Evaluation of skin absorption of inorganic ions with regard to their physicochemical properties

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Étude du passage cutané des ions inorganiques selon leurs propriétés physicochimiques

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(To my parents)

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EVALUATION OF SKIN ABSORPTION OF INORGANIC IONS WITH REGARD TO THEIR PHYSICOCHEMICAL PROPERTIES

Human skin forms a unique interface between the body and the external environment. Its main role is to protect the internal organs from external factors. Its highly hydrophobic outermost layer, *stratum corneum*, has long been believed impermeable for highly hydrophilic compounds, including ions. Several studies proved this concept wrong, and recent research by Paweloszek *et al.* demonstrated the important contribution of facilitated transport in permeation of halide anions.

Skin penetration of anions classified in Hofmeister series (of F^-, Br^-, I^-, SCN, ClO_4^-) alone and in bi- and ternary mixtures in two experimental series was studied *in vitro*. All tested ions permeated viable skin within 24h. Among halides, the presence of F^- reduced the penetration of Br^- and I^- in mixtures, and synergy between Br^- and I^- was observed. Within the second group (I^-, SCN^-, ClO_4^-) the inhibition of ClO_4^- penetration in the presence of other ions was observed.

Finally, the impact of formulation of marketed thermal spring water (TSW) into emulsions (TSW/O, O/TSW, TSW/O/W) and liposomes on skin absorption of Ca^{2+} and Mg^{2+} was evaluated. Liposomes and emulsions promoted retention of Ca^{2+} and Mg^{2+} in skin layers as compared to TSW. Our results prove that the beneficial effects observed during treatment with TSW are associated with penetration of the minerals into and through the skin and are not only a surface action.

In this thesis, we demonstrate the possibility of both anions and cations to penetrate viable skin *in vitro*, and we disclose the effects of mixing and formulating on skin penetration profiles.

Keywords: **skin penetration, inorganic ions, Hofmeister series, Thermal Spring Waters, formulation**

ÉTUDE DU PASSAGE CUTANE DES IONS INORGANIQUES SELON LEURS PROPRIETES PHYSICOCHIMIQUES

La peau constitue une interface unique entre l'organisme et son environnement extérieur. Son rôle principal est de protéger les organes internes contre les facteurs externes. Le *stratum corneum*, est la couche supérieure et très hydrophobe de la peau. Elle était considérée peu perméable pour les molécules hydrophiles chargées tels que les ions inorganiques. Plusieurs études ont démontré le contraire et l'étude récente par Paweloszek et al. a indiqué l'importance du transport actif dans le passage cutanée des ions halogénures.

L'absorption cutanée des ions classés selon la série de Hofmeister (F^-, Br^-, I^-, SCN, ClO_4^-) seuls et en mélanges bi- et ternaires a été évaluée dans deux séries d'expériences *in vitro*. Tous les ions ont pénétré dans la peau viable en 24h. Parmi les halogénures, la présence de F^- a diminué l'absorption de Br^- et de I^- en mélanges, tandis qu'une synergie d'absorption entre Br^- et I^- a été observée. Dans le second groupe (I^-, SCN^-, ClO_4^-), la pénétration cutanée du ClO_4^- a été inhibé par la présence des autres ions.

L'impact de la formulation sur l'absorption cutanée de Ca^{2+} et Mg^{2+} présents dans des eaux thermales (TSW) a été évaluée. Différentes formes galéniques telles que des émulsions (TSW/O, O/TSW, TSW/O/W) et des liposomes ont été étudiées. Les liposomes et les émulsions ont favorisé la rétention de ces ions d'intérêt dans les couches cutanées en comparaison à l'eau thermale pure. Nos résultats ont démontré que les effets bénéfiques associés aux traitements à base de TSW ne sont pas seulement dus à une action superficielle mais à la pénétration des ions dans la peau.

Dans cette thèse nous démontrons la capacité des anions et cations à pénétrer la peau viable *in vitro* et nous mettons en évidence les effets de mélange et de la formulation sur la pénétration cutanée.

Mots clés : **pénétration cutanée, ions inorganiques, série d'Hofmeister, eaux thermales, formulation**

General introduction

Human skin, one of the biggest organs of the body, creates a unique barrier between the internal and external environment of the organism. It provides protection against physical and chemical factors entering the organism on the one hand, and prevents excessive water evaporation- on the other. This primary role of the skin is fulfilled owing to its specific, non-uniform structure. *Stratum corneum*, the outermost skin layer is often considered the main barrier for skin absorption of chemicals. It is built of dead, nuclei-deprived keratinised cells (corneocytes) immersed in the lipidic matrix creating highly hydrophobic barrier. Therefore, skin absorption of xenobiotics is considered limited to the molecules fulfilling "the rule of 5" which implies low molecular weight (< 500 $g \cdot mol^{-1}$), less than 5 H-bond donors, less than 10 H-bond acceptors, and the calculated LogP between 1 and 3 [1, 2]. With regard to these criteria, inorganic ions have long been considered inappropriate candidates to pass through the skin, mainly because of their high hydrophilicity (negative values of Log P) [3]. However, measurable positive outcome observed since many centuries after thermal spring water (TSW)-based therapies (hydro- and balneotherapy) has been contradicting this view [4–8]. These results triggered fundamental research on skin absorption of inorganic ions with the view of toxicological evaluations [9, 10], therapeutic [11, 12] and cosmetic [13, 14] applications.

Above all, it must be noticed that ions are endogenously present in the skin, reflecting the general elemental composition of human body, and are distributed in a specific manner. Several patterns are observed within the tissue for macroelements: (i) Na^+ and Cl^- concentrations increase from *stratum corneum* towards dermis [15–17]; (ii) Ca^{2+} and Mg^{2+} appear as a concentration gradient [18]; (iii) concentrations of K^+ and P are low in the *stratum corneum*, high in the viable epidermis and low again in the dermis [15–17, 19]; (iv) sulphur concentrations are higher in *stratum corneum* than in living skin. Moreover, microelements such as Zn^{2+}, Cu^{2+} and Fe^{2+} as well as trace elements Cr^{3+}, Ni^{2+} and Si can be found in the skin [16, 20]. Ions play important roles in skin homeostasis acting as messengers or cofactors of enzymes involved in regulation of barrier function, wound healing, synthesis collagen and elastin, etc. [21–23].

Depending on the effects that ions exert in the skin, their absorption can be an object of investigation in various fields. Dermal penetration of contact allergens (ex. Ni^{2+}, Co^{2+}, Cr^{3+}), pollutants (Pb, Hg) and radioactive ions (ex. $^{99m}TcO_4^-$, $^{67}Ga^{3+}$, ^{131}I) is studied for toxicological purposes, and to help establishing decontamination protocols in case of accidental exposure [24–26]. Another field of studies where there is clear interest in the impact of ions on the skin is dermocosmetology. Ions found in thermal spring waters are claimed to have beneficial effects on skin condition that strongly depend on the mineral composition. The waters differ in the proportions among bulk minerals

(sodium, calcium, magnesium, potassium, chlorides, sulfates and bicarbonates) as well as in the content of trace elements (zinc, copper, strontium, selenium, iron, manganese, silica, fluoride, bromide, etc.), and are commonly used as active ingredients for cosmetic formulations [4, 27, 28]. Finally, medical applications of salts and ions administered topically [29] or even used as penetration enhancers for other molecules [30, 31] have been described.

Multiple studies report that certain inorganic ions are able to passively cross skin barrier while others are retained within upper skin strata. The individual behaviour of ions depends on their properties: oxidation state, mobility, binding affinities, etc. These characteristics correlate well with their order within Hofmrister series, classifying inorganic ions based on their ability to interact with macromolecules. Ions that are poorly hydrated in aqueous solutions (chaotropes) exhibit weaker interactions with water than water itself ("water-structure breakers"). Thus, they interact strongly with non-polar substances, resulting in their increased solubility (salting-in effect). On the other hand, the presence kosmotropes that interact strongly with water molecules ("water structure makers"), reduces the solubility of non-polar substances (salting-out effect) [32, 33]. For instance, monovalent cations (Na^+, K^+) have rather kosmotropic properties and rarely interact with proteins. Therefore, they are considered very mobile. Divalent Mg^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+} are less mobile due to their possible interactions with proteins (complexation of S and N) or even with phospholipids constituting cell membranes [34, 35]. In the Hofmeister framework they have chaotropic properties [32, 33]. Although no systematic study concerning skin absorption of cations using standardised conditions has been reported so far, the abovementioned parameters could explain why certain cations reach deeper skin layers or even blood (*in vivo*) or acceptor medium (*in vitro*). For instance, Franz cell experiments conducted by Tregear showed measurable flux of Na^+ and K^+ [36], a report on the clinical use of Epson Salt baths revealed significant increase of blood levels of Mg^{2+} and SO_4^{2-} in patients taking regular baths in Epson Salt [14] also demonstrating skin penetration of these ions. On the other hand, several *in vitro* studies showed that trivalent cations, such as Cr^{3+} Al^{3+}, In^{3+} and Ga^{3+} accumulate in the upper skin strata without penetration [13, 24, 37–39].

Available bibliography reporting skin absorption of anions is poorer as compared to that of cations probably because less biological activity in the skin has been described for the negatively charged ions than for cations. So far, skin absorption of PO_4^-, Br^- and I^- has been reported [24, 36]. A recent study on cutaneous penetration of inorganic anions pioneered systematic research in the field. Paweloszek et al. investigated skin absorption of halide anions (F^-, Cl^-, Br^- and I^-) from aqueous solutions and demonstrated that: (i) skin absorption of these anions is proportional to deposited concentration; (ii) the order of total absorbed amounts of anions increased in the following order: $F^- < Cl^- < Br^- < I^-$; (iii) lag time observed for iodide was particularly long (5 h). Additionally, authors

disclosed that facilitated transport contributes to up to 80% of skin absorption of tested anions, indicating that the use of viable skin explants in Franz cell experiments is required for absorption studies of ions [40].

Other than skin condition, various parameters can affect skin absorption of anions. Exogenous ions can encounter the endogenous ones, and might interact with them. As example, a monovalent silver ion Ag^+ applied as an aqueous solution of $AgNO_3$ for treatment of difficult wounds reacts with the endogenous anions (Cl^-, SO_4^{2-}, or PO_4^{3-}) and form insoluble salts that precipitate in the upper layers of damaged skin. Moreover, the counter-ion and a type of vehicle in which salts are incorporated can also influence skin absorption rates of the ion of interest [41].

The aim of this thesis was to study skin absorption of inorganic ions with regard to their physicochemical properties.

The manuscript consists of four chapters: one theoretical and three experimental.

Chapter I includes the bibliographic report in the form of a review (to be submitted in *International Journal of Pharmaceutics)* describing the distribution of water and endogenous ions within the skin and roles that they play in skin homeostasis. Beneficial and harmful effects of skin exposure to exogenous ions for skin health are discussed.

Chapter II is dedicated to the methodological aspect of the thesis. Given a great contribution of active and facilitated transport in skin absorption of ions, it was necessary to establish proper experimental conditions ensuring viability of skin explants over 24 h. This part includes a manuscript of a research communication (published in *International Journal of Cosmetic Science, https://doi.org/10.1111/ics.12581*) providing an insight on design of acceptor medium for maintaining metabolic activity of skin explants in an experiment using Franz cell method.

Chapter III gathers the results of skin absorption of anions classified in the Hofmeister series (F^-, Br^-, I^-, SCN^-, ClO_4^-) from concentrated aqueous solutions. The effects of mixing them in bi- and ternary systems were also evaluated. Two sections corresponding to manuscripts of two articles are included. The first one concerning the effects observed for F^-, Br^-, I^- (under review in *Soft Matter*) and the second related to more chaotropic ions: I^-, SCN^-, ClO_4^- (to be submitted in *International Journal of Pharmaceutics*).

Chapter IV is the applicative part of this research in which we evaluated skin absorption of Ca^{2+} and Mg^{2+} from thermal spring water and the effects of incorporating TSW into model formulations (w/o, o/w, w/o/w emulsions and liposomes). It contains a manuscript submitted to *International Journal of Cosmetic Science).*

In the last section we gather general conclusions of the work presented in the manuscript and give the prospect for further research in the field.

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BIBLIOGRAPHICAL REVIEW

Chapter I

Inorganic ions in the skin: allies or enemies?

Inorganic ions in the skin: allies or enemies?

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Abstract

Skin constitutes a barrier protecting the organism against physical and chemical factors. Therefore, it is constantly exposed to the xenobiotics, including inorganic ions that are ubiquitous in the environment. Some of them play important roles in homeostasis and regulatory functions of the body, also in the skin, while others can be considered dangerous. Many authors have shown that inorganic ions could penetrate inside the skin and induce more or less local effects. In this review, we resume the current knowledge on the effects of skin exposure to inorganic ions. Beneficial effects on skin conditions related to the use of thermal spring waters are discussed together with the application of aluminium in underarm hygienic products and silver salts in treatment of difficult wounds. On the other hand, the potential harmful consequences of dermal exposure to topical sensitizers and harmful heavy ions including radionuclides are discussed.

1 Introduction

Ions take part in various life processes. Some of them are called essential as they are indispensable for growth, reproduction, and maintaining general good health. Others are considered nonessential and may reflect the dietary intake, environmental exposure or even geochemical origins of humans r[1, 2]. Finally, some of them, called trace elements, can be beneficial for health owing their pharmacological action. As evidenced by many authors, the main body elements (99%) are light and have atomic numbers lower than 36, except iodine [3–6] . The most abundant ones are bulk structural elements: oxygen (25.4% wet wt), carbon (9.4% wet wt), hydrogen (62.8% wet wt), and nitrogen (1.4% wet wt). Besides, macrominerals such as sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), chlorine (Cl), phosphorus (P) and sulphur (S) (mainly as phosphates (PO_4^{3-}) and sulfates (SO_4^{2-}) contribute greatly to the elementary composition. All of the non-metals, excluding the inert gases, with atomic numbers smaller than bromide's, are considered essential. Essential trace elements are those that occur in amounts in the range of µg per g or less. Most them are transition metals or are classified in a subdivision of the group, as reported by Frieden (Table I) [5]. Depending on the author, the classification comprises: iron (Fe); zinc (Zn); copper (Cu), fluorine (F); iodine (I); selenium (Se); manganese (Mn); cobalt (Co); molybdenum (Mo) and chromium (Cr). Finally, ultra-trace elements are those for which there is only experimental evidence from animal models: boron (B); vanadium (V); silicon (Si); nickel, (Ni) and arsenic (As) (Table I) [1, 3–6]. All groups of periodic table of elements are represented except from the IIIA and IVA and inert gases (Table I). Consequently, multiple types of chemical interactions with biological tissues are expected [4]. Although some biological effects of nonessential ions are still under debate, the essential ions are known to be indispensable for the maintenance of life and, are active in a certain concentration range. Their deprivation or over consumption may lead to severe damage [7, 8]. Apart from C, N, H and O, which are essential for synthesis of biological structure of the body, some ions have essential functions for the transition of information (Na^+, K^+, Ca^{2+}), catalysis (Fe^{2+}, Cu^{2+}, Zn^{2+} and Mo^{2+}), or electron transfer (Fe^{2+}) [4]. The characteristic electrolyte patterns of both intracellular and extracellular fluids are crucial for cell survival and homeostasis [3, 9]. In body tissues some of the ions are necessary to ensure the electrical activity supporting muscle contractions and to help to maintain the function of nerve cells. At the cellular level, electrolytes produce charged molecules when dissolved in water and contribute to osmotic pressure and movement of water between body compartments. They perform a number of other functions such as the preservation of electrical neutrality or maintaining acid-base balance and plasma isotonicity. They can also act as carriers, messengers, cofactors in enzymes and stabilizers of proteins and lipids. In the human body, sodium is the most prominent cation in extracellular fluids (140 $mmol \cdot L^{-1}$ against 12 $mmol \cdot L^{-1}$ in the

intracellular space) while potassium is its counterpart intracellular cation with 140 $mmol \cdot L^{-1}$ against 3.5 to 5 $mmol \cdot L^{-1}$ in the extracellular space [10]. Chloride is the first body anion, mostly extracellular, and represents 70 % of the total negative ion content [11]. Calcium is the most abundant cation (1.5 %) of total body weight the majority of which (99%) is found in bones and teeth in association with phosphate as hydroxyapatite. Calcium ions are also involved in blood clotting, normal muscle contraction and nerve activity. Magnesium ions, Mg^{2+}, are associated with the normal functioning of muscle and nerve tissue, bone formation and are cofactors of many coenzymes. The detection of any deviation from the normal concentration ranges of these species is useful for the diagnosis of metabolic disorders such as diabetes, liver and renal dysfunction, or cardiac problems [1, 12]. Exposure to all of the abovementioned elements can have either positive or detrimental effects which depend on their concentration and administration route. Finally, other ions of the periodic table of elements may also interfere with the human body because they are present in the environment and may be ingested, inhaled or applied topically. As example, heavier metals such as Cd, Hg, Pb or lanthanides are present in the environment (Earth's crust for example) and are toxic for most organisms.

The skin is the largest organ of the body and plays the role of the frontier between the external environment and the interior of the organism. It represents an attractive route for therapies (topical and systemic) but it may also be an entry gate for hazardous agents. It must preserve "a state of dynamic equilibrium with its surrounding environment and is dependent on a correct intracellular and intercellular environment in achieving an optima proliferation and differentiation" as stated by Lansdwon [12, 13]. Moreover, the skin may be regarded as one of the most important water-storage organs of the body. With a water volume ranging between 623 ml per kg of tissue and 717.7±20.1 g per kg of fat free skin, this volume is considerably larger in the skin (20% of the body water content) than in any other tissue of the body [8, 13, 14]. The first studies aiming at determining the ionic content of the skin were carried on only in the eighties. This is quite amazing given that one of the most important functions of skin is the control of the fluxes of electrolytes and water across this tissue and that "taking the waters" is the most ancient water treatment promising relief to the sick [15, 16]. The distribution of ions in the skin serves as a reference for pathological changes occurring in the tissue as in the case of psoriasis or atopic dermatitis [16]. The combination of hot or cold baths from volcanic or sea water was beneficial for the treatment of numerous diseases including gout, paralysis, chronic pain, or convalescence after surgery. Conversely, some oral treatments or skin exposure may have adverse effects on the skin, for example lithium salts administered orally in psychiatric patients have been recognized to aggravate or provoke psoriasis [17]. Application of mercury salts is still popular due to their skin-lightening effect and causes nephrotic syndrome [18].

Skin sensitization has been reported after exposure to many ions including chromium (IV), cobalt, and nickel [12, 19].

This review attempts to summarize and analyse current knowledge regarding the effects of ions applied on the skin. It is focused upon the passive skin absorption of ions into the skin with a particular emphasis on their effects on human health. Assuming that all ions are dissolved in the tissue water, the distribution of both water and ions in the skin are intimately linked and therefore described in the first place. In the third section, the speciation of ions in biology is recalled. Next, the ion pathways to cross membranes are described. The fifth paragraph focuses on the effects of ions on human health. It mainly concerns the effects of cations on the skin as only few anions are recognized to disturb the skin when applied topically. Iodide is an exception and is described in the last section dedicated to the transport of anions related to their physicochemical properties.

Table I Periodic table of elements: ions essential for life

IA	**IIA**											**IIIA**	**IVA**	**VA**	**VIA**	**VIIA**	**VIIIA**
H																	He
Li	Be											B	C	N	O	F	Ne
Na	Mg	**IIIB**	**IVB**	**VB**	**VIB**	**VIIB**		**VIIIB**			**IB**	**IIB**	Al	Si	P	S	Cl	Ar
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	*Tc*	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
Cs	Ba	La-	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	Ac-	*Rf*	*Db*	*Sg*	*Bh*	*Hs*	*Mt*	*Ds*	*Tg*	*Cn*	*Nh*	*Fl*	*Mc*	*Lv*	*Ts*	*Og*
			La	Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
			Ac	Th	Pa	U	*Np*	*Pu*	*Am*	*Cm*	*Bk*	*Cf*	*Es*	*Fm*	*Md*	*No*	*Lr*

Bulk elements

Trace elements

Possible trace elements

2 Water distribution in the skin

The distribution and movement of water in the skin is important for maintaining the physical properties of the tissue such as permeability and flexibility. The presence of water regulates also the activity of enzymes and cells and is the solvent for many inorganic ions necessary for skin homeostasis.

Its distribution is widely used as a convenient marker of the boundary between adjacent layers which allows the measurement of the thickness of the *stratum corneum* by Confocal Raman Microspectroscopy (CRM).

The water distribution in the skin has been extensively studied by many authors and with the application of different methods both *in vitro* and *in vivo* (Table II) [15, 20–24]. The semi quantitative water profile must be carefully considered as it is based on two assumptions: the frozen-hydrated cryosections are uniformly thick, and uniform lateral shrinkage occurs throughout the section during freeze-drying process [15, 25, 26]. These assumptions are also applicable for the distribution of ions in the skin.

Table II summarises available data on water distribution in the skin layers. It can be noticed that the *stratum corneum* has the lowest water content (10 to 35 % expressed as percent of total weight under the ambient conditions of relative humidity ranging from 0 to 75%) contrary to the viable epidermis and dermis which contain 65 – 70% of water [15, 25, 26]. A discontinuity was also noticed between the *stratum corneum* and the *stratum granulosum*, the latter containing water rich viable cells. Some authors have noticed that the free water (water non-hydrogen bonded to biomolecules but hydrogen bonded to other water molecules) is located in the viable epidermis and dermis comparatively *to stratum corneum* (30-33% water bound to biomolecules like proteins) or the reticular dermis [25]. This bound water is incorporated in the multi-lamellar arrangement of the *stratum corneum* lipids and exerts a water-holding function [25, 27–29]. Finally, the hypodermis contains low percentage of water.

Von Zglinicki found an average water content of the *stratum corneum* quite higher (54%) as compared to Warner (15-40%) [15, 26] and a hydration of the dermis not so different from that of the viable epidermis. This result is in accordance with Richard et al. [30] who used Magnetic Resonance Imaging based on the variations of the relaxation times T1 and T2. The T1 relaxation times, which are related to the total water content are in the same range between both layers. The decrease of water proton mobility decreases with T2 relaxation times. The very short T2 value obtained for dermis has therefore been related to a motional constraint of protons probably due to a highly ordered structure of water around collagen fibrils. Very short T2 values are also reported in

the *stratum corneum,* which is not surprising considering that it is a dry layer composed of dead cells with water bound in keratins [31].

Table II Estimation of the water content in the skin

	Von Zglinicki et al. [26] **% of dry weight**	**Warner et al. [15]** **% of dry weight**	**Caspers et al. [32]** **% of wet tissue**
Method	Hydrated and freeze-dried samples	-	In vivo (6 volunteers)
Apparatus	X-ray microanalysis	-	Confocal Raman Microspectroscopy (CRM)
Location	-	-	forearm
Stratum corneum	54	15-40	35
Viable Epidermis	75	40-65	65
Dermis	75	70-75	65
Hypodermis	75	-	NA

3 Distribution of endogenous ions in the skin

Just as water, ions are sufficiently compartmentalized in the skin to constitute useful indicator of each skin layer [24].

Several authors have studied the elemental distribution of ions as mmoles per kg generally per dry weight (dw). Among the first papers on the body ionic content, Eisele and Eichelberger examined fat-free skin after cryo-freezing, followed by a desiccation in an oven and chemical analysis [13]. The skin samples were removed from different parts of the body and the following values in $mmol \cdot kg^{-1}$ were reported: Cl^-: 79 ± 4.8, Na^+: 93 ± 8, K^+: 16.47 ± 3.36, Ca^{2+}: 2.68 ± 0.52, Mg^{2+} 2.13±0.3; total nitrogen 45.5 ± 3.8 g and total water: 717.7 ± 20.1 g. Since then, more sophisticated biophysical techniques have been applied, such as ion capture cytochemistry with the oxalate-pyroantimonate-osmium technique [33, 34], X-ray microanalysis (XRMA) [26], particle probe microanalysis [35], electron probe analysis [15], and more recently, three different ion beam techniques methods: Scanning Transmission Ion Microscopy (STIM), Rutherford Backscattering Spectrometry (RBS), and Particle Induced X-ray Emission (PIXE) [36–39]. XRMA is useful to study Na, P, S, Cl and K but is rather limited by the sensitivity threshold for elements such as Mg, Ca, Fe, Cu and Zn. PIXE is a better method for these latter elements [40].

Within the abovementioned studies, there was no gender-related variations. A wide range of values of elemental content have been found which is explained by the differences in sampling methods and by the fact that the entire skin was considered [40]. Moreover, the units used to report results differ among papers and authors generally favoured figures rather than concentration tables to express their data which unables a precise mapping.

This distribution is often given layer by layer but does not always take into account the inter- and extracellular ion repartition together. Movements of diffusible components (e.g., physiologically important ions such as Na^+, K^+, Cl^-, and Ca^{2+}) are always possible but are minimized when fresh skin samples are quench-frozen immediately at very low temperature. Otherwise the results are an average between intra- and extracellular compartments. Table III yields some range from papers in which the data presentation made it possible to report the values in $mmol \cdot kg^{-1}$.

Major results found for cryopreserved skin could be summarised as follows:

- There are several gradients in the skin. Na^+ and Cl^- concentrations increase from the *stratum corneum* to the dermis [26, 39, 40]. Von Zglinicki, noticed a concentration gradient for Na^+ (50 $mmol \cdot kg^{-1}$ dw (SC) – 200 $mmol \cdot kg^{-1}$ dw (D)) and Cl^- (200 m $mmol \cdot kg^{-1}$mol/kg dw (SC) – 500 $mmol \cdot kg^{-1}$ dw (D)) from the *stratum corneum* to the dermis roughly in accordance with the results of Forslind [40]. Both authors found that Cl^- is always the most abundant anion (175 $mmol \cdot kg^{-1}$) which peaks in the dermis because of its extracellular nature. In the *stratum corneum*, Warner et al. found the opposite result with a steep increase of both ions from the *stratum basale* to the surface of the *stratum corneum* [15]. Also Veríssimo reported an inverted gradient with a steep increase of Cl^- from the dermis to the *stratum granulosum* before a sharp decrease in the *stratum corneum* [39].

- The contents of K^+ and P are low in the *stratum corneum*, high in the living cells of the epidermis (~300mmol/kg) (especially in the basal cell layer, and the lower *stratum spinosum*) and low again in the dermis, as expected for the extracellular compartment [26, 39–41]. Warner et al., found a high concentration of K^+ in the outer, and a very low one in the inner *stratum corneum* [15]. The dermo-epidermal junction appears as a frontier after which a drastic decrease of phosphorus content is reported [24]. These results can be explained by the abundance of phospholipids and nucleic acids present in the living epidermis as suggested earlier by Forslind [36, 39, 42]. The authors explain the low contents of K^+ and P in the *stratum corneum* by a recycling of these intracellular solutes within the viable tissue.

Table III Average distribution of elements in mmol·kg^{-1} adapted from Von Zglinicki (VZ) (XRMA) [26] and Forslind et al.[40] (Fo) by XRMA and PIXE. The gradient observed in a layer is given from the bottom up in the same layer. In the study of Von Zglinicki, the samples were shock-frozen and then Freeze-dried. In the paper of Forslind the samples are just quench-frozen.

A: Macrominerals

Ions (mmol·kg^{-1})	**Na^+**		**Cl^-**		**K^+**		**Mg^{2+}**	**Ca^{2+}**		**P**		**S**	
reference	*VZ* [26]	*Fo* [40]	*VZ* [26]	*Fo* [40]	*VZ* [26]	*Fo* [40]	*VZ* [26]	*VZ* [26]	*Fo* [40]	*VZ* [26]	*Fo* [40]	*VZ* [26]	*Fo* [40]
Stratum corneum	75	85	200	60	75	10-25	70	5	20-30	100	32	100	125
Stratum granulosum	-	300-350	-	170-230	-	100-205	-	-	10 -15	-	130	-	90 - 125
Stratum Spinosum	100	695	300	230	200	330	40	5	3 -5.5	275	40	125	62 - 90
Stratum Basale	150	565	425	285	150	360	40	5	4.5	200	515	125	62
Upper Dermis	150	1000	450	315	100	250	25	5	6	100	160	100	93
Reticular dermis	200		475		80		20	5		100		100	

B: Trace elements

Ions in (mmol·kg^{-1})	*Fo* [40]
Fe^{2+}	0.14 – 0.27
Cu^{2+}	0.041-0.073
Zn^{2+}	0.44-0.91

-The K^+/Na^+ ratio compatible with the ion content of cells that are capable of renewing is only found in the *stratum basale* [40]. In the upper layers, the Na^+ cell content increases while the one of K^+ decreases: the authors did not exclude the inward diffusion of salts (Na^+ et Cl^-) from sweat glands in the outer *stratum corneum*. Therefore, lower K^+/Na^+ ratio was found in the *stratum spinosum* compared to the *stratum basale* [40]. This result disagrees with Warner, who found a rather uniform concentration of Na^+ throughout the viable skin so that K^+/Na^+ ratios in the *stratum spinosum* were higher than those found in the *stratum basale*. No final conclusion was drawn by the authors [15].

- Sulfur has the same concentration in the dermis and in the *stratum corneum* but a little bit lower in the living cells of the epidermis [15, 40]. Veríssimo found quite constant values in the SC and the other living *strata* with a sharp increase of concentration at the interface between the *stratum granulosum* and the *stratum corneum* [39]. This result is expected considering that keratinocytes differentiate into corneocytes, and this leads to an increase in the keratin content rich in cysteine [37]. Therefore, the sulfur content is an excellent marker of this layer [24].

- The amount of calcium has been found in quite similar low range in every layer (5 $\mu mol \cdot kg^{-1}$) [26]. These values differ significantly from the results obtained by other teams who reported a concentration gradient increasing from the dermis up to the *stratum corneum.* The authors found around 50 $mmol \cdot kg^{-1}$ in the upper skin layers (*stratum corneum* and *granulosum*). Calcium is particularly highly concentrated in the *stratum corneum*, the epidermal region with the lowest metabolism. There is a consensus to claim that human epidermis displays the highest Ca^{2+} concentration at the *stratum corneum/stratum granulosum* interface. In this layer, the Ca^{2+} localization is high in the extra- and intercellular compartments. In the *stratum spinosum* and *stratum basale*, very low amount of mainly intracellular Ca^{2+} was found. Some authors claimed that there is no calcium in the *stratum corneum* except in case of skin disruption [33, 34] but other teams found high amount in this layer [24, 40].

- Concerning the trace elements, the contents of Zn^{2+} and Fe^{2+} were two-fold higher at the epidermal/dermal interface as compared to the value observed in the *stratum corneum* and dermis [39, 40]. Neither Fe^{2+} nor Zn^{2+} were detectable in the *stratum corneum*. Forslind indicated an approximate ratio of Zn^{2+} content in epidermis/dermis is of 3/1 in human normal skin [40]. This result was in accordance with Lansdown et al. who reported a zinc concentration in epidermis higher than in dermis (50-70 $\mu g \cdot g^{-1}$ dry weight versus around 10 $\mu g \cdot g^{-1}$ dry weight) probably due to the activity of zinc-dependent-RNA and DNA polymerase in the *stratum basale* keratinocytes [43]. An inverse gradient is observed between zinc and calcium across the epidermis. Finally, chromium, iron nickel and silicon appear only in the corneocyte layer [24].

4 Speciation of ions in the skin

In the skin layers, ions are dissolved in water (pH=7). *Stratum corneum* having a pH of 5.5 is the exception. Fraústo da Silva and Williams have analysed the speciation of ions with respect to biological systems [4]. Some interesting statements can be made from the oxidation diagrams for metals and non-metals at pH=7. The importance of the acidity and redox potential of specific cell compartments must be considered. Most of complexes formed by divalent transition metals and proteins are not stable in pH below 6, as in lysosomes for example. In such an acidic medium, the

metal dissociates from the complex, the carrier protein returns outside of the cell while the metallic ion stays in the cytoplasm.

Monovalent ions such as Na^+, K^+ or Cl^- are very mobile. The predominant oxidation state for both Na^+ and K^+ is +1 and thus these metals rarely bound in enzyme reaction centres but they can transfer ionic charges better than fixed ions. Mg^{2+} and Ca^{2+} are considered as semi-mobile as compared to Na^+ and K^+. Oxygen is the preferred donor atom for Na^+ and K^+ while carboxylate and phosphate are the preferred ligands for Mg^{2+} and Ca^{2+}; Zn^{2+} and Ni^{2+} complex easily with S and N. For transition metals: Fe, Cu Co, Mo and Mn more than one oxidation state is possible (Figure 1). They complex very easily, with N and S being their preferred donor atoms except for Fe^{2+} and Mn^{2+} for whom carboxylate and phosphate are preferred ligands. These metals are generally considered static with the exception of Mn^{2+} and Fe^{2+} that are semi-mobile.

