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A Novel Method for Evaluating Water in SC —Visualization of Water Using "Ultra"- Low Temperature DSC—

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1. Introduction

Maintaining skin homeostasis is closely related to the water content within the stratum corneum (SC). Healthy SC contains approximately 20 to 30% water, which plays a crucial role in maintaining skin hydration, flexibility, and barrier function [1].

Although the exact mechanism by which water contributes to the plasticity of the SC remains unclear, the involvement of bound water is indicated [2]. Water in the SC can be broadly classified into two types: "free water" and "bound water." Free water easily evaporates with changes in temperature and humidity, whereas bound water, which molecularly binds to the components of the SC, tends to evaporate less. Consequently, approaches to increase bound water in the SC and methods for quantifying bound water have been actively researched [3-5].

DSC is an established method for quantifying bound water in the SC. This method generally observes and quantifies the crystallization behavior of water at approximately 0°C to -40°C, or the evaporation behavior of water above 0°C. Furthermore, Takenouchi *et al*. have enabled the distinction and quantification of primary bound water, which binds strongly to substances, and secondary bound water, which binds loosely [4]. However, the methods for quantifying primary and secondary bound water vary across literature, and standardized measurement criteria have yet to be established. Moreover, the detailed structure of water in the SC has not been extensively discussed in conventional DSC methods.

In recent years, detailed structural analysis of water has become possible in the field of medical polymer materials. By observing hydrated polymer materials under ultra-low temperature conditions of 0 to -150°C using DSC, a method for classifying water surrounding polymers into three types based on different binding states has been reported [6,7]. Figure 1 illustrates the differences between the newly defined thermodynamic definition of water and the conventional definition of bound water. The most notable point is that water previously defined as "bound water" can be classified into three states in order of binding strength: "non-freezing water (NFW) ", "intermediate water (IW) " and "free water (FW) ".

Recently, the presence of IW on the molecular surfaces of biological components such as proteins and DNA has been suggested [8]. For example, IW is abundant on the surface of vascular endothelial cells, where repulsive forces between IW layer and foreign substances contribute to anti-thrombosis and anti-coagulation effects [9,10].

In this study, we focused on the previously unexplored existence of "intermediate water (IW)" and aimed to optimize the IW within SC to promote healthy skin. We termed the DSC analysis of the SC and cosmetic ingredients at ultra-low temperatures down to -150°C as the "ultra"-low temperature DSC method. This research provides a new perspective on the analysis of IW using the ultra-low temperature DSC method and aims to enhance our understanding of the role of IW within the SC.

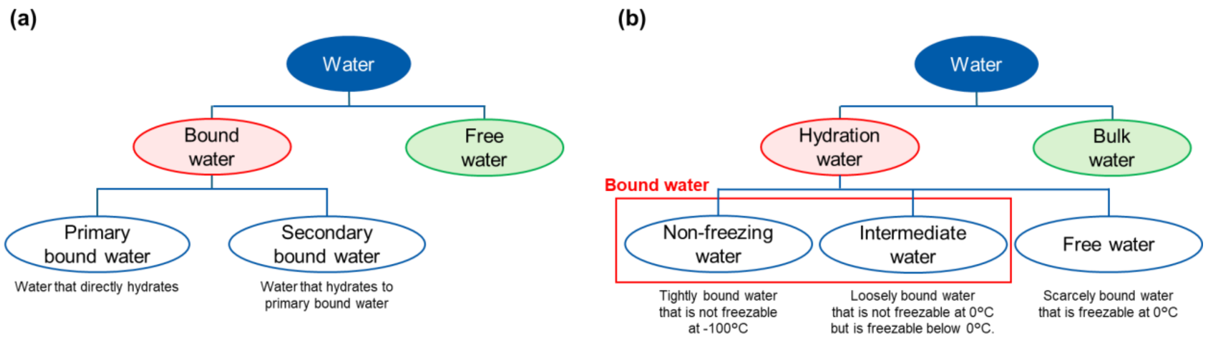

Figure 1. Definitions of water in various fields. **(a)** Conventional classification in the cosmetics field. **(b)** Thermodynamic classification.

