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"Identifying a Distinct NMF Barrier in the Stratum Corneum: Insights into Hydration, Lipid Organization, and Skin Barrier Function"

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1. Introduction

The skin is the body's primary layer in contact with the external environment, acting as a protective barrier against physical, chemical, and biological insults. The outermost layer, the stratum corneum (SC), plays a crucial role in preventing transepidermal water loss and protecting underlying tissues from pathogens and toxins (1). The function of the SC is directly associated with its molecular composition, particularly components such as lipids, urea and proteins, which collectively determine skin hydration and the skin barrier integrity (2).

Traditional methods for assessing the skin's biochemical composition, including tape stripping, biopsies, and cell scrapers, are invasive and lack the capacity to provide accurate, depth-resolved information (3). This limitation evidences the need for non-invasive techniques to evaluate the skin chemical coimposition *in vivo*. In this regard, confocal Raman spectroscopy has emerged as a powerful tool, enabling the harmless, non-invasive measurement of the skin's chemical composition with an unparallel and accurate depth resolution (4). This method performs the simultaneous analysis of multiple skin components and has been implemented in dermatological and cosmetic research (4). However, many existing studies utilizing this technology have been limited by small sample sizes, typically involving fewer than 100 participants (5)(6)(7)(8), therefore limiting the impact of their findings. In this clinical study, we measured the skin chemical composition *in vivo* in a large group of volunteers, offering a more representative analysis of the skin chemical composition and enhancing the reliability and relevance of the results for both scientific and clinical applications.

Among the critical components of the SC, natural moisturizing factor (NMF) plays a key role in maintaining hydration and skin elasticity (9). NMF is a complex mixture of low molecular weight, water-soluble compounds, primarily composed of free amino acids and their derivatives (such

as pyrrolidone carboxylic acid and urocanic acid), along with salts, sugars, and organic acids. These substances are highly hygroscopic, allowing them to attract and retain water, hence supporting the moisture retention in the SC (9). Disruptions in NMF content are associated with various dermatological conditions, including xerosis and atopic dermatitis, emphasizing the importance of maintaining this balance (10). Our study uses *in vivo* confocal Raman spectroscopy to characterize the depth-spatial distribution of NMF and related molecules across the SC in a large cohort. These findings provide new insights into the biochemical organization of the SC, particularly the role of the NMF-associated barrier around 5 microns depth, and highlight the relevance of non-invasive *in vivo* Raman spectroscopy in advancing dermatological research and targeted skincare development.

2. Materials and Methods

2.1 Study subjects and informed consent

A total of 155 volunteers participated in this study (121 volunteers for water analysis, 118 for NMF, urea and protein analysis, 114 for lactate and transuronic acid analysis and 118 for ceramides and fatty acids analysis). Eligible participants were females and males aged 20 to 70 years with normal, dry, or mixed skin, in self-reported good general health. Exclusion criteria included recent surgery in the experimental area; visible skin alterations; ongoing pharmacological or hormonal treatments relevant to skin physiology; diagnosed skin diseases; pregnant or nursing. This clinical study was conducted at the facilities of Bionos Biotech S.L. according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Bionos Biotech S.L. (Date 14/04/2025 and Code number 051 - 2025). The study protocol is in accordance with the Scientific Committee on Consumer Safety (SCCS) guidance. It meets all international standards for research studies involving human subjects, the Good Clinical Practices (ICH - GCP), and World Medical Association. Each volunteer provided signed informed consent before the study, acknowledging its purpose, potential risks and benefits, and limits of liability.

2.2 Analysis of the chemical composition of the skin

Skin hydration and chemical composition were evaluated by Confocal Raman Spectroscopy by using the Gen2 SCA Ultimate (RiverD International B.V., Rotterdam, Netherlands) in the volar forearm of volunteers. This instrument is a confocal Raman system of high sensitivity designed for *in vivo* skin analysis (11) (12). The gen2-SCA Ultimate has two built-in wave class 3B lasers, 671 nm and 785 nm, used for measuring skin hydration (wavenumbers from 2500 to 4000 cm^{-1}) and chemical composition (wavenumbers from 400 to 1800 cm^{-1}), respectively. The laser power complies with the maximum permissible levels for skin as defined by the

international laser safety standard (IEC 60285-1:2007; <30 mW for 785 nm, and <20 mW for 671 nm). Spectra were acquired using a pinhole of 50 µm. The SkinTools software (RiverD International B.V., Rotterdam, Netherlands) was used to analyze the generated spectra and to express the data as mass-% (water), mg/cm^3 (urea) and arbitrary units (rest) of stratum corneum. The thickness of the SC was calculated (in µm) from the water concentration profiles using the SkinTools software (13), in which the SC thickness was defined as the intercept of 2 straight lines delineating the boundary between the SC and the epidermis. Data in the graphs are represented by mean ± standard error of the mean.

