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Research paper

Continuous removal of single cell layers by tape stripping the *stratum corneum* – a histological study

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ABSTRACT

Studies on the penetration of toxicologically or pharmaceutically relevant substances through the skin and, more specifically, through the *stratum corneum* (s.c.) often rely on the well-established method of tape stripping. Tape stripping involves the removal of skin layers by means of adhesive tape, which is usually followed by quantification of dermally applied substances in these layers. However, the amount of s.c. removed by each individual tape strip is still a matter of scientific debate. While some studies imply that the amount of s.c. adhering to each tape strip decreases with increasing depth into the s.c., others observed a constant removal rate. All these studies rely on the quantification of the amount of s.c. captured on individual or pooled tape strips. Here, we present an approach whereby we measured the amount of s.c. remaining on excised porcine skin in the process of tape stripping. Staining and bloating of the s.c. allowed to measure its thickness and to count individual s.c. layers, respectively. Histologically, we show that the s.c. remaining on the skin decreased linearly as a function of strips taken. We found that each tape strip removes about 0.4 µm of s.c., which corresponds to approximately one cellular layer. With a high coefficient of determination ($r^2 > 0.95$), we were able to linearly correlate the thickness of the remaining s.c., the number of remaining cell layers and the number of tape strips applied. Furthermore, we elaborate on possible reasons for the discrepancies reported in the scientific literature regarding the amount of s.c. removed by each tape strip.

1. Introduction

Tape stripping is a frequently used method in skin penetration research, by which the *stratum corneum* (s.c.) is successively removed by means of stripping the skin surface with adhesive tape. Earliest descriptions date back to 1939 [1]. Studies in the past have utilized tape stripping to determine the amount and corresponding concentration profiles of dermally applied substances in the s.c. [2–4], or to investigate how the barrier function of the s.c. decreases with fewer cell layers [5]. Compared to skin biopsies, it represents a method that is less invasive but still applicable in the context of *in vivo* studies [6].

Concentration profiles of substances in the s.c. crucially rely on the removal rate of the s.c. layers, that is, how much of the s.c. is taken off by each tape strip. To determine the removal rate, various methods have been applied previously, including microscopy [7], measurement of the transepidermal water loss (TEWL) [8–9], weighing [10–11], UV/vis-spectrometric characterization of the removed tape strip [12–13], infrared densitometry [14] and measuring the protein content on the removed tape strip [15–17]. Importantly, all these studies rely on the measurement of quantities retrieved from the removed cell layers. Different studies have come to opposing conclusions: While some infer that the amount of s.c. taken off by each tape strip remains constant with successive strips, others show that it decreases with increasing depth.

Colorimetric protein assays, for example, have demonstrated a uniform removal of s.c. layers [15–16]. Comparable results were obtained when the amount of s.c. removed on each tape strip was investigated via laser scanning microscopy [7]. However, several studies that focus on measuring the *pseudo*-absorption (a protologism for non-transmitted light, that is, the total of absorption, scattering, reflection and diffraction) report that the amount of s.c. removed with a tape strip decreases in deeper layers [12–13,17]. Similarly, the mass and the protein content of s.c. removed by each tape strip were found to decrease with increasing

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numbers of tape strips, thereby confirming these findings [9].

On the other hand, variable results were reported when investigating the thickness of the s.c. after tape stripping of freshly excised and frozen human skin [14]. The fresh skin showed a linear decrease of the s.c. thickness with the number of tape strips whereas for the frozen skin of the same donor the decrease was not linear. For a different donor, the results were reversed. However, it was argued that the plateauing observed after a given number of applied tape strips is due to the complete removal of the s.c.

In this study, we contribute to the ongoing investigation on the removal rate of s.c. layers by tape stripping excised porcine skin. Porcine skin has been investigated as a substitute for human skin in various skin penetration studies [2–4,18–20]. It does not significantly differ from human skin in relevant permeation characteristics, including lag time, diffusion behavior of substances in the s.c. [4,18–19] and thickness of the s.c. [4,21–22], suggesting that results obtained with respect to these properties are transferrable to human skin. Porcine skin is therefore recommended by the Scientific Committee on Consumer Safety (SCCS) to be used in skin penetration studies [23]. In contrast to the aforementioned studies, which examined the amount of s.c. captured on each tape strip, we inspected the s.c. that is left behind on the skin. Cryosections of the s.c. at successive points in the tape stripping process were taken and the thickness of the remaining skin was directly measured microscopically after histological staining. Furthermore, the number of cell layers remaining were counted and the results were correlated.

