

The effect of vehicle on skin absorption of Mg²⁺ and Ca²⁺ from thermal spring water

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Received 7 October 2019, Revised 30 January 2020, Accepted 5 February 2020

Keywords: delivery/vectorization/penetration, emulsions, Franz cell, liposomes, skin barrier, thermal spring water

Abstract

OBJECTIVE: Thermal spring waters (TSW) are commonly used as active ingredients in cosmetics. Their biological activities directly depend on the ionic composition of the spring. However, in order to exhibit beneficial properties, the minerals need to reach viable skin layers. The present study addresses the incorporation of marketed TSW in model cosmetic formulations and the impact of the formulation on skin absorption of magnesium and calcium ions that are known to improve skin barrier function.

METHODS: Marketed TSW was introduced into five formulations. Liposomes were prepared using saturated or unsaturated phospholipids mixed with cholesterol by the thin layer evaporation technique. Emulsions water-in-oil (W/O), oil-in-water (O/W) or double: water-in-oil-in-water (W/O/W) were prepared by high-shear mixing. Skin absorption of Mg²⁺ and Ca²⁺ from those formulations was studied *in vitro* using static Franz diffusion cells under infinite dose condition and under occlusion of the apparatus.

RESULTS: Mg²⁺ and Ca²⁺ penetrate skin samples from TSW. Encapsulating TSW into double emulsion (TSW/O/W) increased skin absorption of both cations of interest and kept the Ca²⁺/Mg²⁺ ratio equal to that of TSW in each skin layer. The dermal absorption of Mg²⁺ from the double emulsion departs from both single emulsions. Application of liposome suspension improved the skin absorption of Ca²⁺ while keeping constant that of Mg²⁺, leading to unbalanced Ca²⁺/Mg²⁺ ratio inside skin.

CONCLUSION: The beneficial effects of TSW are not only due to their action on the skin surface. Their active components, especially Ca²⁺ and Mg²⁺ cations, reach viable skin layers in a formulation-dependent manner. The distribution of ions inside skin depends on the type of formulation.

Résumé

OBJECTIFS: Les eaux thermales sont couramment utilisées comme substances actives dans les formulations cosmétiques. Leurs activités biologiques dépendent directement de leur composition en ions. L'action des ions s'exerce à différents niveaux dans la peau, mais bien souvent dans les couches profondes, au-delà du stratum corneum, qu'ils doivent donc atteindre. L'objectif de cet article est d'étudier l'absorption des ions magnésium et calcium, reconnus pour leur effet bénéfique sur la fonction barrière de la peau, depuis différentes formes galéniques formulées avec une eau thermale.

METHODES: Une eau thermale commerciale a été utilisée comme phase aqueuse dans 5 formulations différentes : des liposomes formulés avec des phospholipides saturés et insaturés et du cholestérol ; des émulsions de différents sens, eau thermale/huile (TSW/O) et huile/eau thermale (O/TSW) ; une émulsion multiple eau thermale/huile/eau (TSW/O/W). L'absorption cutanée du calcium et du magnésium a été étudiée depuis ces différentes formulations, en utilisant la méthode des cellules de Franz, en dose infinie, et en fermant les cellules pour prévenir toute évaporation.

RESULTATS: Les ions magnésium et calcium pénètrent dans la peau depuis l'eau thermale, utilisée comme contrôle. L'encapsulation de l'eau thermale dans les gouttelettes internes de l'émulsion double (TSW/O/W) permet de promouvoir la pénétration des deux ions d'intérêt dans chaque couche de la peau tout en respectant le rapport Ca²⁺/Mg²⁺ obtenu avec l'eau thermale, contrairement aux émulsions simples. Les liposomes augmentent la pénétration cutanée des ions calcium, tandis que celle des ions magnésium reste constante, ce qui conduit à des rapports Ca²⁺/Mg²⁺ élevés dans la peau.

CONCLUSION: Les effets thérapeutiques des eaux thermales ne sont pas seulement dus à une action de surface. Les ions comme le calcium et le magnésium pénètrent dans la peau et exercent une action en profondeur qui dépend de la formulation dans laquelle ils sont formulés. En effet, leur distribution ions dépend de la formulation qui les contient.

Introduction

Curative properties of thermal spring waters (TSW) have been known and used in treatment of various skin (psoriasis, eczema, etc.) and systemic diseases (e.g. osteoarthritis, mental stress, sleep disorders) since many centuries [1,2]. 'Taking the waters' is the most ancient water treatment which is 'promising relief to the sick', as written by Elder Pliny in the book XXXI of his Natural History and reported by Jackson [3]. Water cures are very common

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Data included in the manuscript were presented during Skin Forum 23 and 24 September 2019, Reims, France; IFSCC conference 30 September and 1–2 October 2019, Milan, Italy; and Formulation Days – Advances in Formulation of Active Ingredients, Lyon, January 10 and 11 2019.

and strongly embedded in European cultures (France, Italy, Germany, Hungary). The core of balneotherapy has been defined by Gutenbrunner as the 'use for natural mineral waters, gases and peloids (including packs that are local applications of peloids), often in resorts (Spas)' which are located next to the sea (Dead Sea) or thermal sources in European countries [4,5]. The therapeutic use of mineral waters sourced from trapped oceanic waters (Dead Sea, Tiberias waters) and other locations, like Vichy water in France, has been evaluated as beneficial by the Cochrane Library. Such an adjuvant treatment is recommended for dermatologic and rheumatologic diseases, and also for patients with all forms of arthritis [6–8]. A 3-week spa therapy has prolonged, symptomatic effects against osteoarthritis that were maintained for more than 6 months [8].

All TSW contain a significant amount of magnesium, calcium and sulphate ions [9]. It is presently questioned whether the components of TSW are able to cross the hydrophobic barrier of the *stratum corneum* and reach viable layers of epidermis and dermis. Several authors have addressed the permeability of skin for ions, both *in vitro* [10–12] and in clinical trials [13,14], demonstrating that dermal and even transdermal delivery of ions is indeed possible under certain conditions.

