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Quantification of the horny layer using tape stripping and microscopic techniques

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Abstract. Tape stripping is a method well suited for studying the penetration of topically applied substances into the upper part of the skin, the stratum corneum (SC). The amount of topically applied substances removed by each tape strip can be determined by common analytical methods. These amounts have to be correlated with their location within the horny layer in order to determine penetration profiles. Therefore the amount of SC on each removed strip must be determined. In the study reported, this amount was derived from the covering density of the SC cells, the corneocytes, on tape strips using classic microscopic techniques and laser scanning microscopy. The amount of corneocytes determined by both microscopic methods shows a good correlation ($R^2 \pm \text{S.D.} = 0.95 \pm 0.02$) with the pseudo-absorption of the corneocytes, a method usually used to determine the SC amount on the removed strips. These measurements require the application of a special spectrometer, whereas the covering density can easily be determined using widely available microscopic equipment. The penetration of a typical UV filter, butyl methoxydibenzoylmethane, applied at two different formulations, was studied using the microscopic method developed. The calculated penetration profiles show that the applied formulation influences the amount of corneocytes on the removed strips. [DOI: 10.1117/1.1609200]

Keywords: tape stripping; penetration measurements; corneocyte density; microscopy; optical spectroscopy; UV filter.

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1 Introduction

A detailed knowledge concerning the penetration processes of substances into the human skin is of special interest in studies dealing with the effects and the efficacy of dermatological drugs and cosmetic products. The uppermost layer of the skin (stratum corneum) determines the penetration kinetics and the reservoir properties of the skin. This horny layer consists of corneocytes surrounded by lipid layers, and it presents a barrier for substances approaching the body from the outside.

The step-by-step stripping of the human horny layer with adhesive tapes is a well-known method for studying the penetration process of substances.[1–3] After a topical application of substances, a tape strip is pressed onto the treated skin area and than removed. This procedure is repeated several times on the same area of skin. This method allows a complete noninvasive removal of the horny layer. The amount of substance fixed on each tape strip can be detected by common analytical methods after extraction.[4–6] The correlation of these concentrations with their location inside the horny layer is a prerequisite to determining the distribution of the substance within the horny layer. Therefore the amount of corneocytes on each tape strip has to be determined and correlated with the total amount of stratum corneum removed.[7,8] The amount of corneocytes removed by tape stripping depends on the location within the horny layer profile, the volunteer, the body site, the topically applied substance, the pressure applied to the tape before removal, the tape type, and the peeling force.[2,7,9]

A number of methods to determine the amount of corneocytes on tapes have been reported in the literature.[8–13] Weighing of the tape strips before and after skin contact, e.g., as described by Marttin et al.,[9] can be disturbed by exogenous (topically applied substances) and endogenous (interstitial fluid) substances. These substances might also be stuck to the tape strips, resulting in an additional weight increase.[10]

A colorimetric method was developed by Dreher et al.[11] This method is based on staining the proteins of the corneocytes after their extraction from the removed tape strips. Standard solutions from excised human skin were utilized for calibration. This method has limitated application for penetration studies of topically applied substances because the tape strips are destroyed and thus are unavailable for later determination of drug concentrations. The protein absorbance of the corneocytes themselves at 278 nm is less sensitive.[9,12] In addition, it can be disturbed by topically applied substances or components of the adhesive layer with a UV absorbance in this range.[8]

The transepidermal water loss (TEWL) is used as an indirect measure to characterize the thickness of the remaining horny layer.[13–15] This method is inaccurate, especially for tape strips removed from the superficial layers of the stratum corneum. In addition, topically applied emulsions can influence the TEWL.

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An optical method recently described by Weigmann et al.[8] is based on measurement of the pseudoabsorption of the corneocytes in the visible range. The pseudoabsorption is caused by an interaction of corneocyte aggregates with light via scattering, diffraction, and reflection mechanisms. The value of the resulting absorbance can be used as a suitable measure for the amount of corneocytes removed by each tape strip. Therefore a special ultraviolet/visible (UV/VIS) spectrometer was developed by PerkinElmer to obtain a large measuring area of 1 cm^2, which is required for the measurements.