2. Materials and Methods

2.1. DSC Samples

2.1.1. Aqueous Solution

Glycerin was purchased from Sakamoto Yakuhin Kogyo Co., Ltd. (Lot. 231201SB), Butylene Glycol (BG) was purchased from Daicel Corporation (Lot. BGUK-EE-039), Trehalose was purchased from Nagase Viita Co., Ltd. (Lot. 2D111, 24G291), and Sodium PCA was purchased from AJINOMOTO CO., INC. (Lot. 2308059B). All other ingredients were used as purchased. Each ingredient was prepared as an aqueous solution at the specified concentration. Samples of 2-5 mg were placed in DSC aluminum pans and immediately sealed. When preparing samples with concentrations higher than the saturated solution, the saturated solution was placed in the pan, water was evaporated to the desired concentration, and then the pan was sealed.

2.1.2. Co-crystal Solution

Trehalose and Sodium PCA were mixed in arbitrary ratios, pre-frozen at -80°C in an ethanol bath, and then processed with a freeze dryer (FDU-2110, TOKYO RIKAKIKAI CO., LTD.) to produce co-crystals. The samples were vacuum degassed and stored frozen. The samples were prepared as aqueous solutions at the specified concentrations for measurements.

2.1.3. Stratum Corneum

SC sheets derived from the abdomen of Caucasian women were purchased from Biopredic International. After removing water from the SC sheets at a humidity of less than 10%, they were punched out using a 3 mm biopsy punch. A 1-2 mg piece of the SC sheet was placed in an aluminum pan, water was added to cover the entire sheet, and the pan was sealed and left to stand for more than one day.

2.2. Ultra-Low Temperature DSC

The amount of hydrated water in the samples was analyzed using a DSC (NEXTA DSC 600, Hitachi High-Tech Corporation). The samples were set to 25°C and then cooled at a rate of 5°C per minute to -100 or -120°C (cooling process). After holding the samples for 10 minutes, they were heated at a rate of 5°C per minute to 25°C (heating process). After the measurement, holes were made in the pans, and the samples were dried in a vacuum oven to remove water, and their weight was measured. This allowed for the determination of the water content (W_c) in the samples, where the water content is defined as follows:

$$W_c = m_1 – m_0 / m_1 \times 100$$

m_1 is the mass of the aqueous solution, m_0 is the mass of the solute placed on the pan. Hydrated water is classified into three states based on the definition in Figure 1: non-freezing water (NFW), intermediate water (IW), and free water (FW). The water content in the samples is expressed as follows:

$$W_c=W_{NFW}+W_{IW}+W_{FW}$$

W_{NFW}, W_{IW} and W_{FW} are the amount of NFW, IW and FW. The exothermic reactions occurring during the cooling and heating processes indicate the low-temperature crystallization of water, while the endothermic reactions occurring during the heating process indicate the melting of ice. The peaks of exothermic and endothermic reactions correspond to the low-temperature crystallization enthalpy (ΔH_{cc}) and the melting enthalpy (ΔH_m) of water, respectively. Using the enthalpy of water (334 J/mg), W_{NFW}, W_{IW} and W_{FW} can be expressed as follows:

$$W_{IW} = (\Delta H_{cc} / 334)$$

$$W_{FW} = (\Delta H_m / 334) - W_{IW}$$

$$W_{NFW} = W_c – (W_{IW} + W_{FW})$$

2.3. Structural Analysis of Cocrystals

The freeze-dried products of Trehalose and Sodium PCA were analyzed for their crystal structure using XRD measurements (XRDynamic500, Anton Paar GmbH). At room temperature, the samples were filled into the XRD cell and quickly smoothed. Using Cu Kα radiation (wavelength 0.154 nm), measurements were taken in the range of 2θ = 2°-70° with a step size of 0.01°. To eliminate the effects of surface irregularities on the samples, the parallel beam method was selected for the beam shape.

