Natural regulation of skin hydration, a biological model to improve cosmetics efficiency and sustainability through biomimicry

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ABSTRACT

Background: We developed innovative formulations designed to reduce their environmental impact, while improving their efficiency by mimicking *Stratum Corneum* (SC) hydration regulation process: increase the water residence and transit time within the SC and decrease water loss.

Objectives: To characterize formulations ability to imitate the SC hydration process and evaluate their benefit on skin hydration in a clinical trial.

Methods: A dynamic functional analysis of SC's various components was used to improve biocompatibility while identifying natural hydration regulation mechanisms. Mass Spectrometry imaging (MSI) was used to compare formulation ingredients with SC components. X-ray diffractions were used to analyze the formulations' impact on the organization of the SC lipids. Skin hydration and tolerance were evaluated in vivo.

Results: The MSI demonstrated the product ability to successfully penetrate the SC and reach expected compartments. The X-rays diffraction showed the formulations ability to increase the relative proportion of orthorhombic crystalized lipids, characteristic of one lever of the SC hydration regulation process by increasing barrier function of the SC.

In *in vivo* studies, a high level of hydration was maintained over 24 h after application with an intense and "very good hydration".

Conclusions: Formulations were well-tolerated and increased skin hydration *in vivo* as formulations were designed to imitate SC hydration regulation process. Results show a successful intake of various ingredients, leading to an increase in the SC hydration and a reinforced skin barrier. Bioinspiration process used to design these products allowed reduction of their environment impact, consistent with Biomimicry definition[1].

Key words : biomimetic formulation, hydration, sensitive skin

Introduction

One of the principal functions of the skin is to protect the body against external environment by providing an effective epidermal barrier function, not only against external factors, but also to prevent water loss from the body and in particular from the skin[2]. In the skin, gradient of water and homeostasis are crucial for maintaining physiological state and function [3]. If there are still many unknowns, hydration levels are recognized to affect not only visible microscopic parameters such as the suppleness and softness of skin, but also molecular parameters, enzyme activities and cellular signaling within the epidermis. As a result, facing various troubles, like sensitive skin, erythema, etc., being able to provide a product to reinforce hydration and barrier function with a good tolerance is at the center of attention for dermo cosmetic research[4,5].

As all other modern industries, the dermo-cosmetic sector current challenges led us to combine these consumers' needs with the highest consideration for the environmental impact of our products and take part of the global change towards sustainability.

To reach these objectives Pierre Fabre have been focusing on biomimicry as an approach to solve problem while learning from living systems ability to be sustainable. Facing the adaptations needed to implement a whole process based on the strict definition of biomimicry[1], we started by implementing as many key methodological elements as possible while remaining within the bioinspiration[1].

As a first step towards biomimicry, we first focused on a bioinspired cream and balm. Thus, the design of these products is inspired by the regulatory processes that ensure an optimal hydration gradient in the skin, and more specifically on the structural and dynamic properties of the Stratum Corneum (SC) to increase the water residence and transit time in the SC along with a decrease in water exit.

Through this bioinspired approach, we've limited to a minimum the number of additional ingredients, respecting the sobriety principle widely spread in living system.

Through *in vitro* and *in vivo* clinical studies, we then evaluated the benefits of these bioinspired products on the two above mentioned main requirements: how to improve the skin barrier function and adequate hydration to reach consumer's needs while limiting to a minimum the environmental impact of our products. *In vitro* methods allowed for a mechanistic understanding of the effect on the *stratum corneum* and epidermis, while the *in vivo* clinical studies confirmed the effect on skin hydration, as well as the tolerance and efficacy in soothing effects of sensitive and reactive skin.

In vitro methods including scanning electron microscopy (SEM) imaging and X-ray diffraction were used to analyze the formulations themselves, as well as the *stratum corneum* (SC) lipids and the impact of the formulations on the SC structure. SC lipids are organized in stacked bilayers, partly covalently bound to corneocyte and partly present as free lipids. The structure and special organization of the matrix can be visualized using X-ray diffraction. small-angle X-ray scattering (SAXS) provides information on the inter-bilayer distance in the multi-lamellar lipid structures, whereas wide-angle X-ray scattering (WAXS) provides information on the in-plane crystalline arrangement of the lipids (lattice type)[6].