The present investigation was aimed at finding new methods for quantifying the amount of stratum corneum removed on single tape strips, which could be utilized in every dermatological or pharmacological institution. Laser scanning microscopy and common light microscopy, in combination with an automatic image analysis software, were tested for this purpose. Both methods were evaluated by comparing the covering density of the corneocytes on the tape strips, determined microscopically, with the pseudoabsorption of the corneocytes. The constant thickness of the corneocyte aggregates removed from different depths of the horny layer was the pivotal prerequisite for using the covering density. Finally, the application of the microscopic method was demonstrated by determining the penetration profile of a chemical UV filter, butyl methoxydibenzoylmethane, applied at two different formulations.

2 Materials and Methods

2.1 *Tissue*

2.1.1 *Human skin*

The basic experiments, using laser scanning and light microscopy, were performed on tape strips removed from seven healthy human male and female volunteers, aged between 29 and 43 years. During a period of 1 h between the topical application of emulsions and the beginning of tape stripping, the volunteer rested without sweating and without covering the test area with textiles. All volunteers had given signed informed consent. Ethical approval for these experiments was obtained from the ethics committee of the Charité University Hospital.

2.1.2 *Porcine skin*

Porcine ear skin is a good model for human skin.[16] Thus pig ears were used for the repetition of the measurements in order to evaluate the newly developed method statistically. The studies were carried out using ears of pigs slaughtered on the day of the experiments. The hair of the ears was carefully removed by scissors. The ears were washed with water and dried using tissue. Approval for these experiments was obtained from the Veterinary Board of Control, Berlin Treptow—Köpenick.

2.2 *Topical Application of Emulsions*

An oil in water (o/w) emulsion containing 0.2% of the fluorescent dye curcumin (Merck-Schuchardt, Hohenbrunn, Germany) was used in the laser scanning microscopy experiments. (An o/w emulsion is a dispersion of oil droplets into an aqueous medium using emulsifiers.) The penetration studies were performed applying the o/w emulsion and an oil containing 1.5% of the UV filter substance butyl methoxydibenzoylmethane (Merck, Darmstadt, Germany). The tape stripping was started 1 h after application of the emulsions.

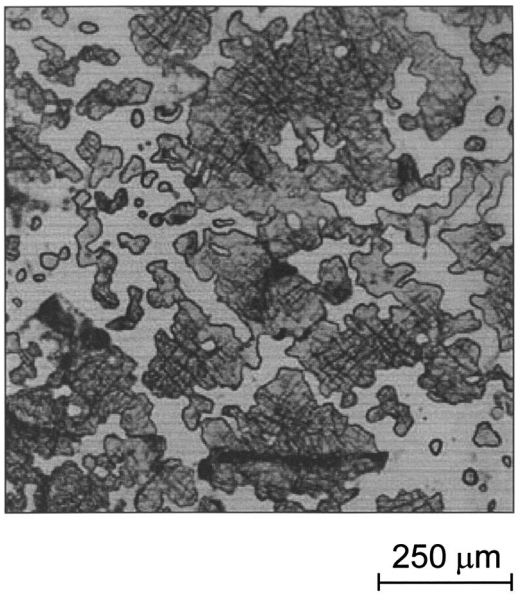

Fig. 1 Distribution of human corneocytes on a tape strip (tape 1) measured with a laser scanning microscope.

The emulsions (2 mg/cm^2) were homogeneously distributed on a skin area of 5×6 cm^2 on the flexor forearms of human volunteers or on pig ears, as described previously.[8] The emulsion was applied with a stump syringe inside the marked area and distributed homogeneously with a gloved finger previously saturated with the emulsion.

2.3 *Tape Stripping Protocol*

The tape strips were removed from the skin areas as described in detail by Weigmann et al.[8] The adhesive tape (*tesa* film No. 5529, Beiersdorf, Hamburg, Germany) was pressed onto the surface of the skin 10 times with a roller. A piece of paper was placed between the tape and the roller to avoid a transfer of emulsion from the adjacent skin part to the adhesive film. The tape strip was removed with one quick movement and fixed to a slide frame. The tape stripping procedure was repeated 10 to 30 times on the same skin area.

