Development of a percutaneous penetration predictive model with chromatographic and spectroscopic tools

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JURY

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Résumé

Le stratum corneum (SC), couche supérieure de l'épiderme, est composé principalement de cornéocytes entourés d'une matrice lipidique. Cette structure particulière confère au SC son rôle de barrière et protège l'organisme de la perte en eau, de la pénétration de substances exogènes et de l'irradiation ultra-violette (UV). La matrice lipidique du SC est constituée de trois lipides majeurs : les céramides, les acides gras et le cholestérol organisés en phase cristalline. Cette matrice est la principale voie de pénétration des molécules exogènes à travers la peau. L'estimation de l'absorption cutanée pour l'analyse du risque des produits cosmétiques est basée sur les recommandations de l'Organisation de Coopération et de Développement Économiques (OCDE) qui prend en compte les propriétés physicochimiques des molécules i.e. Log Pow (lipophilie) et MW (masse moléculaire). En effet, l'OCDE considère une absorption de 100% pour une molécule ayant une MW inférieure à 500g/mol et un Log Pow compris en -1 et +4. En dehors de ces valeurs, l'OCDE applique une estimation de 10%. Hors, cette estimation est bien souvent loin de la réalité et a besoin d'être affinée. Notre travail s'est focalisé sur le développement d'un critère d'évaluation de la pénétration cutanée afin de moduler les données de l'OCDE par trois approches différentes : chromatographie d'affinité, spectroscopie de fluorescence et microspectroscopie infra-rouge à transformée de Fourier (FTIR) avec une source synchrotron.

Etant donné que les propriétés barrières de la peau sont étroitement liées à la composition en céramides du SC, les méthodes développées en chromatographie d'affinité et spectroscopie de fluorescence se sont focalisées sur l'interaction céramide-molécules. Un critère prédictif de la pénétration percutanée a été défini avec chacune de ces méthodes : α et ΔI. La troisième méthodologie a consisté à développer un autre critère (S_{index}) par microspectroscopie FTIR avec une source synchrotron. La distribution cutanée des molécules a été suivie sur coupes microtomées de biopsies humaines. A partir de S_{index}, une cartographie prédictive de la pénétration percutanée des molécules a été établie. Notre design expérimental comprenait des molécules (filtres UV, conservateurs, actifs cosmétiques) avec des Log Pow et MW variés (cf annexe 1). La pénétration cutanée de ces molécules a été étudiée avec une méthode de référence : cellules de Franz couplées à la chromatographie. Ces données de référence ont servi à valider les modèles et critères prédictifs développés.

Ce travail a permis d'explorer de nouvelles pistes pour l'étude prédictive de la pénétration percutanée et de développer ainsi de nouveaux critères. Utilisés en complément des propriétés physicochimiques des molécules, ces nouveaux critères permettent d'affiner l'estimation de la pénétration cutanée de molécules exogènes pour l'analyse du risque.

Mots clés : pénétration percutanée, céramides, spectroscopie de fluorescence, chromatographie, microspectroscopie FTIR, synchrotron

Abstract

The stratum corneum (SC) is the upper skin layer and due to its particular composition, corneocytes embedded in a lipidic matrix, it owns a role of barrier function and protects our body against water loss, penetration of exogenous molecules and UV irradiation. Its lipidic matrix is composed of three major lipids: fatty acids, cholesterol and ceramides, organised in liquid crystalline phase. This high cohesion creates cement between corneocytes. This cement is the principal pathway taken by the exogenous molecules to penetrate the skin. Percutaneous penetration estimation of cosmetic products is today based on the Organisation for Economic Co-operation and Development (OECD) recommendations, regarding molecules structural characteristics i.e. Log Pow (polarity) and MW (molecular weight). The OECD claims that 100% dermal absorption may be assumed if the exogenous molecule molecular mass is lower than 500 g/mol and Log Pow ranged between -1 and +4. Besides these values, a 10% coefficient is applied. This approach is sometimes far from reality. Our work focused on developing new evaluation criteria of percutaneous penetration from three different approaches: affinity chromatography, fluorescence spectroscopy and FTIR microspectroscopy with a synchrotron source in order to modulate OECD predictions.

Considering that skin barrier properties are closely linked to ceramide composition and conformation within the SC, two methods were developed to study the interaction between ceramides and exogenous molecules by affinity chromatography and fluorescence spectroscopy. A predictive criterion of percutaneous penetration was developed from each of these methods: α and ΔI. The third methodology consisted of developing a predictive criterion, $S_{index,}$ by FTIR microspectroscopy with a synchrotron source, on microtomized cuts of human skin biopsies. A predictive cartography was build from S_{index}. Our experimental design included exogenous molecules (e.g. UV filters, preservatives, cosmetic actives) with various Log Pow and MW (cf annexe 1). Molecules skin penetration was studied with a Franz cell device coupled to HPLC analysis. These results served as reference data to validate our predictive models and criteria.

This work permitted to set up new methods for predicting skin penetration of exogenous molecules and to develop complementary predictive criterion to Log Pow and MW. These new criterion will serve to modulate OECD predictions.

Key words: percutaneous penetration, ceramides, fluorescence spectroscopy, chromatography, FTIR microspectroscopy, synchrotron

Abréviations

AB: avobenzone
Ac: acetone
ACN: acetonitril
AD: atopic dermatitis
ANN: Artificial neural network
BP: butyl paraben
BP3: benzophenone 3
BPA: benzophenone A
CAF: caffeine
CER: ceramide
Ch: chloroform
CL: chloroxylenol
ComDim: Codimensial analysis
DB: diobenzone
DE: dermis
DPH: 1,6-diphenyl-1,3,5-hexatriene
EA: ethyl acetate
EP: epidermis
EP: ethyl paraben
EPtot: total epidermis
FTIR: Fourier transformed infrared
HPLC: high performance liquid chromatography
Ifluo: fluorescence intensity
IR: irgansan
Kp: permeation coefficient
LIP: cutaneous lipids, EA: ethyl acetate
LMR: linear multiple regression
LOD: limit of detection
Log Pow: partition coefficient
LOQ: limit of quantification
m-THPP: 5,10,15,20-tetrakis (3-hydroxyphenyl)-21H,23H-porphirine
MeOH: methanol
Mol: exogenous molecules
MP: methyl paraben
MW: molecular mass
OC: octocrylene
OECD: Organization for Economic Co-operation and development
OS: octisalate
OX: octinoxate
PA: palmitic acid
PAMPA: parallel artificial membrane permeability assay
PCA: principal component analysis
PP: propyl paraben
PRED: prednisolone
QRPR: Quantitative retention permeability relationships
QSAR: Quantitative Structure Activity Relationships
S/R: specific band/reference band
SAXD:
SC: stratum corneum
SCCS: Scientific Committee on Consumer Safety
SDS: sodium dodecyl sulfate
SLS: sodium laury sulfate
TS: tape stripping

Sommaire

Introduction Générale

Le stratum corneum (SC), couche supérieure de l'épiderme, est constitué principalement de cornéocytes, cellules dépourvues de noyaux, entourés d'une matrice lipidique. Cette matrice est composée de céramides, acides gras et cholestérol. Depuis la découverte de la fonction barrière de la peau au niveau du SC au milieu des années 1940, il a rapidement été montré que les lipides détenaient un rôle majeur dans la protection contre la pénétration percutanée de molécules exogènes. La voie principale de la pénétration des molécules exogènes se trouve à travers le ciment lipidique intercornéocytaire, dont l'organisation supramoléculaire est à l'origine de voies de passage polaires et apolaires.

Les laboratoires se doivent de prouver l'innocuité de leurs produits cosmétiques ou pharmaceutiques. Dans le but d'assurer la sécurité des produits appliqués sur la peau, la réglementation impose des tests obligatoires avant toute commercialisation. En effet, si un produit à action revendiquée locale est absorbé au niveau du derme, il peut alors passer dans la circulation sanguine et présenter un risque potentiel pour le consommateur. Depuis l'interdiction des tests sur animaux pour les produits cosmétiques, le test principal pour étudier la pénétration cutanée de molécules est réalisé sur biopsies cutanées par cellules de Franz. Même si cette technique est très efficace, elle rencontre certaines limites dues par exemple aux différents types de peau utilisés (animale ou humaine), au traitement des biopsies (dermatomée, entière) ou au véhicule choisi pour tester l'absorption d'une molécule. Par exemple, les études réalisées sur l'analyse du risque et la pénétration cutanée des parabènes sont difficiles à comparer en raison des différentes formulations choisies car plus les parabènes sont solubles dans leur véhicule, plus ils sont retenus et pénètrent peu dans la peau. De plus, les résultats des différentes études ex vivo dépendent des conditions expérimentales. Il est donc compliqué de tirer des conclusions précises sur la pénétration d'une molécule à risque potentiel lorsque que les conditions expérimentales sont difficilement comparables entres elles.

Pour remédier à ces facteurs de variabilité, de nombreux modèles mathématiques ont été développés afin d'établir un modèle prédictif de la pénétration cutanée. Seuls certains d'entre eux ont été validés par l'OCDE comme le modèle QSAR de Potts et

Guy. Les modèles validés se basent principalement sur les propriétés physicochimiques des molécules, leur lipophilie (Log Pow) et leur masse moléculaire (MW). De nombreuses études ont mis en doute la fiabilité de ces modèles car bien souvent ces modèles se retrouvent peu corrélés aux données de pénétration cutanée.

Dans ce travail nous avons donc ainsi décidé de développer de nouveaux critères de l'absorption cutanée afin d'affiner les recommandations de l'OCDE. Ces nouveaux critères permettraient, utilisés en compélement du Log Pow et MW, de mieux estimer la pénétration de molécules exogènes. Trois méthodologies ont été développer afin de définir ces nouvaux critères : en chromatographie d'affinité, spectroscopie de fluorescence et microscpectroscopie FTIR couplée à une source synchrotron.

