Measuring the effects of topical moisturizers on changes in stratum corneum thickness, water gradients and hydration *in vivo*

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Accepted for publication
23 March 2008

Key words
confocal Raman spectroscopy, corneometer, optical coherence tomography, niacinamide, stratum corneum, water content and thickness

Conflicts of interest
J.M.C., A.S., P.B., C.M., J.K. and P.J.M. are employees of Procter and Gamble who funded this study and is the manufacturer of formulation A. A.V.R. is a consultant to Procter and Gamble.

DOI 10.1111/j.1365-2133.2008.08703.x

Summary

Background Moisturizers are the most commonly used topically applied product for the treatment of dry skin conditions. They affect many properties and functions of the stratum corneum but some moisturizers have been reported to be detrimental to barrier function. Stratum corneum barrier function is a composite of its total structure and thickness but few studies have taken this into account. As a biosensor, the stratum corneum (SC) will change its structure in response to treatment and a swelling effect has been clearly demonstrated by skin hydration. Recently several moisturizing agents have been shown to have an effect on SC swelling behaviour with conflicting results. However, there is a paucity of data reported for measuring the effects of long-term usage of moisturizers on SC thickness *in vivo* as, until recently, traditional techniques did not have the resolution to measure the effects of moisturizers on nonpalmoplantar body sites. The development of confocal Raman spectroscopy for use in human subjects provides noninvasive, real-time, *in vivo* measurement of SC water concentration profiles and we have also used this state of the art equipment to measure the effect of the long-term use of moisturizers on SC thickness for the first time.
Objectives To validate the use of confocal Raman spectroscopy (CRS) to measure SC thickness and then use it to investigate the short- and long-term effects of moisturizers (one of which is known to improve SC barrier function) on SC thickness, water gradients and hydration.
Methods Two studies were conducted: (i) to validate the use of CRS for measuring SC thickness through comparison with optical coherence tomography (OCT); and (ii) once validated to use CRS to measure the long-term effects of three commercially available moisturizers (A, B, C) on SC thickness and water gradients, together with total hydration, over a 3-week period (2 weeks of treatment and 1 week regression) and compare the spectroscopy-derived hydration value with instrumentally derived capacitance hydration values.
Results (i) A strong, positive correlation in SC thickness was obtained between CRS and OCT (OCT-derived thickness = 0·96 × CRS-derived thickness, r^2 = 0·93; $P < 0·0001$). OCT was shown, however, to have a lower resolution than CRS in distinguishing SC thickness on thinner nonpalmoplantar body sites. Using the CRS method, differences in SC thickness were readily apparent on different body sites (cheek 12·8 ± 0·9 μm, volar forearm 18·0 ± 3·9 μm, leg 22·0 ± 6·9 μm). (ii) Examining the effects of moisturizers in a blinded, randomized 3-week study in human volunteers (n = 14) demonstrated that only one commercially available formulation (A) changed SC water gradients, thickness and hydration as measured by CRS. These hydration data did not directly correlate with capacitance hydration values.

Conclusions (i) *In vivo* CRS was validated as a technique to measure SC thickness on both palmoplantar and, particularly, on nonpalmoplantar skin sites. (ii) Moisturizers improve skin moisturization but in this study only formulation A improved SC thickness, water gradients and hydration as measured by CRS. We hypothesize that this was due to compositional differences between the products. We believe that niacinamide (nicotinamide, vitamin B_3) is probably contributing significantly to this effect, as it has been proven to increase epidermal lipogenesis and SC barrier function in other studies. These results show that by using CRS, we were able for the first time to determine the effect of moisturizer on multiple SC barrier endpoints including SC thickness, and water content as a function of depth and total SC water content.

Dry and scaly skin is the most common symptom of dermatological disorders.[1] Many factors are involved in its precipitation and a 'dry skin cycle' has been recently proposed for cosmetic dry skin conditions.[2] Epidermal hyperproliferation and defective differentiation are classic features of these conditions which lead to an immature stratum corneum (SC) with inferior barrier, hydration and desquamatory properties.

This condition is most commonly treated with moisturizers and measurement of SC hydration is usually measured using equipment that relies on changes in SC electrical properties[3] while changes in SC barrier function are measured typically by transepidermal water loss measurements (TEWL).[4] However, it has been reported that some moisturizers are more effective than others on both parameters.[5] The longer-term effects of moisturizers on SC barrier function have recently received attention, as some studies have reported that continued treatment of normal skin with certain moisturizers may increase susceptibility to irritants[6] and, especially, to nickel.[7] In this respect, Berardesca *et al.*[8] demonstrated that slightly alkaline moisturizers (pH 8·2) applied over 4 weeks can impair barrier function. More recently, Buraczewska *et al.*[9] demonstrated very effectively that the long-term effects of moisturizers on SC barrier function are determined by their composition and, that while some moisturizers can strengthen SC barrier function, others can actually weaken it. For example, certain emulsifiers have been shown to both augment and compromise SC barrier function.[10]