Among cations of interest, Ca^{2+} and Mg^{2+} have been recognized for their benefits in skin barrier recovery [15]. High concentrations of endogenous Ca^{2+} and Mg^{2+} are present in the uppermost epidermis. They are distributed according to a concentration gradient increasing in the epidermis from the *stratum basale* up to the *stratum granulosum*. In case of external damage, this ionic gradient is perturbed which is a signal for skin barrier repair driven by lipid exocytosis from lamellar bodies [16–20]. Denda *et al.* reported that application of ionomycin, a calcium ionophore, in cultured human keratinocytes delayed the barrier repair by increasing the intracellular calcium concentration. In their *in vivo* study on rats exposed to ionomycin, the authors showed that the lipid domains at the *stratum corneum*–*stratum granulosum* interface were relatively thin and skin contained many unsecreted lipids inside lamellar bodies [19]. Another study disclosed the effects of mixed magnesium and calcium salts with varying molar ratio [15]. Salt solutions having Mg^{2+} -to- Ca^{2+} ratio higher than one accelerate barrier recovery in a more efficient way than solutions of each cation separately. The solution of CaCl_2 (10 mM) alone delays recovery of *stratum corneum* in mice. Dietary recommendations encourage daily intake of calcium and magnesium in a ratio close to 2, which disagrees with the findings of Denda *et al.*, who recommended an inverted molar ratio ($\text{Mg}^{2+} : \text{Ca}^{2+} > 1$) for acceleration of skin barrier recovery. Magnesium, and probably calcium, penetrates the skin preferentially through the follicular pathway [12]. Moreover, 'encapsulation' in various vehicles may modulate the distribution of both ions, thereby modifying the $\text{Ca}^{2+} : \text{Mg}^{2+}$ molar ratio that controls lipid release from lamellar bodies [15,19].

Calcium- and magnesium-rich TSW are known to improve skin barrier function and accelerate wound healing [20,21]. Moreover, they have soothing and protective properties in sensitive skin (antioxidant or anti-ageing) that are enhanced by the presence of trace elements such as selenium, strontium and zinc [22–24]. These properties have been demonstrated in many studies using human keratinocytes, fibroblasts or other response-appropriate cell lines [6,25,26]. Therefore, they are considered as active substances when used in a cosmetic product. Skincare products such as emulsions or lotions containing TSW as aqueous phase are present on the market, which claim soothing and hydration properties.

However, the absorption profiles of these cations applied together, as in the case of TSW, are not documented. Moreover, TSW in spray form may cause secondary skin dehydration. Evaporation of TSW at the skin surface increases osmotic pressure and leads to crystallization of salts that cause greater water loss from deep skin layers and skin dryness in general. Therefore, TSW-based skincare products are developed to overcome the drawbacks of a simple spray form, allowing incorporation of other types of active cosmetic ingredients in one product. As discussed by Otto *et al.* [27], dermal delivery of molecules depends strongly not only on their physicochemical properties, but also on the formulation in which it is incorporated. Though several TSW-based cosmetic formulations have been claimed, no skin absorption of ions from such products has been reported so far. The present study aimed at investigating the dermal penetration of TSW rich in Ca^{2+} and Mg^{2+} entrapped in different model formulations: classical emulsions, double emulsions and liposomes. Experiments were carried on using static Franz diffusion cells after infinite dosing of tested formulations and under occlusion of the system.

This method allows *in vitro* measuring of the delivery of chemicals from the surface of a skin explant to an acceptor medium that mimics the bloodstream. It is useful to compare the delivery of molecules into and through the skin from various formulations.

Liposomes are known to enhance skin permeation of entrapped drugs and improve skin repair [28,29]. Phospholipids containing high amounts of phosphatidylcholine (>90%) such as hydrogenated phosphatidylcholine or pure phosphatidylcholine promote penetration into epidermis by altering the barrier properties of the lipid medium of the *stratum corneum* [30]. Moreover, liposomes formulated from unsaturated phospholipids were shown to be more efficient for skin delivery than those composed of saturated phospholipids. To investigate the impact of phospholipid type used for vesicle preparation on skin absorption of cations, unsaturated and saturated phospholipids were used (LPM-90H and LPM-90G, respectively). Simple water-in-oil (W/O) and oil-in-water (O/W) were studied as the model cosmetic formulations varying in the availability of hydrophilic molecules. In W/O emulsions, ions in water are entrapped in the dispersed phase so that their release is modified, whereas O/W formulations are characterised by immediate availability of ions in the continuous phase. In the latter case, the presence of oil component could modify skin absorption. Water-in-oil-in-water (W/O/W) multiple emulsions are composed of both W/O and O/W droplets. The oil phase makes a barrier between both aqueous phases and may lead to a sustained release of the cations from the internal water droplets as reported by Ferreira *et al.* [31] for highly hydrophilic glucose. Additionally, multiple emulsions may stabilize active ingredients and contribute to a better skin hydration [32].

Materials and methods

Materials

Marketed, mixed sulphate–chloride thermal spring water (TSW) was purchased at a local pharmacy. Medium chain triglycerides (MCT, Labrafac Lipophile WL 1349) were kindly provided by Gattefossé, (Gattefossé, Saint-Priest, France). Polyglyceryl-4-polyricinoleate (PGPR, Dermofeel PGPR®) was a generous gift from Evonik (Evonik, Essen, Germany). Glyceryl Citrate/Lactate/Linoleate/Oleate (G-CLLO, IMWITOR®375) was obtained from IOI Oleo (IOI Oleo GmbH, Hamburg, Germany). Hydrogenated phosphatidylcholine

Table I Characterization of TSW. Values in standard font are given according to the supplier, and values in italics are the measurements made by our own

Parameters	Mineral content (mmol L ⁻¹)			
		Cations	Anions	
Spring water temperature (°C)	62			
Dry residue (g L ⁻¹)	6.86 6.72	Li ⁺	0.14	F ⁻ 0.11 0.11
Conductivity (25°C, mS cm ⁻¹)	9	Na ⁺	69.8	Cl ⁻ 87.7
Osmolality (mOsm kg ⁻¹)	180	K ⁺	3.40	Br ⁻ 0.09
	'isotonic against skin cells'		3.46	0.02
pH	5.5 7.04	Mg ²⁺	4.36	NO ₃ ⁻ 0.04 3.50 >0.01
Mg ²⁺ : Ca ²⁺	1 : 3 1 : 3	Ca ²⁺	13.4	SO ₄ ²⁻ 31.3 10.3 22.6

(>90%) (Phospholipon[®] 90H) and pure phosphatidylcholine (>94%) (Phospholipon[®] 90G) were kind gifts from Lipoid GmbH (Lipoid, GmbH, Ludwigshafen Germany). Cholesterol, Certified Multielement Ion Chromatography Anion Standard Solution, Certified Multielement Ion Chromatography Cation Standard Solution, nitric acid (HNO₃) and dipicolinic acid were purchased from Sigma-Aldrich (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Betaine (trimethylglycine), sodium hydrogen carbonate (NaHCO₃), sodium carbonate (Na₂CO₃), sodium dihydrogen phosphate (NaH₂PO₄), potassium dihydrogen phosphate (KH₂PO₄) and glucose were purchased from Acros Organics, (Acros Organics, Illkirch, France). Ultrapure water with resistivity >18 MΩ cm at 25°C was used in all experiments.