De nombreux travaux ont montré le rôle primordial des céramides dans la barrière cutanée et de récentes études se sont focalisées sur ces lipides afin d'étudier leur rôle dans la pénétration cutanée dans le but de développer de nouvelles méthodologies prédictives pour l'analyse du risque. Jusqu'alors, l'interaction entre les céramides du SC et des molécules exogènes a été très peu étudiée. En première partie de ce travail, l'interaction céramides du SC/molécules exogènes a donc ainsi été exploitée *in-vitro* afin de définir de nouveaux critères prédictifs de la pénétration percutanée par chromatographie d'affinité sur phase stationnaire de carbone graphite poreux (PGC) modifiée par un céramide. En deuxième partie, le même type d'approche a été réalisé en spectroscopie de fluorescence à l'aide de sondes de fluorescence. Enfin, une troisième méthode prédictive en microspectrospie FTIR avec une source synchrotron a été développée *ex-vivo* afin d'établir un critère de la prédiction de la pénétration cutanée prenant en compte l'ensemble du tissu cutané et non une interaction spécifique avec un céramide. Afin de valider les approches développées, la pénétration percutanée des molécules d'intérêt, principalement des filtres UV et conservateurs (cf annexe 1), a été évaluée avec une méthode de référence *ex vivo* (les cellules de Franz) utilisant des biopsies humaines.

Un premier protocole d'étude de l'interaction d'un céramide avec des molécules exogènes par chromatographie d'affinité a été mis au point. En se basant sur le modèle QSPR des colonnes IAM, servant à prédire l'absorption gastro intestinale, nous avons imprégné une colonne carbone graphite poreux de céramides. Une fois

cette imprégnation réalisée, nous avons étudié les différents temps de rétention des molécules étudiées. Un critère α calculé à partir des variations des temps de rétention a été comparé aux données de pénétration cutanée de référence obtenues avec des cellules de Franz.
Puis, un protocole d'étude a été établi par spectroscopie de fluorescence fondé sur la variation de l'intensité de fluorescence en fonction de l'interaction céramides/molécules exogènes. La lipophilie de la sonde étudiée a limité la sélection des molécules à celles présentant une lipophilie relativement élevée. A partir de ces résultats un critère de la fluorescence, ΔI, a été calculé et comparé aux données de pénétration cutanée de référence en cellules de Franz.

Enfin, une mesure semi-quantitative des molécules exogènes dans les différents compartiments cutanés de biopsies abdominales après 22h de contact a été effectuée par microspectroscopie FTIR avec une source synchrotron. Les données de distribution tissulaire ont permis, après analyse chimiométrique, de sélectionner un critère, S_{index}, sur la base duquel il est possible d'établir une cartographie S_{index} / Log Pow prédictive de la pénétration percutanée.

Les apports et les limites de ces différentes approches ont été ensuite discutés.

L'objectif final serait, après sélection du critère le plus pertinent résultant de ces études de faisabilité, de constituer une nouvelle base de données regroupant la valeur de ce critère prédictif calculé sur un grand ensemble de molécules d'intérêt cosmétique. Cela nécessitera l'élaboration d'un protocole d'étude strictement défini et identique pour toutes les molécules. En plus du Log Pow, il existe des bases de données sur le coefficicient de perméabilité, Kp, mais, plus que le flux transcutané de substances, ce qui nous intéresse dans ce travail est la prévision de la distribution cutanée de molécules pour une analyse du risque à priori, avant le développement de la formulation.

Etude Bibliographique

Chapitre 1

Role of skin lipids in barrier function and percutaneous penetration: From emerging concepts to the discovery of the leading role of ceramides

I- INTRODUCTION

Since the discovery of the skin barrier function localization in the SC and its particular organization, corneocytes imbedded in a lipidic matric, intercellular lipids were rapidly shown to retain a major role in skin homeostasis and in protection against percutaneous penetration. Recently, with the banning of animal testing for cosmetics and the need to discover economic and effective methods to assess the risk of new topical ingredients, interest in understanding lipids interaction (more particularly ceramides) with exogenous molecules, has became a major challenge to develop the most effective predictive tools. This review summarizes first, the emerging concepts of the barrier function and the discovery of the importance of SC lipids in skin permeability, and then it focuses on the importance of SC ceramides in skin homeostasis and the growing interest to understand ceramides role in percutaneous penetration for the development of alternative methods to the classic permeability tests.

II- ROLE OF LIPIDS IN SKIN BARRIER FUNCTION AND PERCUTANEOUS PENETRATION

1- Emerging concepts of importance of skin lipids for barrier function

a- Great Depression: First interest in skin lipid composition

When the world was facing the Great Depression in the early thirties, new concepts on skin lipids emerged. In 1932, Kooyman described lipids as "The general term applied to substances utilized by the living cell that are insoluble in water and soluble in the ordinary fat solvents and that have some real or potential relationship to the fatty acids as esters" [1]. At this time, the presence of phospholipids was characteristic of active tissues. Scientist already demonstrated the keratinization process. Keratinization is the skin process in which cells from stratum granulosum are displaced toward SC and undergo a change of activity with the disappearance of their organelles and synthesis of keratin, the major structural protein of epidermis, nails,

and hair. A need emerged to understand if there was a change of lipids content within epidermal cells during keratinization.

Kooyman analyzed variation of phospholipid content during keratinization. Analysis were made with skin from humans sole or palm in order to have a skin surface without sebaceous glands that could influence epidermal lipid content [2]. Values of phospholipids, cholesterol and fatty acids content were determined with methods developed during the decade that helped to profile lipidic classes [3,4]. Kooyman found that phospholipid/cholesterol ratio, from palm and sole, was ten times higher in the basal layers compared to the horny layers. Half of the cholesterol was found to disappear during keratinization. Fatty acids were found to be highly unsaturated in stratum germinatum and became more saturated in SC. Not until 1975 were ceramides found in SC.

b- Glorious thirties: SC is the protective barrier

Even though all lipids classes were still not yet identified, the concept of a barrier located in the SC started to be consider by Winsor in 1944 [5]. Winsor removed abdominal SC from patients with sandpaper. The removal with sand paper was done at different pressures from soft to hard in order to remove a little or more SC. Skin with a greater quantity of SC removed had a transepidermal water loss (TEWL) as high as skin with no epidermis. Measurements of TEWL were made at this time in a small chamber with a method developed by Neumann [6]. With this observation, Winsor offered the concept that SC may be the skin layer mainly responsible for the inhibition of water diffusion. Blank described the SC as a thin layer with the tape stripping method, a method just previously developed by Wolf and Pinkus [7–10]. Tape stripping is a simple and efficient method where the cell layers of the stratum corneum are successively removed from the same skin area using adhesive films. After removing the first layers of SC with 8 tapes, Blank observed no increased of water loss until 8 strips, when there was an abrupt diffusion rate increase (figure 1) [11].

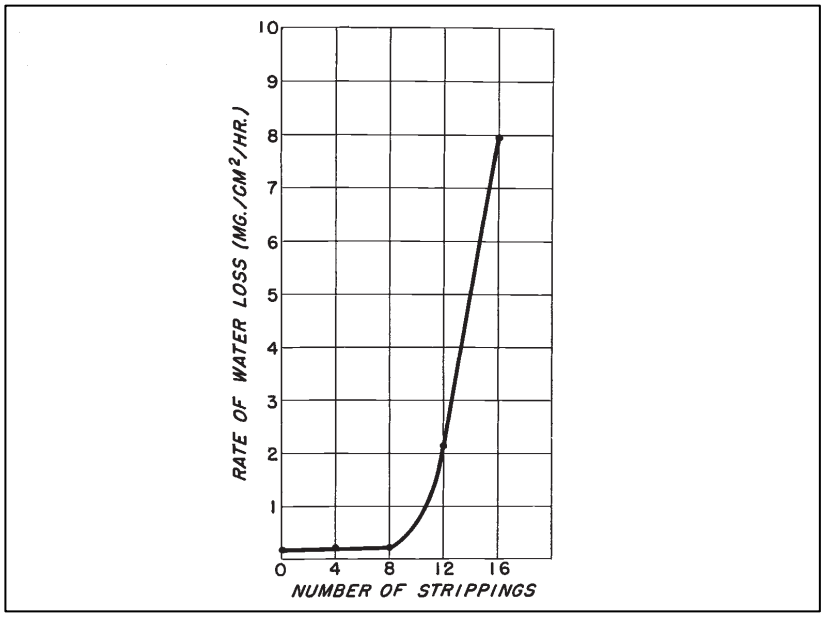


Figure 1: Showing the effect of tape stripping on TEWL. After 8 tapes, TEWL significantly increased [11].

This observation suggested that the major barrier against water loss in skin over most areas of the body is not the entire cornified epithelium itself but is a thin layer near the base of the SC.

c- Nineteen Seventies: Role of skin lipids in barrier function

With the development of diffusion chamber and lipids profiling by thin layer chromatography, Sweeney and Downing observed in 1970 seven classes of lipids from mouse skin extracted with organic solvents [12–14]. They correlated the type of lipids extracted with its influence on water diffusion but didn't find a correlation.
Breathnach determined the role of skin lipids in the barrier function by freeze fracture replication of epidermal cells [15]. He showed that the material present within intercellular spaces of the SC represented lamellar granules with an intercellular interval of 11-14 nm (figure 2) and suggested that this lamellar phase constitutes a stronger cohesive bond between the cells and give SC its barrier properties.