Two complementary mass spectrometry imaging techniques, atmospheric-pressure matrix-assisted laser desorption/ionization high-resolution mass spectrometry (AP-MALDI-HRMS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS), were also used to compare the ingredients of the formulations with SC components, and to detect their distribution on and in the skin after topical application[7].

Clinical studies were performed to evaluate the effect of the formulations on skin hydration, as well as investigate potential adverse skin effects, such as irritation and sensitization. Changes in skin hydration were measured according to the Hydration Index over 24 h after topical application. A clinical study was conducted to evaluate the sensory profile (a subjective assessment of various descriptors for touch, appearance and feeling).

In addition to these results on the products' ability to reach their functional objectives, we conducted the evaluation of the product's environmental impact through the Pierre Fabre's Green Impact Index. The robustness and reliability of the method have been evaluated and endorsed by the independent

body AFNOR Certification and has shown to respect the highest standards established by the company.

The results of all these experimental phases show that this design process led to products that answer our requirements on both clinical efficiency and environmental impacts.

Finally, this paper presents the limitations and coming steps to reach biomimicry, as defined by the ISO 18458:2015 norm[1], as a way to further improve our sustainable design process and take an active part in the transition towards a dermo-cosmetic industry answering the need for the highest environmental and consumer considerations.

Methods

Bioinspired design process

The bioinspired design process used in this study derives from the biology-push biomimetic design process defined in the ISO 18458:2015 norm[1].

Indeed, we performed a functional analysis of a biological model, the human SC, described the mechanism of interest, and performed a direct transfer of some part of this model to our products.

We then performed an analysis of the environmental impact of the product through Pierre Fabre Green Impact Index in order to get as close as possible to Biomimicry[1].

Mass Spectrometry imaging (MSI)

Skin treatment and cryo-section preparation

Human skin of 3 Caucasian female donors (46 ± 18 years old) were supplied by L'Union Clinics (L'Union, France) and Toulouse hospital (Toulouse, France).

Skin preparation and MSI analysis has been described previously[8].

Briefly, fresh human skin was cut using a dermatome and punched out. Formulations were applied at 2 mg/cm² to the surface of the skin and skin samples were incubated at 37°C with 5% CO_2 for 30 h. Punch-biopsies were frozen in liquid nitrogen and stored at -80ºC prior to sectioning. Samples were sliced into 10µm thick sections using a Cryo-Ultramicrotome Leica EM FC6 (Leica Microsystems GmbH, Germany). Optical images of the tissue sections were taken using an Olympus BX51 Microscope (Olympus, Belgium) in black field mode.

Two MSI techniques were performed: AP-MALDI-HRMS and ToF-SIMS for the analysis of formulation applied to *ex vivo* skin. Different MSI techniques have varying characteristics especially when it comes to achievable lateral resolution and give complementary answers.

AP-MALDI-HRMS analysis

Dried sections were coated with HCCA matrix using an HTX TM-sprayer (HTX Technologies LLC, USA). MALDI analysis was performed using an AP-MALDI UHR ion source (Masstech Inc., USA) coupled to an LTQ/Orbitrap Elite high-resolution mass spectrometer (Thermo-Fisher Scientific, USA) in positive ion mode. Spectra were acquired with a 1000 ms injection time, over a mass range of 200 – 1500 Da and at a mass resolution of 60k@ m/z 400. Data analysis and visualization was performed with Thermo Xcalibur 2.2 and MultimagingTM software (ImaBiotech, France). Optical/MSI overlays were performed using Multimaging HD import module.

ToF-SIMS imaging

ToF-SIMS analysis was performed using an TOF.SIMS 5 (IONTOF GmbH, Germany) with a 25kV Bi3+ primary analysis beam. Dried skin sections were analyzed in burst alignment, delayed extraction, positive ion mode with a total primary ion dose of 1×10^{12} ions/cm². Spectra were recorded over a mass range of 1 – 1000 Da and at a mass resolution of 5000@ m/z 300. Data analysis and visualization was performed using SurfaceLab 7 (IONTOF GmbH, Germany).

