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"A novel skin penetration evaluation method for cream containing tranexamic acid: Raman imaging using multi-variate curve resolution-alternating least squares analysis"

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1. Introduction

Skin is the largest organ in the human body. In adults, the skin has a surface area of 1.6 m^2 and accounts for approximately 16% of body weight. The skin plays roles in maintaining homeostasis, including thermoregulation and protection against external harmful substances. Regarding the physical barrier function of the skin, the stratum corneum is the most important layer. Active ingredients incorporated into cosmetics possess specific physiological activities that act directly or indirectly on the structure and function of the skin. Sufficient efficacy of active ingredients is expected upon their penetration through the stratum corneum and subsequent arrival at the target site. For example, vitamin C derivatives inhibit melanin formation, hyaluronic acid has a water retention effect, and retinoid has epidermal keratinization modulating effects. Active ingredients contribute to the improvement of specific skin conditions based on their unique mechanisms of action [1-4]. Visualization is a useful tool for understanding the behavior of these components in the skin, and visualization techniques have facilitated the evaluation of simple systems. However, because it is difficult to isolate the spectrum of a target component from multiple components, using conventional methods for multi-component and highly viscous creams remains challenging [5]. In addition, skin penetration evaluations are often optimized for liquid formulations. In such cases, it is difficult to apply such methods to creams.

Standard methods for the evaluation of skin penetration include a quantitative skin penetration method using Franz cells and an imaging method using fluorescent labeling [6, 7]. However, using the former method, it is difficult to determine the distribution of the target substance within the skin. Although this distribution can be determined using the latter method, it is necessary to change the molecular weight of the source material. To address these issues, we employed Raman imaging, an imaging technique using Raman scattering that provides more detailed molecular information. Raman imaging has the advantages of being non-destructive and allowing sensitive determination of the molecular structure. However, with a

multi-component structure such as skin, there are limits to detecting spectra specific to the target component. Multivariate curve resolution–alternating least squares (MCR-ALS) isolates Raman spectra associated with multiple components to enable imaging.

Given this background, in the present study, we aimed to investigate Raman imaging of tranexamic acid (TXA), which has traditionally been difficult to detect, by combining MCR-ALS and Raman spectroscopy. TXA is used in dermatology, but its water-soluble component has low skin penetration. Therefore, its ability to reach the target site needs to be improved [8]. In addition, TXA yields a low intensity of Raman spectrum, so a finer understanding of its penetration behavior is needed. Therefore, we performed imaging of the skin penetration behavior of TXA in cream formulations using MCR-ALS.

2. Materials and Methods

2.1. Human Reconstructed Epidermis (HRE)

EpiDerm™ human-reconstructed in vitro epidermal tissues (0.6 cm^2 of each) were purchased from Mattek (Ashland, MA, USA) .HRE were maintained in EPI-100-ASY culture medium according to the manufacturer's instructions [9].

2.2. Tranexamic Acid (TXA) Aqueous Solution and Cream Formulations

Aqueous tranexamic acid (TXA) solutions were prepared by diluting TXA with purified water to solutions of 0 %, 0.3 %, 2.0 %, and 4.0 % (w/w). Similarly, water-soluble components were adjusted so that the TXA concentration became 0 %, 0.3 %, 2.0 %, and 4.0 % (w/w), and then mixed with oil-soluble components to prepare cream formulations. Furthermore, polyglyceryl diisostearate and bis-ethoxydiglycol cyclohexane-1,4-dicarboxylate, known as penetration enhancers, were mixed into each aqueous solution or cream formulation to prepare aqueous solutions or cream formulations containing penetration enhancers [10].

2.3. Preparation of Cream-Applied HRE

25 µL of each prepared TXA-containing cream formulation was applied to the HRE using a sterile cotton swab and incubated at room temperature for the respective time periods. After a predetermined time, the HRE surface was gently cleaned with a dry cotton swab. The HRE was then frozen at -20 °C and 10–30 µm thick sections were obtained by cryo-microtome.

