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# *"Intermediate" water is beneficial to the skin -Unveiling the important role of a long-overlooked state of water-*

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## 1. Introduction

"Water" is an indispensable element for skin moisturization, and as a result, there are numerous research reports related to water. In particular, studies on bound water in the stratum corneum have been conducted for a long time [1, 2], with reports also linking the application of glycerin and poly(2-methacryloyloxyethyl phosphorylcholine) to improvements of the barrier function in relation to bound water [3, 4].

In recent years, in the research of hydrogels made from medical polymer materials, a classification method using thermodynamic approaches has been proposed to analyze the state of water in more detail, with reports indicating that water can be classified into three states: non-freezing water, intermediate water, and free water [5]. Among these, the binding state of intermediate water, characterized by loose binding, has been shown to play an important role at the interfaces of biological systems [6-8], leading to an increasing recognition of the significance of this new state of water.

Meanwhile, methods for measuring skin have evolved significantly where in vivo confocal Raman spectroscopy can non-invasively provide depthwise information regarding moisture levels, lipids, amino acids, and other constituents in the skin [9]. Additionally, methods have been developed that leverage peak separation techniques in Raman spectra to identify 3 to 5 different types of water states based on differences in O-H stretching vibrations, allowing for the non-invasive analysis of both moisture content and the binding states of water [10, 11]. Figure 1 illustrates the classification of water states using Raman spectroscopy. To date, weakly bound water was not clearly defined in traditional classifications, but thermodynamic classification can evaluate it as intermediate water.

In studies involving human skin using in vivo confocal Raman spectroscopy, attempts have been made to classify the moisture states in the stratum corneum into five binding states of water (free water, DDA (slightly bound), DA (weakly bound), DDAA (strongly bound), and

DAA (tightly bound)) [12]. It has been revealed that younger individuals tend to have higher DA/DDAA ratios compared to older individuals [13]. Reports have indicated that the changes in moisture retention function due to aging are related to structural changes in stratum corneum proteins [14], suggesting that by inhibiting structural changes in these proteins or by maintaining a high DA/DDAA state in the stratum corneum, it may be possible to achieve healthy skin akin to younger individuals.

With the proposal of new classification methods through thermodynamic analysis and Raman spectroscopy, the existence of previously overlooked states of water (intermediate water and weakly bound water) is likely to play an important role in maintaining skin homeostasis. Therefore, in this study, we aimed to clarify the roles of these states of water in the skin, focusing on the age-related differences in DA/DDAA, and confirmed the impact of skincare ingredients on the increase of DA/DDAA in the stratum corneum through Raman spectral analysis.