2. Methods

2.1. Porcine skin

The pigskin used in this study was provided by the Charité, Universitätsmedizin Berlin. The skin was taken from the flank of female pigs, which was demonstrated to be comparable to frequently used skin from pig ears [24]. The pigs were sacrificed shortly before the skin was removed in an unrelated surgical experiment that did not influence the integrity of the skin. The excised skin was transported to our institute on ice, sheared, cut into pieces of approximately 10 × 20 cm with a scalpel, and frozen and stored at −20 °C until further use for a maximum of 12 months.

2.2. Preparation and quality control of skin samples

In preparation for tape stripping experiments, the frozen skin pieces were partially thawed and a skin sheet of 500 μm thickness was cut off using a dermatome (Aesculap AG, Tuttlingen, Germany). Sections of 25 mm diameter were punched out of the skin sheets with a stencil. Their integrity was verified visually and by measuring the TEWL. For TEWL measurements, the sections were laid atop the receptor chamber of a Franz diffusion cell (PermeGear, Hellertown, Pennsylvania, United States) filled with Dulbecco's phosphate-buffered saline (DPBS, pH 7.4, PAN Biotech, Aidenbach, Germany) containing 50 $mg \cdot ml^{-1}$ bovine serum albumin (BSA, Biomol, Hamburg, Germany). It was ensured that no air bubbles were present directly under the skin specimen in the receptor solution. The TEWL was measured using the AquaFlux device AF200 (Biox systems Ltd, London, United Kingdom), in accordance with OECD Guideline 428 [25]. Skin punches were excluded and replaced if the TEWL exceeded 10 $g \cdot m^{-2} \cdot h^{-1}$ [26]. The skin pieces were removed from their respective Franz diffusion cell and dried with a clean precision wipe (Kimtech Science, Kimberly-Clark, Irving, Texas, United States).

2.3. Tape stripping

The samples were cut in four sections with a scalpel and then stripped with tape strips (kristall-klar, tesafilm, tesa, Norderstedt, Germany) up to 20 times, increasing the number of tape strips by two between each sample. The tape consisted of biaxially oriented polypropylene with water-based acrylate as an adhesive [27] and has been used in previous studies for the same purpose [2–3,28]. We were able to confirm the polypropylene polymer by online-coupled pyrolysis–gas chromatography–mass spectrometry (see the Supporting Information, Figure S1). The stripping protocol was as follows: The tape strip was laid atop the skin, ensuring that the contact between the skin and the strip was complete. The tape strip was pressed onto the skin with the backside of curved tweezers by stroking the strip two times in each perpendicular direction, taking care to apply constant pressure (this procedure was carried out by the same person throughout the study). Then, the strip was removed in a single swift movement. Six replicate skin samples were used to obtain data for each investigated number of applied tape strips.

2.4. Sample preparation for histology

The skin sections were frozen in optimal cutting temperature compound (Tissue-Tek O.C.T., Sakura, Staufen, Germany) using liquid nitrogen (Linde, Pullach, Germany). Of each frozen skin section, multiple cross sections of 5 μm thickness were cut using a cryo-microtome (HM 550 OP, Thermo Fisher Scientific, Waltham, Massachusetts, United States) and laid onto microscopy slides (Epredia SuperFrost Plus, Thermo Fisher Scientific). The slides were then submerged in acetone (Sigma-Aldrich, Darmstadt, Germany) at −20 °C for 20 min to remove the optimal cutting temperature compound.

2.5. HE-Stain

For staining with hematoxylin and eosin (HE-stain), half of the slides were placed into freshly filtered (folded filters 3 hw, Ahlstrom Munksjö, Helsinki, Finland) Mayer's acidic hematoxylin solution (Carl Roth, Karlsruhe, Germany) at room temperature for 20 min. The slides were rinsed with desalinated water and then transferred into Scott's tap water substitute (23.8 mM sodium hydrogen carbonate (Sigma-Aldrich, Saint-Louis, Missouri, United States) and 166 mM magnesium sulfate heptahydrate (Carl Roth) in desalinated water, pH 8.3) for 12 min. The slides were rinsed with desalinated water and then placed into a freshly filtered (folded filters 3 hw, Ahlstrom Munksjö) 1% aqueous Eosin Y solution (Carl Roth) for 3 min. The slides were rinsed again with desalinated water and then fixed with a coverslip using FluorSave reagent (Millipore Corporation, Billerica, Massachusetts, United States). These slides were used to measure the s.c. thickness by optical microscopy (see section *Optical microscopy*).