2.4. Time-of-flight mass spectrometer (TOF-SIMS)

Following the method described above, HRE treated with TXA and aqueous solution containing penetration enhancers, and subsequently sectioned, were analyzed using TOF-SIMS (time-of-flight mass spectrometer, ION-TOF GmbH, Münster). TOF-SIMS spectra were acquired in negative ion detection mode by selecting the ionization peak of TXA (m/z 156), and mapping analysis was performed over an area of 200 µm × 200 µm (N = 3) [11].

2.5. Confocal Raman microscope

Raman spectra were measured from thin sections fixed on glass slides using a confocal Raman microscope (Nanophoton, Osaka). Measurements were recorded using a 100× objective operating with a numerical aperture of 0.9. The laser wavelength was 532 nm, the laser power was 40 mW, and the integration time was 1s. Detection was performed using a CCD detector

cooled to -75°C by a Peltier element. Spectra were recorded in the spectral range of 450–1900 cm^{-1}. Skin frozen sections were scanned in region of 70 µm (Y-axis) by 5 µm (X-axis), with a scanning step size of 0.5 µm in both the x and y directions.

2.6. Multivariate curve resolution-alternating least squares (MCR-ALS)

Raman spectral data were pretreated with the Igor Pro software (WaveMetrics, Inc., Lake Oswego, OR, USA). Indene spectra were utilized for the wavenumber calibration standard. All spectra acquired in the Raman mapping measurements were combined into a single matrix, on which singular value decomposition (SVD) was performed for noise reduction prior to the MCR-ALS.

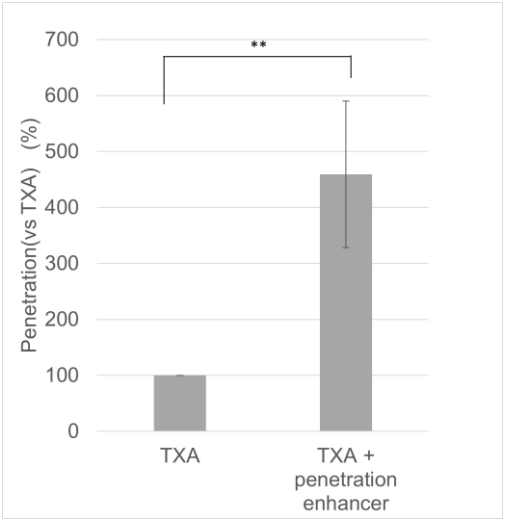
The MCR-ALS method employed in this study was based on previous work [12, 13]. A semi-supervised approach was implemented to address the challenges of extracting weak Raman signals of TXA from complex skin tissue samples. First, we obtained reference spectra of TXA by applying MCR-ALS to concentration-dependent datasets of TXA solutions and creams. Simultaneously, we extracted the intrinsic spectral components of the skin by performing spectral decomposition on datasets from skin samples without TXA.

These reference spectra were then introduced in the MCR-ALS algorithm to guide the decomposition process. This semi-supervised spectral decomposition approach allows for the isolation of weak TXA signals from the complex skin matrix background, which is difficult to achieve with conventional univariate analysis methods. The MCR-ALS analysis was performed using in-house developed scripts in Python.

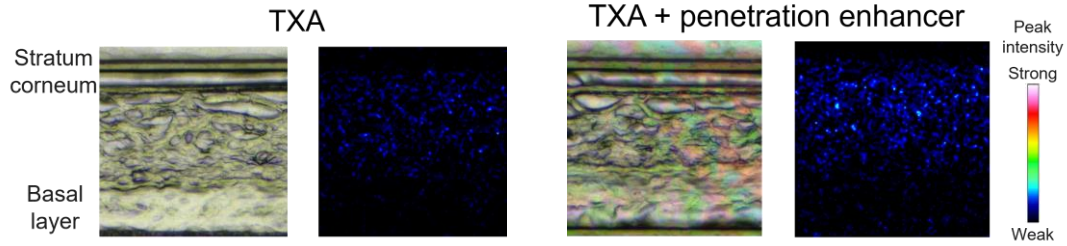
3. Results

3.1. *Permeability Screening of Tranexamic Acid (TXA) Aqueous Solutions Using Penetration Enhancers (Deep Delivery Formulation)*

