INTRODUCTION

Free radicals are generated in the skin as a result of the influence of various external factors, for example UV,[1,2] VIS,[3] and IR‐[4,5] irradiation of sunlight, temperature increase,[6] the exposure to exogenous chemical agents,[7,8] etc. They are also generated by the influence of the internal factors, such as the cell's metabolism,[9,10] skin ageing[11] and skin diseases including inflammations and skin cancer.[12] The stratum corneum (SC), the multifunctional outermost skin layer, maintains the skin barrier function mainly provided by the lateral organization of intercellular lipids (ICL).[14]
flux through the skin\textsuperscript{[15]} and contains an abundant amount of antioxidants,\textsuperscript{[16–18]} which quench the generated free radicals. One group of cutaneous lipophilic antioxidants is carotenoids (\(\alpha\), \(\beta\), \(\gamma\), \(\xi\)-carotenes and lycopene\textsuperscript{[19]}), which have conjugated carbon‐carbon double bonds in their molecular structure (nine for \(\alpha\), \(\beta\), \(\gamma\), \(\xi\)-carotenes and 11 for lycopene),\textsuperscript{[20]} determining their antioxidative properties in neutralization of reactive oxygen species.\textsuperscript{[21–24]}

Carotenoids serve as marker substances of the entire antioxidative status of the epidermis in vivo.\textsuperscript{[25,26]} The most frequent carotenoids in the human epidermis are \(\beta\)-carotene and lycopene,\textsuperscript{[19]} whose concentration can be measured in vivo and non‐ invasively using resonance and non‐resonance Raman spectroscopy, multiple spatially resolved reflection spectroscopy and skin colour (yellowness) measurements.\textsuperscript{[27–29]} The antioxidative marker properties of epidermal carotenoids and the ability of their quick non‐ invasive determination in the skin in vivo opened up new directions in investigating the redox processes in the skin in vivo. Many studies show protective effects of carotenoids on the skin.\textsuperscript{[30–32]}

The metabolism of carotenoids in the human organism is a complex process. As humans cannot synthesize carotenoids, their concentration in the organism is completely dependent on their nutritional intake and digestibility. The digested carotenoids are transported and accumulated mainly in adipose tissue, liver and blood.\textsuperscript{[33]} Darvin et al.\textsuperscript{[34–36]} showed that the carotenoid concentration in the skin is strongly dependent on the individual lifestyle conditions, including nutrition and free radical‐ induced stress factors.

There are two pathways for carotenoids to reach the SC. In the first pathway (from inside to outside), the carotenoids reach the SC via blood, lymph and subcutaneous fat from the side of viable epidermis during the proliferation of keratinocytes.\textsuperscript{[33,37]} In the second pathway (outside to inside), the carotenoids are secreted via eccrine sweat glands or sebaceous glands to the skin surface\textsuperscript{[38,39]} and penetrate from the surface into the SC, saturating the superficial SC layers.\textsuperscript{[40]} The same process is also known for lipophilic vitamin E.\textsuperscript{[41,42]}

As carotenoids are lipophilic substances, they are incorporated in lipid bilayers and could potentially influence the organization and fluidity of lipid membranes and their physical properties.\textsuperscript{[43,44]}

In this, in vivo study the interaction between the carotenoids and the molecular structure of ICL in the SC was investigated depth‐ dependently by using confocal Raman microscopy (CRM). The main objective of this study was to investigate the depth‐dependent concentration of carotenoids by the precise measurement of the carotenoid‐ related Raman peak at \(\approx 1524\) cm\(^{-1}\) with error‐ reducing preprocessing methods. Furthermore, the relationship between the molecular structure of carotenoids, their aggregation and the presence of carotenoids inside lipid lamellas was analysed.

2 | MATERIALS AND METHODS

2.1 | Confocal Raman microscopy (CRM)

Confocal Raman microscopic measurements were performed using the skin composition analyzer (River Diagnostics, Model 3510) designed for in vivo measurements. A 785 nm laser (20 mW on the skin surface, measurement time 5 seconds) was used to analyse probes in the fingerprint (FP: 400–2000 cm\(^{-1}\)) and a 671 nm laser (17 mW on the skin surface, measurement time 1 second) was used for analysis in the high wavenumber (HWN: 2000–4000 cm\(^{-1}\)) regions. Raman spectra were recorded from above the skin surface down to the depth of 40 \(\mu\)m at 2 \(\mu\)m increment. The spatial depth and spectral resolutions were \(\pm 5\) \(\mu\)m and 2 cm\(^{-1}\), respectively.