2.6. Safranin stain

For safranin staining, the other half of the slides were treated according to an adapted protocol [29]. The slides were placed into a 1% aqueous solution of safranin (Carl Roth) for 1 min. The slides were then taken out of the solution, rinsed off with desalinated water and treated with a drop of a 2% solution of potassium hydroxide (AppliChem, Darmstadt, Germany) in desalinated water applied directly onto the skin cross sections in order to bloat the skin cells for better visualization of individual cell layers. After 20 s, a cover slip was gently pressed onto the slides, which were then immediately examined under the microscope. These slides were used to count the s.c. layers remaining after tape stripping.

2.7. Optical microscopy

In optical microscopy, the skin sections obtained with both staining methods were subjected to 10, 20 and 40-fold magnification (fluorescence microscope BX51, Olympus, Shinjuku City, Tokyo, Japan) and photographs were taken (SC50, Olympus).

The measurement of the s.c. thickness was carried out with the HE-

stained slides using CellSens standard 3.1 software (Olympus). In order to count the cell layers, the safranin-stained slides were used. Each measurement was carried out at least five times per analyzed skin sample. The measurements were performed at different positions and with different cross-sections of the same sample.

The data were analyzed using the statistical programming language R (version 4.2.1). Means and standard deviations were calculated based on all individual measurements.

2.8. Control sample (compression test)

To ensure that the measured decrease in s.c. thickness is not due to compression during tape stripping, the stripping procedure was mimicked with six skin sections. A tape strip was laid atop each skin section and the strip was pressed on with tweezers as described above. Pressing was repeated twenty times without removing the tape strip in between. Finally, the tape strips were removed in a single swift movement. The samples were then prepared for optical microscopy as described above. The individual thickness values of untreated s.c. and the control specimen were compared with the Wilcoxon rank-sum test. This test was used because the data of the compression test were not normally distributed according to a Shapiro–Wilk test ($p = 0.369 > 0.05$). The hypothesis of the Wilcoxon rank-sum test was that the two values (total thickness of s.c. vs. thickness of s.c. after compression test) are not equal, the null hypothesis assumed that the two values cannot be distinguished.

3. Results

The amount of s.c. removed after every second tape strip was determined on cryo-sections of individual skin samples in two ways. First, the thickness of the remaining s.c. on the porcine skin specimen was measured by optical microscopy after HE-staining. Second, the number of remaining s.c. layers was counted after staining with safranin and bloating the cells with a potassium hydroxide solution to better distinguish individual cell layers during microscopy.

3.1. Stratum corneum thickness

For the thickness of intact s.c. before tape stripping, we determined a value of $\delta_{sc} = 11.0 \pm 2.0$ µm. After 20 tape strips, δ_{sc} was reduced to 3.23 ± 0.76 µm (Fig. 1 and Table 1). The values for the thickness of full s.c. are in good agreement with previous studies. For example, a thickness of 11.1 to 17.4 µm was measured for healthy human s.c. [4,21], whereas a value of 10.8 ± 2.3 µm was reported for porcine s.c. [4].

The control skin sample – mimicking the compression following 20 tape strips – had a thickness of 10.6 ± 1.5 µm, which is not statistically different from δ_{sc} when no tape stripping was applied (p-value = 0.304 > 0.05). Furthermore, since the tape strip used to mimic the compression had to be removed before microscopy, the skin samples had been stripped once, explaining the slightly lower value.

The s.c. thickness, δ_{sc}, can be correlated with the number of tape strips applied, n_{TS}, with a linear function (Fig. 3a, $r^2 = 0.975$) described by:

$$\delta_{sc} = -(0.38 \pm 0.02)\ \mu m \cdot n_{TS} + (10.83 \pm 0.23)\ \mu m. \quad (1)$$

Equation (1) can be interpreted as each tape strip reducing the s.c. thickness on the skin specimen by 0.38 ± 0.02 µm on average.