Screening was conducted to enhance the permeation of TXA into the deeper layers of the epidermis using penetration enhancers, aiming for a deep delivery formulation. Human Reconstructed Epidermis (HRE) was employed for the screening. Aqueous solutions containing TXA and various penetration enhancers were applied to the HRE, and the permeated TXA within the epidermis was detected using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). The ion intensity of the TXA ionization peak (m/z 156) is shown in a graph (Figure 1a). Despite its small molecular weight, the permeability of TXA across the stratum corneum was low due to its hydrophilic nature (Figure 1b, left). Therefore, by screening penetration enhancers, it was confirmed that the combination of TXA with specific penetration enhancers significantly enhanced its permeation into the epidermis (Figure 1b, right).



(a) Results of measurement of TXA in HRE

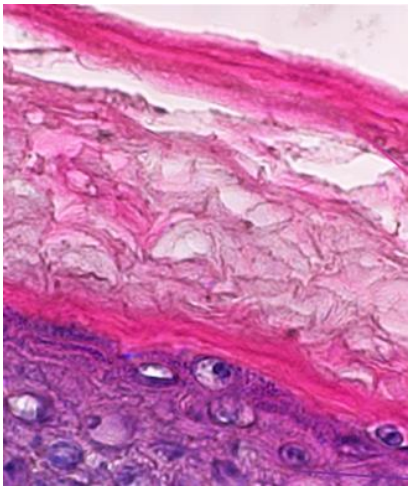


(b) TOF-SIMS images

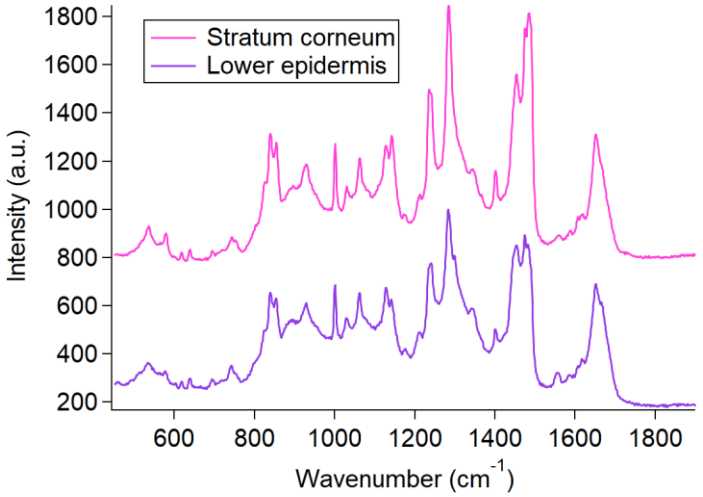
Figure 1. Penetration of tranexamic acid (TXA) determined by TOF-SIMS.
(a) TXA and penetration aids aqueous solution were added to a HRE. It was found that the aqueous solution containing the penetration enhancer promoted the penetration of TXA. n = 3, unpaired t-test, ** $p < 0.01$; (b) Optical micrograph (left) and TOF-SIMS images (right).

3.2. Stained Images and Raman Spectra Results of HRE

An image of Hematoxylin and Eosin (HE) stained section of HRE is shown in Figure 2a. The HRE for the permeation study exhibited increased keratinization and a higher barrier function compared to normal skin. Raman spectra were also acquired from different regions (Figure 2b). The spectra were obtained in the range of 450–1900 cm^{-1} (fingerprint region) using a 532 nm laser. Characteristic peaks of skin tissue, such as those for proteins including phenylalanine (1003 cm^{-1}, ring breathing), CH_2/CH_3 deformation modes (1440-1460 cm^{-1}), and amide I band (1650-1680 cm^{-1}, C=O stretching) were identified. Due to its nature as a biological tissue, the HRE did not yield a uniform spectrum, and variations in the intensity of characteristic peaks were observed depending on the region.