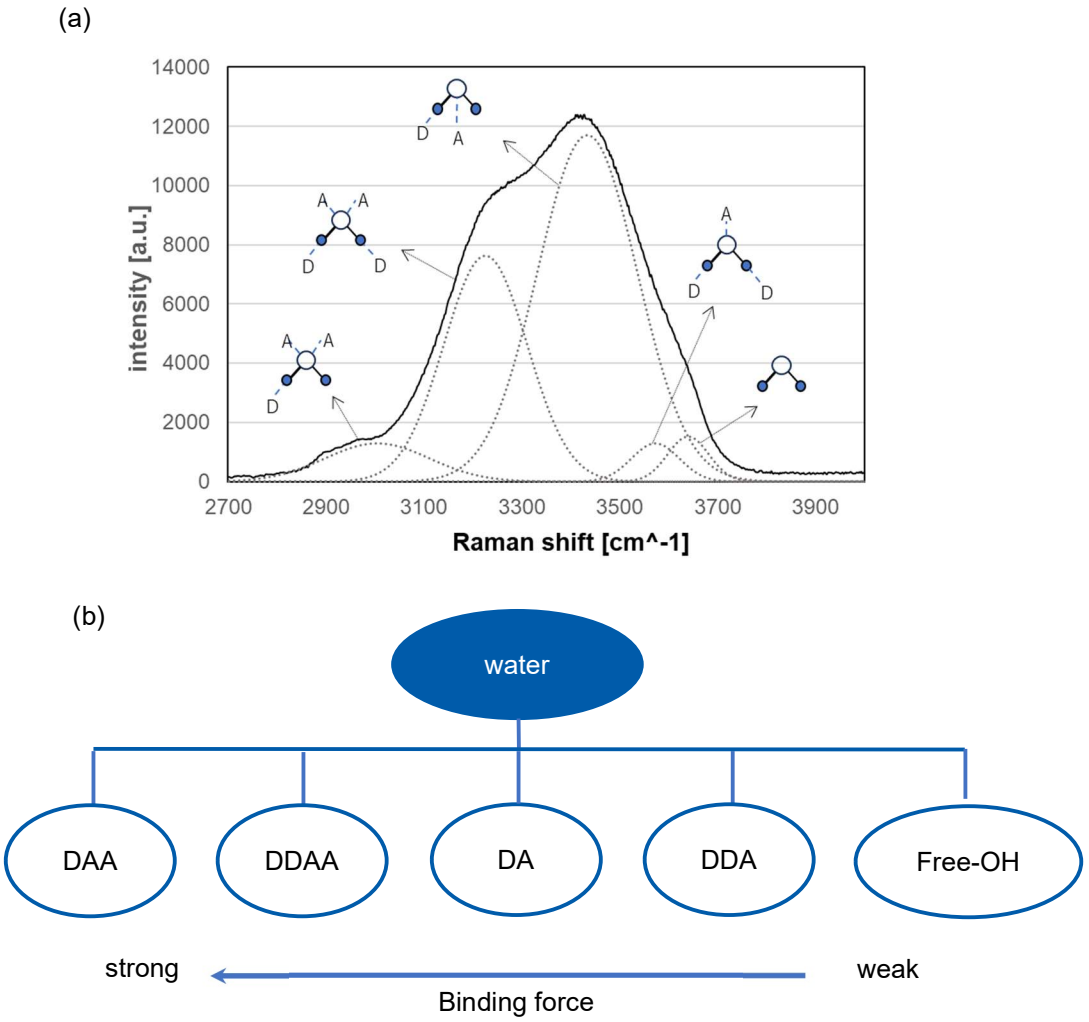


**Figure 1:** (a)The obtained Raman spectrum (solid line) was separated into five peaks (dashed line) through spectral analysis. (b)In water dimer, consisting of two nonequivalent water molecules, the hydrogen bonding can be differentiated into D (proton donor) and A (proton acceptor).

## 2. Materials and Methods

### 2.1 Material Evaluation

Glycerin, BG, Sodium PCA, Ectoine, Maltose, and Trehalose were prepared as samples at concentrations of 10, 20, 40, and 60 wt% a.q. Each sample was measured using an in vivo confocal Raman spectrometer (gen2-SCA, RiverD International B.V., Netherlands) in moisture content measurement mode with a laser wavelength of 617 nm. The measurements were conducted in a controlled laboratory environment at a temperature of 22 ± 3°C and a humidity of 40-60%.

### 2.2 Human Study

These studies were conducted in accordance with the principles of the Declaration of Helsinki. Six volunteers (three males and three females) participated in the study. The equipment used, settings, and laboratory environment were consistent with those described in section 2.1. The study protocol is outlined below.

A 1 cm × 1 cm area was marked on the inner forearm, which had been washed only with purified water. After a 15-minute acclimatization period, the initial state was measured. A cotton patch, cut to 1 cm × 1 cm and saturated with the formulation, was then applied, and it was occluded using a transparent film (30 μm thick, made from stretchable polyurethane film). After two hours, the cotton and film were removed, and without rubbing the skin, the formulation on the skin surface was wiped away with a lint-free laboratory wiper, followed by measurement of the skin after application. The applied sample consisted of 20% trehalose, 20% Sodium PCA, and 60% purified water.

Moisture content from the skin surface to a depth of 20 μm was measured in increments of 0.5 μm. The acquired data were analyzed using Skin Tools (RiverD International B.V., Netherlands). The thickness of the stratum corneum at each point was automatically calculated by the device based on the ratio of keratin to moisture content.