3.2. Stratum corneum layers

Intact porcine s.c. before tape stripping consisted of 27.06 ± 3.21 cellular layers, n_{sc}, on average. This number was reduced to 7.72 ± 2.59 layers after 20 tape strips (Fig. 2 and Table 1). A previous report found 21 ± 5 cell layers for full thickness s.c. of porcine ear skin [30]. In that study, an average of 13 ± 3 cell layers were removed by stripping the skin 20 times, compared to about 20 cell layers removed here.

A correlation of n_{sc} and n_{TS} was also derived. The resulting linear function (Fig. 3b, $r^2 = 0.968$) is described by:

$$n_{sc} = -(0.94 \pm 0.05) \cdot n_{TS} + (26.99 \pm 0.63). \quad (2)$$

Equation (2) can be interpreted as 0.94 ± 0.05 cell layers of the s.c. being removed with each tape strip on average.

As described above, we found that not all of the s.c. layers were removed after 20 tape strips. Equations (1) and (2), however, allow estimating from their respective x-axis intercepts how many tape strips would have been required to achieve this. The resulting values of 28.50 and 27.71 tape strips from Equations (1) and (2), respectively, are in excellent agreement with each other (deviation < 3 %). This validates both equations as they support the same conclusion although being derived from different data sets obtained with different and independent methods (measurement of thickness vs. bloating and counting).

When the two data sets (δ_{sc} and n_{sc}) were directly correlated, we found a linear relationship (Fig. 3c, $r^2 = 0.977$) described by:

$$\delta_{sc} = (0.40 \pm 0.02)\ \mu m \cdot n_{sc} + (0.05 \pm 0.36)\ \mu m. \quad (3)$$

Importantly, the slope of Equation (3) can be interpreted as the thickness of a single s.c. layer. Thus, each cell layer adds on average 0.40 ± 0.02 µm to the thickness of the s.c. The function almost perfectly intercepts the origin, further validating the equation as with no remaining

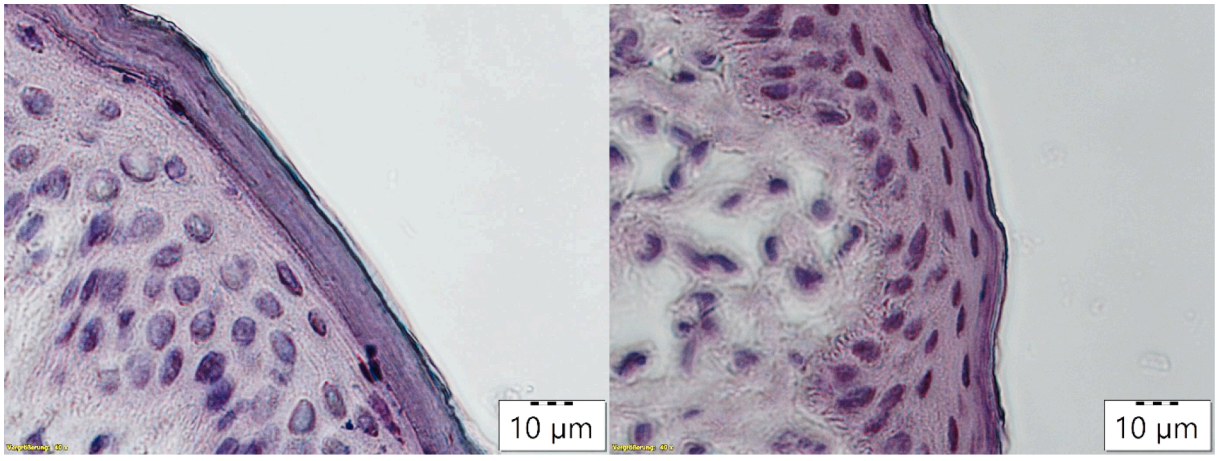

Fig. 1. Microscopy image of the upper most skin layers after hematoxylin-eosin (HE) stain at 40-fold magnification. Left: before tape stripping; right: after 20 tape strips taken off.

