Non-invasive Depth Profiling of Base Cosmetic Formulations in the Skin Using Handheld Confocal Raman Spectroscopy

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Abstract

Ceramide-based and aqueous-based moisturizers have distinct absorption and retention characteristics in skin. Conventional methods like Transepidermal Water Loss (TEWL) and Corneometry are limited by environmental factors, and no current in vivo method measures ceramide content in skin. This research explores the use of a handheld Confocal Raman Spectroscopy (CRS) system to non-invasively analyze the absorption kinetics of these moisturizers. Measurements were taken on 40 subjects, including healthy individuals (n = 20) and those with atopic dermatitis (n = 20). Results showed that ceramide-based creams retained ceramide in the stratum corneum for longer periods, particularly 24 hours post-application, compared to aqueous-based creams. Validation against Liquid Chromatography-Mass Spectrometry (LCMS) for ceramide NP in four healthy subjects showed a strong correlation (r = 0.96). These findings suggest that handheld CRS can improve the evaluation of skincare formulations, advancing the development of personalized skincare solutions.

Keywords: Noninvasive Measurements, Confocal Raman Spectroscopy, Permeation, Pharmacokinetics, Skincare.

1. Introduction

The skin, our body's largest organ, serves as a vital protective barrier against environmental aggressors, pathogens, and physical injuries. This intricate shield comprises of multiple layers, each designed to safeguard internal systems while maintaining overall health and homeostasis [1,2]. However, in the domain of clinical dermatology and cosmetic science, this protective

barrier presents a unique paradox. While essential for protection, it also poses a significant challenge for the effective delivery of therapeutic agents and active ingredients in topical treatments [3-5]. The development of therapeutic and cosmetic products often centers around the need to overcome or bypass this natural barrier, enabling the penetration of active ingredients to the targeted depths within the skin.

Ceramide-based and aqueous-based creams are fundamental components in dermatological and cosmetic formulations, each tailored to meet specific skincare needs. Ceramide-based creams are particularly effective in restoring the skin barrier and improving hydration by seamlessly integrating into the lipid matrix of the stratum corneum [6,7]. This integration supports the skin's natural defenses, promoting resilience and reducing susceptibility to environmental stressors and signs of aging. On the other hand, creams with an aqueous base are known for their lighter texture, which enables quicker absorption [8]. This property makes aqueous-based creams ideal for delivering active ingredients rapidly into the skin, enhancing their efficacy in treating the skin without the heaviness associated with more lipid-rich formulations. Research focusing on the optimal balance and penetration capabilities of these ingredients is essential, not only for improving product performance but also for ensuring consumer safety, making the study of these foundational elements a critical area of interest within both medical and cosmetic research communities.

Several established methods have been used in research and clinical settings to measure water and ceramide levels in the skin. For water measurements, in vivo methods such as Transepidermal Water Loss (TEWL) provide an indirect assessment of skin barrier function by measuring water evaporation. However, TEWL is sensitive to environmental factors and does not measure actual water content [9-11]. Corneometry, popular for its non-invasive assessment of skin hydration, measures the capacitance of the skin to infer hydration levels. This method is restricted to the superficial layers and influenced by external variables like skin surface condition [12-14]. For ceramide measurements, ex vivo methods such as Liquid Chromatography-Mass Spectrometry (LCMS) provide accurate quantification but require skin biopsies, which are invasive, and the use of sophisticated laboratory equipment, making them unsuitable for real-time or point-of-care applications [15-17]. Currently, in vivo methods for ceramide content measurement are not well-established. Hence, there is a need for a non-invasive, in vivo measurement technique that can accurately assess both water and ceramide content in the skin while providing layer-specific analysis to offer detailed insights into their absorption kinetics across various skin depths.

Confocal Raman Spectroscopy (CRS) emerges as a promising solution to evaluate ceramide and water content throughout all skin layers, addressing the limitations of traditional methods. This technique utilizes laser light to probe the molecular composition of the skin, enabling non-invasive, in-depth analysis of biochemical components such as ceramides and hydration levels [18-20]. By focusing the laser at different depths, CRS provides detailed profiles of the skin's stratigraphy, offering insights into the distribution of molecules across the sublayers of the skin’s epidermis. This capability not only eliminates the need for invasive sampling but also delivers precise, layer-specific data on the penetrative depths of both cosmetic and clinical products. Moreover, CRS has been extensively utilized in research to assess skin inflammatory

conditions such as atopic dermatitis (AD) and psoriasis, demonstrating its effectiveness in quantifying biochemical alterations associated with complex skin diseases [21-23]. This makes CRS an invaluable tool in assessing how effectively cosmetic and clinical products deliver active ingredients, enhancing our ability to develop and evaluate treatments that optimally interact with the skin's structures.

In this study, we use an in-house-developed handheld CRS system to explore the absorption kinetics of a ceramide-based and an aqueous-based moisturizer on the skin. By conducting measurements before and after application of these moisturizers, we could analyze the ceramide and water content across the various layers of the stratum corneum. The flexibility and portability of our CRS system allow for non-invasive, in-situ measurements, providing real-time insights into how these moisturizers affects the skin, including changes in hydration levels and ceramide content across time. Additionally, we validate our results through correlation with the gold standard LCMS measurements to verify the reliability of our findings. This approach not only helps in understanding the immediate effects of moisturizer application but also assesses its long-term impact on skin health and barrier function, particularly ceramides, which are crucial in managing inflammatory skin conditions. Through this detailed layer-specific analysis, this study seeks to contribute significantly to the optimization of skincare formulations, enhancing their efficacy and the potential for personalized skin care solutions.

