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Kinetics of carotenoid distribution in human skin *in vivo* after exogenous stress: disinfectant and wIRA-induced carotenoid depletion recovers from outside to inside

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Abstract. The human organism has developed a protection system against the destructive effect of free radicals. The aim of the present study was to investigate the extent of exogenous stress factors such as disinfectant and IR-A radiation on the skin, and their influence on the kinetics of carotenoids distribution during the recovery process. Ten healthy volunteers were assessed with resonance spectroscopy using an Argon-laser at 488 nm to excite the carotenoids *in vivo*. Additionally, Raman-confocal-micro-spectroscopy measurements were performed using a model 3510 Skin Composition Analyzer with spatially resolved measurements down to 30 μm . The measurements were performed at a baseline of 20, 40, 60, and 120 min after an external stressor consisting either of water-filtered infrared A (wIRA) with 150 mW/cm^2 or 1 ml/cm^2 of an alcoholic disinfectant. Both Raman methods were capable to detect the infrared-induced depletion of carotenoids. Only Raman-microspectroscopy could reveal the carotenoids decrease after topical disinfectant application. The carotenoid-depletion started at the surface. After 60 min, recovery starts at the surface while deeper parts were still depleted. The disinfectant- and wIRA-induced carotenoid depletion in the epidermis recovers from outside to inside and probably delivered by sweat and sebaceous glands. We could show that the Raman microscopic spectroscopy is suited to analyze the carotenoid kinetic of stress effects and recovery. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3555183]

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1 Introduction

The skin is the barrier to the environment and represents a multifunctional and self-regulating barrier to exogenous influences and topically applied compounds.^{1,2} UV exposure from the sun and contact to hazardous substances produce free radicals in human skin.³⁻⁶ These highly reactive molecules can damage the skin on a cellular and molecular level.⁷⁻⁹ The consequences can be skin irritation, collagen and elastin destruction,^{10,11} skin aging,¹²⁻¹⁴ and also skin cancer.^{15,16} The human organism has developed a protection system against the destructive action of the free radicals in the form of the antioxidative potential. Antioxidants can neutralize free radicals on and in the skin before they can cause damage.¹⁷ The antioxidative network of the human skin consists of substances such as vitamins, enzymes, and carotenoids,¹⁸⁻²¹ which might act synergistically.²²⁻²⁴

The carotenoids are important components of the human cutaneous protective system.^{25,26} Beta-carotene and lycopene represent 70% of the total carotenoids of the skin.²⁷ Most of the antioxidants cannot be produced by the human body itself and have to be supplied by nutrition. In particular, fruit and vegetables contain a high amount of antioxidants.^{28,29}

Antioxidant substances form a protection chain in human organisms. This means that they protect each other from damage caused by free radicals. Therefore, carotenoids can be used as marker substances for the antioxidative network in general.^{19,30,31} Topical and systemic administration of antioxidants has shown to exert positive effects on epidermal and dermal functions.³²⁻³⁶ Carotenoids can be detected noninvasively in the human skin by resonance Raman spectroscopy measurements.³⁷⁻³⁹ The investigation of the kinetics of the carotenoids in the skin of 10 volunteers during the course of 1 year, showed that different individuals have different antioxidant levels in their skin, reflecting their lifestyle.³⁰ Nutrition, rich in fruit and vegetables, leads to a high antioxidant concentration in the skin. In contrast, stress and stress factors such as illness and sleep deprivation, as well as alcohol abuse and nicotine consumption, strongly reduce the antioxidative network of the skin. In this study, it was reported that, for instance, the uptake of high amounts of alcohol reduce the antioxidative capacity of the skin within 24 h, while the increase in the initial antioxidative level takes several days. The Raman microspectroscopy is a measurement device designed for noninvasive depth-resolved assessment of specific compounds such as water, natural moisturizing factor, lipids, antioxidants, and topically applied products in the epidermis in humans *in vivo*.⁴⁰⁻⁴³ A specific feature

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of the Raman microscopy is the measurement of concentration profiles over the upper 30 to 50 μm of the skin. To this end, an automated series of Raman spectra is measured in small discrete steps (typically 2 to 5 μm) from the skin surface down to a specified depth.