2.2 | Volunteers

Eleven healthy Caucasian volunteers (10 male and 1 female) aged from 25 to 56 (average 34) years took part in this study. The volunteers were instructed not to utilize any skincare products on the forearms before the experiments and not to bath or shower at least 4 hours before the beginning of the measurements. The volunteers had given their written informed consent. Approval for the measurements had been obtained from the Ethics Committee of the Charité – Universitätsmedizin Berlin and all the procedures complied with the Declaration of Helsinki.

2.3 | Data analysis

2.3.1 | Determination of the SC thickness

The mass percentage of water in the SC was calculated by the ratio of the area under the curve (AUC) of the OH vibration band of water (3350–3550 cm\(^{-1}\)) to the CH\(_3\) vibration band of keratin (2910–2965 cm\(^{-1}\)). A linear baseline was applied between 2720 and 3790 cm\(^{-1}\) to remove the fluorescence background.\textsuperscript{[47]}

The SC thickness was calculated by the distance from the skin surface to the boundary between the SC and the stratum granulosum (SG), which in turn, was determined by the depth profile of water in the SC.\textsuperscript{[48]} The first derivative of the water profile was calculated, and the boundary between the SC and the SG was determined as the point where it reached 0.5.\textsuperscript{[49]} In this study, all SC depths were normalized by their thickness, in order to compare the physiological parameters of the SC between the skin samples with different SC thickness. The entire SC thickness (0%–100%) was interpolated to 10% increments.

2.3.2 | Normalization of depth‐dependent signal attenuation

In order to compensate the signal attenuation with increasing depth in the SC, it is necessary to set internal normalization criteria. Gniadecka et al.\textsuperscript{[50]} suggested that the 1450 cm\(^{-1}\) band intensity can be used as a reference, because it originates from protein chains (keratin) and does not take part in intermolecular interactions. However, this band also contains a contribution of ICL, that is, ceramides and free fatty acids.\textsuperscript{[51,52]} Recently, it was shown that both keratin‐related Raman peaks at 1450 and 1650 cm\(^{-1}\) can be used for normalization of the depth‐dependent signal attenuation.\textsuperscript{[53]}

In the present study, the normalization on the Amide I peak centred at 1650 cm\(^{-1}\) has been used. For calculating the intensity
of the peak at 1650 cm\(^{-1}\), a linear baseline subtraction was applied between 1538 and 1770 cm\(^{-1}\) and the AUC was calculated in the 1634-1678 cm\(^{-1}\) range (Figure 1).

### 2.3.3 Determination of the skin surface

The skin surface was determined by using the intensity of the Amide I band at 1650 cm\(^{-1}\).[54,55] The skin surface (0 μm) was determined to be at the position where the intensity of the peak around 1650 cm\(^{-1}\) reached half of its maximum value.

### 2.3.4 Lateral/lamellar organization of the intercellular lipids (ICL) in the SC

The lateral organization of ICL, which directly determines the skin barrier function provided by the SC, was calculated by the ratio of the lipid-related Raman peak intensities in the HWN region (\(I_{2880}/I_{2850}\)) using a specially developed algorithm which excludes the contribution of keratin in the Raman spectrum.[56] Thus, higher values correspond to the increase in orthorhombic state (higher-ordered ICL), while lower values correspond to the increase in hexagonal state (lower-ordered ICL).