2.4 *Laser Scanning Microscopic Measurements*

The thickness of the corneocyte aggregates removed by tape stripping was determined by confocal laser scanning microscopy (LSM 2000, Carl Zeiss, Jena, Germany) using an excitation at 488 nm. Therefore an o/w emulsion containing 0.2% of the fluorescent dye curcumin was applied on the skin in order to make the corneocytes visible. After a penetration time of 1 h, the tape strips were removed from different depths of the stratum corneum.

Figure 1 shows a laser scanning microscope (LSM) image of the typical distribution of the corneocyte aggregates on a tape strip removed from human skin. The contours of the corneocyte aggregates can be seen clearly. The average thickness of the corneocytes on the tape strips was determined by scanning the focus of the LSM through the tape strips. A

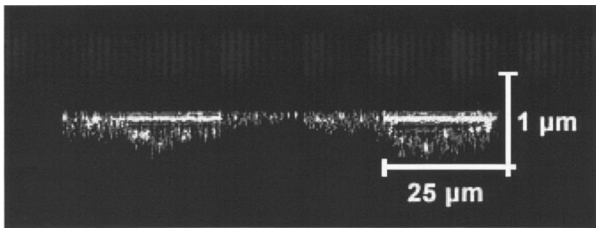

Fig. 2 Cross-section of corneocytes on a removed tape strip. The corneocytes were covered with fluorescent dye.

typical image of the cross-sections of two corneocyte aggregates collected on the adhesive tape is presented in Fig. 2. The dimension of the corneocyte aggregates can be clearly recognized owing to the fluorescence of the dye covering the surface of the corneocytes.

The density of the corneocytes on the tape strips was also analyzed using the standard LSM software program. Figure 3 shows the density of the corneocytes on the removed tape strips determined by laser scanning microscopy. The contour of each corneocyte aggregate on different subareas of a tape strip was marked manually; the area A_i of the aggregate was calculated by the software program. The sum of all areas ΣA_i was related to the total area of the complete image A_{tape}, representing the relative covering density D of the corneocytes.

$$D = \frac{\Sigma A_i}{A_{\text{tape}}} \times 100\%.$$

The procedure was repeated until a total measuring area of 1 cm^2 for each tape had been analyzed. The average corneocyte density was determined by calculating the mean value of all values for D measured on this total area.

2.5 *Determination of the Covering Density Using Light Microscopy*

The covering rate of human corneocytes on tape strips was determined using a light microscope with transmitted light (System microscope BX60, Olympus Optical Co., Hamburg, Germany) in the phase contrast mode (objective UPLFL4X/0.13, Olympus Optical Co., Hamburg, Germany). The microscope was equipped with a digital camera (SIS ColorView12, Soft Imaging System GmbH, Münster, Germany) and the appropriate software (analySIS AUTO 3.1, Soft Imaging System GmbH). The phase contrast mode used offers the possibility of evaluating the images of removed tape strips for the covering density of the corneocyte aggregates. These densities were determined by relating the dark areas (areas covered by corneocytes) to the total sizes of corresponding images [Fig. 4(a)]. The software analysis AUTO 3.1 was used to distinguish between the corneocyte aggregates and the background by an alteration in the contrast of the image [Fig. 4(b)]. A histogram of the image was calculated automatically. A threshold level easy to identify in this histogram was chosen to mark only the corneocytes. This calculation was performed on ten subareas of 3.68 mm^2 inside a total area of 4 cm^2 for each tape strip owing to the nonhomogeneous distribution of the corneocytes on the removed strips. An average value of the corneocyte density was calculated. This procedure was performed frequently image by image.