TSW physicochemical characterization

The composition of the TSW marketed product was identified by ionic chromatography (Table I). Analysis of the analyte was restricted to ions of interest. Traces of oligo elements such as zinc and strontium reported as components by the supplier were not quantified in the present study.

Controls of osmolality, pH, conductivity and dry residue were performed. Osmolality and pH were evaluated using an Osmomat 030 (Cryoscopic osmometer; Gantec, Berlin, Germany) and a pH meter (pHenomenal[®] pH, VWR equipped with an electrode pH 11769798, Fisher Scientific, France), respectively. Conductivity was measured using a Conductivity Meter (CDM210; MeterLab[®], Villeurbanne, France). The mineralization level was evaluated by gravimetric analysis after evaporation of water from 10 mL TSW using a rotary evaporator (200 mbar, 70°C, Rotavapor[®] R-205; Büchi, Flawil, Switzerland).

Formulation manufacture

The compositions of formulations (Table II) were designed so that the final concentrations of Mg²⁺ and Ca²⁺ in each formulation were kept constant. There was no other source of Mg²⁺ and Ca²⁺ (nor other ions) in the formulations. Classical TSW/O and O/TSW emulsions contained 60% *m/m* of TSW. O/TSW emulsion was prepared by the heat-heat process: TSW containing G-CLLO and oil were heated up to 60°C; both phases were mixed with an IKA T25 digital Ultra-Turrax[®] (Imlab, Lille, France) at 20,000 rpm for 5 min and then cooled down to room temperature. TSW/O emulsion was prepared at room temperature by mixing the oil and aqueous phases using an IKA T25 digital Ultra-Turrax[®] at 20,000 rpm for 5 min. TSW was dispersed in the oily phase in which PGPR was dissolved beforehand.

The composition of TSW/O/W emulsion was based on the TSW/O primary emulsion. TSW/O/W double emulsion was prepared at room temperature using a two-step method. In the first step, TSW/O was prepared as described above. The TSW used in the formulation was concentrated twice using a rotary evaporator (200 mbar; 70°C; Rotavapor[®] R-205; Büchi, Flawil, Switzerland) and filtered through Whatman[®] Uniflo[®] 0.45-μm syringe filters (Sigma-Aldrich, Saint-Quentin-Fallavier, France) prior to use to ensure the same concentration of calcium and magnesium ions in the final formulation. This procedure led to an increase in osmolality of TSW from 180 to 360 mOsm kg⁻¹. In order to avoid instability of the multiple emulsion (over-swelling because of differences in osmotic pressure), betaine (300 mmol kg⁻¹) was added to the continuous phase containing G-CLLO (hydrophilic surfactant) as an osmotic agent that did not change the ionic strength of the solution. In the second step, the primary emulsion was dispersed in the external aqueous phase using an IKA T25 digital Ultra-Turrax[®] at 5000 rpm for 1 min. Two liposome suspensions containing 60% (*m/m*) TSW

Table II Composition of the formulations. Values are given as % *m/m* of the final preparation

Emulsions				Liposomes			
Components	O/TSW	TSW/O	TSW/O/W	Components	90H (saturated)	90G (unsaturated)	
TSW	60	60	60 ^a	50% primary TSW/O	Phospholipid	2.5	2.5
MCT	35	35	35		Cholesterol	0.5	0.5
PGPR	0	5	5		TSW (60%)	97	97
G-CLLO	5	0	0	5			
H ₂ O isotonic	0	0	0	45			

^aPercentage of double-concentrated TSW used in the formulation. Theoretical contents of Mg²⁺ and Ca²⁺ were 2.7 and 8.0 mmol kg⁻¹, respectively.

were prepared by the thin lipid film hydration method [33] using saturated (LPM-90H) or unsaturated (LPM-90G) phospholipids. Saturated (Phospholipon® 90H) or unsaturated (Phospholipon® 90G) phospholipids and cholesterol were solubilised in 15 mL of mixture of chloroform, diethyl ether and methanol (7 : 7 : 1, v/v/v). Evaporation of the organic solvents under vacuum (300 mbar, 60°C) led to the thin lipid layer formation. TSW (diluted for the final concentration of 60%) was used to rehydrate the lipids. Then, the dispersion was vortexed for 5 min and warmed in a water bath at 60°C for 5 min. The cycle was repeated three times and led to the formation of a primary suspension of multilamellar liposomes. This was then sonicated using an ultrasonic disperser Sonics VibraCell equipped with a 25 mm shaft working at 20 kHz, 500 W power and 40 % of the full amplitude (Bioblock Scientific, France) for 12 min with ice cooling.

Physicochemical characterizations

Granulometric analysis

Size (z-average diameter) and width of size distribution (PDI) of liposomes were measured by means of dynamic light scattering (Malvern Zetasizer Nano ZS®, Orsay, France). Samples were diluted with ultrapure water prior to measurements and analysed at 25°C at a scattering angle of 173°. pH was measured on fresh formulations.

Droplet size range in emulsions O/TSW, TSW/O and TSW/O/W was assessed from diluted samples by optical microscopy imaging using 100-fold magnification of the objective (Leica Microsystems, Wetzlar, Germany).

Cryogenic-transmission electron microscopy

Liposomes were analysed by means of cryogenic-transmission electron microscopy (Cryo-TEM) at the 'Centre Technologique des Microstructures' (CTμ, facility of the University of Lyon 1). Liposomes were deposited onto 300 mesh holey carbon films (Quantifoil R2/1) and quench-frozen in liquid ethane using a cryo-plunge workstation (made at Laboratoire de Physique des Solides, Orsay, France). Prepared samples were then mounted on a pre-cooled Gatan 626 specimen holder, transferred in the microscope (Philips CM120; Thermo Fisher Scientific, Waltham, MA, USA) and observed at an accelerating voltage of 120 kV.

Viscosity measurements

Viscosity of emulsions was measured at 25°C using MRC 302 Rheometer (Anton Paar, Courtaboeuf, France) 24 h after preparation using a cone-plate measuring device of 25 mm diameter, at the shear strain rate of 300 s⁻¹.

pH measurements

pH was measured for the formulations with aqueous continuous phase (LPM-90H, LPM-90G, O/TSW and TSW/O/W) using pH meter (pHEnomenal® pH, VWR equipped with an electrode pH 11769798; Fisher Scientific, Illkirch, France).