2.4. Human Trial (SC Hydration Measurement)

A three-day continuous use trial was conducted with 9 subjects (mean age, 40.4±12.2 y.o.; 6 females and 3 males). After facial cleansing, subjects acclimated for 20 minutes under conditions of 21°C and 45% humidity. We measured the initial SC hydration levels and obtained the moisture content images using a moisture imaging system (Epsilon E 100, Biox Systems Ltd.). Subjects applied the test product twice daily. After three days, hydration levels were measured again. The average usage was 5.00±0.38 g.

3. Results and Discussion

3.1. Ultra-Low Temperature DSC

Figure 2 shows the heating chart from ultra-low temperature DSC (-100 or 120°C to 25°C) of glycerin aqueous solutions. Since water melts at 0°C, an endothermic peak appears near 0°C in DSC. As glycerin concentration increases, the melting peak shifts to lower temperatures because water molecules bind to glycerin, disturbing the ice crystal structure and causing it to melt at temperatures below 0°C. Therefore, ultra-low temperature DSC chart can be used to determine the state of water near glycerin. The endothermic reaction at 0°C in the 20% glycerin solution indicates the presence of FW with weak binding strength. When glycerin concentration is 22 to 60%, all water melts below 0°C, indicating the absence of FW and the presence of IW and NFW around glycerin. The absence of crystallization and melting peaks at concentrations above 65% suggests strongly bound water that cannot freeze, classified as NFW. In summary, water near glycerin can be categorized as follows (Figure 3):

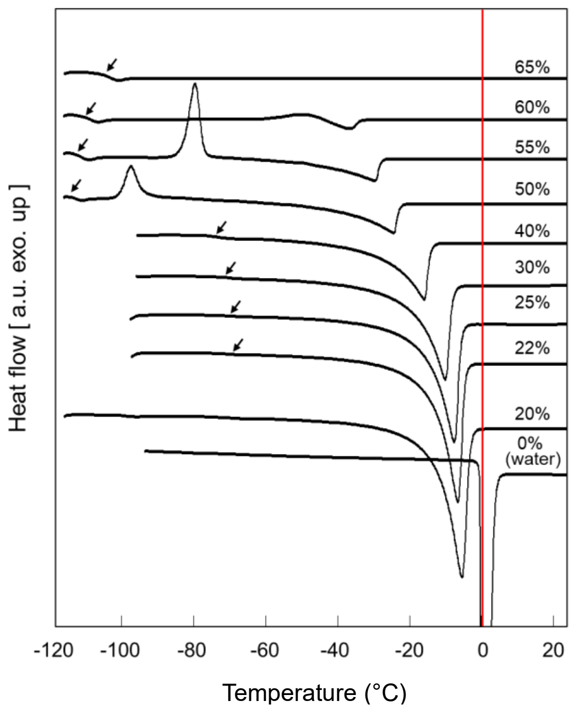

Figure 2. Stacked ultra-low temperature DSC heating curves of glycerin aqueous solutions ranging from 0 to 70% (-100 or 120°C to 25°C). The red line indicates the 0°C. The arrows show the glass transition point (T_g) of the water.

Traditionally, DSC used for the quantification of water have generally been observed in the range of approximately 0 to -40°C , or above 0°C [4,5]. However, there are many cosmetic ingredients, such as glycerin, that form low-temperature crystals below -40°C. Additionally, the baseline shift observed below -100°C indicates the glass transition point of the water-glycerin system. Water that crystallizes at low temperatures during the cooling process undergoes glass transition during the heating process, enhancing molecular mobility before melting. Therefore, the glass transition temperature (T_g) is also an important piece of information for observing the state of water. By using ultra-low temperature DSC, which observes temperatures far below -40°C, it has become possible to reveal the presence of IW that was previously overlooked and to gain a deeper understanding of the binding state of water.