X-ray diffraction

Normal human SC samples from two donors were prepared as described previously

. SC samples were placed on a polymeric support and 5 mg/cm^2 of the formulation was applied topically. After 45 min, treated and non-treated SC were analysed. Global and Z-analysis diffraction analysis were carried out. SAXS and WAXS were visualized, and diffraction patterns were acquired in the scanning mode (vertical step was 10 μm and the horizontal step was 75 μm).

Data were processed using ESRF FIT2D software. The intensity profiles corresponded to angular profiles integrated on ± 5° around the meridian (SAXS profiles) and ± 10° around the equator (WAXS profiles). The intensity profiles were represented as a function of the scattering vector length s (Å-1). The distance between crystallographic planes d (Å) was obtained by d = 1/s.. An increase in intensity or lipid enrichment (e.g., by the formulation), correlates with a lipid barrier reinforcement and nourishing effect, respectively.

Clinical studies: Hydration Index (HI)

The HI was measured in 26 female volunteers (between 22 and 64 years old) whithout no hair or dehydrated skin on the front of the forearms (HI ≤ 45). A single standardized topical application of the formulation (2 mg/cm²) was performed and massaging until fully absorbed. The efficacy outcome measured was the change in cutaneous capacitance (Hydration Index = HI) between the treated and the untreated control. The measurements were carried out before application of the product (H0) then 1, 2, 4, 6 and 24 h after application using a Corneometer® (Model 820 MPA5, Courage and Khazaka, Germany). Responses to the product were assessed by analysis of covariance, based on the changes between T0 and each interval. The statistical significance levels were: non-significant: NS ($p>0.1$), * significant ($p<0.05$), **highly significant ($p<0.001$).

Clinical studies: skin tolerance

Skin tolerance was measured with 32 volunteers (94% female, 6% male) between the ages of 22 and 64 years for the balm and with 30 volunteers (91% female, 9% male) between the ages of 20 and 67 years for the cream. The type of facial skin was: 100% reactive sensitive skin (declarative) and 59% dry skin, 41% very dry skin for the subjects' group that tested the balm and 22% dry skin, 19% normal skin, 19% oily skin, 22% combination skin with dry tendency, 19% combination skin with oily tendency for the subjects' group that tested the cream. A stinging test was performed before the first product application to confirm subject's inclusion. Each subject was given a dermatological and ophthalmic clinical evaluation before and after the first application. The cream and balm formulations were applied to the face, eye area, and neck at least twice a day for 3 weeks. After this time, the dermatological evaluation was repeated, and the subjects asked to fill in a cosmetic acceptability questionnaire. Formulation-related reactions were recorded as the number of subjects with functional and/or physical signs.

Green impact index

The Green Impact Index is a tool for information and rating the environmental and societal impact of cosmetics and family health products.

It was designed by Pierre Fabre to evaluate all of its products; the robustness and reliability of the method have been evaluated and endorsed by the independent body AFNOR Certification.

A total of 20 criteria are screened to give the product an A, B, C or D score. A score of A or B meaning that the product can be considered eco-socio-designed because it meets a sufficient number of environmental or societal performance criteria based on recognized benchmarks

Conscious Care Approach

Pierre Fabre created Conscious Care approach, a people-centric movement committed to the environment, scientific progress, and better living, through the conception of dermo-cosmetics products on a total of 8 technical criteria :

- develop products that meet the needs of our customers and patients, while also ensuring that they are pleasant to use to encourage compliance.
- design « clean » formulas by selecting ingredients with scientifically proven benefits and safety.
- ensure that the number of ingredients is minimized, nothing is superfluous.
- develop formulas with a minimal environmental impact.
- prioritize natural-origin ingredients and environmentally friendly manufacturing processes
- eco-designed packaging to help reduce environmental impact.
- forge positive relationships with our suppliers and prioritize Made in France.
- Publish our eco-social scores: Green Impact Index and clearly identifying the role of our ingredients.

Results and Discussion

Functional analysis of biological system of interest

SC has been shown not only to play an essential role in the skin barrier function but also in the maintenance of the hydration level of the skin. Mainly, this mechanism can be considered through four main functions, themselves highly coordinated to form the overall SC hydration regulatory system:

- Control of water inflow, which impacts the amount of water that enters the system.
- Control of water outflow, which impacts the amount of water that leaves the system.
- Control of the water interactions with the SC components, which impacts the residence time of water and so the amount of water available within the skin at a given point in time (Inflow-Outflow).
- Control of the distance travelled by water within the SC layers, which impacts the transit time of water, and so act as a second parameter of the amount of water available within the skin at a given point in time (Inflow-Outflow).