2.6 *UV/VIS Spectroscopic Measurements*

UV/VIS spectroscopic measurements were performed with a modified double-beam spectrometer (Lambda 20, Perkin Elmer, Überlingen, Germany) with a quadratic beam of 1×1 cm^2. These measurements were used to determine the pseudoabsorption of the corneocytes as a standard value for evaluation of the microscopic methods. In these experiments, the pseudoabsorption of the corneocytes was determined at

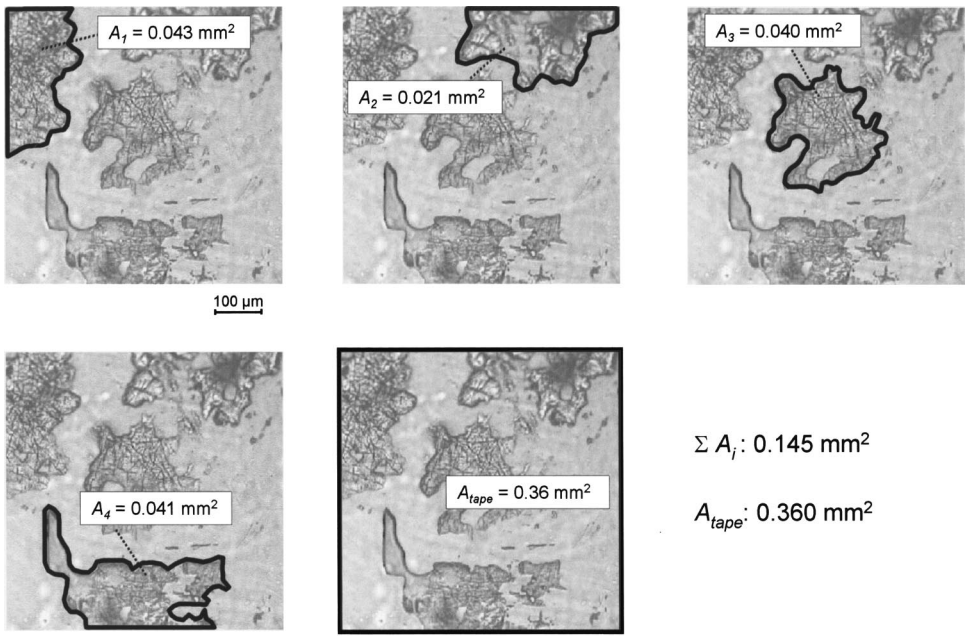

Fig. 3 Determination of the covering density of human corneocytes on a tape strip (No. 4) using laser scanning microscopy.