### 2.3 Data Analysis

Spectral analysis was performed using the software GRAMS AI (Thermo Fisher Scientific Inc., USA) to separate the peaks. First, baseline correction was applied to the obtained Raman spectra. The baseline was manually adjusted based on the linear and stable portion on the high wavenumber side beyond 3800 $cm^{-1}$. Then, the region of approximately 3000 to 3800 $cm^{-1}$, which was necessary for the analysis in this study, was manually extracted, and the peak fitting function was used to separate it into five peaks. The peak tops were specified to correspond to 3005, 3226, 3434, 3573, and 3640 $cm^{-1}$ based on prior literature [12]. To prevent analysis issues, positive peak area values were specified for all peaks. The accuracy of peak separation was visually confirmed by ensuring that the "measured spectrum" and the "summed spectrum of the separated five peaks" did not significantly deviate from each other.

In this research, the focus was on analyzing the ratio of DDAA to DA (DA/DDAA). Additionally, the sum of the two peaks with weak binding (DDA + Free OH) and the overall moisture content (ALL = DAA + DDAA + DA + DDA + Free OH) was also calculated.

### 2.4 Significance Testing

In the material evaluation, changes in DA/DDAA values with concentration were evaluated using a Student's t-test. In the human evaluation, the DA/DDAA values before and after the application of the formulation were assessed using a paired t-test. A p-value ≤ 0.05 was

considered "significant," a p-value ≤ 0.1 indicated "a tendency toward significance," and a p-value > 0.1 was regarded as "no significant tendency."

## 3. Results and Discussion

### 3.1 Spectral Analysis by Raman Spectroscopy

The method of analyzing the results obtained from Raman spectral analysis will be explained using BG as an example. Figure 2 shows the data obtained from the analysis, separated using the method described in section 2.3. These peaks indicate DAA, DDAA, DA, DDA, and Free OH from the low wavenumber side. Additionally, analyses were conducted for each concentration (20 wt%, 40 wt%, and 60 wt%) using a similar method, and the area values of each peak were calculated.

Figure 3 presents the values of DA/DDAA and DDA + Free OH/ALL. DA/DDAA reflects the ratio of weak binding to strong binding, while DDA + Free OH/ALL indicates the proportion of free water. As the concentration of the raw material increases, both DA/DDAA and DDA + Free OH/ALL values tend to decrease. This trend aligns with the decrease in the proportion of water, indicating that free water and water with weak binding are diminishing as the concentration increases.

Furthermore, a study analyzing ice reported that the proportion of DDAA increased with the cooling of water [15], suggesting that suppression of OH stretching indicates stronger binding, and therefore, it is believed that this evaluation accurately reflects the state of water.

Using this method, an evaluation of commonly used cosmetic ingredients was conducted.