Skin permeation studies

Skin sample preparation

Flank skin excised from young female pigs (32 ± 2 kg) sacrificed at Léon Bérard Medical Centre (Lyon, France) was used as a model

for human skin given the similarities between these species in terms of barrier function for ion absorption, *stratum corneum* and full skin thickness, hair follicle density as well as ion composition [34–36]. Freshly excised tissue samples were used immediately after harvesting (<1 h). The subcutaneous adipose tissues were carefully removed with a scalpel, and the final thickness was measured using a Micrometer (Mitutoyo, Roissy en France, France); then, samples were cut into round sections of 3 cm². Skin integrity was checked by measuring the Trans Epidermal Water Loss (TEWL) (Tewameter TM210; Monaderm, Monaco). Samples having TEWL values above 15 g h⁻¹ m⁻² after 1 min were discarded.

Skin absorption study for Ca²⁺ and Mg²⁺ distribution after 24 h

Skin absorption studies were performed in static diffusion cells according to OECD guidelines [37]. Full-thickness, freshly excised skin samples were mounted in two-chamber Franz glass cells (exposure area 2.54 cm²) with the *stratum corneum* facing the donor chamber.

Infinite doses of 1 g of freshly prepared formulation (LPM-90H, LPM-90G, TSW/O, O/TSW, TSW/O/W) and TSW (60%) as a control containing 1.0 μmol cm⁻² Mg²⁺ and 3.2 μmol cm⁻² Ca²⁺ were applied on the skin surface. The acceptor compartment was filled with 10 mL of the survival medium designed to provide high metabolic activity of the skin samples during experiments. This was isotonic against skin cells (betaine 250 mmol L⁻¹) but contained the minimal amount of ions so that its composition did not interfere with the experimental set-up. Glucose (1 g L⁻¹) provided the energetic fuel for skin cells and phosphate buffer ensured physiological pH over the exposure to formulations [38].

Franz cells were placed in a water bath at 37°C with magnetic stirring so that the surface of skin was 32°C after heat loss. After 24-h exposure under occlusive conditions, the cells were dismantled. The non-absorbed fraction was removed from the skin surface by washing the donor chamber thrice with 1% solution of the polyethylene glycol *tert*-octylphenyl ether octoxynol-9 non-ionic surfactant (Triton X-100; Sigma-Aldrich, Saint-Quentin-Fallavier, France). The glass donor chamber and the skin surface were then whipped carefully with cellulose tissue paper. After, skin layers were separated as follows: *stratum corneum* (SC) was removed using cyanoacrylate glue (Loctite SuperGlue-3, Henkel) spread on a glass plate. The viable epidermis (VE) was separated from the dermis (D) by heat treatment (45 s in water at 60°C). The ions were extracted from the cellulose whip paper and each skin layer by sonication for 30 min at 60 Hz followed by extraction overnight (17 h) in a water/dichloromethane (1 : 1 v/v) mixture under magnetic stirring [36]. The addition of the organic solvent, which is not soluble in water and has higher density than water, allowed easier separation of aqueous phase from the lipidic fraction extracted from the emulsions and skin samples. Recovered quantity of ions was quantified by ion chromatography for the donor solution, acceptor medium (AM) and different skin layers after filtration of the aqueous fractions of samples through 0.45-μm Whatman® Uniflo® syringe filters. The amount extracted from the whip paper was added to that of the donor solution, yielding the amount of the donor compartment (DC). The mass balance calculations were performed to ensure complete recovery of applied products. The full absorbed recovered amount (Q_{abs}) corresponded to the sum of the recovered amounts in SC, VE, D and AM. For each experiment, $n \geq 8$ replications were performed. To assess the amounts of endogenous Ca²⁺ and Mg²⁺, blank experiments (with no product applied on the skin surface) were carried on for each donor animal. The donor

chamber was left empty, and rinsing step was skipped while dismantling the cells. The rest of manipulations were performed as described for skin absorption studies. The amounts of Ca^{2+} and Mg^{2+} eluted to AM and recovered inside the skin layers after 24 h were quantified by ion chromatography and subtracted from the experimental values of samples, so that only the exogenous fraction was considered.

Ion chromatography

Ion contents of TSW, blank and formulation-exposed skin samples together with AM and DC were evaluated using ion chromatography (930 Compact IC Flex; Metrohm, Villebon Courtaboeuf, France).

Anions were analysed at 35°C using Metrosep A Supp 5 250/4.0 column with an adapted pre-column (Metrosep A Supp 5 Guard/4.0). Standard eluent (1.0 mmol L⁻¹ NaHCO₃ and 3.2 mmol L⁻¹ Na₂CO₃) was used.

Cation analysis of TSW samples was performed using a Metrosep C6 250/4.0 column with an adequate pre-column (Metrosep C 6 Guard/4.0) at the temperature of 45°C. Mobile phase was 1.7 mmol L⁻¹ HNO₃ and 1.7 mmol L⁻¹ dipicolinic acid.

Injection volume of 20 µL was used for the analyses of TSW. Calibration curves measured by diluting Certified Multielement Ion Chromatography Anion Standard Solution (10.0 mg kg⁻¹ ± 0.2% of each anion: F⁻, Cl⁻, Br⁻, NO₃⁻, PO₄³⁻, SO₄²⁻) and Certified Multielement Ion Chromatography Cation Standard Solution (10.0 mg kg⁻¹ ± 0.2% of each cation: Li⁺, Na⁺, K⁺, Mg²⁺, Ca²⁺) were linear in the range from 0.25 to 10.0 mg kg⁻¹ for both cations and anions ($R^2 = 0.999$). For analyses of samples from skin absorption experiments (DC, SC, VE, D, AM), a higher concentration of the eluent was used (2.27 mmol L⁻¹ HNO₃ and 2.27 mmol L⁻¹ dipicolinic acid) and the injection volume was 100 µL. The calibration curve from a mixture of MgSO₄·7H₂O (Acros Organics) and CaCl₂·2H₂O (Cooper, France) was linear in the range from 0.5 to 50 mg L⁻¹ for Mg²⁺ and Ca²⁺ ($R^2 = 0.999$). The limits of detection (LOD) and quantification (LOQ) were 0.15 and 0.5 mg L⁻¹, respectively. All results of analyses were converted into molar concentrations.