	The behavior of crystallization and melting peaks		
	With peak The melting peak overlaps at 0° C	With peak The melting peak is below 0° C	Without peak
Glycerin aqueous solutions	- 20%	22% - 60%	65% -
The state of water molecules	FW+IW+NFW	IW+NFW	NFW

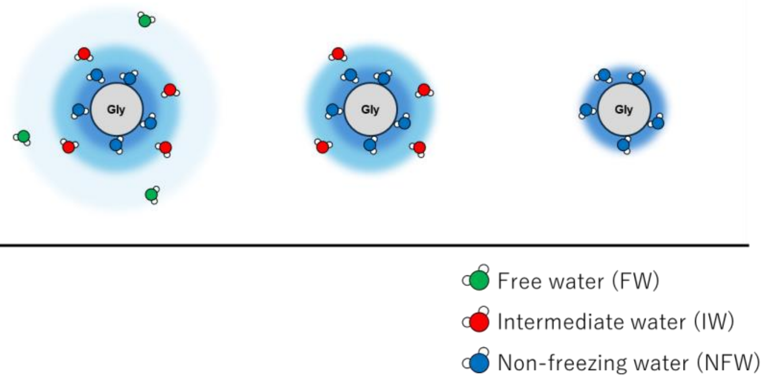

Figure 3. The state of water surrounding glycerin can be inferred from the behavior of melting peaks in ultra-low temperature DSC measurements.

3.2. Screening Raw Materials

We measured moisturizers using ultra-low temperature DSC and quantified IW and NFW.

(a) Glycerin
The phase diagram of glycerin is shown in Figure 4(a). As mentioned above, glycerin retains IW in aqueous solutions of 22-60%. In other words, the water content at which glycerin contains intermediate water (C_{IW}) is 40-78%. Additionally, the maximum amount of intermediate water that glycerin can retain (M_{IWmax}) was 10.79 mol.
(b) Polyols
Next, other commonly used polyols besides glycerin were analyzed. The phase diagram of BG is shown in Figure 4(b). For BG, the C_{IW} is 43-75% (25-57% aqueous solution), and its IW retention range is narrower than glycerin. The M_{IWmax} of BG was 7.79 mol, indicating that BG retains less IW than glycerin. Assuming that each component continues to retain IW on the skin or within the SC when applied, glycerin, with a higher M_{IWmax}, can provide more IW to the skin than BG. Furthermore, since glycerin has a wider IW region than BG, it is expected to provide IW for a longer period during the evaporation process on the skin. Thus, both M_{IWmax} and C_{IW} are crucial for evaluating raw materials, and components with high M_{IWmax} and wide C_{IW} are preferable. Other polyols (Pentylene Glycol, Dipropylene Glycol, Diglycerin, etc.) were also analyzed, and none had both a higher M_{IWmax} amount and a wider C_{IW} than Glycerin.
(c) Sugar and sugar derivatives
Next, sugar and sugar derivatives were analyzed. The phase diagram of trehalose is shown in Figure 4(c). Trehalose has an M_{IWmax} of 15.13 mol and a C_{IW} of 29-49% (51-71% aqueous solution), retaining more IW than glycerin but with a narrower IW region. The binding sites of NFW and IW have been largely identified in previous research [6]. NFW is likely to bind to hydroxyl groups and ether oxygen, while IW is likely to form hydrogen bonds with carbonyl oxygen and NFW. Molecules with more hydroxyl groups tend to have a higher M_{NFWmax}. For

example, PPG-14 Polyglyceryl-2 Ether, with many hydroxyls and ether oxygen groups, has an M_{NFWmax} of 34.72 mol and an M_{IWmax} of 26.74 mol, making it rich in NFW. In contrast, sugars such as trehalose, despite having many hydroxyl groups, tend to have a lower M_{NFWmax} relative to M_{IWmax}. This suggests that the stereochemical configuration of the sugar backbone contributes to intermediate water retention. The ability of trehalose, which has long been widely used as a food additive, is speculated to be due to its capacity to retain a significantly larger amount of intermediate water compared to NFW.