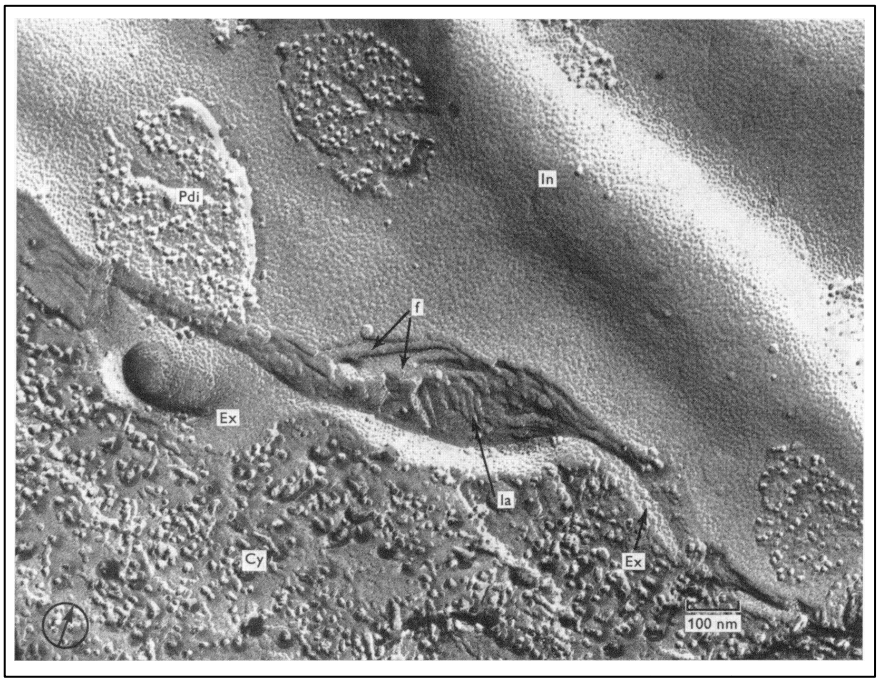


Figure 2: Replica from a deep level of SC illustrates lamellated material (la) between cells, (Cy) cytoplasm, fracture face of plasma membrane towards this exterior (Ex) or interior (In) [15]

This study demonstrated the importance of skin lipids present in the intercellular spaces for the barrier function and the skin impermeability to water diffusion. Scheuplein demonstrated the location of the skin impermeability in the SC and the fact that the permeation through it was a passive phenomena rather than a biological one [16–20].

In 1975, Michaels, elucidated skin characteristics, besides its composition and microstructure, for its permeability and selectivity of permeation [21]. He showed that there are two pathways for molecules to penetrate through the skin barrier. One pathway is through the intercellular lipids that he named “L”, the second pathway is through the cells and proteins, that he named “P”. Within each phase, the permeating species is assumed to have a characteristic solubility and diffusivity (figure 3). This simplified model of SC is famously known as the “Brick and Mortar model”, where the cells are the brick and intercellular lipids the mortar.

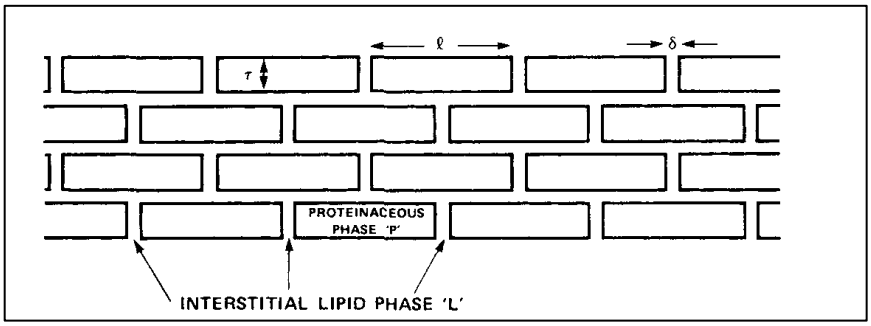


Figure 3: Idealized model of SC from Michaels, famously known as the "brick and mortar model". Two pathways are proposed for molecules to penetrate through the skin barrier: one pathway is through the intercellular lipids that he named "L", the second pathway is through the cells and proteins, that he named "P" [21].

The interstitial lipid barrier to permeation in the SC is comprised of two-dimensionally ordered, lipid bilayer membrane. Michaels defined that the maximum attainable rate of permeation of a specific drug through intact skin will depend primarily upon of the water solubility of the drug and the oil/water partition coefficient of the drug, which largely governs the specific permeability of the skin to that compound.

Elias, with the freeze section technique, highlighted at the same period that the intercellular region in the SC were rich in lipids and had a role in percutaneous penetration [22]. Thin-sections of intact mouse skin followed by a freeze-fracture permitted delineate barrier sites within keratinizing epithelia. The intercellular regions of the SC were shown to be expanded, structurally complex and lipid-rich region that may play an important role in percutaneous transport. In parallel, epidermal sheets perfused in vitro with electron-dense tracers confirmed the impermeable nature of intact SC and it lipophilic nature. Water-soluble tracers couldn't penetrate the cornified cells or to the intercellular spaces except where cells were detached. However, he showed that with the use of lipid solvents and surfactants, the same hydrophilic tracers penetrate though intercellular pathways. These agents cause increased cell separation, presumably through extraction of intercellular lipid-rich material.

Before 1975, there was little quantitative data on epidermal lipids and ceramides had not been identified as a skin lipid component [23,24]. With the development of a new chromatographic protocol to separate lipids and the application of a method developed by Kisic to determine ceramides by estimation of long chain base [25,26],

Gray managed in 1975, after lipid extraction, to profile ceramides as another minor component of the epidermis in pig, human and rat. He also quantified the different skin lipids profile; before his work, other studies were mostly semiquantitative [27]. Phospholipids in pig, human, and rat skin cells accounted for respectively, 62%, 53%, and 35% of the total lipids. Phosphatidylcholine (34-38%), phosphatidylethanolamine (18-23%), and sphingomyelin (17-21%) were major compounds in all species. He demonstrated in pig skin that phospholipids, which predominated in the basal and spinous cells, accounted for only 21% of the total lipids in the granular cells and less than 0.1% in the SC [28]. The proportion of ceramides was much higher in the SC (17%) and granular cells (9%) than in the basal and spinous layers (1%).

Elias showed the origin of SC lipids by freeze fracture of mouse SC and lipids components analysis [29]. He showed that lamellar bodies that secreted glycolipids and free sterols metabolized in free fatty acids, ceramides and cholesterol (figure 4). These three lipids coalesced with free sterols to form broad bilayers of the SC interstices. He suggested that this unique lipidic composition and organization in the SC suit the functions as the principal epidermal permeability barrier (figure 5).

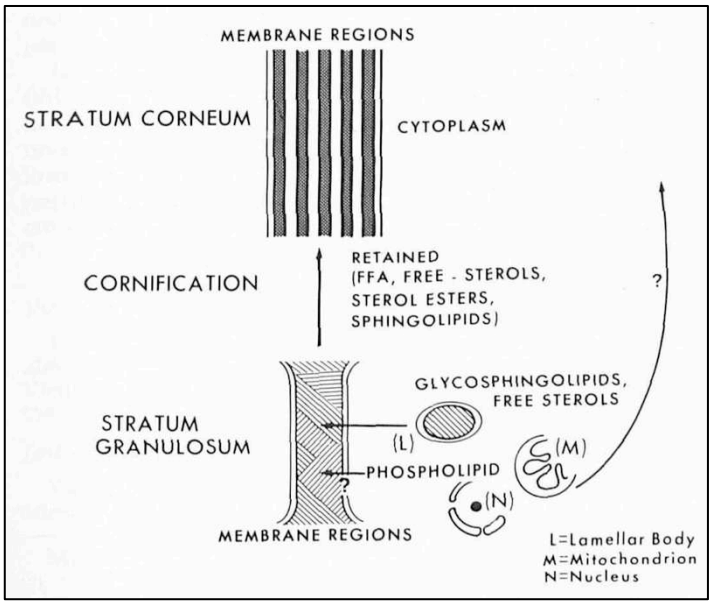


Figure 4: Model of precursors and products of intercellular lipids in SC by Elias [29].

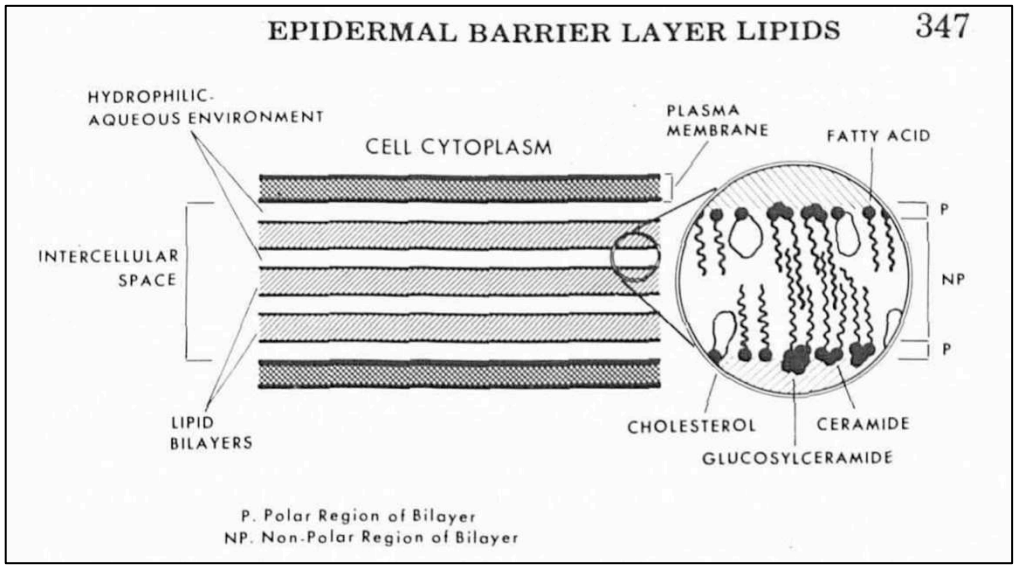


Figure 5: Composition of the SC lipidic bilayer in intercellular spaces proposed by Elias [29].