Statistical analysis

Results followed a normal distribution, and the variances of pairs of data sets were equal. Absorbed amounts from various formulations were compared using an appropriate Student's *t*-test using XLSTAT version 2014.5.03 (Addinsoft, Paris, France). Significance level was set at $P < 0.05$.

Results and discussion

Skin penetration studies were conducted on the samples with final thickness of 1.29 ± 0.02 mm and average TEWL value of 11.0 ± 0.3 g h⁻¹ m⁻².

Skin absorption profiles of Mg²⁺ and Ca²⁺ from three types of emulsions (O/TSW, TSW/O/W, TSW/O) and liposome suspensions composed of saturated and unsaturated phospholipids were investigated. The concentrations of Mg²⁺ and Ca²⁺ present in the aqueous phase were kept constant (2.7 and 8.0 mmol kg⁻¹, respectively). All three emulsion types were prepared using PEG-free emulsifiers of vegetable-based raw materials at the concentration of 5% *m/m* for the final product. The choice of emulsifiers was dictated by the current trends in the cosmetic market towards products of natural origin. PGPR is a lipophilic (hydrophilic–lipophilic balance (HLB) = 1.5) non-ionic emulsifier for W/O emulsions that is widely used in food products [39] and has recently entered the personal care industry. It is also known to be used for further introduction of primary W/O emulsion into multiple emulsions (W/O/W) [40]. Thus, it allowed preparation of a TSW/O/W emulsion based on the recipe of single a TSW/O emulsion with high TSW content. G-CLLO was chosen as a hydrophilic emulsifier (HLB = 11) for the stabilization of the O/TSW emulsion and the secondary emulsion of the TSW/O/W system [41].

Liposomes were prepared by thin layer rehydration technique followed by sonication leading to the formation of unilamellar vesicles. Saturated and unsaturated lipids (LPM-90H and LPM-90G, respectively) were used because of their different penetration enhancement potentials that have been reported previously [42].

Physicochemical characterizations of all formulations are reported in Table III. Cryo-TEM observations of liposomes showed a collection of small unilamellar vesicles (Fig. 1). Their mean size was smaller than 100 nm. Aggregation of phosphatidylcholine vesicles by divalent cations like Mg²⁺ or Ca²⁺ was not observed, which was in agreement with Düzgüneş *et al.* [43] who did not notice aggregation or fusion of vesicles up to 10 mM Ca²⁺ and 100 mM NaCl. Optical microscopy allowed observation of the three emulsion types (Fig. 1). Single emulsions showed well-separated spherical droplets with a mean diameter in the 1–2 µm range estimated from the pictures. The TSW/O/W double emulsion showed larger droplets including smaller droplets of TSW inside their oil core. The O/TSW emulsion was fluid (twice the viscosity of pure water), which was expected for a well-stabilized emulsion containing 40% dispersed phase (MCT + G-CLLO). The TSW/O/W double emulsion was more viscous owing to its higher volume fraction of dispersed phase reaching 55% (50% primary TSW/O emulsion + 5% G-CLLO). These results show that the G-CLLO emulsifier

Table III Physicochemical characterization of liposomes made of saturated and unsaturated phospholipids (LPM-90H and LPM-90G, respectively) and three emulsion types: O/TSW, TSW/O/W and TSW/O. Mean size of emulsion droplets was estimated from optical microscopy observations; those for liposomes were measured by dynamic light scattering (mean size and PDI)

	LPM-90H	LPM-90G	O/TSW	TSW/O/W	TSW/O
Size (µm)	0.08 ± 0.01	0.07 ± 0.01	1–2	5–20	1–2
PDI	0.2 ± 0.02	0.2 ± 0.02			
Viscosity (mPa s)	–	–	1.6 ± 0.1	6.8 ± 0.3	197 ± 13
<i>T</i> = 25°C, strain rate = 300 s ⁻¹					
pH	7.5	7.6	5.3	5.5	–

was efficient at the stabilization of emulsions, so that droplets of dispersed phase were independent of each other. The viscosity of the TSW/O emulsion was high (about 7 times that of pure MCT oil) because of the fairly high concentration of dispersed phase (TSW + PGPR) of 65% approaching the theoretical limit for random packing of hard spheres [44].

Skin absorption studies were performed by exposure of skin explants for 24 h under infinite dose conditions. These are not considered 'in-use conditions', but they were chosen to maximize the effects of applied dose and allow clearer differentiation between

endogenous and exogenous cations. The mass balance calculations indicated the recovery of $100 \pm 15\%$ of initially applied dose proving that the extraction method allowed complete ion recovery. As expected after infinite dosing conditions, the majority of ion content (80–90%) was recovered from the donor chamber (data not shown).

Figure 2 and Table IV show the total absorbed amounts of Ca^{2+} and Mg^{2+} in AM + D + VE + SC (Q_{abs} in nmol cm^{-2}) and their distribution in skin layers and AM. It can be noticed that the Q_{abs} of Ca^{2+} was higher than the one of Mg^{2+} , which was expected based

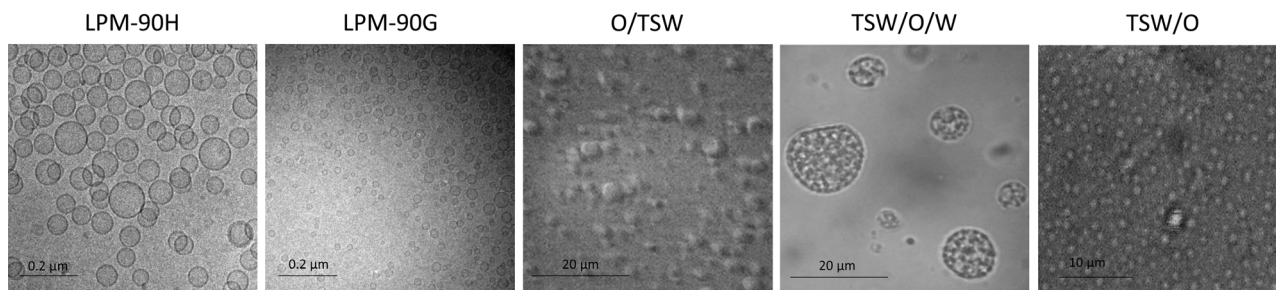


Figure 1 Microscopic images of formulations. Pictures of LPM-90H and LPM-90G were taken by cryo-TEM. Optical microscopy pictures were taken for O/TSW, TSW/O/W and TSW/O.