The lamellar organization of ICL was determined by analysing the ratio of the lipid-related Raman peak intensities in the FP region (\(I_{1080}/(I_{1060}+I_{1130})\)). The lamellar organization of ICL represents the gauche/trans-conformation or loose/compact states of the ICL, originating from the vibrations of the lipid carbon skeleton (–C–C–) and being particularly sensitive to the conformational changes of hydrocarbon chains.[57,58] In detail, while the peaks at 1080 cm\(^{-1}\) are sensitive to the gauche-conformation, the sum of the peaks at 1060 and 1130 cm\(^{-1}\) are sensitive to the trans-conformation of ICL. Thus, a lower value indicates a higher trans-conformation/compact state of ICL in the SC and vice versa. This is also indirectly related to the crystallographic properties of ICL (hexagonal/orthorhombic organization), that is, higher values correspond to the increase in hexagonal state (lower-ordered ICL), while lower values correspond to the increase in orthorhombic state (higher-ordered ICL).[52]

### 2.3.5 The determination of the carotenoid concentration

Carotenoids have three prominent Raman peaks at 1005, 1159 and 1524 cm\(^{-1}\) originating from the C–CH\(_3\), =C–C= and –C=C– group vibrations of methyl, respectively. To calculate the depth profile of the carotenoid concentration in the SC, the non-linear multivariable fitting method is often used.[58-40,59,60] Because this method is mainly focused on the minimization of the global error for the overall spectral region (400-1800 cm\(^{-1}\)),[47] the spectral changes in the specific small region, for example in the vicinity of the 1524 cm\(^{-1}\) peak position, are not sensitively reflected in the calculation of the carotenoid concentration. A simpler method to determine the carotenoid concentration in the skin is to calculate the AUC of the carotenoid peak at \(\approx 1524\) cm\(^{-1}\), which was used in resonance Raman spectroscopy of human skin.[19,61] In this study, the depth-dependent profile of carotenoids in the SC by measuring the AUC of the C=C Raman peak at \(\approx 1524\) cm\(^{-1}\) was investigated. Since the excitation wavelength of CRM (785 nm) is non-resonant for carotenoids, the intensity of this peak is small and often comparable to the noise. Therefore, a special error-reducing preprocessing was applied as follows: (a) Linear baseline removal was performed in the 1500-1542 cm\(^{-1}\) range (Figure 1); (b) For smoothing, local regression using weighted linear least squares regression of a second-order polynomial model is applied in the 1480-1580 cm\(^{-1}\) range for better visibility; (c) A Gaussian function is fitted to the smoothed curve with constrained non-linear regression. Finally, the intensity of the Raman peak at \(\approx 1524\) cm\(^{-1}\) was calculated as an area under the Gaussian curve (AUGC) and its maximum position was additionally evaluated (Figure 1).

**FIGURE 1** Raman spectrum of human stratum corneum in vivo in the fingerprint region with the baseline removal procedure. Inset shows a smoothing and Gaussian band fitting procedure for a precise calculation of the carotenoid-related Raman band intensity at \(\approx 1524\) cm\(^{-1}\).
2.3.6 | The determination of the carotenoid aggregation

The maximum position of the peak around 1524 cm\(^{-1}\) is considered as a parameter describing the aggregation state of carotenoids within the lipid membrane bilayers.\(^{[62]}\) It was found, that when the carotenoid molecules interact strongly with the lipid molecules in the ICL lamellar structures, it caused disaggregation of carotenoids, shifting the maximum position of the 1524 cm\(^{-1}\) peak towards lower wavenumbers. Inversely, when the carotenoid molecules aggregated and interacted only weakly with ICL lamellar structures, the maximum position of the peak at 1524 cm\(^{-1}\) shifted towards the higher wavenumbers. Here, the maximum position of the Gaussian band around 1524 cm\(^{-1}\) was calculated from the Gaussian fitting procedure depth-dependently and used for the determination of the molecular structure of carotenoids in the lipid phase in the entire SC. Kutuzov et al.\(^{[44,45]}\) indicated that the \(I_{1524}/I_{1160}\) intensity ratio correlates with the trans/gauche-conformation in the order of lipids that contain carotenoids in the myelin nerve membrane. Direct translation of this method to the SC is not applicable, as the peak at 1160 cm\(^{-1}\) originates not only from carotenoids but also from keratin.\(^{[63,64]}\) Also, the carotenoid peak at 1005 cm\(^{-1}\) is very small due to non-resonant excitation (785 nm) of carotenoids in the skin and is very close to the peak at 1003 cm\(^{-1}\) that originates predominantly from urea and phenylalanine of the keratin.\(^{[65]}\) The separation of the above-mentioned contributions is currently not possible. Therefore, the 1005 and 1160 cm\(^{-1}\) peaks were not considered in this study, and the intensity of 1524 cm\(^{-1}\) Raman peak was analysed.