Grayson showed in early 80's that 80% of the SC lipids were located in the intercellular space and only 3% in the cytoplasm [30]. Also this distribution is homogenous in the whole SC. Solvent can remove these lipids. In consequence, changes in SC lipids and organization were supposed to have possibly a serious impact on the barrier function and percutaneous penetration.

d- Nineties: Importance of lamellar secretion for barrier repair

In the early nineties, concepts emerged about the importance of the lipid structure within the SC for the maintenance the barrier function. Lamellar body secretion is activated after barrier disruption [31]. The consequence of this secretion is the migration of these lamellar bodies at the uppermost cellular layer, and the dumping of their content into the intercellular spaces at the granular-cornified cell interface. Sphingolipids were shown to have a major role in this barrier function. Holleran demonstrated that the synthesis of sphingolipids was increased after barrier disruption in mouse skin. This increase stopped once the level of sphingolipids normalized [32]. Holleran also assessed the impact of sphingolipid synthesis inhibition on epidermal barrier recovery after acetone treatment. After barrier alteration, the action of the inhibition of the synthesis slowed barrier recovery of a delay of more than six hours. However an external application of ceramides permit barrier to recover. These studies demonstrated the important role of sphingolipids for barrier homeostasis.

After the extrusion of lamellar bodies at the stratum granulosum/SC interface their content is secreted into the intercellular spaces. There, the polar lipid precursors, glycosphingolipids, are enzymatically converted into nonpolar products, sphingolipids, and assembled into lamellar structures surrounding the corneocytes [33].

Holleran studies demonstrated the importance of the skin lipids nature for barrier permeability. Some scientists thought at this time that the barrier homeostasis did not depend on the lipid nature [34,35]. Other studies reinforced the importance of the lipid nature on the barrier function [36,37].

e- Comments

It was a combination of histological and analytical methods that leaded to the understanding of the SC organization and composition. For example histological methods such as freeze fracture cryoscopy helped first to localize the lipids in the intercellular spaces. In complement, analytical methods development permitted to quantify and to discover the unique lipidic composition of the SC. The main methodology used for lipid profiling was by extraction of epidermal lipids with different organic solvents and analysis by thin layer chromatography.

Most of these experiments were realized in vitro rather than in vivo. Even for water loss measurement and tape stripping, experiments were run on SC sheets or excised skin. The skin type came from different man or animal cadaver or from human blisters. Most studies were done on mice skin.

The use of mouse epidermis can be discussed because several studies showed differences between human and other skin models. Bronaugh tested the penetration of three compounds on human, rat, mice, hairless mice and pig skins [38]. He noted that the thickness of the SC was greater in pig skin and the thinnest in mouse skin and their thickness did not correlate with permeation rate. Differences in skin absorption may be in fact related to the animal skin structure. The lipid composition can vary significantly between the models as Gray showed it previously [39]. Simon discussed the variation between hairless mouse and human skin models [40]. For example, the physicochemical properties of the compound tested for percutaneous penetration have to be taken in account for the model choice. The testing of new molecules directly on mouse skin may be at risk if we cannot correlate the result to previous experiment on human.

2- Intercellular lipids organization and composition

a- Organization

Since the fifties, X-ray diffraction was a very useful technique to study lipid organization within SC. This technique led scientist to think in a first time that intercellular lipids were organized as a similar pattern surrounded keratin filaments. Whereas later, intercellular lipids were revealed to be organized in lamellar phase and arranged in two crystalline coexisting lamellar phases with repeat distances of 13 and 6 nm, by small cross section of the small angle X-ray diffraction (SAXD). These lamellar phases are referred to as the long periodicity phase (LPP) and the short periodicity phase (SPP). The presence of this phase plays an important role in skin barrier [41–43].

Cutaneous lipids status can be described by infrared descriptors. Vibrational methods such as FTIR, showed the organization of SC lipids in orthorhombic packing. This packing indicates that lipids within the lamellae are very densely packed, essential for a proper skin barrier function. Vibration of CH_2 (νCH_2) from skin lipids originate an infrared signal around 2850 cm^{-1} that is specific of lipids organization and correlated to cutaneous barrier status [44]. Skin νCH_2 stretching mode is comprised between 2847 and 2856 cm^{-1} and describes an orthorhombic, hexagonal or disorganized packing of cutaneous lipids (figure 6) [45]. An orthorhombic lipid organization reflects a compact lipid organization related a normal barrier status. If lipids are closer to a disorganized state, barrier is defective.

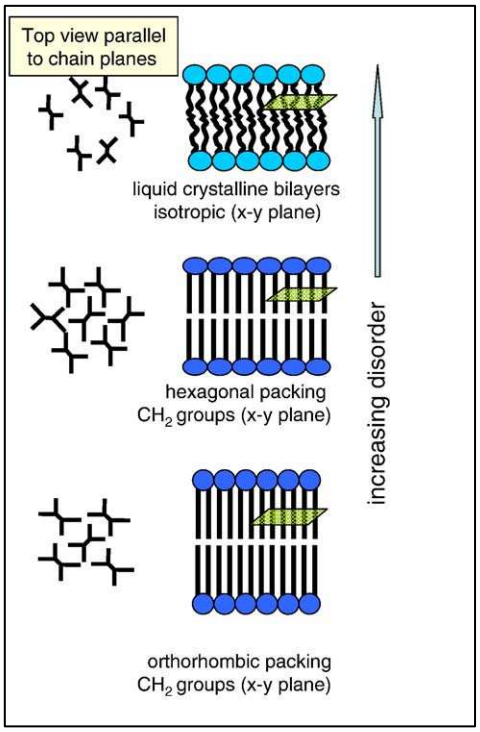


Figure 6: Schema of the conformational ordered (bottom) and disordered (top) lipid phases thought to simultaneously exist in the lipid matrix of the SC [45].

Iwai recently determined the molecular organization of the skin's lipid matrix in situ, in its near-native state by sing a methodological approach combining very high magnification cryoelectron microscopy (EM) of vitreous skin section defocus series, molecular modeling, and EM simulation [46]. He found that the lipids are organized in stacked bilayers of fully extended ceramides (CERs) with cholesterol molecules associated with the CER sphingoid moiety. This arrangement rationalizes the skin's low permeability toward water and toward hydrophilic and lipophilic substances, as well as the skin barrier's robustness toward hydration and dehydration, environmental temperature and pressure changes, stretching, compression, bending, and shearing.

b- Composition

The major lipid classes in SC are ceramides, fatty acids and cholestorol. Many studies reported an approximate amount of SC extracellular lipids of 50% ceramides, 35% cholesterol and 15% fatty acids in human [47] .

3- Intercellular lipids role in percutaneous penetration

a- Importance of lipids in percutaneous penetration

Since the early eighties, lipids have been shown to have a major impact on percutaneous penetration. Elias showed the importance of lipids for governing the skin permeability on legs and abdomen human skin [48]. He compared the penetration of water and salicylic acid through these two skins compartments. After determining thickness by measuring the thinnest sectioned region, skin layers and lipid amount of these skins, he examined most influenced factor that controlled the penetration. He found that the skin leg was more permeable to these molecules than abdominal skin. This observation was not related to the SC thickness or cell layers but rather to the lipid composition (Table 1).

Table 1: Average penetration in relation to the lipid composition [48].

Penetration	Leg[b]	Abdomen[b]	Significance
Water (mM/cm^2/24 hr)	8.1 ± 2.6	3.2 ± 0.8	$p < 0.0025$
Salicylic acid (μM/cm^2/24 hr)	5.7 ± 1.8	3.6 ± 0.6	$p < 0.1$
Lipid (% wet weight)	3.0%	6.8%	

[a] Lipid expressed as percent of lipid weight/tissue wet weight.
[b] Number of samples (abdomen = 15; leg = 6).

Smith showed the importance of lipids in the barrier permeability. He removed intercellular lipids and disaggregated SC cells from human calf and plant [49]. He then added lipids to reaggregate the cells and measured at the same time the TEWL. He found that the quantity of lipids in the intercellular space was inversely proportional to the water permeability. A higher flux was observed when cells were reaggregated with plantar lipids rather than calf lipids, showing the importance of the lipid organization and composition in the permeability. Recently, a positive correlation was found between total epidermal lipid composition and percutaneous permeation [50,51]. Epidermal lipid composition from four species (dog, cattle, pig, and rat) were positively correlated with percutaneous permeation rates of four non steroidal anti-inflammatory drugs with a octanol–water partition coefficient log P > 2. However, Stahl did not find a particular lipid type that especially affected skin permeability.

These studies showed the importance of the lipids in the skin permeability and absorption, however non of them highlighted the particular role of a lipid class.

b- Intercellular pathway

Because of the importance of lipids in percutaneous penetration, it was relevant to identify penetration pathways of molecules through the SC. Scheuplein identified three routes of penetration for exogenous molecules: transcellular, intercellular or follicular [16]. Barry illustrated two potential routes for drug permeation (figure 7), between the cells (intercellular route) or through the protein filled cells and across lipid-rich regions in tandem (transcellular route) [52].