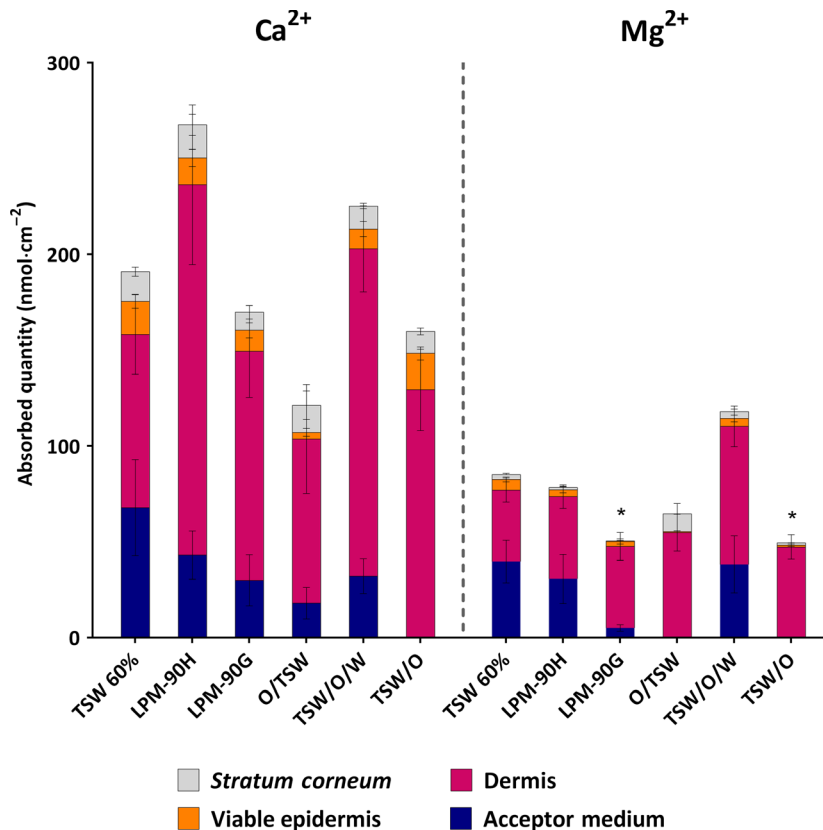


Figure 2 Distribution of Ca^{2+} and Mg^{2+} in the skin layers after 24-h exposure to emulsions (O/TSW; TSW/O), liposomes (LPM-90H and LPM-90G), the multiple emulsion (TSW/O/W) and TSW 60% as a reference. Superimposed bars correspond to total absorbed amount (Q_{abs}). Values represent mean \pm SEM from 8 experiments. Asterisk (*) indicates statistically significant difference obtained between given sample and TSW 60% in the *t*-test ($P < 0.05$).

on the composition of the TSW. Moreover, skin absorption profiles differed depending also on the formulation type. Statistically significant differences were difficult to obtain which were related to the variability typical for the results of skin absorption experiments.

Q_{abs} values were generally not altered by incorporation of TSW in various formulations except for Mg^{2+} , loaded in LPM-90G and TSW/O which led to a significantly lower Q_{abs} . Actually, in the case of Ca^{2+} a trend to higher Q_{abs} was observed after application of LPM-90H and TSW/O/W and a lower Q_{abs} in the case of the O/TSW emulsion, but these differences were not statistically significant. Further analysis of Ca^{2+} distribution in the skin layers after 24-h exposure revealed a strong correlation between the Q_{abs} values and the amounts extracted from the dermis for the LPM-90H and TSW/O/W formulations (Fig. 2 and Table IV). The same observation can be made for Mg^{2+} when loaded in TSW/O/W emulsion. We can therefore conclude from the results shown in Fig. 2 that the application of a double emulsion led to a significantly stronger retention of both cations within this layer as compared to the control.

The mean amounts of calcium reaching the AM followed the order in which the lipophilic character of formulations increased as follows: TSW60% > liposomes > TSW/O/W > O/TSW > TSW/O which was an expected result. Calcium and magnesium ions did not permeate from the TSW/O emulsion where TSW was entrapped in the inner core of the droplets. These results were also expected since it is well-known that the continuous lipophilic phase delays penetration of hydrophilic drugs (glucose [31], caffeine [45]) because of their unfavourable partition coefficient between oil and water that retains them in the inner aqueous droplets of W/O emulsions. However, a more surprising result was obtained for O/TSW since Mg^{2+} was not recovered in the acceptor medium at all with calcium reaching the AM in a very low amount. Even though O/TSW was the only emulsion in which TSW was not encapsulated and the concentrations of Ca^{2+} and Mg^{2+} in the external aqueous phase were higher than in the control, the absorption rates of both cations tended to be lower with high recovery from upper skin layers. An explanation is the higher oil fraction in these single emulsions comparatively to the other formulations. This high oil fraction remains on the skin, after coalescence of the emulsion droplets on the skin surface, causing occlusion regardless of the type of emulsion (O/W or W/O). This slows down the cation release. The main difference between both cations is their concentration being three-fold higher for calcium than magnesium. This can explain why some detectable calcium amount reached the AM comparatively to magnesium during the time of the experiment.

Finally, the distribution of Ca^{2+} in the SC and VE did not differ significantly among the vehicles except for the O/TSW formulation in the latter layer for both cations. This was the result of a previous observation: the lowest total amount of cations absorbed from O/TSW (Fig. 2 and Table IV).

As it can be noticed from these results, separate Mg^{2+} and Ca^{2+} cations do permeate through the skin, in spite of the electrostatic barrier against permeation of the positively charged (at low pH) *stratum corneum* (Table IV, Fig. 2). This is in agreement with several authors who reported the permeation of these inorganic cations in spite of their unfavourable physicochemical properties [11,12,46]. Both Mg^{2+} and Ca^{2+} are considered hard metal ions of low polarizability because they retain valence electrons close to their nucleus. Hard cations can easily associate with hard bases bearing oxygen (e.g. water), nitrogen (e.g. amines, ammonia) and phosphate [47]. Extensive literature reports show that divalent cations easily bind to glycolipids, phospholipids, carboxylate,

Table IV Results of dermal absorption of Ca^{2+} and Mg^{2+} from the emulsions and liposomes in nmol cm^{-2} . The values represent the mean values of 8 experiments and are expressed as mean \pm SEM. $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratios (R) were calculated based on the mean values obtained for each skin layer

