds.[19] Recent investigations have shown that the carotenoids could serve as marker substances for the entire antioxidant network of the human skin.[20]

Antioxidants including carotenoids are widely used in the cosmetic industry to reduce the free radical-induced negative effects on the skin[21–24] and for skin therapy.[2,25,26]

High-performance liquid chromatography (HPLC) is a widely used gold standard method for determination of carotenoids in the skin.[27] This method is invasive because it is based on the analyses of tissue biopsy samples. For analyzing the kinetics of carotenoids in the skin noninvasive methods should be applied. Optical methods are well-suited noninvasive methods in this context.

As the fluorescence efficiency of carotenoids, which was determined to be (10^{-4} to 10^{-5}),[28,29] is very low, they cannot be detected in the skin by any fluorescence analyses. Other optical methods such as resonance Raman spectroscopy, Raman microscopy, reflection spectroscopy, and skin color measurements could be applied for *in vivo* determination of carotenoids in mammalian skin. These methods will be discussed below.

2 Resonance Raman Spectroscopy

Resonance Raman spectroscopy of cutaneous carotenoids was reported by Hata et al.[30] in 2000. Later, in 2003 to 2004, the two-wavelength excitation scheme was proposed to separate the most dominating cutaneous carotenoids beta-carotene and lycopene in human skin.[17,31] At the same time, an improved measurement stability was proposed by utilizing the broad excitation laser spot on the skin surface, which averages the influence of cutaneous inhomogeneities, the typical size of which is usually 1 to 2 mm.[32] One year later, based on the achieved stability, a two-wavelength excitation-based setup was developed for noninvasive determination of carotenoids in human skin.[33,34]

The carotenoids alpha-, beta-, gamma-, sigma-carotene, lutein, zeaxanthin, lycopene, and their isomers, which are

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found at different concentrations in the skin, have approximately the same absorption values in the blue spectral range (for example, at 488 nm). In the green range of the optical spectrum, only lycopene showed high absorption values compared with other carotenoids, with the maximal difference obtained at 516 nm.[35] This is due to the quantity of conjugated carbon double-bonds C═C in the structure of carotenoid molecules between 9 (carotenes, lutein, zeaxanthin) and 11 (only lycopene), which constitute their absorption properties.[36] Absorption spectra of the solutions of beta-carotene and lycopene in ethanol are shown in Fig. 1. Nevertheless, it should be taken into consideration that the real absorption spectra of carotenoids in the skin could be slightly shifted toward longer wavelengths.[37] As a result of the different absorption values, the Raman efficiencies will also be different and strongly correlate with the absorption spectra of investigated molecules. Moreover, depending on the amount of carbon double-bonds, the efficiency of Raman scattering for lycopene will be 11/9 times higher than for other carotenoids. However, in practical application, this difference could be detectable only under the excitation that is far from the absorption maxima of carotenoids (nonresonance excitation). In the case of resonance regime of excitation, the difference in the amount of carbon double-bounds is negligible and overloaded by the highly dominated resonance-induced enhancement of Raman signal.[38]

The efficiency of Raman scattering for the carotenoids is strongly dependent on their absorption, and, as a result, on the excitation wavelength. As a result of the different absorption values for beta-carotene and lycopene in the blue and the green spectral ranges (for example, wavelengths 488 and 514.5 nm shown on Fig. 1), the Raman efficacies will also be different, strongly reflecting the absorption abilities of carotenoids.

As a result, carotenoid Raman lines lying at a wavelength of 527.2 nm (wavelength corresponding to the Raman shift of 1523 cm^{-1} belonging to the C═C stretching vibration of carotenoids excited by the radiation at a wavelength of 488 nm) and at a wavelength of 558.3 nm (wavelength corresponding to the Raman shift of 1523 cm^{-1} belonging to the C═C stretching vibration of carotenoids excited by the radiation at a wavelength of 514.5 nm) were analyzed for their intensity. Based on the fact that the most prominent cutaneous carotenoids are beta-carotene and lycopene,[17] their concentrations could be determined by the two-wavelength excitation scheme.[17,33,34]