It has been shown that the antioxidant, vitamin E, is delivered with sweat production and via sebaceous glands from inside the human body onto the skin surface.^{44,45} The same pathway was proposed for carotenoids^{46,47} from inside the human body onto the skin surface.

The aim of the present study was to investigate the distribution of the carotenoids in the skin, under the influence of different skin stress factors. Previous studies using resonance Raman spectroscopy had revealed that the concentration of the carotenoids decreases after IR irradiation.^{36,48–50} Water-filtered infrared (wIRA) penetrates even deeper into the tissue than regular IR.^{50–54} In a first series of experiments, the *in vivo* irradiation of the skin with IR light was used to influence the antioxidative capacity of the stratum corneum and the living epidermis. IR light was used because it penetrates several millimeters deep into the tissue. Carotenoid decreases are associated to the production of free radicals in human skin.^{36,48–50}

In a second series of experiments, the changes in the distribution of the carotenoids in the human skin were investigated after topical disinfection of the skin surface with an alcoholic solution. In this particular case, the irritation process acts only on the skin surface.

Two different methods of Raman spectroscopic measurements were used in this study.

With resonance Raman spectroscopy, the average concentration of the carotenoids in a tissue volume with a depth up to 150 μm was investigated. This means that integral information of the carotenoid concentration in the stratum corneum (SC), the epidermis, and parts of the dermis were obtained. The blood vessels were only marginally reached by this method. In contrast to the resonance Raman spectroscopy, the Raman microscopy was used to investigate the depth distribution of the carotenoids in these tissue layers with a depth resolution of 5 μm . From the analysis of the kinetics of the antioxidant levels after irradiation and disinfection of the skin, information about the penetration pathways of antioxidant substances from inside the body onto the skin surface was obtained and discussed in the present paper.

2 Materials and Methods

2.1 Volunteers

The investigations were carried out on 10 healthy volunteers aged between 25 and 50 years on the forearm. Permission for the study had been obtained from the Ethics Committee of the Charité–Universitätsmedizin Berlin.

2.2 Infrared Irradiation Sources

For IR-A irradiation, a water-filtered IR-A radiator was used (Hydrosun, Medizintechnik GmbH, Mühlheim, Germany) emitting light in the spectral range from 590 to 1400 nm. The radiation intensity was approximately 200 mW/cm². The total radiation intensity in the IR-A spectral range was 150 mW/cm². The light source was used at a distance of 25 cm to the skin.

2.3 Disinfection

Topical application of the disinfectant SoftaseptN (B. Braun Melsungen AG, Melsungen, Germany) was used to influence the antioxidative network of the skin. 100 ml Softasept contains as active ingredients ethanol 74.1 g, 2-Propanol 10.0 g, and, in addition, purified water. The disinfectant was applied onto the skin surface using the spray applicator, as usually done in the operation theater. An average amount of 1 mL/cm² was applied.

2.4 Delivery of Antioxidant Substances with the Sweat

The sweat production of the volunteers was stimulated by physical activity. Droplets of sweat were collected from the forehead and transferred onto microscopic slides. The sweat was analyzed by Raman microscopy and spectroscopy.

2.5 Resonance Raman Spectroscopy

The resonance Raman spectroscopy (RRS) method and the measuring system used in the study has been described in detail by our group.³⁸ An Argon laser at a wavelength of 488 nm was used to resonantly excite the carotenoids in the skin. The Argon laser irradiation was transferred into optical fibers, which connect the basic control station with a hand piece containing the optical system. The hand piece could be positioned on any body site of the volunteers. The intensity of a strong Raman peak at 1521 cm⁻¹, which corresponded to carbon-carbon double-bond vibrations of carotenoid molecules, was analyzed. Thus, under the resonance conditions of excitation at 488 nm, the Raman peak was measured at 527.2 nm. The receiving filter was chosen to transmit the light in the range between 522 and 562 nm to counteract the influence of laser excitation lines on Raman measurements. For the substantial decreasing of a high influence of pigmentation and heterogeneities of the skin on RRS measurements, a broad excitation spot 6.5 mm in diameter was used on the skin surface.