(**a**) Hematoxylin and eosin (HE) stained section of HRE



(**b**) Raman spectra of the stratum corneum and lower epidermis

Figure 2. Raman spectra of HRE. (a) HE stained section of HRE.; (b) Raman spectra of stratum corneum and lower epidermis.

3.3. Raman Spectroscopy Results of TXA Crystal and Aqueous Solutions

Raman spectra of TXA and its aqueous solutions (0%, 0.3%, 2.0%, 4.0%) were acquired (Figure 3). Multiple peaks specific to TXA were present in the fingerprint region (450–1900 cm^{-1}). Characteristic peaks at 780 cm^{-1}, 1035 cm^{-1}, 1162 cm^{-1}, and 1466 cm^{-1} were identified. The spectral features differed between the crystalline and aqueous states of TXA. When examining the permeation behavior of the compound, it was considered necessary to select which spectrum to use as an indicator for imaging.

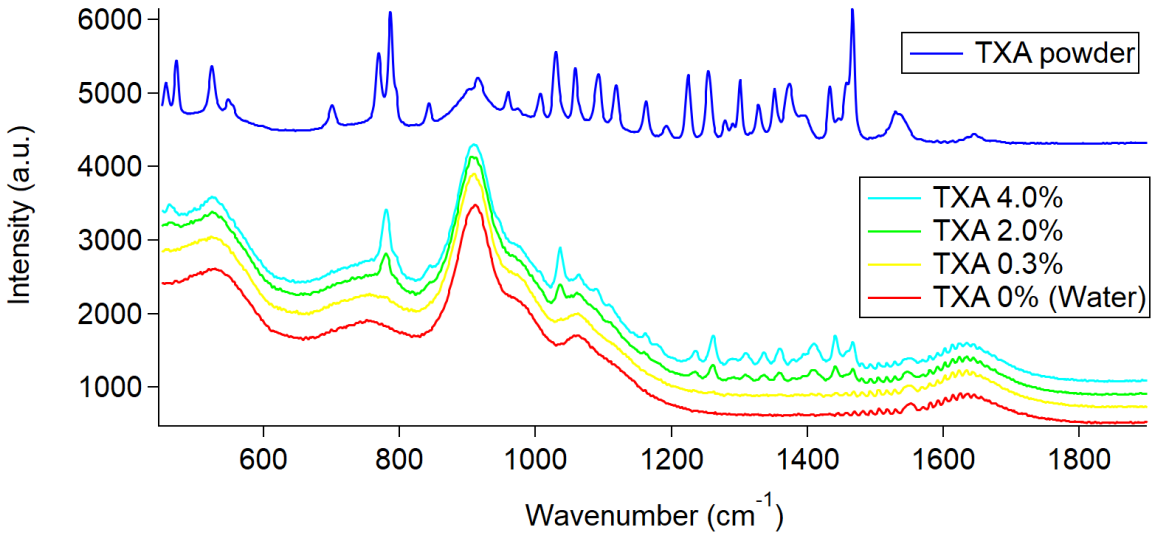


Figure3. Spectrum of TXA. Spectrum of TXA powder (blue) and TXA aqueous solution (0%: red, 0.3%: yellow, 2%: green, 4%: light blue)

3.4. Raman Spectroscopy Results of Pure TXA Spectrum and TXA Containing Cream Formulation

The pure spectrum of TXA was extracted from the Raman spectra of TXA aqueous solutions using Multivariate Curve Resolution (MCR). TXA 2.0% cream was compared with TXA 0% cream, and the characteristic peaks derived from TXA (780 cm^{-1}, 1035 cm^{-1}) were found to be different. The characteristics peaks for TXA in the creams were examined, and it was found that while the intensities were different, the spectrum peaks matched those derived from TXA in aqueous solution by MCR (Figure 4).