Glycerol is a 'classic' humectant used in a majority of commercial moisturizers and its efficacy in improving SC hydration and condition is well established,[11] although the utility of glycerol-based moisturizers in the treatment of dry skin resulting from atopic dermatitis seems limited.[12] Glycerol-driven efficacy may involve augmentation of SC barrier function, although the data are mixed. Appa *et al.*,[13] for example, demonstrated that glycerol-containing moisturizers continue to improve barrier function for at least a week after cessation of treatment and an acceleration of SC barrier recovery in damage models and dry skin conditions was confirmed by Fluhr *et al.*[14] and Gloor and Gehring.[15] Conversely, others have found no SC barrier efficacy with glycerol-based moisturizers.[16–19] In light of this mixed experience with humectant-only approaches, researchers have turned to other technologies to supplement SC-barrier activity. For example, urea has proved to have a useful barrier efficacy beyond glycerol.[5,18] Likewise, α-hydroxy acids have also been shown to improve barrier function,[8] alleviate dry skin and increase epidermal lipogenesis.[20] Most recently, glycerol and niacinamide-containing formulations have been shown to be more effective than other commercially available moisturizers in alleviating dry skin conditions and improving SC barrier function.[2,21] In this respect, niacinamide was shown to increase the levels of SC ceramides by influencing epidermal differentiation during the regeneration of new SC.[22]

Central to their efficacy, moisturizers are designed to improve SC hydration and the effects of this on SC ultrastructure have recently been studied. For example, Orth *et al.*[23,24] demonstrated that topical application of pure glycerol and petrolatum had no effect on the swelling of the SC whereas aqueous solutions of glycerol did. The differential swelling characteristics of the SC when exposed to water and salt solutions have also been demonstrated by other authors.[25–28] When in water or exposed to high humidity conditions, the SC swells and expands to a greater extent in the plane perpendicular to the skin surface rather than in a lateral dimension. This, naturally, will have an impact on the tortuosity of the SC and, as such, can certainly influence SC barrier function. Most recently, using cryoscanning electron microscopy, Caussin *et al.*[29] demonstrated in elegant, short-term *in vitro* studies that moisturizer ingredients can have differing effects on SC water content and thickness. Whereas, concentrated lipophilic moisturizers were found to increase SC water content, concentrated hydrophilic moisturizers could also reduce SC thickness. Furthermore, whereas a 5% glycerol solution was found to increase SC thickness by approximately 25%, Fluhr *et al.*[30] reported a shrinkage and reduction of the surface area of corneocytes using 9% glycerol. From this work, therefore, it can be seen that there is a need to determine the effects of moisturizers on SC thicknesses as a core component of their efficacy.

Current industry standard methodologies for determining moisturizer efficacy use a variety of noninvasive electrical (capacitance, conductance or impedance[31,32]) or spectroscopic (infrared spectroscopy[32]) measures to determine skin hydration. While these techniques are rapid, allow high throughput

in the context of a clinical study, and may correlate to some extent with visual dryness assessments,[32] they give no quantitative indication of the actual water distribution in the tissue or SC thickness. Recently, confocal Raman spectroscopy (CRS) was developed to obtain water concentration profiles real-time and *in vivo*.[33–37] Skin water profiles are obtained by combining the principle of confocal microscopy with Raman spectroscopy. Raman spectroscopy provides chemical analysis while confocal microscopy allows this information to be determined from a small, discrete volume within the SC. Thus, SC hydration is measured by 'optically sectioning' skin tissue and expressing the relative water content as a function of depth. Water concentration is calculated from the ratio of the water signal to the combined signal from water and protein within the skin. This method has also been used to estimate differences in SC thickness *in vivo* at different body sites and during ageing[38,39] and, recently, to evaluate the effects of water and moisturizing ingredients on SC hydration, but only after short-term treatment.[40,41] This method has never been used to evaluate the effects of moisturizers on SC thickness or swelling *in vivo*. There is a need, therefore, to study the long-term effects of moisturizers on SC water gradient and thickness using these techniques. There is also a need to validate the SC thickness measure obtained by analysis of CRS-derived water gradients using a separate, objective technique.

To understand the long-term use of moisturizers on SC water gradients, hydration and thickness, therefore, we used CRS to compare the use of one commercially available moisturizer (A; previously reported to alleviate dry skin and to improve SC barrier function over several weeks of application) with two others, known only to alleviate dry skin (B, C).[21] Total SC water content values obtained by CRS were compared with values obtained via capacitance measurement (Corneometer™). To facilitate this, we first had to validate the use of CRS to measure SC thickness and this was achieved via direct comparison with optical coherence tomography (OCT) at a variety of body sites.

Methods

Stratum corneum hydration and thickness measurements: confocal Raman microspectroscopy

In vivo CRS measurements were carried out using a RiverDiagnostics 3510 Skin Analyzer (RiverDiagnostics, Rotterdam, the Netherlands). This is a state-of-the-art inverted confocal Raman microspectrometer equipped with two fibre-coupled diode pumped lasers operating at two wavelengths: at 671 nm to obtain measurements in the high wave number region (2500–4000 cm^{-1}), and at 785 nm to collect fingerprint spectra (400–2000 cm^{-1}).[33–36] The laser light (a 1-μm spot of ≤ 20 mW power) is focused at the skin via an oil-immersion microscope objective (NA 0·86, focal length 5·8 mm, working distance 1·5 mm) at a well-defined depth, controlled by a high-precision piezoelectric focusing drive. Light scattered by the tissue is collected through the same objective, and the Raman scattered light is focused onto an optical fibre (25 μm diameter), acting as the confocal pinhole and rejecting signals from out-of-focus regions in the skin. An air-cooled, high-sensitivity back-illuminated, deep-depletion CCD camera (1024 × 128 pixels) detects the Raman signal at a 5 cm^{-1} spectral resolution out to 1000 nm. The axial resolution of the instrument is ~4 μm. Measurement capture time was 1 s per point within the scan (total time per scan 21 s).