Figure 2 shows the principle two-wavelength scheme of the experimental setup used for the noninvasive determination of beta-carotene and lycopene concentrations in human skin [Fig. 2(a)] and its photo [Fig. 2(b)]. The radiation of the argon laser (1) emitting in the multimode regime was collimated by the lens system (2), filtered (3) in a narrow optical band to obtain a desirable wavelength of 488 or 514.5 nm, and focused onto an optical fiber (4). This fiber is connected to an excitation channel (5) of the optical imaging system (6), where the exciting beam is widened by the lens system and focused onto the skin (7). To decrease the influence of inhomogeneity and pigmentation of the skin, the excitation beam was extended, achieving 6.5 mm in diameter on the skin surface. The measurements show that, in this case, the fluctuations caused by the mentioned

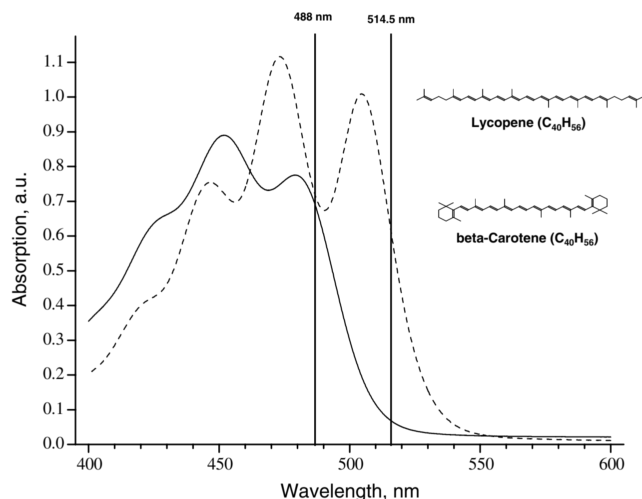
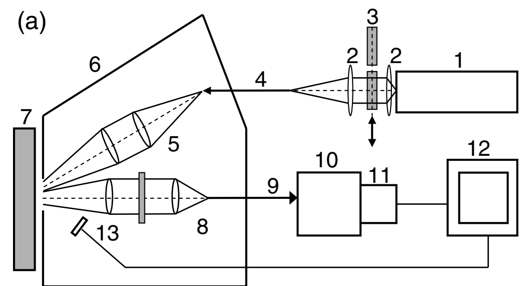
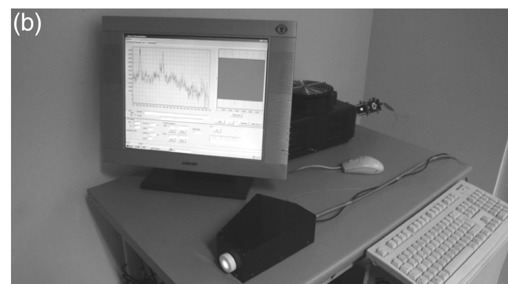


Fig. 1 Absorption spectra of solutions of beta-carotene (solid line) and lycopene (dotted line) in ethanol.





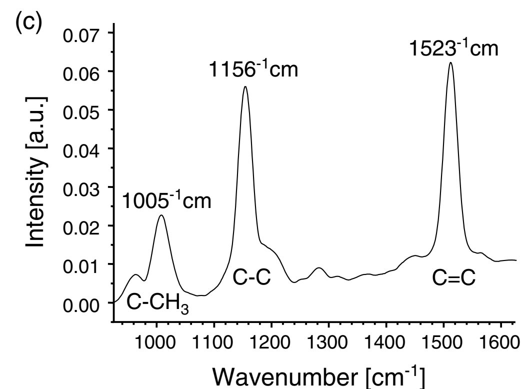


Fig. 2 Resonance Raman spectroscopy-based setup used for the noninvasive determination of the concentrations of beta-carotene and lycopene in human skin. Principle scheme (a) (taken from;[41] photo (b) and typical Raman spectrum obtained *in vivo* from human skin under the excitation at 514.5 nm after the smoothing procedure and background subtraction (c). (1) argon laser (488 nm, 514.5 nm); (2) lens system; (3) filters (488 nm, 514.5 nm); (4) optical fiber; (5) excitation channel; (6) optical imaging system; (7) skin; (8) receiving optical channel; (9) bundle of seven optical fibers; (10) spectrometer; (11) charge-coupled device camera; (12) computer; (13) photo detector.