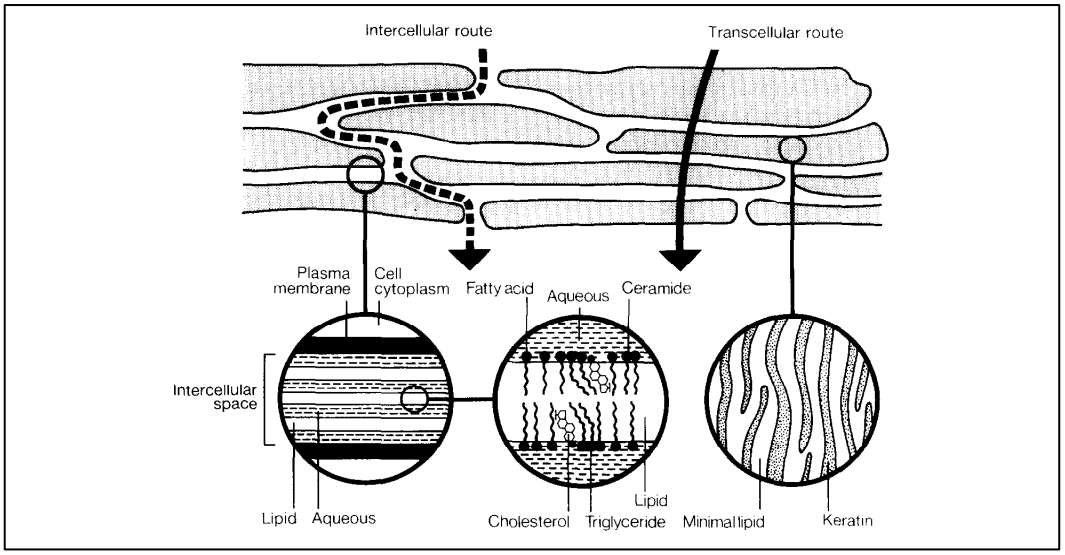


Figure 7: Suggested route of drug penetration through human SC, proposed by Barry [52].

Mali showed that the main pathway of permeation is mostly through the intercellular space rather than the intracellular or follicular pathway [53]. Guy insisted on the pathway of penetration through the SC lipids rather than the corneocytes. He also related the penetration mostly to the molecule Log Pow rather than to the MW [54]. Many studies related the predominance of the intercellular pathway on the intracellular. Nemanic showed the diffusion of molecules throught the intercellular pathway by perfusing n-butanol into human SC sheets. The n-butanol, despite being a hydrophilic molecule (Log Pow = 0,8) was revealed to be mostly distributed in the intercellular space (figure 8) [55].

Figure 8: Presence on n-butanol (black) revealed in intercellular spaces of SC by in situ precipitation [55].

Squier studied the penetration and distribution of radiolabelled cholesterol, ethanol and water on porcine skin. Skin cuts were examined by microscopic autoradiography or electron microscopic autoradiography. These molecules were predominantly present into the intercellular space [56].

Golden showed that a change in the extracellular lipid structure results in an enhancement of the skin absorption flux of a transdermal drug [57]. This observation suggests that penetration is related to the lipids structure within the intercellular space.

c- Interaction of exogenous molecules with the SC lipids

The only interaction demonstrated was besides lipophily, hydrogen bonding. An hydrogen bond is the electromagnetic attractive interaction of a hydrogen atom and an electronegative atom that come from another molecule. Potts suggested that molecules permeability through the SC was mainly due to hydrogen bonding between the molecules and the polar head of SC lipids. Mathematical models were build based on molecular volume (MV), hydrogen bond donor (Hd) and acceptor (Ha) activity [58]. The results of the regression analysis show that a model that includes only the permeant's MV, Hd and Ha can explain 94% of the variability in the data. Roberts observed also that H-bonding have a dominant effect on the percutaneous penetration

in complement of the molecular weight and solvent partition coefficient [59–61]. The hydrogen bonding between the molecule and the SC lipids can have a retardant effect on the penetration.

d- Effect of delipidization and barrier disruption

Many scientists examined the effect of delipidization on the skin permeation in order to confirm the intercellular pathway and the importance of skin lipids for governing the penetration.

Abrams looked at the effect on skin barrier of organic solvents applied on skin biopsies at different duration [62]. The barrier disruption was followed by measuring the TEWL. Some solvent mixtures such as chloforme:methanol (Ch/Me) [2:1] followed by hexane:methanol (He/Me) [2:3] caused the greatest barrier disruption when compared to untreated skin or water. These two mixtures also extracted a comparable quantity of lipids, the greatest compared to other solvents. However, despite that these two mixtures extracted a similar amount of lipids, biopsies had different TEWL variations and no extracted lipids seemed to be relevant for being implied in the variation of TEWL. Abrams suggested that SC lipids are not the only elements responsible of the barrier function.

Sznitowska looked at the penetration of baclofen in aqueous or ethanolic saturated solutions on skin that was pretreated with methanol:chloroform (Me:Ch) (1:1) or acetone:chloroform (Ac:Ch) (1:1) mixtures, or with these solvents followed by 0,2% sodium lauryl sulfate (SLS) [63]. The ethanol and the SLS did not increase the flux. However the delipidization with Me:Ch (1:1) increased penetration. The effect of delipidization depended on the polarity of the solvents used, and any enhancement in penetration was observed only when the skin was pretreated with the Me:Ch (1:1) mixture, which extracted polar lipids. Sznitowska suggested that the polar pathway may be located in the intercellular space and comprises aqueous regions surrounded by polar lipids, which create the walls of such microchannels [63].

It was suggested that delipidization also influenced the penetration of drugs. The diffusion coefficient increased of almost ten times in a delipidized SC for the permeation of a hydrophilic drug [64]. Permeability coefficients were increased for hydrophilic solutes like water, urea, and inulin after skin treatment with Ch:Me (2:1) [65]. Their permeability coefficients increased with increasing lipid extraction. Rastogi

shares the same conclusion as Sznitowska, that the extraction of intercellular lipids creates additional free volume to enhance the permeability of hydrophilic solutes through the epidermis.

Tsai investigated the effect of barrier disruption with acetone on the penetration for molecules with various lipophily [66]. The effect of compound lipophilicity was studied using sucrose, caffeine, hydrocortisone, estradiol, and progesterone as model compounds. The results demonstrated that permeability barrier disruption by acetone treatment significantly enhanced the permeability of the skin to compounds, including sucrose, caffeine and hydrocortisone. Acetone treatment, however, didn't appear to alter the percutaneous penetration of highly lipophilic compounds, such as estradiol and progesterone. This study seems coherent with Sznitowska who considered the mixture (Ac:Ch) (1:1) not appropriate for delipidization.

e- Penetration enhancers

In contrary to delipidization and barrier disruption, enhancers should have a short reversible action on the skin barrier. They are required to be non-toxic, to work rapidly and barrier properties should returned rapidly once they are removed from the skin. Many enhancers can be found, the most common ones are water, sulphoxides (ex: DMSO), azone, pyrrolidones, fatty acids, alcohols, surfactants, urea essential oils, phospholipids (ex: liposomes) and solvent at high concentration [67,68]. Terpenes were also shown to be good enhancers [69]. They are generally considered to be less toxic, with a lower irritancy, compared to surfactants and other synthetic skin penetration enhancers.

Enchancers have different way of modifying the intercellular lipids in order to increase the penetration of exogenous molecules (figure 9) [68].

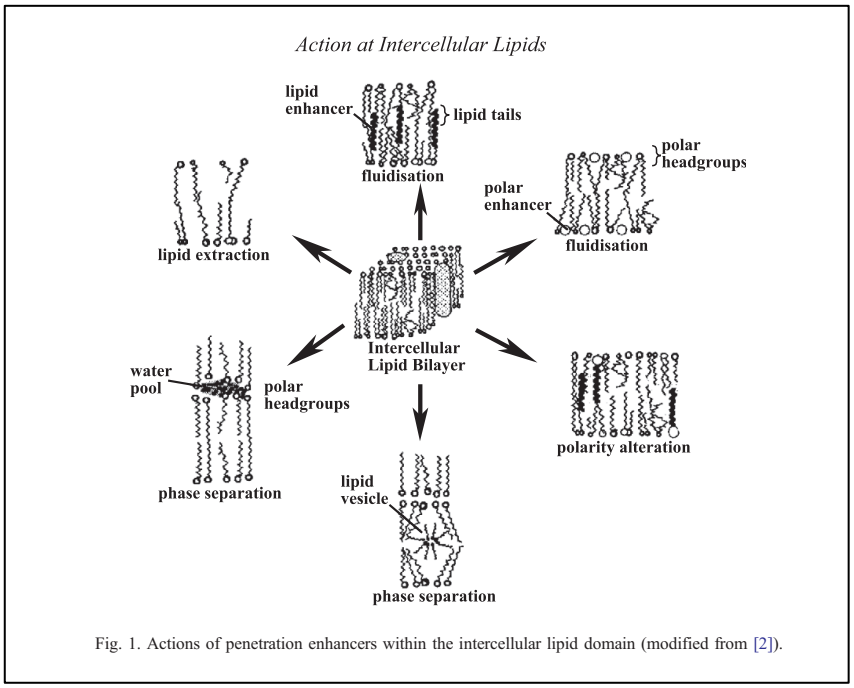


Fig. 1. Actions of penetration enhancers within the intercellular lipid domain (modified from [2]).

Figure 9: Actions of penetration enhancers within intercellular lipids [68]

It was shown by FTIR that sodium lauryl sulfate (SLS) reduces the amount of orthorhombic phase in the SC and increases the amount of hexagonally packed lipid at physiologically relevant temperatures [70].

The effect of enhancers acting on the intercellular lipids increased the fact that SC play a major in the penetration of exogenous molecules and that a modification of their structure, organization or quantity can affect drastically the permeation and the barrier function.