Skin water content measurements were obtained from the 671 nm laser. Scans of 2-μm steps over a total depth of 40 μm into the tissue were collected. After each measurement, the laser was positioned on a different skin area and the measurement repeated. In total, eight scans were collected for each treatment site, at each time point. Skin hydration measured by CRS was determined by taking the ratio of the integrated signals of water, i.e. the O–H stretching vibration region between 3350 and 3550 cm^{-1} ([water]) to that of protein, i.e. the $-CH_3$ stretching vibration from 2910 to 2965 cm^{-1} ([protein]).[33–36] A correction factor as determined by Caspers *et al.*[35] was used to normalize the spectral response of water and protein relative to their mass ratio (the 'constant' that appears in the formula below). Percentage water within the SC at a given point is calculated using the following formula:

$$\text{Percentage water} = \text{constant} * [\text{water}]/([\text{water}] + [\text{protein}]).$$

Percentage water is defined, therefore, as being the mass of water per mass of wet tissue. Percentage water values were plotted as a function of depth to generate a water profile across the SC.

To determine an average water concentration for each location at each time point, obvious outliers (arising for example from scanning through heterogeneous structures, such as skin appendages including hair follicles, sebaceous glands, etc., or profiles recorded while the panellists were moving) were removed. Then, an average water profile was fitted through the remaining data, using a customized algorithm based on a four-parameter Weibull curve (written in Matlab; The Mathworks, Natick, MA, U.S.A.). The upper 'levelling-off-point' of each profile was determined by a gradient threshold method from the Weibull algorithm by calculating the location where the gradient reached a value of 0·5 moving from the midpoint of the curve (Fig. 1). This point was identified as the theoretical boundary of the SC (see CRS-OCT comparison section below) and served as the endpoint of the SC hydration profile. The area-under-the curve values (AUC) were determined by integrating each hydration profile from the skin surface (x = 0 μm on the profile) to each individual SC boundary (point c in Fig. 1), and used to express the total SC water content.

Stratum corneum thickness measurements: optical coherence tomography

OCT was conducted using a SkinDex 300 instrument (ISIS Optronics GmbH, Mannheim, Germany). This uses a 1300-nm laser (power < 20 mW) to produce a 1 mm wide by

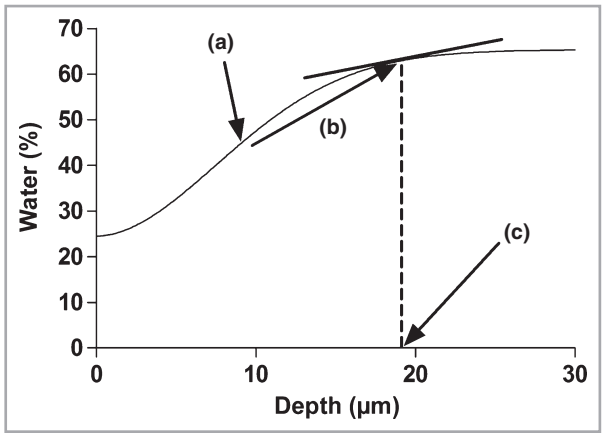

Fig 1. Calculation of stratum corneum (SC) thickness from the water profile. The algorithm calculates the point where the gradient equals 0.5, working from the middle of the curve inwards (i.e. deeper in the tissue; b). The depth at this point corresponds to the base of the SC (c).

0·9 mm deep cross-sectional image through the skin. Spatial resolution was 3 μm (lateral) × 5 μm (depth). Images were collected in three-dimensional enhanced mode and exported as bmp files. SC thickness was determined using a custom image analysis algorithm written in Optimas™ 6.5 (Media Cybernetics LP, Silver Spring, MD, U.S.A.). Three images were taken at each site (panellists were repositioned between each image) from which a mean SC thickness value was calculated after identifying apparent upper and lower SC margins.

Stratum corneum hydration measurements: corneometry

Capacitance measurements were taken using a CM820 Corneometer (Courage & Khazaka, Cologne, Germany) using the recommendations of Berardesca.[3] Readings were taken before the Raman hydration profiles to minimize any effect of having the skin in contact with the CRS window. An average three readings in close proximity were taken for each site per time point.

Human studies

All subjects came from the Procter and Gamble site in Egham, U.K. and the studies were approved by the Procter and Gamble Safety and Regulatory group. All studies complied with the Guidelines for Medical Experiments in nonpatient human volunteers published by the Association of the British Pharmaceutical Industry (ABPI) and the World Medical Association's Declaration of Helsinki (2000) concerning biomedical research involving human subjects. Each subject provided written informed consent and received a copy of the International Nomenclature of Cosmetic Ingredients (INCI) ingredient list for each product. For all studies, panellists were acclimatized for 15 min in a temperature-controlled room (21 ± 1 °C, humidity 50 ± 10%) prior to measurements.

Measurements of stratum corneum thickness

This study was conducted to attempt to validate CRS-derived SC thickness measures vs. those derived from OCT. Nine panellists (five men, four women, aged between 28 and 50 years, mean age 35 years) participated in this study. To obtain a range of SC thickness, measurements were taken from several anatomical sites – the cheek, volar forearm, outer and lower leg and palm. Each site was wiped with a dry optical tissue (Edmund Optics, Barrington, NJ, U.S.A.) to remove superficial sebum and any other surface contamination before Raman measurements were taken. SC thickness values were determined as described above. OCT measurements were performed last, a drop of clear, water-based gel being used to couple the OCT instrument to the skin. Three OCT images were taken at each location and mean SC thickness values were calculated for each site using a custom written image analysis package and manually selecting features which corresponded to the top and bottom of the SC (as described above).