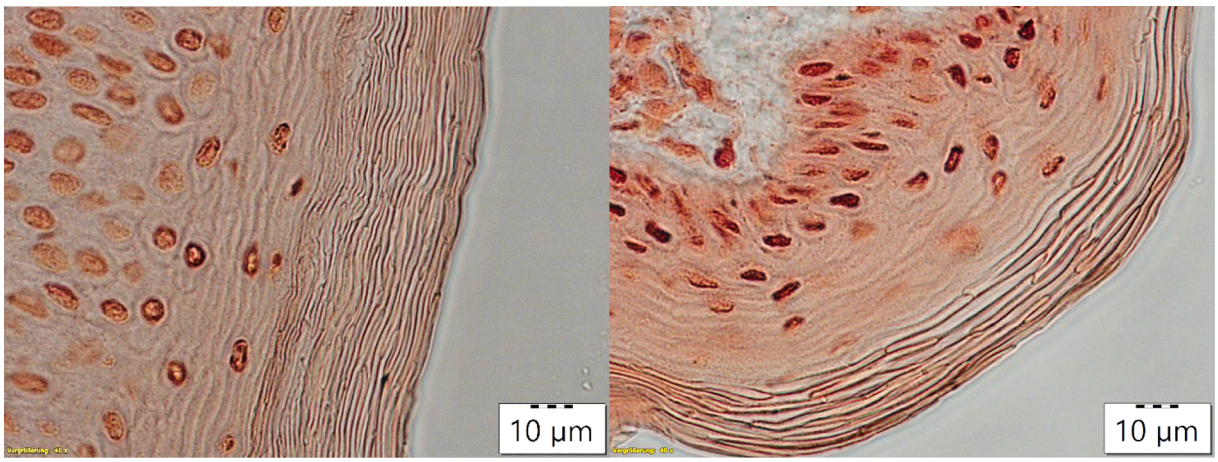

Fig. 2. Microscopy image of the upper most skin layers after safranin stain and bloating with 2% KOH_{aq} at 40-fold magnification. Left: before tape stripping; right: after 20 tape strips taken off.

Table 1
Thickness and number of layers of the *stratum corneum* (s.c.) after a given number of tape strips.

tape strips	thickness s.c. [µm]	layers s.c.
0	11.03 ± 1.99	27.06 ± 3.21
2	9.91 ± 1.90	23.09 ± 2.35
4	8.58 ± 1.35	22.57 ± 2.36
6	8.88 ± 1.64	23.20 ± 1.61
8	7.90 ± 1.02	20.54 ± 3.19
10	7.20 ± 1.49	17.57 ± 4.56
12	6.93 ± 1.48	16.40 ± 4.26
14	5.45 ± 1.48	14.46 ± 4.07
16	4.23 ± 0.98	11.14 ± 3.76
18	3.96 ± 1.15	9.33 ± 3.05
20	3.23 ± 0.76	7.72 ± 2.59
compression test: 1	10.61 ± 1.48	23.37 ± 3.00

cell layers the s.c. thickness should be zero.

4. Discussion

In previous studies, the number of applied tape strips is usually correlated with the amount of s.c. that is removed, be it by determining the mass, the cumulative *pseudo*-absorption or other measurable quantities associated with individual or pooled tape strips. These values are then converted into the depth attained within the s.c. In this study, however, the remaining s.c. layers and the s.c. thickness were directly measured. From our data and the corresponding linear regression (Equation (3)), we found that a cellular layer of porcine s.c. is on average 0.40 ± 0.02 µm thick. This is in approximate agreement with results obtained from human skin by scanning laser microscopy imaging that revealed a thickness of 0.5 µm [7]. The similarity of these results (within 20% of each other) derived by two different techniques illustrates that in terms of thickness of the s.c. layers, porcine skin is a suitable surrogate for human skin.

We report a linear correlation between the number of tape strips applied and the thickness of the remaining s.c. on the skin specimen