	TSW 60%		LPM-90H		LPM-90G		O/TSW		TSW/O/W		TSW/O							
	Ca^{2+}	Mg^{2+}	R	Ca^{2+}	Mg^{2+}	R	Ca^{2+}	Mg^{2+}	R	Ca^{2+}	Mg^{2+}	R						
Q_{abs}	190 \pm 38.5	84 \pm 11	2.3	267 \pm 53	78 \pm 20	3.4	169 \pm 27	50 \pm 7.4	3.4	117 \pm 41	66 \pm 16	1.8	171 \pm 23	72 \pm 11	2.4	160 \pm 22	50 \pm 6	3.2
AM	68 \pm 25	40 \pm 11	1.7	43 \pm 13	31 \pm 13	1.4	30 \pm 13	5.0 \pm 1.7	6.0	18 \pm 8	0	/	32 \pm 9	38 \pm 15	0.8	0	0	/
D	90 \pm 21	37 \pm 6	2.4	193 \pm 42	43 \pm 6	4.5	120 \pm 24	55 \pm 7	2.2	86 \pm 28	55 \pm 9.6	1.6	171 \pm 23	72 \pm 10	2.4	129 \pm 21	47 \pm 6	2.7
VE	17 \pm 4	5.5 \pm 1	3.0	14 \pm 4.5	3.6 \pm 1.6	3.9	11 \pm 3.9	2.6 \pm 1.3	4.2	3.5 \pm 2	0.5 \pm 0.5	7.0	10 \pm 4	4.2 \pm 2	2.4	19 \pm 3	1.0 \pm 0.6	19
SC	15 \pm 2	2.6 \pm 0.7	5.8	17 \pm 5.5	1.2 \pm 0.7	14.2	9.4 \pm 3.5	0.2 \pm 0.2	47	14 \pm 7	9.3 \pm 5	1.5	12 \pm 1	3.6 \pm 1	3.3	11.4 \pm 2	1.2 \pm 0.1	9.5

Statistically significant differences ($P < 0.05$ in t -test when compared with TSW 60%): the values which are lower than for TSW 60% are shown in *italic*; the values which are higher than for TSW 60% are shown in **bold**

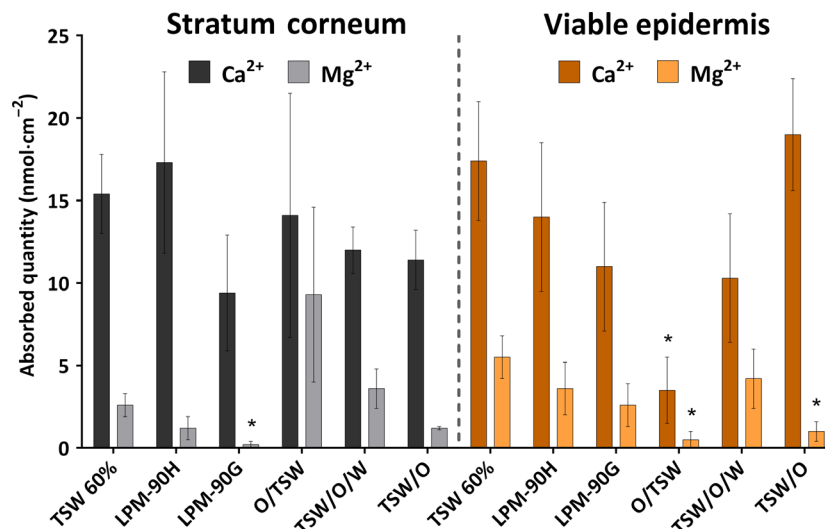


Figure 3 Distribution of Ca^{2+} and Mg^{2+} in the epidermal layers (SC and VE) after 24-h exposure. Values represent mean \pm SEM from 8 experiments. Asterisk (*) indicates statistically significant difference obtained between given sample and TSW60% in the *t*-test ($P < 0.05$).

phosphate groups and carbonyl groups of *sn*-2 phospholipid chains [48–51]. Moreover, their ability to permeate skin samples may be explained by the long exposure time and hydration of the skin during the experiment with water being a penetration enhancer for hydrophilic substances in some parts of the *stratum corneum*. Van Hal *et al.* observed swelling of corneocytes under water exposure and a smoother structure of the intercellular bilayers with the presence of water pools for skin explants immersed in PBS for 48 h. This may shorten the length of the diffusional path in the *stratum corneum* between corneocytes. Additionally, small hydrophilic substances may profit from the presence of water in the intercellular layer to diffuse [52–54]. Kosmotrope ions being strongly hydrated and tightly bound to water may benefit from skin hydration to cross the *stratum corneum*. Even though water creates water pools in the *stratum corneum* after long exposure time, a part of the *stratum corneum* does not participate in water pool formation. Thus, this deeper region over which lipid lamellae are formed still acts as a barrier [53]. Given the cosmetic application of TSW-based formulations and their specific action on skin barrier enhancement, the ideal formulation would enhance retention of Ca^{2+} and Mg^{2+} within the *stratum corneum* and viable epidermis that are the target sites for the skin repair mechanisms (lipid synthesis and the acceleration of exocytosis of lamellar bodies) [15,19]. This desired retention enhancement, however, should be equal for both cations; otherwise, the initial $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratio (3 : 1) can change and lead to a reverse effect of inhibition of skin recovery, as reported by Denda *et al.* [15]. Considering the total quantity retained in the skin, the $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratio was comprised between 1.8 and 3.4 depending on the formulation (Table IV), and given the variability of the data expressed as *sem* values (Table IV), it was not different of the initial 3 : 1 ratio reported in TSW (Table I). This result is in agreement with previous papers showing a clear correlation between the concentration of ions and their flux [10,12]. However, a closer observation of the results in the skin layers shows that the ratios differ depending on the formulations.

In the upper epidermal layers, where the cations influence the skin repair mechanisms, some differences in their distribution arise

depending on the formulations. In the *stratum corneum*, the increase in the ratio recovered from the skin layers was caused by the variations in Mg^{2+} as the values recovered for Ca^{2+} were comparable among all the formulations. Consequently, it was the dermal absorption of Mg^{2+} that drove the $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratios in this layer (Fig. 3). The ratio for the classical emulsions was equal to 1.5 and 9.5 in the *stratum corneum*, respectively, for the O/TSW and TSW/O. In the viable epidermis, the ratio was very far from the 3 : 1 ratio. It was larger than 7 in the viable epidermis regardless of the type of emulsion. It reached 19 for the TSW/O emulsion. High $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratios were recorded in viable epidermis precisely for the formulations that tended to slow down the absorption of Mg^{2+} . It is worth noticing that in the experimental set-up applied within this study, ion transport in viable skin layers occurs through ion-specific transporters [55,56]. Physiological manifestation of this phenomena is shown by the regulation of the ratio in the deeper layers in the absence of skin damage [57]. It can be hypothesized, on one hand that when skin absorption is slow (TSW/O and O/TSW), the ion channels and ion transporters are efficient enough to pump the cations into deeper skin layers according to their concentration gradient. Observed low concentrations of Mg^{2+} in the viable epidermis could be attributed to the efficacy of this mechanism. Accumulation of Mg^{2+} could occur in VE for the formulations, which accelerated skin absorption as a result of saturation of these ion transporters.