The redox potentials are restricted to the systems H^+/H_2 and O_2/OH^- ranging between -0.4 and +0.8 V above and below which water is reduced to H_2 or oxidised to O_2. Hence, metal ions have only a small range of stable oxidation (iron: +2, +3; copper +1, +2) while non-metals may take a wider range of values (i.e. S: –2 to +6; carbon: –4 to +4) (Figure 1).

Some of the ions, independently of their classification as metal or non-metals, can only occur in one very stable oxidation state. This is the case of alkali and alkaline-earth metals restricted to the oxidation state +1 or +2 and zinc and cadmium restricted to the +2 oxidation state.

Finally, phosphorus, boron and silicon are only present in the highest oxidation states, +3, +4 and +5 respectively as borate, silica and phosphate. Chlorine only exists as chloride and is never bound covalently [4].

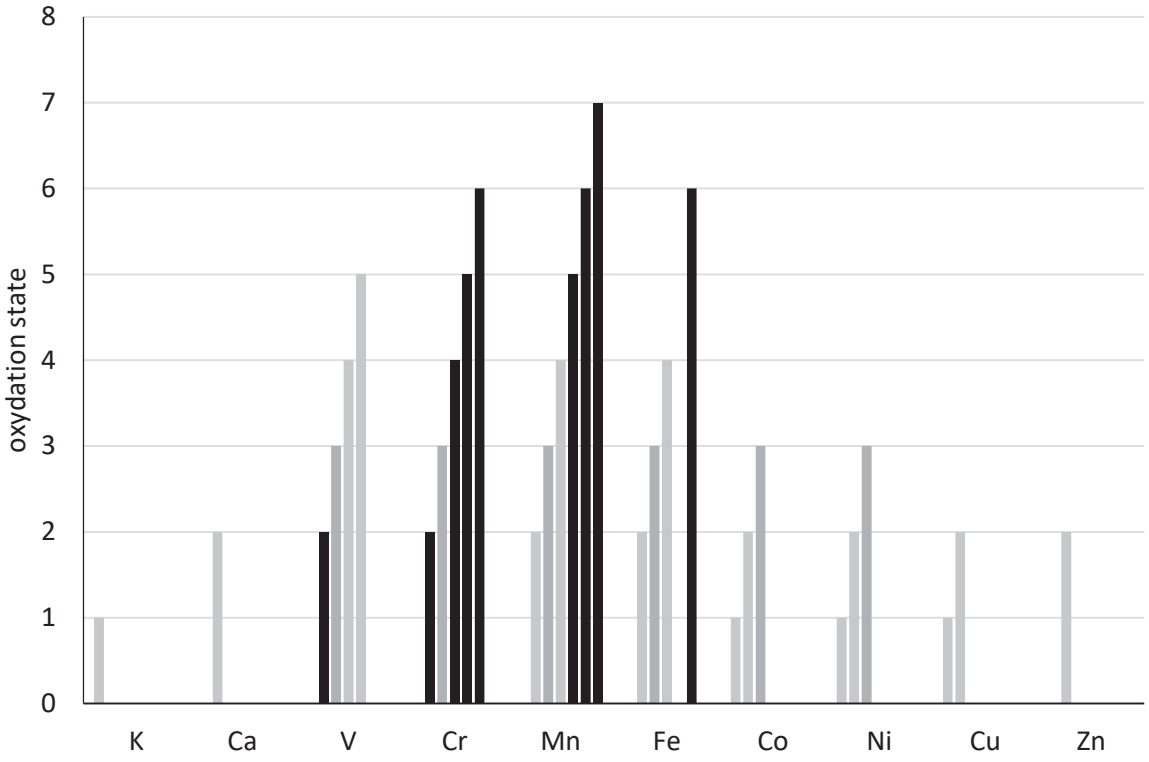

Figure 1 Oxidation state patterns of metals from K to Zn in aqueous solution. The black bars correspond to oxidation states not available in biology (adapted from [4])

5 Transport of ions in the skin

As seen above, the distribution of water and endogenous ions in the skin is not homogeneous. The local administration of ions from aqueous solution to cure skin diseases is compromised by the outermost skin layer, *stratum corneum,* which is often considered the main, if not unique, barrier for permeation of xenobiotics in general.

It is built of dead, nuclei-deprived keratinised cells (corneocytes) immersed in the lipidic matrix making it highly lipophilic. This barrier strongly limits the transport of these low molecular weight hydrophilic molecules especially under their ionized state [15, 20, 22, 23].The pathway for ion transport is also controversial [44]. Two passive mechanisms of passage through *stratum corneum* are possible: via trans- and intracellular route [45].

5.1 Passive transport

The intercellular pathway (Fig. 2-B) is the continuous route for absorption of molecules and it had been thoroughly studied over the past decades [46, 47]. It is the predominant route for transport within *stratum corneum*. The organisation of lipid bilayers within the intercellular space enables the transport through the lipidic core, as well as via polar headgroups pathways [48, 49].

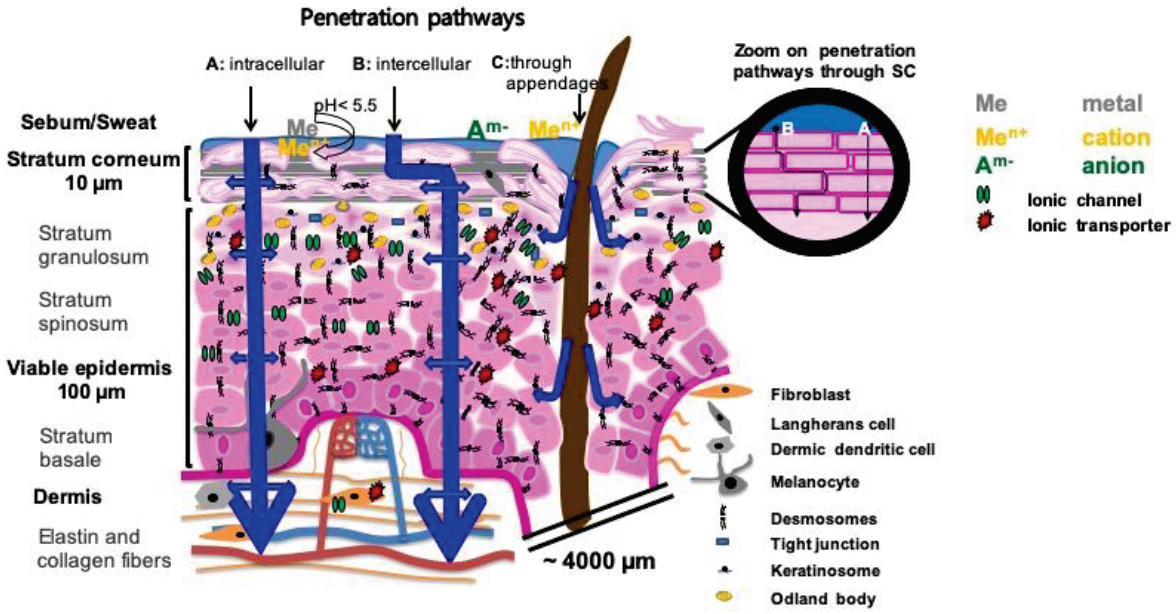

Figure 2 Schematic representation of three passive skin absorption pathways. three penetration pathways: intracellular, intercellular and follicular. The upper right inset is a close-up of the *stratum corneum* showing the intracellular pathway and the tortuous intercellular pathway. Adapted from [45].

The intracellular route (Fig. 2-A) is sometimes considered as a polar pathway within the *stratum corneum* [49]. The Natural Moisturizing Factor entrapped in the cytoplasm confers a slightly hydrated intracellular matrix providing a polar environment. However, transcellular absorption requires multiple partitioning of the molecule between this polar environment and lipidic external matrix.

The last permeation pathway within the horny skin layer leads through the hair follicles (Fig. 2-C). Scheuplein [48] suggested that the contribution of this route is negligible compared to the intercellular one. More recently, hair follicles have been considered as "weak spots" in the skin barrier enabling faster penetration in the skin. Depending on the skin site their contribution to the dermal absorption cannot be neglected, for example on the forehead [50]. Transfollicular route is now considered as an attractive target for nanoparticle-based delivery and its role as long-term reservoirs for drug delivery has been extensively studied [51–53]. Recently, Chandrasekaran et al. reported significant contribution of follicular pathway for the skin absorption of magnesium cations [54].

5.2 Facilitated transport[1]

Although the passage mechanisms of the majority of studied xenobiotics are restricted to the above-mentioned pathways, skin absorption of inorganic ions involves complementary routes of transport present in the lower skin layers (viable epidermis and dermis) (Fig.2). Membrane proteins can create channels or receptors for the specific passage of ions. Their activity can be associated with energy expenditure.

5.2.1 Cations

The first group of exchangers gathers the transport proteins that help to maintain the cationic gradients in the mammalian cells in general. Epithelial sodium channel (ENaC) actively transports hydrogen outside the cells in exchange to sodium. In the skin, it allows resorption of Na^+ excreted initially with the sweat. It is strongly expressed in viable epidermal layers as well as in sebaceous glands, eccrine glands and arrector pili smooth muscle cells [55]. Lithium and ammonium cations are non-physiological substrates that can be transported through this channel [56, 57]. Na+/K+ ATPase is essential for maintaining the homeostasis of all cells, including skin cells. It allows active resorption of $2K^+$ inside the cell in exchange to $3Na^+$ and is therefore involved in regulation of cationic gradients and osmotic pressure, resting and action potentials, water-electric balance, and transport of sugars and amino acids [58]. Additionally, the pump binds magnesium cations that are cofactors of the active site [59]. A similar role is attributed to $Na^+/Ca^2 – K^+$ exchanger (NCKX) which transports both K^+ and Ca^{2+} inwards while evacuating Na^+ to the extracellular medium [60]. Lamason et al. found that the levels of expression of *SLC24A5* gene encoding this exchanger, are correlated with skin pigmentation processes [61].

Calcium plays a crucial role in intracellular signalling (secondary messenger) so that its concentrations both inside the cells and in the extracellular fluid need to be precisely controlled. For this purpose, Ca^{2+} ATPase helps cells to remove excessive Ca^{2+} to the extracellular matrix. On the other hand, the inward flux of calcium is controlled by second messenger-operated channels (SMOC), receptor-operated channels (ROC) and voltage operated channels (VOC) [62]. Magnesium is another divalent cation of a big importance for the cells. It is mainly present in the intracellular medium and plays important roles in cell signalling, metabolism, growth and proliferation. Magnesium transporters (MgtE) help to maintain homeostasis related to its concentrations but as they are not very selective, they can also facilitate the transfer of other divalent cations (Ba^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}) [63].

[1] Apart from given bibliography, the evidence of presence all the proteins mentioned in this paragraph was found either by gene expression or immunostaining of skin tissues (Proteinatlas.org).

Other types of transport proteins are much less specific and allow the flux of various divalent cations.

The initial role of divalent metal transporter (DMT) is the transport of Fe^{2+} and H^{+}. Although it is primarily expressed in the intestine where it regulates Fe^{2+} absorption from the digestive tract, Pereira-Suárez et al. linked its overexpression in skin biopsies of patients suffering from leprosy to its impotence in pathogen-defence mechanisms [64]. Depending on the pH it may also transport other ions, such as Mn^{2+}, Co^{2+}, Ni^{2+} and possibly Zn^{2+} [65].

Zinc is an essential microelement that is involved in enzymatic and structural functions of various proteins. Two transporters regulate its cellular concentrations: the ZnT pumps the zinc outside the cell to decrease cytoplasmic zinc concentrations and the ZIP creates the inwards flux of Zn^{2+} concentrations [66]. In the skin cells however, the ZIP family is predominant which may lead to facilitated absorption of Zn^{2+} and other divalent cations Cd^{2+}, Mn^{2+}, Fe^{2+} [67].

5.2.2 Anions

Chloride channels and transporters are the main group of proteins that are controlling anion flux in the skin cells. Lin and Gruenstein identified three main pathways of chloride transport in human fibroblasts. The first one was 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) insensitive route which could be partially inhibited by anthracene-9-carboxylic acid and that accounted for 20% of total flux (calcium-activated chloride channels, CaCC). The second described pathway was cation-coupled transport relying on the simultaneous presence of Na^{+}, K^{+} and Cl^{-} yielding to 25% of the total efflux (Na^{+}/Cl^{-} co-transporter, NCC; Na^{+}/Cl^{-} co-transporter NKCC; K^{+}/Cl^{-} co-transporter, KCC). Finally, up to 50% of the total transport occurred via anion exchangers that could be inhibited by DIDS (chloride channels, CLC) [68]. Shortly after, Mastrocola et al. identified the chloride transport pathways in human keratinocytes. In that case, authors determined two predominat mechanisms: an anion exchange and an electrically conductive pathway whereas the cation-coupled transport was found to be negligible in case of this cell line. The transport via exchangers accounted for 50% of the total efflux that was in line with the results found for the fibroblasts. The CCL were found yield approximately 40% of the Cl^{-} flux [69].

The NCC, NKCC and KCC represent the channels that are not tissue specific and thus are expressed in all types of cells. Their role is to maintain the osmotic pressure and ionic concentration gradients. The anion exchanger (AE) helps, in particular, in regulating the intracellular pH as its main substrates are bicarbonate (secretion) and chloride (uptake) (for review, see Nilius and Droogmans [70]). Additionally to those chloride-focused transporters, the gene encoding Na^{-}/I^{-} symporter (NIS) whose primary role is to pump iodide ions inside the thyroid cells, has been detected in skin samples [71].

Apart from I^-, NIS transports other monovalent anions in the following affinity order: ClO_4^- > ReO_4^- > I^- ≥ SCN^- > ClO_3^- >> Br^- [72].

Members of Chloride Channel family (CLC) are voltage-dependent channels and react on the changes in transmembrane electric field. The other substrates include NO_3^- > Cl^- > Br^- > I^- [73]. Braun et al. in their study of murine expression of CLCA5 member of this family suggested that in the skin, CLCA5 may be involved in maturation and keratinization of squamous epithelial cells [74]. Soon after, a similar conclusion was drawn by Plog et al. in the case of porcine CLCA2 [75].

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a is a cAMP- activated chloride channel of great importance in the skin. It regulates electrolyte and fluid secretion as well as absorption in the sweat glands [76]. Chloride is not its only substrate and the difference between CFTR and CLC is the affinity to other monovalent anions : I^- > NO^{3-} > Br^- > Cl^- > F^- [77].

Osmotic swelling resulting in a decrease of the intracellular ionic strength can also trigger the ionic flux through volume regulated anion channels (VRAC). Just as CFTR, they are able to transport various monovalent anions, with the following affinities: SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- > gluconate [70]. Raoux and collegues stuied the behaviour of normal human keratinocytes in the presence of haptylamine. They proposed that VRAC participate in the mechanisms underlying contact dermatitis caused by exposure to tested molecule, which actually is an inhibitor of the channel [78].

Ca^{2+}- activated Cl^- channels (CaCC/CLCA) react on an increase in intracellular Ca^{2+}. The expression of CLCA2 in human and pig skin was thoroughly studied. In human samples the protein was detected in alongside the basal epithelial cells [79]. The porcine homologue was detected exclusively in the mature keratinocytes of the epidermis and in the inner root sheath of hair follicles [75]. Both authors linked the occurrence site with its structural role in epithelial stratification.

Glycine receptor (GlyR) and γ-aminobutyric acid receptor (GABAR) channels represent the ligand-gated channels and are activated by binding glycine or GABA respectively. The affinity of both receptors to the monovalent anions follows the same order as in the case of CFTR and VRAC (SCN^- > I^- > Br^- > Cl^- > F^-) [80].

6 Effects of skin exposure to inorganic cations

Available literature addressing dermal absorption of cations and their impact on the skin homeostasis is considerably richer than that of anions. Therefore, the following paragraph is focused on the beneficial and detrimental effects related to dermal exposure to cations.

A large number of ions may enter the skin either by systemic transport: gastrointestinal absorption or inhalation of dusts and vapours or directly after dermal exposure. In this review, the effects of direct application or absorption of ions through topical exposure are reported.

6.1 Curative effects

This section gathers the beneficial health effects of ions after topical application with the focus on ions found in thermal spring waters. Aluminium salts used in underarm hygiene products and silver salts applied in the wound treatment are also considered. Titanium dioxide and zinc oxide, although widely used in sunscreen formulations and make-up products, are excluded from the considerations as they are not soluble in water and thus cannot be absorbed through healthy skin in the blood circulation.

6.1.1 TSW-components

Thermal Spring Waters (TSW) are naturally mineralised waters that share three main characteristics: spring origin, bacteriological pureness, and therapeutic potentials. Their common feature is the abundance in calcium and magnesium which is due to reactions and exchanges with the rocks surrounding the spring. Enrichment in calcium and magnesium occurs in exchange to sodium and potassium (that usually still occur in high concentrations) [81]. The main anions present in the TSW are chlorides, sulfates and bicarbonates. The waters differ in the proportions among those bulk minerals as well as in the content of trace elements, such as zinc, copper, strontium, selenium, iron, manganese, silica, fluoride, bromide, etc. [82–84]. Since the Antiquity, bathing was regarded as more than a simple cleansing procedure. Waters rich in sulphur have been popular to cure psoriasis and to help wound healing [85]. Recently, the clinical trials examined by the Cochrane library focused on the effects of balneotherapy on chronic painful diseases, as rheumatoid arthritis for example [86, 87]. Extensive research related to curative treatments of chronic dermatological diseases like ichthyosis, psoriasis and atopic dermatitis by hydrotherapy has been investigated since it has significant benefits on the quality of patients' lives [88–91]. Huang et al. have recently published a review on the clinical applications of TSW on the skin, especially their immunomodulatory effect on inflammatory and autoimmune dermatologic diseases. According to the authors, the balneologic benefits of the Dead Sea Water, a very salty medium, are attributed to four variables: sunny climate (330 days/year) with moderate to high temperatures, low humidity, high barometric pressure with oxygenation of the air increased by 10% and low pollen count [92].

The evaporation of the water creates an aerosol that could be inhaled. Mud-packs and bathing, sometimes in association with phototherapy, are used to relieve and soothe inflammatory lesions [93, 94]. Several studies proved such a combined treatment more efficient in psoriatic patients than

phototherapy alone [90, 94, 95]. Proksch et al. have evidenced that bathing in Dead Sea waters led to enhancement of *stratum corneum* hydration, improvement of skin barrier function and reduction of skin roughness and inflammation in patients suffering from atopic dermatitis. Authors attributed these effects to high content of magnesium [96].

These clinical data are supported by the fundamental research on the skin absorption of ions associated to their effects at the cellular level [97–100].

An *in vivo* experiment, in which shaved guinea pigs were "bathed" for 30 minutes in a solution of radiolabelled salts mimicking the composition of Dead Sea Water, was carried by Shani et al. as an extension of a clinical study [101]. The results revealed high concentrations of ions retained in the skin as well as their significantly higher levels in various organs (ex. spleen, bones, heart) [101]. More recently, a report on the use of Epson Salt baths revealed significant increase of blood levels of Mg^{2+} and SO_4^{2-} in patients taking regular baths in Epson Salt [102]. Along with these clinical assays, several *in vitro* studies carried on using diffusion cells were reported. Tregear evaluated skin absorption of radiolabelled cations ($^{24}Na^+$ and $^{42}K^+$) from aqueous solutions using 3 skin models *in vitro* (rabbit, pig and human) and *in vivo* (human). His findings proved that both cations penetrated through the skin. However, the *in vitro* absorption of Na^+ was slower than *in vivo,* and K^+ penetrated faster through the rabbit skin as compared to porcine model [103]. Skin absorption of copper and zinc after finite dosing of various topical formulations was also evidenced on human skin model. Both cations were retained in the skin rather than penetrated through the tissue and the percentage of recovery from epidermis was higher for Cu^{2+} than Zn^{2+} [104]. After developing the novel method of blocking the follicular absorption route, Chandrasekaran and colleagues disclosed that these appendages play an important role in skin absorption of magnesium ions *in vitro* [54]. Finally, our last study carried on viable porcine skin explants demonstrated measurable absorption of Ca^{2+} and Mg^{2+} from a marketed TSW. Both cations diffused through the skin samples after direct application of TSW and when the TSW was incorporated into model cosmetic formulations [105].

Not surprisingly, these results triggered further research aiming at understanding the mechanisms underlying observed biological activities. Table IV summarises the effects on skin observed after application of various ions.

As it can be seen from the table, the vast majority of beneficial effects of TSW on skin is due to its cationic composition.

Potassium is an abundant intracellular cation present in all living cells whose particular role in the skin is regulating the epidermal barrier homeostasis (along with Ca^{2+}). Skin barrier disruption was found to decrease the endogenous concentrations of K^+ in epidermis [106] which is consistent with

its role in barrier repair. Later on, Denda and colleagues indicated the importance of K^+ flux in the skin barrier recovery. In their study authors observed that alterations of activity of K^+ channels significantly affected skin barrier homeostasis: application of blockers of the K^+ channels inhibited skin recovery while treatment with the molecules opening the same channels or classified as K^+ ionophore resulted in acceleration in skin recovery. Proposed mechanism of this regulation is based on the variations in intracellular K^+ that either stimulates (low K^+) or inhibits (high K^+) secretion of lamellar bodies [107].

Calcium is an important ion in cell signalling (secondary messenger). In the skin, it is distributed according to a concentration gradient in the epidermis in a way that the concentrations increase from the *stratum basale* till the *stratum granulosum*. This gradient, in case of an external damage, is perturbed and activates skin repair mechanisms [106, 108]. Thus, its topical application may lead to the improvement of skin barrier [109]. Ca^{2+} was shown to play an important role in regulation of keratinocyte differentiation [110, 111]. Denda et al. showed that its influx and into keratinocytes induces the exocytosis of lamellar bodies and improves skin barrier function [112].

A concentration gradient of magnesium is present within the epidermal layers and is similar to the one observed for calcium. Its role in the induction of skin barrier repair mechanisms is also analogical to the one described for Ca^{2+} [108]. Topical application of some magnesium salts improved skin barrier recovery in hairless mice after tape stripping. However, observed effects depended on the counter-anion and association of magnesium with calcium salts [109]. Moreover, exogenous magnesium cations were shown to specifically inhibit Langerhans cells function and, by consequence, account to the anti-inflammatory properties of magnesium salt solutions observed after topical applications of Dead Sea waters [113].

Selenium is a trace element that can be found in some TSW. The research carried on skin fibroblasts cultured in selenite-supplemented medium disclosed its protective properties against UV-A radiation even at low concentrations (0.1 $mg \cdot L^{-1}$). The survival rate of Se-cultured cells was significantly higher than in the control group. Authors attributed this action to enzymatic induction of glutathione peroxidase and the protection of cell membranes [114]. Moysan et al. also performed their study on human fibroblasts cultured in foetal calf serum depleted medium (2%) that was enriched either by selenite alone or by the addition of Se-rich TSW (La Roche Posay). The observations from this study agreed with Leccia et al. [115]. Authors confirmed a protective effect of Se on dividing fibroblasts after exposure to UV-A radiation, that was linked to the protection of dividing or quiescent fibroblasts against lipid peroxidation, and increased glutathione peroxidase activity in fibroblasts [115]. Similar results were obtained when TSW was used instead of a simple aqueous solution of

selenite, which led to the conclusion that the capacity of La Roche Posay TSW to protect against UV-A radiation is due to its Se content. No protective effect was observed in case of UV-B exposure.

Celerier et al. observed that the addition of Se into the culture medium of reconstituted skin resulted in decreased production of inflammatory cytokines, namely IL-1 and IL-6 both at the intra- and extracellular levels. Authors linked their observations with the results of Leccia et al. [114] and Moysan et al. [115], and suggested that the mechanism of protection against inflammatory *in situ* reactions is due to the ability of selenium to catalyse the decomposition of reactive forms of oxygen [116]. In the same study, the inflammatory properties of TSW containing Se and $Sr^{2+,}$ as well as strontium alone were evidenced. The effects observed for an aqueous solution of strontium were less evident and limited to the inhibition of IL-6. La Roche Posay TSW exhibited intermediate cytokine inhibition as it contained both minerals [116].

In their investigation of the impact of trace elements on the expression of integrins crucial for skin wound healing processes, Tenaud and colleagues noted that manganese gluconate could modify the expression of integrins that are transmembrane receptors responsible for communication between cell and environment. They play a critical role in the regulation of adhesion, migration, proliferation, and differentiation of cells. They are necessary for the formation and maintenance of tissue structure integrity. Thus, modification of their expression may modulate wound healing mechanisms (for review on their roles in the skin see [117]). Manganese salt induced the expression of integrins $\beta 1$ and $\alpha 6$ in suprabasal keratinocytes. Observed effects were concentration-dependent and occurred both in the cell culture (keratinocytes) and in reconstructed skin samples. Authors did not notice increased expression of integrin $\alpha 3$ which, combined with noted activation of integrin $\alpha 6$, led to the conclusion that the effect of manganese was much more pronounced in keratinocytes in differentiation phase than those in the proliferation phase, and that it enhanced the migration and dermo-epidermal adhesion in the final healing phase [118].

Both copper and zinc described below are trace elements found in TSW certainly contributing to their overall health and beauty properties. Their beneficial effects observed after topical application have been known since many centuries and resulted in the development of widely used Dalibour cream ($CuSO_4$, $ZnSO_4$, ZnO, camphor, lanolin, water and vaseline), a medicine available in a number of countries worldwide and zinc or Lasaar paste. Dalibour cream as well as other (dermo)cosmetics based on the association of Zn^{2+} and Cu^{2+} present soothing properties and are used to calm irritated and damaged skin. As mentioned above, skin absorption from copper and zinc soluble salts are considered poor through human skin *in vitro* but their application remains interesting in case of wound healing [104, 119].

The main biological activity of copper is enhancement of wound healing processes that have been described by many authors. Tenaud et al. disclosed that incubation of keratinocytes or reconstituted skin in a medium supplemented with copper gluconate was less active on the integrins involved in the keratinocyte proliferation phase. On the other hand, the inductive action of copper gluconate was more specific towards differentiated keratinocytes leading to enhanced expression of α6, β1 and α2 integrins. Similarly to manganese, Cu^{2+} was found to play an important role in the final wound healing phase. These findings agreed with the results obtained *in vivo* on wounded rats. Simeon et al. investigated the efficacy of a tripeptide-Cu^{2+} complex (Glycyl-L-Histidyl-L-Lysine-Cu^{2+}, GHK-Cu) on activation of matrix metalloproteinases and their impact on wound healing *in vivo*. The study was carried on wounded rat model and demonstrated that tested complex did modulate the expression of various metalloproteinases at different stages of healing which could alter the wound remodelling [120].

Later on, the impact of the same complex on cultured fibroblasts and keratinocytes was evaluated. GHK-Cu treated irradiated fibroblasts produced more growth factors (β-FGF and VEGF) as compared to the controls. This can be beneficial in the early stages of wound healing processes [121]. In keratinocyte culture, the tripeptide increased the proliferative potential of keratinocytes by regulating extracellular matrix proteins such as α6 and β1 integrins [122].

Apart from its undeniable benefits in wound healing processes, copper also enhances the production of collagen and elastin fibres in the skin. Clinical tests of cosmetic products containing copper-zinc malonate reported significant reduction of wrinkles after 8-week treatment with the product. This result was supported by molecular and histological data showing regeneration of elastic fibre network in treated sites which was due to enhanced synthesis of tropoelastin [123].

Zinc seems to be a truly versatile cation when it comes to its biological activity within the skin. Zinc is located both intra- and extracellularly in epidermis while in dermal tissue it is complexed with proteins. Its primary role is to stabilize cell membrane. It is also a cofactor with an important role in mitosis migration and maturation of cells [43]. Many zinc salts are used in medicine and cosmetic: (i) zinc undecylenate as an antifungal agent, (ii) zinc pyrithione as an active agent for the control of dandruff, seborrheic dermatitis and scalp itch and, (iii) zinc acetate, zinc sulfate, zinc gluconate to soothe the skin with antimicrobial and seboregulatory effect, (iv) finally, nanoparticles of zinc oxide are applied for skin protection against UVA and UVB radiations [124]. Microparticles of ZnO are used to protect the skin of wetness, and as soothing and antimicrobial agent in case of diaper rash. In the pH lower than 6, ZnO can release Zn^{2+} ions that exhibit antimicrobial and soothing properties. As example, in contact with acidic sweat ZnO hydrolyses to Zn^{2+} cations having protective effects against inflammation mediated by the UV radiations. Indeed, Leccia et al. in their *in vitro* study

demonstrated that addition of $ZnCl_2$ to cell culture of UV-A irradiated fibroblasts protected the cells against radiation and increased their survival rate [114]. The role of zinc at the cellular level in wound healing processes has been recently reviewed by Lin et al. [125]. It was shown to be an important modulator of all the stages of the cicatrisation process. Just after wounding, zinc is involved in haemostasis. It enhances platelet activity and aggregation during blood clotting. Moreover, platelets and zinc are suggested to play an important role in initiating the inflammatory phase of wound healing. In the latter, Zn^{2+} reduces oxidative stress markers, ensures migration of neutrophils and leukocytes to infected/damaged sites, enhances neutrophilic phagocytosis and has an effect on cytokine production. In the next proliferation phase, zinc stimulates T lymphocytes that regulate and suppress inflammation. Zinc regulates the expression of metalloproteinases involved in remodelling of extracellular matrix operating within the last stage of wound healing. They modulate growth factor activation, cleavage, degradation and composition of extracellular matrix, processing of cell-cell junctional adhesion molecules, cytokines and cell surface receptors and cell-matrix signalling during different stages of wound healing [125].

Zinc-based products are commonly used in the treatment of acne due to several mechanisms of action. Firstly, Zn^{2+} has significant anti-inflammatory potential. As demonstrated by Yamaoka et al, the presence of Zn^{2+} in keratinocyte culture supresses cytokine-induced iNOS expression and IFN-γ or TNF-α induced NO production [126]. This, combined with its sebosupressive efficacy based inhibition of 5-α-reductase activity justifies its efficacy in anti-acne treatments [127–129]. Additionally, it has demonstrated anti-microbial activity against *S. aureus, P. aeruginosa, C. albicans* and *A. brasiliensis* that is maintained in the topical formulations [130].

Finally, zinc in association with copper, enhances regeneration of elastic fibres within dermis which results in the reduction of wrinkles [123].