Measurements of the effect of moisturizers on stratum corneum hydration and thickness

Fourteen panellists with normal volar forearm skin participated in this study (seven men, seven women, aged between 23 and 55 years, mean age 32 years). All subjects underwent a 1-week washout phase, abstaining from moisturizer usage. Three cosmetic moisturizers (A, B, C), one of which contained niacinamide (A), and one nontreatment control site (U) were used in this study. Core formula ingredients are as follows for each product (taken in order from the respective INCI lists): moisturizer A – water, glycerol, niacinamide, petrolatum, isopropyl isosterate (IPIS), stearic acid, polyethylene glycol (PEG)-100 stearate; moisturizer B – water, white petrolatum, glycerol, stearic acid; moisturizer C – water, glycerol, petrolatum, stearic acid.

The participants were instructed not to use any other moisturizers over the course of the study, and to refrain from caffeinated drinks for at least 3 h before any measurement. This was to exclude possible changes in skin water levels due to the pharmacological effect of caffeine.[42]

After baseline readings, 50 μL of product was applied to 25 cm^2 sites to give an application of 2 μL cm^{-2} on the volar forearms of the panellists, according to a predetermined randomization. Panellists returned for measurement 24 h later, using the same procedure for measurement. From this point on, panellists applied the treatments themselves twice daily at home for the following 2 weeks. On the measurement days, panellists refrained from applying product in the morning. Thus, minimal contamination of skin spectra with product was ensured. After 2 weeks, the treatment was stopped and a final assessment of skin water content made after a 1-week regression period (panellists refrained from using any other moisturizing products on their forearms during this time).

Statistical analysis

The treatment effects on Raman-derived SC thickness and total hydration (AUC) and Corneometer hydration were compared using a two-way ANOVA procedure, using treatment, site and subject as the main effects with the baseline readings as covariate. Errors were plotted as the least significant difference (LSD) at the 95% confidence level.

Results

Skin Raman spectra

Typical skin spectra for the high wave number region, collected at a single scan at an exposure time of 1 s is given in Figure 2. The high wave number spectrum shows characteristic O–H and $-CH_3$ stretching vibrations at 3390 cm^{-1} and 2935 cm^{-1}, respectively. Scans in this region are used to calculate the percentage water within the SC as reported here.

Confocal Raman spectroscopy–optical coherence tomography comparison to determine the stratum corneum boundary

The location of the SC boundary as a function of the CRS water concentration profiles was confirmed by comparing SC thicknesses from a number of different body sites obtained directly by OCT and CRS (Fig. 3a,b). Linear regression through the data shows a strong positive correlation between SC thickness derived from CRS and OCT (OCT thickness = 0·9603 × CRS thickness, $r^2 = 0{\cdot}9339$; $P < 0{\cdot}0001$). Expanding the area to the lower left of Figure 3a, corresponding to the thinner skin sites of the body (volar forearm, cheek and outside of lower leg) show how the dynamic range is compressed for the OCT method in this region (Fig. 3b). It can be seen that all of the OCT-derived SC thicknesses are between 9 and 15 μm while the CRS-derived thicknesses vary between 12 and 30 μm. For panellists who had cheek, forearm and leg measures, CRS ranked the sites, in terms of SC thickness, as follows: cheek < forearm < leg (cheek 12·8 ± 0·9 μm, volar forearm 18·0 ± 3·9 μm, leg 22·0 ± 6·9 μm), whereas OCT gave very similar readings for these three different locations (cheek 11·1 ± 1·8 μm, volar forearm 10·4 ± 0·9 μm, leg 13·7 ± 1·4 μm).

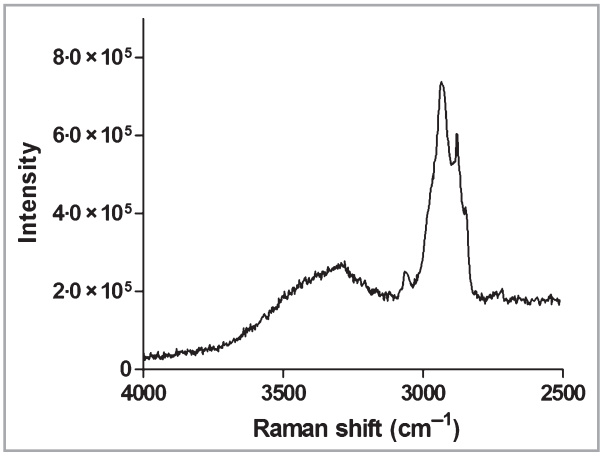

Fig 2. Typical background-corrected Raman spectra from a single scan of human skin *in vivo*, collected at ~4 μm depth in the high wave number region (671 nm laser excitation, exposure 1 s).