3. Results

3.1. Skin hydration

Water concentration in the skin increased progressively with depth, showing a characteristic gradient from the outer surface inward (Figure 1a). The transition point in the hydration curve, corresponding to a marked rise in water content, was used to estimate the stratum corneum (SC) thickness. Across all volunteers, the average SC thickness was 16.1 ± 0.4 µm, as shown in the bar graph, with moderate interindividual variability reflected in the standard error (Figure 1b). Note that the living epidermis layer is characterized by a high water content (around 65-70%), required for the enzymatic reactions *in vivo*.

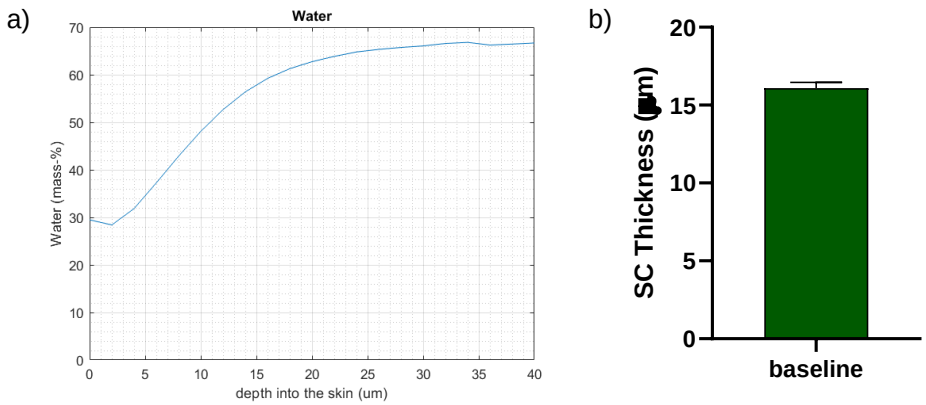

Figure 1. (a) The averaged percentage of water as a function of depth is represented. Vertical line indicates the thickness of the SC. (b) Graphical representation of the SC thickness (µm). The Mean and S.E.M. are shown.

3.2. Natural Moisturizing Factor

The SC has a critical function of preventing the transepidermal water loss (TEWL). This fact is evidenced by the lower water content in the skin surface (28.4 ± 0.5%) compared to the epidermis (~ 65%). The NMF profile exhibited a clear peak at 4 µm depth, indicating a localized

concentration of NMFs in the upper stratum corneum (Figure 2). Beyond this depth, NMF levels declined sharply, reaching minimal values past 20 µm (Figure 2). The presence of this peak supports the hypothesis of a chemically-defined barrier zone within the SC, potentially critical for preventing TEWL and maintaining skin hydration.

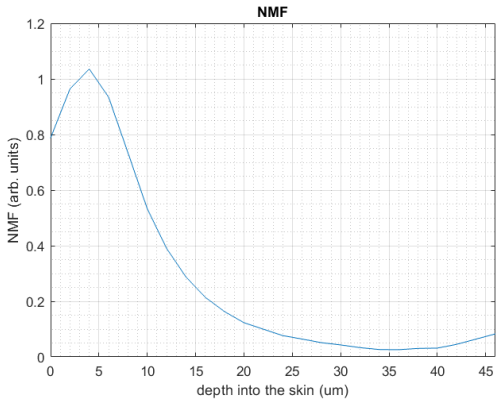

Figure 2. The averaged amount of NMF of the skin as a function of depth is represented in arbitrary units.

3.3. NMF individual components

The concentration of ceramides and fatty acids was highest at the outermost surface of the SC (0 µm) and gradually decreased with increasing depth (Figure 3a). This profile is different from that of NMF, which presented a peak deeper within the SC (Figure 2). The high lipid concentration at the surface reflects their structural role in the intercellular lipid matrix, essential for barrier integrity and protection against external aggressors. Similarly, urea displayed a surface-localized peak, with the highest levels detected at the outermost layer (0 µm), followed by a rapid decline toward the deeper regions of the SC (Figure 3b). This parallel distribution suggests that both lipids and urea are concentrated at the skin surface, likely contributing to avoiding water by evaporation and establishing the initial hydration gradient, in contrast to the deeper, water-retaining role of NMF.