The biomimetic process led us to analyze, for each function, what were the key parameters allowing the SC to manage water flow at a given point in time.

Control of…	Biological strategies	Abstracted principles (AP)
…water flow entering the system.	Water diffuses from the epidermis into the SC following the hydration gradient[9,10].	AP1: The system uses gradient of osmotic properties to generate a flow of liquid.
…water exiting the system.	Sebum covers the surface of the SC to prevent water outflow through hydrophobic interactions.	AP2: In his external surface, the system is covered with occlusive hydrophobic molecules to prevent water outflow.
…water interactions with the SC components.	SC cells contain highly hydrophilic compounds (NMF, hyaluronic acids, glycerol) generating an osmotic gradient leading water to flow within corneocytes[11].	AP3: The system is composed of numerous compartmented sub-systems, themselves filled with humectants molecules to increase water attraction and trapping.
	SC intercellular space is filled with lipidic lamellar sheets mainly composed of saturated free fatty acids, ceramides, and cholesterol. Permeability depends on the level of organization, decreasing from liquid to hexagonal to orthorhombic	AP4: The space in between sub-systems is filled with occlusive and emollient hydrophobic molecules highly organized into multi lamellar sheets. The more packed the molecules are, the less water can flow through the layers

	structure [Ananthapadmanabhan, 2013] [Duplan, 2018].	and so reach the external boundary to leave the system.
…the distance travelled by water within the SC layers.	Corneocytes size, SC thickness, and number of corneodesmosomes have a direct impact on the distance (intercellular route) water has to travel to exit the SC.	AP5: Sub-systems' envelopes and their tight interlinks create a physical route for water to flow through the system. As a result, the thickness of the system, the size of the sub-systems and the number of links between them are all impacting the length and complexity of the path water must take to exit the system.

This table allowed us to identify our model's key functional parameters. From a bioinspiration perspective, it gives us information on how to design a system able to manage internal water flow. However, our objective is to design products allowing the regulation of another system's water flow, here the SC. Thus, the key mechanism we want to imitate is the way the SC uses and coordinate the above-mentioned functions to return to an optimal hydration state after a perturbation.

As a results, we focused on currently available basic research data, to formalize a partial model focusing on the control of water interactions with the SC components:

- First, Ca2+ acts as a key informative pathway leading to the secretion of lamellar bodies from the corneo-epidermal junction when facing dry conditions[11,12]. This secretion improves the SC barrier through the intake of lipids precursors (of free fatty acids, cholesterol, and ceramides) and associated enzyme, allowing the reinforcement of the intercellular lipidic lamellar sheets[13]. Indeed, these additional lipidic resources are known to better fill the intercellular spaces and to drive lamellar sheet lateral organization toward an orthorhombic crystalized form, which shows a highly repulsive behavior with water (regulation mechanism based on AP4).
- Secondly, dry conditions lead to filaggrin's proteolysis into free amino acids which are main NMF[5]. Thus, these molecules highly increase SC interaction with water and prevent it from flowing out (regulation mechanism based on AP3).

Based on these two levers, we designed products which compositions mimic both the increase of lipidic and NMF content in the SC as closely as possible. In vitro and in vivo evaluations then confirmed that providing only molecules existing in the natural skin allows the SC to take them in charge without disrupting its structural integrity.

In vitro evaluations

MSI techniques

Using mass spectrometry, a global overview of the composition of the formulations showed similarities with SC and epidermis composition. For example, they all contained mono-, di- and triglycerides. for skin treated with the cream and balm formulations, and the SC and epidermis of non-treated skin. Fig. 1 shows the marked similarity between skin treated with formulations and reference skin with different monoglycerides and diglycerides recovered but also cholesterol and ceramides fragments.