III- CERAMIDES: THEIR ROLE IN BARRIER FUNCTION AND PERCUTANEOUS PENETRATION

1- Context

Regarding the three lipidic classes within SC, ceramides play a moajor role in the supramolecular organization of the lipidic matrix. Orthorhombic, hexagonal and disorganized supramolecular organizations depend mostly of ceramides type and any ceramides modification can in consequence have a serious impact on barrier function. Iway recently determined that ceramide bilayer organization led the lipidic matrix to

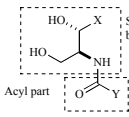
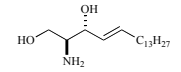
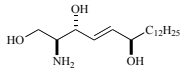
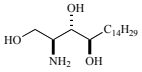
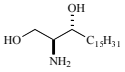
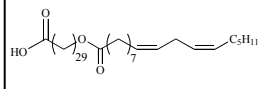
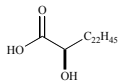
be largely impermeable to water, as well as to both hydrophilic and lipophilic substances, because of the condensed structure and the presence of alternating lipophilic (alkyl chain) and hydrophilic (headgroup) regions [46].

2- Importance of ceramides in skin barrier function

a- SC ceramides

Ceramides, the major lipidic class present in the SC, consist of a sphingoid base N-acylated by a long chain of fatty acid [71]. Eleven classes of ceramides have been determined showed in table 2 [72]. Their structural variety results from possible combination between 4 types of sphingoid base (sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) or dihydrosphingosine (DS)) and 3 types fatty acids (non-hydroxyacids, α or ω hydroxyacids, esterified (EO)) [71,73]. The acyl chain length is variable from 18 to 32 carbons. In every lipidic class, molecules vary regarding their carbon number and unsaturations.

Table 2: Structure and nomenclature of skin ceramides [72].

	 Sphingosine (S) **1**	 6-Hydroxy-sphingosine (H) **2**	 Phyto-sphingosine (P) **3**	 Dihydro-sphingosine (DS) **4**
 Lignoceric acid (N) **5**	Ceramide NS (Ceramide 2)	Ceramide NH (Ceramide 8)	Ceramide NP (Ceramide 3)	Ceramide NDS
 30-(Linoleyloxy)-triacontanoic acid (EO) **6**	Ceramide EOS (Ceramide 1)	Ceramide EOH (Ceramide 4)	Ceramide EOP (Ceramide 9)	
 (*R*)-2-Hydroxytetracosanoic acid (A) **7**	Ceramide AS (Ceramide 5)	Ceramide AH (Ceramide 7)	Ceramide AP (Ceramide 6)	Ceramide ADS

Masukawa developed a method by normal-phase liquid chromatography-electrospray ionization-mass spectrometry to quantify ceramides from tape stripping of human cheek or forearm SC [74]. In forearm, ceramide 3, 8, 6 and 7 were the most detected. In

cheek ceramides were detected at a lower level, most detected were ceramides 7 and 6 (figure 10).

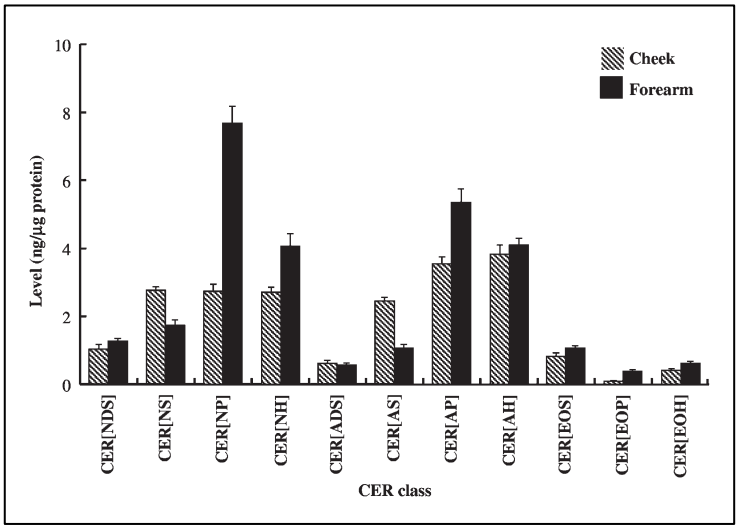


Figure 10: Average levels of ceramide classes in cheek and forearm SC [74].

b- Ceramides and the barrier function

Chain length of ceramides have an impact on the barrier function [75]. Novotny showed that ceramides with a 4- to 8-carbon acyl chain increase skin permeability for drugs up to 10.8 times with maximum effect at a 6-carbon acyl chain. Joo observed a correlation between the chain length and the barrier function [76]. He studied the combination of ceramide 2 and its correlation with the barrier function. Ceramide 2 chain length in human SC from healthy human volunteers were measured and was correlated to the TEWL of each subject. Subjects with shorter chain ceramides chain length add a more deficient barrier function, showed by an increased of TEWL. Joo suggested that longer chain fatty acid produces stronger Van der Waals forces between molecules and resultantly tighter molecular stacking.

Bowstra showed that ceramide 1 may be a major lipid to maintain the barrier homeostasis due to its structure and long acyl chain length [77]. She highlighted the importance of ceramide 1 structure in the lamellar structure organization with different lipidic mixtures.

c- Ceramides and skin disease: example of atopic dermatitis

The study of atopic dermatitis (AD), the most common inflammatory skin disease in the general population, helped to highlight the crucial importance of the presence of ceramides to maintain the barrier function. Some evidences that reinforce the fact that ceramides are essential to maintain the barrier function are that their deficiency have been related for over twenties years to atopic dermatitis [78].

AD is a chronic inflammatory skin disorder and the most frequent skin disease in children [79]. Non-lesional skin from AD patients also exhibits a defective permeability barrier that is reflected in an increase in transepidermal water loss (TEWL) [80,81]. AD skin also presents a defect in skin permeability against exogenous molecules that probably leads to a greater penetration of allergens [82,83].

In 1991 Yamamoto extracted SC lipids from AD patients in order to ascertain if any relation existed between AD skin and SC lipids [84]. Lipid classes were analyzed by high-performance thin-layer chromatography. The relative amounts of stratum corneum lipid classes did not differ statistically between AD patients and controls; however, the proportion of ceramides 1 was slightly lower in AD patients. Ceramide 1 was the most reduced ceramide in AD skin. This observation was also made by Imokawa, after tape stripping and extraction of ceramides from tapes in healthy and AD patients [85]. He observed a decrease amount of ceramides in AD patients in lesional and non lesional skin and that Ceramide 1 was the most reduced in both skins. This study showed that significant changes in the proportion of the ceramide fraction might cause the structurally impaired lipid lamellar sheets in SC, resulting in the diminished water-barrier function in AD patients. Ceramide 3 has also an important role in the barrier function. Di Nardo observed a correlation between a decrease of ceramide 3 in AD patients and an increase of TEWL [86]. No correlation between TEWL and ceramides type was found in healthy patient skins [87,88].

The importance of ceramides in barrier function led scientists to develop new drug in order to improve the barrier function in AD patients. Whereas steroids are communly use to treat AD, researchers studied the impact of ceramides application on disease skin. The application of a lipid class alone does not help recovery however, Man observed on murine skin that an application of equimolar mixture of ceramides, cholesterol and fatty acids skin lipids allows recovery of a normal TEWL. Later he showed the barrier recovery is accelerated when the ratio of any of these lipids is

increased up to 3-folds [89,90]. Chamlin tested a ceramide dominant moisturizer was tested on the skin of AD children [91]. The moisturizer contained 2,1 % ceramides, 0,8% free fatty acids and 0,8% cholesterol. During the study, TEWL was controlled as well as hydration, determined by electrical capacitance and SC integrity determined by sequential tape stripping. Both SC integrity and hydration also improved during therapy. The ultrastructure of the SC, treated with ceramide-dominant emollient, showed extracellular lamellar membranes that were absent in AD skin before treatment.

3- Ceramides and percutaneous penetration

a- Ceramides interaction with enhancers

The study of ceramides interaction with enhancers helped to understand the possible interaction of these lipids with exogenous molecules and their impact on the skin permeability.

The action of penetration modifiers was first suggested to infer with H-bonding within SC lipids. Hadgraft suggested that binding between ceramide 6 and exogenous molecules should represent the strongest intermolecular binding possible between SC lipids due to its structure [92]. Based on Hadgraft theory, Kaushik investigated the interaction between ceramides and exogenous molecules by chemical modeling [93]. Modeling studies revealed that two penetration enhancers, laurocapram and DMBIS, are capable of forming one-sided H-bonding instead of two sides that in consequence increase the penetration. However, DMMCBI and TBDOC formed multiple two-sided H-bonds with ceramide 6 suggesting retardation behaviors. All penetrations enhancer structure are presented in figure 11.

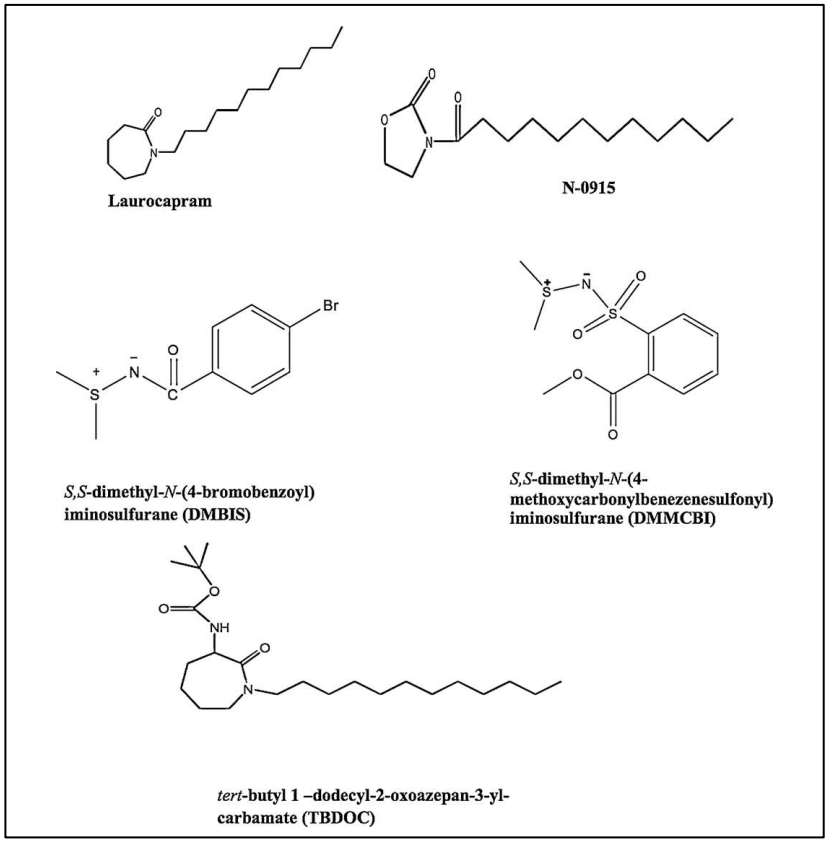


Figure 11: Structures of penetration enhancers [93]

De La Maza examined the interaction between a penetration enhancer, sodium dodecyl sulfate (SDS) and ceramide III. Liposomes were formed with SC lipids such as cholesterol, cholesteryl-sulfate (Chl-S) and palmitic acid (PA) with a proportion of ceramide III ranging from 30 to 50 %. Liposomes served to mimic the SC lipids. Fluorescent probes were entrapped inside the liposome. An interaction between the SDS and liposomes could break the liposome structure and in consequence released the probe. A fluorescence increase was then observed. The stronger the liposome was, the less fluorescence signal was detected. The author observed that liposome containing the highest proportion of ceramide III, 50%, was more resistant to the SDS interaction [94].