2. Methods

2.1 Ethics and study population

The clinical study was approved by the Domain Specific Review Board (DSRB) of National Health Group, Singapore (Ref:2020/00266) and the Agency for Science, Technology and Research Institutional Review Board (Ref:2020/158).

A total of 40 subjects aged over 21 with Fitzpatrick skin type III and below were recruited and consented over a one-year period. Among them, 20 were patients at the National Skin Centre with confirmed atopic dermatitis, while the remaining 20 were healthy volunteers without known chronic skin conditions. Only de-identified data relevant to the analysis was sent to the team at A*STAR.

2.2 Study protocol

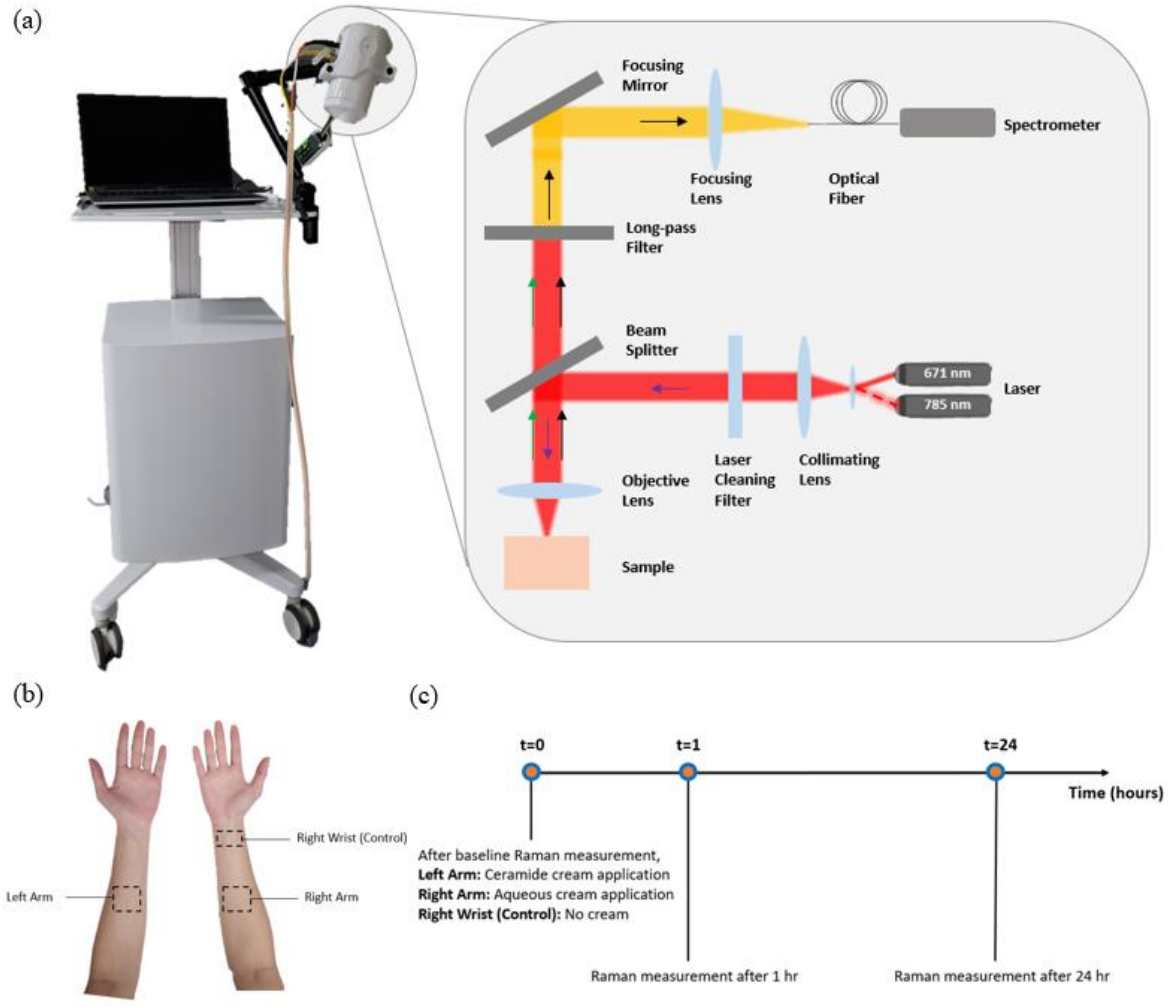

Figure 1. Clinical Study Setup. (a) Handheld confocal Raman system. (b) Location of CRS measurements. (c) Clinical study timeline.