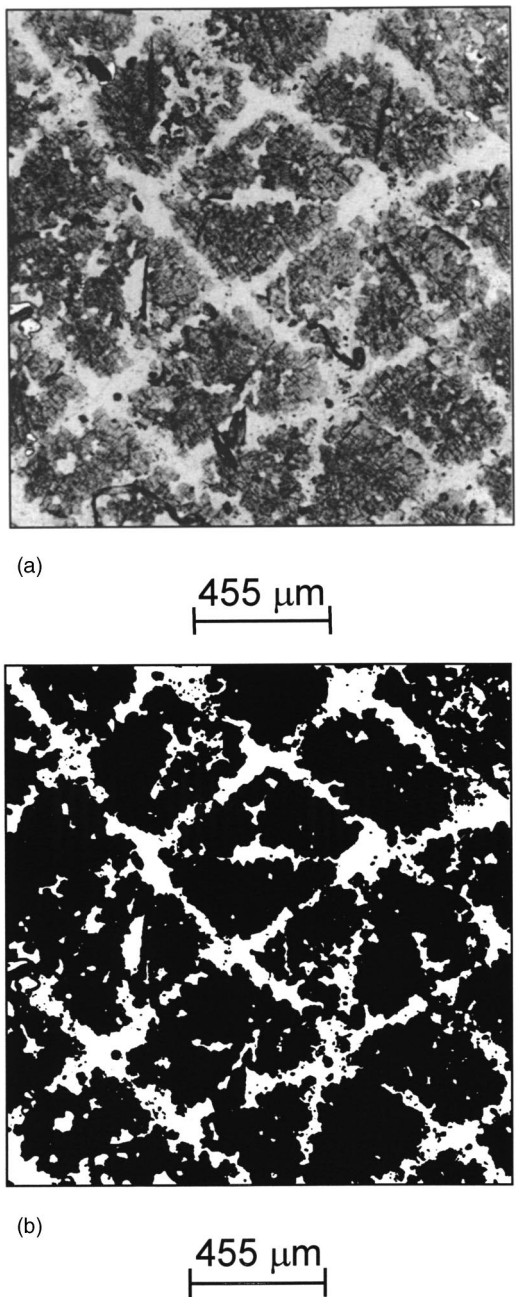

Fig. 4 The distribution of corneocytes on a tape strip detected by light microscopy (No. 6). (a) Detected in the mode of phase contrast. (b) Evaluated by the contrast software program (resulting relative covering density of the horny layer particles, 59.7%).

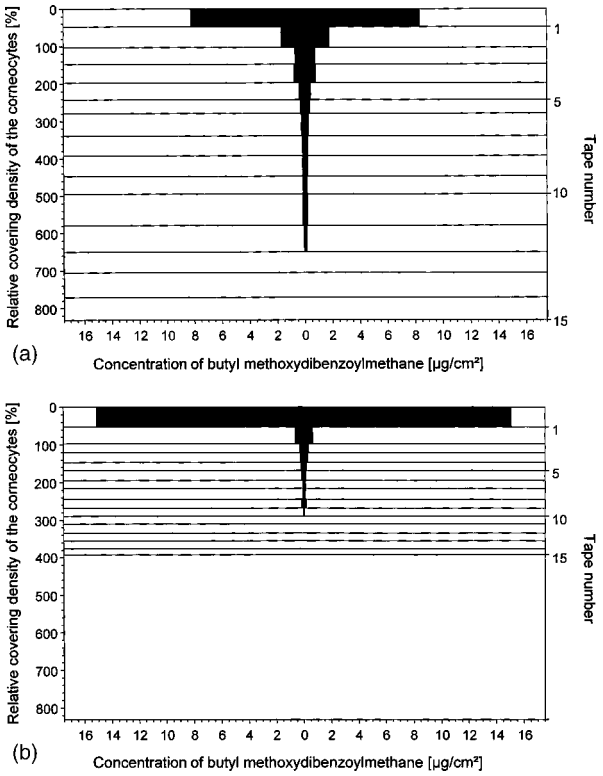

Fig. 5 Penetration profiles of butyl methoxydibenzoylmethane; based on the calculated horny layer profile determined via the relative covering density and on the concentration of butyl methoxydibenzoylmethane, 1 h after application to the forearm. (a) Applied with an o/w emulsion. (b) Applied with an oil.

430 nm. In principle, all wavelengths can be used that are not disturbed by absorption bands of exogenous and endogenous compounds.[8] The removed tape strips were measured using an empty tape as reference. After determining the amount of corneocytes, the UV/VIS spectroscopic measurements were used to determine the amount of topically applied UV filter substance.

The edges of the tape strips were cut in order to achieve defined areas of 1.5×4.3 cm^2. Then the strips were put into tubes filled with 6.45 ml ethanol (Uvasol, Merck, Darmstadt, Germany) and treated for 5 min in an ultrasonic bath (Sonorex Super RK 102H, Bandelin Electronic, Berlin, Germany). The solutions were purified by centrifugation (5 min at 4000 cycles/s, centrifuge MR 1812, Jouan GmbH, Unterfaching, Germany) to separate small horny-layer particles. The uppermost part was decanted with a pipette into a quartz cell with a length of 10 mm or 1 mm (Hellma, Jena, Germany).

The UV/VIS spectra of the solutions were measured between 240 and 500 nm using the pure solvent as a reference. The concentration of butyl methoxydibenzoylmethane was calculated on the basis of a calibration curve of UV filter substance in ethanol using the determined absorption maximum at 358 nm.

2.7 *Calculation of Penetration Profiles*

The penetration profiles were calculated based on the determination of the concentration of butyl methoxydibenzoylmethane and the amount of the removed corneocyte aggregates on each tape strip (Fig. 5). The strips were removed after topical application and penetration of the o/w emulsion or the oil containing the UV filter.