distorting factors do not exceed 10% of the Raman signal, which is quite acceptable. The power density of the laser radiation on the skin surface did not exceed 50 mW/cm^2, which is within the admitted margin. The geometry of the receiving optical channel (8) eliminated the light directly reflected from the skin. The light directly reflected from the skin was recorded by a photo detector (13) and used then for the normalization according to the skin type (skin of type I reflected more light than skin of type IV). Nevertheless, if Raman measurements are performed on the thenal skin area, where the epidermis is thick enough (up to 600 μm),[39] almost no corrections are needed for measurement of volunteers with different skin types. Measurement of other skin areas where the epidermal thickness is less than 200 μm (average penetration depth of blue and green light into the skin) is usually associated with the influence of blood chromophores and melanin, which reduce the Raman scattering efficiency. To minimize the blood influence, the heightened pressure could be applied onto the skin that locally interrupts the blood flow and decreases the absorption of blood chromophores.[40] This procedure, however, does not influence the cutaneous melanin. The radiation scattered by the skin, which included the elastic scattering at the excitation wavelength, the fluorescence, and the Stokes Raman scattering, was filtered and collected by the lens system at the entrance of the bundle of seven optical fibers (9). The strong illumination caused by the elastic scattering was removed by filtering the radiation in the wavelength range between 522 and 562 nm. The filtered signal was delivered to the entrance slit of spectrometer (10), which is connected to a charge-coupled device camera (11). The obtained spectrum was displayed and analyzed on the computer (12). The typical Raman spectrum measured on the palm area of a healthy volunteer is shown in Fig. 2(c). To determine the intensity of Raman peaks, after the spectrum smoothing procedure, a Gauss curve approximation was used.

The light-emitting diodes (LED) could also be used as sources of excitation.[42] The combination of excitation LEDs and narrow bandpass detection filters give a possibility to distinguish between carotenoid Raman peak and fluorescence background resulting in a repeatability higher than 10% on human skin. This scheme provides rapid spectral switching in excitation and detection channels for the purpose of carotenoid detection in human skin.

The advantage of the Raman spectroscopic method is its measurement quickness (complete measurement lasted 3 s, usually not exceeding 5 s) and its high sensitivity, which permits the detection of low concentrations of carotenoids in mammalian skin. If the low-intensity Raman signal, the intensity of which is comparable with the noise, should be measured in the skin, the decrease of fluorescence background is needed to increase the measurement accuracy. For this purpose the effect of photobleaching can be utilized.[43] In this case, the skin is illuminated for a few minutes with excitation laser light, which causes the fluorescence background to decrease without any influences on the intensity of carotenoid Raman peaks.[44] In practical application necessity of the photobleaching procedure is very uncommon. This procedure is usually noticeable during the Raman measurements of the stomach and back skin areas, where the carotenoid concentration is small and intensity of fluorescence achieves maximum values. The transportability of Raman spectroscopic device could also be mentioned as an advantage.

The main limitation of two-wavelength excitation scheme is that other carotenoids, such as lutein, zeaxanthin, alpha-, gamma-, and sigma-carotenes can exert an influence on the Raman measurements because of the similar absorption spectra to beta-carotene and, as a result, approximately the same Raman scattering efficacies under the excitation at 488 nm. Also, a reabsorption of Raman scattered light at 527.2 nm by lycopene cannot be excluded.

To eliminate the above-mentioned limitations, the one-wavelength excitation schemes could also be used for measurement of carotenoids in the skin.

The wavelength of 488 nm resonantly excites all cutaneous carotenoids simultaneously. Thus, analyzing only the intensity of a subsequent Raman peak at 527.2 nm, the information about the concentration of all carotenoids existing in the skin is provided. The presence of reabsorption of Raman scattered light at 527.2 nm by cutaneous lycopene cannot be excluded. The shift of excitation wavelength toward longer wavelengths does not prove beneficial as the absorption of carotenoids and, consequently, the Raman efficiency, are substantially reduced in this case.