(d) Sodium PCA

Figure 4(d) shows the phase diagram of Sodium PCA, which has an M_{IWmax} of 20.73 mol and a C_{IW} of 55-80% (20-45% aqueous solution). This indicates that Sodium PCA retains more IW than glycerin but has a narrower IW region, similar to trehalose. Trehalose retains IW in low water content regions, while Sodium PCA retains IW in high water content regions. Molecules with sodium ions tend to retain more NFW and IW, especially under high water content conditions (e.g., sodium lactate). Sodium PCA, a key component of natural moisturizing factors (NMF), is present in the SC at a few percent (NMF makes up 30% of the SC, and PCA constitutes 12% of NMF). The high M_{IWmax} of Sodium PCA suggests it plays a crucial role in water retention in the SC.

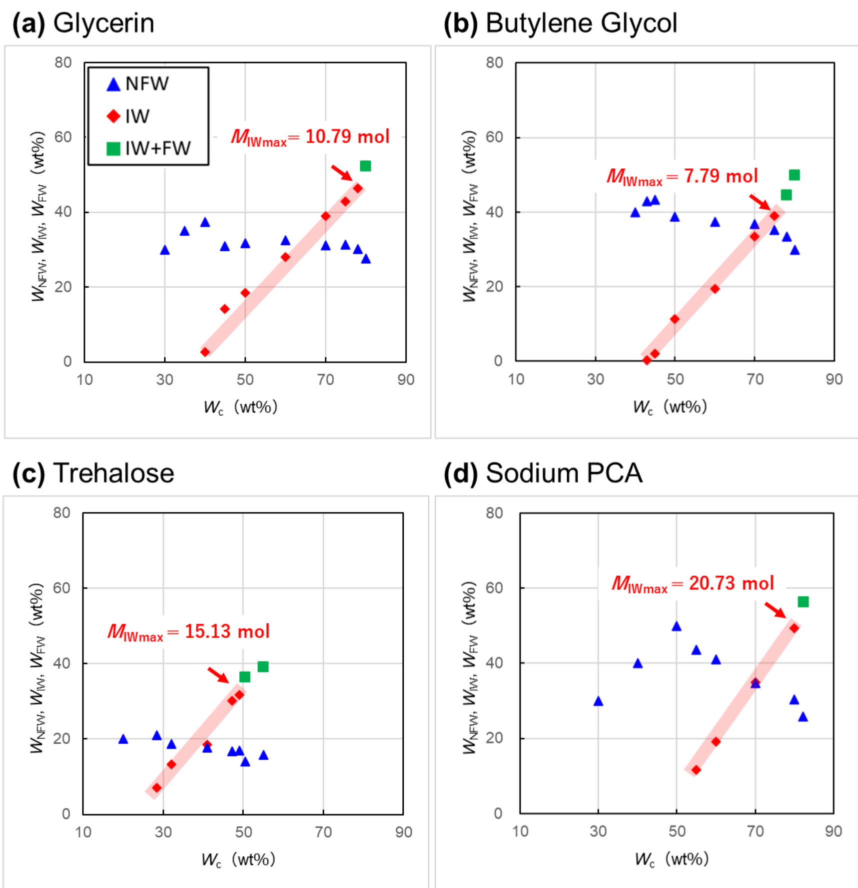

Figure 4. Phase diagrams of **(a)** Glycerin, **(b)** Butylene Glycol, **(c)** Trehalose and **(d)** Sodium PCA where the blue markers represent the amount of non-freezing water (W_{NFW}), the red markers represent the amount of intermediate water (W_{IW}), and the green markers represent the amount of intermediate water and free water (W_{IW} + W_{FW}). W_C represents the water content of the system. The red arrows indicate the maximum amount of IW that a substance can retain (M_{IWmax}). The concentration conditions at which each raw material holds IW (C_{IW}) are highlighted in red.