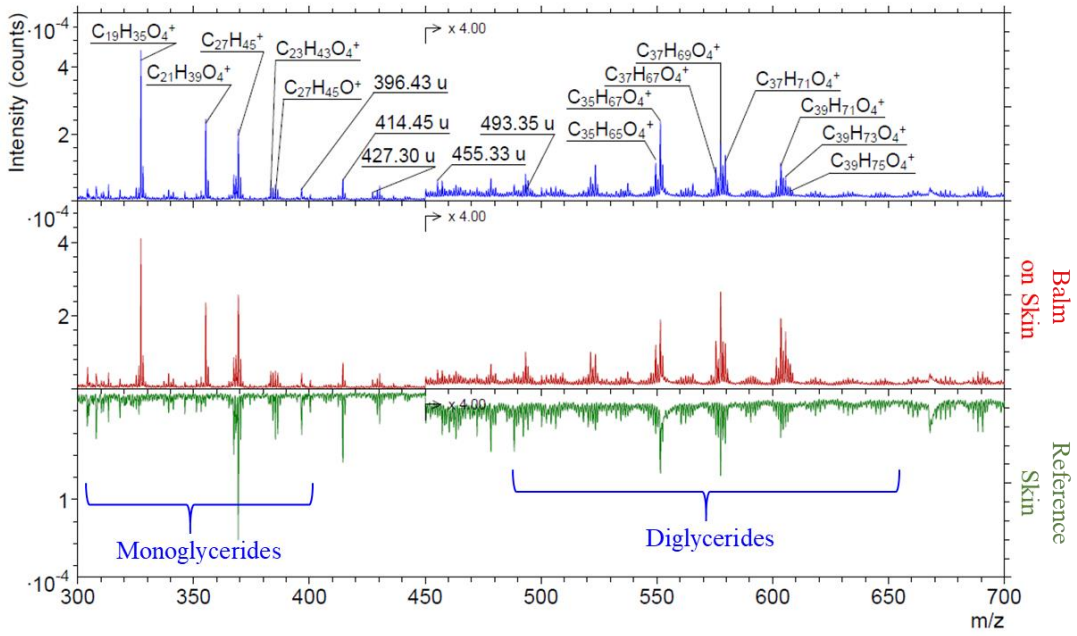

Fig.1. ToF-SIMS spectra of cream, balm and the SC + epidermis over an m/z range of 400 to 700.

The penetration of the formulation components was analyzed by MSI. MSI allowed clear detection of the different layers of the skin using endogenous markers of each skin layers like ceramides and phospholipids. Penetration of the formulation ingredients did occur, especially monoglycerides, diglycerides and triglycerides (Fig. 2 A and B), which were detected mainly in the SC but also in the viable layers of the epidermis. The penetration of these components was higher after application of the balm compared to the cream, in particular, into the epidermis. MALDI imaging is superior to ToF-SIMS at detecting higher masses and shows penetration, especially of the triglyceride components into deeper skin layers.