The other possibility is the measurement of only cutaneous lycopene independent of other carotenoids. Using the green excitation wavelength at around 516 nm (Argon laser line at 514.5 nm is well-suited), the strong excitation of lycopene occurs. Other carotenoids are also excited, but they are in the dip of the absorption curve at 514.5 nm (Fig. 1). Taking into consideration the high enhancement of Raman signal under the resonance regime of excitation, it could be concluded that mostly lycopene is excited and the contribution of other carotenoids is around 6% in this case.[33] Moreover, the use of an excitation wavelength at 514.5 nm guarantees the absence of the effect of reabsorption by lycopene at 558.3 nm. The absence of reabsorption at 558.3 nm indicates that Raman measurements are independent from the influence of other carotenoids existing in the skin.[35]

Strong correlations were obtained between HPLC and resonance Raman spectroscopic measurements, reported by various working groups.[17,33,45]

At the moment, internationally available one- or two-wavelength excitation Raman devices provide measurement stability better than 10% and differ in measurement time and excitation spot size.

3 Raman Microscopy

Raman microscopy of human skin was introduced by Caspers et al.[46,47] as a combined *in vivo* confocal Raman spectroscopy and confocal microscopy of human skin. This method was used for determination of the molecular concentration profiles in the skin with the focus on the determination of water distribution. In 2009, Lademann et al.[14] showed that Raman microscopy of cutaneous carotenoids (natural and topically applied) could be performed *in vivo* under the nonresonant conditions of excitation in the near-infrared spectral range (excitation wavelength 785 nm), where the penetration depth into the skin is higher compared to visible light. The concentration of all cutaneous carotenoids was determined without separation by the intensity of prominent Raman peak at 1523 cm^{-1}. The utilization of the near-resonance Raman microscopy for measurement of the cutaneous carotenoids has also been reported under the excitation at 532 nm.[48,49]

The principal block diagram and the photo of the setup used for *in vivo* measurement of the carotenoid distribution in the human epidermis are shown in Fig. 3(a) and 3(b). The laser

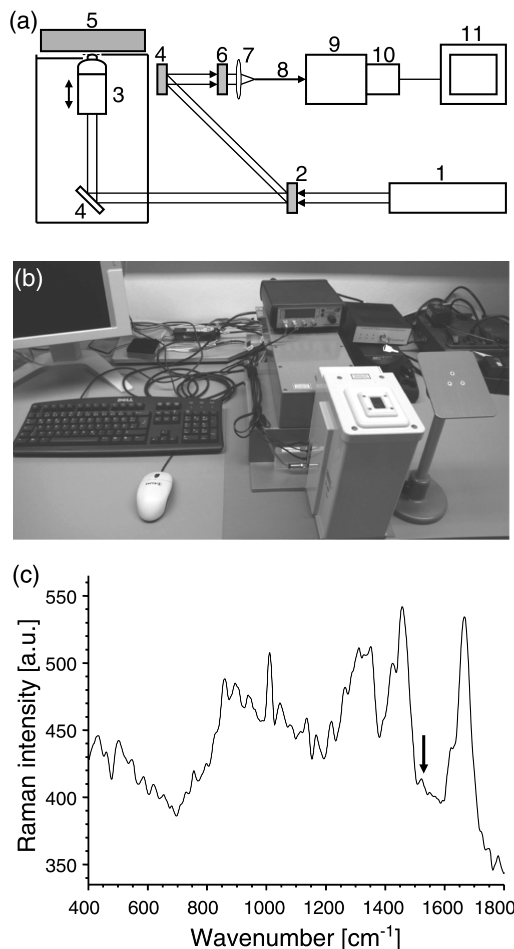


Fig. 3 Raman microscope used for *in vivo* measurement of carotenoid distribution in epidermis of human skin. Principal block diagram (a); photo (b); and typical Raman spectrum obtained *in vivo* from the depth of 10 μm in human skin (c). (1) laser (785 nm); (2) short-pass filter; (3) microscope objective; (4) mirrors; (5) skin; (6) laser rejection filter; (7) focusing system; (8) optical fiber; (9) spectrometer; (10) charge-coupled device camera; (11) computer.