The penetration depth of the laser irradiation was approximately 150 μm . In this way, mainly the SC, epidermis, and small parts of the dermis were analyzed in an integral manner. A differentiation between different tissue layers cannot be obtained with this method.

2.6 Raman Microspectroscopy

Raman microspectroscopy measurements were performed using a model 3510 Skin Composition Analyzer (River Diagnostics, Rotterdam, The Netherlands). The axial spatial resolution was 5 μm and the laser excitation wavelength was 785 nm. The utilization of near-infrared excitation light was provided because of the “window” of wavelengths that are able to pass through tissue up to millimeters deep, which is important for microscopic measurements. Thus, carotenoids were excited nonresonantly. Detailed Raman profiles of the carotenoids at 1521 cm⁻¹ were obtained across the stratum corneum by measuring Raman fingerprint spectra (400 to 1800 cm⁻¹) from the skin surface down to a depth of 30 μm in 2 μm increments. The method has

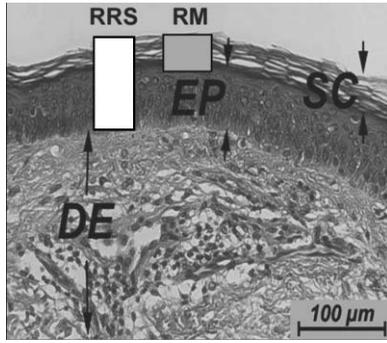


Fig. 1 Comparison of the skin penetration depths obtained by RRS—white bar and confocal Raman microscopy (CRM)—gray bar are shown in a histological section in comparison to the SC, the epidermis (EP), and the dermis (DE) (mag bar = 100 μm).

previously been described in detail.^{40,41} The comparison of the depth resolution of the two Raman methods used in this study is illustrated in Fig. 1. In the present study the beta-carotene content was assessed as a marker for the antioxidative network of human skin.

2.7 Statistics

A comparison of two groups was performed using the non-parametric Wilcoxon rank test. Prism 3 (GraphPad, San Diego, CA) was used for statistical testing. P-values <0.05 were set as statistical significant.

2.8 Study Protocol

The resonance Raman spectroscopic and confocal Raman microscopic measurements were performed on the 10 volunteers before the stress factors (disinfection, IR irradiation) were applied and 20, 40, 60, and 120 min as well as 24 h after treatment. Three repeat measurements were performed in each of the 5 s long measurement segment. Room temperature was 22°C (+/-1°C) at 50% relative humidity (+/-5%) under controlled room conditions.

3 Results

3.1 IR Irradiation

3.1.1 Resonance Raman spectroscopy

Figure 2 shows a typical kinetic curve of carotenoids in the skin before and after IR irradiation. The carotenoid concentration is markedly reduced immediately after IR irradiation. Subsequently, it took approximately 24 h. before the original level of the initial antioxidant concentration was re-established.

3.1.2 Raman microscopic spectroscopy

Taking into consideration the results of the resonance spectroscopic measurements, the Raman microspectroscopic analyses were carried out before IR irradiation and 30 minutes after irradiation when the minimal level of the carotenoid concentration in the skin had been detected.⁵⁵ A typical curve is presented in Fig. 3 before and after wIRA irradiation. The concentration of carotenoids in the stratum corneum was reduced after IR ir-