For each subject, skin physiology (TEWL and pH) and CRS measurements were taken prior to any moisturizer application. A vapometer (Delfin Technologies) provided TEWL readings by measuring the relative humidity in a chamber while a pH meter (Mettler Toledo Solutions) was used to determine the skin's acidity. CRS measurements were performed using an in-house handheld flexible CRS system (Figure 1a). A ceramide-based moisturizer was applied to the left arm and an aqueous-based moisturizer to the right arm as shown in Figure 1b. The clinical trial was conducted in a blinded manner for both the subjects and the analysis team. All skin physiology and CRS measurements were repeated on the left arm, right arm, and a third control location on the right wrist without moisturizers, 1 hour, and 24 hours after application, as summarized in Figure 1c. Finally, biopsies were taken from four subjects to validate the ceramide composition of the skin compared to the data obtained using the in-house CRS system.

2.3 CRS measurements

The in-house handheld CRS system [24] features dual-wavelength excitation at fingerprint region of 785 nm (IPS), and high wavenumber region of 671 nm (CrystaLaser), coupled with

a near-infrared spectrograph (Kymera 193i) and a back-illuminated CCD camera (iDus 416, Andor). The system employs a single-mode fiber for excitation and a multimode fiber for signal collection, all housed within a flexible handheld probe. This probe is equipped with a motor to control the movement of the objective lens, allowing for precise focus at different depths within the skin. The CRS system measures 17 axial depths with a mechanical step size of 5 µm. Each depth measurement requires an acquisition time of 5 seconds, with a total measurement time of 2-3 minutes, including positioning of the probe on the subject's arm, which aligns with the site of physiological measurements. To minimize artifacts, patients were asked to lie down and place their hand on a stable surface during the measurement process. This positioning helped reduce motion and stabilize the skin area being measured, ensuring more accurate data acquisition.

2.4 Data processing

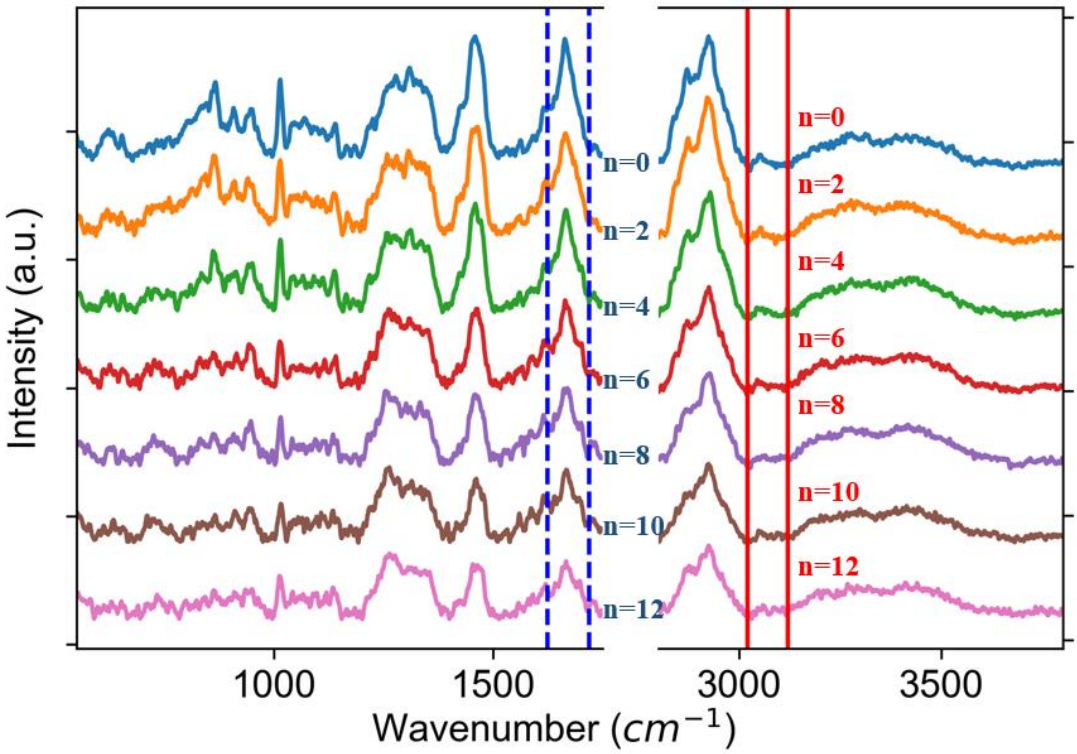

Figure 2. Processed CRS spectra in fingerprint region (left) and high wavenumber region (right). Blue (dotted line) and red (solid line) are the keratin peaks used to determine the start of the stratum corneum (n=0). Note that only alternate spectra are displayed.

The Raman spectra obtained from the in-house CRS system were preprocessed using several steps. First, background removal was performed using asymmetric least squares, followed by noise removal with a Savitzky-Golay smoothing filter. Spectral data deemed poor due to subject motion were excluded from the analysis.

For the fingerprint region, the spectral layer with the maximum keratin peak at 1625 to 1720 nm was identified as the first layer of the stratum corneum for each subject as shown in Figure 2 (blue dotted line). Non-negative least squares fitting was employed to match linear combinations of the fingerprint region (1450 to 1520 nm) spectra with individual constituents

from an existing Raman library. This library included ceramide NP ((9Z)-N-[(2S,3S,4R)-1,3,4-trihydroxyoctadecan-2-yl]octadec-9-enamide), lactic acid, pyrrolidone carboxylic acid, urocanic acid, urea, melanin, cholesterol, and keratin, which mimic the skin's composition. For the high wavenumber region, the spectral layer with the maximum keratin peak at 3020 to 3120 nm was considered the first layer of the stratum corneum as shown in Figure 2 (red solid line). Spectral unmixing was performed for watermass using water constituent spectrum, focusing on the intensity region of 3350 to 3550 nm.