operating in the near-infrared or visible spectral range (1) is focused on the skin (5) by a microscope objective (3) located under the transparent CaF_2 window. The light scattered by the skin is collected by the same objective and reflected by the mirrors (4) and by the short-pass filter (2). The scattered light is filtered by a laser rejection filter (6) and focused (7) onto an optical fiber (8) connected to the spectrometer (9), which is equipped with a charge-coupled device camera (10). The obtained spectrum in the fingerprint range (400 to 1800 cm^{-1}) was displayed and analyzed on the computer (11). Figure 3(c) shows the typical Raman spectrum obtained from a depth of 10 μm in human forearm skin. The sensitivity of this method allows measuring the distribution of carotenoids in different anatomical locations of human skin up to a depth of 30 μm into the epidermis.[50] The Raman intensity of carotenoids was corrected to the Raman intensity of keratin, which is the dominant dry mass fraction in the stratum corneum, depending on the depth. The Raman microscope has been described previously in detail by Caspers et al.[46,47]

The main advantage of the Raman microscopic method is that it permits the determination of the axial distribution of carotenoids in the skin at accuracy higher than 5 μm, which is not possible with other methods.

The limitation of this method for measurement of carotenoids is the high absorption, scattering, and anisotropy factor of the skin in the visible spectral range[51] that substantially decreased the investigated depths. Moreover, the randomly inhomogeneously distributed chromophores influence the optical properties μ_a (absorption coefficient) and μ_s' (effective scattering coefficient) of skin layers. Cutaneous chromophores could also reabsorb the light, thus influencing the final results. In the near-infrared range, a reduction of absorption and scattering is possible, but it substantially deteriorates the Raman scattering efficiency of carotenoids due to the absence of their absorption in the near-infrared range. Therefore, not all volunteers can be measured with this method, as low concentrations of cutaneous carotenoids remain undetectable. The penetration profile of topically applied carotenoids could be investigated without exceptions in all volunteers, independent of their individual carotenoid concentration.[14]

The high price, bulky size of the device and time-consuming measurement procedure could be mentioned as main disadvantages.

4 Reflection Spectroscopy

Reflection spectroscopy of carotenoids is based on the registration of the radiation reflected by the skin, the irradiation occurring in the spectral range of maximal absorption of carotenoids (i.e., in the blue-green range of the spectrum). Although the reflection measurements of the skin have been known for a long time, the measurements of cutaneous carotenoids was mentioned not before 1964 by Buckley and Grum.[52] In 1998, Stahl et al.[53] used reflection spectroscopic measurements for determining the influence of beta-carotene rich supplementation on the carotenoid concentration in human skin. In 2001, Niedorf[54] presented reflection spectroscopy-based determination of carotenoids in bovine udder skin. A strong correlation ($R = 0.711$) was obtained in comparison with HPLC measurements. Despite the strong correlations obtained in the above-mentioned studies, no further analyses of carotenoids in human and animal skin were reported by these groups, probably because of the wide spread of the measured values, which usually overloaded the expected effects.

The reason for such dispersion could be the high penetration depth of the excitation light into the skin[55] and the presence of cutaneous chromophores, such as melanin, flavin adenine dinucleotide (FAD), reduced FAD ($FADH_2$), deoxy-haemoglobin, or oxy-haemoglobin and bilirubin, which are inhomogeneously distributed in the skin,[56,57] absorb the light in the blue-green range of the spectrum, and thus potentially influence the final reflectance spectra.

To eliminate or substantially reduce the influence of chromophores, which are mostly distributed in the basal level and in the dermis of the skin, a light-emitting diode-based miniaturized spectroscopic system (MSS) was developed to detect back-reflected light from depths not exceeding 200 μm, which corresponds with a depth of epidermis on the thenal areas in humans. For this purpose, the light barrier, which is the distance between the windows of excitation and receiving channels, was designed on the measurement surface of MSS. Figure 4 shows the principal scheme [Fig. 4(a)] and photo of the MSS device [Fig. 4(b)]. The light barrier constrains the light to travel through the skin (2) up to a depth of 200 μm and prevents the light from