Table IV Biologically active ions found in TSW and their mechanisms of action

	ion	Details	ref
Skin barrier enhancement	**Ca^{2+}**	Hairless mice, acceleration of exocytosis of lipid- containing lamellar bodies	[112]
	K^{+}	Hairless mice, modulation of excretion of lamellar bodies	[107]
	Mg^{2+}	hairless mice, no particular mechanism proposed	[109]
	Cl^{-}	Hairless mice, acceleration of exocytosis of lipid- containing lamellar bodies	[112]
Anti-inflammation	**Mg^{2+}**	Specific inhibition of the antigen-presenting capacity of Langerhans cells	[113]
	Zn^{2+}	Suppression of cytokine-induced iNOS expression and IFN-γ or TNF-α-induced NO production	[126]
	Se	Reconstituted skin from biopsies (healthy and inflamed); moderate inhibitory effect on inflammatory cytokine production, particularly IL-6.	[116]
	Sr^{2+}	Reconstituted skin from biopsies (healthy and inflamed), moderate (smaller than Se) inhibitory effect on inflammatory cytokine production, particularly IL-6.	[116]
Wound healing	**Mn^{2+}**	Keratinocyte culture and reconstituted skin; enhancement of expression integrins specific for the late phase of wound healing	[118]
	Cu^{2+}	Keratinocyte culture and reconstituted skin; enhancement of expression integrins specific for the late phase of wound healing	[118]
		Wounded rats, induction of metalloproteinases involved in wound remodelling	[120]
		Irradiated fibroblasts, increased production of βFGF and VEGF in cells treated with GHK-Cu	[121]
	Zn^{2+}	Regulation of all stages of wound healing processes	[125]
Elastin/collagen production enhancement	**Zn^{2+}**	Clinical study, enhanced synthesis of tropoelastin leading to regeneration of elastic fibres within dermis	[123]
	Cu^{2+}		
UV protection	**Se**	Fibroblasts, UV-A radiation cultured in Se or TSW-supplemented medium presented higher survival rate	[114]
		Fibroblasts exposed to UV-A radiation cultured in Se-supplemented medium presented higher survival rate (inhibition of lipid peroxidation)	[115]
	Zn^{2+} & ZnO	Fibroblasts: UV-A radiation cultured in $ZnCl_2$ or TSW-supplemented medium presented higher survival rate	[114]
Anti-acne	**Zn^{2+}**	Sebosupression (inhibition of 5 α-reductase activity)	[127–129]
	ZnO	Anti-microbial	[130]

6.1.2 Aluminium

Aluminium compounds are used as inorganic very soluble salts (chloride, nitrate, sulfate, chlorate), partially soluble (acetate, benzoate and lactate) and as organic salts with a high molecular weight and poor solubility. Aluminium salts have been successfully used in antiperspirant products owing to the capacity of Al^{3+} to bind –SH groups within epidermal keratin resulting in an obstruction of appendages (sweat ducts, hair follicles and sebaceous glands) [131, 132]. Some of them are used in toothpastes (Aluminium fluoride), as colorants, lakes and pigments (Aluminium silicate, Aluminium hydroxide (Al_2O_3)) or as viscosity agents (magnesium aluminium silicate). Medical applications of aluminium include its use as adjuvant in vaccines (aluminium phosphate and aluminium hydroxide) and, of course, as a symptomatic treatment against pathological hyperhidrosis [133]. The risk assessment of aluminium salts, especially those used in deodorants and antiperspirants, is continuously evaluated [134]. Aluminium chloride and aluminium trichloride are unstable in water and form the hexahydrate ($Al(H_2O)_6Cl^{3+}$) which gives rise to $Al(OH)_3$ with release of HCl and heat. Therefore, $AlCl_3$ is very corrosive. To minimize this effect, hydrated aluminium chlorides which are partially hydrolysed are used, as aluminium chlorohydrate, for example. Double salts of zirconium and aluminium (Aluminium zirconium tetra- or pentachlorohydrate) and the well-known "Alum stone" are also used. Some of them are restricted (Annex III) in the Regulation on cosmetic products 1223/EC. Aluminium neurotoxicity has first been reported a consequence of inhalation of aluminium dusts and in patients suffering from a kidney failure who were under dialysis but never after the topical exposure. The cumulative dose gave rise to encephalopathies. A lot of controversy arose about its use in cosmetic products after the hypothesis given by Darbre in the *Eur. J. of Cancer Prevent.*, claiming that "Underarm cosmetics are a cause of breast cancer" in 2001 [135]. This publication triggered the research on the link between the use of antiperspirants and deodorants and the risk of cancer. As a result, numerous case reports and clinical trials were published. Some of them claimed there was no correlation between hygiene habits of examined subjects and the occurrence of disease [136, 137]. Simultaneously, other teams disclosed the mechanisms of Al^{3+} action in breast cancer [138, 139] and published clinical studies proving the initial hypothesis. The morbidity of breast cancer was linked to the use of aluminium-based underarm cosmetics either in the population that started such hygiene patterns at early age (pre-puberty) or removed the auxiliary hair while using Al^{3+}-based underarm products [140–142]. This scientific discussion was then supported by the research focusing on the skin absorption of aluminium. Chronologically, the first study aiming at assessing skin absorption of Al was carried on by Anane et al. dates back to 1995 when aluminium was not yet suspected to increase the incidence of breast cancer but was possibly related to the development of neurodegenerative diseases. The *in vitro* study was performed on

healthy shaved mice that underwent daily applications of low concentrations of aqueous solutions of aluminium chloride for 130 days. Authors demonstrated a significant retention of aluminium in brain (hippocampus) combined with an increase in concentrations of Al^{3+} detected in urine and serum as compared to non-treated animals [143]. Given the anatomical differences between murine and human skin (namely the thickness and number of hair follicles per cm^2), the following tests were carried as either clinical studies or with the human skin explants. Within their *in vitro* (Franz cell) investigation of skin absorption of aluminium from commercial products (aerosol, stick and roll-on forms), Pineau and colleagues verified the impact of the physio-pathological state of the skin using both intact and damaged (tape-stripped to mimic "after-shaving" conditions) skin models. Authors evidenced significantly higher absorption of aluminium within the skin layers when the product was applied on damaged skin. Regardless the skin condition, the permeated dose (recovered in acceptor fluid) after 24 h was negligible. The majority of applied doses was recovered from *stratum corneum* and viable epidermis [144]. These results agreed with an earlier clinical study, in which a radiolabelled (^{26}Al) aluminium chlorohydrate (ACH) was applied in the axillary zone in male and female volunteers. The application of the product was unique within the study and was followed by hair removal (shaving with an electric razor). The attention was paid so as to avoid application to damaged skin. The treated axilla was kept under an occlusive bandage during 6 consecutive days and the application area was stripped twice. The results showed the absorption levels of 3.6 µg (estimated by the renal clearance) of Al over 2 weeks that corresponded to 2.5% of the dietary intake. Higher absorption rates in women were attributed to the damages of the skin which occurred within the experiment and led to a conclusion that the use of antiperspirants on damaged skin should be avoided [145]. This preliminary study examined the dermal absorption of aluminium after a single application without taking account daily use of underarm products in real life. Additionally, the authors confirmed the loss of 50-70 % of applied dose within the manipulations. The most recent clinical pharmacokinetic study on dermal absorption of Al used the "state- of- the- art techniques" to investigate the effects of daily exposure to ^{26}Al-based underarm cosmetics. Tested conditions involved various regimens: application of ^{26}Al-radiolabelled ACH. The results confirmed that the antiperspirants play a minor role in the total systemic exposure to the aluminium salts. Moreover, shaving or daily use did not influence the absorbed quantities of ^{26}Al [146].

6.1.3 Silver ions

Silver is not a trace metal and serves no physiological role in the human body. It exhibits three oxidation states: Ag (I), Ag (II) and Ag (III) but the only stable and biologically active ion is Ag^+. Different chemical forms of silver, for example metallic silver particles (nano or not), inorganic salts with different solubility ($AgNO_3$, AgCl) and organic complexes (silver proteins for example), are used

in many industries [147–149]. Because Ag^+ is only active in solution, the role of its carrier is crucial. Silver derivatives are widely used as antimicrobial agents, with an activity proportional to the concentration of Ag (I), in dentistry (dental amalgams), in medical devices, pharmaceutical products and cosmetics. Exposure to silver exhibits low toxicity in the human body except chronic ingestion or inhalation. The systemic absorption of silver-based drug may be toxic and cause local argyria of the skin which is the most common manifestation of toxicity. Numerous cases of argyria have been described in literature and were related to the ingestion of metallic silver or silver compounds over periods of months or even years. A well-known case is that of an American woman who has been using nose drops for viral colds or allergy for years. Her skin had a characteristic irreversibly grey or blue-grey discoloration. Silver granules concentrate in basement membranes and elastic fibres surrounding sweat glands [150, 151]. *In vivo* evidence of argyria associated with modern silver wound dressings applied topically has never been reported [152]. Aqueous solution of silver nitrate has been used since many years in treatment of difficult wounds. Silver ions complex with proteins in skin wounds or, with free anions that are abundant in the skin: chloride, phosphate or sulfate to form precipitates. This limits the availability of silver ions in the wound bed. Actually, silver nitrate has a very high ionizing capacity as compared to other silver compounds, and binds strongly to electron donor groups of biological molecules containing thiol groups, oxygen and nitrogen. The nature of the medium (salt and protein content) can have a great influence on the silver ion levels [149, 153]. Silver nitrate is caustic and has astringent properties. Its main side effect is related to reduction of colourless Ag (I) to black Ag (0). To avoid this reaction, pharmacists precipitated silver in the form of silver proteinate or colloidal solution. The addition of sulfadiazine to stabilize Ag(I) was very useful and “marked the renaissance of the use of silver in wound care”, as written by Lansdown [149]. Sulfadiazine maintains Ag(I) in a stable form avoiding its reduction to Ag(0). A cream containing 1% silver sulfadiazine and 0.2% chlorhexidine used for the treatment of serious burns which is a well-known silver preparation. This combination is also used in bandages, for treatments of burn wounds and traumatic injuries of humans [150]. Absorption through the skin of silver ion is very limited even in the case of skin injury. Lansdown et al. have studied the absorption of silver nitrate in wound therapy. Silver accumulated in sweat gland ducts and hair follicles but the new epithelialized tissue did not exhibit any staining suggesting that the silver bound to epidermal cells was eliminated with wound exudate, and epidermal cell debris [154]. Absorption through intact skin has been estimated as low as 1%/5h by Skog and Wahlberg for concentrations of ^{110m}Ag ranging between 0.00048 M and 4.87 M [155]. This low absorption was confirmed by other teams [147, 149, 156]. Silver salts or colloidal silver are used in personal skin care products as preservatives or colorants in make-up, face masks or face care products, cleansers, antiperspirants or chemical exfoliating products, for example. Silver nitrate is reported in the Annex III of the European Regulation only for colouring eyelashes and

eyebrows with the following wording of “conditions of use and warning” clearly indicated on the packaging: “Contains silver nitrate. Rinse eyes immediately if product comes into contact with them”. Silver particles are also authorized as colorant in cosmetic products (Annex IV, CI 77820). Silver chloride deposited on titanium dioxide can be used as preservative (Annex V). In this latter case, the maximum concentration in ready to use preparation is 0.004% as AgCl or 20% AgCl (w/w) on TiO_2. It must not be used in products for children under 3 years of age in oral products and in eye or lip products [134]. Ag^+ nanoparticles have been produced by different means with various shapes and sizes. The improvement of antibacterial activity depends on the size and shape with the triangular nanoparticles displaying better activity compared to rod-shaped particles [148, 157]. Concerning this type of colloidal silver nanoparticles, the conclusions of the opinion of the Scientific Committee on consumer Safety on colloidal silver (nano) were inconclusive due to insufficient data, especially concerning dermal absorption of such nanomaterials [158].

6.2 Ions as toxic agents

The main toxic effects reported after skin exposure to cations are irritancy, immunological changes leading to delayed hypersensitivity, carcinogenicity and systemic effects being the results of percutaneous absorption. Within this category we can distinguish sensitizers: Ni, Co and Cr and metal ions which are highly toxic and cause long-term damages: As, Cd, Hg, Pb, Sb and lanthanides [12, 19, 159].

Endogenous ions are essential for many processes that involve extensive cell proliferation in childhood. In the adulthood however, only some tissues are still proliferative with the epidermis that renews within 28 days, as an example. It is therefore vulnerable to toxicity or deficiency of substances in its close environment. These days, dermatologists and toxicologists are able to study carefully the hazards associated with vast arrays of ions because metals exposure has changed from an occupational to a consumer problem. For example, chromium allergy is related to occupational exposure to cement, metals and to leather consumer goods. Thyssen and Menné reported that leather exposure among chromium- allergic patients increased from 24.1% between 1989-1994 to 45.5% during 1995-2007. Cobalt allergy is associated to exposure to jewellery, alloys metals, prostheses, dental materials and paints. In the same way, higher prevalence of cobalt allergy has been observed in pierced people in comparison to non-pierced ones. The use of all these metals, especially Cr (VI), leads to serious health concerns in tattooed individuals because most pigments used in tattoo ink formulations are originally produced for other applications (such as textiles, dyes, car paints, and plastic pigments). Additionally, tattoos are persistent whereas consumers affected by other goods (jewellery, creams or textiles) can easily remove them to avoid the contact with allergen. Ink products contain numerous impurities such as Cr (VI), Cr oxides, but also nickel, copper and

cobalt, iron oxides, aromatic amines in azo-colorants, and polycyclic aromatic hydrocarbons in carbon black [160].

6.2.1 Sensitizers: nickel, chromium III and VI and cobalt

Nickel, cobalt, iron, palladium and platinum belong to the group VIIIB and chromium to VIB of periodic table of Mendeleev. As transition elements, these metals generally exhibit high density, high melting point, magnetic properties, variable valence, and the formation of stable coordination complexes. Thanks to these properties, they are applied in the modern industry in the form of alloys which are widely used to produce jewellery and medical prostheses [12].

The most stable valence states are trivalent Cr (Cr (III)) and hexavalent Cr (Cr (VI)). Cr (III) is an essential nutrient while Cr (VI) is produced by industrial processes and is used in the manufacture of dyes and inks, in leather tanning, textile industries and as cement and wood preservative [161]. Cr (VI) is highly toxic for human health. It is classified as human carcinogen (category1) and induces allergies at lower exposure levels than most Cr (III).

In biological systems, +2 oxidation state is the most prevalent form of nickel and cobalt. These metals dissolve easily in acidic pH of sweat upon contact with the skin [162]. Nickel turns to a greenish hexahydrated $[Ni(H_2O)6]^{2+}$ in aqueous medium at pH=7 (see Denkhaus and Salnikov for review [163]). Although nickel, chromium and cobalt are considered as trace nutrients, they are the most frequent and strong sensitizers compared to other ions like iron, silver or gold [164]. They cause Allergic Contact Dermatitis, and Allergic Contact Urticaria. Cross allergy to nickel and cobalt is very common. It is estimated that up to 17% of women and 3% of men are nickel-allergic, whereas only 1-3% are allergic to cobalt and chromium (see Thyssen and Menné for review [159]). Nickel, cobalt and even palladium interact directly with the specific histidine residues in the human Toll-like receptor 4 (TLR4), which normally acts as an innate immune receptor for bacterial lipopolysaccharide. Therefore, it activates the intracellular pro-inflammatory signalling pathways by mimicking pathogen-associated molecular patterns such as TNFα, IL-8 and IL-6 [164]. Nickel and chromium (VI) may bind to the major histocompatibility molecules (MHC) and, as a consequence, activate the cytotoxic responses of T-cell. This induces T-lymphocyte mediated delayed hypersensitivity reaction which occurs after previous exposure and sensitisation to the metal [164]. To exert these damages, nickel, chromium and cobalt do penetrate into the skin. Skin absorption of metals is closely related to the capacity of the sweat to oxidise them. This penetration has been modelled as being skin-thickness and concentration-dependent [165].

Because nickel is ubiquitous (as the 24^{th} element in the Earth's crus) and people are constantly exposed to it, its skin absorption has been thoroughly studied. Nickel, as $NiCl_2$, has been found to

penetrate in the epidermis and to create a local reservoir due to its affinity to the tissue. Nickel sulfate and nickel chloride hexahydrate permeate human skin easily: much better under occlusion than in non-occluded conditions. For nickel chloride, 4% of applied dose permeated under occlusion against 0.23% in non-occluded conditions and after a very long lag-time (50h). Fullerton et al. noticed a better permeation of $NiCl_2$ compared to $NiSO_4$ (50 times faster). Additionally, a considerable amount is bound to proteins (between 13% and 43% for nickel chloride and 4 to 7% for nickel sulfate) [166]. Nickel ions bound to the dermis may act as a reservoir, and probably the threshold concentration producing an allergic reaction is related to the actual amount retained in epidermal tissue [167]. Given that sweating enhances solubilisation of nickel, Menné et al., evaluated the patch test reactivity to nickel alloys corroded for a period of 6 weeks at 30°C with a synthetic sweat composed of demineralized water, 0.5% sodium chloride, 0.1% lactic acid and 0.1% urea and a pH adjusted to 6.5 with ammonium hydroxide. Discs of metals were applied *in vivo* to 267 patients with known or suspected allergy to nickel sulfate [168]. Authors found that allergic reactions were linearly correlated with the amount of nickel released from discs. Roughly, alloys with a nickel release exceeding 1$\mu g \cdot cm^{-2}$/week gave a strong patch test reaction while those with a release less than 0.5 $\mu g \cdot cm^{-2}$/week were poor sensitizers.

Larese et al. extended the experiment by measuring the dermal absorption of nickel but also that of cobalt and chromium using the Franz cell method. For that purpose, they dissolved nickel, cobalt and chromium powder and potassium dichromate in a synthetic sweat at pH 6.5 (50 $g \cdot L^{-1}$). After 30 min the authors found 27.1 $mg \cdot L^{-1}$ of nickel, 33.3 $mg \cdot L^{-1}$ of cobalt and no chromium in the donor phase. The fluxes were 0.0165 $\mu g \cdot cm^{-2} \cdot h^{-1}$ for nickel, 7.29 $\mu g \cdot cm^{-2} \cdot h^{-1}$ for potassium dichromate and 0.123 $\mu g\ cm^{-2} \cdot h^{-1}$ for cobalt. The lag times were shorter than those reported by Fullerton (14 h for Ni^{2+} and $K_2Cr_2O_7$ and only 1 h for Co^{2+}). The donor media were not the same: water in the paper of Fullerton and a synthetic sweat in the papers of Larese et al. which can explain observed differences [169, 170]. No Cr was detectable from the powder, maybe because Cr powder cannot be oxidised by synthetic sweat as opposed to Cr salts that pass quickly into the skin [170, 171]. Gammelgaard et al., who studied the permeation of chromium salts by the Franz cell method, found that chromium (VI) permeates better than chromium (III) [172]. However, it does not penetrate well through the skin but accumulates in skin strata [173–175]. Chromium applied onto the skin as chromate is reduced to chromic ions (Cr^{3+}) by tissue proteins containing sulfhydryl groups and accumulates in the epidermis and dermis [176]. Cr (VI) as chromate or dichromate is seen to cross the skin unchanged showing that the skin has a limited capacity to reduce the chromate ion. Indeed, Samitz and Katz [177] estimated that one gram of skin can reduce 1 mg of dichromate to Cr^{3+}.

Finally, contact sensitization to platinum is increasingly reported because it is used extensively in jewellery and, like silver and gold, is reported to be an occasional sensitizer.

The decrease of the prevalence of metal allergy decreases with regulatory interventions. For instance, in Europe the prevalence of nickel allergy has decreased since the adoption of the "nickel directive EN 1811:2011+A1:2015", and the European REACH regulations [159, 178]. In the same way the E.U. REACH regulation 301/2014 restricted Cr (VI) content in leather products (<3 ppm) [134].

6.2.2 Harmful effects of heavy metal ions

Mercury, arsenic and lead are the most toxic elements present in the atmosphere, food and water, in our ecosystem. Actually, mercury (as well as arsenic oxide) in all forms alter the cellular functions by denaturation of the structure of proteins and by binding sulfhydryl and selenohydryl groups [179, 180]. Inorganic mercury exists under several forms: (i) metallic mercury present in dental amalgam, thermometers, incandescent lights, batteries and the incineration of medical waste; (ii) mercury vapour (Hg0); (iii) mercurous (Hg_2^{2+}), the famous Calomel, historically used as slight laxative; (iv) and mercuric salts (Hg^{2+}). Organic mercurials include mercury bound to a chemical group, ex. methyl, ethyl or phenyl groups like in thiomersal, for example. Methyl mercury is the most toxic mercury compound because it absorbs easily through biological membranes, and is excreted very slowly (>70days). *In vivo,* methyl mercury is demethylated to elemental mercury in the brain and its preferential form is Hg^{2+} [180]. This latter product has been widely used as an active agent for its antiseptic properties in biocides or as preservative in many drugs and cosmetics (thiomersal, Annex V of the cosmetic regulation N°1223/2009). Unfortunately, mercuric compounds (very often as $HgCl_2$, a certain oil-soluble salt, ammoniated mercury and mercurous oxide) are commonly misused as skin-lightening agents, especially in dark-skinned women from sub-Saharan Africa [181–183]. Mercury bleaches the skin probably excreting an effect on antityrosinase but paradoxally (as noticed by Olumide et al. [181]) chronic exposure darkens the skin by dispersion of metal granules which give to the skin a recognizable slate-grey pigmentation [162, 183]. Mercuric salts can penetrate the skin with an absorption that increases proportionally to applied concentration. Once in bloodstream, mercury can easily cross other biological membranes such as the blood-brain barrier which can lead to deposition of Hg in its metallic form [155, 173]. Chronic topical exposure to Hg salts can result is neurologic, renal (nephrotic syndrome), and dermal toxicity [182, 184]. Other possible cutaneous damages include burning of the face, contact dermatitis, flushing, erythroderma, purpura, and gingivostomatitis [161].

Some heavy ions have also been studied for their radioactive properties. Radioactive sources are used in various fields of nuclear industry but also for medical purposes to prepare

radiopharmaceuticals. A radiopharmaceutical is defined as a drug containing a radionuclide. Some of them, labelled with gamma-emitting isotopes, are used in for diagnostic purposes, for example ^{99m}Tc, ^{67}Ga, ^{111}In, ^{131}I and ^{51}Cr. Therapeutic radiopharmaceuticals are meant to deliver a dose of cytotoxic radiation to the tumour cells while avoiding radiation damage in normal tissue. They are generally low β-emitters and their radiation penetrates quite deeply in the tissue (2-12 mm), which is particularly important for the treatment of solid tumours. The choice of radiopharmaceuticals depends on their half-life, radiation type, other particulate radiation emissions, energy and biodistribution characteristics.

In the nuclear industry, thermal fission occurs when fissile elements are fabricated as nuclear fuel or reprocessed. This process results in formation of α-emitting radionuclides, such as: uranium (^{235}U), plutonium (^{239}Pu, ^{241}Pu) or americium (^{241}Am) whose nuclei contain odd numbers of neutrons. During the processing of nuclear fuels for recycling purposes, workers manipulate different actinide forms from extraction processes, or discharges of nuclear plants that may be a potential source of contamination [185]. Alpha particles are characterised by high radiation energy and a very short range (40 µm in tissue) [186, 187]. ^{137}Cs and ^{90}Sr nuclides are respectively β/γ emitter and pure β emitter and therefore belong to the most dangerous radionuclides of high toxicity. They are components of the long-lived fraction of fission products and are frequently involved in nuclear accidents. Indeed, Sr^{2+} is homologous to calcium in terms of chemical properties and thus it can compete with Ca^{2+} in the bone mineralisation processes [188]. The limitation of the dose is of great importance for the workers manipulating high doses of radioactive products in industrial and hospital sectors. Gamma rays and β-emitters are highly penetrating radiations while α-emitter may locally lead to very high dose in the case of repeated occupational exposure [176, 186, 189]. The critical site of radiation resulting from topical contamination is the basal layer of epidermal cells. The radiation dose reaching the basal layer involves predominantly beta and gamma radiations [188]. Grappin et al. reported a survey of 548 exposure cases which occurred in France between 1970-2003 and showed that approximately 53.5% of them included skin exposure to actinides (uranium, plutonium and americium and fission products as ^{137}Cs or ^{90}Sr), 17% of which concerned contamination of healthy skin [189]. More recently, Bérard et al. [190] reported that between 2003 and 2007, 100 to 150 cases including skin injuries occurred each year. Given that most contamination happens on the hands, the skin penetration of such heavy ions has been studied to develop effective countermeasures and decontamination guidelines.

^{241} Am, ^{239}Pu and ^{233}U have been studied *in vitro* using the Franz cell method or *in vivo* on rats by many researchers. These actinides are soluble in acidic medium. Americium is solely trivalent in water while plutonium would be tetravalent (Pu (IV)). Various uranyl isotopes are available but the

most stable is ^{235}U. Uranium, plutonium and americium, in contact with the skin, form polymerized hydroxide particles $(UO_2)_x(OH)_y^{(2x-y)+}$, $Pu(OH)_z^{(4-z)+}$, $Am(OH)^{(3-x)}$ which are similar to the insoluble particles met in most human contamination cases, as reported by Tymen et al. [187] and Beall and Allard [191]. In consequence, more than 90% of the applied activity is concentrated at the surface and in the epidermis [187]. Petitot found a lag time of 4 h and a steady state flux of 18.05 ± 3.75 $ng \cdot cm^{-2} \cdot h^{-1}$ after application of 100 µL of 60 $\mu mol \cdot L^{-1}$ uranyl nitrate solution (0.1N HNO_3) containing 500 Bq ^{233}U on intact pig skin [192]. Americium contamination has been rarely studied and the most recent results were obtained by Tazrart et al. on fresh and frozen pig skin explants using the *in vitro* Franz diffusion cell method [185, 186]. In these studies, 4.1 kBq of ^{241} Am were deposited in the donor chamber (270 µL on 0.9 cm^2). The acceptor fluid of the Franz cell was collected after 4 h, and the distribution in the skin layers after 24 h was evaluated. The results showed that 90% of the initial activity was recovered at the skin surface and 0.016 % and 0.003 % in the acceptor medium for the fresh and frozen skin respectively. This difference was statistically significant. Interestingly, even after washing of the *stratum corneum* with water or the use of a chelating agent, 8 to 10% of the activity remained "stuck" within the *stratum corneum* layer. The same behaviour was also observed in other studies for plutonium and uranium [187, 193].

The skin penetration of $^{137}Cs^+$ and $^{60}Co^{2+}$ through abdominal rat skin has been studied by Koprda et al. and was shown to be proportional to the time for 5-7 hours of exposure. These heavy metal cations form covalent bonds with side chain polar groups of peptides (-COOH, -OH, and -NH2) and phospholipids. They can also form multinuclear chelates [176, 194, 195].

Concerning radionuclides used for diagnostic purposes, Bolzinger et al. have shown a low permeation of indium and gallium [176]. These metal ions are stable only in the +3 oxidation state, due to the formation of insoluble hydroxides at pH=5.5 on the skin surface, as reported by Reichert et al. [194]. Technetium (^{99m}Tc), on the other hand, permeated faster through the skin from the pertechnetate anion (TcO_4^-). It was suggested that there was no effective chemistry to attach ^{99m}Tc to biomolecules in the skin under its oxidation state (VII) [176].

7 Skin absorption of Hofmeister ions and their impact on absorption of other molecules

Three columns of the periodic table of the elements: IA, IIA and, VIIA must be considered specifically as they form abbreviated Hofmeister series (Fig. 3) These ions display common physicochemical properties with two main classes: strongly hydrated (small size, high surface charge density) and weakly hydrated (large size, low surface charge density) species relative to the strength of water–water interactions, as written by Collins et al. [196]. Chaotropic ions are poorly hydrated in aqueous

solutions and thus called “water-structure breakers”. They exhibit weaker interactions with water than water itself so that they interact strongly with non-polar substances, resulting in their increased solubility (salting-in effect). On the other hand, kosmotropes interact strongly with water molecules and reduce the solubility of non-polar substances (salting-out effect). Alternatively, these ions are called “water structure makers”.

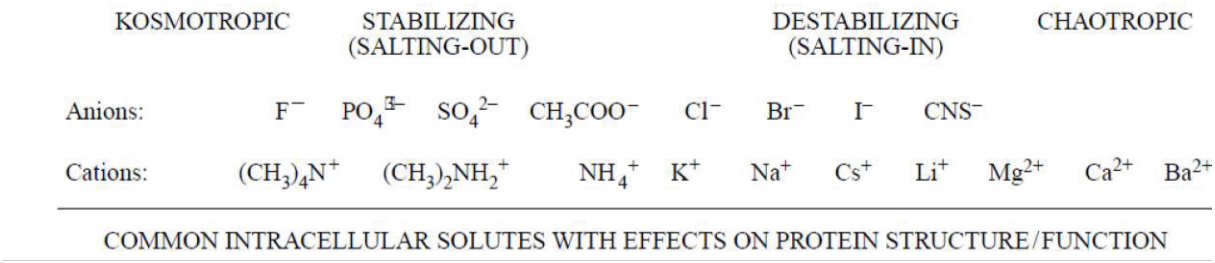

Figure 3 Hofmeister series of ions [197]

Considering these specific characteristics, some attempts were done to investigate the properties of the ions classified in Hofmeister series in terms of their own skin absorption as well as their effects on skin penetration of other substances.

Tregear investigated *in vitro* and *in vivo* skin penetration of radiolabelled Br^- (^{82}Br), Na^+ (^{24}Na) and PO_4^{3-} (^{32}P) using different skin models (human and rabbit (*in vitro* and *in vivo*), pig (*in vitro*)). Despite the differences in the flux depending on the model, all skin types used for *in vitro* and *in vivo* protocols were permeable to ions. The steady penetration rates of Na^+ and Br^- through the skin were proportional to the concentration difference of the ion across the skin in accordance to the Fick’s law. There were no significant differences between the penetration of different ions through the same skin model [103]. Our team investigated percutaneous penetration of radioactive iodide (^{125}I) in the context of skin contamination. Iodide penetrated full thickness pig skin over 24 h and had a 5-hour lag time [176]. Iodide is an anion of a great biological and pharmaceutical interest. It plays a central role in thyroid physiology and synthesis of thyroid hormones: triiodothyronine (T_3) and thyroxine (T_4). Thus, its systemic supplementation may be required in the case of hypothyroidism [198]. On the other hand, radioactive iodine is a useful tool in the treatment of thyroid cancer and hyperthyroidism [199]. Furthermore, iodine-based aseptic are commonly used topically in the treatment wounds difficult to heal and in pre-surgical preparations [200]. Because of these applications skin absorption of iodide has been thoroughly studied using Franz cell method. Lou et al. focused on transdermal delivery for iodide ions and enhancement of their penetration from various w/o microemulsions that could be an alternative route of administration. They demonstrated measurable skin penetration of iodide from KI aqueous solution that was significantly improved when the solution was incorporated in microemulsions. However, authors provided no information

concerning the retention of the molecule within skin layers which could account to even greater absorption rates. The experiments used human excised skin and was carried over 24h [200]. In that study, the lag time observed for iodide was also 5 h which was due to its high retention in *stratum corneum* in the early phase of absorption study.

Our recent investigation showed that within the lower concentration range the flux of anion through the skin is proportional to applied concentration of I^- but in the case of highly concentrated solutions, the flux is correlated to the activity of the salt solution [201]. This did not agree with Nygren et al., who studied radiocontamination by ^{131}I. Authors reported linear correlation between flux and tested concentration of iodide. This disagreement is due to difference in the preparation and storage of skin samples (epidermal sheets prepared from frozen-stored explants *vs* full thickness fresh skin). Thus, the observations in the last study were only related to passive transport of the ion. Nygren et al. also noticed differences in absorption of iodide through human epidermal sheets depending on the acceptor medium used. Higher flux was obtained when a mixture of water an ethanol (1:1) was used as compared to PBS. The lag time of only 5 minutes observed in that study which was also due to the use of split frozen-stored skin model. Additionally, the preparation of epidermal discs included heating up to 60°C that could affect the structure of *stratum corneum* lipids [202]. The distribution of anion in the skin layers after 6 h exposure time revealed that there was no retention in the skin [176].

These results were then extended in the works by Paweloszek et al. who studied skin absorption of non-radioactive halide ions (F^-, Cl^-, Br^- and I^-) as an important part of the Hofmeister series. The ions deposited as aqueous solutions of sodium salts permeated pig and human skin in a concentration-dependent manner [203]. Moreover, tested anions had significantly higher penetration rates when fresh skin samples were used as compared to frozen-stored explants. These results, evidenced for both human and porcine skin, indicated that facilitated transport can contribute to the skin penetration of anions. Indeed, this thesis was confirmed by applying chloride channel blocker on fresh skin prior to its exposure to the salt solutions. Penetration rates in this experiment were similar to the ones obtained on frozen-stored skin [204]. Finally, Tarnowska et al. tested skin permeation of the most chaotropic anions thiocyanate and perchlorate (also applied as aqueous solutions of sodium salts). These anions are known as endocrine disruptors as they can inhibit iodine uptake in the thyroid leading to insufficient synthesis of thyroid hormones [205]. They were found to penetrate through viable skin *in vitro* over 24 h. The total absorbed amount of perchlorate was comparable to that of iodide while thiocyanate penetrated less [201, 206].

Within the series of anions whose skin absorption was studied by Paweloszek et al. there are species that have some demonstrated biological effects. Chloride plays an important role in the skin biology.

Denda et al. evidenced that GABA and glycine that activate the chloride channels accelerated skin barrier repair in hairless mice after tape stripping and acetone treatment. The application of chloride ionophore accelerated the exocytosis of lipid- containing lamellar bodies and thus the process of skin barrier repair. Mechanism suggested by the authors involved blocking the outward movement of the cell membrane and accelerated fusion of the lamellar body and cell membrane due to the repolarisation of cell membrane by the Cl^- efflux [112].

Skin exposure to high doses fluoride, especially when related to skin absorption of the ion, may lead to reduced collagen content, as shown in rats and rabbits but these effects were not observed for lower doses. Indeed, the mechanism of toxicity of sodium fluoride is known to cause deficient collagen fibres due to inadequate cross-links. Additionally, high doses of NaF were found to disturb terminal differentiation of cultured keratinocytes which was manifested by decreased keratin expression and failing in stratification that could impair skin barrier function [207, 208].