In the first part of the study, only the average of the first five depths for each subject was analyzed for changes in ceramide and hydration levels. The primary interest was in examining how the moisturizers affect the barrier function of the skin. Additionally, a two-sided Mann-Whitney-Wilcoxon test was used for comparison across the subject groups to assess the statistical significance of the observed differences.

In the second part of the study, the relative ceramide and water mass values were compared between two categories—superficial and deeper layers—to evaluate the moisturizers' penetration and retention properties. The average of the first five layers for each subject was taken as the superficial layer, while the subsequent five layers were considered the deeper layers.

2.5 LCMS measurement

For separation of the epidermis from the dermis, biopsies were incubated with 500 µL of 0.25% trypsin (catalog no: 25200056, Thermo Fisher Scientific,) for 12 hours at 4°C. To quantify ceramides, the samples were extracted twice with 150 µL of 1:1 butanol:methanol mixture containing internal standards comprising 0.2–0.4 µM of deuterated ceramides (100× dilution of catalog no: 330713, Avanti Polar Lipids). Samples were then dried and reconstituted in 50 µL of 9:1 acetonitrile:water solution. A calibration curve was constructed using a standardized mixture of 4 ceramides (Catalog no: 330712, Avanti Polar Lipids), and 1 µL of all samples were injected into the LC-MS system for analysis (Vanquish™ Duo coupled to TSQ Quantis™, Thermo Fisher Scientific). The liquid chromatography was performed using a Waters ACQUITY Amide column (130Å, 1.7 µm, 2.1 mm × 100 mm). The mobile phases consisted of 10 mM ammonium acetate in 95% water and 5% acetonitrile (solvent A) and 10 mM ammonium acetate in 5% water and 95% acetonitrile (solvent B). Mobile phases were delivered at a flow rate of 0.4 mL/min. The gradient program was as follows: linear gradient from 100% B to 20% B (0.0–4.0 minutes), isocratic at 20% B (4.0–5.0 minutes), linear gradient from 20% B to 100% B (5.0–5.1 minutes), and isocratic at 100% B (5.0–7.0 minutes). The mass spectrometry was operated in electrospray ionization (ESI) positive mode and the source conditions are as follows: spray voltage 4500V, sheath gas (arb.) 50, aux gas (arb.) 18, sweep gas (arb.) 0, ion transfer tube temp. 325°C, vaporizer temp. 230°C. The multiple reaction monitoring (MRM) transitions and compound-dependent MS parameters of the analytes are summarized in Table S1.

2.6 Correlation with LCMS measurements

Pearson's correlation coefficient and the corresponding p-value were used to evaluate the association between ceramide levels measured by CRS and those obtained via LCMS in four healthy subjects. The LCMS ceramide values represent the sum of four specific ceramide compounds, as detailed in Table S1, which were quantified relative to internal standards.