Guillard studied the interaction of penetration enhancers on ceramides films by FTIR [95]. Lipophilic enhancers such as oleic acid and limonene presented a direct fluidizing action on the alkyl chains and an indirect action on the polar head groups resulting in a more spacing lipid packing. Hydrophilic enhancers such as ethanol and DMSO, had no interaction on the lipid bilayer but show a complex action on the polar headgroup,

weakening the H-bonds. All the enhancers studied interacted with the ceramides that in consequences may increased in vivo the skin permeability of exogenous molecules. Merle studied the influence of ethanol on ceramides films [96]. Ethanol significantly decreased hydrogen bonding for ceramide IIIa and IIIb. New hydrogen bondings were formed between ethanol and the polar headgroup of the ceramides. In consequences, ethanol decreased the coherence between ceramides. The same was further observed ex vivo on abdominal human skin biopsies.

All these studies showed the consequences of the enhancers interaction with ceramides. The pertubation of the ceramides structure within the SC led to an icrease of skin absorption and in a desorganization of the lipidic bilayers. These studies reinforced the theory that ceramides in maintaining skin barrier functionnal and impermeable to exogenous molecules.

b- Impact of ceramides chain length on percutaneous penetration

Novotny and Janusova showed in vitro the importance of ceramides chain length on skin penetration [75,97]. They synthesized short-chain analogues of ceramide 2 from 2 to 12 carbons and evaluated their effects on the skin permeability of two drugs (Indomethacin and theophylin). Donor samples consisted of a suspension of the two drugs with 1% of ceramides. This suspension was then dropped on pig ear skin disposed on a Franz cell device. Ceramides with a 4 to 8 carbon acyl chain were able to increase skin permeability for both drugs up to ten times with maximum effect at a 6-carbon acyl chain. Janusova reported the effects of a series of ceramides having natural 18C sphingosine and varying acyl chain length on the skin permeability, measured by two drugs, indomethacin and theophylin with the same method [97]. Skin permeability increasing with the short chain ceramides was not linear but showed a maximum at 4–6C chains. The ceramide chain length was confirmed here to be crucial for against skin absorption.

c- Ceramides model membranes: a useful predictive tool to understand their impact on percutaneous penetration

The development of alternative methods to animal testing for assessing the percutaneous penetration of new cosmetic ingredients is a very current topic. Many

scientists are working on the development of artificial membranes that would mimic the skin barrier and most of these membranes are only composed of skin lipids. The interest of these membranes is that they can provide more information about role of lipids by controlling their amount and testing the role of each, such as ceramides, in percutaneous penetration.

For example, the membrane PAMPA (parallel artificial membrane permeability assay), first developed for predicting gastrointestinal absorption [98], is now developed to mimic the main barrier properties of human SC for assessing skin absorption. Ottaviani developed a PAMPA skin membrane composed of silicone oil and isopropyl myristate (propan-2-yl tetradecanoate) [99]. He found that high permeable compounds through human skin are differentiated by PAMPA into two groups, namely, compounds trapped in the artificial membrane and compounds not retained by the membrane. For a restricted set of compounds, the membrane retention determined in this new in vitro system reflects the affinity of compounds for the SC. Sinko ameliorated this model [100]. He synthetized the cetramide; an artifical ceramide analog with similar structure and properties to natural SC ceramides. Different certramides, with chain length varying from C8 to C18, were tested in mixture with varying ratio of cholesterol and stearic acid. Relevant correlation were found for membranes with certramide:cholesterol:stearic acid at 60:20:20 and cetramide chain length varying from C8-C16 up to C18-C18.

Ochalek confirmed the interest of using membrane for determining in the future the impact of each SC ceramide on the percutaneous penetration [101]. He confirmed with different analytical techniques (i.e. SAXD, HPTLC, ESEM, confocal Raman imaging, ATR-FTIR spectroscopy) that the lipids within the membrane composed of artificial Ceramide 6 or 1 with Chol, PA and Ch-S were arranged similarly to the lipids in the human SC. The diffusion studies using small hydrophilic compound revealed that the artificial lipid system used in the experiment showed strong barrier properties. Proposed approach with simple lipid model systems can be used in the future to study the impact of the different ceramide species on the diffusion and penetration of drugs and other substances of interest [102,103].

Molecular simulation of model membranes is also a new consideration from scientist to understand the impact of skin lipids. Molecular simulation was made on simple models of skin lipids that permitted the calculation of the permeability of molecules and to illustrate the mechanisms of action of the penetration enhancers [104]. Molecular

simulations are for now restricted to model membrane due to the molecular complexity of the human SC. The situation is, however, rapidly evolving and both experiments and simulations are increasingly utilizing ceramide-based model membranes.

IV- CONCLUSION

Today, with the banned of animal testing for cosmetic research and the development of new tools to predict the percutaneous penetration of new molecules for the risk assessment, interest of developing economic alternatives methods keep increasing. Since the knowledge evolution of skin barrier properties and composition, it is becoming very clear that ceramides detained a major role in cutaneous homeostasis and in the skin permeability.

However, ceramides role in percutaneous penetration and their interaction with exogenous molecules need to be more defined in order to build strong alternative method. During the last couple years, many methods were developed in order to understand better this role with various investigative tools and brings many new keys that will probably lead soon to very good predictive methods in alternative to animal testing and the use of skin biopsies.

Chapitre 2

Percutaneous penetration and risk analysis of cosmetic products

I- INTRODUCTION

According to the European Commission, a cosmetic product is defined as "Any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odors and/or protecting them or keeping them in good condition." [105]. In order to ensure cosmetics safety, some tests are compulsory before to launch a product on the market. The chapter summarizes the main skin absorption reglementations for cosmetics analysis in Europe and presents the most common predictive tools developed.

II- RISK ASSESSMENT OF COSMETICS: EVALUATION OF THEIR SKIN PERMEATION

1- Cosmetics risk analysis

The dermal absorption of cosmetics can cause systemic activity and be in consequences at risk for consumers. Some medical cases of the consequences of dermal absorption of exogenous molecules were reported by Alikhan [106]. As explained in chapter 1, the skin does not represent a complete barrier and allows the penetration of exogenous molecules though three possible pathways: intercellular, follicular and intracellular. The main factors that influence the percutaneous absorption of chemicals through the skin are: the "structure" of the skin; the physico-chemical characteristics of the penetrant; the physico-chemical characteristics of the penetrant vehicle and the dosing conditions [107]. That is why the skin distribution and absorption of a chemical needs to be clearly evaluate.

2- Evaluation of skin absorption

a- Definitions

The purpose of dermal absorption studies of cosmetic ingredients is to obtain qualitative and/or quantitative information on the amounts that may enter, under in-use conditions, into the systemic compartment of the human body. The OECD defines the percutaneous/dermal absorption process into three steps [108]:

- Penetration: which is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum;
- Permeation: which is the penetration through one layer into another, which is both functionally and structurally different from the first layer;
- Resorption: which is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment.

b- Skin absorption method

With the ban of animal testing, today the most common way to assess ex vivo cosmetics skin permeation is from human or animal biopsies. To evaluate transdermal absorption of a molecule, the most relevant membrane is human skin. The most frequent source of human skin is from plastic abdominal surgery. However, its availability is limited and animal skin is therefore frequently used. A wide range of animal models is used in replacement for human skin to evaluate percutaneous permeation of molecules. These include mostly porcine, mouse, rat and guinea pig. However, with ban of animal testing for cosmetics, porcine skin from slaughterhouse is frequently used in replacement of human skin. Porcine ear skin is particularly well-suited for permeation studies and gives comparable results to human skin [109,110]. Skin absorption studies can be made with different skin treatments. Skin can be used in full thickness, or the dermis can be cut (dermatome). Also SC can be removed by tape stripping.

35 years ago, Dr Tom Franz developed the Franz cell device, a cell diffusion tool to assess the permeation of exogenous molecules ex vivo. Franz cells revolutionized drug development for topical formulations. This device utilizes human or animal skin

(figure 1). This method can help to have both pharmacokinetic and diffusion profiles of molecules by withdrawing liquid receptor samples and also by analyzing molecules concentrations profiles within skin at the end of experiment [111].