As chaotropic anions are the “sticky ions” that bind to non-polar lipids or proteins and swell the extracellular lipid matrix of *stratum corneum* by a salting-in effect (by expelling water), they can contribute to hydration of lipid bilayers and then facilitate the skin absorption of drugs into the *stratum corneum*. Chronologically the first study disclosing a significant improvement in skin absorption of neostigmine bromide, a positively charged organic model molecule, in the presence of NaCl and LiCl was reported by Michael-Baruch et al. [209]. Indeed, the drug did not permeate split skin from a simple aqueous solution (values below detection limit). The addition of NaCl and LiCl increased drug penetration rates in a concentration-dependent manner. The dependence of the permeability coefficient on inorganic ion concentration was biphasic. The first phase was attributed to a decrease in the viscosity of lamellar phase caused by the influx of salting-in cations which participated in the hydration of the lamellar phase. The second phase was ascribed to a Donnan-like effect where the poorly diffusible cation (Na^+ and Li^+) enhances the partitioning in the favour of the more diffusible ion, neostigmine [209]. In another study, Ko et al. tested the impact of different sodium salts of anions classified in the Hofmeister series (NaF, NaCl, NaBr, $NaNO_3$, NaI, NaSCN, $NaClO_4$) on skin penetration of salicylic acid. The experiments were carried on using excised mouse skin mounted in Franz cells. The permeation-enhancing activities of tested salts only partially followed the ion order within the Hofmeister series. More chaotropic anions (Br^-, I^- and SCN^-) improved the flux of tested molecule while the kosmotropic ones either did not have the effect (Cl^- and ClO_4^-) or decreased (F^-, SO_4^{2-}) permeation rates. For the ions classified as permeation enhancers, concentration dependence of the effect was reported up to 2 M. Further increasing of the salt concentration did not cause additional improvement of salicylic acid skin permeation. Authors confirmed these results by further investigation *in vivo*. Indeed, addition of NaI or NaBr to the

vaseline ointment significantly increased the transdermal permeation of drug through rabbit skin. Authors suggested that these effects were due to alteration of the thermodynamic activity of salicylic acid in the donor solution in the presence of bromide and iodide [210]. Finally, our recent studies investigated how mixing Hofmeister anions in bi- and ternary systems could affect their skin absorption *in vitro*. Two series of anions were tested using the Franz cell method while maintaining pork skin explants viable allowing facilitated transport. The first series included halide ions and their mixtures obtained in such a way that the total concentration of salts in deposited solution was constant. The results of skin absorption were expressed as an absorption coefficient which is the ratio of the total absorbed amount after 24 h exposure to the activity in the donor solution ($k_{abs}=Q_{abs}/\gamma c$). Such conversion allowed direct comparison among k_{abs} values regardless different anion concentrations deposited in the donor compartment of Franz cell system. These studies revealed strong synergy in between iodide and bromide in binary mixtures. Addition of fluoride was either inert or slightly reduced overall penetration of other anions. This was clearly confirmed by statistical analysis. Skin absorption of halides from ternary mixtures was constant [201].

The second series of anions, investigated skin absorption of chaotropic anions: I^-, SCN^- and ClO_4^- alone, in bi- and ternary mixtures keeping anion concentrations constant. In this case, the total absorbed quantity of thiocyanate was the highest when applied in a ternary mixture. On the other hand, perchlorate had the highest permeation rates when applied alone. For iodide only a trend indicating its reduced absorption from I^-+SCN^- mixture was observed but the difference was not statistically significant [206].

8 Conclusions

The effects of inorganic ions on human health depend on the type of ion and its dose. It is evident that bulk and macroelements are essential for healthy grow, development and functioning of the organism. Micro- and trace elements are necessary for proper functioning but their excessive administration can lead to serious adverse effects. Xenobiotic ions could also have either beneficial (ex. Ag^+) or harmful effects on the organism (ex. lanthanides). In various studies, skin has been shown to be a potential route of entry of these inorganic ions. Depending on the physicochemical properties of ions, some of them permeate into the tissue while others can penetrate into the bloodstream. Generally, the penetration is easier for anions and monovalent cations, while polyvalent ones tend to be retained within skin layers. Cations exert more biological functions in the skin than anion, thus the beneficial of thermal spring waters depend on their cationic composition. Also, harmful effects are often due to excessive exposure to cations of heavier metals.

9 References

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EXPERIMENTAL SECTION

Chapter II

Survival media for maintaining viability of skin explants during in vitro absorption experiments

1 Problem statement

In vitro experiments are useful tools enabling evaluation of dermal absorption and percutaneous penetration of various compounds for skin absorption studies, safety assessment procedures and decontamination efficacy assessments. The purpose of skin absorption studies is to provide information on bioavailability by means of qualitative and quantitative measurements of passage of a substance through the skin. Main advantages of *in vitro* over *in vivo* protocols include limited use of living animals (forbidden for cosmetic tests in EU), the possibility of increasing replications of measurements and overcoming problems caused by the variability of health and physiological conditions of studied subjects [1].

A well-adapted *in vitro* protocol provides a more or less accurate prediction of *in vivo* skin absorption, depending on the physicochemical properties of the tested compound and the animal skin model used. The site of action of a tested molecule can be either located within the skin layers: epidermis and dermis (in case of topical administration) or inside the body so that the substance should reach the systemic circulation (transdermal administration route). Ross *et al.* provided a comprehensive comparison of predictions of *in vivo* dermal absorption based on a collection of *in vitro* data published in the literature [2].

The commonly applied protocol uses Franz diffusion cells consisting of a donor and a receptor compartment separated by a biological (skin) or a synthetic membrane (Fig. 1) [3]. The Franz cells are immersed in a thermoregulated water bath, providing a stable skin surface temperature of 32°C. Skin samples are placed in the cell with *stratum corneum* facing the donor chamber where the substance-loaded formulation is applied. The lower reciever chamber is filled with the acceptor solution that has to ensure adequate solubility of the compound such that sink conditions are satisfied throughout the study. For hydrophilic compounds, isotonic saline or buffered isotonic saline (pH 7.4) are

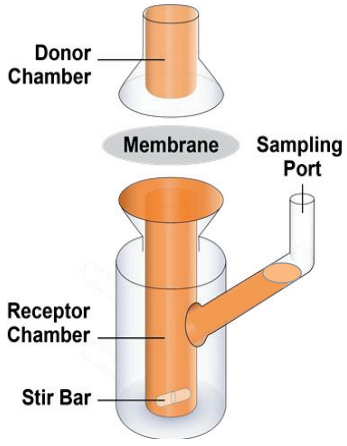

Figure 1 The design of a vertical static Franz diffusion cell used for skin absorption experiments [3].

proposed as physiological environments mimicking the blood circulation. The routine test has been extensively studied, and guidelines for conducting experiments have been published to design correct skin absorption experiments ensuring reliable results [1, 4].

Most routine skin penetration studies are performed using skin explants that had been stored frozen, assuming that skin absorption of xenobiotics is a passive diffusion process [5].

With regard to current knowledge, considering skin only as a simple passive barrier which limits the transport of molecules owing to the highly hydrophobic structure of its outermost layer- *stratum corneum*- seems irrelevant. Considering skin as a "huge and highly active biofactory" as suggested by Choung *et al*. [6] will be much more appropriate.

Skin actively participates in the first-line of defence against exogenous substances [7]; it exhibits neuroendocrine and steroidogenic activities leading to the synthesis and excretion of many substances such as neurotransmitters, neuropeptides, hormones and vitamin D_3 [8–10]. Several studies have demonstrated significant biotransformation of tested compounds such as parabens [11], benzo[α]pyrene [12] or cinnamic derivatives [13] inside fresh (viable) skin that occurred due to the abundance of enzymes both of the first and the second phase metabolism [14–17].

In this thesis, we focused on skin absorption of ions. A wide range of ion channels and transporters responsible for facilitated transport of both cations [18–20] and anions [21–23] have been found in the skin or in the cell cultures of keratinocytes and fibroblasts. These structures contribute to up to 80% of ionic transport through viable skin [24]. The studies of such facilitated transport require maintaining skin explants viable over the course of experiments. Unfortunately, given the character of tested molecules and analytical method applied within the research (ion chromatography) the use of commonly chosen survival medium (HHBSS or DMPBS [25]) which have high concentrations of salts was not possible. Thus, the objective of the first experimental chapter was to design a survival medium which could be used as an acceptor fluid in the experimental setup. The conditions that needed to be satisfied were: (i) minimal content of ions that could interfere with ion chromatography analyses; (ii) efficacy in maintaining skin metabolic activity over 24 h. In the research communication below, we, therefore, studied several compositions of such a survival medium. We tackle some practical issues arising while performing in vitro skin absorption experiments on viable explants related to the limited number of Franz cells that can be treated at the same time and the irregular availability of skin samples. Storing fresh skin explants in the refrigerator at 4°C overnight before starting a kinetic study of skin absorption in the morning seems to be a common practise in such circumstances. Hence, in the last part of the paper, we evaluated the impact of short-time (17 h) preservation of skin explants at 4°C.

HIGHLIGHTS OF THE CHAPTER

- We defined the conditions necessary for maintaining skin explants viable over 24 h: physiological pH, isotonic composition and glucose. Acidic pH was found to be the most detrimental to skin viability *in vitro.*
- BG medium satisfied requirements imposed by experimental setup and thus was used in all skin absorption experiments.
- Short-time storage at low temperature affected skin metabolic function. Hence, all presented studies were performed on freshly-excised skin explants.
- Presented study on skin survival in the experimental conditions was extended in order to evaluate the impact of common additives (non-ionic surfactant and ethanol) used in acceptor media when hydrophobic molecules are being tested. The addition of up to 1% of surfactant seems to be a good compromise for such tests. However, higher concentrations of surfactant and ethanol (50%) considerably affect skin viability *in vitro*.

Formulation of survival acceptor medium able to maintain the viability of skin explants over in vitro dermal experiments

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Abstract

OBJECTIVE: *In vitro* assessments of skin absorption of xenobiotics are essential for toxicological evaluations and bioavailability studies of cosmetic and pharmaceutical ingredients. Since skin metabolism can greatly contribute to xenobiotic absorption, experiments need to be performed with skin explants kept viable in suitable survival media. Existing protocols for non-viable skin are modified to consider those conditions. The objective was to design a survival medium used as an acceptor fluid in Franz cells for testing cutaneous penetration of hydrophilic or lipophilic molecules. Their metabolism inside skin may be investigated under the same conditions. The determining factors involved in survival mechanisms *in vitro* are discussed. The consequences of short-term skin preservation at 4°C were also evaluated.

METHODS: The metabolic activity of fresh skin samples mounted in Franz cells were studied by measurement of lactate release over 24 h in order to assess the impacts of pH, buffering, osmolality, ionic strength, initial glucose supply, and the addition of ethanol or nonionic surfactant in the acceptor part of Franz cells.

CONCLUSION: Survival media must maintain physiological pH (>5.5), be isotonic with skin cells (300 $mOsm \cdot kg^{-1}$) and contain at least 0.5 $g \cdot L^{-1}$ glucose. Several compositions able to preserve skin metabolism are reported. Storage of skin explants overnight at 4°C impairs skin metabolic activity.

The present work provides guidelines for designing survival media according to constraints related to the scientific requirements of the experiments.

2 Introduction

Dermal absorption studies provide information on the bioavailability of xenobiotics, pharmaceutical or cosmetic active substances by means of qualitative and quantitative measurements. The aim of such studies is the evaluation of their passage through skin for the sake of safety assessments or the optimization of cosmetic formulations towards better targeting of the active substance to a specific skin site [4, 26, 27].

Usually, skin absorption studies are performed on non-viable split-, full-thickness skin or epidermal membranes (although viable skin is preferred) because the *stratum corneum*, the uppermost layer of the skin made of dead keratinized cells, is considered the main barrier against skin penetration. The complex organization of this horny skin layer is the main rate-limiting step against molecules absorption [5]. Most studies are performed on skin explants that had been stored frozen and thawed the day of experiment.

However, skin is a living tissue having its own metabolism [6, 28]. Therefore, *in vitro* studies using frozen skin may not provide full information on dermal absorption of compounds that are transported through metabolic pathways or that undergo biotransformation inside skin [1]. Significant biotransformation of tested compounds such as parabens [11], testosterone [29] or catechines [30] occurs in fresh (viable) skin owing to the presence of enzymes from the major biotransformation pathways [16, 31]. Sintov observed faster penetration rates for caffeine and diclofenac in frozen skin that he attributed to alterations of skin barrier functions during freezing [32]. Conversely, Paweloszek *et al.* disclosed enhanced permeation of ions in fresh, viable skin related to the contribution of facilitated transport [24]. Existing guidelines provide scant information on how to ensure sink conditions while maintaining skin viability. Bronaugh *et al.* addressed in a review paper current knowledge on skin viability [33]. The majority of survival media used to support skin viability throughout experiments are either buffers for biological use supplemented with glucose at 1 $g \cdot L^{-1}$ [25, 34, 35] or broth cell culture media [25, 36]. Some authors using commercially available culture media included higher concentrations of glucose, reaching 4.5 $g\ L^{-1}$ [36, 37], but no data are available on how such changes in glucose concentration affect skin viability. Therefore, the present study addresses the role of physicochemical parameters such as pH, buffering capacity, osmolality, ionic strength and glucose concentration in maintaining skin viability.

Another challenge is to design an acceptor fluid that ensures sink conditions for skin penetration experiments of highly hydrophobic compounds without affecting skin barrier function. For this

purpose, ethanol [38] or surfactants [39] are commonly added to the acceptor fluid. Since their impact on skin metabolism remains unknown, the influence on skin viability of addition of 50% ethanol and nonionic surfactant (1 and 5%) into acceptor medium was investigated.

Finally, a practical issue arising from the limited number of Franz cells that can be treated simultaneously and the irregular availability of fresh skin samples was addressed. It is sometimes necessary to store freshly excised skin explants in the refrigerator at 4°C overnight before starting a kinetic study of skin absorption. The OECD guideline reports that "as a general guidance, freshly excised skin should be used within 24 h, but the acceptable storage period may vary depending on the enzyme system involved in metabolisation and storage temperatures" [4, 31]. Hence, the impact of short-term (17 h) preservation of skin explants at 4°C was studied.

3 Materials and methods

3.1 Chemicals

Sodium sulfate (Na_2SO_4), calcium sulfate ($CaSO_4$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), sodium hydrogen carbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and betaine (trimethylglycine) were purchased from Acros Organics (Illkirch, France). Ethanol 96°, glucose, sodium chloride (NaCl) and potassium chloride (KCl) were obtained from Fisher Scientific (Illkirch, France). Oleth-20 (polyoxyethylene(20) oleyl ether) was provided by **Commercial Química Massó (Lyon, France).** Calcium chloride ($CaCl_2$) was from Cooper (Melun, France), and the Lactate Assay Kit MAK064 was from Sigma-Aldrich (St Quentin Fallavier, France). Ultrapure water with resistivity > 18 MΩ·cm at 25°C was used in all experiments.

3.2 Methods

3.2.1 Media preparation

The survival media compositions are presented in Tables I and II. All the solutions were analysed in terms of pH (pHenomenal® pH, VWR equipped with an electrode pH 11769798, Fisher Scientific, France) and osmolality (Osmomat 030 Cryoscopic osmometer, Gantec, Berlin, Germany), filtered through Whatman® Nylon membrane filters with pore size of 0.45 µm (Sigma-Aldrich, St Quentin Fallavier, France) and degassed in an ultrasonic bath (Elmasonic S 50 R, Elma Schmidbauer Gmb, Singen, Germany) for 5 min prior to use.

Table I Compositions of survival media. All concentrations are given as $mg \cdot L^{-1}$.

	DMPBS Dulbecco's Modified Phosphate-Buffered Saline [25]	**SM** Salt-rich Medium	**BG** Betaine-Glucose	**WGB** Water-Glucose phosphate-Buffered	**SSG** Serum Saline Glucose [35]	**WG** Water-Glucose [35]	**PBSG** Phosphate-Buffered Saline supplemented with Glucose [25]
NaCl	8000	18000 (Na_2SO_4)	-	-	9000	-	7000
KCl	200	-	-	-	-	-	-
$CaCl_2$	100	84 ($CaSO_4 \cdot 2H_2O$)	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	121	64	-	-	-	-	-
Glucose	1000	1000	1000	1000	1000	1000	1000
$NaHCO_3$	-	350	350	-	-	-	-
$NaH_2PO_4 \cdot 2H_2O$	-	-	-	-	-	-	8970
Na_2HPO_4	1150	48	48	48	-	-	-
KH_2PO_4	200	60	60	60	-	-	-
Betaine	-	-	2990	-	-	-	-

Table II Modifications to BG survival medium.

A: variation of the initial concentration of glucose in BG medium. B: BG medium modified to test hydrophobic molecules. All concentrations are given in $mg \cdot L^{-1}$

	A				B		
	BG-0.5	**BG-0.75**	**BG-1.0**	**BG-2.0**	**BG + Oleth-20 1%**	**BG + Oleth-20 5%**	**BG + EtOH 50%**
Glucose	500	750	1000	2000	1000	1000	1000
$NaHCO_3$	350	350	350	350	350	350	350
Na_2HPO_4	48	48	48	48	48	48	48
KH_2PO_4	60	60	60	60	60	60	60
Betaine	2990	2990	2990	2990	2990	2990	2990
Oleth-20	-	-	-	-	100	500	-
Ethanol 96°	-	-	-	-	-	-	5000

3.2.2 Skin sample preparation

Full-thickness porcine flank skin explants were obtained as medical waste from five young female pigs (30 ± 1 kg), sacrificed at the CERMEP Laboratory, University Claude Bernard Lyon 1 (Lyon, France).

Freshly excised tissue samples were used immediately after harvesting to study the efficacy of different survival media in maintaining skin viability. For experiments aiming at evaluating the impact of short-term storage, harvested flanks (including subcutaneous adipose tissue and underlying layer of muscles) were stored in a refrigerator (4°C) for 17 h. The samples were brought back to 25°C over 1 h. The bristles were cut with electrical clippers, and the explants were rinsed with water. The subcutaneous adipose tissues were carefully removed with a scalpel to yield a final thickness of 1.38 ± 0.02 mm (micrometre Mitutoyo). Skin integrity was checked by measuring the Trans Epidermal Water Loss (TEWL; Tewameter TM210, Monaderm, Monaco). Samples having TEWL values above 15 $g \cdot h^{-1} \cdot m^{-2}$ after 1 min were discarded. The average TEWL value of skin samples used for experiments was 12.0 ± 0.4 $g \cdot h^{-1} \cdot m^{-2}$ at 25°C.

3.2.3 Skin viability

Skin viability was assessed though measuring the efficiency of anaerobic glycolysis. This is the predominant energetic pathway operating in the viable epidermis [25]. Lactate dehydrogenase

catalyses the transformation of glucose into L(+)-lactate as the end-product of anaerobic glycolysis. L(+)-lactate production was measured as an indicator of skin viability.

Full-thickness skin samples were mounted into two-chamber static Franz glass cells [3] (exposure area 2.54 cm^2) with the *stratum corneum* facing the donor chamber that was left empty, while the acceptor compartment was filled with 10 mL of survival medium or pure water to asses endogenous lactate concentrations. Mounted Franz cells were placed in a water bath at 37°C with magnetic stirring so that the surface of the skin was 32°C owing to heat loss. After 30 min of stabilisation, the acceptor fluids were entirely replaced with fresh media. To assess skin viability in various media, 250 µL aliquots of acceptor fluid were collected at defined times (3, 6, 16 and 24 h) and replaced by an equivalent volume of fresh medium. Lactate concentration in the acceptor fluid fractions was determined by a colourimetric enzymatic assay using a commercial Lactate Assay Kit as advised in OECD guidelines [4]. The absorbance of samples was measured at 570 nm wavelength using a Multiskan EX plate reader (Thermo Fisher Scientific, Villebon sur Yvette, France). Three samples of acceptor medium per experimental condition per time point were diluted to ensure that measured concentration was within the linear range of calibration curves (0.4 - 2 $mmol \cdot L^{-1}$). Solution of reaction mix was used as negative control; its absorbance was subtracted from the ones of investigated samples.

3.2.4 pH evaluation

pH was controlled for fresh media and at the end of 24h experiment using pH-meter (pHenomenal® pH, VWR equipped with an electrode pH 11769798, Fisher Scientific, France).

3.2.5 Statistical analysis

The mean and standard error of the mean (*sem*) of n = 3 determinations were calculated. Statistical comparisons were made using the Student's t-test (two-sample assuming equal variances) and analysis of variance (ANOVA, single factor) with the level of significance at $p < 0.05$.

4 Results and discussion

There is a big gap between research interest in skin metabolic functions, including new approaches in skin delivery of prodrugs [34, 40], and the few studies that assess the suitability of survival media for *in vitro* assays. This work aims at determining the minimum requirements of an effective survival medium for studies performed on freshly excised skin. In their early work, Collier *et al.* [25] concluded that supplementation of a complex cell culture medium with vitamins and amino acids was not required to maintain skin metabolic activity for 24 h. Hank's Balanced Salt Solution (HBSS) supplemented with glucose, and an antibiotic became one of the most commonly used medium for maintaining skin viability. Then, addition of antibiotics was not necessary for experiments performed

over a short time (24 h) [35]. In this study, various media compositions were studied to assess the role of each ingredient related to physicochemical properties such as pH, osmolality, ionic strength and glucose supplementation (Table I). Based on the available literature, the reference medium DMPBS was selected as a positive control able to maintain skin viability in a similar experimental setup, and PBSG was chosen as a negative control that did not maintain skin viability in the article by Collier *et al.* [25]. Modifications were subsequently introduced to test changes in osmolality, and the presence and type of buffering system. Various aqueous solutions of glucose were tested at first: glucose alone [35], glucose-supplemented saline [35], buffered glucose solution, and more complex media (BG and SM) based on the HBSS composition [35]. They also contained glucose and different agents, ensuring iso-osmolal conditions together with the buffer system (Table I). Finally, the impact of the initial glucose concentration in the BG medium and that of common additives used for skin penetration experiments of hydrophobic compounds was evaluated (Table II).

4.1 Impact of pH control

Anaerobic glycolysis leading to lactate production is the predominant energetic pathway for skin cells in this experimental setup [25], and could acidify the external medium due to the efflux of lactate. The first measurement of lactate released to the medium was performed after 3 h of exposure. Such a delay was necessary to have enough of accumulated lactate allowing accurate measurement, even in case of low lactate production. To verify the impact of initial pH and strength of buffer system on lactate production, the following media were tested: DMPBS, BG, and SM (buffered at pH 7.4), SSG (not buffered, physiological pH), WGB (buffered at pH 6.8), WG (not buffered, pH 5.8) and PBSG (buffered at pH 4.5). After 24 h exposure, pH was lowered in all cases, except for PBSG which had an initial acidic pH and the respective final pH values were 5.6, 5.7, 5.3 and 4.2. This indicates that the amount of lactic acid produced over exposure time was larger than the capacity of buffers used for DMPBS, SM, BG and WGB. This trend was also present in non-buffered solutions with the final pH values of 4.9 for SSG and WG. These pH changes can be correlated with the cumulative lactate production over 24 h (Fig. 2). DMPBS, SM and BG maintained skin metabolism for 24 h as indicated by the cumulative lactate release, which linearly increased with respect to time. SSG and WG did not maintain skin viability since lactate release rates in SSG and hypotonic WG were negligible and corresponded to the release of endogenous lactate in deionised water which caused immediate pH decrease. The difference between those two groups was statistically significant during the whole experiment (Fig.2). The medium of intermediate activity, WGB (a buffered equivalent of WG), maintained metabolic activity only for the first 6 h, where no statistically significant differences were observed as compared to DMPBS. Weak lactate production afterwards was related to the buffer

saturation. Finally, skin samples in PBSG did not show any metabolic activity throughout the experiment, due to its low initial pH.

Anaerobic glycolysis requires a lactate efflux mediated by proton-coupled symporters or monocarboxylate transporters (MCT) and causes a pH decrease. The low external pH of the extracellular medium induces the inhibition of MCT-mediated lactate efflux [41]. This results in the accumulation of lactate inside the cells and subsequent inhibition of glycolytic enzyme activity through a feedback mechanism [42]. This mechanism is irreversible, as observed in our previous studies investigating lactate release in SSG and WG using a protocol with regular media renewal, which showed no resumption of lactate efflux despite re-establishing physiological pH after 1 h [35]. Hence, maintaining a pH close to physiological throughout the whole experiment is crucial for the effectiveness of the survival medium. This may be achieved with a highly buffered medium or regular renewal of medium during experiment. Using a flow-through system allows a continuous renewal of survival medium that maintains pH conditions constant.

4.2 Impact of osmolality

Osmolality measurements of all media were performed on fresh solutions. DMPBS, PBSG, SM, BG and SSG were isotonic with plasma, exhibiting osmolality values of 260, 326, 297, 308, and 287 mOsm·kg^{1}, respectively. WGB and WG were hypotonic (9 and 7 mOsm·kg^{-1}).

The media that maintained high metabolic activity of skin samples for 24h (Fig. 2), did not ensure isotonic conditions in identical ways (Table 1): DMPBS contained a significant amount of NaCl, which is commonly used to increase the osmolality of solutions; SM osmolality of 300 mOsm·kg^{-1} was ensured by replacing NaCl with Na_2SO_4 ; BG contained minimum amount of ionic species, and the zwitterionic betaine (250 mM) ensured osmolality without increasing ionic strength [43]. Similar profiles of lactate release for DMPBS, SM and BG indicated that these isotonic formulations *had no differential effect. SSG did not maintain skin viability despite being isotonic. The buffered but hypotonic and slightly acidic WGB medium had partial efficacy. These results, once again, showed the importance of pH control over the course of the experiment.*

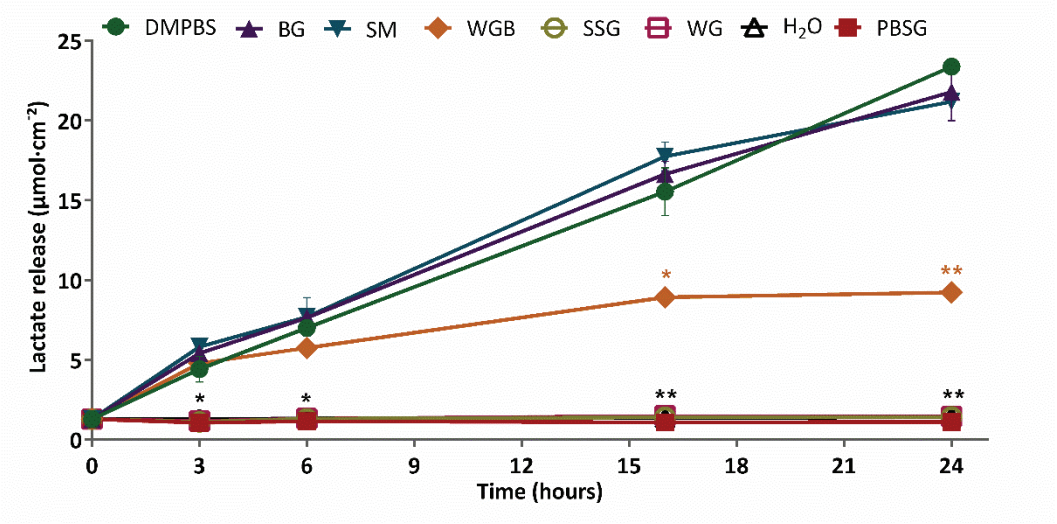

Figure 2 Impact survival medium composition on skin viability. The graph illustrates cumulated values of released lactate in µmol·cm-2 of skin area at different time points. Values represent mean ± sem carried out on n = 3 skin explants. Statistically significant differences between given medium and DMPBS (positive control) are denoted by single asterisk (*) for $p < 0.05$ and double asterisk (**) for $p < 0.01$

There were noticeable differences of lactate release between the present results of the DMPBS positive control and those reported by Collier *et al.* [25]. Collier *et al.* used flow-through Franz cells in contrast to the static system presented here. The rate of lactate release calculated as the slope of the linear cumulated release with respect to time was 0.97 µmol·cm^{-2}·h^{-1}, two-fold lower than that reported by Collier *et al.* (1.96 µmol·cm^{-2}·h^{-1}) [25]. A previous study on skin viability using pork flank explants and an intermediate protocol where the survival medium was regularly renewed during the experiment gave a lactate release rate of 1.13 µmol·cm^{-2}·h^{-1} in DMPBS [35], comparable to the present result. Thus, regular renewal of the survival medium, which made the static experiment close to flow-through conditions, did not significantly change the rate of lactate release, showing that the difference between Collier's study and the present results was primarily due to the different animal models (pig *versus* rat).

4.3 Impact of initial glucose concentration

It was necessary to check whether the glucose concentration of 1 g·L^{-1} that was not renewed during experiments provided a sufficient energy source to skin samples over 24 h. Within the glucose concentration range from 0.5 to 2.0 g·L^{-1} in BG medium, there was no influence of the available glucose concentration on its conversion rate into lactate. 0.5 g·L^{-1} was above the glucose concentration for saturation.

4.4 Impact of additives used in receptor fluid for penetration studies of hydrophobic molecules

The acceptor medium was modified by the addition of ethanol or surfactants to fulfil sink conditions for molecules of poor solubility in water. Bronaugh and Stewart [44] proposed a nonionic surfactant, PEG-20 oleyl ether (Oleth-20), in order to increase the solubility of cinnamyl anthranilate acetyl ethyl tetramethyl tetralin in an *in vitro* experiment and to improve the correlation with an *in vivo* study. The addition of a surfactant was safe with regards to the skin barrier function and improved the correlation between *in vitro* and *in vivo* results. In another study on the skin penetration of cypermethrin, Scott and Ramsey [38] concluded that 50% addition of ethanol was the only acceptor solution enabling satisfactory *in vitro* - *in vivo* correlation.

In the present case, the amount of lactate released after 24 h of exposure was significantly lower in media containing 50% ethanol and 5% Oleth-20 as compared to BG (Fig. 3). In both cases, a reduction of about 50% was observed compared with the reference BG medium. However, the addition of 1% nonionic surfactant maintained 80% of the viability, which was a good compromise for testing hydrophobic compounds. These findings are consistent with the propensity of both ethanol and nonionic surfactants to destabilise cell membranes. Ethanol is not only known to enhance skin penetration [45]; but it is also a biocidal agent at high concentrations (70%) due to its capacity to disrupt cellular membranes in bacteria [46] and artificial models of lipid bilayers [47]. Prolonged

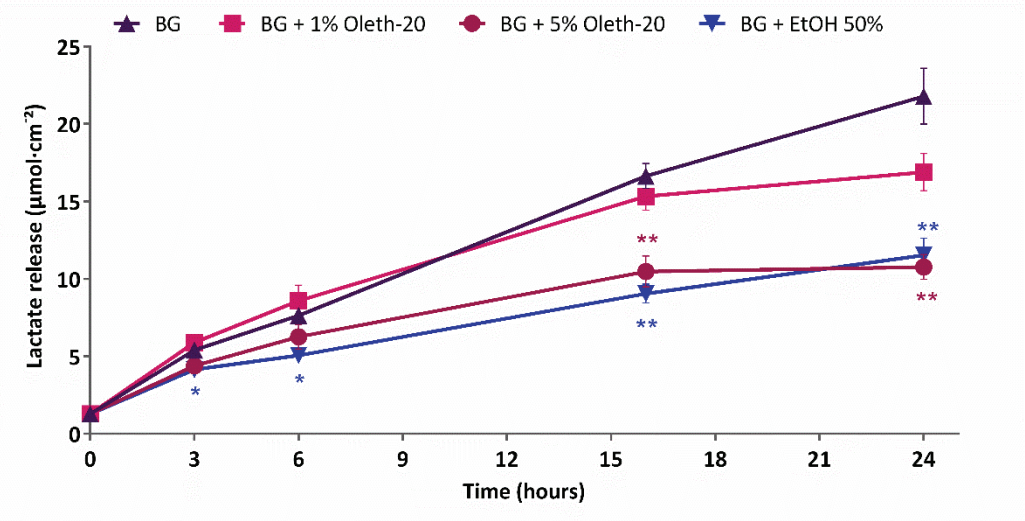

Figure 3 The impact on skin viability of common additives to acceptor fluids used for cutaneous penetration experiments of hydrophobic compounds. The graph illustrates cumulated values of released lactate in µmol·cm-2 of skin sample at different time points. Values represent mean ± sem carried out on n = 3 skin explants. Statistically significant differences between given medium and BG are denoted by single asterisk (*) for $p < 0.05$ and double asterisk (**) for $p < 0.01$.

exposure time (24 h) leads to considerable leakage of alcohol into skin and may cause cell death. Similarly, it has been reported that nonionic ether surfactants such as Oleth-20, Beheneth-25 or Ceteth-10 favour IL-1α release proving their irritancy potential. They were also toxic for cells of reconstructed human epidermis (viability lower than 5% in MTT test) [32].