The amount of corneocytes was determined for each removed tape strip using light microscopy. The amount of butyl methoxydibenzoylmethane on each strip was determined spectroscopically. Since sunscreens are usually localized in the upper part of the horny layer,[6,17] only fifteen tape strips were removed. The relative thickness of the removed horny layer is characterized by the sum of the covering densities of corneocytes on these fifteen tape strips. Every strip was re-

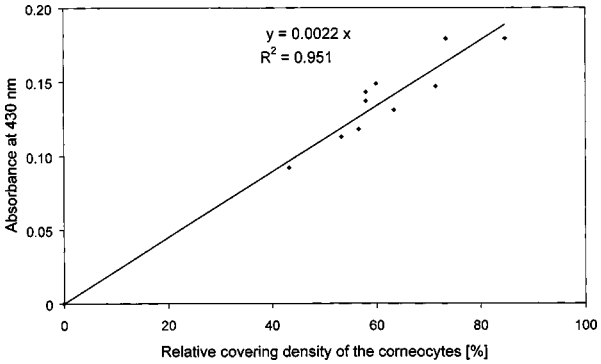

Fig. 6 Correlation between the pseudoabsorption at 430 nm and the tape area covered by human corneocytes, determined by laser scanning microscopy (tape strips 1 to 10).

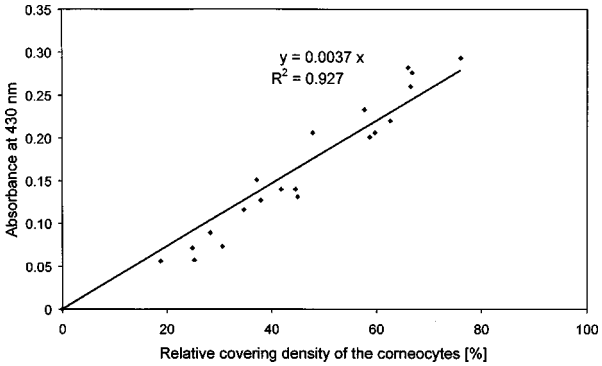

Fig. 7 Correlation between the pseudoabsorption at 430 nm and the tape area covered by corneocytes using light microscopy (tapes 1 to 20).

lated to its position in the removed part of the horny layer. The horizontal lines in Fig. 5 correspond to the tape strip number. The distance between these lines is determined by the amount of corneocytes on the corresponding tape strips. The horny layer profile represents a cut through the removed part of the horny layer, which allows the determination of the local position of each tape inside this profile.[8]

3 Results

3.1 *Determination of the Thickness of the Corneocytes*

The average thickness of the corneocytes on the tape strips was determined by scanning the focus of the LSM through the tape strips. A typical image of the cross-sections of two corneocyte aggregates attached on the adhesive tape is presented in Fig. 2. The dimension of the corneocyte aggregates can be clearly recognized owing to the fluorescence of the dye covering the surface of the corneocytes. The average thickness of the corneocyte aggregates was nearly identical on all thirty tape strips removed from different depths of the horny layer. The average thickness determined on six volunteers was ca. 0.5 μm. A stack of two or more corneocytes was not observed.

3.2 *Determination of the Covering Density Using Laser Scanning Microscopy*

The density of the corneocytes on the removed tape strips was determined by laser scanning microscopy (see Fig. 3). The resulting relative covering densities (D) were compared with the pseudoabsorption of the corneocytes measured with a modified UV/VIS spectrometer at 430 nm. The correlation of both quantities is shown in Fig. 6. A high correlation coefficient of $R^2 = 0.95$ was obtained.

3.3 *Determination of the Covering Density Using Light Microscopy*

The covering rate of human corneocytes on tape strips was determined using a light microscope in the phase contrast mode. The average density of the corneocytes on each tape strip was correlated with the pseudoabsorption at 430 nm. The results are presented in Fig. 7 for a series of twenty tapes removed from human skin. A correlation coefficient R^2 of 0.93 was obtained. The procedure was repeated and evaluated statistically for eight series of twenty tape strips each. These experiments were carried out on porcine skin. The mean correlation coefficient and the standard deviation from the eight experiments were calculated as $R^2 \pm \text{S.D.} = 0.95 \pm 0.02$.