2.4 | Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 and Matlab R2015a (The MathWorks, Inc). In order to test for normal distribution, a Jarque-Bera test was performed. In order to test for differences between the values of two skin depths, a paired student’s \(t\) test was applied. \(P < .05\) was considered as “different”, \(P < .01\) as “strongly different” and \(P < .1\) as “trend” between two skin depths.

3 | RESULTS

3.1 | Distribution and aggregation of carotenoids in the SC

In order to calculate the concentration of carotenoids correctly and to take the depth-dependent attenuation of the Raman intensity into account, it is necessary to normalize the signals using an internal normalization reference. Figure 2A shows the ratios of the AUGC of the 1524 cm\(^{-1}\) band to the AUC of the peak at 1650 cm\(^{-1}\) interpolated to the SC depth in 10% increments taking the non-homogeneous distribution of keratin throughout the SC into consideration.\(^{[53]}\)

The semi-quantitative depth profile of the carotenoid concentration (Figure 2A) is higher at the superficial SC layers and reaches a minimum at 20% SC depth (\(P < .01\) between 0% and 20%). This profile supports the hypothesis that the carotenoids can be supplied from the outer side of the skin being delivered with sweat and/or sebum secretion,\(^{[38–40,66]}\) which is similar to previously reported outside to inside delivery pathway of vitamin E.\(^{[41]}\) Lipophilic carotenoids are present in the sweat together with the protein complexes,\(^{[66]}\) that is carotenoids are aggregated, which can hinder the further penetration into the SC compared to unbound carotenoids. The decrease in carotenoid concentration at the 10%-20% SC depths might be caused by the limited penetration and by free radicals generated by the sunlight or other exogenous factors. The concentration of carotenoids increases again towards the deeper layers of the SC (\(P < .05\) between 20% and 30%-100% SC depth). This suggests that there is also a carotenoid diffusion/flux from the papillary dermis side which is rich in
blood and lymph capillaries. On the basal layer of the epidermis, the carotenoids diffuse from the blood and/or lymph flow to basal keratinocytes and start to move towards the SG. In the SG cells, the lipophilic carotenoids are most likely located inside the lipid-rich lamellar granules, but could also be located in the membranes and cytoplasm. Then, the carotenoids could be released together with the extrusion of lamellar granules into the intercellular space at the boundary between the SC and the SG. Released lipids are structurally organized in the lamellas and form a low-permeable ICL barrier at the bottom of the SC, and lipophilic carotenoids are located in the intercellular space of the SC inside the lamellas. Thus, Figure 2A clearly shows a non-homogeneous distribution of the carotenoid concentration in the SC which is likely the result of two different delivery pathways—from inside to outside (due to corneocyte proliferation) and from outside to inside (via the sweat/sebum secretion). The main role of carotenoids in this respect is to provide an antioxidant defense of the lamellas against the lipid oxidation induced by destructive action of free radicals.

Figure 2B shows the in vivo depth profile of the maximum position of the Gaussian band around 1524 cm⁻¹, which is sensitive to the aggregation state of carotenoids in the lipid lamellas. At the superficial layers 0%-20% SC depths, the maximum position is highest (P < .01 between 0%-20% and 40%-80% SC depths), which shows that the carotenoid molecules are highly aggregated at the superficial SC layers (0%-20% SC depths). In the centre of the SC, the maximum position shifts towards lower wavenumbers, reaching a minimum at 40%-60% SC depths, which shows maximal disaggregation of carotenoid molecules. At the 90%-100% SC depth, the 1524 cm⁻¹ peak position increases again (P < .01 between 40%-50% SC depths and 90%-100% SC depths), showing increased aggregation of carotenoids.