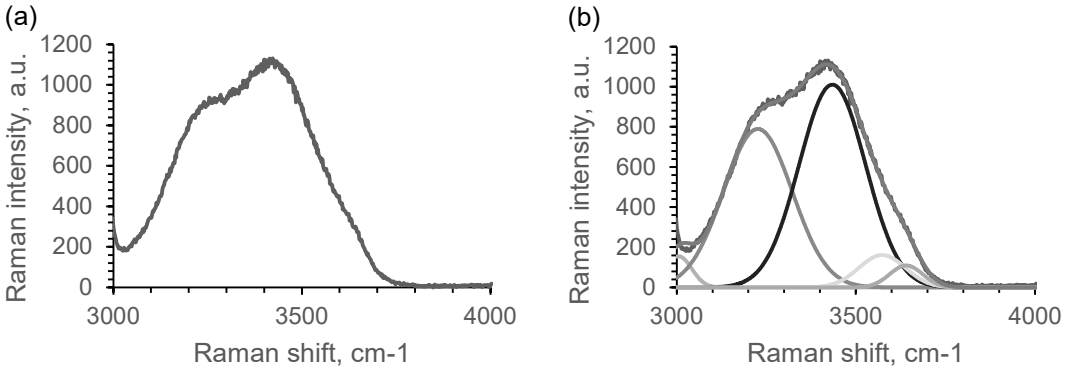


**Figure 2:** (a) Raman Spectrum of BG at 20 wt%, (b) Peak Separation of the Same Spectrum Data through Curve Fitting.

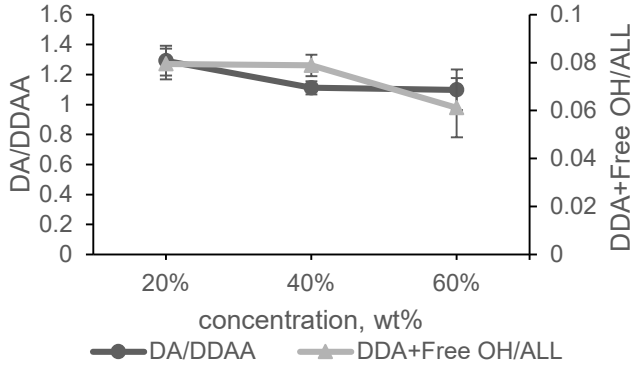


**Figure 3:** DA/DDAA and DAA + Free OH/ALL at Different BG Concentrations.

### 3.2 Material Evaluation

Similar measurements and analyses were conducted on polyols commonly used as moisturizing agents in cosmetics (glycerin, BG), amino acid-based moisturizers (Sodium PCA, Ectoine), and disaccharides (maltose, trehalose). Analyses were performed for each ingredient at concentrations of 20, 40, 60, and 80 wt%, and the values of DA/DDAA and DDA + Free OH/ALL were calculated (Figure 4). However, due to solubility limitations, trehalose was analyzed at a maximum concentration of 40 wt%. Additionally, considering that healthy stratum corneum contains approximately over 30% moisture [16], in this study, a maximum concentration of 60 wt% for the raw materials was employed.