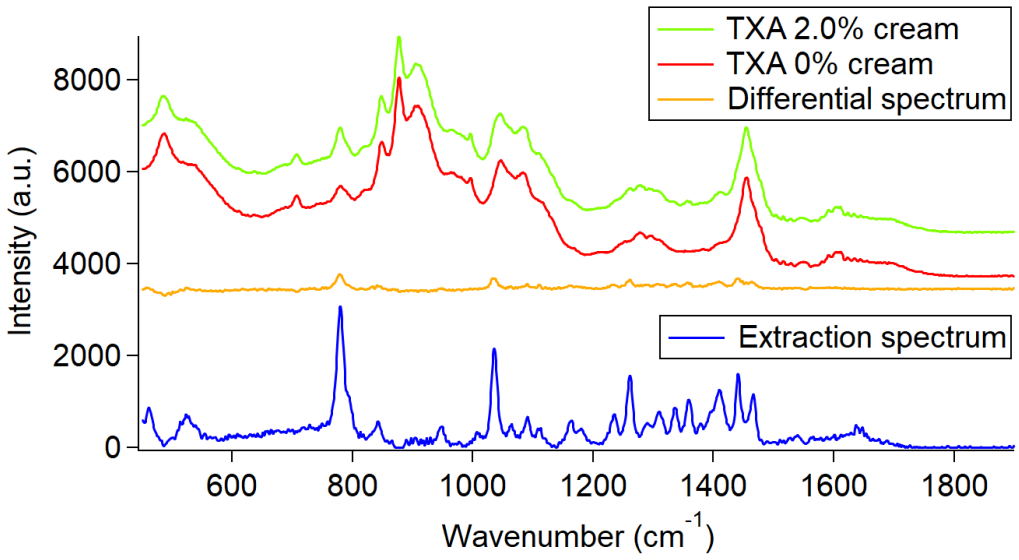
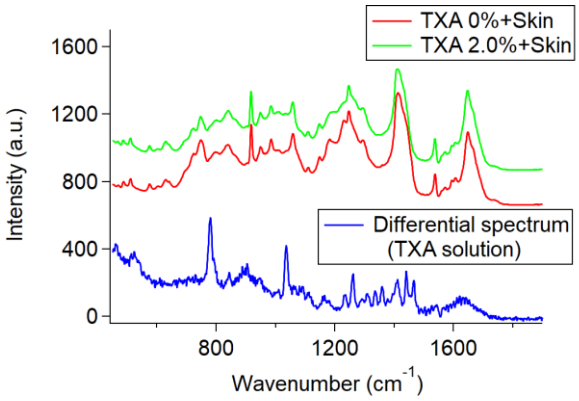


Figure 4. Extracted Spectrum of TXA. Spectrum of TXA cream (0%: red, 2.0%: green) and spectrum extracted from the concentration gradient of TXA aqueous solution (blue)

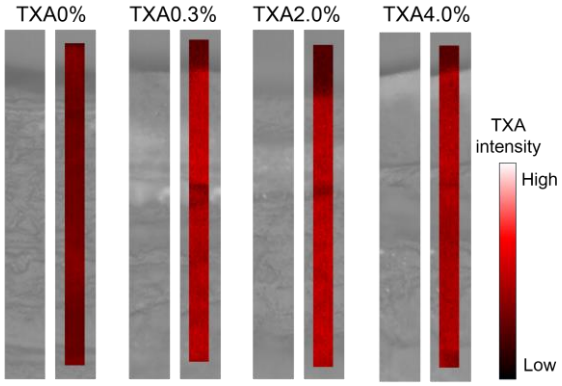
3.5. Evaluation of penetration of TXA cream