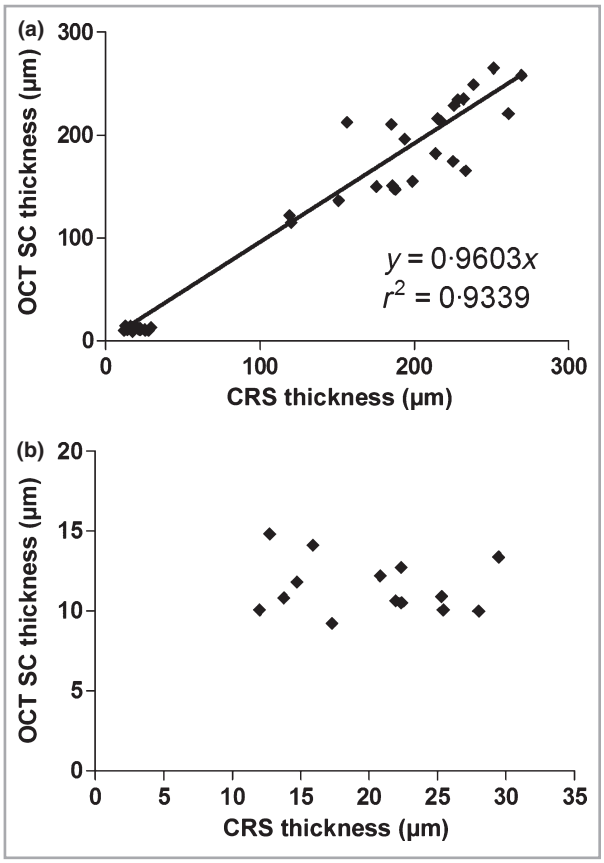

Fig 3. (a) Comparison of optical coherence tomography (OCT)- and confocal Raman spectroscopy (CRS)-derived stratum corneum (SC) thicknesses at a variety of body sites. (b) Comparison of OCT- and CRS-derived SC thicknesses on volar forearm, cheek and outside of lower leg (complete dataset).

Skin hydration using different cosmetic treatments

Application of moisturizers on the skin was expected to increase the water content of the SC and/or to change the shape of the water profile. For illustration, average water profiles from each treatment are given in Figure 4. All hydration profiles start at 20–30% water content at 0 μm depth (the SC surface) and rise in a sigmoidal curve to 60–70% water, where they plateau. While all water profiles at baseline and 1 day treatment show the same shape, differences in shape start to appear after 1 week of treatment. After 2 weeks, notable differences are observed, e.g. treatment A induced a laterally 'stretched' profile at 2 weeks' treatment which is still evident

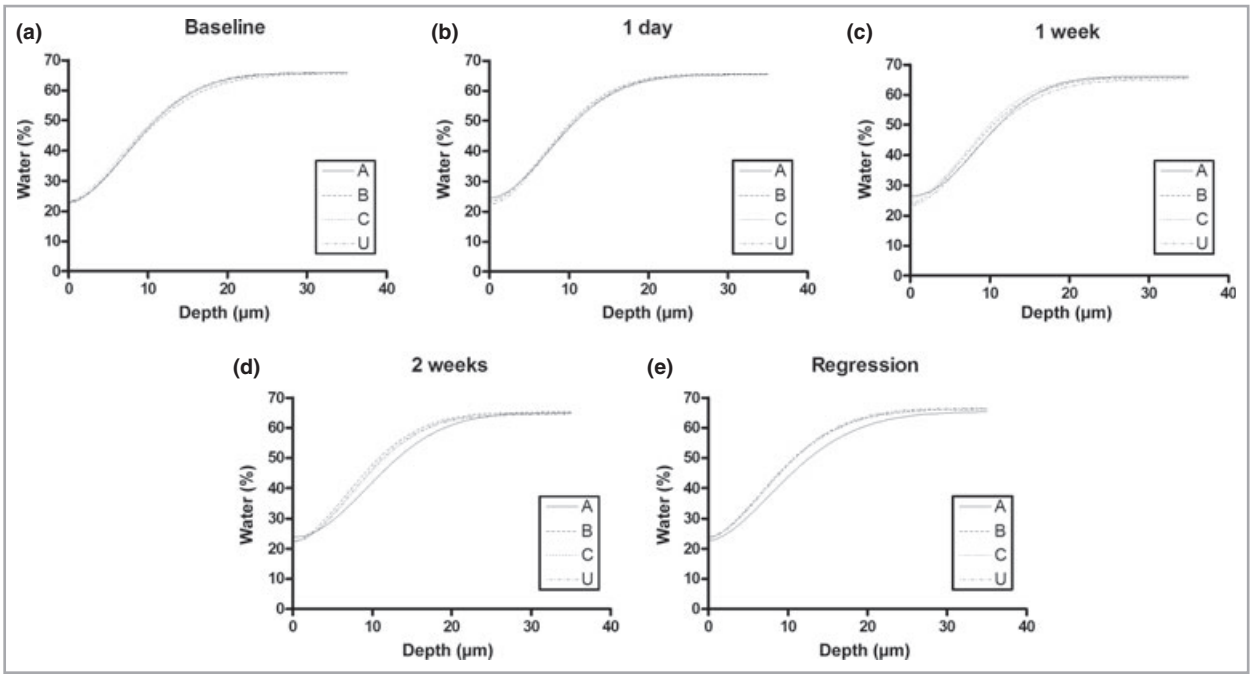

Fig 4. Average water profiles over the course of the study. A, B, C, formulations A, B and C; U, nontreatment control.

after regression. As the zero depth on the graph corresponds to the SC surface this different shape appears to indicate, therefore, an increase in SC thickness induced by product A.

After 2 weeks' treatment, the increase in SC thickness induced by product A was significantly different from the other two products and the untreated control site ($P = 0{\cdot}0121$), and this difference remained at the 1-week regression time point ($P = 0{\cdot}0162$). Data corrected for within-subject untreated control have also been presented to remove day to day variation caused by changes in the environment (Fig. 5a,b). This shows that the effect seen for product A after 2 weeks of usage remains even after the 1-week regression phase.

Total water content of the SC can be calculated from the AUC (integration between $x = 0$ μm and the calculated SC levelling-off point). Figure 6a,b illustrates that, concomitant with the increase in SC thickness, total skin water content increases significantly with treatment A after 2 weeks of product usage and the 1-week regression ($P = 0{\cdot}0275$ and $P = 0{\cdot}0435$, respectively). When corrected for the untreated control data, total SC water content remains significantly elevated at the 1-week regression time point.

Changes from baseline for Corneometer readings taken during the study are given in Figure 7a,b. It should be noted here that, as with the CRS measurements, Corneometer readings were taken at least 12 h after product application.