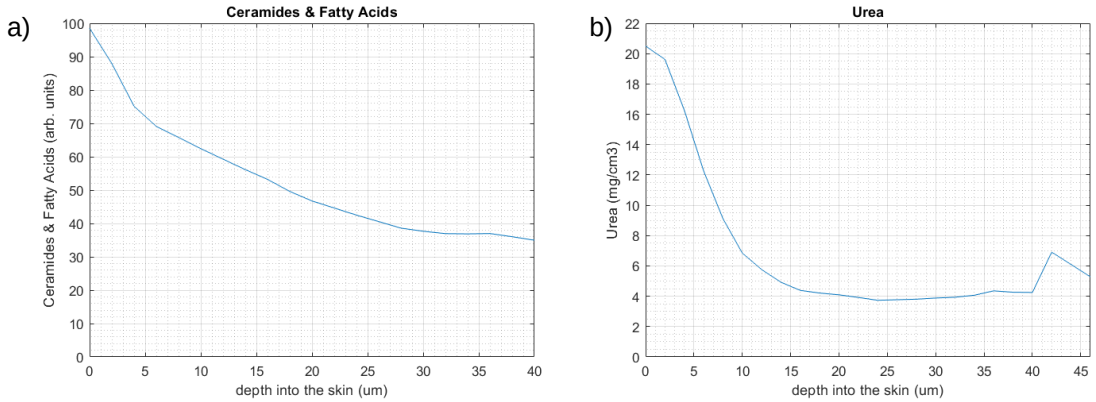

Figure 3. The averaged amount of ceramides and fatty acids (a) and urea (b) of the skin as a function of depth is represented in arbitrary units.

The protein profile showed a peak at 4 µm, matching the depth at which NMF concentration was highest (Figure 4a). This co-localization suggests a potential interaction or coordinated organization of structural proteins and NMFs within the upper SC, possibly contributing to the formation of a biochemical barrier zone. In contrast, lactate levels peaked at the skin surface (0 µm) and declined rapidly with depth (Figure 4b). A slight secondary rise was observed beyond 30 µm, potentially reflecting increased metabolic activity in the viable epidermis, where keratinocyte metabolism contributes to lactate production (Figure 4b). Lactate was measured at pH 4, which reflects the natural acidic conditions of the SC. This acidic environment is essential for enzymatic regulation, microbial defense, and lipid processing in the skin. Therefore, quantifying lactate at this physiologically relevant pH offers important insights into barrier homeostasis and the skin's acid environment.

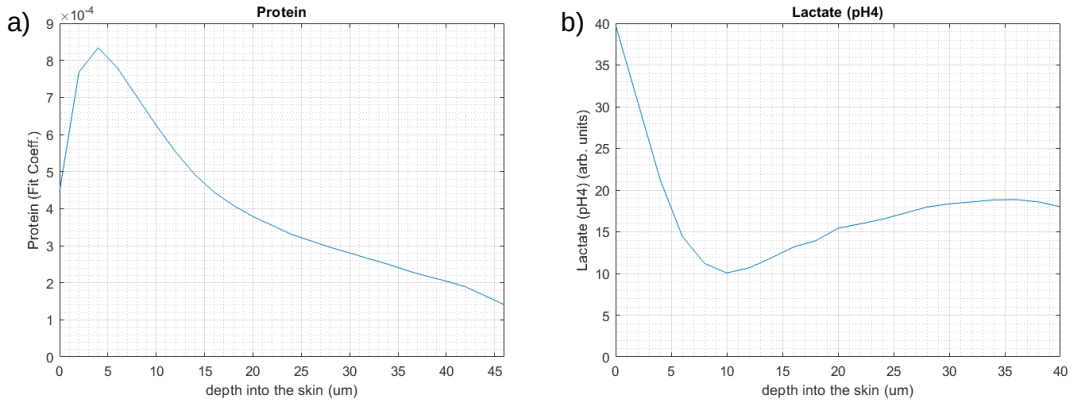

Figure 4. The averaged amount of ceramides and proteins (a) and lactate (pH4) (b) of the skin as a function of depth is represented in arbitrary units.

Both profiles of trans-urocanic acid (UCA), measured at pH 2 and pH 5, showed a consistent peak between 4 and 8 µm depth (Figure 4). This localization is comparable to that of NMF and protein, further supporting the existence of a functionally specialized region within the upper stratum corneum. Measuring trans-urocanic acid at different pH levels provides insight into its protonation-dependent behavior, as it can exist in different ionic forms depending on the local skin pH. These forms may influence its UV-absorbing capacity, immunomodulatory properties, and interaction with other components of the acid environment of the skin. The distinct profiles observed at pH 2 and pH 5 help characterize how UCA behaves across microenvironments in the SC, where pH gradients naturally occur from surface to deeper layers.