3.3. Evaluation of IW in Co-Crystals

Trehalose and Sodium PCA retain significant amounts of IW, but under narrow conditions. Trehalose retains IW at low water content, while Sodium PCA retains it at high water content. When mixed in a 1:1 ratio and freeze-dried, XRD patterns shown in Figure 5 indicated that simple mixture resulted in peaks from individual crystal structures. In contrast, freeze-drying led to new peaks, suggesting a different crystal structure. Figure 6 shows the phase diagrams of IW and NFW amounts for both the simple mixture and the freeze-dried product. As a result, it was revealed that the M_{IWmax} of the simple mixture was the average of the two components, with a slightly expanded C_{IW}. The freeze-dried product exhibited increased M_{IWmax} and expanded C_{IW} to 33-73%.Co-crystallization during freeze-drying, involving hydrogen bonding between Trehalose's -OH groups and Sodium PCA's carboxyl groups, as well as ionic bonding with sodium ions, is expected. Due to the disappearance of those structures that strongly bind to water, the binding strength with water is loosened, leading to an increase in the amount of IW.

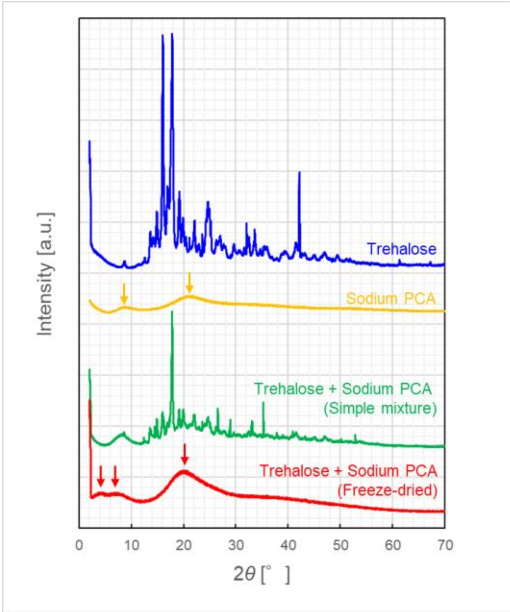

Figure 5. The diffraction peaks obtained from XRD measurements. The measurements were conducted in the 2θ range of 2°-70° with a step size of 0.01°. The arrows indicate the characteristic peaks for each sample.

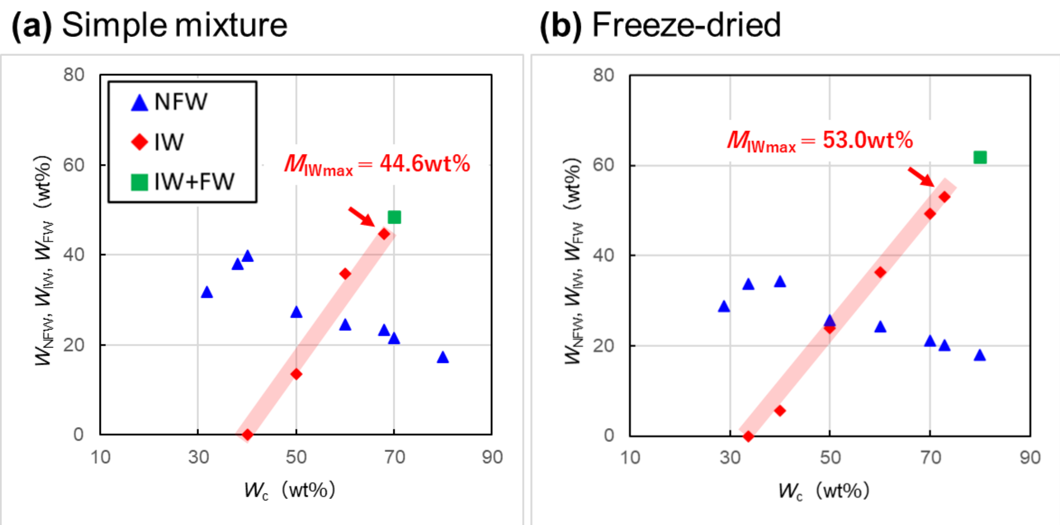

Figure 6. Phase diagrams of **(a)** simple mixture and **(b)** freeze-dried product of trehalose and PCA-Na mixed at a weight ratio of 1:1. The blue markers represent W_{NFW}, the red markers represent W_{IW}, and the green markers represent W_{IW} + W_{FW}. W_{C} represents the water content of the system. The red arrows indicate M_{IWmax}. C_{IW} are highlighted in red.