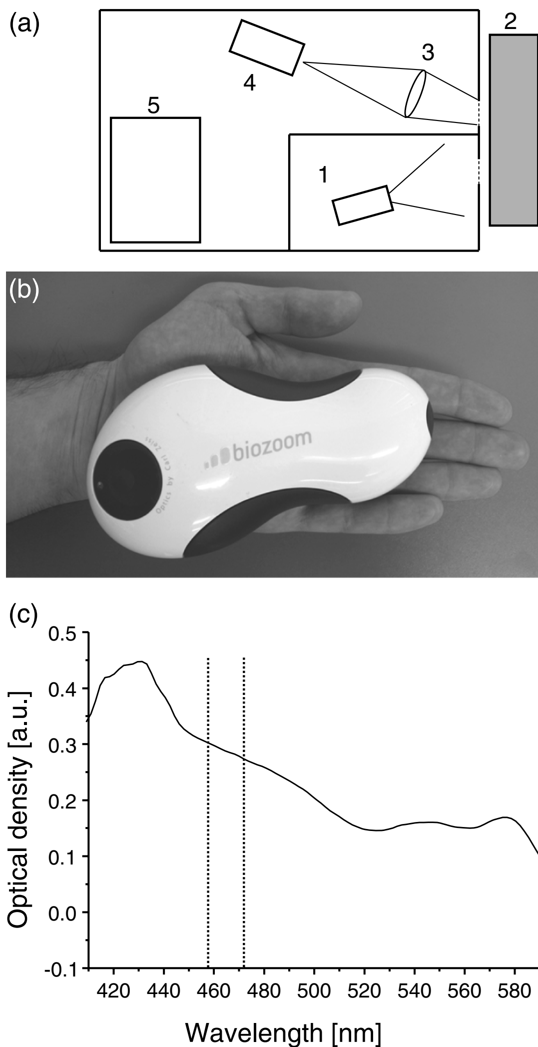


Fig. 4 Reflection spectroscopy-based miniaturized spectroscopic system for measurement of cutaneous carotenoids. Optical scheme (a); photo (b); and typical reflectance spectrum obtained from human skin (c). (1) light-emitting diode (440 to 490 nm); (2) skin; (3) focusing system; (4) replicated holographic grating spectrometer; (5) Bluetooth system.

being directly reflected back by the skin surface as used in other setups applying reflectance spectroscopy.[58–60] Thus the MSS based on reflection spectroscopic measurements uses the tissue below the surface as a diffusely reflecting substrate. A light-emitting diode (1) emitting a bright spectrum ranging from 440 to 490 nm was used as a source of excitation. The back-reflected light focused (3) onto the replicated holographic grating spectrometer (4). The small dip in the diffuse reflected spectrum measured in the range between 458 and 472 nm was associated with the absorption of cutaneous carotenoids and recalculated to their concentration [Fig. 4(c)]. The selection of this range was made experimentally by comparison of reflection spectra with the purpose to exclude possible influence of other chromophores and the shift in carotenoid absorption to longer wavelengths in the skin. The final reflectance spectrum was transmitted through the Bluetooth system (5) on the personal computer or mobile phone equipped with the adapted software. The optimization and subsequent calibration of the MSS were performed *in vivo* and *in vitro* using resonance Raman spectroscopy. Resonance Raman spectroscopy has been used for MSS calibration because this method is well correlated with HPLC measurements and is highly specific for measurements of carotenoids in human and animal skin. Strong correlations were obtained for human thenal skin areas *in vivo* ($R = 0.88$) and for bovine udder skin areas *in vitro* ($R = 0.81$). The reflection spectroscopy-based MSS was recently described in detail by our working group.[57]

Another possibility to perform measurements on different skin areas independent of the thickness of epidermis is based on pressure mediated reflection spectroscopy.[61] Applied external pressure temporarily squeezes blood out of the illuminated skin volume making epidermal carotenoids detectable in the range between 460 and 500 nm. The limitation of this method lies in the influence of melanin, which also absorbs in this optical range and is achieved in the case of skin areas with thin epidermis making measurements skin type dependent.