3. Results

3.1 Skin physiology

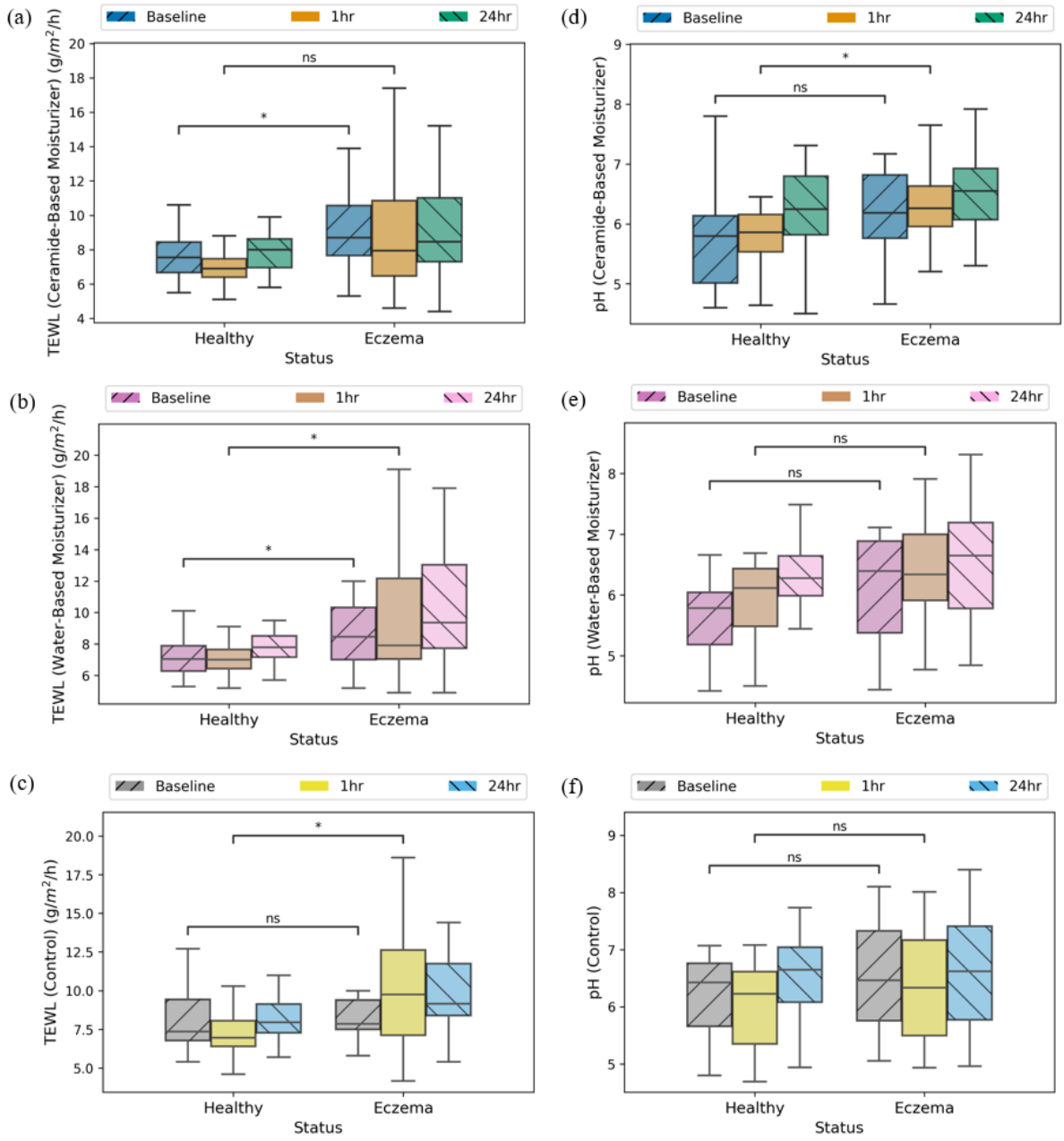

Figure 3. Skin physiology of various moisturizer application across timepoints. TEWL Values: (a) Ceramide-Based Moisturizer, (b) Water-Based Moisturizer, (c) Control. pH Values: (d) Ceramide-Based Moisturizer, (e) Water-Based Moisturizer, (f) Control. For each body region, 20 subjects were measured

at three time points - Baseline (before cream application), 1 hour, and 24 hours after application. Boxplots display the minimum, lower quartile (Q1), median (Q2), upper quartile (Q3), and maximum values. Statistical significance was evaluated using the two-sided Mann-Whitney-Wilcoxon test, with p-values annotated as follows: *: $0.01 < p <= 0.05$ and ns: $p > 0.05$.

In general, the TEWL of eczema subjects were higher than healthy subjects across all measured locations as shown in Figure 3a to 3c. The difference between healthy and eczema subjects at baseline and 1 hour were significant for both the left and right arm. It can also be observed that at the 1hour timepoint, the TEWL decreased in the left and right arm, which were applied with ceramide-based cream and aqueous-cream respectively. When an additional two-sided Mann-Whitney-Wilcoxon test was performed within each group, no significant differences were found between the time points. Consequently, these results are not displayed in the figure, as they did not demonstrate statistically significant changes over time within the individual groups.

Figure 3d to 3f illustrates the pH measurements of the skin for both healthy and eczema subjects. It can be observed that eczema subjects consistently have higher pH levels compared to healthy subjects. The pH of the skin increases over time for both groups on the left and right arms after the application of ceramide-based and aqueous-based creams, respectively. At baseline, there is no significant difference in pH between eczema and healthy subjects across all measured locations. However, there is a slight difference in pH between the two groups at the 1hour-mark on the left arm.

Overall, Figure 3 indicate that it is challenging to determine distinct hydration and pH changes between healthy and eczema subjects, as the observed differences are not consistently significant across time points and conditions.