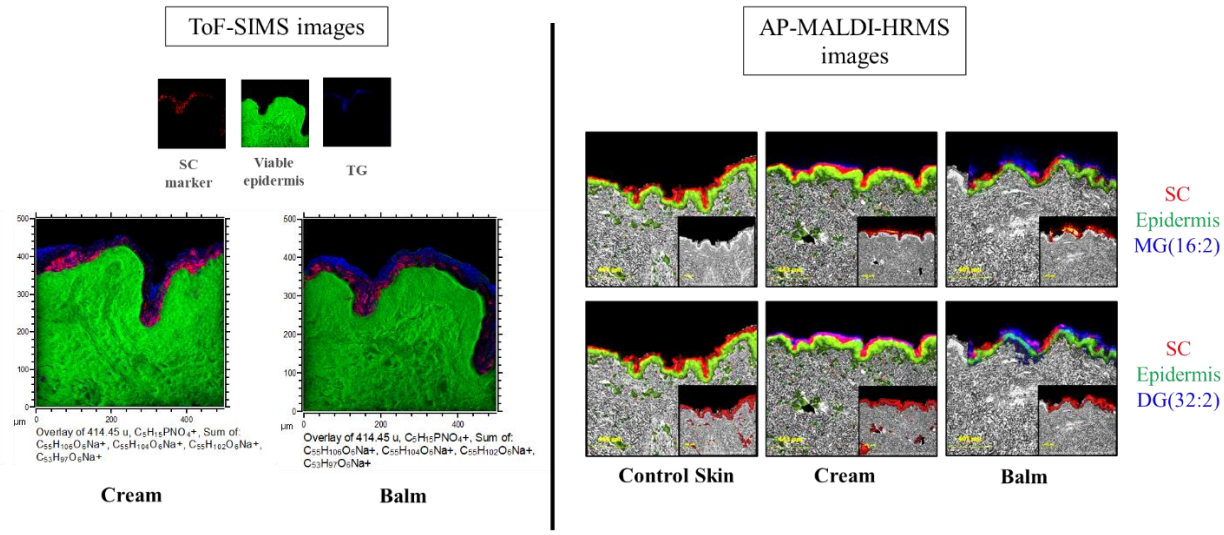

Fig.2.A. ToF-SIMS image analysis of penetration of triglyceride formulation components after application of the cream (left-hand side) and balm (right-hand side) to *ex vivo* skin. SC marker: ceramide fragment [$C_{26}H_{56}NO_2^+$], m/z 414.43 in red; Viable epidermis: PC-headgroup [$C_5H_{15}PNO_4^+$] m/z 184.07 in green and summed-up triglyceride species in blue.

Fig.2.B. AP-MALDI-HRMS image analysis of penetration of monoglyceride, diglyceride formulation components after application of the cream (left-hand side) and balm (right-hand side). The SC is highlighted by a ceramide fragment [$C_{26}H_{56}NO_2^+$], m/z 414.43 in red, the epidermis with lipid species PE(36:2) [$C_{41}H_{79}NO_8P^+$], m/z 744.55 in green and representative mono- and diglyceride species in blue.

WAXS and SAXS analysis

The global WAXS profile of the formulations analysed on bulk superimposed with the SC. The lipid organization of the formulations was similar to the SC. The broad WAXS peak (4.6 Å) related to the amount of the fluid part or amorphous phase, was present in both formulations. The narrow WAXS peaks at 4.1 and 3.7 Å, indicating the amount of crystallized part (hexagonal to orthorhombic) and the structure of the lattice system were also similar across formulations and the SC.

The global profiles comparing the SC without and with application of the cream and balm formulations are shown in Fig. 3A. In WAXS, there was a slight change of the relative intensity of the peaks at 4.1 and 3.7 Å, which indicated a change in the crystalized structure towards orthorhombic lattice. This effect was more intense for the balm than for the cream. The amorphous lipids were also increased with both formulations.

The Z-profile (Fig. 3B) in WAXS, showed an increase in the signal from amorphous lipids at the SC surface (green curve) at ~4.6 Å and an extra signal from amorphous matter at 17.5 Å. There was also an increase of the relative intensity of the peaks at 4.1 and 3.7 Å. The balance between lipids crystalized in hexagonal and orthorhombic was moved to a more hexagonal lattice structure for the cream. There was an intense SAXS peak at 59 Å, which is likely to be due to the crystallization of the formulation inside the SC in lamellar stacking orientated mainly parallel to the SC plane. Overall, these indicate that the formulations did not modify the lamellar organization of the SC lipids but they did increase the relative proportion of the crystalized lipids in the orthorhombic lattice and some of the amorphous lipids.

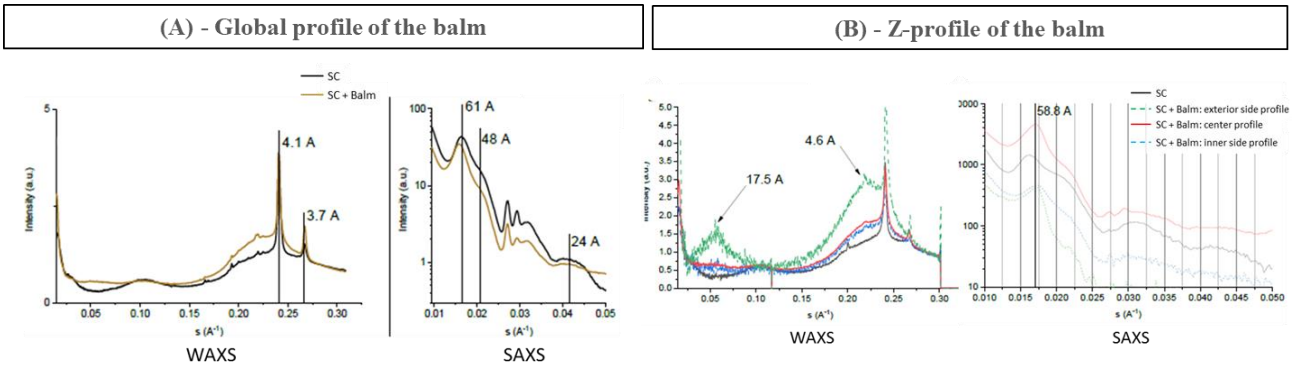

Fig.3. The global profiles (a and b) and Z profiles (c and d) of the SC with and without application of the cream (a and c) and balm (b and d) formulations