In the current study, the detailed crystal structure of the freeze-dried product of Trehalose and Sodium PCA has not been fully elucidated. Therefore, more precise analytical methods will be employed in future research to further identify the crystal structure.

3.4. Evaluation of Intermediate Water in Human SC

We observed the state of water in the SC of young individuals (20-30 y.o., *n*=3) and aged individuals (50-60 y.o., *n*=3). Figure 7(a) shows the ultra-low temperature DSC charts with a water content of approximately 20-30%. Crystallization of IW-derived water was confirmed in both groups. Young individuals' SC showed a sharp peak during cooling, while aged individuals' SC showed a gentle peak and a lower melting peak during heating. These differences are due to the crystalline structure of water. Weakly bound water forms a hexagonal structure and crystallizes rapidly around 0°C, while non-hexagonal structures crystallize slowly at lower temperatures, causing gentler peaks. Although there were no significant differences, young individuals tended to have more IW and less NFW. This suggests that aged individuals' SC binds water more strongly, while young individuals' SC binds water more loosely. NFW is strongly bound to substances and does not freeze even at -100°C, acting as part of the substance rather than as water. IW, which is loosely bound, can function as a solvent while constantly exchanging with bulk water. Thus, "loosely" bound water with solvent effects is key to skin hydration, barrier function, and cellular activities.

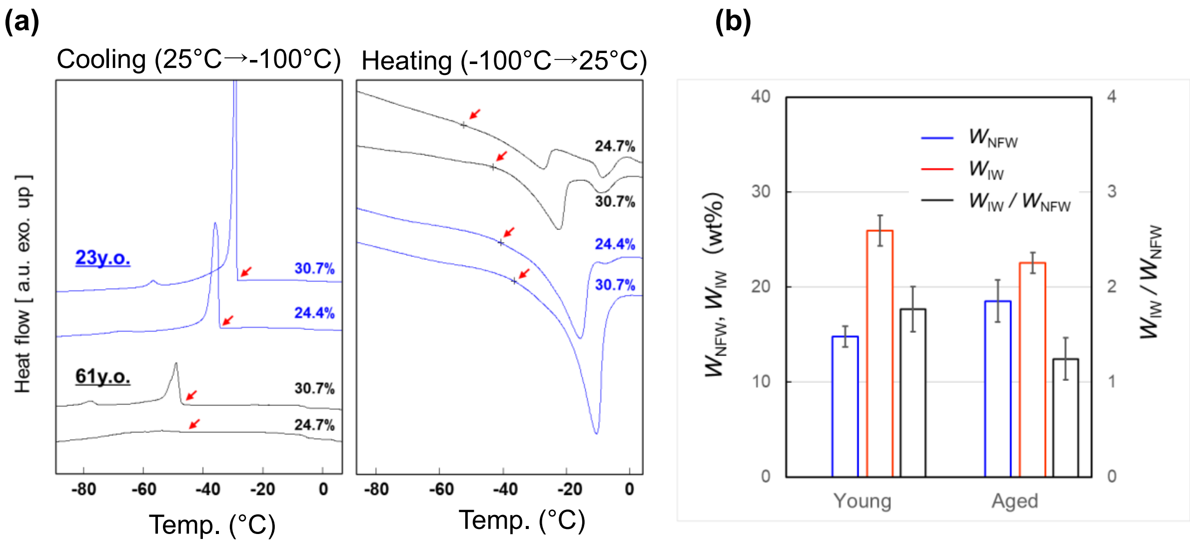

Figure 7. (a) Ultra-low temperature DSC charts at a water content of approximately 20-30%, measured from the SC of individuals 23 y.o. and 61 y.o. The red arrows indicate the point at which freezing or melting begins. **(b)** The average value of NFW, IW, IW/NFW at a water content of approximately 40% in the SC of young and aged individuals represented in blue, red, and black, respectively.