4.5 Influence of short-time storage of skin explants at 4°C

Due to limitations in the availability of fresh skin samples and the number of Franz cells that can be run simultaneously, the problem of short-time skin preservation arises. Comparison of viability of fresh and cold-preserved skin samples stored for 17 h at 4°C was done by kinetic measurements of lactate release at 37°C in DMPBS, BG and SM (Fig. 4). Cold storage caused significant loss of skin viability during the first hours of exposure to survival medium where the loss of lactate production reached 70%. Skin samples slowly recovered some metabolic activity such that the mean loss was only 23% after 24 h exposure to the survival medium. Such recovery not as efficient as for other tissues [48]. This agrees with Castagnoli *et al.* who measured 25% drop of viability within the first day of storage at 4°C [37] and Ge *et al.* [49] who reported 10% and 20% loss of viability in DMEM and normal saline, respectively, during storage at 4°C for one day. The observed recovery delay could be related to a transient inhibition of the anaerobic glycolysis during storage at 4°C.

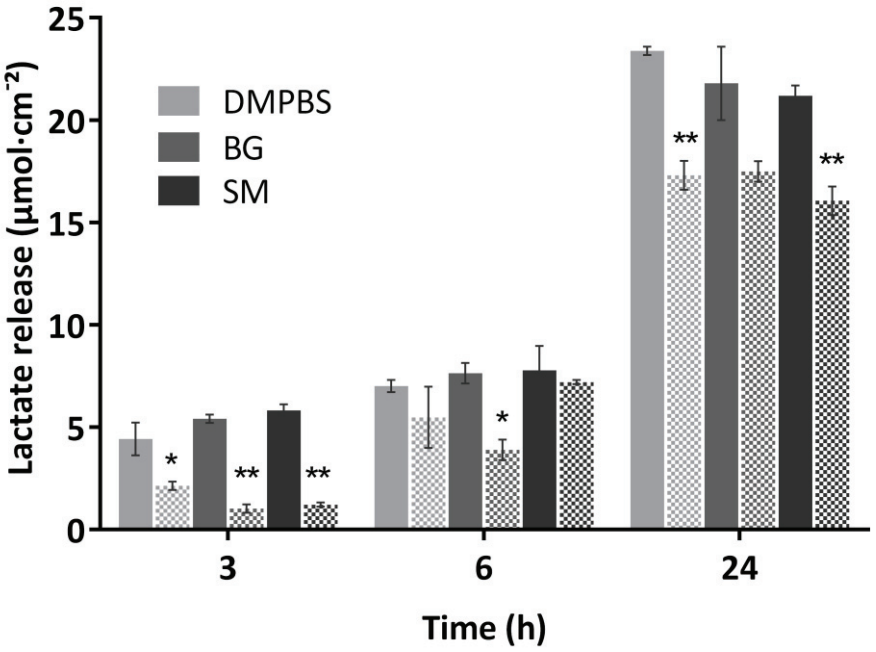

Figure 4 Impact of storage of skin samples at 4°C (17 h) on lactate release into survival media at 32°C at different exposure times. Full bars: skin used immediately after harvesting, hatched bars: skin stored at 4°C overnight. Values represent mean ± sem carried out on n = 3 skin explants. Statistically significant differences between lactate release in skin samples used immediately and skin samples stored in 4°C overnight in the same medium are denoted by single asterisk (*) for $p < 0.05$ and double asterisk (**) for $p < 0.01$.

5 Conclusions

The main features of survival medium for *in vitro* skin absorption studies using skin explants have been systematically studied, and guidelines are given. The key factor determining skin metabolic activity was the significant pH reduction caused by high lactate production into a medium of too low buffering capacity. Efficient survival media able to maintain skin viability over 24 h *in vitro* were isotonic and isohydric with plasma and buffered to prevent pH decrease. Physiological pH ensured by the presence of a buffer is a discriminating parameter. The initial glucose supply should be above 0.5 $g \cdot L^{-1}$, confirming the suitablity of usual concentrations of 1 $g \cdot L^{-1}$. Such findings provide guidelines for modifying survival media according to constraints related to the scientific requirements of the experiments. Minimising ionic content in the solution or adding a surfactant and/or ethanol for skin absorption experiments of hydrophobic compounds are examples of how a survival medium may be adapted to manage experimental conditions. Short-term storage of skin explants at low temperatures should be considered with caution because of possible alterations of skin metabolic activity within the first hours of experiments.

Conflict of interests

The authors have no conflicts of interest to declare.

Acknowledgements

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Chapter III

Skin absorption of anions from concentrated aqueous solutions. Effects of mixing.

1 Problem statement

Human skin forms a unique barrier separating the body from the external environment. At first glance, its outermost lipidic layer, *stratum corneum*, seems impermeable for inorganic ions- very small hydrophilic molecules with high charge density [1]. Multiple studies have proven this concept wrong. However, the majority of available literature focuses on functions of cations in skin homeostasis and their dermal absorption. This trend has been driven by the traditional use of thermal spring waters, whose properties depend mainly on the cationic composition, in the treatment of various skin and systemic diseases with measurable outcome. Other well-known applications of mineral salts where cations exhibit medicinal properties are silver (for its anti-bacterial properties), zinc and cooper, often combined (ex. Dalibour water and cream) [2] used to treat mild skin irritations and burns.

The interest concerning skin absorption of anions grew quite recently and resulted in several important findings. Previous research carried by our group demonstrated that radionuclides used for diagnostic purposes can indeed be absorbed after dermal exposure [3]. Later on, Paweloszek et al. [4, 5] investigated percutaneous penetration of halide anions (F^-, Cl^-, Br^- and I^-). These anions are comprised within Hofmeister series which classifies ions interacting with biological systems. Paweloszek's systematic approach of testing a series of homologous ions sharing certain characteristics and varying the others was quite unique in the field of skin absorption studies of ions. His studies revealed concentration-dependent flux of ions through skin samples and showed that the Hofmeister properties could affect the permeation rates of tested ions. Total absorbed quantities of anions, when applied in the same mass concentrations (ppm) of ions, showed the lowest flux for fluoride and the highest for iodide, respecting the kosmotrope-chaotrope properties of Hofmeister series.

Within this chapter, we present two publications extending Paweloszek's research.

The first one investigates the effects of mixing halide anions (F^-, Br^- and I^-) in bi- and ternary systems on skin absorption of individual anions. The experiments were carried on according to mixing plane where the final ionic strength of tested solutions was kept constant. This allowed further quantitative statistical analysis using lattice {3,2} simplex-lattice design model.

The second manuscript included in this chapter is dedicated to the research on skin absorption of anions classified as chaotropes within Hofmeister series: thiocyanate and perchlorate. Iodide was included in this study as the link between both series. Because all of them are able to interact with sodium-iodide symporter (NIS), which transports iodide in the thyroid gland and thus participates in the biosynthesis of thyroid hormones, the anions can be considered as endocrine disrupting

chemicals (EDCs). The effects of mixing of I^-, SCN^- and ClO_4^- in bi- and ternary mixtures on their skin absorption is also studied.

HIGHLIGHTS OF THE CHAPTER

- Total absorbed amount of an anion from a concentrated aqueous salt solution after infinite dosing is proportional to the activity of the solution, as the usual relationship Q_{abs}-concentration is not linear within high concentration range.
- We defined an absorption coefficient (k_{abs}) as the ratio of the total absorbed amount after 24 h exposure to the activity in the donor solution: $k_{abs} = Q_{abs}/\gamma C$ that allowed direct comparison between different experimental conditions, in which either concentrations of anions (halides) or the ionic strength of donor solutions (EDCs) varied.
- The values of k_{abs} were comparable for all tested anions except thiocyanate which had lower absorption coefficient.
- Association of ions in bi- and ternary mixtures can modulate their absorption profiles.
- A synergy in skin absorption between bromide and iodide was evidenced by means of quantitative statistical analysis.
- Association of hydrophobic anions among each other and with endogenous cations can modulate their skin absorption profiles.

Skin absorption of mixed halide anions from concentrated aqueous solutions

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Abstract

Non-ideal behaviour of mixed ions has been disclosed in skin absorption experiments of mixed halide solutions in excised pig skin. Comparison of skin absorption of pure and mixed ions showed enhanced penetration of hydrophobic halide ions from mixed solutions. An experimental design and statistical analysis using Scheffé {3,2} simplex-lattice allowed investigating the full ternary diagram of anion mixtures of fluoride, bromide and iodide. Synergism in mixed solutions was observed for hydrophobic bromide and iodide anions. A refined analysis highlighting specific interactions was made by considering the ratio of the absorbed amount to the ion activity instead of the direct measured absorbed amount. Statistical analysis discarded non-significant effects and disclosed specific interactions. It was proposed that enhanced absorption from mixed solution involved the formation of neutral complex species of mixed bromide and iodide with endogenous magnesium or calcium inside *stratum corneum*.

2 Introduction

Skin plays a key role in separation of human body from external environment. This is mainly due to *stratum corneum*, the outermost layer made of dead cells (corneocytes) surrounded by a lipid intercellular medium. *Stratum corneum* is organised into a brick-and-mortar-like structure where the bricks are corneocytes and the mortar is made of the intercellular lipids. Such highly hydrophobic structure protects body from excessive water loss from one hand and from pathogen and xenobiotic absorption from the other. Because of this hydrophobic barrier, skin absorption of simple inorganic ions has long been believed limited, or even impossible [1]. However, several recent studies have proved this concept wrong [3, 6]. Recent work by Paweloszek *et al.* shed new light on the possible mechanisms of halide ion transport across the skin including facilitated transport in viable skin by ionic channels and ion transporters [5]. In fact, skin absorption for ionic species is much more complex than just a passive diffusion described for various hydrophobic molecules because of the complex skin structure organised in layers. Outermost poorly hydrated *stratum corneum* with high content of lipids could indeed act as a simple passive barrier. On the other hand, viable epidermis is characterised by higher water (up to 70%) content and presence of living cells producing variety of proteins [7, 8]. Similar water content can be found in dermis, whose protein composition includes mainly collagen and elastin. Therefore, various types of skin-ion interactions seem possible. Ions interacting with biological systems have been classified in Hofmeister series (F^-, Cl^-, Br^-, NO_3^-, I^-, SCN^-, ClO_4^-) based on their interactions with macromolecules in aqueous solutions [9].

Ions binding to organic materials have been related to the “salting in” or “salting out” properties of ions [10]. The Hofmeister series classifies ions that are strongly hydrated in aqueous solutions as kosmotropic. They are also called “water-structure makers”. The solubility of neutral organic molecules in the presence of kosmotropes is reduced (salting out effect). On the other hand, chaotropic ions are poorly hydrated in aqueous solutions (“water-structure breakers”). They interact strongly with non-polar substances that results in their increased solubility (salting-in effect) [9, 11–14]. The properties described by Hofmeister series determine various physicochemical phenomena occurring in biological systems. For instance, binding of anions to model phospholipid bilayers was found to increase following the Hofmeister series of anions and binding of chaotropic ions was reported to affect the lipid packing within bilayers [15, 16]. Similar effects can be expected within *stratum corneum*. Within viable skin layers, ions-proteins interactions are operating. These may include non-specific effects based on the capacity to either stabilise or break water structure. These phenomena were proposed to explain differences in swelling properties of collagen in aqueous solutions of sodium salts of Hofmeister anions that follow the order $F^- < Cl^- < Br^- < SCN^-$ [17]. Specific binding of anions to transport proteins [18–21] following the same order is also highly possible in

viable tissues. These effects have already been disclosed by Paweloszek *et al.* [4] who studied skin absorption of simple model ionic aqueous solutions and showed significant skin penetration of halide anions from aqueous solutions following the Hofmeister series. Moreover, a significant contribution of facilitated transport of tested ions in viable skin was observed [5]. Beyond these observations, the detailed physicochemical phenomena involved still remain an open issue. It is worth noticing that such different phenomena as anion adsorption/absorption to hydrophobic *stratum corneum* and facilitated anion transport by proteins depend on the nature of anions in the same order: the Hofmeister series.

The present work addresses mixed solutions of anions where non-ideal mixing (interactions with ions of different chemical nature) influence skin absorption. In line with present knowledge regarding skin absorption of ionic species, the objective of this paper is to conduct fundamental studies investigating skin absorption of mixed ionic species in order to gain better understanding of penetration mechanisms and disclose the barriers against this penetration.

Some clues regarding such effects of ionic mixtures have been given in studies of skin absorption of organic molecules from ionic solutions. Thus, Michael-Baruch *et al.* [22] investigated skin penetration of neostigmine in the presence of LiCl and NaCl. Both salts enhanced the permeation of the drug in a concentration-dependent manner, the skin being completely non-permeable without addition of salts. Soon after, Ko *et al.* studied transdermal penetration of salicylic acid from mixed water-ethanol medium at pH 2 containing sodium salts of inorganic anions classified in lyotropic Hofmeister series (NaF, NaCl, NaBr, NaI, Na_2SO_4, $NaClO_4$, $NaNO_3$, NaSCN). They reported increased skin penetration of tested molecule in the presence of NaBr, NaI and NaSCN while other salts had no or a reverse effect on cutaneous absorption of salicylic acid [23]. As a whole, the Hofmeister series was not followed. These two earlier works are only clues for us. Indeed, neostigmine is a cationic drug that obviously interacts in bulk aqueous solution with inorganic anions of opposite charge following the Hofmeister series. Any interaction with skin might not have a contribution to the effects in such case; the authors ascribed the effects to interactions in solution [22]. Salicylic acid is mostly a neutral organic molecule in the experimental conditions of Ko *et al.* Indeed, salicylic acid is 90% in its neutral acidic form at pH 2, owing to its p*K*a of 2.98; the fraction of neutral from is probably even higher in the mixture of water/ethanol (70/30) that does not correspond to “real life” because of the lower dielectric constant and ion hydration.

The present work addresses absorption of halide ions from concentrated aqueous solutions of single or mixed sodium halides (NaF, NaBr, NaI) into freshly-excised skin while keeping its metabolism in a suitable survival medium [24]. Chloride was excluded from the study because its high endogenous concentration in skin causes analysis issues [5, 25]. Indeed, chloride is the most abundant anion in

the body, especially in extracellular medium; it represents 70% of the whole negative ions [26]. Skin absorption of salts (NaF, NaBr, NaI) in binary and ternary mixtures is studied to disclose the enhancing and decreasing effects of halides on one another's skin absorption. Experimental results were exploited in a {3,2} simplex-lattice design model allowing quantitative statistical analysis.

3 Experimental

3.1 Materials and methods

3.1.1 Chemicals

Sodium fluoride (NaF), sodium bromide (NaBr) and sodium iodide (NaI) were purchased from Fisher Scientific (Illkirch, France). Aqueous solutions of salts were prepared in ultrapure water (resistivity > 18 MΩ·cm at 25°C).

3.1.2 Methods

3.1.2.1 Skin samples preparation

Full-thickness porcine flank skin explants were obtained from five young female pigs (30 ± 1 kg), sacrificed at the École de Chirurgie, University Claude Bernard Lyon 1 (Lyon, France). Freshly excised tissue samples were used immediately (< 1 h) to evaluate skin absorption of anions in aqueous solutions. The bristles were cut with an electrical clipper, and the explants were rinsed in water. The subcutaneous adipose tissue was carefully removed using a scalpel. Skin samples were prepared to the final thickness of 1.35 ± 0.02 mm (Micrometer Mitutoyo) and cut into round sections of 3 cm². Skin integrity was assessed by measuring the Trans Epidermal Water Loss (TEWL) (Tewameter TM210, Monaderm, Monaco). Samples presenting TEWL values larger than 15 $g \cdot h^{-1} \cdot m^{-2}$ after 1 minute measurements were discarded. The average TEWL values of skin samples used for experiments was 9.85 ± 0.50 $g \cdot h^{-1} \cdot m^{-2}$.

3.1.2.2 Permeation and ion distribution in skin layers after 24 h exposure

The permeation study was carried out for 24 h in static Franz diffusion cells. Skin samples were mounted into two-chamber glass cells [27] (exposure area = 2.54 cm²) with *stratum corneum* facing up. Acceptor compartment was filled with 10 mL of salt-free survival medium ensuring skin viability over the duration of the experiment [24]. Mounted Franz cells were placed in a water bath at 37°C under magnetic stirring. Such conditions provided stable temperature of the skin surface of 32°C due to heat loss. After 30 min of stabilisation, the acceptor fluid was entirely replaced and 1 mL of aqueous solution of sodium halides was deposed on the skin surface. Aqueous solutions of NaF, NaBr and NaI alone and in binary mixtures (NaF + NaBr, NaF + NaI, NaBr + NaI) at the concentration of 500 $mmol \cdot L^{-1}$ of each salt alone, 250 $mmol \cdot L^{-1}$ in a binary and 167 $mmol \cdot L^{-1}$ in a ternary mixture were tested.

At the end of the experiment, Franz cells were dismantled and skin layers were separated. *Stratum corneum* (SC) was removed using cyanoacrylate glue (Loctite SuperGlue-3, Henkel) spread on a glass plate, according to the method of cyanoacrylate surface biopsies [28]. Then, the viable epidermis (VE) was separated from the dermis (D) by immersing the sample in water at 60°C for 45 s. The extraction of ions from each skin layer was performed by sonication for 30 min at 60 Hz followed by further extraction with a water/dichloromethane (1:1 v/v) mixture for 17 h. Recovered quantities of ions were evaluated in the donor medium (DM), the acceptor medium (AM) and in the different skin layers: *stratum corneum*, viable epidermis and dermis. The amounts coming from endogenous ions were measured in a control experiment performed in the same conditions as the samples with acceptor medium present but without donor fluid. This background was subtracted from the absorbed amounts of the sample experiments. All measurements were performed on 9 replicates with skin pieces excised from 5 different pigs according to the OECD guidelines [29].

3.1.2.3 Analysis of ions concentrations

Collected samples were analysed using ion chromatography (930 Compact IC Flex, Metrohm, Switzerland) equipped with a chemical suppressor and conductivity detection. Elution solvent was 8 mmol·L^{-1} sodium carbonate (Fisher Scientific, Illkirch, France) in ultrapure water. Metrosep A Supp 5 250/4.0 column with an adequate pre-column at a temperature of 35°C was used for all analyses. Calibration curve was prepared as a ternary mixture of all given salts. It was in the linear range from 0.06 to 2000 µmol·L^{-1} (R^2 = 0.999).

3.1.3 Data reduction

Results were expressed as quantity recovered in µmol·cm^{-2}. The mean value and standard error of the mean (*sem*) were calculated from the n = 9 repetitions of experiment. Q_{abs} corresponds to the absorbed amounts of ions recovered in SC + VE + D + AM.

3.1.3.1 Statistical analysis

Comparison of formulations for their significant differences was performed with a Student *t*-test. Significance level was set at $p < 0.05$.

3.1.3.2 Statistical model for ion mixtures

A mixture design approach was used to investigate the influence of the ionic composition on the skin permeation of each ion through the skin. Such approach allows to regularly screen the entire investigated experimental domain while limiting the number of experiments to be carried out.

The experimental domain corresponds to the full ternary diagram defined by the mole fractions of the three ions F^-, Br^- and I^- ranging from zero to unity, all blends among the ions being possible. This type of experimental domain is called a simplex. Scheffé first introduced in the early 60s the simplex-

lattice designs dedicated to mixtures. These designs allow accommodating a polynomial equation that describes the studied phenomenon over the entire simplex region [30, 31]. A {3,2} simplex-lattice was used here. It consisted of the three vertices and the three binary blends located at the midpoints of the edges of the simplex represented by green dots in Figure 3. According to the Scheffé's design, six aqueous solutions were tested: three single-anion solutions NaF, NaBr and NaI at the concentration of 500 mmol·L^{-1} each, (x_F,x_{Br},x_I) = (1,0,0), (0,1,0) and (0,0,1) respectively, and three binary mixtures made of equal molar amounts of both ions, (x_F,x_{Br},x_I) = (1/2,1/2,0), (1/2,1/2,0) and (0,1/2,1/2), as previously described in the experimental part (Figure 3).

The {3,2} simplex-lattice allows estimating the six coefficients of a {3,2} polynomial equation; i.e. a second-degree model for a three-component system is written as follows:

$$\hat{y} = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \qquad \textit{Eq. 1}$$

where $\hat{y}$ is the predicted response for each observed anion, x_i are the mole fractions of components *i* in the mixture (with $x_F + x_{Br} + x_I = 1$) and the parameters b_ix_i and $b_{ij}x_ix_j$ are respectively the linear and nonlinear mixing terms of the model. Responses can be either measured as absorbed amounts per unit skin area (Q_{abs} for F^-, Br^- and I^-), or as other parameters derived from Q_{abs} such as the ratio of Q_{abs} to ion activity.

An additional experiment corresponding to the centroid of the simplex (ternary mixture of NaF, NaBr and NaI at 167 mmol·L^{-1} each, (x_F,x_{Br},x_I) = (1/3,1/3,1/3), has been added as check point to test the adequacy of the model.

Multiple linear regression calculations, analysis of variance (ANOVA) and statistical analyses were performed with Umetrics MODDE 12.0 software (Umetrics, Umeå, Sweden).

4 Results and discussion

4.1 Experimental results

Skin penetration of ions has long been believed limited and most of available research on the subject considers cations far more often than anions. Previous study by Paweloszek *et al.* [4] initiated the research on cutaneous absorption of halide anions by investigating skin absorption of NaF, NaCl, NaBr and NaI from aqueous solutions at equal mass concentrations. In the present study, molar concentrations were set equal as they are more closely related to thermodynamic parameters of ions such as activity and chemical potential. Also, we decided to exclude chlorides from experiments for the sake of feasibility and reproducibility. Indeed, chloride anions are abundant endogenous ions in skin tissue and the presence of high contents of endogenous molecules cause analytical problems related to distinguishing the endo- and exogenous populations [5, 25].

4.1.1 Effect of ionic concentration

The effect of ionic concentration is the first issue. Indeed, a higher concentration in the donor creates a larger concentration gradient through the skin towards the acceptor. The amount of ions penetrating skin was very low with respect to the amount in the donor solution, so that ion absorption did not significantly deplete the donor; the experiment was under "infinite dose" conditions where the concentration in the donor remained constant. The concentration in the acceptor also increased very little compared to that of the donor. As a consequence, the concentration gradient that drove the passive diffusion was kept constant over the whole experiment duration. Thus, changing the concentration in the donor changes the concentration gradient to the same extent. Under such conditions, the flux of passive diffusion should be proportional to the concentration in the donor, unless other effects are operating. As shown in Figure 1, the total absorbed amount of iodide ions penetrating skin actually did not vary linearly with respect to the concentration. It curved downwards at the highest concentrations. Since the ionic concentrations were fairly high, the activity should be better considered than the concentration. The electrostatic contribution to the activity coefficient, γ, for a 1:1 electrolyte at 32°C is given by the Debye-Hückel equation

$$\ln(\gamma) = -\frac{z^2 e^3 {N_{Av}}^{1/2}}{4\pi\,(\varepsilon_0 \varepsilon\, kT)^{3/2}} \sqrt{\frac{10^3}{2} \sum_{ions} z_i{}^2 C_i} = -1.132\sqrt{C} \qquad \textit{Eq. 2}$$

The relationship was linear when activity was considered instead of the concentration (Figure 1). The same behaviour was observed with fluoride and bromide ions. Since the Debye-Hückel theory only considers electrostatic interactions between ionic species, this linear relationship showed that only non-specific electrostatic interactions were operating in bulk aqueous solution. Possible specific interactions between ions in bulk aqueous solution were negligible and did not contribute to skin absorption. Any non-ideal behaviour of ions adsorption from mixed ionic solutions should come from specific interactions operating inside skin.

As a consequence, it makes sense to consider the ratio of the absorbed amount to ion activity when experiments at different ionic strengths are compared. Similar ratio has been introduced a long time ago as the permeation coefficient k_p defined as the ratio of the permeation flux in steady state regime to the concentration in the donor medium [32]. We define here an absorption coefficient as the ratio of the total absorbed amount after 24 h exposure to the activity in the donor solution as:

$$k_{\mathrm{abs}} = \frac{Q_{\mathrm{abs}}}{\gamma C} \qquad \textit{Eq. 3}$$

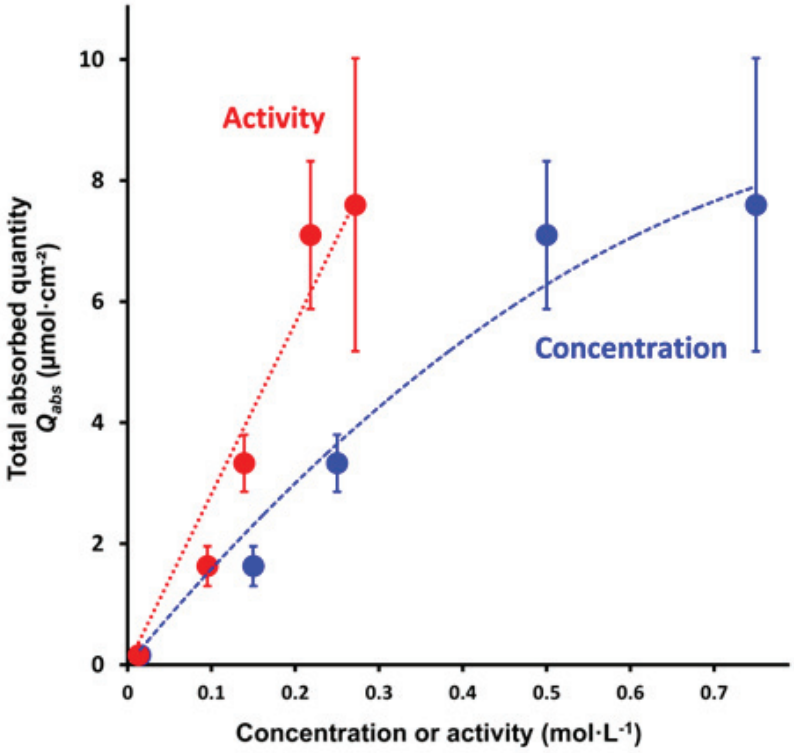

Figure 1 Total absorbed amount of iodide ions (µmol·cm^{-2}) after 24 h exposure to sodium iodide solutions plotted as a function of iodide concentration and iodide activity.

4.1.2 Ion absorption of single and mixed anions

The total absorbed quantity of halide anions tested alone at 500 mM did not differ significantly (Figure 2). The same was observed for concentrations of 250 mM (data not shown). The present results differ from the ones reported previously [4] where a definite increase of skin penetration with respect to the chaotropic character of anions (F^- < Cl^- < Br^- < I^-) was observed. This can be explained by different ionic concentrations of the solutions as Paweloszek *et al.* studied solutions containing the same mass concentration (ppm) for each anion. Given large differences in molar masses of sodium salts of F (38 g·mol^{-1}), Br (80 g·mol^{-1}) and I (127 g·mol^{-1}), it is easy to notice that conversion into molar concentrations makes the variations weaker. Moreover, the survival medium used as the acceptor medium differed between the experiments. Paweloszek *et al.* [4] used ultrapure water supplemented with glucose only, which created a flux of ions towards the hypotonic acceptor solution. The acceptor medium here was replaced by an isotonic composition in the present study, ensuring equilibrium of osmotic pressures between skin cells and acceptor and thus reduced the difference between the donor and acceptor, and longer viability of skin samples [24].

Investigation of skin permeability for anions in binary mixtures revealed significant differences in their absorption depending on the composition. Synergistic effects were observed for mixtures of NaBr and NaI. The values of k_{abs} of both anions were significantly higher in the case of paired salts than alone. These observations agree with Ko *et al.* [23] who studied the effects of addition of NaF, NaCl, NaBr, NaI on skin penetration of salicylic acid. They found that addition of NaBr or NaI to the vaseline-based formulation significantly increased penetration of an organic compound through murine skin *in vitro* and rabbit skin *in vivo* while the addition of NaF and NaCl decreased its absorption. Similarly, we noticed significantly reduced k_{abs} values for NaI when mixed with NaF. Skin

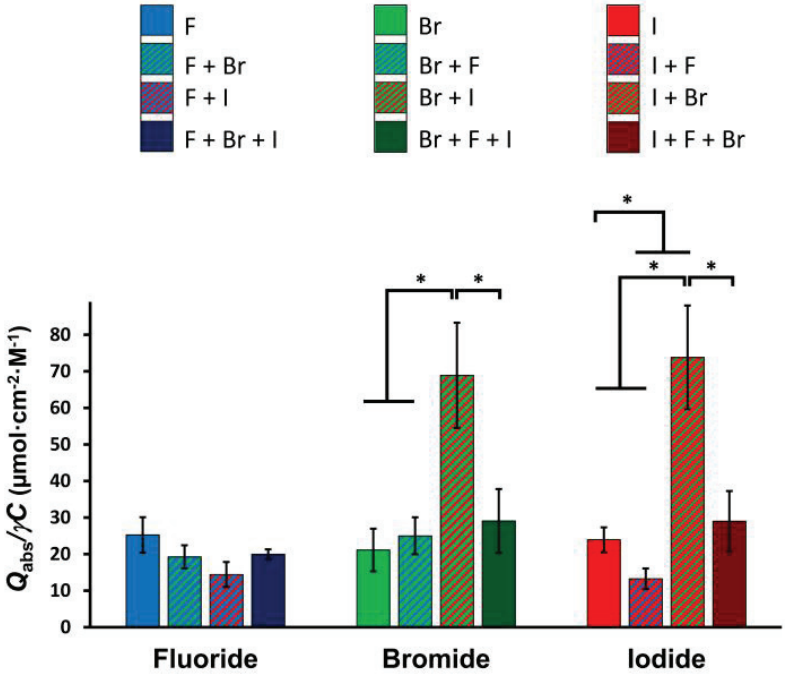

Figure 2 Absorption coefficient $k_{abs} = Q_{abs}$/activity (µmol·cm^{-2}·M^{-1}) after 24 h exposure time for skin exposed to binary and ternary mixtures of halide salts compared single halide salt as control. Data represent *mean ± sem* carried out on *n = 9* individual skin explants taken from 5 different pigs. Asterisks (*) indicate $p < 0.05$ in t-test analysis.

penetration of fluoride was also impaired in the presence of NaBr and NaI.

Ko *et al.* [23] also observed that given salts increased the amount of sterol leached out of the excised skin impairing the initial hydrophobic barrier. This led to conclusion that enhanced penetration of salicylic acid in presence of NaBr or NaI might be due to reduction of the barrier function by disorganizing crystalline lipids of *stratum corneum*, allowing its better swelling. According to Elden [17], lyotropic swelling of collagen also occurs in the order: $F^- < Cl^- < Br^- < SCN^-$. The main presumption from these studies is that penetrating chaotropic ions disorganize the skin barrier and

make it more permeable to penetrating molecules, either ionic or nonionic. There is no definite proof of such phenomenon, however. The present study goes deeper in the mechanisms by considering the distribution of anions in skin layers: *stratum corneum*, viable epidermis, dermis, and acceptor medium (SC, VE, D and AM). Indeed, anion distribution in skin layers after 24 h (Figure 3) was also altered depending on the composition of tested solution. Applying Br^- and I^- simultaneously led to increased recovery in AM as compared to Br^- and I^- alone. This was associated with reduced values obtained for D, VE and especially SC indicating lower tendency for accumulation within the tissues and higher permeability of tested tissue without accumulation. These observations contradict the postulated idea of a disorganization of skin structure by the presence of absorbed anions. Indeed, changing the properties of skin layers would require accumulation of ions inside them.