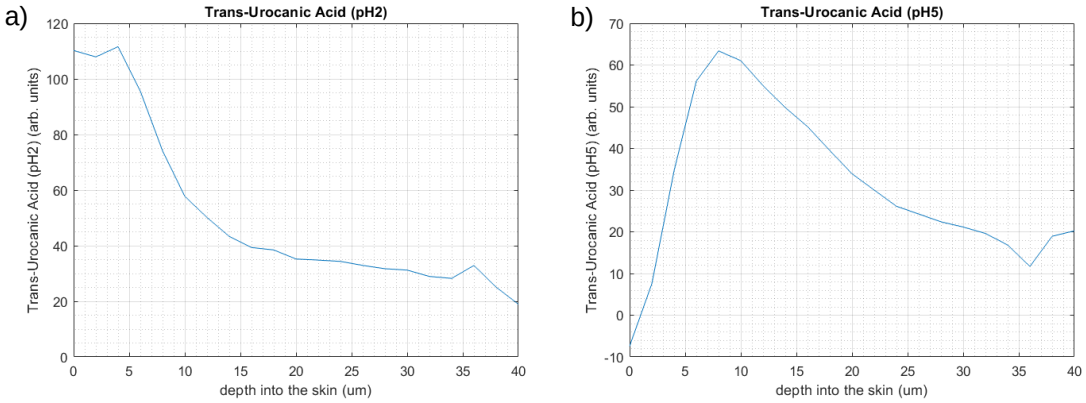

Figure 4. The averaged amount of trans-urocanic acid at pH2 (a) and pH5 (b) of the skin as a function of depth is represented in arbitrary units.

4. Discussion

The stratum corneum (SC) serves as the skin's outermost barrier, and its protective efficacy is not only dependent on structural integrity, but also on the spatial organization of its molecular components (1)(2). Our results are in agreement with the concept that the SC is not chemically homogeneous but stratified (1)(2), with a particularly relevant transition zone observed at 4 µm depth. This region, characterized by a sharp peak in NMF concentration, consistently appeared across our large cohort of subjects and represents a biochemically active barrier crucial for limiting transepidermal water loss (TEWL). Raman spectroscopy enabled the precise identification of this NMF layer, revealing a functional layer within the SC where hydration levels and biochemical profiles change significantly.

In this NMF layer, we also observed a notable co-localization of proteins and NMF, suggesting a potential interaction of structural proteins and NMF components within the upper region of the SC, possibly contributing to the formation of a biochemical barrier zone. In contrast, components such as ceramides, fatty acids, and urea exhibited maximal concentrations at the SC surface (0 µm), followed by a gradual decline with depth. This parallel distribution of ceramides, fatty acids and urea suggests that lipids and urea are concentrated at the skin surface, likely contributing to avoiding water loss by evaporation and establishing the initial hydration gradient, in contrast to the deeper, water-retaining role of NMF (2). Additionally, lactate showed a strong surface-localized peak. This acidic environment is essential for enzymatic regulation, microbial defense, and lipid processing in the skin (14). Therefore, quantifying lactate at this physiologically relevant pH offers important insights into barrier homeostasis and the skin's acid environment.

The use of *in vivo* confocal Raman spectroscopy is essential for identifying these depth-dependent biochemical patterns (11)(12). This non-invasive, layer-specific method allowed us to detect accurate molecular gradients, such as the peak of trans-urocanic acid between 4 and 8 µm, measured at both pH 2 and pH 5. This localization of the trans-urocanic acid is comparable to that of NMF and protein, further supporting the existence of a functionally specialized region within the upper SC. Moreover, the high number of subjects participating in this study adds significant value to these findings. While most previous studies with confocal Raman spectroscopy involved fewer than 50 participants, our study offers robust, population-relevant insights that enhance both the biological understanding and translational potential of SC biochemical profiling.

The implications of this work are broad. From a dermatological and cosmetic science perspective, recognizing the stratified nature of skin components opens new opportunities for targeted skincare formulations, such as those designed to reinforce the mid-depth NMF layer or the surface lipid layer. This approach supports the future of personalized skincare and more effective barrier-supportive products. Future research should investigate how dynamic factors, such as treatments, aging, disease, environmental exposures, ethnicity, and seasonal changes, affect the biochemical stratification observed in the SC.

5. Conclusion

- The stratum corneum shows clear biochemical stratification, with a distinct NMF-rich zone at 4 µm depth.
- NMF and proteins co-localize in this zone, suggesting a functionally important biochemical barrier.
- Lipids, urea, and lactate are most concentrated at the skin surface, indicating their role in surface barrier function and hydration.
- Using *in vivo* confocal Raman spectroscopy on a large cohort enabled precise identification of depth-dependent molecular patterns, lending robustness and broader relevance to the findings.

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