3.2 Biochemical components obtained using CRS

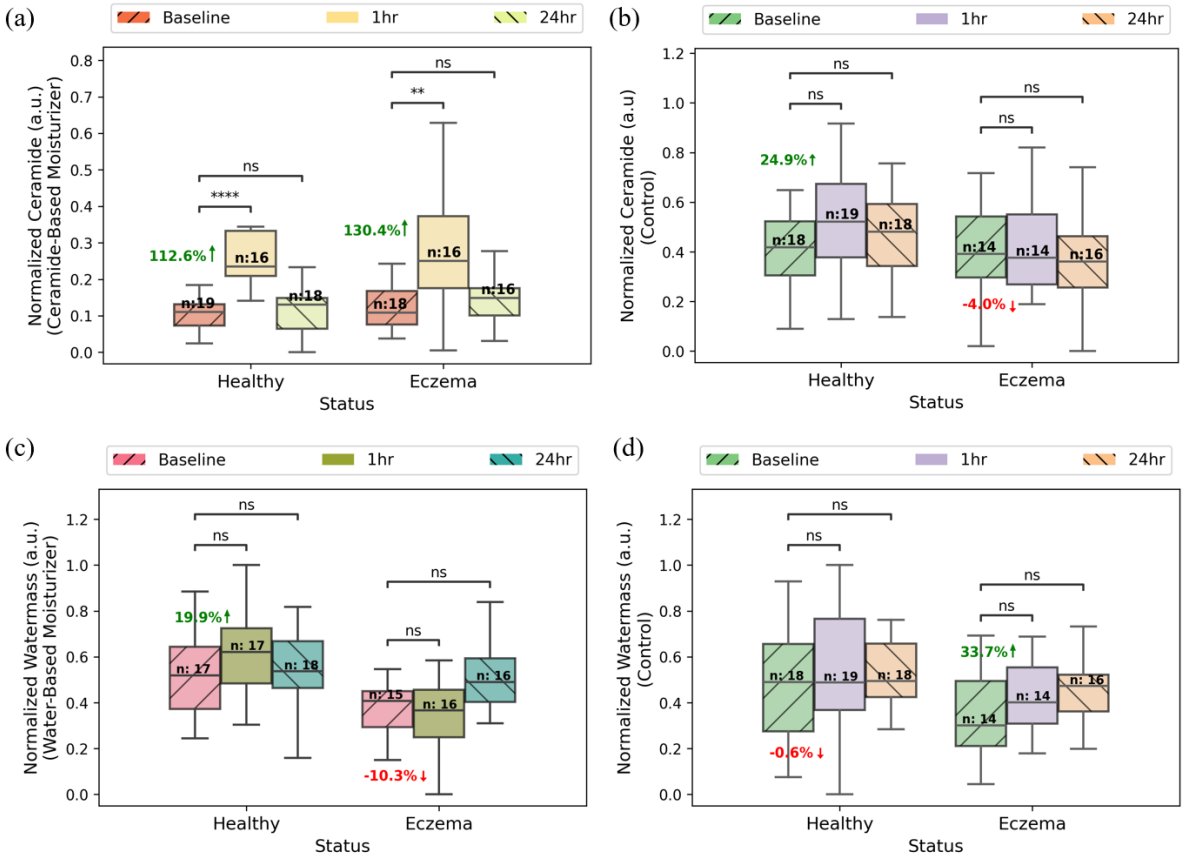

Figure 4. CRS components after spectral unmixing of various moisturizer application across timepoints. Ceramide Values: (a) Ceramide-Based Moisturizer (b) Control. Watermass Values: (c) Water-Based Moisturizer (d) Control. Values shown for the indicated number of subjects were measured at three time points - Baseline (before cream application), 1 hour after application, and 24 hours after application. Boxplots display the minimum, lower quartile (Q1), median (Q2), upper quartile (Q3), and maximum values. Statistical significance was evaluated using the two-sided Mann-Whitney-Wilcoxon test, with p-values annotated as follows: ****: $p <= 0.0001$; ***: $0.0001 < p <= 0.001$; **: $0.001 < p <= 0.01$; *: $0.01 < p <= 0.05$; ns: $p > 0.05$.

Figure 4 presents the changes in ceramide and watermass levels, obtained using CRS, for both healthy and eczema subjects after the application of ceramide-based and aqueous-based creams. The median ceramide level in the left arm of healthy subjects increased significantly by 112.6% at 1 hour after applying the ceramide-based cream compared to the baseline value. For eczema subjects, the corresponding increase was even more pronounced at 130.4% at 1 hour, showing a significant change (Figure 4a). In contrast, Figure 4b indicate no significant changes in ceramide levels on the control region across all time points, when no cream was applied.

For watermass levels, healthy subjects exhibited a 19.9% increase in the right arm at 1 hour after the application of the aqueous-based cream, while eczema subjects experienced a 10.3% decrease in their median watermass values (Figure 4c). However, neither of these changes were significant at the 1-hour mark. Similarly, there were no significant changes in watermass on the control region across all time points when no cream was applied. Overall, there was no

significant difference between the baseline and 24-hour measurements for all locations, as expected, since the ceramide and water levels returned to baseline values after 24 hours.

3.3 Depth profiling of biochemical components

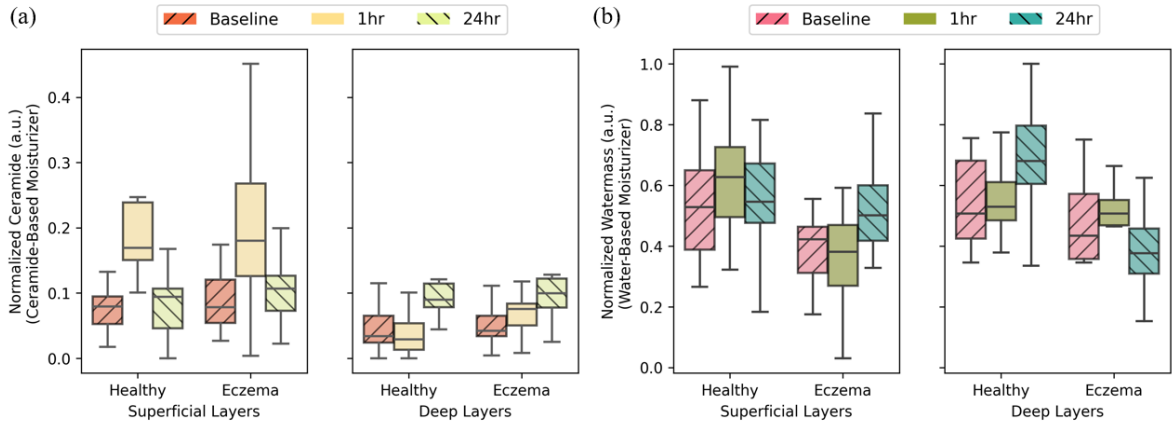

Figure 5. Depth profile of ceramide and watermass. (a) Ceramide levels for ceramide-based moisturizer of healthy and eczema subjects at Baseline, 1 hour, and 24 hours after ceramide cream application, in both superficial and deep skin layers. (b) Watermass levels for water-based moisturizer of healthy and eczema subjects at Baseline, 1 hour, and 24 hours after aqueous cream application, in both superficial and deep skin layers.