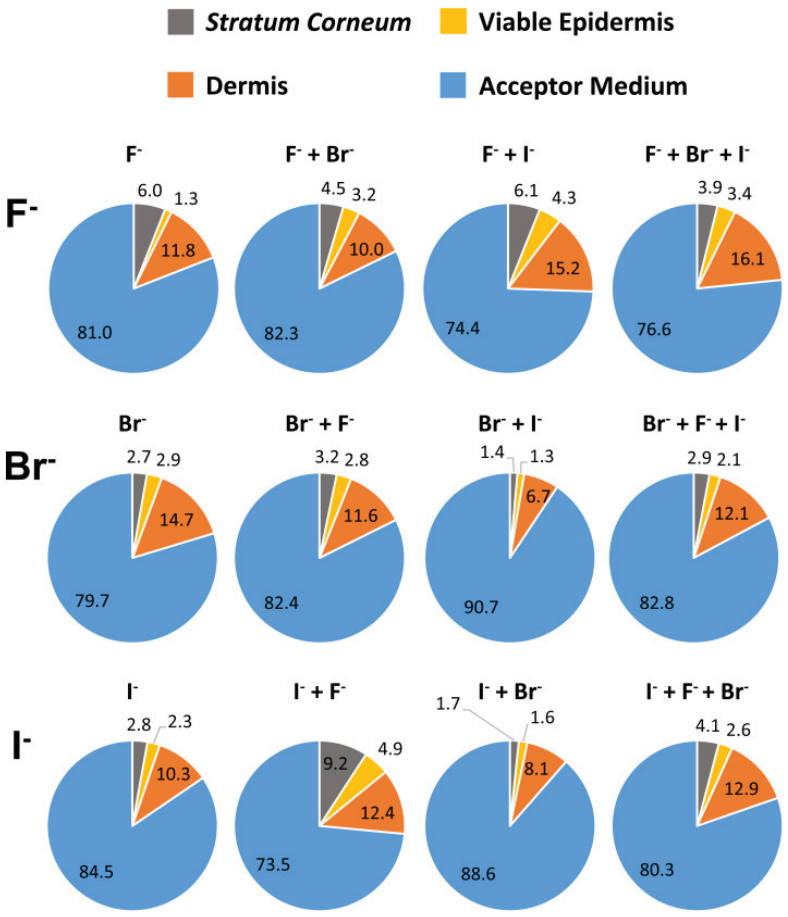

Figure 3 Distribution of halide anions in skin layers after 24 h exposure. Data represent mean value of absorbed amount expressed as a percentage of Q_{abs}. Results are means of n = 9 independent measurements.

On the other hand, the trends are reversed for iodide distribution in the presence of NaF: AM recovery slightly decreased for mixed ions and absorbed amount in SC increased. This effect was less pronounced in the case of NaF + NaBr.

The observed effects are quite complex as they result from several contributions: interactions between ions in the bulk donor solution, adsorption of ions to the skin surface, transfer into *stratum corneum* and passive diffusion inside it, facilitated transport in the viable epidermis with the help of membrane proteins in keratinocytes (ion channels, ion transporters).

Interactions in bulk solutions are non-specific since they can be accounted for by the Debye-Hückel activity coefficient. Facilitated transport in viable skin is predominant as Paweloszek *et al.* disclosed a large difference of skin absorption between viable and non-viable skin [5]. Passive diffusion through *stratum corneum* is necessary for ions to reach viable epidermis where facilitated transport operates. So that most elementary steps for ion absorption are interdependent. In addition, the majority of transport phenomena depend on ion type in the same way according to Hofmeister series. The best strategy for addressing such a complexity is a global approach undertaken here using an experimental design.

4.2 Statistical Modelling

Another way to exploit experimental results is fitting them to statistical models. The benefit input of such data analysis is a rigorous statistical framework. The outcome is a quantitative parametric assessment of non-ideal behaviour through the coefficients of the Scheffé {3,2} polynomial equation (Eq. 1). The $\hat{y}$-responses are either absorbed amounts Q_{abs} or absorption coefficients $k_{abs} = Q_{abs}/\gamma C$. They must fully fill a matrix containing absorption data of ions. Some of them are absorption data of ions that are not present in the solution of mixed ions. As example, a binary mixture of fluoride and bromide does not contain iodide, but the matrix for iodide absorption data also contains information on iodide absorption from a mixed solution of fluoride and bromide. This is mandatory to fully fill the $\hat{y}$-response matrix in the mathematical framework of Scheffé simplex-lattice design. This is easy to manage when Q_{abs} is considered as a response: Q_{abs} is zero when the anion is not present. When responses are absorption coefficients in the present analysis, the correct values are $\lim_{C\to 0}\left(\frac{Q_{abs}}{\gamma C}\right)$. They were obtained by extrapolation from experiments at several concentrations. Although those concentrations were fairly high (> 100 mM) for ensuring enough experimental accuracy, extrapolation to infinite dilution was quite robust, owing to the almost linear dependence of Q_{abs} with respect to C in lower concentration range. The approach was first to determine the coefficients of the polynomial using the six experiments of the {3,2} simplex-lattice design for the skin absorption of each anion. Since there are mixtures where some ions are absent, their coefficient in the

polynomial is undetermined, and it has been removed from the polynomial model. As example, because there is no iodide in a binary mixture of fluoride and bromide, the term containing the coefficient b_{FBr} was removed from the polynomial pertaining to Q_{abs} or k_{abs} of iodide. The modified Eq. 1 for Q_{abs} and k_{abs} became the set of Eqs 4 to 9:

$$Q_{abs}(F^-) = a_F x_F + a_{Br} x_{Br} + a_I x_I + a_{FBr} x_F x_{Br} + a_{FI} x_F x_I \quad \textit{Eq. 4}$$

$$Q_{abs}(Br^-) = a_F x_F + a_{Br} x_{Br} + a_I x_I + a_{FBr} x_F x_{Br} + a_{BrI} x_{Br} x_I \quad \textit{Eq. 5}$$

$$Q_{abs}(I^-) = a_F x_F + a_{Br} x_{Br} + a_I x_I + a_{FI} x_F x_I + a_{BrI} x_{Br} x_I \quad \textit{Eq. 6}$$

$$k_{abs}(F^-) = b_F x_F + b_{Br} x_{Br} + b_I x_I + b_{FBr} x_F x_{Br} + b_{FI} x_F x_I \quad \textit{Eq. 7}$$

$$k_{abs}(Br^-) = b_F x_F + b_{Br} x_{Br} + b_I x_I + b_{FBr} x_F x_{Br} + b_{BrI} x_{Br} x_I \quad \textit{Eq. 8}$$

$$k_{abs}(I^-) = b_F x_F + b_{Br} x_{Br} + b_I x_I + b_{FI} x_F x_I + b_{BrI} x_{Br} x_I \quad \textit{Eq. 9}$$

Qabs was first analysed because this was the actually measured parameter during experiments. The models for Qabs (Eqs 4, 5 and 6) were first used to predict the amounts of absorbed ions for the centroid experiment (ternary mixture) as a validation tools of the model: residuals of 0.5,-1.5 and -1.3 µmol·cm-2 respectively, obtained for Qabs(F-), Qabs(Br-) and Qabs(I-) were considered satisfactory compared to the experimental standard deviation. The centroid experiment was then used in combination with the 6 experiments of the simplex-lattice design to refit the models to the full experimental data. The best statistical coefficients of the models are presented in Table I. For each model, ANOVA analysis indicated the high significance of the fits ($p < 0.05$); and the corresponding determination coefficient R^2 greater than 0.97 (Table I) also showed their satisfactory adequacy.

Table I Coefficients a_{ij} (µmol·cm^{-2}) of the {3,2} polynomial models of Q_{abs} (Eqs 4, 5 and 6) together with the corresponding determination coefficient R^2, standard deviation (*SD*) and *p*-value of the ANOVA test between brackets. All values are given with two significant digits with no consideration to their statistical significance.

Ions (i,j)	$Q_{abs}(F^-)$ a_{ij} (*SD*, *p*)	$Q_{abs}(Br^-)$ a_{ij} (*SD*, *p*)	$Q_{abs}(I^-)$ a_{ij} (*SD*, *p*)
F^-	**6.26** (0.3, 0.001)	**-0.08** (0.9, 0.93)	**-0.07** (0.7, 0.93)
Br^-	**0.03** (0.3, 0.93)	**7.80** (0.9, 0.01)	**-0.07** (0.7, 0.93)
I^-	**0.03** (0.3, 0.93)	**-0.08** (0.9, 0.93)	**7.20** (0.78, 0.01)
F^-/Br^-	**-3.53** (1.4, 0.13)	**-6.50** (4.3, 0.27)	-
F^-/I^-	**-5.65** (1.4, 0.06)	-	**-10.1** (3.6, 0.11)
Br^-/I^-	-	**12.7** (4.3, 0.10)	**16.3** (3.6, 0.05)
R^2	**0.994**	**0.975**	**0.983**

The predictions of the models are presented on a classical ternary diagram in an equilateral triangle in which sides are axes for mole fractions of ions (Figure 4) and the colour scale stands for Q_{abs}.

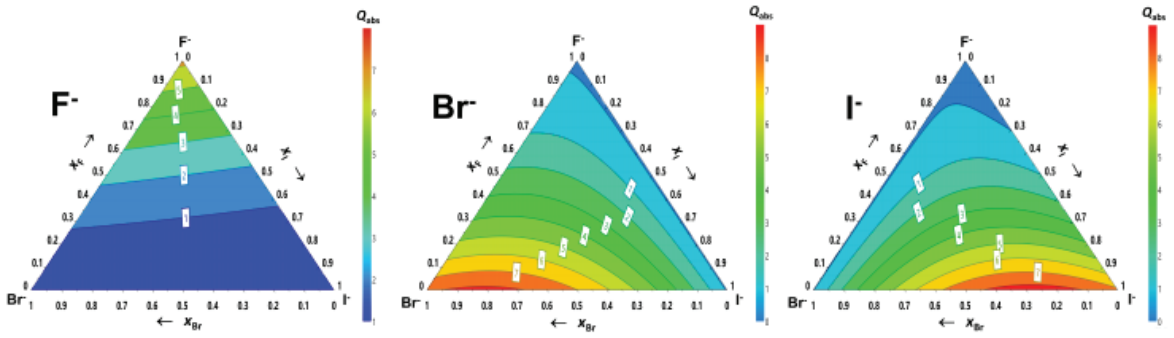

Figure 4 Contour plots of the predicted absorbed amounts (Q_{abs}, µmol·cm^{-2}) of F-, Br- and I- ions after 24 h exposure in the composition space of ionic mixtures expressed in mole fractions.

The variation of Q_{abs} of F^- in Figure 4 shows a continuous increase of Q_{abs} as the mole fraction of fluoride increases, which is the obvious consequence of the increase of the fluoride concentration in the donor solution. The iso-Q_{abs} lines are almost horizontal, showing that the effects of bromide and iodide ions are identical. As a whole, there is no specific effect of mixed anions. Conversely, absorptions of bromide and iodide anions show a maximum in the presence of the other whereas there is no apparent effect of fluoride. The statistical analysis disclosed a specific interaction between bromide and iodide that caused enhanced skin absorption of these anions. The maxima of absorption occur when the solution is rich in the observed anion because of the obvious effect of the higher concentration of observed anion as the corner of pure anion is approached. This analysis considering Q_{abs} includes contributions of the concentration of observed anions and specific interactions with their partners. A quantitative approach is to consider the a_{ij} coefficients (Table I) that have statistical significance. The a_F, a_{Br} and a_I coefficients of the same anions (e.g. a_F for Q_{abs} of F^-) are highly significant ($p \leq 0.01$) and positive. They reflect the obvious effects of increased absorption as a function of concentration. The a_F, a_{Br} and a_I coefficients of different anions (e.g. a_F for Q_{abs} of Br^-) are of poor significance (*SD* is larger than the coefficient value and $p = 0.93$). Their value close to zero would mean an absence of interaction; but these values are statistically not significant. a_{FBr}, and a_{FI} negative coefficients are also not significant owing the large *SD* and *p*-value. The striking feature is the large and significant positive values of a_{BrI}, showing a specific interaction between bromide and iodide anions.

The global statistical analysis of skin absorption of mixed anions disclosed a specific interaction between them, but it does not allow neither inferring the nature of the interactions, nor defining the

location of them in the complex layered structure of skin (donor solution, *stratum corneum*, viable epidermis and dermis). A refined analysis was considering the absorption coefficient $k_{abs} = Q_{abs}/\gamma C$ in the place of Q_{abs}. This suppresses the effects of the concentration of observed anion and electrostatic interactions in the donor solution through the Debye-Hückel activity coefficient. The same statistical analysis as for Q_{abs} was made. Results are given in Table II and Figure 5.

Table II Coefficients b_{ij} (µmol·cm^{-2}·M^{-1}) of the {3,2} polynomial models of k_{abs} (Eqs 7, 8 and 9) together with the corresponding determination coefficient R^2, standard deviation (*SD*) and *p*-value of the ANOVA test between brackets. All values are given with two significant digits with no consideration to their statistical significance.

Ions (i,j)	$k_{abs}(F^-)$ b_{ij} (*SD*, *p*)	$k_{abs}(Br^-)$ b_{ij} (*SD*, *p*)	$k_{abs}(I^-)$ b_{ij} (*SD*, *p*)
F^-	**26** (2.7, 0.011)	**28** (8.2, 0.075)	**28** (6.8, 0.053)
Br^-	**20** (2.5, 0.015)	**36** (9.0, 0.056)	**28** (6.8, 0.053)
I^-	**20** (2.5, 0.015)	**28** (8.2, 0.075)	**33** (7.4, 0.046)
F^-/Br^-	**-19** (12, 0.26)	**-48** (41, 0.36)	-
F^-/I^-	**-28** (12, 0.15)	-	**-86** (34, 0.13)
Br^-/I^-	-	**127** (41, 0.091)	**156** (34, 0.044)
R^2	**0.81**	**0.88**	**0.95**

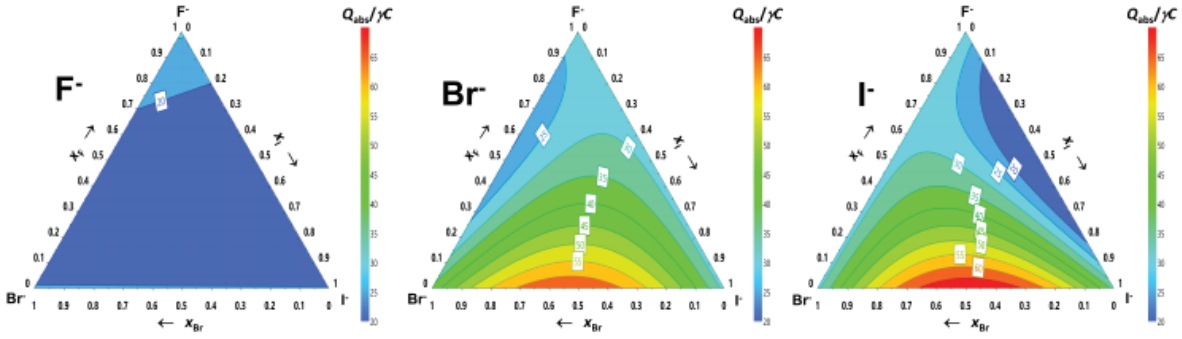

Figure 5 Contour plots of the predicted absorption coefficients (k_{abs}, µmol·cm^{-2}·M^{-1}) of F^-, Br^- and I^- ions after 24 h exposure in the composition space of ionic mixtures expressed in mole fractions.

Substitution k_{abs} for Q_{abs} eliminates the effects of ion concentrations in a similar way to a background flattening process in image analysis. The general consequence is a loss of statistical significance of all b_{ij} coefficients (see R^2 values in Table II). But experimental data has been cleaned from the effects of ion concentration and non-specific electrostatic interactions, so that the sole effects of specific interactions remain. The map for fluoride appears flat with a constant value of k_{abs}= 20 µmol·cm^{-2}·M^{-1}. This feature shows the overall absence of specific interactions with fluoride. Conversely again, the

k_{abs} maps of bromide and iodide anions show a maximum at the centre of the bottom side. Substitution of k_{abs} for Q_{abs} shifted the location of the absorption maximum at the bottom side of the triangle from close to the vertex of adsorbed ion to the mid-point of the Br-I axis, (x_F,x_{Br},x_I) = (0,1/2,1/2). Such symmetrical position suggests a 1:1 stoichiometry of specific interaction between bromide and iodide anions that could not be detected in the raw Q_{abs} map. Many of the b_{ij} coefficients (Table II) have poor statistical significance. The b_F, b_{Br} and b_I coefficients are of high significance ($p < 0.05$). Though the usual maximum p-value for claiming significance is $p < 0.05$, this constraint was released to $p < 0.1$ in the present case. Under this condition, b_{BrI} assumes a high positive value of medium significance, and the negative b_{FBr} and b_{FI} coefficients are not significant. A negative b_{ij} coefficient would mean that the simultaneous presence of two ions has a lesser influence than that of one ion alone. The origin would be interactions between these two ions that decrease their interactions with a third one. However, the negative b_{ij} coefficients have a low statistical significance, so that the reality of these cross-effects remains purely speculative. Though the values of b_{FBr} and b_{FI} did not have statistical significance, they assumed large negative values that may give a contribution to the statistical analysis. Thus, it is clear that skin absorption of fluoride was not influenced by the presence of other ions, so that the positive values of b_F contradicted the overall features of the F^- map of k_{abs}. It was suspected that the effects of the positive values of b_F and the negative values of b_{FBr} and b_{FI} compensated such that the balance is null. Because of the lack of statistical significance of b_{FBr} and b_{FI}, it may be considered that the positive values of b_F are a bias coming from a too large variability of skin absorption measurements. Such variability is intrinsic of experiments performed on skin and living matter in general.

Refinement of the analysis by considering k_{abs} may allow discrimination between the ion effects coming from alteration of the medium (ionic strength, dielectric properties…) through the b_F, b_{Br} and b_I coefficients, and from specific interactions through the b_{FBr}, b_{FI} and b_{BrI} coefficients. This was partly successful and led to supplementary conclusions:

- The k_{abs} map of F^- shows that the positive values of b_F, b_{Br} and b_I being all of the same order of magnitude (20 to 30 $\mu mol \cdot cm^{-2} \cdot M^{-1}$) cannot be considered as a non-specific effect of the presence of other ions inside skin.

- The high positive value of b_{BrI} indicates a strong specific interaction between bromide and iodide.

A specific interaction operating between bromide and iodide was already inferred from the raw experimental data. The statistical analysis showed that this was the sole significant specific interaction.

4.3 Discussion

The location of disclosed interactions is difficult to discuss in details despite the distribution of ions stored in the different skin layers has been measured (Figure 3). As the main feature, very few figures show significant differences. The main contributions to differences of total absorbed amount Q_{abs} come from the amounts that reached the acceptor medium. The very small differences in SC suggest that SC is just a passive diffusion barrier where permeation is only driven by the concentration gradient. There is no accumulation of ions inside SC, showing that there is no specific binding of anions to materials of SC. There are also very few significant differences of ion amounts in viable epidermis and dermis; but it is known that these skin layers are not passive diffusion barriers and that they contain ion transporters contributing a lot to the overall absorption [4]. The absorbed amounts per unit skin area can be converted into concentrations in mmol·L^{-1} taking the thickness of SC, VE and D as 20 µm, 50 µm and 1.25 mm. There is a smooth concentration gradient along skin depth in all cases (Figure 6). One consequence is that concentrations in different layers are interdependent. There is no indication of accumulation coming from specific interactions with skin materials. Ion distributions within skin layers do not indicate SC or VE being ion-specific barrier because of their lack of significant differences.

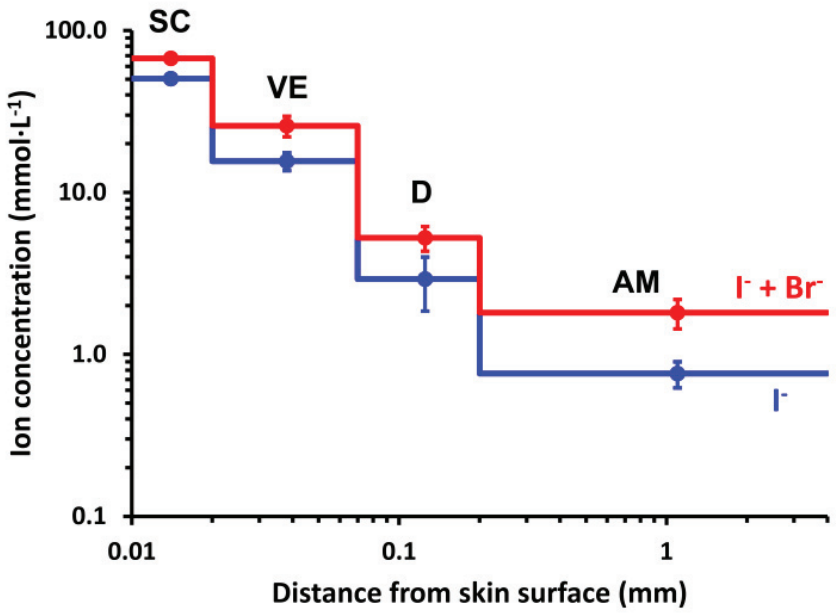

Figure 6 Concentration profile of iodide ions inside skin layers (mmol·L^{-1}) as a function of depth showing the concentration gradient from the surface to the acceptor medium. Concentrations are taken homogeneous in each skin layer, *Stratum corneum* (SC), Viable epidermis (VE), Dermis (D) and Acceptor medium (AM).

The nature of interactions operating in skin absorption experiments cannot be inferred from the present data that remains a global approach. Non-specific electrostatic interactions are present in the concentrated donor solution; they have been accounted for in the Debye-Hückel activity coefficient. Ions absorption cannot contribute to electrostatic effects inside skin because the concentrations of penetrating ions inside skin layers were low with respect to the concentrations of endogenous ions [5]. As example taking the thickness of viable epidermis as 50 µm and that of dermis as 1.3 mm, the mean concentration of endogenous chloride in viable epidermis and dermis are respectively 200 $mmol \cdot L^{-1}$ and 15 $mmol \cdot L^{-1}$; the concentrations of sodium are three times larger and those of potassium are also larger than those of chloride [5]. All presently measured anions concentrations were less than 50 $mmol \cdot L^{-1}$ in viable epidermis and less than 10 $mmol \cdot L^{-1}$ in dermis.

A specific interaction between bromide and iodide ions should be considered. These two anions are chaotropic (hydrophobic) in the Hofmeister series of halides. This property comes from their large size and high polarizability that allow for their association by strong attractive dispersion interactions. Their poor hydration is also a parameter that contributes to their ability to association. Hydration of ions [33, 34] and dispersion interactions [35] are the two main rationalizing factors for the Hofmeister series. As outlined early by Fajans [36], both phenomena are interrelated as they originate from the size and polarizability of ions [37–39]. Skin absorption of pure halides did not exactly follow the Hofmeister series. The reason for this, presumably, is the complexity of penetration paths into skin that some contribution are: passive diffusion through *stratum corneum*, facilitated transport by ion channels and proteins in viable epidermis [4], diffusion in the gradients of ion concentration and osmotic pressure in dermis. As both Hofmeister and anti-Hofmeister behaviours have been observed [40, 41], complexity may lead to compensation of effects and final reversal of the global balance. Bromide and iodide are chaotropic and this property allows for dispersive attractions between them. Electrostatic repulsions act against association of Br^- and I^- anions, so that a cation is necessarily in the association. A possible complex species may result from the association of Br^-, I^- and Na^+ ions as the $NaBrI^-$ monovalent anion. Another option is the association with endogenous magnesium or calcium cations as the neutral MgBrI and CaBrI species. Indeed, magnesium and calcium are abundant endogenous ions inside skin, with the presence of a Ca^{2+} gradient from the *stratum basale* to the *stratum granulosum* where it peaks before declining in *stratum corneum* [42]. On the basis of size and polarizability of ionic partners, Mg^{2+}, Ca^{2+}, Br^- and I^- [37–39], such complex association with magnesium or calcium looks quite a likely mechanism for anions to pass through the *stratum corneum*. The same effect might occur with pure Br^- or I^- ions. The stronger effect with mixed ions probably comes from entropic stabilization of the hetero-association with respect to homo-association.

5 Conclusions

Skin absorption of mixed halide ions shows synergistic effects between hydrophobic bromide and iodide anions. This has been clearly disclosed by a statistical analysis of absorption experiments on excised pig skin mounted in diffusion Franz cells. Statistical analysis allowed identification of interacting anions pairs from skin absorption experiments that show a large variability. Considering the ratio of the absorbed amounts to the ion activity discards the obvious contribution of ion concentration and the non-specific effects of electrostatic interactions in the aqueous donor medium; this highlights the effects of specific interactions.

It has been inferred that polarizable hydrophobic anions associate in the *stratum corneum* as complex species together with cations, in particular with magnesium and calcium cations, in order to counterbalance their electrostatic repulsions.

The present results together with our previous work [4], definitely shows that ionic species do penetrate skin although it was believed for a long time that such very hydrophilic species could pass through the hydrophobic barrier of *stratum corneum*. Practical ionic solutions such as thermal spring waters used in cosmetic formulations are often complex mixtures where deviations from ideal behaviour should be taken in consideration.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Skin absorption of thyroid-disrupting anions (I^-, SCN^-, $ClO4^-$) from concentrated aqueous solutions. Effects of mixing.

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6 Introduction

Thyroid is an endocrine gland whose main role is to produce triiodothyronine (T_3) and thyroxine (T_4). Iodide ions are required for synthesis of these hormones and are transported from the bloodstream to the thyroid by means of sodium/iodide symporter (NIS) [43].

Thyroid hormones simulate metabolism and regulate growth and development of the organism. Therefore, the disruption of their normal production leads to severe health issues that can be generally classified in two categories: hyperthyroidism associated with overproduction of T_3 and T_4 and hypothyroidism characterised by subphysiological excretion of thyroid hormones. Hyperthyroidism can be caused by gland inflammation, autoimmunological (Graves') disease or cancer. Its treatment involves anti-thyroid medications and radioactive iodine to slow the production of thyroid hormones. On the other hand, among the possible causes of insufficient excretion of thyroid hormones congenital disease, iodide deficiency, pituitary disorder can be named. Treatment of these conditions requires substitutional treatment [44].

Recently, the impact of endocrine disrupting chemicals (EDCs) on the development of thyroid diseases is widely discussed. EDCs are exogenous molecules that interfere with the normal hormone homeostasis in the human body, commonly considered to be compounds that are agonists or antagonists of physiologically circulating hormones [45, 46].

Ions that could interact with NIS, especially perchlorate and thiocyanate, are of our particular interest within this work. In fact, NIS is able to transport a variety of monovalent anions with the specific order of affinities: ClO_4^- >> ReO_4^->> I^- ≥ SCN^- > ClO_3^- >> Br^- [47]. Both thiocyanate and perchlorate are competitive inhibitors of iodide uptake and thus can be considered as thyroid disruptors.

These anions are naturally present in the environment in low concentrations. Although thiocyanate is ubiquitous in the environment, mainly in animals' body fluids as the non-toxic metabolite of cyanide produced by plants [48], its main source for contamination for general population is cigarette smoke [49]. Moreover, cyanides and thiocyanates are formed or used in several industrial processes, for example gold mining, production of coke in steel factories, herbicide and insecticide production, dyeing, photofinishing, acrylic fibre production, etc. [50].

Naturally occurring perchlorate is very rare and can be formed in the atmospheric processes. It has been detected as a contamination of Chilean Nitrate, historically used as fertilizer [51]. A great majority of perchlorate is of the anthropologic origin. Owing its oxidizing properties, it has been used in the production of rocket fuel, firework, flares or airbags [52].

Radioactive iodide has several biomedical applications that depend on the radioisotope but can also be considered as EDC. ^{123}I is used as a medical tracer in nuclear imaging while ^{131}I is applied in treatment of hyperthyroidism and in higher doses in thyroid cancer therapy [53, 54]. Accidental exposure to this isotope could lead to the development of cancer [50].

Pregnant and lactating women with adverse effects observed in the offspring, and adolescents have been identified as the most thyroid-disrupting sensitive subpopulations reflecting the time periods of extensive growth and development of the organism [55–57].

General population is exposed to NIS inhibitors that are administered by digestion (contaminated water or food) or by inhalation (cigarette smoke). The same routes of entry can be named for the radioactive iodide in case of a nuclear accident. However, the occupational exposure for people working with NIS inhibitors mentioned here may include topical exposure.

Recent research showed that skin absorption of both cations and anions is possible [4, 58, 59]. Our previous studies demonstrated that up to 80% of halide ions passage across the skin occurs thanks to facilitated transport [5]. Sodium-iodide symporter, apart from chloride channels, is suspected to participate in this transport as its mRNA has been detected in skin samples [20]. We have also reported that skin absorption of anions through viable skin is proportional to the ion activity within high concentration range. Finally, a synergy of equimolar mixtures of bromide and iodide, two fairly chaotropic halide ions, in skin absorption experiments has been reported [60].

This paper continues our previous work and focuses on investigating skin penetration of chaotropic anions that can interact with NIS (I^-, SCN^-, ClO_4^-) from concentrated solutions of sodium salts. The effects of mixing of the abovementioned salts in binary and ternary systems are studied.

7 Materials and methods

7.1 Chemicals

Sodium iodide (NaI), sodium thiocyanate (NaSCN) and sodium perchlorate ($NaClO_4$) were purchased from Fisher Scientific (Illkirch, France). Aqueous solutions of salts were prepared in ultrapure water (resistivity > 18 MΩ·cm at 25°C).

7.2 Methods

7.2.1 Skin samples preparation

Full-thickness porcine flank skin explants were obtained from five young female pigs (30 ± 1 kg), sacrificed at the École de Chirurgie, University Claude Bernard Lyon 1 (Lyon, France). Freshly excised tissue samples were used immediately (< 1 h) to evaluate skin absorption of anions from aqueous

solutions. The bristles were cut with an electrical clipper, and the explants were rinsed in water. The subcutaneous adipose tissue was carefully removed using a scalpel. Skin samples were prepared to the final thickness of 1.31 ± 0.02 mm (Micrometer Mitutoyo) and cut into round sections of 3 cm². Skin integrity was assessed by measuring the Trans Epidermal Water Loss (TEWL) (Tewameter TM210, Monaderm, Monaco). Samples presenting TEWL values larger than 15 $g \cdot h^{-1} \cdot m^{-2}$ after 1 min measurements were discarded. The average TEWL values of skin samples used for experiments was 9.85 ± 0.50 $g \cdot h^{-1} \cdot m^{-2}$.

7.2.2 Permeation and ion distribution in skin layers after 24 h exposure

The permeation study was carried out for 24 h in static Franz diffusion cells according to OECD guidelines [29]. Skin samples were mounted into two-chamber glass cells (exposure area = 2.54 cm²) with *stratum corneum* facing up. Acceptor compartment was filled with 10 mL of salt-free survival medium ensuring skin viability over the duration of the experiment [24]. Mounted Franz cells were placed in a water bath at 37°C under magnetic stirring. Such conditions provided a temperature of the skin surface of 32°C due to heat loss. After 30 min of stabilisation, the acceptor fluid was entirely replaced and 1 mL of aqueous solution of sodium halides was deposed on the skin surface. Aqueous solutions of NaI, NaSCN and $NaClO_4$ alone, in binary (NaI + NaSCN, NaI + $NaClO_4$, NaSCN + $NaClO_4$), and ternary (NaI + NaSCN + $NaClO_4$) mixtures at the concentration of 250 $mmol \cdot L^{-1}$ of each salt were tested. At the end of the experiment, Franz cells were dismantled and skin layers were separated. *Stratum corneum* (SC) was removed using cyanoacrylate glue (Loctite SuperGlue-3, Henkel) spread on a glass plate, according to the method of cyanoacrylate surface biopsies [28]. Then, the viable epidermis (VE) was separated from the dermis (D) by immersing the sample in water at 60°C for 45 s. The extraction of ions from each skin layer was performed by sonication for 30 min at 60 Hz followed by further extraction with a water/dichloromethane (1:1 v/v) mixture for 17 h. Recovered quantities of ions were evaluated in the donor medium (DM), the acceptor medium (AM) and the different skin layers: *stratum corneum*, viable epidermis and dermis. All measurements were performed on 9 replicates with skin pieces excised from 5 different pigs according to the OECD guidelines [29].

7.2.3 Analysis of ion concentrations

Collected samples were analysed using ion chromatography (930 Compact IC Flex, Metrohm, Switzerland) equipped with a chemical suppressor and conductivity detection. Elution solvent was 8 $mmol \cdot L^{-1}$ sodium carbonate (Fisher Scientific, Illkirch, France) in ultrapure water. Metrosep A Supp 5 250/4.0 column with an adequate pre-column at a temperature of 68°C was used for all analyses. Calibration curve was prepared as a ternary mixture of all given salts. It was in the linear range from

0.06 to 2000 µmol·L^{-1} (R^2 = 0.999). The limits of detection (LOD) and quantification (LOQ) were 0.06 and 0.02 µmol·L^{-1}, respectively.