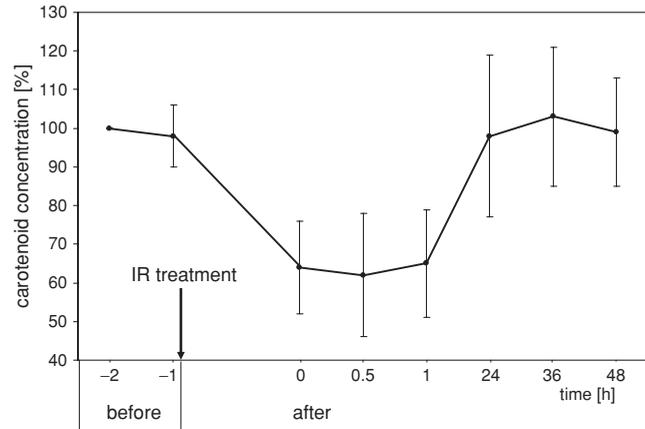


Fig. 2 Kinetics of the degradation of dermal carotenoids after the IR-A irradiation on the volar forearm of human skin *in vivo* at different time points (2 h prior to and up to 48 h after the IR radiation) ($n = 10$; mean +/-SD).

radiation, while the shape of the distribution profile remained unchanged. Figure 4 shows comparative results of the resonance Raman spectroscopic and Raman microscopic measurements of the carotenoid concentration of all volunteers before and 30 min after IR irradiation. For better comparison, the initial values of the carotenoid concentration of all volunteers had been normalized to 100%.

3.2 Disinfection

3.2.1 Resonance Raman spectroscopy

Within 2 h after disinfection of the skin, no relevant changes in the carotenoid concentration were observed with resonance Raman spectroscopy.

3.2.2 Raman microscopic spectroscopy

Measuring the distribution of the beta-carotene concentration at different depths of the SC by Raman microscopy before application of the disinfectant, it was found that the highest concentration of the carotenoids could be detected in the uppermost cell layers of the stratum corneum. Typical results are presented in Fig. 5. In deeper parts of the stratum corneum, the carotenoid concentration was lower than at the surface. Twenty minutes after disinfection, the amount of carotenoids in the upper 25% of the stratum corneum was markedly reduced, while almost no changes were detectable in the deeper SC parts. After 40 min., the depletion in the upper stratum corneum continues. In the upper 50% of the stratum corneum, the carotenoid concentration was further reduced. The kinetics of the carotenoid depletion corresponds well to the expected penetration kinetics of the disinfectants. After 60 min., the carotenoid depletion in the deeper parts of the stratum corneum continued while at the skin surface recovery of the carotenoid reservoir has already started. After 2 h, the original distribution of the carotenoid concentration in the SC measured at baseline (before application of the disinfectant) had been re-established in all SC layers (data not shown).

Raman spectra of carotenoids were measured in an aqueous solution [Fig. 6(a)]. Furthermore, Raman spectra of carotenoids