Figure 5 demonstrates the penetration depth of ceramide and watermass following the application of creams, a crucial factor in cosmetic and medical applications. Figure 5a reveals that ceramide levels are higher in the superficial layers (0-25 μm) than in the deeper layers (25-50 μm) for both healthy and eczema subjects. At the 1-hour mark, there is an expected increase in ceramide levels in the superficial layers after the ceramide cream application for both groups. However, it is important to note that the standard deviation is quite large for eczema subjects, indicating a high variability in response. No observable changes in ceramide levels were detected in the deeper layers for either group at this time point. Interestingly, at the 24-hour mark, ceramide levels in the deeper layers increased for both healthy and eczema subjects, potentially indicating the retention of cream in the deeper layers.

In terms of watermass, healthy subjects generally exhibit higher levels in both superficial and deeper layers compared to eczema subjects as shown in Figure 5b. At the 24-hour mark, watermass is higher in the deeper layers compared to the superficial layer for healthy subjects, while for eczema subjects, it is higher in the superficial layers. This indicates a differential retention of watermass between the two groups over time.

3.4 Correlation with clinical standard

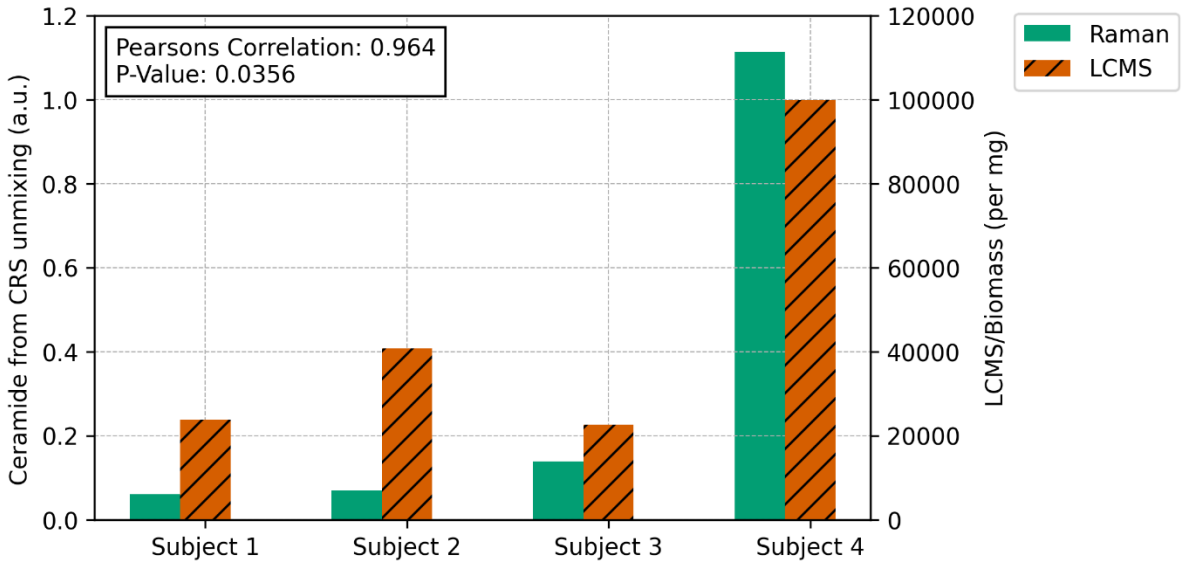

Figure 6. Correlation between CRS and LCMS measurements to evaluate the ceramide level in healthy subjects. Bar plot compares the overall ceramide content of 4 healthy subjects obtained from CRS and chemically obtained values from LCMS normalized against their respective biopsy mass. Pearson's correlation coefficient was calculated and displayed with its respective p-value.

To validate the effectiveness and accuracy of our CRS system, we compared the ceramide content obtained from four subjects using CRS with data obtained from LCMS, as shown in Figure 6. The analysis revealed a robust correlation between the two methods, with a Pearson correlation coefficient of 0.96, indicating a strong linear relationship between the ceramide levels measured by CRS and LCMS. The associated p-value of 3.56×10^{-2} further supports the statistical significance of this correlation, suggesting that the CRS system reliably mirrors the ceramide quantification achieved by the more established LCMS technique.

4. Discussion

This study explored the biochemical impact of ceramide-based and aqueous-based moisturizers on the skin over 24 hours. Eczema subjects exhibited higher TEWL and pH levels compared to healthy subjects. These findings align with the known compromised barrier function in eczema-affected skin, which leads to increased water loss (higher TEWL) [25] and a higher pH due to reduced production of skin acidifiers [26]. However, additional two-sided Mann-Whitney-Wilcoxon tests conducted within each group revealed no significant differences between the time points, indicating variability and a lack of robustness in detecting temporal changes in hydration levels and pH. This lack of significant results highlights the limitation of current method in accurately monitoring skin condition changes over time, emphasizing the need for more sensitive and reliable measurement tools to evaluate the effects of topical treatments.