7.2.4 Data analysis

Results were expressed as the mean and the standard error of the mean (*sem*) from n ≥ 9 repetitions of the experiment as concentration in µmol·cm^{-2}; Q_{abs} values correspond to the absorbed amount of ions recovered in SC + VE + D + AM.

8 Results and discussion

Skin absorption of ions has long been believed limited because of their hydrophilic character. Such highly hydrated species of low molecular weight were considered unable to cross very lipophilic *stratum corneum*. So far, skin absorption and interactions with cations have received more attention than that of anions. Various biological activities, both beneficial and harmful for health that have been reported for cations are responsible for this disproportion [61–63]. Previous research dedicated to skin absorption of halide ions (F^-, Cl^-, Br^- and I^-) disclosed significant (up to 80%) contribution of facilitated transport of these anions through viable skin [5]. Additionally, we reported that skin absorption of anions from highly concentrated aqueous solutions is proportional to the activity of the anion and not, as suggested before, to its concentration. Also, a strong synergy was observed between more hydrophobic anions: bromide and iodide [60]. Present study is the first one to investigate skin absorption of thiocyanate and perchlorate. Because both anions are inhibitors of NIS, whose mRNA has been detected in the skin samples [20], iodide was also included in the study. The concentrations of salts applied here were of the same range as in our previous work and similarly, the effects of mixing the salts were studied [60]. The results of total absorbed quantities of anions are shown in Figure 7. The data is represented as an absorption coefficient being the ratio of the total absorbed amount after 24 h exposure to the activity in the donor solution ($k_{abs} = Q_{abs}/\gamma c$), as reported in our previous work [60]. Such a correction allowed direct comparison of data obtained for different tested conditions despite varying ionic strength of investigated solutions. It could be noticed that thiocyanate had the significantly lower penetration rate as an individual anion when compared to iodide and perchlorate. Its skin absorption was not affected by mixing in either of binary systems, but was higher in the ternary mixture (Fig. 7). The reverse behaviour can be noticed for perchlorate. Its k_{abs} was the highest for an individual anion while incorporating it in the binary mixtures negatively affected its skin absorption rates. When tested in the ternary system, its penetration tended to be lower than for ClO_4^- alone and higher than that of binary systems. However, these differences are not statistically significant. Similarly, iodide absorption tended to be lower in binary systems and equal in ternary mixture as compared to I^- tested alone.

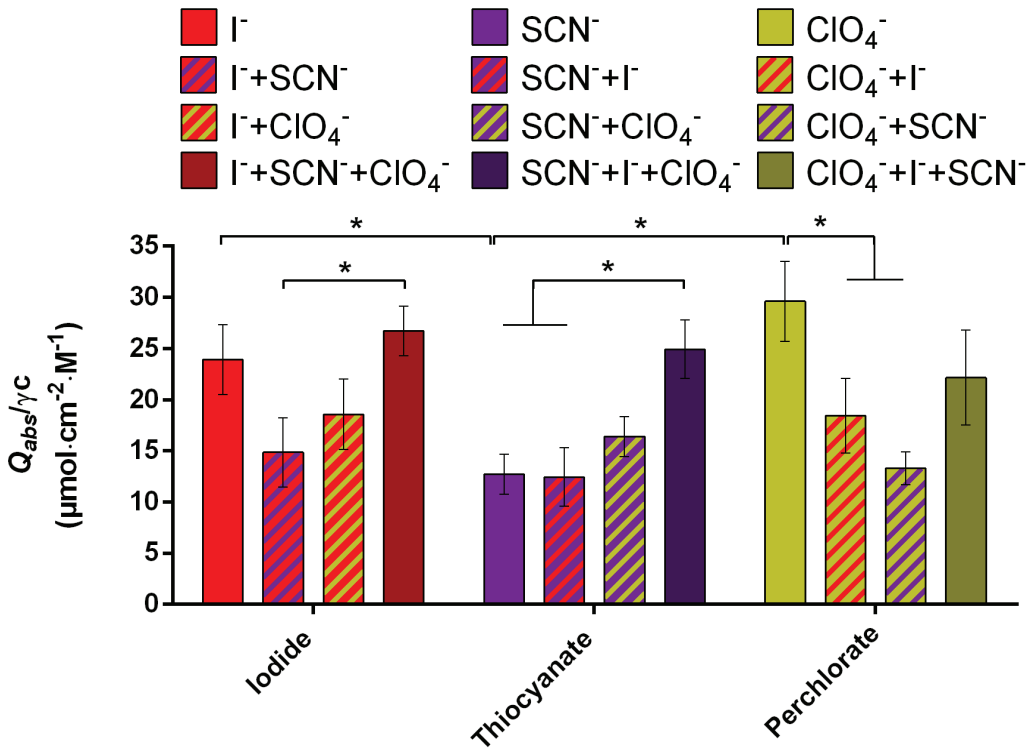

Figure 7 Absorption coefficient $k_{abs} = Q_{abs}/activity$ (µmol·cm^{-2}·M^{-1}) after 24 h exposure time for skin exposed to binary and ternary mixtures of salts compared to single salt as control. Data represent *mean ± sem* carried out on *n = 9* individual skin explants taken from 5 different pigs. Asterisks (*) indicate $p < 0.05$ in t-test analysis.

In none of our studies investigating skin absorption of Hofmeister, the characteristic order of interactions was preserved. This is probably a result of multiple interactions occurring at different skin layers, as described below. Based on the previous results demonstrating strong synergy in skin penetration of halide ions occurring between bromide and iodide, similar effects could have been expected for even more chaotropic anions tested here.

The distribution of ions into the skin layers and acceptor medium after 24 h exposure is shown in Figure 8. Very few statistically significant differences occurred within *stratum corneum*, which is the outermost unselective skin barrier. However, for thiocyanate significantly higher retention from the ternary mixture as compared to other conditions was observed. These differences were maintained in the viable epidermis, where the same could be noticed for iodide and to some extent for perchlorate, however for the latter the trend is not statistically significant. Interestingly, the recovered amounts of ClO_4^- and SCN^- from a binary mixture of these two anions are lower as compared to the other experimental conditions. In dermis, the differences observed in other skin layers for SCN^- are maintained. Moreover, the absorption of thiocyanate applied as a single anion is lower than that of iodide. The values recovered from AM as well as the differences observed in that fraction reflect quite accurately the observations made for the Q_{abs}. Indeed, the absorbed amounts of

anions into AM correlate directly with the Q_{abs} values accounting for over 75% of total absorbed quantity. It is worth noticing that ClO_4^- when applied in the ternary mixture, tends to be retained within the upper skin layers which affects its permeation to AM where the highest perchlorate recovery was obtained for the anion applied alone.

The differences in absorbed quantities of tested anions recovered from the skin layers and AM are due to the character of tested molecules as well as the properties of each of the skin layers. *Stratum corneum* being a lipidic barrier is not expected to be selective. The differences observed here can occur as a result of interactions of tested anions in the aqueous solution. The anions tested here are, according to the Hofmeister series, weakly hydrated. Such "sticky ions" can potentially interact with each other in an aqueous medium especially in high concentrations [64]. In such a way, a creation of more complex species by association of anions among each other or with the endogenous cations (Ca^{2+} and Mg^{2+}), as we had suggested before is a possible scenario [60]. As a consequence, the passage through *stratum corneum*, the lipophilic barrier of the skin could be altered depending on the composition of the complexes.

The nature of interactions changes in viable epidermis that is the first hydrophilic skin layer. As demonstrated before, the intracellular transport pathway for ions is privileged in viable skin thanks to the presence of ionic channels and receptors located in keratinocytes [5]. Most of them are not specific and thus can transport various monovalent anions with different affinities. Cystic fibrosis transmembrane conductance regulator (CFTR), volume regulated anion channels (VRAC), glycine receptor (GlyR) and γ-aminobutyric acid receptor (GABAR) have a similar affinity to monovalent anions which follows the order of Hofmeister series: $SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^-$ > gluconate [19, 65–68]. More extended spectrum of anions able to interact with NIS which is also susceptible to be present within the skin has been reported: $ClO_4^- >> ReO_4^- >> I^- \geq SCN^- > ClO_3^- >> Br^-$ [20, 47]. For Chloride Channel family (CLC) this order is reversed $NO_3^- > Cl^- > Br^- > I^-$ [69]. The application of inifinite dose of highly concentrated ionic solutions probably led to saturation of these channels and the effects observed in viable episermis are the sum of passive and facilitated transport. These mechanisms of facilitated transport are still present in dermis. However, within this layer supposedly the main type of interactions would concern binding of the ions to collagen and elastine. A study investigating swelling properties of collagen in aqueous solutions of sodium salts of Hofmeister reported the following order $F^- < Cl^- < Br^- < SCN^-$ [17] as for other types of proteins [70].

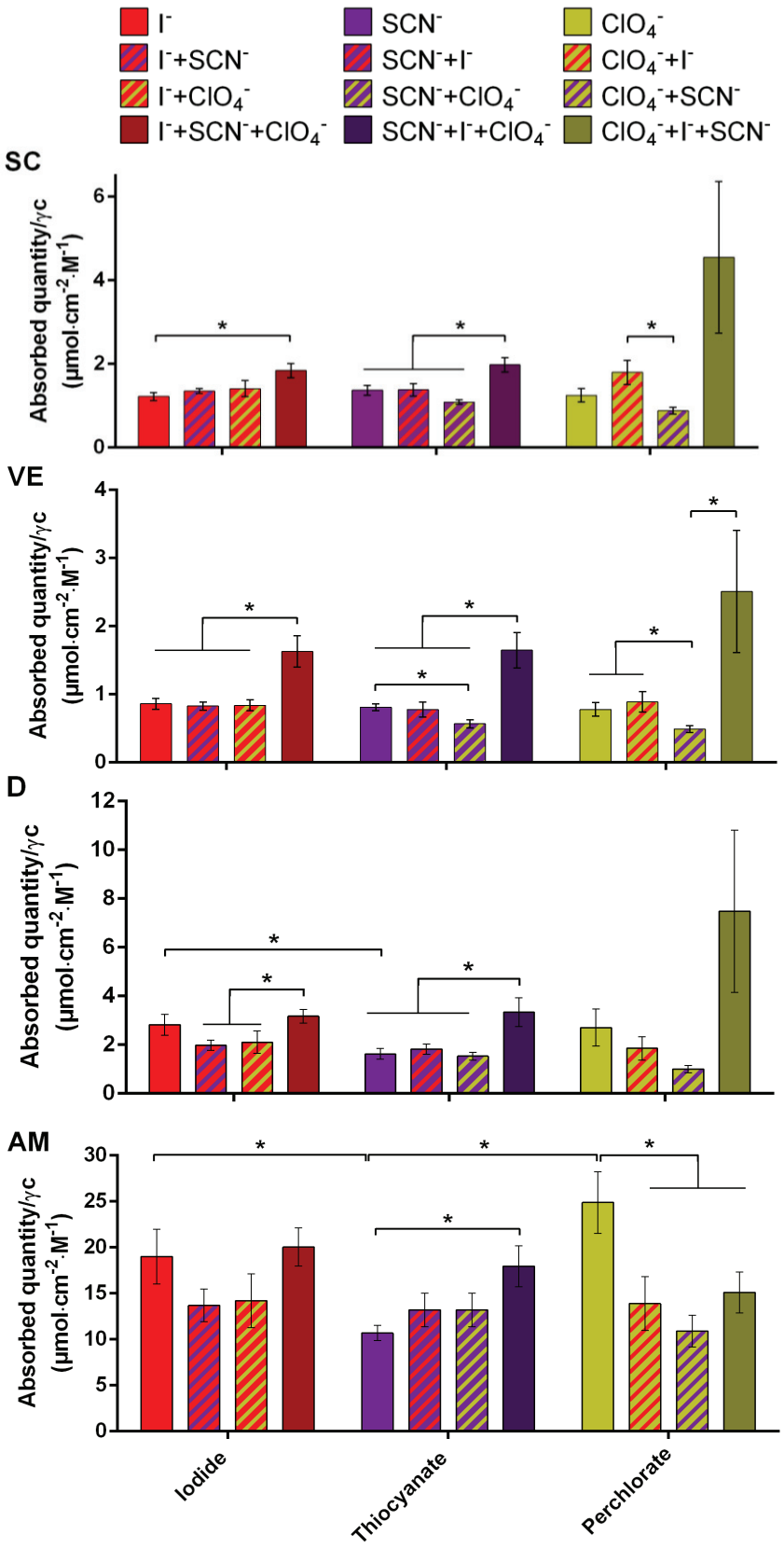

Figure 8 Distribution of anions in the skin layers and AM after 24 h exposure to to binary and ternary mixtures of salts compared to single salt as control expressed as absorbed quantity/activity of anion (µmol·cm^{-2}·M^{-1}) Data represent *mean ± sem* carried out on *n = 9* individual skin explants taken from 5 different pigs. Asterisks (*) indicate $p < 0.05$ in t-test analysis.

9 Conclusions

Present study is the first one that addresses skin absorption of chaotropic anions classified as endocrine disrupting chemicals (I^-, SCN^-, ClO_4^-). 24 h skin exposure to concentrated aqueous solutions and infinite doses led to measurable skin absorption for all tested anions in different mixing variants. As expected for a skin absorption study in such conditions, the total absorbed quantities of anions were quite low (~3% of applied dose). Given very high initial concentration, it might be presumed that topical accidental exposure to salts mentioned in this study should not affect the thyroid function. However, the possibility of these anions permeate skin should be taken into account in occupational risk assessments as topical route could contribute to the total exposure especially if the latter occurs on daily basis.

The mechanisms of passage of anions into and through the skin are not fully understood. Various processes and interactions occurring in different skin layers and depending on the physical-chemical properties of given anion and the skin layer contribute to the final results.

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Chapter IV

The effect of vehicle on skin absorption of Mg2+ and Ca2+ from Thermal Spring Water

1 Problem statement

Thermal Spring Waters (TSW) are naturally occurring mineralised waters which have been used in treatment of various skin and systemic conditions since antiquity [1–3]. Until now they are commonly used in treatment of numerous diseases. For example, the use of TSW is approved for patients with all the forms of arthritis (including psoriatic arthritis) and are recognized to improve musculoskeletal disorders and to relieve pain in suffering patients more efficiently than simple aqueous solutions of specific salt solution [4–8]. Balneotherapy (spa bathing) was recently reported to improve psychological health by significant reduction of insomnia, pain and even depression together with improvement of mood in general in healthy elderly patients [9].

Although back in times, TSW were mainly associated with spa baths, they gained their current "fame" thanks to their beneficial effects on the skin. As adjuvant therapy, they are helpful in treatment of psoriasis associated or not to UVB exposure or atopic dermatitis [10–16]. Apart from medicinal use, TSW-based dermocosmetics are popular solutions for management of "problematic" skin covering vide range of conditions, such as rosacea, sensitive/atopic, acne-prone, aged. The versatility of the applications of TSW depends mainly of their ionic composition (see Table IV p. 30) and temperature of the spring (for balneotherapy).

TSW, being complex mixtures of inorganic salts are natural solutions, for which the non-ideal behaviour in skin absorption experiments could be observed. Claims concerning their dermocosmetic activity are based on either clinical studies or cell culture experiments disclosing mechanisms of action of given ions or TSW [6, 10, 17–20]. The missing link between these two experimental conditions is the dermal absorption study. So far, skin penetration of multiple cations has been evaluated for toxicological or pharmaceutical purposes however, available literature reports mainly studies where single cations were studied. Moreover, nearly all these studies were carried on using an aqueous solution of salts and not actual cosmetic formulations or their models.

Therefore, this chapter includes a manuscript of an article which focuses on the use of Thermal Spring Waters (TSW) in cosmetic formulations. A highly-mineralised marketed TSW was chosen based on the ionic content, which allowed the analyses of skin absorption. The cations of our particular interest were Ca^{2+} and Mg^{2+} presenting the concentration ratio Ca^{2+}: Mg^{2+} of 3:1. These two cations are endogenous in the skin where they create concentration gradients [21]. Their concentrations ratio (Ca^{2+}: Mg^{2+}) in the healthy skin varies form 2:1 to 1:2 depending on the skin layer [22]. They are involved in skin barrier enhancement and recovery as the disruption of well-established concentration gradient is a signal for excretion of the lamellar bodies [23, 24].

Apart from simple study of skin penetration of these cations from the full TSW, the impact of model formulations: emulsions TSW/O, O/TSW and TSW/OW and liposome suspension on dermal absorption of Ca^{2+} and Mg^{2+} was also evaluated.

HIGHLIGHTS OF THE CHAPTER

- Model cosmetic formulations: two liposome suspensions (using saturated and unsaturated phospholipids) and emulsions TSW/O, O/TSW, TSW/OW stabilised with row vegetable material-derived emulsifiers were obtained and characterised in terms of physicochemical properties.
- Skin absorption studies using viable pig skin explants proved that Ca^{2+} and Mg^{2+} could penetrate in deeper skin layers and reach the acceptor medium from marketed TSW.
- Tested model formulations were able to modulate skin absorption rates and cation retention within different skin layers.
- Double TSW/O/W emulsion was the most efficient formulation which increased the overall skin absorption of both cations, simultaneously maintaining the initial Ca^{2+} to Mg^{2+} ratio (3:1).

The effect of vehicle on skin absorption of Mg^{2+} and Ca^{2+} from Thermal Spring Water

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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 Mg^{2+} and Ca^{2+} from those formulations was studied in vitro using static Franz diffusion cells under infinite dose condition and under occlusion of the apparatus.

RESULTS: Mg^{2+} and Ca^{2+} penetrate skin samples from TSW. Encapsulating TSW into double emulsion (TSW/O/W) increased skin absorption of both cations of interest and kept the Ca^{2+} /Mg^{2+} ratio equal to that of TSW in each skin layer. The dermal absorption of Mg^{2+} from the double emulsion departs from both single emulsions. Application of liposome suspension improved the skin absorption of Ca2+ while keeping constant that of Mg^{2+}, leading to unbalanced Ca^{2+}/Mg^{2+} ratio inside skin.

CONCLUSION: The beneficial effects of TSW are not only due to their action on the skin surface. Their active components, especially Ca^{2+} and Mg^{2+} cations reach viable skin layers in a formulation-dependent manner. The distribution of ions inside skin depends on the type of formulation.

2 Introduction

Curative properties of thermal spring waters (TSW) have been known and used in treatment of various skin (psoriasis, eczema etc.) and systemic diseases (ex. osteoarthritis, mental stress, sleep disorders) since many centuries [2, 3]. “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 [25]. Water cures are very common 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 [4, 5, 8]. A 3-week spa therapy has prolonged, symptomatic effects against osteoarthritis that were maintained for more than 6 months [8].

All TSW contain significant amount of magnesium, calcium, and sulfate ions [14]. 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* [27–29] and in clinical trials [30, 31], 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 [23]. 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 lipids exocytosis from lamellar bodies [19, 21, 24, 32, 33]. 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 [23]. 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

(Mg^{2+}:Ca^{2+} > 1) for acceleration of skin barrier recovery. Magnesium, and probably calcium, penetrate the skin preferentially through the follicular pathway [29]. Moreover, “encapsulation” in various vehicles may modulate the distribution of both ions, thereby modifying the Ca^{2+}:Mg^{2+} molar ratio that controls lipids release from lamellar bodies [19, 23].

Calcium and magnesium-rich TSW are known to improve skin barrier function and accelerate wound healing [21, 34]. 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 [35–37]. These properties have been demonstrated in many studies using human keratinocytes, fibroblasts or other response-appropriate cell lines [4, 38, 39]. Therefore, they are considered as active substances when used in a cosmetic product. Skin care 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 skin care 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.*, dermal delivery of molecules depends strongly not only on their physicochemical properties, but also on the formulation in which it is incorporated [40]. Though several TSW-based cosmetic formulations have been claimed, no skin absorption of ions from such products has been reported so far. 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 [41, 42]. 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* [43]. Moreover, liposomes formulated from unsataturated phospholipids were shown to be more efficient for skin delivery than these 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 a direct availability of hydrophilic molecules. In W/O emulsions, ions in water are entrapped in the dispersed phase so that their release is modified, while 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.* for highly hydrophilic glucose [44]. Additionally, multiple emulsions may stabilize active ingredients and contribute to a better skin hydration [45].

3 Materials and Methods

3.1 Materials

Marketed, mixed sulfate-chloride thermal spring water 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 (> 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 ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium dihydrogen phosphate (NaH_2PO_4), 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.

3.2 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.

Table I Characterisation of TSW. Values in standard font are given according to the supplier, values in italics are the present measurements

PARAMETERS		**MINERAL CONTENT (mmol·L^{-1})**			
SPRING WATER TEMPERATURE (°C)	- 62	**Cations**		**Anions**	
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* 75.2	Cl^-	*87.7* 56.9
OSMOLALITY (mOsm·kg^{-1})	*180* "isotonic against skin cells"	K^+	*3.40* 3.46	Br^-	*0.09* 0.02
pH	*5.5* 7.04	Mg^{2+}	*4.36* 3.50	NO_3^-	*0.04* >0.01
Mg^{2+}:Ca^{2+}	*1:3* 1:3	Ca^{2+}	*13.4* 10.3	SO_4^{2-}	*31.3* 22.6

Controls of osmolality, pH, conductivity, and dry residue were performed. Osmolality and pH were evaluated using and 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 mineralisation 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, Switzerland).

3.3 Formulations manufacture

The compositions of formulations (Table II) were designed so that the final concentrations of Mg^{2+} and Ca^{2+} in each formulation were kept constant. There was no other source of Mg^{2+} and Ca^{2+} (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.

Table II Composition of the formulations. Values are given as % m/m of the final preparation. Asterisk (*) refers to the percentage of double-concentrated TSW used in the formulation. Theoretical contents of Mg^{2+} and Ca^{2+} were 2.7 and 8.0 mmol·kg^{-1} respectively.

EMULSIONS					LIPOSOMES		
Components	**O/TSW**	**TSW/O**	**TSW/O/W**		**Components**	**90H**	**90G**
TSW	60	60	60*	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_2O isotonic	0	0	0	45			

The composition of TSW/O/W emulsion was based on the TSW/O primary emulsion. TSW/O/W double emulsions were 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 0.45 µm syringe filters (WhatmanTM Uniflo) 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 due to 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 5,000 rpm for 1 min. Two liposome suspensions containing 60% (m/m) TSW were prepared by the thin lipid film hydration method [46] using saturated (LPM-90H) or unsaturated (LPM-90G) phospholipids. Saturated (Phospholipon® 90H) or unsaturated (Phospholipon® 90G) phospholipids and cholesterol were solubilized 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.

3.4 Physicochemical characterizations

3.4.1 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).

3.4.2 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 precooled 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.

3.4.3 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}.

3.4.4 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).

3.5 Skin permeation studies

3.5.1 Skin samples 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 [22, 47, 48]. 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 $g \cdot h^{-1} \cdot m^{-2}$ after 1 min were discarded.

3.5.2 Skin absorption study for Ca^{2+} and Mg^{2+} distribution after 24 h

Skin absorption studies were performed in static diffusion cells according to OECD guidelines [49]. 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 $\mu mol \cdot cm^{-2}$ Mg^{2+} and 3.2 $\mu mol \cdot cm^{-2}$ Ca^{2+} 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 $mmol \cdot L^{-1}$) but contained the minimal amount of ions so that its composition did not interfere with the experimental setup. Glucose (1 $g \cdot L^{-1}$) provided the energetic fuel for skin cells and phosphate buffer ensured physiological pH over the exposure to formulations [50].

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** nonionic surfactant (Triton X-100; Sigma Aldrich, 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 [22]. 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 syringe filters (Whatman™ Uniflo). 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 Ca^{2+} and Mg^{2+}, 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.

3.6 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, Switzerland).

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} $NaHCO_3$ and 3.2 mmol·L^{-1} Na_2CO_3) 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} HNO_3; 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^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) and Certified Multielement Ion Chromatography Cation Standard Solution (10.0 mg·kg^{-1} ± 0.2% of each cation: Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}) 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} HNO_3 and 2.27 mmol·L^{-1} dipicolinic acid) and the injection volume was 100 µL. The calibration curve from a mixture of $MgSO_4 \cdot 7H_2O$ (Acros Organics, France) and $CaCl_2 \cdot 2H_2O$ (Cooper, France) was linear in the range from 0.5 to 50 mg·L^{-1} for Mg^{2+} and Ca^{2+} (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.

3.7 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 t-test using XLSTAT Version 2014.5.03 (Addinsoft, Paris, France). Significance level was set at $p < 0.05$

4 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 $g \cdot h^{-1} \cdot m^{-2}$.

Skin absorption profiles of Mg^{2+} and Ca^{2+} 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 Mg^{2+} and Ca^{2+} present in the aqueous phase were kept constant (2.7 and 8.0 $mmol \cdot kg^{-1}$ 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) nonionic emulsifier for W/O emulsions that is widely used in food products [51] 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) [52]. 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 [53].

Liposomes were prepared by thin layer rehydration technique followed by sonication leading to 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 [54].

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 Mg^{2+} or Ca^{2+}, was not observed, which was in agreement with Düzgüneş *et al.* who did not notice aggregation or fusion of vesicles up to 10 mM Ca^{2+} and 100 mM NaCl [55]. 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 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 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 [56].

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 ± 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).

Table III Physicochemical characterisation of liposomes made of saturated and unsaturated phospholipids (LPM-90H and LPM-90G respectively) and 3 emulsion types: O/TSW, TSW/O/W and TSW/O. Mean size of emulsion droplets was estimated from optical microscopy observations; those of liposomes were measured by dynamic light scattering.

	LPM-90H	LPM-90G	O/TSW	TSW/O/W	TSW/O
Size (µm)	0.09	0.08	1-2	5-20	1-2
Viscosity (mPa·s) T = 25°C, strain rate = 300 s^{-1}	---	---	1.6 ± 0.1	6.8 ± 0.3	197 ± 13
pH	7.5	7.6	5.3	5.5	---

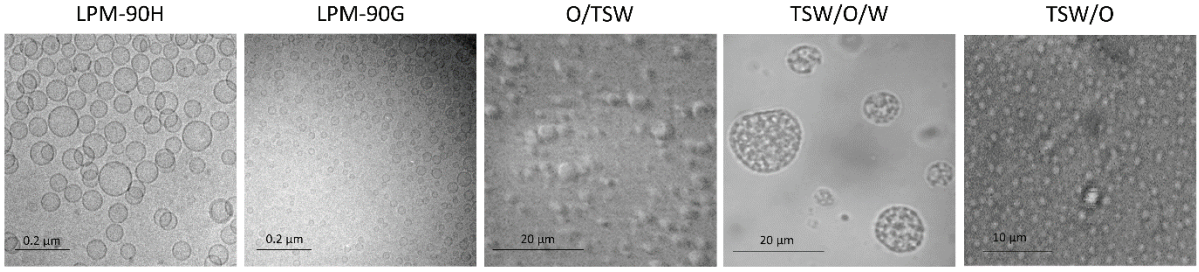

Figure 1 Microscopic images of formulations. Pictures of LPM-90H and LPM-90G were taken by cryo-TEM. Optical microscopy pictures were taken for O/TSW, TSW/O/W and TSW/O.

Figure 2 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 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 (Figure 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 Figure 2 that the application of a direct 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: 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 [44], caffeine [57]) due to 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 3-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.

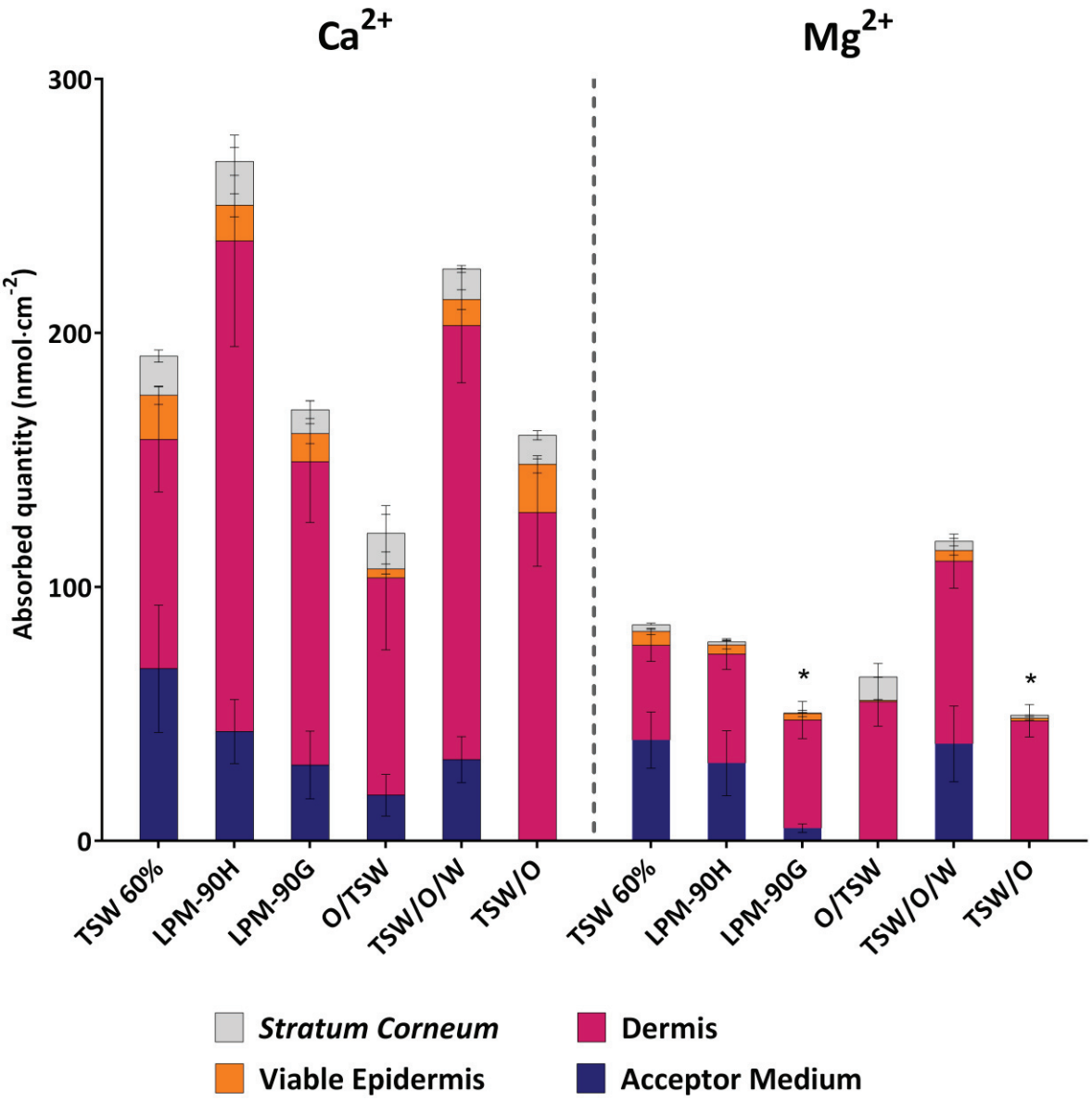

Figure 2 Distribution of Ca^{2+} and Mg^{2+} in the skin layers after 24 h exposure to formulations or TSW 60% as reference. Superimposed bars correspond to total absorbed amount (Q_{abs}). Values represent mean ± *sem* from 8 experiments. Asterisk (*) indicates statistically significant difference obtained between given sample and TSW 60% in the t-test ($p < 0.05$).

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 [28, 29, 58]. 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 [59]. Extensive literature reports show that divalent cations easily bind to glycolipids, phospholipids, carboxylate, phosphate groups and carbonyl groups of *sn-2* phospholipid chains [60–63]. 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 ~~a~~ 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 [64–66]. 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 pools formation. Thus, this deeper region over which lipid lamellae are formed still acts as a barrier [65]. 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) [19, 23]. This desired retention enhancement, however should be equal for both cations otherwise the initial Ca^{2+}:Mg^{2+} ratio (3:1) can change and lead to a reverse effect of inhibition of skin recovery, as reported by Denda *et al.* [23]. Considering the total quantity retained in the skin, the Ca^{2+}: 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 [27, 29]. However, a closer observation of the results in the skin layers show 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 Ca^{2+}: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 Ca^{2+}: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 setup applied within this study, ion transport in viable skin layers occurs through ion-specific transporters [67, 68]. Physiological manifestation of this phenomena is shown by the regulation of the ratio in the deeper layers in the absence of skin damage [69]. 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.