Next, we attempted to evaluate penetration by using a Raman microscope with greater resolution to detect TXA in skin to which TXA had been applied. First, formulations of cream containing different concentrations of TXA (0%, 0.3%, 2.0%, 4.0%) were prepared and applied to HRE, sections were created, and the TXA present in the skin was detected. Detection was carried out by acquiring the Raman spectrum of the section and performing imaging with the characteristic peaks derived from TXA (780 cm–1) (Figure 5a). However, the characteristic TXA peaks were in the same position as the peaks derived from HRE, so that TXA imaging was not possible (Figure 5b). Next, TXA imaging was performed from the same Raman spectrum using the pure spectrum of TXA shown in Figure 4. MCR-ALS was used for this imaging. As a result, spectra with intensity correlated with the concentration of TXA were obtained. (Figure 5c and 5d) TXA creams containing a penetration enhancer were prepared and evaluated using the method shown in Figure 5c to confirm that the penetration enhancer improved the penetration of TXA. Using the method in Figure 5c, we prepared and evaluated cream formulations containing a penetration enhancer and TXA to confirm that the penetration enhancer improves the permeability of TXA in the cream formulation as well.

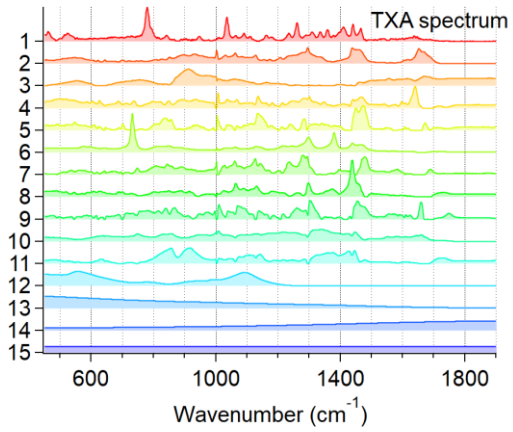
The amount of penetration enhancer used in the evaluation was chosen based on the results of the penetration enhancer formulation screening. Although there was no significant difference with N=6 (p = 0.054), the cream formulation containing the penetration enhancer showed a tendency for higher permeability of TXA (Figure. 6a and 6b).



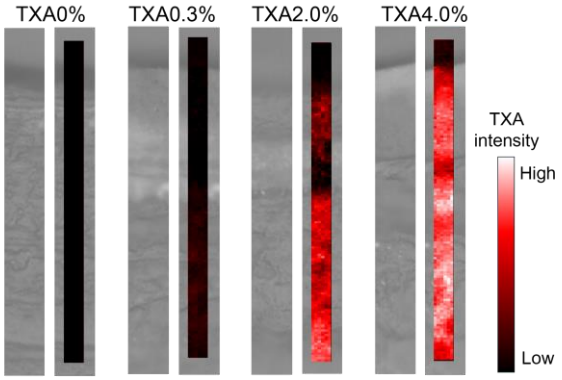
(**a**)Raman spectra of characteristic TXA peaks



(**b**)Imaging of Characteristic TXA spectrum

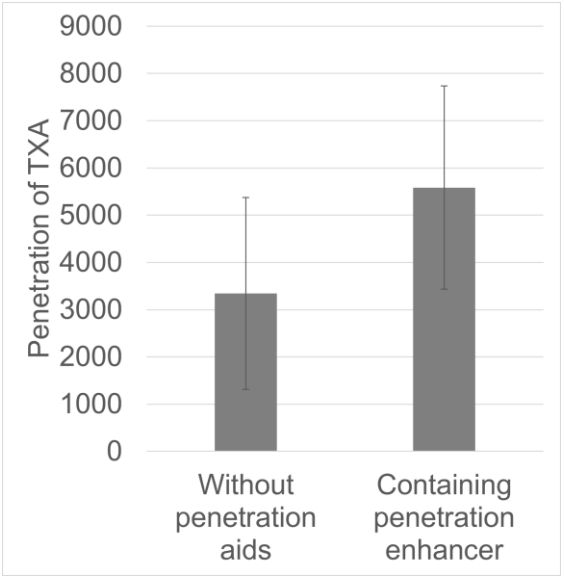


(**c**) Spectra resolved by MCR-ALS.