The main advantages of reflection spectroscopy-based devices in comparison to Raman devices are the relatively low price, compact size, and light weight. Moreover, the application of the battery-based MSS permits measuring humans or animals outside the clinic independent of the presence of electric mains.

The most important limitation of the application of this method in humans is that reflection measurements with MSS could only be performed on the thenal or plantar skin areas where the epidermis is thick enough and the influence of the above-mentioned chromophores is less pronounced. Measurements on animal skin could be performed *in vivo* on the hairless areas with thick epidermis; for instance, udder skin. Contrary to the large Raman setups, the size of the MSS is that of a computer mouse and can be directly controlled by the Bluetooth system of a laptop or mobile phone.

5 Skin Color Measurements

Human skin color is predominantly determined by the cutaneous pigments hemoglobin, bilirubin, and melanin. Due to their absorption properties, epidermal carotenoids are also strongly contributing to the coloration of epidermis[15,62,63] and, as a result, the total skin color. As an example, carotenoderma is a well-known harmless phenomenon characterized by strong yellow pigmentation of the skin, resulting from the deposition of carotenoids in the stratum corneum.[64,65]

The skin color measurements, which are based on spectrophotometric reflection measurements,[66] were applied on human skin to determine the carotenoid concentration. The yellow component of the human skin color, which quantified by the b^* value of the $L^*a^*b^*$ color-opponent space (where L^* represents lightness and a^* and b^* values represent degrees of redness and yellowness, respectively), was shown to significantly correlate with the concentration of cutaneous carotenoids determined by reflection spectroscopy.[55] The increase in the b^* value was significantly associated with the carotenoid increase in human skin due to the increase in fruit and vegetable intake.[67] As a source of excitation, broad visible spectrum illuminated lamps or light-emitting diodes are usually used. The light back-reflected from the skin is collected for a tristimulus color analysis in the red (~630 nm), green (~560 nm), and blue (~450 nm) ranges of the spectra using the $L^*a^*b^*$ color system, as determined by the Commission Internationale de l'Eclairage (CIE).[66,68]

Like the reflection spectroscopic method, the skin color measurement method is favorable for its low price, compact size, light weight, and independence from the electric mains.

The limitations of this method are similar to the limitations mentioned for reflection spectroscopy of carotenoids. Because cutaneous melanin and blood could significantly contribute to the b^* value, the determination of carotenoids with skin-color measurement methods should be performed on the thenal or plantar skin areas, where the epidermis is thick enough, and the influence of melanin and blood are less pronounced. Moreover, the determination of low carotenoid concentrations in the skin with this method can be as difficult as in the case of reflection spectroscopic measurements.

6 Discussion and Outlook

The results obtained with the noninvasive optical methods for determination of cutaneous carotenoids open up wide prospects to measure their kinetics in the skin and investigate the influence of stress factors on the redox status of the skin *in vivo*.

Measurement of different skin areas in humans revealed the areas with the highest concentration of carotenoids, which are most convenient for the detection. It was found that the highest concentration of carotenoids is found in the skin, where the density of sweat glands is high (i.e., on the thenal and plantar areas and on the forehead.)[34] No significant differences between ethnic groups (Caucasian, Asian, and African) measured on the thenal area have been determined.[69]

Different authors reported that a carotenoid-rich nutrition and supplements significantly increase the carotenoid concentration in human skin.[45,53,67,70–73] An increase was measured one to three days after the start of carotenoid-rich supplementation.[74,75] A significant correlation was also found between total blood versus cutaneous carotenoids in humans,[76] female cattle with a moderate to obese body condition[77] and between the carotenoid content in the blood and skin microcirculation.[78]

On the contrary, cutaneous carotenoids in humans are degraded following the influence of stress factors of any type: *inter alia*, disinfection,[79] sun irradiation,[80–82] illness,[74,83] endurance exercises,[84] consumption of high amounts of alcohol,[85] etc.[74] The decrease occurs relatively quickly, within a few of hours, while the subsequent recovery is a more prolonged process, which requires a number of days before leveling and varies individually. Taking into consideration the conditions, under which carotenoids could manifest pro-oxidative activity,[86–89] the best protection strategies have been determined.[90,91]