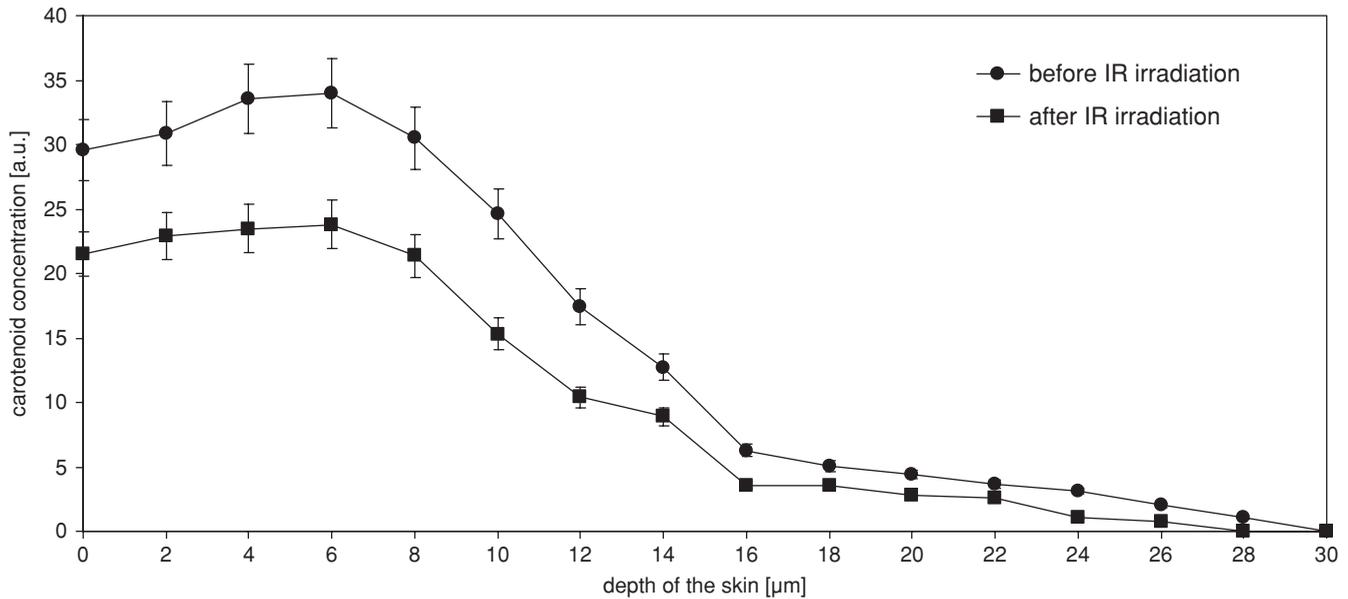


Fig. 3 Relative distribution of the natural carotenoids in the stratum corneum determined on the volar forearm by the use of confocal Raman microscopy (CRM). 1 - before IR irradiation; 2- after IR irradiation ($n = 10$; mean \pm SD).

in the skin with a characteristic peak at 1521 cm^{-1} were assessed [Fig. 6(b)].

3.3 Delivery of Antioxidant Substances With the Sweat

Despite the small amount of sweat obtained from the volunteers, well detectable amounts of carotenoids (signal/noise ratio 8/1) could be found in the samples (data not shown). The same results were previously obtained by the use of resonance Raman spectroscopy.⁴³

4 Discussion

Two different methods of Raman spectroscopic measurements were used in this study: resonance Raman spectroscopy and Raman microspectroscopy. With resonance Raman spectroscopy, the average concentration of the carotenoids in a tissue volume with a depth up to $150\text{ }\mu\text{m}$ was investigated. This means that integral information of the carotenoid concentration in the SC, the epidermis, and parts of the dermis were obtained. The blood vessels were only marginally reached by this method.

Raman microscopy was used to investigate the depth distribution of the carotenoids in these tissue layers, offering a depth resolution of $5\text{ }\mu\text{m}$. From the analysis of the kinetics of the

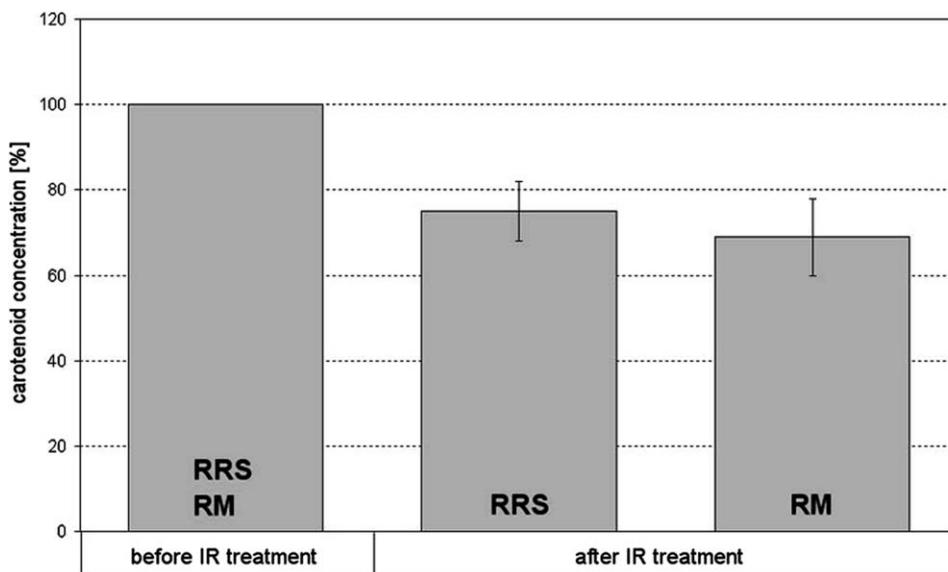


Fig. 4 Normalized (to baseline values = 100% carotenoid concentration). mean concentration of dermal carotenoids before and 30 min after IR irradiation of the volar forearm skin determined for the entire epidermis with RRS and at the depth of $6\text{ }\mu\text{m}$ with confocal Raman microscopy (CRM). ($n = 10$; mean \pm SD).