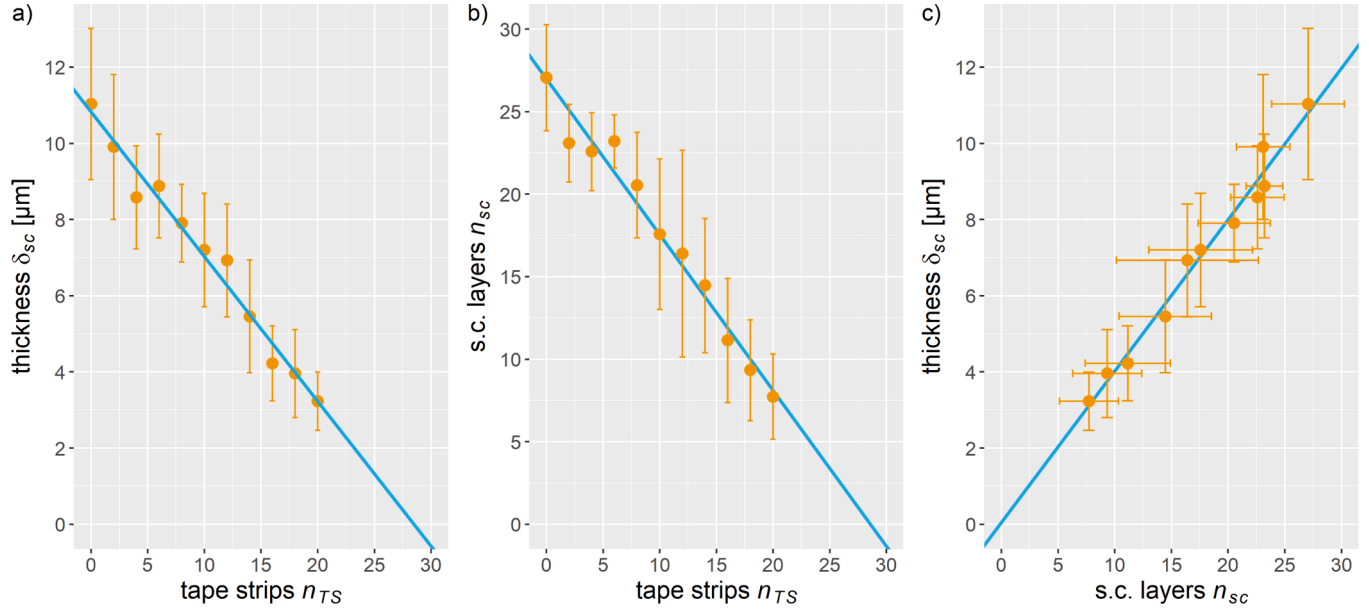

Fig. 3. Graphical representation of tape stripping data: a) thickness of the *stratum corneum* (s.c.), δ_{sc}, vs. the number of tape strips taken off, n_{TS}; b) number of s.c. layers, n_{sc}, vs. n_{TS}; c) δ_{sc} vs. n_{sc}. Individual values are reported in Table 1.

(Equation (1) and Fig. 3a), starting with the first tape strip. The data correspond to 0.38 ± 0.02 μm being removed by each tape strip, which is significantly higher than a value reported previously (0.22 ± 0.02 μm) [9]. However, the latter value was derived from the protein content on the removed tape strips. As discussed below, this might lead to an underestimation of the reduction in thickness of the s.c. by tape stripping.

Of note, our data indicate that the *stratum disjunctum* (s.d.), which represents the outer most layer of the epidermis, is removed efficiently. However, in dermal absorption studies, substances that are applied onto the skin can accumulate in the folds and furrows of the s.d. Furthermore, the s.d. is subject to constant desquamation so that substances in this layer are less likely to become bioavailable. For these reasons, the guidance of the European Food Safety Authority (EFSA) on dermal absorption recommends to exclude the first tape strip from analysis [31].

Apart from the thickness of the s.c., we also quantified the number of remaining s.c. layers after tape stripping. By linear regression of our data (Equation (2) and Fig. 3b), we found that each tape strip removed on average 0.94 ± 0.05 cellular layers, which is about one layer per tape strip. The data obtained from individual tape strips indicate that this value remained constant over at least 20 tape strips. Thus, outer s.c. layers, including those of the s.d., were removed with the same efficiency as deeper lying ones. This is in line with studies that have measured the cumulative amount of s.c. removed. For example, it was reported that the cumulative mass of s.c. removed increased linearly with the number of tape strips as determined by a colorimetric protein assay [15]. Another study concluded similarly when using a gravimetric approach to determine the number of removed s.c. layers [32].