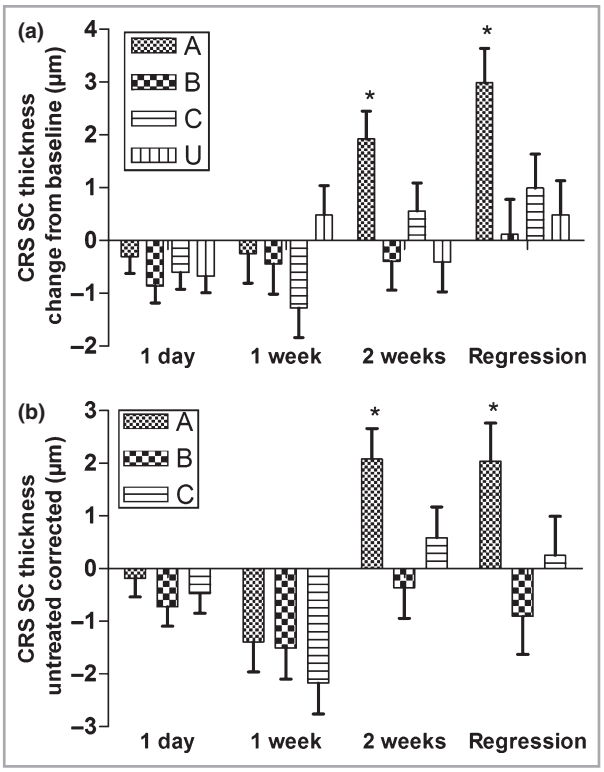

Fig 5. (a) Change in confocal Raman spectroscopy (CRS)-derived stratum corneum (SC) thickness during the moisturization study (means and SEM). (b) Change in CRS-derived SC thickness during the moisturization study corrected to the untreated control values (means and SEM). *Significant difference from the other treatment regimes, $P < 0.05$. A, B, C, formulations A, B and C; U, nontreatment control.

Discussion

All moisturizers alleviate dry skin when formulated appropriately. However, in recent years it has become apparent that

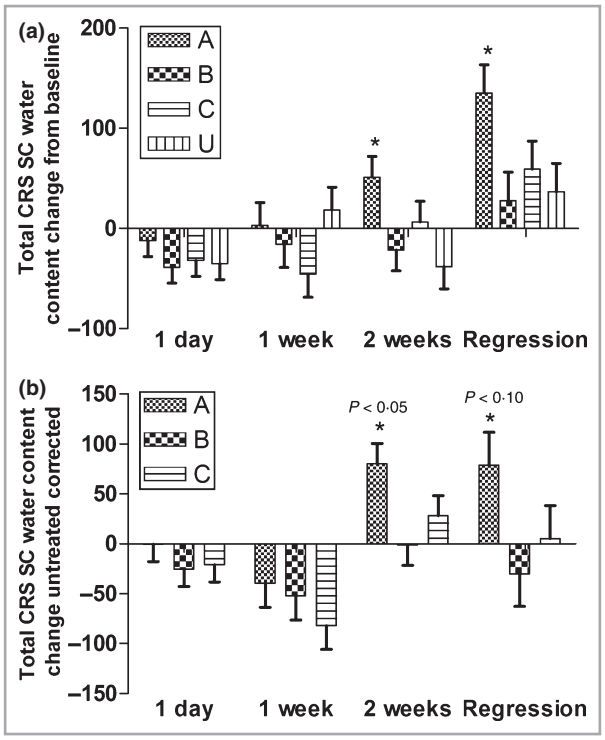

Fig 6. (a) Change in total confocal Raman spectroscopy (CRS)-derived stratum corneum (SC) water levels during the moisturization study (means and SEM). (b) Change in total CRS-derived SC water levels during the moisturization study corrected to the untreated control values (means and SEM). *Significant difference from the other treatment regimes, $P < 0.05$. A, B, C, formulations A, B and C; U, nontreatment control.

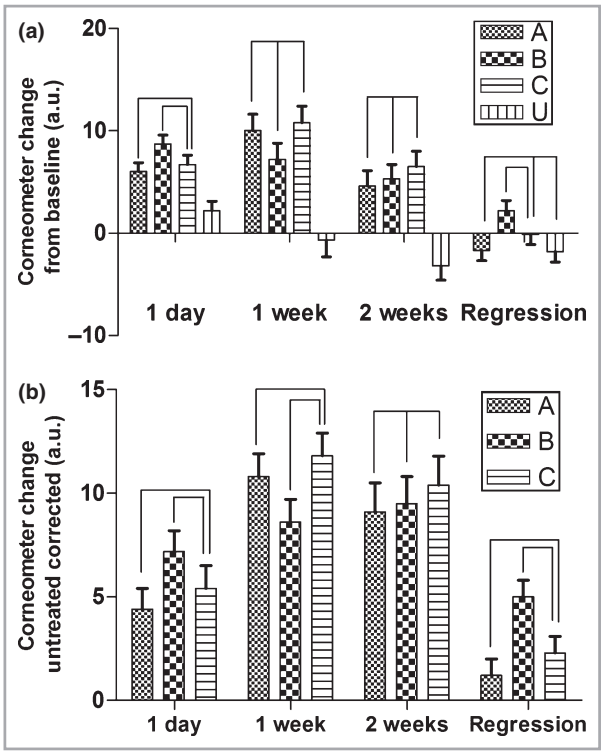

Fig 7. (a) Change in Corneometer values during the moisturization study (means and SEM). (b) Change in Corneometer values during the moisturization study corrected to the untreated control values (means and SEM). Homogeneous statistical groupings are given ($P < 0.05$). A, B, C, formulations A, B and C; U, nontreatment control; a.u., arbitrary unit.