3.5. Human Trial of Co-crystal

When a model lotion containing the co-crystal of Trehalose and Sodium PCA, as shown in Table 1, was applied to the skin, SC hydration levels approximately doubled after three days of continuous use, showing a significant increase (p<0.01). The co-crystal of Trehalose and Sodium PCA has a wide C_{IW} of 33-73% and a high M_{IWmax}. Therefore, it is believed that it retains a large amount of intermediate water for a long time during the process of penetrating the SC immediately after being applied to the skin, resulting in an increase in SC hydration levels.

Table 1. Formulation of a model lotion

Sample Information	
Butylene Glycol	5.0%
PEG-60 Hydrogenated Castor Oil	0.1%
Trehalose / Sodium PCA (Co-crystallization)	6.0%
Water	Up to 100%

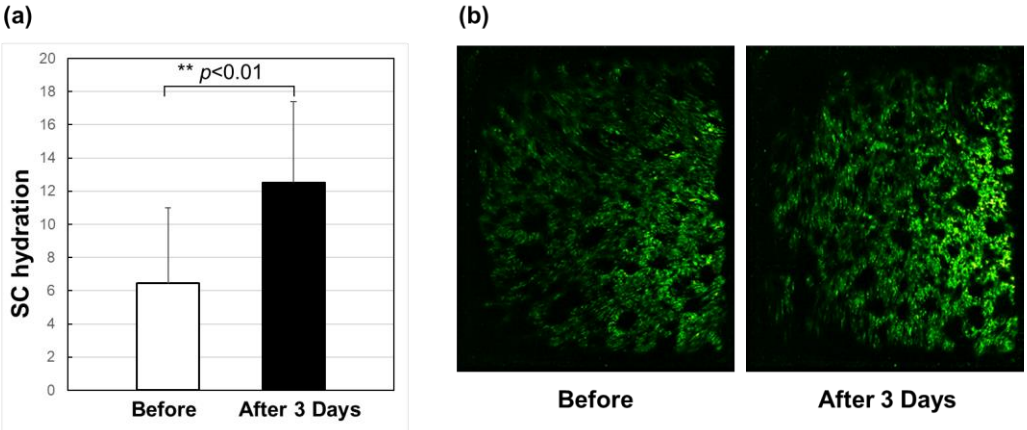

Figure 8. SC hydration levels **(a)** and moisture content images **(b)** obtained using a moisture imaging system (Epsilon E 100, Biox Systems Ltd.) before and after the trial. The average usage was 5.00±0.38 g.

4. Conclusion

In this study, we used a novel thermal analysis method called "ultra"-low temperature DSC to analyze the binding states of water in the SC. We discovered that water previously recognized as "bound water" actually exists in three states: non-freezing water (NFW), intermediate water (IW), and free water (FW). The analysis revealed the presence of IW in the SC and further inferred that there are differences in the binding states between younger and older individuals, with younger individuals having more loosely bound IW. We believe that this "loosely" bound water acts as a solvent around SC, mediating various chemical reactions and maintaining a healthy SC state. IW is constantly exchanged between the SC and the atmosphere, making it difficult to capture its actual state, and its existence was unexplored. However, in this study, its presence was confirmed under ultra-low temperature conditions of 25°C to -100°C, and it was found to be quantifiable in a very simple manner.

Furthermore, this study evaluated moisturizing ingredients using ultra-low temperature DSC and found that Trehalose and Sodium PCA retained more IW than glycerin. The application of trehalose and sodium PCA mixtures suggested an increase in SC moisture content. This is believed to be due to the application of cosmetics that retain more IW, supplementing loosely bound water on the skin and within the SC.

Previously, moisturizing approaches aimed at simply increasing the "amount of bound water" in the SC. However, this study revealed the diversity of water states, suggesting the need for new moisturizing approaches that focus on the "quality of bound water" in the SC. Moving forward, we will conduct further detailed analyses of the binding states of water in the SC and examine how the moisture state in the SC changes with the application of cosmetics. Additionally, we will evaluate the effects of IW on moisturizing, elasticity, and cellular activity from a broad perspective.

This moisturizing approach will lead to the development of novel cosmetic formulations that fundamentally understand the moisture state desired by the SC and to appropriately replenish it.

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