3.4 *Calculation of Penetration Profiles*

The penetration of the UV filter butyl methoxydibenzoylmethane applied in two different formulations was investigated. The penetration profiles are presented in Fig. 5. The concentration of butyl methoxydibenzoylmethane is depicted by the length of the black bars. In the case of the oil [Fig. 5(b)], the main amount of the UV filter is localized on the skin surface and in the uppermost part of the stratum corneum, whereas the o/w emulsion [Fig. 5(a)] causes a deeper penetration of the UV filter. In the case of this emulsion, a higher amount of corneocytes was removed by the tape strips than after application of the oil.

4 Discussion

A constant thickness of the corneocyte aggregates of about 0.5 μm was found, even if they were removed from different depths of the horny layer. This result corresponds to the results presented by Bouwstra et al.[18] and Marks and Barton,[19] who investigated the thickness of the corneocytes in different depths of the horny layer by electron microscopy. The constant thickness of the corneocytes is an important prerequisite to successfully utilizing spectroscopic methods for quantification of the stratum corneum removed. A stack effect of the corneocytes on the tape strips was not observed.

A good correlation between the corneocyte density on the tape strip detected by LSM and the established method of pseudoabsorption of the corneocyte aggregates[6,8,10] was found (see Fig. 6). This correlation shows that the covering density of the corneocytes on the tape strips can be used as a tool to quantify the corneocytes.

The results obtained by LSM measurements can also be obtained by replacing these time-consuming measurements with the fast and commonly accessible method of light microscopy. The data obtained by light microscopy also show a good correlation with the pseudoabsorption of the corneocytes at 430 nm (see Fig. 7). This result was confirmed by the high correlation coefficients ($R^2 \pm \text{S.D.} = 0.95 \pm 0.02$) obtained in a series of experiments that were performed on porcine skin,

which is a suitable model for human skin.[16] These good correlations indicate that light microscopy is well suited for determining the relative amount of the horny layer particles removed by tape stripping.

The spectroscopic method is based on a homogeneous distribution of the corneocytes on the tape strips. Therefore it is expected to be limited to use on healthy skin. In contrast, the microscopic method could also be used to study the horny layer of patients with skin diseases, such as psoriasis and ichthyosis.

For penetration measurements, it is only necessary to determine the relative thickness of the horny layer because the absolute thickness differs for different volunteers and for different body sites.[2,7,20] Even the same volunteer has a different thickness of the horny layer in winter and summer.[21] The penetration profiles obtained for different volunteers using the relative thickness are comparable, and differences caused by different formulations can be clearly detected.[8,17]

The penetration profiles obtained using this microscopic method show the distribution of the topically applied butyl methoxydibenzoylmethane inside the horny layer (see Fig. 5). In both cases, the fifteen tape strips were sufficient to remove the topically applied UV filter. As seen in Fig. 5, the distribution of butyl methoxydibenzoylmethane, as well as the amount of corneocytes removed with each tape strip, depended on the formulation used for the application. In the case of the oil, the main amount of the UV filter was localized on the skin surface and in the uppermost part, where it has the best protection properties. In contrast, the o/w emulsion caused a deeper penetration of the UV filter. In addition, the o/w emulsion led to a higher amount of corneocytes on the tape strips than in the oil. This effect can be caused by reduced cohesive forces between the corneocyte layers or by reduced adhesive properties of the tape. A similar behavior was observed for untreated skin using different types of tapes.[2]

Summarizing the results, the tape stripping procedure in combination with microscopic techniques is a suitable method for determining the amount of corneocytes removed. Therefore this method can be recommended for the qualitative and quantitative analysis of the penetration of topically applied substances. Another advantage of this new method is the use of a widely available device. This combined method allows the determination of the amounts of removed corneocytes and topically applied substances on the same tape strip. The correlation of tape strips with the depth of the horny layer removed is a prerequisite for comparing penetration profiles and the penetration kinetics of topically applied substances.

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