Unlike existing methods, CRS measurements were able to effectively capture changes in ceramide levels over time, particularly highlighting the efficacy of ceramide-based creams in both healthy and eczema-affected skin. The significant increase in ceramide levels at the 1-

hour mark suggests that the ceramide-based cream is well absorbed and retained in the skin. In contrast, there were no significant changes in ceramide levels on the control region (right wrist) across all time points when no cream was applied, confirming that the ceramide-based cream specifically influenced the treated area.

For watermass levels, changes were observed in healthy and eczema subjects 1 hour after the application of the aqueous-based cream. However, neither of these changes was significant at the 1-hour mark. The variability in watermass changes, particularly the non-significant decrease observed in eczema subjects, may be attributed to the differing severity (mild to severe) among the subjects. More severe cases of eczema could respond differently to the aqueous-based cream, potentially explaining the observed variation. Another potential reason for the variability in watermass changes could be environmental conditions, which might cause the aqueous component of the cream to evaporate more quickly, thus impacting its effectiveness.

One important finding during depth profiling was that skin treated with ceramide cream showed increased ceramide content in the deeper layers after 24 hours. This suggests that ceramide cream takes longer to penetrate the skin but remains there longer compared to aqueous-based cream, which penetrates more quickly but does not retain as long at the 24-hour mark. This is commonly seen in base formulations, where more complex molecules, such as ceramides, often require more time to penetrate the skin barrier but offer longer-lasting benefits once they do [27]. Conversely, formulations with smaller, simpler molecules, like those in aqueous-based creams, penetrate quickly but are metabolized or evaporate faster, leading to shorter retention times. This suggest that ceramide cream reduces the need for frequent emollient applications, whereas aqueous-based creams, which evaporate more quickly, require more frequent reapplication.

When comparing the ceramide levels in healthy subjects measured using CRS compared to the gold standard LCMS, we obtained a strong and significant correlation. However, it is important to note that obtaining punch biopsies from healthy subjects was challenging, which limited our sample size to four subjects. We acknowledge that this small sample size is not significant, but it still provides valuable preliminary validation of the CRS system's capability to reliably measure ceramide levels in the skin, comparable to the established LCMS technique.

It is also noteworthy to mention that the ceramide cream used in the clinical trial contains a range of ceramide subclasses. However, our spectral Raman library currently supports spectral unmixing only for ceramide NP. This limitation may introduce some potential confounding factors, as the diverse ceramide subclasses in the cream could influence the overall ceramide dynamics differently. Specifically, the interactions of these additional ceramide subclasses with the skin might vary, potentially affecting their absorption, retention, and efficacy in ways not fully captured by our current analysis.

In addition, this study currently includes only patients with Fitzpatrick skin types III and lower. In patients with darker skin tones (Fitzpatrick types IV and above), the higher melanin content in the forearm absorbs more light, reducing the signal-to-noise ratio and making accurate measurements challenging.

5. Conclusion

In conclusion, this study provides valuable insights into the skin absorption kinetics of ceramide-based and aqueous-based moisturizers on the skin over a 24-hour period. While existing skin physiology methods, such as TEWL and pH measurements, demonstrated limitations in robustness and sensitivity, our in-house-developed CRS system proved effective in detecting and quantifying changes in ceramide levels across different time points and depths. The CRS system's ability to capture the prolonged effects of ceramide-based creams and their deeper skin penetration highlights its potential as a tool for cosmetic and clinical assessments. Despite the promising results, challenges such as the small sample size for LCMS validation acknowledges the need for further research to comprehensively validate CRS against established methods. Future work should further stratify subjects into different eczema severity levels to enhance the understanding of how severity impacts the efficacy of moisturizers. To gain a comprehensive understanding of the ceramide cream's impact on skin hydration and barrier function, we are currently expanding our spectral Raman library to include a broader range of ceramide subclasses. Additionally, we are actively working on improving the measurement accuracy for individuals with darker skin tones by optimizing the laser and detector configurations to address challenges related to melanin absorption. This will allow us to enhance the precision of measurements across a more diverse patient population. Overall, this study validates the necessity for advanced measurement tools and reinforces the CRS system's potential in optimizing skincare formulations and enhancing personalized skincare solutions, such as determining the frequency of application for various formulations.

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Author contributions

The conceptualization of the study was carried out by Bi Renzhe, Valerie Xinhui Teo, and U. S. Dinish. Subject recruitment was managed by Steven Tien Guan Thng. Bi Renzhe was involved in the hardware development of CRS. Valerie Xinhui Teo, Bi Renzhe, and U. S. Dinish were responsible for the formal analysis and interpretation of the data. Lee Sze Han and James Chan were involved in the LCMS analysis of the clinical samples. The original draft of the manuscript was prepared by Valerie Xinhui Teo while U. S. Dinish reviewed and edited it. Supervision was provided by U. S. Dinish, Steven Tien Guan Thng, and Malini Olivo.

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Declarations

Competing interest

The authors have no conflict of interests or financial disclosures to declare.

Ethics approval

The clinical study was approved by the Domain Specific Review Board (DSRB) of National Health Group, Singapore and the Agency for Science, Technology and Research Institutional Review Board (IRB).

DSRB Ref: 2020/00266
A*STAR IRB Ref: 2020/158

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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