different moisturizers can have different effects on the SC and the epidermis (for review see Loden[5]). Naturally, in the short term, moisturizers will increase SC hydration[5,12,21,37,40,41] and in the medium term improve desquamation,[11,43] but in the longer term, it appears some can actually compromise SC barrier function[6–10,16] whereas others can strengthen it.[12,14,18,20,21] *In vitro*[25–29] and *in vivo*[23,24] studies also demonstrated that moisturizing ingredients can influence SC thickness. As a result, it is becoming increasingly apparent that not only is there a need for longer-term studies to evaluate the effect of moisturizers, but that measurements more sophisticated than 'traditional' electrical parameters are needed to understand their effects more completely.[9] To examine the effects of moisturizers on SC thickness, water gradients and total SC hydration we used CRS to compare the effects of a formulation (A) which is known to improve SC barrier function and desquamation better than two other commercially available moisturizers (formulations B and C).[21]

We first needed to validate the use of CRS for measuring SC thickness as the work of Egawa *et al.*[38,39] was not published when this current study was commenced. Moreover, these authors offer no formal validation data, establishing a direct relationship between their CRS-derived SC thickness data and values derived from other measurements. It should be noted that we have also used a different (and, we believe, a more rigorous) approach in calculating SC thickness from the water profile measurements. In this present work, we took sets of up to eight profiles from each measurement site and analysed these together, rather than studying each water profile in isolation. This is because Raman water profiles are a point measure, given the laser spot size of approximately 1 μm. As the skin is far from homogeneous, it is relatively easy to include in the measure a feature which is not representative of the SC, e.g. a hair follicle, sebaceous gland or sweat duct. We applied a mathematical model to the cloud of data points representing each site, which we were able to do by combining the data from multiple measurements. Using this method, a strong positive correlation was observed between SC thickness derived from CRS and OCT. However, CRS was shown to be significantly more sensitive than OCT in the measurement of thinner SC sites (approximately 15 μm and under). This is important, as SC thickness values of < 15 μm are common across much of the body. The limitations of OCT measurement of thinner skin sites has also been noted recently using *in vivo* laser scanning fluorescence microscopy.[44,45] We believe, therefore, that the results of this present study demonstrate that CRS provides a new rapid, accurate and sensitive means of measuring SC thickness *in vivo*.

On the first day after starting product application in the moisturizer treatment study there is little difference in CRS-derived water profiles between any of the treatments or the untreated control site. During the first week of treatment there was a numerical diminution in SC thickness. Although this was not statistically significant it could have been be due to the osmotic effects of glycerol (which was present in all three formulations). When examining the effects of moisturizers on SC hydration and swelling, Caussin *et al.*[29] reported that changes in SC swelling can occur. Lipophilic moisturizers increased SC thickness whereas hydrophilic moisturizers tended to reduce SC thickness. This apparent SC thinning may be due to osmotic effects of the moisturizing ingredients (used at high concentration); the work of Fluhr *et al.*[30] describing the effect of glycerol on reducing corneocyte surface area concurs. However, some inconsistencies still occur where *in vitro*[29] and *in vivo*[23,24] increased swelling was reported for solutions of glycerol. It is also possible that, within this first week of treatment, there was some activation of SC protease activity (simply by elevated water activity), resulting in more efficient desquamation and an ensuing reduction in SC thickness. This effect prompts further investigation.

In the second week of the study, formulation A induced a statistically significant increase in SC thickness (2 μm mean increase, corresponding to an approximate 10% increase in thickness). This could mean that a cell layer initially located at a depth of, for example, 10 μm in the baseline profile is now found at a different depth in the tissue. It should be noted, therefore, that water content measurements at absolute depths are not comparable between time points in a study where SC thickness may change or vary. It is more meaningful to extract information regarding the total thickness of the SC and express water measurement derivatives as a function of this (e.g. our use of total SC water content). Considering SC thickness first of all, it can be seen that, after 2 weeks of treatment, product A produced a significantly greater increase in this measurement than the other two treatments and the untreated site ($P = 0{\cdot}0121$), and this difference remained at the 1-week regression time point ($P = 0{\cdot}0162$). Of note, increases in SC thickness have also been reported by Jacobson *et al.*[46] using a lipophilic niacin derivative.

Concomitant with this increase in SC thickness, total skin water content as measured by CRS increased significantly with treatment A after 2 weeks of product usage and remained elevated at the 1-week regression time point. No such effect was observed for treatments B and C. These data did not, however, correspond with Corneometer measurements taken at the same time points. Significantly increased Corneometer values were observed for all treatments and, indeed, values remained elevated throughout the 2-week treatment phase. Corneometer values also remained elevated for all treatments at the 1-week regression (although all values were significantly lower than those at the 2-week treatment time point – an effect observed in other regression studies.[17,18] Considering the ingredients present in all three formulations, the capacitance effects noted are probably due to the high dielectric constant of glycerol.[47,48] It appears from the CRS hydration profiles presented herein and their relative difference to corresponding Corneometer values, however, that capacitance does not reflect total SC water content. This raises the question as to where the capacitance signal is coming from within the skin and what moieties are driving changes in this measurement in the context of treatment with a moisturizer. These questions are the subject of ongoing research in our laboratories.