Clinical evaluations

Hydration Index

After application of both formulations, the HI was statistically increased by 65.1% (cream) and 60.2% (balm) compared the hydration level before application (H0) (Fig. 4). A high level of hydration was maintained over 4 h after application, such the HI of skin treated with cream or balm was at least 55% higher than at H0 over this time. The HI declined over the following 20 h but was still statistically significantly higher than the untreated control skin or H0 (11.7% (cream) and 12.9% (balm) compared to H0).

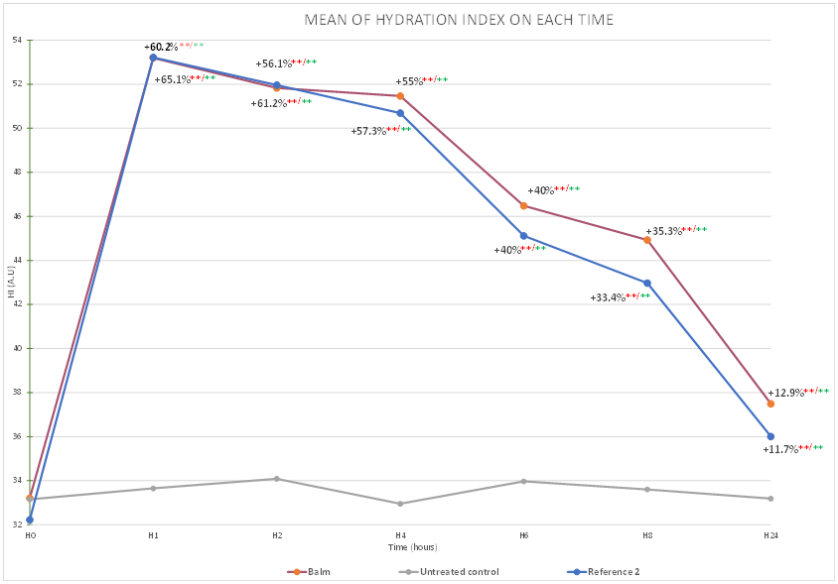

Fig.4. Hydration index (HI) of skin of subjects after application of the cream (blue symbols) and balm (orange symbols) formulations.

Skin tolerance

In the tolerance studies, both formulations (balm and cream) exhibited excellent skin tolerance after twice daily applications for 3 weeks and could be labelled as "High Tolerance" (i.e. no reaction occurred during the study). The subjects of the in-use tolerance studies appreciated the soothing properties of both formulations (skin comfortable, soothed and relieved) with an agreement rating more over 90% immediately after application (Fig.5). The long-lasting soothing effect was sustained for the balm after a 21-day use period at the same level and decrease slightly to 87% (skin comfortable) and 83% (skin soothed and relieved) for the cream.

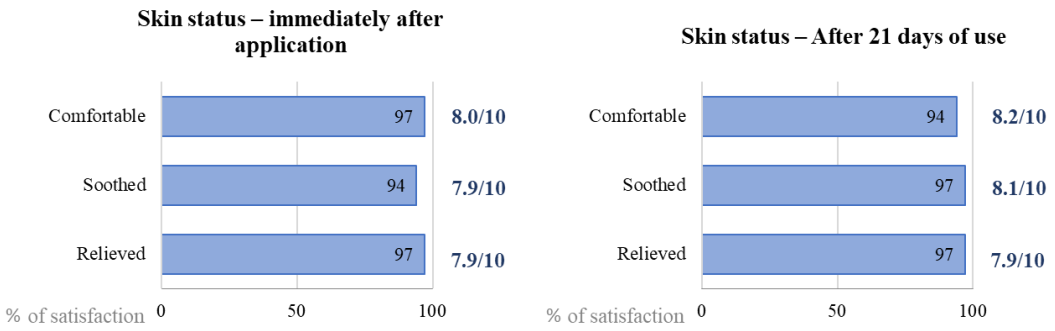

Fig.5. Appreciation of the cosmetic acceptability during the in-use tolerance studies (Balm formulation example)

Environmental impact and Green Impact Index

Both products have been evaluated and received a B Ranking: its formula is composed of 98% natural origin ingredients in sterile cosmetics and manufactured in a factory certified ISO 14001 for environmental management.