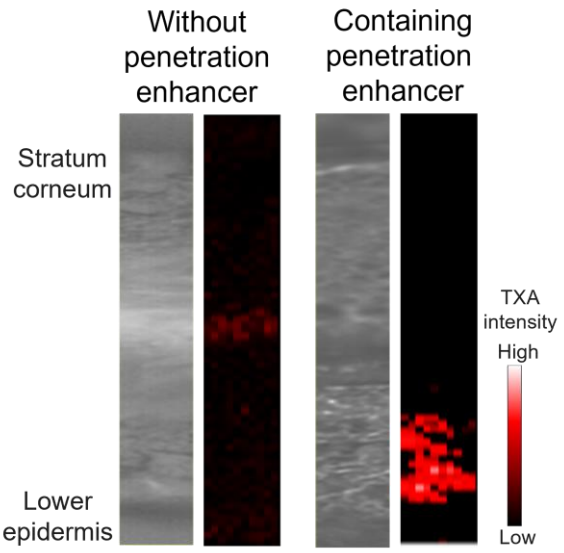


(**d**) Images of TXA using MCR-ALS

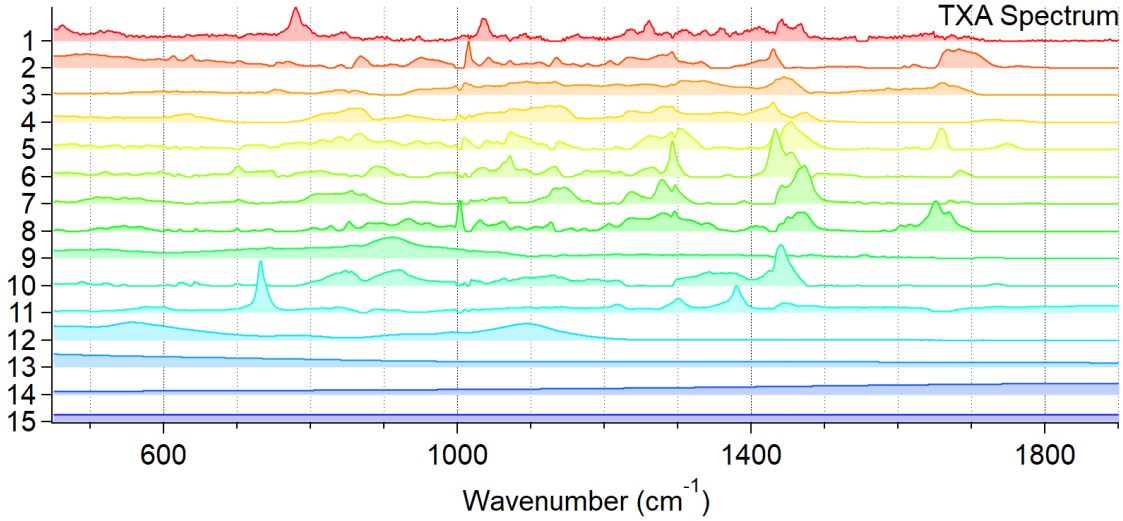
Figure 5. Comparison of single-peak imaging and MCR-ALS imaging.
(a) Raman spectra of HRE models applied with TXA and characteristic peaks of TXA solution.; (b) Imaging results using the characteristic peaks of TXA solution.; (c) Decomposed Raman spectra of 15 components by MCR-ALS. (Red: spectrum of TXA); (d) Raman images of TXA spectrum.



(**a**) Penetration of TXA



(**b**) Images of TXA using MCR-ALS



(**c**) Spectra resolved by MCR-ALS.