The unexpected effect (i.e., the generation of reactive oxygen species in human skin subsequent to the irradiation with infrared light was predicted based on the significant degradation of cutaneous carotenoids.)[41] Later, this assumption was confirmed for infrared-A[92] and near-infrared[81,93] irradiations using electron paramagnetic resonance spectroscopy and other analytical methods.[94,95]

The distribution of carotenoids in the epidermis was analyzed with the help of Raman microscopy. It was found that the highest concentration is in the top layer of epidermis (i.e., in the stratum corneum), which could be explained by the delivery of carotenoids with sebum and/or sweat secretion onto the skin surface and its subsequent penetration into the superficial layers of stratum corneum.[50] The same mechanism had been previously observed for vitamin E.[96] It was additionally shown that after the infrared-A and disinfection-induced depletion of cutaneous carotenoids, they recovered from outside to inside giving evidence to have been delivered with the sweat and/or sebaceous glands.[97]

The results obtained on human skin with the use of Raman spectroscopic methods are completely summarized in the following review articles.[98,99]

Reflection spectroscopy-based miniaturized spectroscopic system, due to its compact size and lightweight, is also well suited for carotenoid measurements on cattle. The obtained results show that bovine udder skin is well suited for such measurements.[100] Carotenoids showed highly significant differences between individual animals, although they were kept under the same environmental conditions and were fed the same diet.[101] A statistically significant increase in carotenoid concentration in bovine udder skin subsequent to recovery followed after abomasal displacement surgery and a decline in cutaneous carotenoids in cows with a fatal outcome were measured,[102] and promise the successful application of noninvasive methods in veterinary practice.

All noninvasive measurements of cutaneous carotenoids were performed on the easily accessible skin areas (i.e., palm, forearm, forehead, and back). The development of a 488-nm excitation-based single-fiber resonance-enhanced Raman spectroscope for analysis of carotenoid concentrations in the hard-to-reach areas, such as upper aerodigestive tract[103] open up wide prospects to analyze endoscopically the buccal mucosa, anterior floor of mouth, oral tongue, and soft palate. Carotenoids were also measured with Raman microscopy in the dried human saliva under the excitation at 532 nm *ex vivo*.[104] Recently, Pudney et al.[105] developed a Raman microscope tailored to *in vivo* measurement on the hard-to-reach body sites, such as the mouth, axilla, and scalp. The carotenoid distribution profile of these tissues could be investigated *in vivo* in the near future.

High scattering of the skin, which could considerably vary inter individually, can influence the results of all above-mentioned measurements.[106,107] The reproducibility of the measurements can be improved by separating the absorption and scattering properties using for instance inverse Monte Carlo simulations,[108,109] whereby changes in the scattering have less influence than changes in the absorption. Nevertheless, a high reproducibility of the cutaneous carotenoid measurements has been obtained *in vivo* by the Raman spectroscopic measurements[35] and by the reflection spectroscopic measurements.[57,101] The obtained stability was determined by the standard deviation of the measured values, which did not exceed 10%.

Most of the results obtained noninvasively were correlated with the results obtained by HPLC analysis. It is hypothesized that once the biopsies are taken, their contact with oxygen and environment as well as probe preparation (crumbling), which is necessary for HPLC measurements, might influence the concentration of carotenoids in the skin sample. The number of carotenoids destroyed during these procedures is, in reality, unknown. Therefore, it could be estimated that carotenoid concentrations in the skin samples measured with HPLC are less than the concentrations in living skin and thus understated. In this regard, it could be enough to measure cutaneous carotenoids in arbitrary units providing relative data on different skin areas to evaluate the kinetics of carotenoids in the skin.

It can be concluded that the above-mentioned optical methods are well suited for noninvasive measurements of carotenoid antioxidant substances in mammalian skin.

To obtain a better comparability of the results obtained with optical methods, it could be recommended to compare results measured on the same skin areas (for example, only on the palm) and on the group of volunteers with the same skin

types. In the case of animal investigations, skin areas should be selected that contain colorless or low-pigmented subtle hairs. These areas should be purely shaved before the carotenoid measurements will be performed.

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