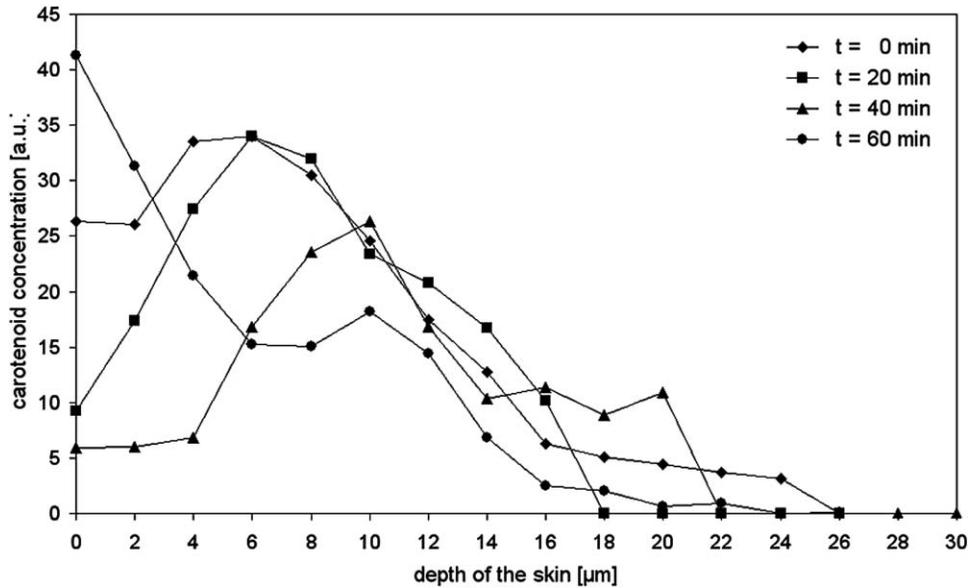


Fig. 5 Typical distribution of the carotenoid concentration profiles across the stratum corneum at baseline ($t = 0$) and 20, 40, and 60 min after application of the disinfectant. Measurements were done *in vivo* by confocal Raman microscopy on the volar forearm.

antioxidant levels after irradiation and disinfection of the skin, information about the penetration pathways of antioxidant substances from inside the body onto the skin surface was obtained and discussed in the present paper.

Irradiating the skin with IR light, the integral antioxidant network has a diminishing impact on the antioxidative capacity in the SC and in the epidermis. This can be explained by IR irradiation induced production of free radicals in the human skin.^{36,48,50,55} Antioxidants represent a defense system of the human organism against the destructive action of free radicals. They neutralize the reactive molecules before they damage skin functions.^{17,19} If the number of free radicals is above a critical level, the antioxidants including carotenoids are destroyed. The process of destruction takes only a few minutes, but it may take several days before the original antioxidative network is re-established. Changes in the distribution of the carotenoids in different skin layers, measured by Raman microscopy, showed that the concentration of the carotenoids was strongly reduced

after IR radiation, but the profile of the distribution did not change (see Fig. 3). This can be explained by the fact that IR light penetrates deeply into the tissue, up to several millimeters. As expected, the carotenoid concentration in the stratum corneum of approximately 20 μm thickness is equally reduced in different depths. Hence, the shape of the concentration profile remains unaltered.