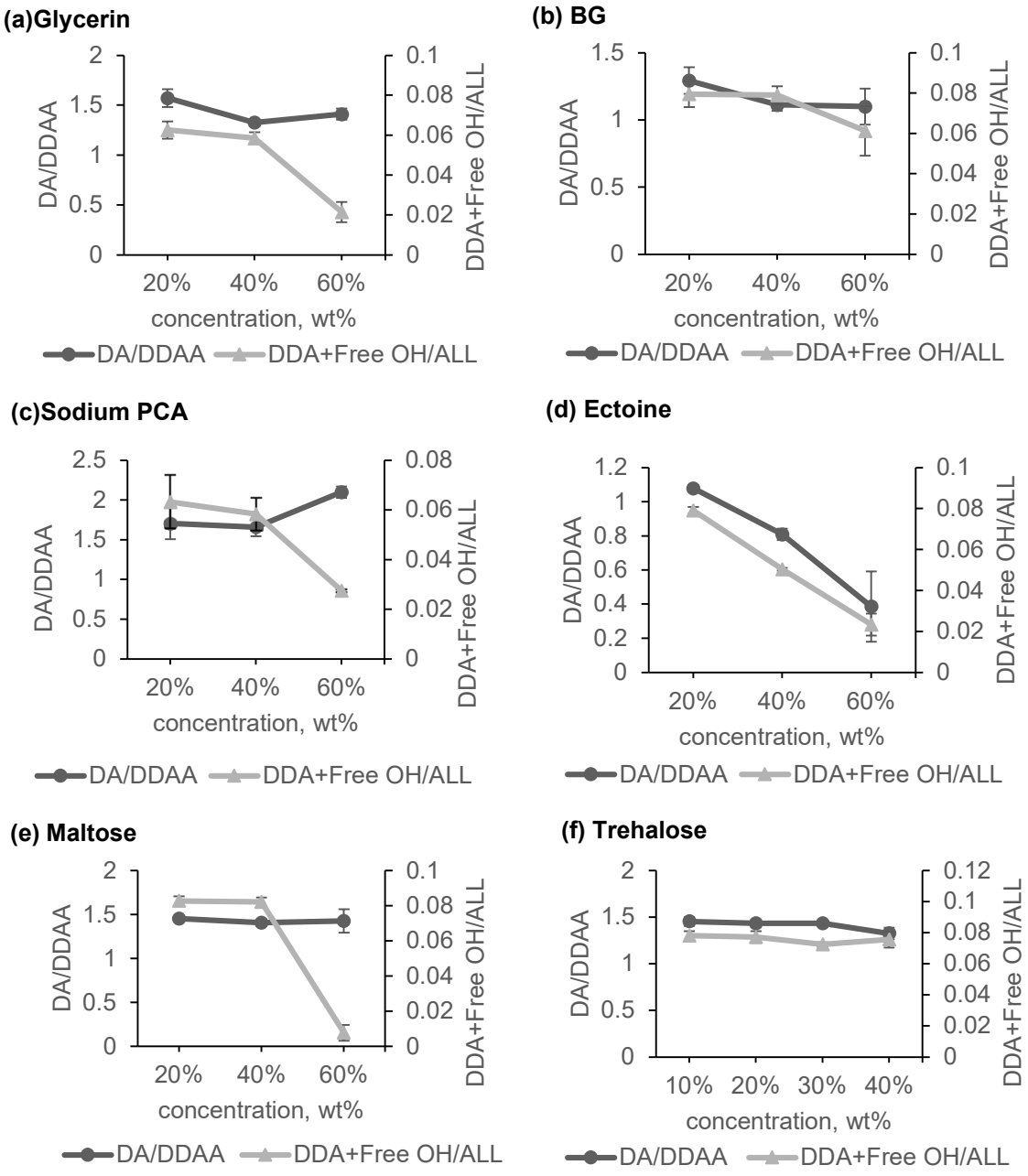


**Figure 4:** DA/DDAA and DAA + Free OH/ALL at Each Concentration for Each Ingredient (a) Glycerin, (b) BG, (c) Sodium PCA, (d) Ectoine, (e) Maltose, (f) Trehalose.

The results of DA/DDAA and (DDA + Free OH)/All for each ingredient are presented in Figure 4.

Regarding DA/DDAA, glycerin, BG, and Ectoine showed a significant decrease with higher concentrations, while Sodium PCA exhibited a significant increase. In contrast, no significant differences were observed for maltose and trehalose across concentrations. The results for the sugars within the measured range remained constant; however, it is anticipated that at higher concentration levels, they will exhibit a decrease similar to that of the other ingredients. Sodium PCA, on the other hand, showed opposite results. This is thought to be related to the suppression of hydrogen bonding by the salt, which leads to an increase in weak binding.

For (DDA + Free OH)/All, a general trend was observed where all ingredients demonstrated a decreasing tendency with increasing concentrations.

In light of the above results, we conducted human evaluations using compositions that were expected to yield higher DA/DDAA values. The water contained in the formulation decreases upon application to the skin due to evaporation and absorption, leading to concentration. Therefore, Sodium PCA, which possesses a high DA/DDAA value at higher concentrations, was selected. Additionally, by mixing it with an ingredient that does not change significantly in DA/DDAA values with concentration, trehalose was chosen with the expectation of stabilizing the DA/DDAA values at a high level. Consequently, a mixture of Sodium PCA and trehalose was used (Figure 5).

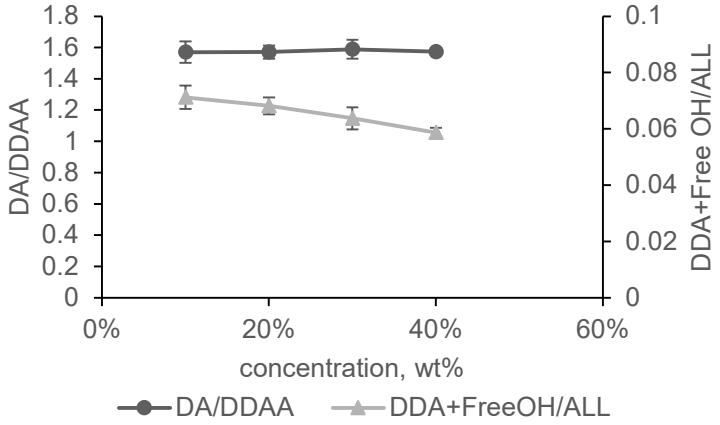


**Figure 5:** DA/DDAA and DAA + Free OH/ALL of the Mixture Containing 20 wt% Sodium PCA and 20 wt% Trehalose.

## 3.3 Human Evaluation

A formulation containing 20% Sodium PCA and 20% Trehalose was applied, and the DA/DDAA values at different depths in the stratum corneum before and after application were assessed using an in vivo confocal Raman spectrometer (Figure 6).