3.2 | The depth profiles of lamellar and lateral organization of ICL

Figure 3 shows the depth-dependent profiles of the lamellar (Figure 3A) and the lateral (Figure 3B) organization of ICL in the SC in vivo. The profile of lamellar organization reaches a minimum at the 30%-50% SC depth. That is the value at = 40% SC depths is lower than at 0%-20% SC depth (P < .01) and at 70%-100% SC depths (P < .01). The minimum value of lamellar organization at 40% SC depth indicates the prevalence of trans-conformation of C–C skeleton vibrations of ICL in comparison to other SC depths, and thereby indirectly confirms the highest lateral organization of ICL in these SC depths. Meanwhile, the upper and lower layers of the SC show less-ordered ICL, characterized by the prevalence of the gauche-conformation of C–C skeleton vibrations of ICL (Figure 3A). The lateral organization of ICL shows a similar tendency of ICL organization as the lamellar organization (Figure 3B). The highest value at 30%-40% SC depths shows more higher-ordered orthorhombic lateral ICL organization. Meanwhile, in the upper and lower layers of the SC, ICL has a less-ordered/hexagonal lateral organization. This coincides well with our previous in vivo results and with the results obtained ex vivo using the X-ray diffraction method.

4 | DISCUSSION

Taking into account that surrounding water molecules facilitate the aggregation of carotenoids and that lipids are in most disordered states at the 0%-10% and 80%-100% SC depths (Figure 3), the lipid-carotenoid interaction will be smaller at these depths. Thus, carotenoids are in an aggregated state at 0%-20% and 90%-100% SC depth (Figure 2B). In the bottom layers of the SC, where the carotenoid aggregation is maximal, it can also be explained by the maximal concentration of water, while at the superficial layers, where the water has a minimal concentration, it can be explained by the water-rich sweat. In the middle SC layers at approx. 40%-60% SC depth, carotenoids are highly disaggregated (Figure 2B). The lateral ICL organization is highest in the middle SC layers at approx. 20%-40% SC depth (Figure 3A,B). This correlation can be explained as follows: At the skin surface, the carotenoids are mostly delivered with sweat/sebum in the aggregated form. Then, the carotenoid molecules penetrate through the intercellular space and...
reach depths of maximal 20% SC thickness, which is confirmed by the lowest carotenoid concentration in this SC depth (Figure 2A). ICL are most densely packed (Figure 3A,B) at ≈20%-40% SC depth. At these SC depths, the carotenoid molecules should be rearranged in the space of lipid bilayers. The highly ordered and densely packed lipid molecules intensify the lipid-carotenoid interactions and weaken the carotenoid-carotenoid interaction in the lipid bilayers inside the lamellas and thus enhance the disaggregation of carotenoids, which is detected by lowering the maximum peak position at 1524 cm⁻¹ (Figure 2B). This disaggregation also coincides with previous results, regarding the incorporation of carotenoids in membrane’s lipid bilayers.[62]

At the deeper layers of the SC, 80%-100% SC depths, where the lipid concentration is lowest,[73] the carotenoid-lipid interaction will be weakened and the carotenoids have a tendency to aggregate. The carotenoids β-carotene and lycopene are plane molecules which are oriented horizontally inside the lipid membranes.[74,75] Based on this knowledge, the experimental results suggested the carotenoid-lipid interaction mechanism, which is schematically shown in Figure 4.

As the lipophilic carotenoid molecules β-carotene, lycopene and their isomers are oriented parallel inside the lipid lamellas, they probably interact with the hydrophobic tail of the lipid molecules. Thus, the skeleton properties of the carotenoid molecules, which are characteristic of the stabilization of the lipid membrane, could be assumed and should be addressed in future investigations.

In this study, the application of the adapted method for measuring carotenoid depth profiles in the human SC in vivo was demonstrated. The results support the conjecture of two penetration pathways of carotenoids in the SC—from inside to outside (during the proliferation of the keratinocytes) and from outside to inside (due to secretion via sweat glands and/or sebaceous glands to the skin surface). Carotenoids are highly aggregated in the superficial SC depths and close to the boundary with SG, while in the middle SC depths carotenoids remain in the disaggregated form.

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**CONFLICT OF INTEREST**

The authors have declared no conflicting interests.

**AUTHORS CONTRIBUTION**

CSC, JS and MED performed the research, analysed data and contributed to the development of the methodology. JRR performed data analysis. MED and JL conceived the experiments and designed the research. All authors have contributed in preparation of the manuscript.

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