In contrast, disinfectants act initially on the surface of the skin and in the first layers of the SC. After topical application, the disinfectant starts to penetrate into the SC. This effect can be observed by the reduction of the concentration profile of the carotenoids in the skin by Raman microscopy. The deeper the penetration of the disinfectant into the SC, the deeper the reduction of the carotenoids could be observed. Sixty minutes after topical treatment, not only a reduction of the carotenoid concentration in deeper parts of the SC could be detected, but also a replenishment of the upper corneocyte layers with carotenoids.

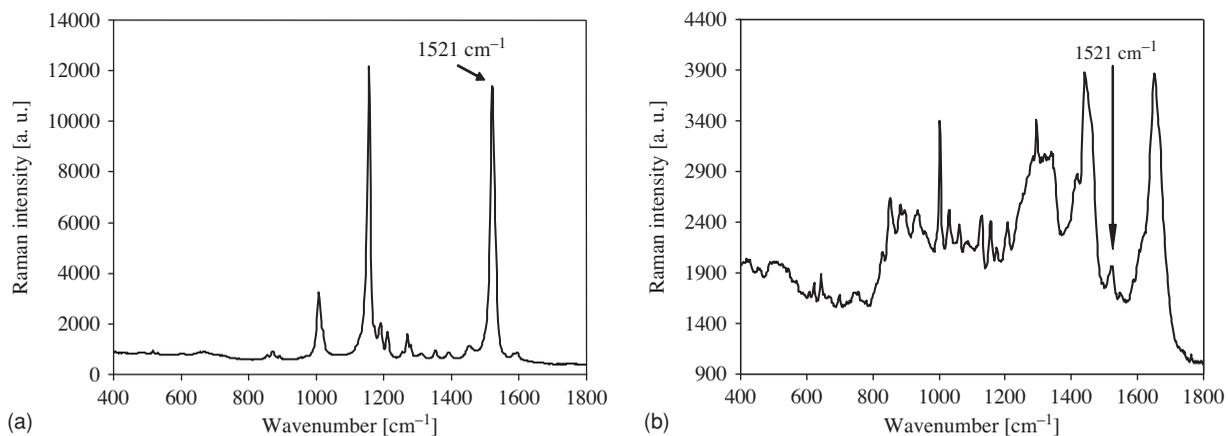


Fig. 6 Raman spectrum of carotenoids in an aqueous solution (a) and Raman spectrum of carotenoids in the skin (b) with a characteristic peak at 1521 cm^{-1} .

Sebaceous glands are predominately located in the face, back, and chest. Sweat glands are present on the entire body surface and their highest density on the palms and soles, arm pits, face, chest, and back of the body. Their major function is regulation of the body temperature. It can be expected that the recovery process is caused by the delivery of carotenoids with the sweat and sebum secretion out of the body onto the skin surface. This hypothesis is supported by the fact that the highest concentration of the carotenoids in the skin of the volunteers is always detected on skin areas with a high density of sweat and sebaceous glands.⁵⁶ Additionally, Thiele et al. demonstrated that the antioxidant substance, vitamin E, was delivered with the sebum onto the skin surface.^{45,57} This hypothesis of sweat, being a key source of surface antioxidants, could be confirmed in this study by the detection of carotenoids in separate sweat drops of the volunteers. The resonant Raman spectroscopic measurements did not reveal the effect of the depleting and refilling of the SC with antioxidants. This method measures the integral concentration of the carotenoids in the SC, the epidermis, and in parts of the dermis with a tissue probing volume approximately 30 times bigger than the tissue volume analyzed by Raman microspectroscopy. Consequently, the highly localized changes in carotenoid content only marginally affect the total carotenoid content in the skin and remain undetected.

In summary, this study demonstrates that nonconfocal resonant Raman spectroscopy provides a very sensitive method to monitor variations in the integral carotenoid content in the skin as a result of stress factors *in vivo*. We also demonstrate that nonresonant confocal Raman microscopy provides a sensitive method to monitor the more localized changes in carotenoid levels as a result of induced skin stress factors. In particular, this method enables analysis of the kinetics of stress effects and recovery of the carotenoid-based antioxidative network of the skin *in vivo*.

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