Liposomes have been chosen as vehicles because of their various benefits in skin delivery. Several authors observed increased skin absorption of molecules from liposome suspensions as compared to control solutions. Higher penetration rates were reported for the compositions which included unsaturated phospholipids [42,58]. Results obtained for both types of liposomes departed from expectations based on literature data. In the present study, only slight enhancement of Ca^{2+} absorption was observed from LPM-90H liposomes, whereas LPM-90G did not promote absorption of Ca^{2+} and even delayed significantly the Mg^{2+} release into deep skin layers. The reason for the disagreement between our results and those reported in the literature is probably the different nature of tested

ions. Hydrophilic organic compounds, such as caffeine, do not interact with the lipids of the formulation and the penetration enhancement reported for this molecule is due to the ability of phosphatidylcholine to modulate skin barrier properties [42]. In present study, divalent cations contained in a multi-ionic mixture of TSW can interact with phospholipid bilayers since they can bind to phosphate groups [59,60]. Also *et al.* demonstrated that Ca^{2+} and Mg^{2+} can bind to completion to the phospholipid membranes and that they are located inside the bilayer. Mg^{2+} was found to bind closer to the phosphate group and to coordinate with water oxygen, whereas Ca^{2+} was adjacent to the glycerol group [61]. Such a deep penetration of ions into the polar regions of the membrane led by swelling due to water revealed an increase in the lamellar repeat distance. The origin may be the electrical charge coming from divalent cation bonding that causes an electrostatic repulsion between bilayers together with the water uptake by hydration of bound cations [62]. Some authors reported that an increase in the number of water molecules bound per lipid molecule can better explain the swelling phenomena in the following order $\text{Fe}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Zn}^{2+}$, corresponding to the Hofmeister series [61]. For dipalmitoylphosphatidylcholine (DPPC), swelling has been reported until a ratio DPPC : $\text{Ca}^{2+} = 1 : 0.14 \text{ mol mol}^{-1}$ as in the present study [63]. The release of the magnesium ion is greatly affected when Mg^{2+} is entrapped in both liposomes comparatively to calcium. Therefore, we observed an imbalance of $\text{Ca}^{2+} : \text{Mg}^{2+}$ in the SC and VE with $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratios larger than 10.

The consequences of an imbalance in calcium to magnesium ions ratio >2.6 – 2.8 or <2 in humans have been recently reviewed by Rosanoff *et al.* [64]. Unbalanced dietary intake of these two ions was shown to have serious health consequences. For example, increased serum $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratio was associated with an increased risk of prostate or colorectal cancer [64]. Lee *et al.* [65] have shown that high concentrations of Ca^{2+} associated with low Mg^{2+} can result in a detrimental effect in the brain and suggested that administration of Mg^{2+} could be an strategy for reducing neuroinflammation caused by elevated Ca^{2+} in degenerative neurological disorders.

The TSW/O/W double emulsion was the only formulation which maintained the 3 : 1 ratio in all skin layers, especially in the SC which was not the case in other formulations and the control.

TSW/O/W had a specific behaviour as a part of cations reached the acceptor medium. The double emulsion used by Ferreira *et al.* [31] had an intermediate glucose release profile ranging between those of the O/W and the W/O emulsions. In this study, the TSW/O/W promoted the absorption of both cations and even a fraction of Mg^{2+} was recovered in the acceptor medium which was not the case for simple emulsions. This can be explained by the fact that this double emulsion combines both hydrophilic and hydrophobic emulsifiers (HLB of the mixture being close to 5) that interact better with the lipidic fraction of *stratum corneum* than

individual emulsifiers in simple emulsions. Such a mixture can act as a skin absorption enhancer.

Conclusions

The present study aimed at investigating the impact of a vehicle, in which TSW was formulated, on skin absorption profiles of magnesium and calcium, the two cations of particular interest because of their biological activity in the skin tissue.

In the first place, we developed and characterized liposomal suspensions and concentrated emulsions (simple: water-in-oil, oil-in-water or double: water-in-oil-in-water) using vegetable raw material-derived emulsifiers. These were used as model formulations for skin absorption experiments. The infinite dosing conditions under occlusion were applied here for two reasons. Firstly, in the case of balneotherapy, the whole body can be immersed in the TSW for a short time, whereas the clinical application of mud packs is practised under occlusion. Both types of treatment gave measurable beneficial effects on health indicating the ion absorption through the skin. Secondly, this paper should be considered as an exploratory study of the subject. Therefore, we extended the contact time to 24 h to maximize the potential skin permeation.

It has been demonstrated that in applied conditions, Ca^{2+} and Mg^{2+} ions can indeed penetrate inside the skin and, in case of some formulations, through the skin. These results suggest that the beneficial effects of TSW, so far observed in cell culture of keratinocytes or fibroblasts, could possibly occur in viable skin as ions constituting the mineral water are able to reach the different skin layers (SC, VE and D) from the TSW itself. Furthermore, the tests performed on models of cosmetic formulations disclosed that ions can be released from the formulations and then penetrate into the skin. Liposomes, contrary to what has been reported for other molecules, did not enhance significantly skin absorption of Mg^{2+} , but they improved the skin absorption profiles of Ca^{2+} leading to an imbalance of the $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratio. On the other hand, the TSW/O/W increased skin absorption (Q_{abs}) in case of both cations of interest as compared to control (TSW60%), and it also respected the $\text{Ca}^{2+} : \text{Mg}^{2+}$ ratio.

To mimic the typical cosmetic application, the study investigating skin penetration under 'in-use conditions' (finite dose without occlusion) should be performed in the future.

Acknowledgements

The authors are thankful to Pierre-Yves Dugas (University of Lyon 1, C2P2 UMR 5265) for cryo-TEM observations at the 'Centre Technologique des Microstructures' (CTμ – University of Lyon 1). The help of Léon Bérard Medical Centre in providing skin explants is also acknowledged. Ministère de l'Enseignement Supérieur et de la Recherche (France) is also acknowledged for financial support of MT.

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