To rationalize the different effects of the products on CRS-derived SC thickness, water gradient and water content, it is necessary to consider the potential effect of the different ingredients in the formulations. Although glycerol was present in all three formulations, formulations B and C had no effect on SC total hydration and thickness after 2 weeks of treatment and during regression. We can, therefore, discount the effect of glycerol moderating epidermal differentiation (e.g. through its delivery into the keratinocyte by the aquaporin 3 transport system,[49,50] despite its likely penetration into the skin.[51]) IPIS and petrolatum are both present in formulation A. IPIS has been reported to increase SC thickness acutely *in vitro*.[29] Petrolatum in combination with glycerol has been shown to alleviate dry skin conditions[11] and was reported to accelerate barrier recovery after acetone damage to the skin. Ghadially *et al.*,[52] however, reported that it was restricted to the SC interstices while Mao-Qiang *et al.*[53] showed that inhibitors of lamellar body exocytosis and organellogenesis did not moderate its activity, pointing to a more probable surface effect (and not an influence on epidermal lipogenesis). Lastly, if SC swelling was a mechanism attributable to these ingredients in this study, it is an effect that would be expected within the first day of treatment. IPIS and petrolatum can, therefore, be discounted as the causative agents behind the SC thickening observed with formulation A.

TEWL is a well-established endpoint used to measure SC barrier function (and would be expected to decrease with a thicker SC with corresponding increased tortuosity). Emulsifiers are other possible candidates for the SC thickening noted but, as reported by Barany *et al.*[10] the emulsifiers used in each of these products (stearic acid, glycerylstearate and PEG-100 stearate) have no reported effect on SC barrier function as measured by TEWL. Glycerol has also been shown to improve barrier function in damaged skin models using a TEWL measure[14,15,21] but formulations B and C had no apparent effect on SC thickness in this present study. Overall, therefore, it seems unlikely that these cosmetic formulation ingredients are responsible for this effect. We believe that niacinamide (nicotinamide, vitamin B_3), present only in formulation A, is almost certainly the agent responsible for these SC effects.

In a vehicle-controlled study, niacinamide was also shown to single-variably and significantly improve SC barrier function in a sodium lauryl sulphate (SLS) challenge model.[54] In other studies[21] we have shown that, while all three moisturizers tested here alleviate visible dry skin condition and improve barrier function (as measured by TEWL), as the symptoms of dry skin are alleviated, moisturizer A was the most effective and, moreover, only moisturizer A improved SC barrier

function after SLS and tape stripping challenges after treatment for 3 or 4 weeks. Moreover, Tanno *et al.*[22] reported that niacinamide increased keratinocyte lipogenesis and, in a controlled 4-week study, demonstrated that it increased SC ceramide and fatty acid levels with an associated significant reduction in TEWL. This present study did not employ a vehicle control and, therefore, we cannot with confidence attribute the SC effects observed to niacinamide. Nevertheless, we do believe that, by improving keratinocyte differentiation and increasing ceramide synthesis and, thus, improving SC barrier function, that this ingredient is responsible for the improvements in SC hydration and thickness observed. Of note, the effects on SC thickness and hydration noted with formulation A were still present at the 1-week regression time point and, for future consideration, it will be interesting to establish the longevity of these effects and at what point baseline conditions are re-established.

In this study, relative changes in SC hydration, as measured by CRS, were not mirrored by Corneometer capacitance measurements. Others have noted similar discrepancies using other measurements and endpoints. For example Loden *et al.*[17,18] could not demonstrate any changes in capacitance in the treatment of atopic dermatitis with glycerol and urea creams, even though decreased skin scaling and improved skin barrier function were apparent. Equally, Breternitz *et al.*[12] reported that, while capacitance values could be increased by topically applied glycerol creams, there was no apparent effect on local severity scores, SC cohesion and integrity. Furthermore, Caussin *et al.*[29] compared the ability of moisturizing ingredients to swell the SC with the measures of hydration (by Corneometry and near-infrared spectroscopy) by Weichers and Barlow.[55] These workers reported that the spectroscopy measurements appeared to correlate with the increased SC swelling attributed to lipophilic moisturizers and speculated that these may form a surface isolating layer preventing effective measurement of hydration by the flat-faced capacitor employed by the Corneometer.

Although an older Corneometer model (CM 820) was employed in these studies, we have no reason to believe that this affects the interpretation of the data, as excellent correlations between the Corneometer CM820 and later CM825 models have been reported by Clarys *et al.*[56] and Fluhr *et al.*[57,58] Indeed, Fluhr *et al.*[58] compared both instruments and reported that 'the older (Corneometer) showed comparable measurement qualities (to the newer Corneometer)'. Although we believe that electrical measurements continue to have their place in the arsenal of noninvasive bioengineering methods available to us, we also believe that our data adds to the growing evidence of the limitations of these measures.[32] CRS, in contrast, offers a new, rapid, semiquantitative, depth-resolved measurement of SC water content that also allows an accurate estimate of SC thickness.

In conclusion, CRS has been used to compare the long-term effect of moisturizer usage on human skin *in vivo* for the first time. The approach presented here allows a previously unavailable quantitative simultaneous measurement of SC depth and hydration comparison of different treatments, and enables biological changes in SC barrier thickness in relation to product usage to be taken into account. This technique has been validated against a known measure of skin thickness (OCT). From the investigation presented here, it appears that the hydration state of the skin cannot adequately be described by a single technique but, rather, a number of complementary techniques are required for a complete description. It is clear, however, that CRS represents a powerful new class of measurement with significant advantage over electrical measures, allowing more sensitive measures and a step-change in information about SC water content. The advent of this new technology seems timely as we consider the development of moisturizers that truly augment SC barrier function, such as that described in this report.

Acknowledgment

The work was sponsored by Procter and Gamble Technical Centres Ltd.

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