On the other hand, variable results were obtained in a different study, with linear dependencies in some cases and plateauing observed in others [14]. It was hypothesized that for samples where the penetration depth into the s.c. plateaus, the s.c. is composed of fewer layers at the point probed. Two related reports also indicate a decrease of the amount of s.c. cells adhering to each tape strip with deeper cell layers [12,17]. Yet, in one case, data were provided for one replicate only [17]. In the other study, the deviation from linearity only starts after about 30 tape strips [12], which, according to our extrapolations (Fig. 3), would already result in the complete removal of the s.c. It is possible, however, that the observed differences are due to the different origin (human vs. porcine) and location (forearm vs. flank) of the skin samples. In a regression averaging data from 240 samples [9], the amount of s.c. removed was quantified by measuring the protein content and the weight of s.c.-related material on the tape strips. Deviations from a linear course were explained by the stronger cohesion between cells in deeper s.c. layers. However, the observed trend could also originate from a different effect.

Apart from water, proteins are the largest contributor to the mass of human s.c. (25–55% mass fraction) [33], whereas the lipid content is found to be between 2% and 10% [33–35]. The density of proteins is usually higher than 1.4 g·cm^{-3} [36]. This is also reflected in the density of dried s.c. which ranges from 1.4 ± 0.18 g·cm^{-3} [37] to 1.54 ± 0.30 g·cm^{-3} [38]. However, the density of the s.c. is frequently assumed to be about 1 g·cm^{-3} [4,9], because the s.c. is considered to be an aqueous matrix. This is despite *in vivo* confocal Raman spectroscopy of infant s.c. revealed that the protein content in the layers of the s.c. decreases from the surface of the s.c. to the viable epidermis/s.c. boundary layer [33]. Thus, it is questionable to consider the s.c. as a homogeneous aqueous matrix, given that the protein content is a major contributor to its mass. These results point to a higher density of s.c. layers lying closer to the surface of the skin. This is corroborated by observations that higher protein content correlates with higher area-specific mass of the s.c. [9,39]. In summary, these results indicate that a decreasing protein amount and, hence, a decreasing mass of the removed s.c. material on the tape strips do not necessarily correspond to less s.c. layers being stripped. In fact, this may be explained by the lower protein content and mass of layers located deeper within the s.c.

For gravimetric approaches, the evaporation of water off the tape strips between stripping and weighing could be a second contributing factor diminishing the expected mass of the removed s.c. Tape strips of deeper s.c. layers with higher water content will lose more mass due to this effect if evaporation is exhaustive.

Scanning laser microscopy measurements of the s.c. have also shown that the s.c. is relatively homogeneous regarding the thickness of individual cell layers. The average thickness of an s.c. aggregate on a tape strip was found to be independent of the depth in the s.c. [7]. Cryo-transmission electron microscopy (cryo-TEM) – a technique allowing imaging closer to the native condition than conventional TEM – showed that the thickness of the s.c. varies very little and only increases near the *stratum granulosum* [40–41]. We hypothesize that the often-cited increased cohesion in deeper layers is only relevant for those s.c. layers in direct contact with the *stratum granulosum*, and that the decreasing amount of s.c. removed per tape strip reported in many studies is at least partially due to changes in water and protein content of individual s.c. layers.

5. Conclusion

Using the well-established HE-stain, it was possible to directly measure the thickness of the remaining s.c. on excised porcine skin by optical microscopy, instead of quantifying the amount of removed s.c. on the tape strips. Thereby, potential inaccuracies associated with indirect detection methods that may, for example, result from the change in density of the s.c. as a function of depth, are circumvented. The same strategy has been used for the quantification of the remaining cell layers using a well-known histological method that involves bloating of the individual cells, thus making them better visible in light microscopy.

With this *in vitro* approach, we have conclusively shown that in excised pigskin the s.c. is indeed removed linearly, at least up to 20 tape strips. Each tape strip removes approximately 0.4 μm of s.c. which corresponds to about one cell layer on average. We have demonstrated that these results are in good agreement with existing literature on human skin, including *in vivo* studies [7,40–41], highlighting the relevance of our approach.

We envisage that future studies can benefit from the information presented in this work. The histological determination of the s.c. thickness and quantity of cell layers of scraps remaining after punching of skin samples is a quick, easy and reliable method that is practicable in many scientific and medical laboratories. With the removal rates reported here, researchers performing *in vitro* skin penetration studies could derive quick estimates of how deep in the s.c. a given concentration of analyte is found by counting the number of tape strips retrieved.

Author contributions

KS developed the project. KS and AR wrote the manuscript with main contributions from KS. KS and GO carried out the experimental work. KS, GO and AR analyzed the data. All authors contributed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.04.022.

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