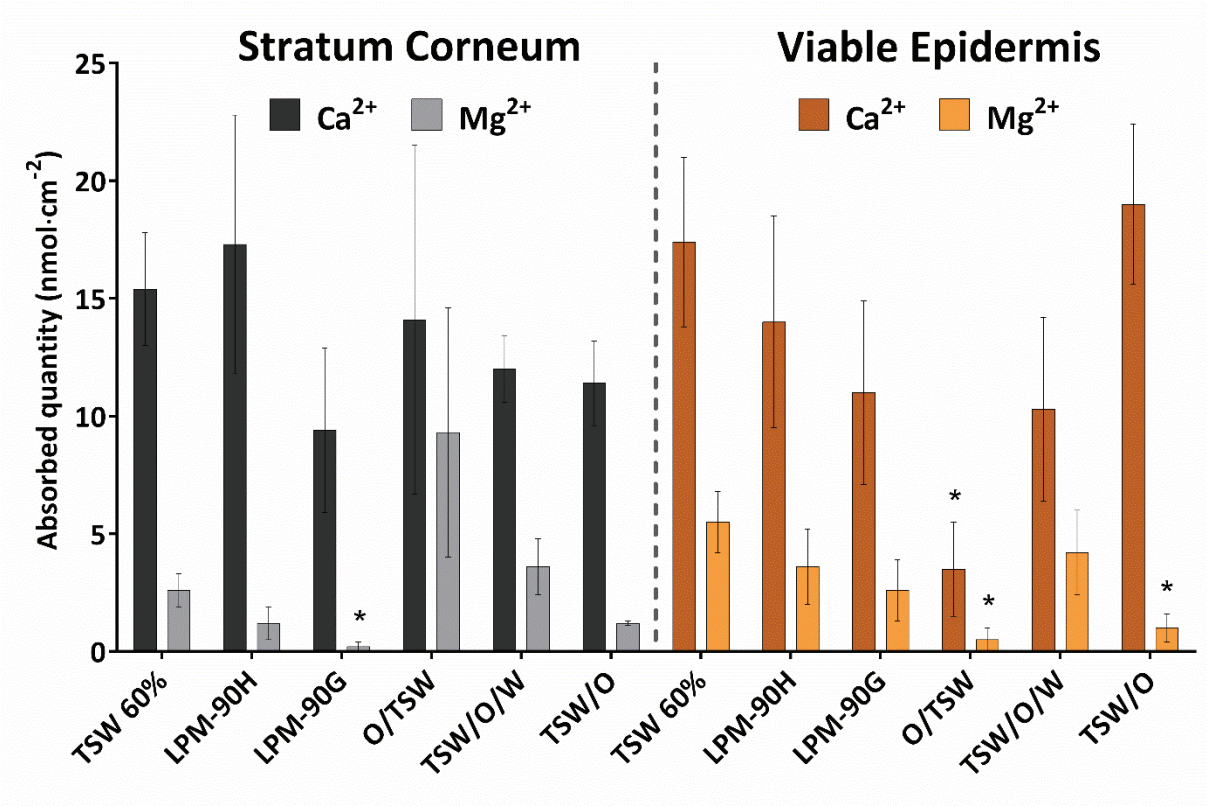

Figure 3 Distribution of Ca^{2+} and Mg^{2+} in the epidermal layers (SC and VE) after 24 h exposure. Values represent mean ± *sem* from 8 experiments. Asterisk (*) indicates statistically significant difference obtained between given sample and TSW60% in the t-test ($p < 0.05$).

Liposomes have been chosen as vehicles due to 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 [54, 70]. 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, while 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 [54]. 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 [71, 72]. Alsop *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, while Ca^{2+} was adjacent to the glycerol group [73]. Such a deep penetration of ions into the polar regions of the

membrane led by swelling due to water revealed an increase of the lamellar repeat distance. The origin may be the electrical charge coming from divalent cation bonding that cause an electrostatic repulsion between bilayers together with the water uptake by hydration of bound cations [74]. 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 $Fe^{2+} > Mg^{2+} > Ca^{2+} > Zn^{2+}$, corresponding to the Hofmeister series [73]. For dipalmitoylphosphatidylcholine (DPPC), swelling has been reported until a ratio DPPC: Ca^{2+} = 1:0.14 mol/mol as in the present study [75]. 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 Ca^{2+}:Mg^{2+} in the SC and VE with Ca^{2+}: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 has been recently reviewed by Rosanoff *et al.* [76]. Unbalanced dietary intake of these two ions was shown to have serious health consequences. For example, increased serum Ca^{2+}:Mg^{2+} ratio was associated with an increased risk of prostate or colorectal cancer [76]. Lee *et al.* 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 [77].

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.* [44] 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 interacts better with the lipidic fraction of *stratum corneum* than individual emulsifiers in simple emulsions. Such a mixture can act as a skin absorption enhancer.

Table IV Results of dermal absorption of Ca^{2+} and Mg^{2+} from the emulsions and liposomes in nmol·cm^{-2}. Values represent mean ± *sem*. Ca^{2+}: Mg^{2+} ratios (R) were calculated based on the mean values obtained for each skin layer. The values being significantly different ($p < 0.05$ in t-test) than the ones obtained for TSW 60% are framed: in red when lower than for TSW 60%, and in green when higher than for TSW 60%.

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

5 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 due to their biological activity in the skin tissue.

In the first place, we developed and characterised 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 while 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 maximise 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 unbalance of the Ca^{2+}: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 Ca^{2+}: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.

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General discussion and prospects

General discussion

The aim of this thesis was to study skin absorption of inorganic ions with regard to their physicochemical properties.

Given the recent discovery by Paweloszek *et al*. demonstrating that up to 80% of ion transport occurs with the help of specific transporters, this main task implied working with viable skin samples. Such an approach imposed the use of survival medium that could be used in Franz cells for testing skin absorption through metabolically viable samples. So far, complex mixtures of inorganic salts such as *Dulbecco Modified Phosphate-Buffered Saline* (DMPBS), HEPES-Buffered Hanks Balanced Salt Solution (HHBSS) [1, 2] or even broth cell culture media [3, 4] have been commonly used. These media are indeed efficient in terms of maintaining skin viability but they all contain significant concentrations of salts that could cause analytical problems within our experiments. Therefore, in Chapter II we presented the work focused on understanding the crucial parameters of medium composition for skin survival. We have established a betaine-based survival medium that meets the basic requirements for efficacy: physiological pH controlled over time with a buffer system, glucose supplementation and isotonicity with skin cells. This “salt-free” medium has been used thereafter for all skin absorption experiments within the thesis. The research on the properties of an adequate survival medium for Franz cell protocol has been extended to take into account the needs implied by testing skin absorption of poorly water soluble molecules. We have evaluated the impact of additives (non-ionic surfactant [5] and ethanol [6]) commonly used to ensure sink conditions in the latter case. The results showed that the choice of the additive should be careful because such an alteration of medium composition can compromise its efficacy. Addition of non-ionic surfactant (Oleth-20) at the concentration up to 1% seems to be a good compromise to ensure sink conditions in studies of *in vitro* absorption of poorly water soluble molecules that are prone to be either metabolised or transported actively within skin. Finally, we tackled the common laboratory issue related to the availability of fresh skin explants. In the last part of the work we verified the impact of overnight “dry” storage of skin explants in 4°C on the metabolic activity of the samples. We observed impairment of skin metabolism especially within the first 6 h of experimentation. To deal with this inconvenience other types of protocols including “wet” preservation where tissue explants are soaked in a glucose-supplemented fluid or increasing storage temperature by several degrees could be tested in the future to optimise the preservation conditions. These results are included in a research communication entitled *Formulation of survival acceptor medium able to maintain the viability of skin explants over in vitro dermal experiments* [7] published in International Journal of

Cosmetic Science, and give an insight on how the composition of survival medium can be tailored depending on the specific experimental needs.

Within the works presented in Chapters III and IV, skin absorption of inorganic ions from non-ideal aqueous solutions has been studied. This is the first study in which skin absorption of ions was investigated using a Franz cell method with an acceptor medium allowing survival of the tissue over the course of experiment. In such an experimental setup all of the skin penetration routes (transcellular, intracellular and transfollicular within *stratum corneum*; transcellular with the contribution of channels and transporters in viable skin layers) were active. According to the literature, the intracellular pathway is characterised by the specific organisation of lipid bilayers which enables the transport through the lipidic core, as well as via the polar headgroups pathways [8, 9]. Therefore, there seems to exist an effective route for ions to cross the outermost skin layer. Another, less plausible theory, would be that ions could pass via transcellular route sometimes considered as a polar pathway within the *stratum corneum* [9]. Even though slightly hydrated intracellular matrix of corneocytes could provide the polar environment, such a pathway would require multiple partitioning of the molecule between this polar environment and lipidic external matrix. Once they reach viable layers of epidermis, ions are subjected to active transport operating in the tissue [10]. Follicular pathway, which has been demonstrated to contribute to absorption of cations [11] is also available. Therefore, observed absorption rates are the sum of all the contributing pathways.

In Chapter III we have studied skin absorption of anions included in Hofmeister series from concentrated aqueous solutions. In these conditions, tested anions exhibited non-ideal behaviour where the total skin absorption was proportional to the activity of the aqueous solution instead of being directly correlated to anion concentration. Thermal Spring Waters (TSW) are an example of naturally occurring complex mixture of inorganic ions where the same type of behaviour could occur. Thus, Chapter IV investigated skin absorption of Ca^{2+} and Mg^{2+} from a marketed TSW.

Results presented in Chapter III and Chapter IV demonstrated that all ions of interest were able to pass through the skin from aqueous solutions. However, their absorption profiles and skin distribution are different, if we compare anions and cations that were investigated here. After 24h exposure, the majority (up to 85%) of total absorbed quantity of anions was recovered from acceptor medium while for cations a stronger correlation between Q_{abs} and the amount retained in dermis was observed.

The same pattern has been previously reported by our team for passive (using frozen-stored explants) skin absorption of radiopharmaceuticals [12]. Cationic radionuclides ($^{111}In^{3+}$ and $^{67}Ga^{3+}$)

tended to be retained within upper skin layers rather than penetrated through the explants. The authors attributed such a poor permeation to unfavourable pH at the skin surface that led to hydrolysis of the cations to poorly water-soluble hydroxides ($Ga(OH)_3$ and $In(OH)_3$ respectively). Even though, chromium was deposited as $^{51}CrO_4^{2-}$, in contact with the skin it was partially reduced to $^{51}Cr^{3+}$ which resulted in its higher total absorption than in the case of Ga^{3+} and In^{3+} with high accumulation in skin layers. In the same study, skin absorption of $^{125}I^-$ and $^{99m}TcO_4^-$ was also evaluated. Conversely to cations, these monovalent anions permeated better through skin samples, iodide having longer lag time compared to pertechnetate. The reason for this difference was rather chaotropic character of I^- that interacted with lipids of *stratum corneum*, which caused delayed passage. No such affinity was described for pertechnetate whose penetration was faster [12]. Our results are also in line with the theory of mobility of ions. According to Frausto da Silva [13], monovalent ions including Na^+, K^+ and Cl^- are strongly hydrated and very mobile. They do not bind with biological molecules (ex. enzymes) but they can easily transfer ionic charges. On the other hand, hard metal ions: Mg^{2+} and Ca^{2+} are considered as semi-mobile. They are characterised by lower polarizability as their valence electrons remain close to their nucleus. The slow absorption profile characterised by high retention within dermis can be explained by their strong affinity to glycolipids, phospholipids, carboxylate groups, phosphates, and carbonyl groups of *sn-2* lipid chains [14–17] rather than by the phenomena observed for trivalent cations Ga^{3+} and In^{3+} [12].

Two strategies of modulating passage of ions have been described within this work. For the anions classified in the Hofmeister series (Chapter III) we demonstrated that certain ions can modify the permeation of others from aqueous solutions. A strong synergy was observed between Br^- and I^- while the presence of F^- seemed to inhibit the absorption of other halides. In the second investigated group, the penetration of perchlorate was negatively affected by addition of either I^- or SCN^- or both of these ions. Regarding Ca^{2+} and Mg^{2+}, their skin absorption has been investigated either directly from marketed TSW or after incorporating TSW into different vehicles. Even though formulating of TSW did not considerably affect the total absorbed quantities of investigated cations, their distribution in the skin layers after 24h differed depending on the formulation type. Generally, emulsions and liposomes promoted retention of cations within the skin layers as compared to TSW that permeated to greater extent to the acceptor medium. Liposomes were found to be an inadequate formulation for enhancement of skin delivery of divalent cations. $Ca^{2+}$and Mg^{2+} bind easily to phospholipids constituting liposomes and are not released from this formulation [18, 19]. Moreover, application of all of the formulations except from TSW/O/W led to an increase of the initial Ca^{2+}:Mg^{2+} ratio in the skin layers.

Prospects

Regarding the fundamental studies investigating skin absorption of inorganic ions, the approach presented within this thesis for anions could be extended to cations. Such a study would allow to verify the impact of physicochemical properties of individual ion on their passage.

Because absorption rates observed for ions are the sum of multiple events that occur within their passage through the skin, further studies aiming at better understanding the impact of each of the following steps could be performed to complement our studies:

1. Interactions in aqueous solutions:

   Ions can interact with each other creating pairs and complexes when applied in aqueous solutions, especially at high concentrations. NMR study of binary and ternary mixtures could provide an insight on the behaviour of tested ions before the actual contact with skin samples.
2. Measurements of partition coefficients (water/octanol or water/SC lipids):

   In order to verify whether mixing ions could have an impact on the rate-limiting step of cutaneous absorption, the measurements of partition coefficients and their changes depending on the salt composition in mixtures could be performed. Traditional protocol of determining partition coefficient between water and octanol could be adapted by using an artificial mixture of lipids mimicking the properties of *stratum corneum*.
3. Investigation of other types of interactions inside the skin:

   A challenging task of further investigating the specific interactions between ions alone and in more complicated mixtures with the components of the skin (ex. collagen and elastin within dermis [20]) could also be undertaken.

The extension of the part of this project related to TSW formulation may include:

1. Testing of *in vitro* skin absorption of biologically active ions from new model formulations (ex. gels) and marketed TSW-based products for comparison.
2. Modifying experimental protocol used here to obtain conditions that would better mimic “in use conditions” ex. reduction of exposure time, reduction of dosing (finite dosing would require more sensitive analytical methods).
3. *In vitro* testing of skin absorption of ions from various formulation on atopic dermatitis skin model (damaged skin barrier by stripping).
4. Clinical mini study on the efficacy of model formulations in stimulating skin barrier repair after artificial disruption of the latter.
5. Investigating the impact of formulating TSW together with different active pharmaceutical/ cosmetic ingredients on skin absorption.

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Résumé substantiel

La peau est l'organe le plus lourd du corps humain qui assure une excellente barrière entre l'environnement extérieur et l'intérieur de l'organisme. Elle préserve l'homéostasie cellulaire. Elle comprend trois couches superposées de tissus qui correspondent, de la surface à la profondeur, à l'épiderme, épithélium différencié, kératinisé multi stratifié et kératinisé, au derme, tissu conjonctif, puis à l'hypoderme. Au niveau de l'épiderme, c'est le *stratum corneum*, couche morte hautement différenciée, qui joue le rôle de fonction barrière. Le profil d'hydratation de la peau reflète bien cette organisation en couche. En effet, le teneur en eau varie entre 15-30% dans le *stratum corneum* et augmente jusqu'à 70-80% dans les couches profondes telles que l'épiderme vivant (*stratum granulosum, stratum spinosum* et *stratum basale*) et le derme. L'eau comprend des ions de nature endogène qui sont les ions chlorure, sodium et potassium et qui suivent le gradient aqueux. La couche basale étant la couche proliférative de l'épiderme, une plus forte concentration en phosphate y est ainsi observée. Par ailleurs, le soufre est un atome très concentré dans le *stratum corneum* car c'est un élément structural de la kératine, *via* les acides aminés soufrés, qui est très présent dans les cornéocytes. Les ions calcium et magnésium peuvent être retrouvés dans des couches supérieures de la peau selon un gradient de concentration. Ce dernier peut être perturbé en cas de rupture de la barrière cutanée, qui elle-même, active les mécanismes de réparation cutanée. La peau, étant la barrière externe de l'organisme est donc continuellement exposée aux espèces ioniques exogènes qui peuvent être issues, par exemple, de sels présents dans l'eau courante, dans les produits cosmétiques, ou dans les métaux composants les bijoux. L'exposition volontaire et occasionnelle est liée aux baignades dans l'eau de mer et à la balnéothérapie. L'autre type d'exposition aux ions, est l'exposition professionnelle, liée aux processus industriels ou à l'agriculture avec l'emploi, par exemple, de fertilisants. Enfin, le contact accidentel avec les agents de pollution ou les radio-isotopes peut être aussi imaginé. En plus des ions exogènes, à la surface de la peau on retrouve les ions contenus dans la sueur et sécrétés par les glandes sudoripares eccrines. Par conséquent, la peau est mise en contact avec un mélange plus ou moins élaboré d'ions possédant des propriétés physicochimiques différentes. Ces dernières déterminent les interactions avec les macromolécules et ainsi régissent les activités biologiques.

Parmi les activités biologiques des ions, on peut distinguer différents effets qui peuvent être bénéfiques ou nocifs. Les effets bénéfiques donnés par les composés des eaux thermales peuvent améliorer la fonction barrière (Ca^{2+}, K^{+}, Mg^{2+}), avoir un effet anti-inflammatoire (Mg^{2+}, Zn^{2+}, Se), aider à la cicatrisation (Mn^{2+}, Zn^{2+}, Cu^{2+}) et à la biosynthèse de collagène et élastine (Zn^{2+}, Cu^{2+}), protéger contre le rayonnement UV (Se, Zn^{2+}) et aussi agir contre l'acné (Zn^{2+}). Ces ions peuvent pénétrer dans

la peau et atteindre des couches cutanées plus ou moins profondes. De plus, en cosmétique par exemple, les sels d'aluminium sont utilisés dans les produits anti transpirants et les cations issus des sels d'argent aident à la cicatrisation des plaies chroniques en médecine. Ces sels exercent leurs actions à la surface de la peau ou de la plaie.

Les ions ont également des effets indésirables et on peut distinguer les ions qui sont des allergènes (Ni^{2+}, Co^{2+}, Cr^{2+}, $Cr_2O_7^{2-}$) ou qui émettent des radiations (In^{3+}, Ga^{3+}, ^{235}U, ^{239}Pu, ^{241}Pu, ^{41}Am). Ils sont nocifs sans pénétrer profondément dans la peau. On peut citer, en exemple les dérivés mercuriels qui sont souvent employés en mésusage, comme agents de blanchiment de la peau. Ils sont particulièrement dangereux en vue de leur absorption et toxicité systémique.

Enfin la série d'Hofmeister ($F^- > PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > I^- > SCN^-$) est une classification artificielle et académique qui classifie les anions inorganiques. Leurs propriétés biologiques sont moins étudiées que celles des cations. A ce jour, les chlorures sont connus pour renforcer la barrière cutanée, les fluorures interfèrent dans la réticulation du collagène le rendant de mauvaise qualité et les iodures participent à la synthèse des hormones thyroïdiennes. Les affinités de ces anions envers les protéines de transport sont intéressantes car elles ont tendance à suivre l'ordre inverse de la série d'Hofmeister, comme pour le symporter sodium/iodure (NIS), le « Cystic Fibrosis Transmembrane Regulator » (CFTR), le « Volume-Regulated Anion Channel » (VRAC) et « γ-Aminobutyric acid Receptor» (GABR). Elles suivent l'ordre direct de la série en cas de pénétration via les canaux de chlore (CLC) et les bicouches lipidiques modèles.

L'objectif de cette thèse était l'évaluation de l'absorption cutanée des ions inorganiques en fonction de leurs propriétés physicochimiques. Ce travail est divisé en trois parties. La première porte sur les études et la mise au point de la méthodologie appropriée aux études d'absorption cutanée des ions. La deuxième partie est consacrée aux études de pénétration cutanée des anions classés en série d'Hofmeister et aux effets de mélange. La dernière partie applicative étudie l'impact de la forme galénique sur la pénétration à travers la peau de cations à effet thérapeutique reconnus, le calcium et le magnésium.

La méthodologie appliquée pour ces études comprend les essais en cellules de diffusion de Franz conformes aux règles décrites dans la directive OCDE 428. Ces cellules comprennent un compartiment donneur dans lequel le produit contenant les actifs est déposé, et un compartiment récepteur qui mime le compartiment sanguin. Les explants de peau porcine fraîche et maintenue en viabilité ont été utilisés comme membranes. Les produits testés ont été déposés dans le compartiment donneur de la cellule, en dose infinie, pendant 24h sous occlusion. Au bout du temps

d'exposition, les cellules ont été démontées et les couches cutanées des échantillons ont été séparées. Le contenu du milieu récepteur a été dosé ainsi que la quantité d'actif restée en surface.

Dans la première partie, les mesures de l'activité métabolique des échantillons de peau, montées en cellules de Franz, ont été évaluées grâce aux mesures du lactate produit durant 24h, durée de l'expérience. Cette première partie a permis de choisir un milieu de survie pour maintenir la peau fraîche métaboliquement viable. La quantification des ions dans les deux autres parties expérimentales a été réalisée grâce à la chromatographie ionique. Le bilan massique a été vérifié pour chaque expérience par quantification des ions dans chaque compartiment de la cellule de Franz et la quantité totale absorbée (Q_{abs}) devait correspondre à la somme des valeurs obtenues pour les couches cutanées et le milieu accepteur.

Pour comprendre l'importance de la partie méthodologie qui avait pour but le développement du milieu approprié pour les expériences de pénétration cutanée des ions, il faut comprendre ce qui se passe lors de l'exposition de la peau aux ions exogènes. Comme tous les xénobiotiques, les ions peuvent traverser le *stratum corneum* via une des trois voies. La voie intercellulaire mène directement à travers les cellules. La voie intracellulaire qui est la plus courante, elle, mène dans la matrice extra cellulaire. Enfin, l'entrée par les annexes cutanées (poils, ..) est la voie la plus rapide mais qui ne représente qu'une faible surface de la peau. Une fois que les ions ont traversé le *stratum corneum*, leur passage dans les couches viables de la peau est facilité par les canaux ioniques et par les protéines de transport. La contribution de ce transport facilité pour les ions halogénures a été démontrée récemment par notre équipe. L'étude comparative de la pénétration cutanée des halogénures a été réalisée à travers la peau fraîche, dite viable, et à travers la peau congelée avant usage, dite non-viable. On observe une différence entre ces deux types de peau qui s'explique par la contribution du transport facilité à hauteur de 80% dans la peau viable indépendamment du modèle cutané (humain ou porcin). C'est pour cette raison que, dans ce projet, la peau doit être maintenue en viabilité.

Pour assurer la viabilité cutanée, les protocoles classiques utilisant des explants congelés non viables sont souvent modifiés pour prendre en compte ces conditions particulières. Les milieux de culture cellulaire ou les solutions destinées à l'usage biologique sont souvent utilisés mais ils ne sont pas pertinents pour les études de passage cutané des ions parce qu'ils provoquent de nombreuses interférences analytiques. L'objectif de cette étude est donc d'étudier les conditions nécessaires à appliquer au milieu receveur des cellules de Franz pour maintenir la viabilité des explants, durant les études de pénétration cutanée des ions inorganiques. Le milieu idéal assurerait la suivie de la peau mais contiendrait un minimum de sels. De plus, les modifications possibles pour les études des autres molécules hydrophiles et lipophiles ont été évaluées. Les facteurs déterminants à prendre en compte

pour assurer la viabilité des explants *in vitro* sont discutés. Les conséquences de la conservation des explants cutanés durant une courte durée à 4°C, avant utilisation, ont été également évaluées.

L'impact du pH, des solutions « tampons », de l'osmolalité, de la force ionique, de la concentration initiale en glucose et de l'addition d'éthanol ou de tensioactifs non-ioniques, dans le milieu receveur de la cellule de Franz, a été étudié. Plusieurs compositions ont été testées : les contrôles, les solutions hypotoniques (l'eau glucosée tamponnée « Water-Glucose + Buffer » (WGB) ou l'eau glucosée non tamponnée « Water-Glucose » (WG)), des solutions isotoniques (le sérum physiologique non tamponné supplémenté en glucose « Saline Serum + Glucose » (SSG)), et deux milieux complexes basés sur la composition du « Hank's Balanced Salt Solution » (HBSS) : le milieu salé « Salt-rich Medium» (SM) sans chlorure qui a été remplacé par les ions sulfate et le milieu « Betaine + Glucose » (BG) où les sels étaient substitués par de la bétaïne.

Trois groupes peuvent être distingués, concernant l'efficacité. Les milieux qui maintiennent la viabilité des explants cutanés au même niveau que le milieu contrôle positif pendant 24h (SM et BG) ; les milieux non efficaces (contrôle négatif, WG, SSG) et le milieu intermédiaire- WGB qui n'a été efficace que 6 premiers heures. Ces travaux permettent de mettre en évidence des prérequis pour la formulation de milieux de survie adaptés aux expériences et sont les suivants : un pH physiologique (> 5,5), osmolalité de 300 $mOsm.kg^{-1}$ (isotonique par rapport aux cellules de la peau) et supplémentation du glucose (> 0,5 $g.L^{-1}$). Le milieu BG a été considéré comme optimal pour les expériences de pénétration cutanée car il remplissait tous les critères.

Dans cette même partie l'impact de la conservation des explants cutanés à 4°C, durant une nuit a été évalué. Nous démontrons que cette pratique perturbe l'activité métabolique de la peau.

La deuxième partie de ce travail se concentre sur les études du passage cutané des anions inorganiques classés dans la série d'Hofmeister. La disproportion entre la quantité des données disponibles qui traitent de l'absorption cutanée des cations versus anions était la motivation pour ces études. A ce jour, dans la littérature on peut trouver des études exploratoires d'absorption cutanée des ions phosphate et des ions bromure sur différents modèles de peau. Plusieurs publications concernant la pénétration cutanée des ions iodure sont disponibles. Ces études ont été menées dans le cadre de radiocontamination ou comme une alternative de supplémentation. Les dernières découvertes concernant la pénétration cutanée des ions halogénures ont démontré que la diffusion dépend du type d'ion testé et de leur concentration dans la solution de dépôt. Tous les ions testés jusqu'à présent font partie de la série d'Hofmeister qui classifie les ions en fonction de leur hydratation ainsi que leurs interactions avec les macromolécules présentes dans la solution. Les petits ions fortement hydratés (ex. F^-, Cl^-) sont appelées kosmotropes car ils ont la tendance à

stabiliser les protéines dans les solutions aqueuses. Les ions chaotropes ont une taille plus importante, sont moins hydratés et déstabilisent des protéines en solution (ex. SCN^-, ClO_4^-). Les études antérieures ont démontré que les ions chaotropes peuvent améliorer le passage cutané des autres molécules. Le but de ce projet était de déterminer l'influence de l'effet du mélange des ions sur la pénétration d'un anion ou d'un autre. Des concentrations élevées ont été délibérément choisies pour observer des effets au niveau cutané. En effet, il est reconnu que les espèces chargées ne pénètrent que très peu les membranes. Dans une première série, les ions comme F^-, Br^- et I^- ont été testés seuls ou en mélange puis, les ions comme l'iode, le thiocyanate et le perchlorate de sodium ont été considérés, puisqu'ils constituent la fin de la série d'Hofmeister comprenant les ions les plus chaotropes. Les ions iodure ont été inclus dans les deux séries et faisaient un lien entre les deux séries. Les ions chaotropes peuvent agir au niveau de la synthèse des hormones thyroïdiennes, l'iodure étant le substrat physiologique de la thyroïde et le thiocyanate et le perchlorate de sodium, des inhibiteurs de cette synthèse. Ce sont donc des perturbateurs endocriniens.

Deux scénarios de mélanges ont été testés : pour les halogénures, la force ionique des solutions a été maintenue constante, ce qui a impliqué des changements des concentrations individuelles des ions, tandis que pour les perturbateurs thyroïdiens, les concentrations des ions ont été maintenues constantes, ce qui a provoqué des changements de la force ionique dans différentes solutions.

Les résultats d'absorption cutanée des ions halogénures ont démontré, dans le premier temps, qu'une relation entre le Q_{abs} et la concentration en ion déposée n'est pas une relation linéaire pour des concentrations élevées. Par contre, cette linéarité est retrouvée pour la relation entre le Q_{abs} et l'activité de la solution ionique. Sur la base de cette observation, nous avons introduit le coefficient k_{abs} qui est une fonction de Q_{abs} et de l'activité. Ce coefficient permet la comparaison directe entre les différentes conditions testées pour s'affranchir des différences des concentrations d'ions en solution de dépôt. L'analyse de résultats exprimés en k_{abs} obtenus pour les ions testés séparément ainsi qu'en mélanges bi- et ternaires a démontré une synergie entre les ions bromure et iodure. Ceci est probablement dû aux interactions entre les ions bromure et iodure avec les cations divalents présents dans la peau. Le plan de mélange appliqué dans cette étude a permis le traitement statistique des résultats en utilisant un plan de mélange (le lattice simplex design), ce qui est une nouveauté dans le domaine d'absorption cutanée. Cette analyse a confirmé les effets synergiques observés entre Br^- et I^- et a prouvé le caractère spécifique de ces interactions. Concernant les ions chaotropes étudiés dans cette partie, les différences observées ont été moins évidentes que dans le groupe des halogénures. La valeur de k_{abs} obtenue pour le thiocyanate seul est plus basse que dans tous les autres cas. De plus, les ions perchlorate ont d'avantage pénétré à partir d'une solution simple qu'à partir des mélanges tandis que la situation inverse a été observée pour les ions

thiocyanate. L'analyse de la répartition des ions testés dans les couches cutanées a mis en évidence des différences de distribution entre ces couches. Dans le *stratum corneum*, couche morte non sélective pour la pénétration des ions, peu de différences significatives ont été constatées. Ces dernières ont commencé à apparaître au niveau de l'épiderme viable qui est susceptible de constituer une barrière sélective pour la pénétration des ions, en raison de la présence de transporteurs spécifiques. Les tendances observées dans le derme et dans le milieu receveur étaient les mêmes car ce sont les deux compartiments les plus hydrophiles de la peau.

Dans cette deuxième partie de thèse, nous mettons en évidence le comportement spécifique des ions lorsque des concentrations élevées sont appliquées. Tous les ions testés ont pénétré la peau viable durant 24h d'exposition et une forte synergie entre Br^- et I^- a été démontrée. Pour mieux comprendre les mécanismes déterminants ces effets, les études des interactions entre les ions d'intérêt dans les milieux aqueux, à l'interface peau-solution aqueuse et dans la peau même peuvent être envisageables.

La dernière partie de cette thèse se focalise sur les effets de la formulation des eaux thermales, qui sont des mélanges d'ions et notamment à l'absorption cutanée des ions calcium et de magnésium. 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 cette dernière partie était 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.

Pour cette raison, une eau thermale commerciale (TSW) 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). Les émulsions étudiées ont été préparées à base d'émulsifiants d'origine végétale pour suivre les tendances actuelles du marché cosmétique. 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.

Les résultats obtenus indiquent que les ions magnésium et calcium pénètrent dans la peau depuis l'eau thermale, utilisée comme contrôle. L'encapsulation d'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 Ca^{2+}/Mg^{2+} 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 Ca^{2+}/Mg^{2+}élevés dans la peau.

Au vu de ces résultats on peut supposer que 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 dépend de la formulation qui les contient.

Conferences

Works included in this thesis were presented during several international and national conferences listed below.

International:

IFSCC (30 September- 3 October 2019, Milan, Italy)

Poster: ***The effect of vehicle on skin absorption of Mg^{2+} and Ca^{2+} from Thermal Spring Water***

Skin Forum (23-24 September 2019, Reims, France)

Poster: ***The effect of vehicle on skin absorption of Mg^{2+} and Ca^{2+} from Thermal Spring Water***

ECIS (8-13 September, Louvain, Belgium)

Poster: ***Skin penetration of halide ions from concentrated aqueous mixed solutions***

Formulation Days (18-19 January 2019, Lyon, France)

Talk: ***Effects of thermal spring water formulation on skin absorption of calcium and magnesium sations***

IFSCC (18-21 September 2018, Munich, Germany)

Poster: ***Percutaneous absorption of mixture of ions found in Thermal Spring Waters***

Skin Forum (20-21 June 2018, Tallinn, Estonia)

Talk & poster: ***Skin penetration of thyroid disturbing anions***

BTS Annual Congress (16-18 April 2018, Newcastle, UK)

Poster: ***Skin penetration of thyroid disturbing anions***

National:

Journée régionale des innovations thérapeutiques : avancements et perspectives (27th January 2017, Lyon, France)

Talk: ***Mécanismes de pénétration des ions dans la peau***