From Figure 6, it was observed that at a depth of 10% from the skin surface, the DA/DDAA values tended to be larger before the application compared to after ($p = 0.07$). In contrast, at a depth of 30%, there was a tendency for the DA/DDAA values to be larger after the application compared to before ($p = 0.055$).

The observed decrease in DA/DDAA values in the shallower stratum corneum could be explained by previous reports [17], which indicate that the application of amino acids tends to cause nearby amino acids in the stratum corneum to aggregate. It is probable that Sodium PCA played a similar role in this study, leading to the accumulation of amino acids with high DDAA within the stratum corneum at the surface after application.

Overall, these results suggest that applying a formulation with high DA/DDAA values can lead to an increase in DA/DDAA values in human skin as well.

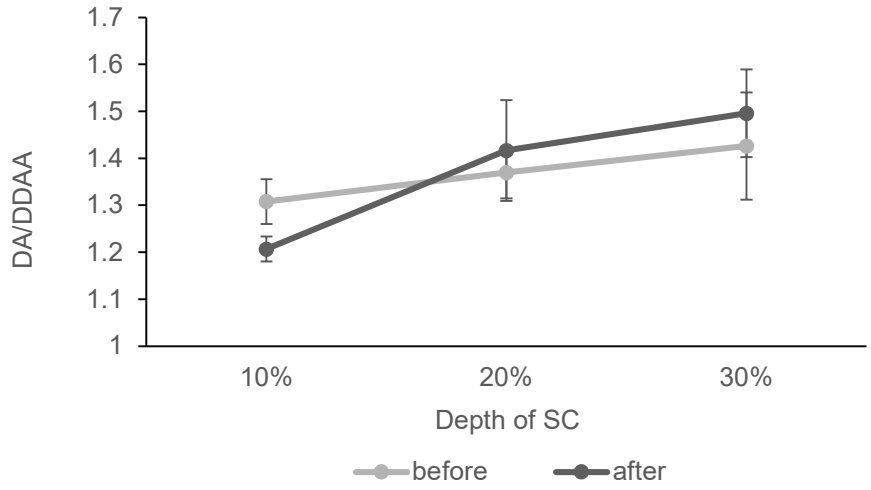


**Figure 6:** DA/DDAA Before and After Sample Application in the Depth Direction of the Stratum Corneum

## 4. Conclusion

We have developed a method using in vivo confocal Raman spectroscopy to further subdivide the "binding state of water," which has previously been categorized collectively as bound water, allowing for evaluation in the depth direction of the stratum corneum. Similarly, we have identified the hydration characteristics of ingredients using this detailed classification, demonstrating the possibility of controlling the state of water in the stratum corneum through the application of skincare agents that utilize these same ingredients.

We believe that previously overlooked weakly bound water in the stratum corneum plays a role in leading to skin conditions characteristic of younger individuals. Therefore, we are confident that this research will contribute not only to the development of ingredients containing abundant weakly bound water, but also to the re-evaluation of commonly used raw materials and the optimal combinations for the effective development of skincare products.

Moreover, by broadening our focus beyond the evaluation within the stratum corneum to include the epidermis and dermis, we recognize that water serves various roles not only as a moisturizing agent but also as a transport medium and reaction site within the body. The surrounding environment varies significantly across different layers of the skin—such as the stratum corneum, granular layer, spiny layer, basement membrane (epidermal stem cells), and dermis. Thus, it is thought that there exists an optimal binding state of water suited to the environment of each layer.

With further advancements in instrumentation in the future, as the accuracy of depth-direction data beyond the stratum corneum improves, it is anticipated that this will lead to the development of skincare products capable of controlling the optimal water state for each skin layer. We also foresee approaches to areas beyond moisture retention, such as targeting epidermal stem cells, leading to unprecedented proposals for skincare.

We plan to continue our research, recognizing that this weakly bound water is a significant factor in determining youthfulness and has the potential to act as a mediator for maintaining various bioactive states.

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