These products are also a good illustration of our Conscious Care approach on the 8 criterias : first of all, tolerance and efficiency, to which we add the principle of the right amount of ingredients and the absence of undesirable substances, but also eco-designed formulas and packaging, naturalness, transparency, as well as local production and sustainable sourcing.

Conclusion

This paper presents a project on eco-socio designed formulations which are able to reinforce the cutaneous barrier and prevent water loss, to maintain adequate hydration and protection of sensitive skins.

To combine clinical efficiency with environmental considerations we wanted to use a biomimicry approach, and because of its key role on skin hydration, we focused on SC hydration regulation process as a model to get inspired from. An evaluation of the products' environmental impact, using the Green Impact Index, was performed to measure the impact of the product (formulation, packaging, manufacturing, etc.). Products received a B index which shows the products can be considered as eco-socio designed.

From a strict biomimicry perspective, several levels for improvement have been identified and compose the next steps on our methodological roadmap.

First, it has been highly difficult to strictly follow the ISO process since the product we designed was inspired from the SC and is also made to be applied on the SC. This situation led to confusions between the use of abstracted principles and the direct transfer of molecules acting on existing molecular pathways. Thus, we focused on performing a thorough bioinspiration to get as close as possible to biomimicry at this stage.

Secondly, the actual mechanism through which our product interact with the SC isn't clear yet. This mechanism can be different from the way SC performs its regulation. Questions on the interactions of the products' components? How do they reach their locations of interest? Do the products NMF enter corneocytes or remain in the intracellular space? Whether lipids directly join the lamellar sheets or are integrated through enzymatic activities? etc. remain to be answered. Nevertheless, results show a clear efficient and interest of our minimal formulation,

In spite of these limitations, we manage to abstract a first part of the model allowing hydration regulation, and we are currently working on additional studies to combine all the interconnected factors (such as pH and Ca^{2+}) and confirm the proper transfer of these mechanisms to our products. Consequently, our products represent a first step towards biomimicry.

Indeed, the SC ability to manage water flow was analyzed and key molecular parameters influencing SC hydration were identified in the literature before the regulation mechanism using those parameters were used to identified key components to add to our product's formulation.

Hence, each ingredient is incorporated because of its involvement in specific functions which are predicted to provide a specific benefit to the skin to alleviate sensitive skin. The components include water, proteins, lipids, sugars and minerals, which together result in a composition that mirrors the skin. Following this logic, we designed a cream and balm with a molecular similarity of 98%.

The similarity of components along with their ability to enter the SC and be addressed by it to predicted location were validated by *in vitro* evaluations (MSI techniques).

The expected consequence of a good integration of the ingredients, more specifically the integration of lipids within lamellar sheet to improve structural packing (orthorhombic lateral organization), was validated through *in vitro* WAXS and SAXS analysis. The resultant increase of the skin hydration was also tested and confirmed by an *in vivo* hydration index measurement.

As a result, thanks to this process, we designed a product with an overall reduced number of molecules, and a low number of molecules foreign to the SC. *In vitro* evaluation showed that such minimal formula can penetrate the SC without disturbing the lipidic lamellar sheets to bring key functional molecules to the SC for it to speed up skin's barrier function recovery and associated proper hydration. *In vivo* clinical evaluation underlined the "high tolerance" for these products.

To conclude, these products meet their objectives as evaluations have validated their eco-socio design along with their clinical performance both *in vitro* and *in vivo* to alleviate sensitive skin.

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Conflict of Interest Statement

Authors are employees of Pierre Fabre Dermo-Cosmetique and Green Mission Pierre Fabre. The authors report no other conflicts of interest in this work.

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