Figure 6. Raman imaging results of TXA after application of cream containing penetration enhancer
(a)The cream containing TXA was applied to HRE tissue, and the penetration of TXA was evaluated 30 minutes later. It was found that the cream containing the penetration enhancer tended to improve the penetration of TXA. n=9, unpaired t-test, p = 0.06.; (b) Raman images using MCR-ALS.; (c) Spectra resolved by MCR-ALS.

4. Discussion

When developing cosmetics and other products that are applied to the skin, it is important to understand the skin penetrating behavior of the active ingredients. The target of the present study, TXA, needs to reach deep into the epidermis [8]. To date, various methods for evaluating penetration into the skin have been developed with the aim of understanding the behavior of active ingredients. These can be broadly classified into quantitative tests (combining diffusion cells or tape stripping with high-performance liquid chromatography) and

semi-quantitative tests (e.g., imaging with fluorescent labeling, TOF-SIMS) [5-7, 11]. However, these methods are generally used for liquid formulations or simple systems and cannot readily be used for the evaluation of products such as creams, which are complex systems with highly viscous formulations.

This study used TOF-SIMS to investigate whether the addition of a penetration enhancer could improve the penetration of TXA aqueous solution (Figure 1). TOF-SIMS is a useful, highly sensitive method of imaging in which a solid surface is irradiated with low-energy primary ions, and the secondary ions that are emitted are analyzed by a time-of-flight mass analyzer. However, it cannot readily be used for multiple component systems such as creams (data not shown).

We therefore focused on Raman imaging, which yields a wealth of molecular information from the active ingredients and skin. Previous studies have reported that it is possible to evaluate the distribution of retinol and flufenamic acid in the skin using Raman imaging [14, 15]. However, in the present study, the peaks derived from TXA were hardly detected at all in the skin (Figure 5b). It is likely that because skin and cream comprise a multi-component system, the weak TXA spectra were buried in other spectra and could not be detected. Previous studies suggest that the detection of a component with strong spectral intensity in a single-component system is technically possible.

In this study, we explored the use of a novel skin permeation evaluation method combining Raman imaging with multivariate curve resolution-alternating least squares (MCR-ALS) analysis. MCR-ALS allows for detailed imaging of various molecular components even from overlapping spectral sets by decomposing the entire spectral region as a pattern, rather than focusing only on a few strong bands. In this study, particularly, clear spectral decomposition was made possible by utilizing the pure spectrum of the target.

For this analysis, it is important to first acquire a reliable pure spectrum of the target. While spectral information can be easily obtained from crystals, the characteristics differ depending on the molecular environment and intermolecular interactions, and in this study, TXA was thought to be closer to a dissociated ionic state in aqueous solution (Figure 2 and 3). Through analysis by MCR-ALS with this pure spectrum as the target, the penetration of TXA was confirmed even in a multi-component system (Figure 5d). Although not statistically significant, the enhancement of permeation by the penetration enhancer was also observed (Figure 6a and 6b). This lack of significant difference was considered to be partly due to variations in the preparation of skin sections. Observation of the sections confirmed that the cross-section of the skin was not perfectly smooth. Since Raman intensity is highest at the outermost surface of the target substance, it is necessary to improve accuracy by creating smooth cross-sections or using autofocus functions. Furthermore, cream formulations contain many components related to permeability, such as polar oils, which may have made the effect of the penetration enhancer less apparent than in aqueous solutions.

In the future, we aim to improve measurement techniques such as section smoothing and autofocus to enable a more accurate understanding of the permeation behavior of components in the skin. In this study, imaging from within the skin became possible even for components with weak Raman spectra. We plan to utilize this as a fundamental technology for designing formulations that deliver various beneficial components to optimal sites, not limited to TXA.

5. Conclusion

The use of Raman imaging and multivariate spectral analysis allows imaging of a range of target components within the skin. We plan to use this technology to gain a better understanding of the behavior of components in the skin to promote the development of products with greater safety and functionality that exceed the expectations of consumers.

6.References

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