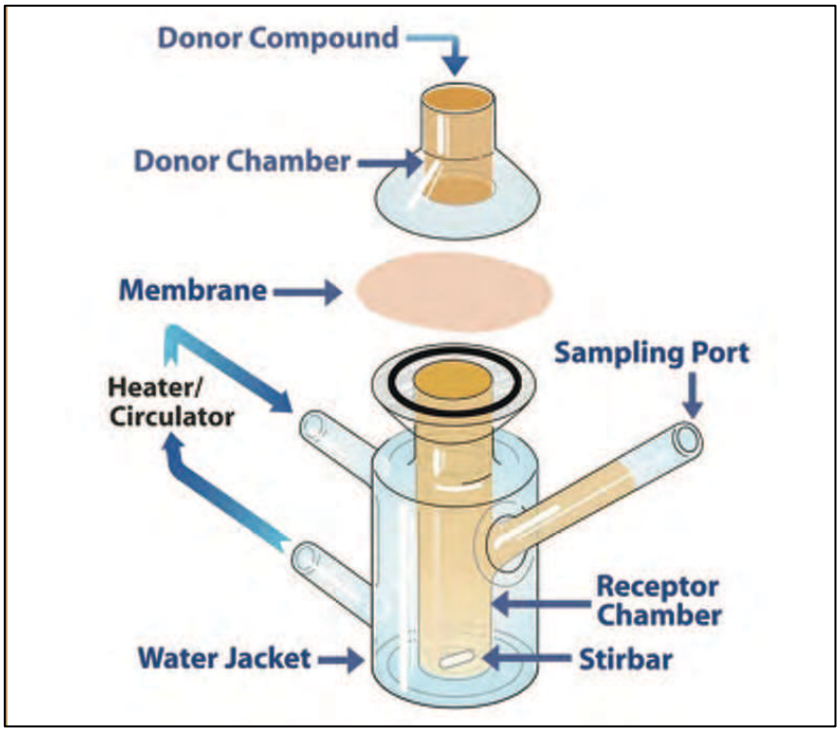


Figure 1: Franz cell device [111].

c- Fick's diffusion law and permeability coefficient

Since the main obstacle of skin permeation of solutes lies in a nonviable SC layer, Fick's diffusion law has generally accepted for the description of skin transport of permeant. According to this law, diffusion is assumed to be a course of mass transfer of individual solutes, driven by random molecular movement and the rate of transport is expressed as [112,113]:

$$\frac{dC}{dt} = Kp.C_0$$

where C_0 is the donor concentration and Kp the permeability coefficient. For non steady state studies, Fick's second law is then required.

The barrier property of the skin is characterized by its permeability coefficient, Kp for

a defined compound [114]:

$$Kp = \frac{D.K}{H}$$

where D is the diffusion coefficient, K is the compound partition coefficient and H is the skin thickness.

Kp provides an estimate of the maximum flux through the skin. However, in real-life occupational exposure to chemicals, such a scenario is rarely encountered, and so this parameter, according to the OCED, is not suitable for a risk assessment that is based on dermal absorption of finite doses. It is difficult to estimate a dermal absorption value from this parameter [114–116].

III- SKIN ABSORPTION GUIDELINES

1- European and International regulations

The Cosmetics Directive (CD) regulates scientific and technical assessment of cosmetic products in Europe. CD, firstly National laws applicable to cosmetic products, have been harmonized at European level in order to facilitate the free movement of these products within the European Union's (EU) internal market. This Directive lays down rules on the composition, labeling and packaging of cosmetic products. It also recently introduces a ban on animal testing and on the marketing of products that have been tested on animals, requires that every cosmetic product placed on the market in Europe is safe to use. The manufacturer must ensure that cosmetic products undergo an expert scientific safety assessment before they are launched for sale. The safety of cosmetics is the prime concern of the EU cosmetics legislation. The Commission is advised by scientific committees on issues related to the safety and allergenic properties of cosmetic products and ingredients. It can take several years for industries to launch a new product on the market and the manufacturer is responsible for testing and safety evaluation of new products. Competent authorities in each Member State are in charge of reviewing the safety assessments and checking products already on the market. The testing of cosmetic products is carried out by national laboratories in accordance with the requirements [117].

2- Skin absorption testing guidance

a-The Organization for Economic Co-operation and Development (OECD) recommendations

The OECD Test Guidelines are a collection of the most relevant internationally agreed test methods used by government, industry and independent laboratories to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. They cover tests for the physical-chemical properties of chemicals, human health effects, environmental effects, and degradation and accumulation in the environment [118].

Principal OECD recommendations [108]*:*

In vitro skin assessment should be conduct on diffusion cells normally unoccluded. The diffusion chamber and skin should be maintained at a constant temperature close to normal skin temperature of 32 °C. Relative humidity should be preferably between 30 and 70%. The use of a physiologically conducive receptor fluid is preferred and should not affect skin preparation integrity. Skin from human or animal sources can be used.

Epidermal membranes, dermatome skin or full thickness skin may be used. It is essential that the skin is properly prepared. As a general guidance, freshly excised skin should be used within 24h. Since skin integrity may start to deteriorate beyond 24 hours, sampling times should not normally exceed 24h. For test substances that penetrate the skin rapidly this may not be necessary but, for test substances that penetrate slowly, longer times may be required.

Under normal conditions of human exposure to chemicals, finite doses are usually encountered. Therefore, an application that mimics human exposure, normally 1-5 mg/cm^2 of skin for a solid should be used.

The skin should be washed of excess test preparation with a relevant cleansing agent, and the rinses collected for analysis. The removal procedure of the test preparation will depend on the expected use condition, and should be justified.

All components of the test system should be analyzed and recovery is to be determined. This includes the donor chamber, the skin surface rinsing, the skin preparation and the receptor fluid/chamber. In some cases, the skin may be

fractionated into the exposed area of skin and area of skin under the cell flange, and into, epidermis and dermis fractions, for separate analysis.

b- Scientific Committee on Consumer Safety (SCCS) recommendations

This Committee provides opinions on health and safety risks of non-food consumer products (e.g. cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products) and services (e.g. tattooing, artificial sun tanning). Their guidance is designed to provide guidance to public authorities and cosmetic industry [119].

The SCCS recommend to follow "OECD Guideline 428 / EC B.45 as close as possible, taking into account the guidance given here. Any deviation from this guideline should be documented and justified by appropriate scientific argumentation."

The SCCS give some precisions regarding OECD guidelines, here some of them [120]:

- Dermal absorption can be expressed as an absolute amount [µg/cm^2 of skin surface] and/or as a percentage of the amount of test substance contained in the intended dose applied per square centimeter of skin surface.
- For a reliable dermal absorption study, 8 skin samples from at least 4 donors should be used.
- The use of cultured or reconstructed human skin models is under development and those systems are not yet advised for in vitro testing on the basis of their insufficient barrier function.
- When epidermal membranes are used for the in vitro dermal absorption study, the reason for this should be justified. Epidermal membranes are sometimes quite fragile and some mass balance techniques (e.g. tape stripping) cannot be applied to this model. It must also be mentioned that epidermal membranes may overestimate human in vivo skin absorption.
- The test compound must be determined in the following compartments:

  - Product excess on the skin
  - Stratum corneum (e.g. adhesive tape strips)
  - Living epidermis (without stratum corneum)
  - Dermis
  - Receptor fluid

IV- MATHEMATICAL PREDICTIVE MODELS OF SKIN ABSORPTION

1- OECD default values

The OECD refers to European authorities recommendation by considering to reduce the 100% dermal absorption default value of a compound to only 10% if the compound molecular weight is greater than 500g/mol and its Log Pow is either below –1 or above 4 [116]. However, the OECD recognizes that the final scientific proof for neither this general assumption nor the arbitrary default numeric value of 10% has been provided (figure 2).

The main advantage of this approach is to get defaults values of a compound skin penetration without any experiments to run. It is a standardized point of view despite the fact that they are different manners to calculate Log Pow. The limit to this recommendation is that it doesn't take into account the compound vehicle. Also these values don't include intermediate situations between the 10 and 100%.

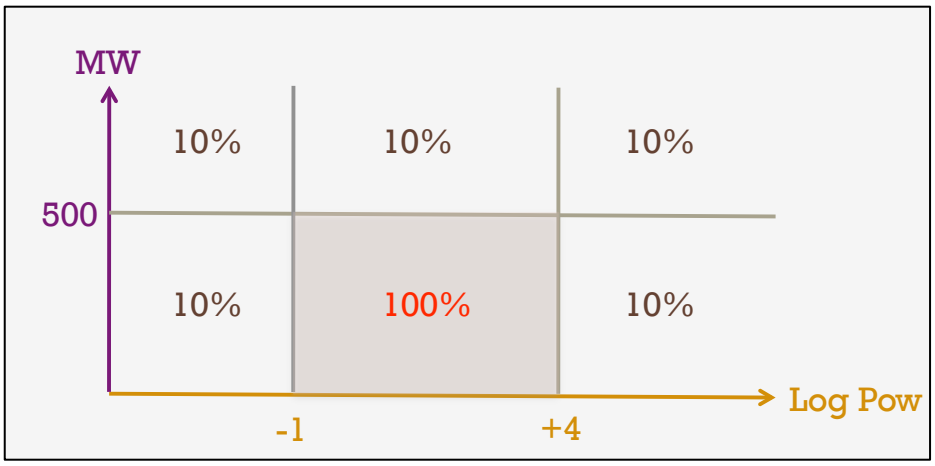


Figure 2: OECD prediction table.

2- Read across

The OECD defines the read across method as “the prediction of skin penetration of a test substance on the basis of experimental data obtained with a ‘similar’ compound, preferably from the same chemical group or class” [116].

The second application is to conclude from existing data for a certain test preparation to dermal absorption of a different preparation containing the same test substance.

The main weakness of this approach is that 'similarity' is not clearly defined and the vehicle impact needs to be taken into account. However no publications could be found on the application of Read-across technic to evaluate it.

3- Mathematical models

a- Empirical models

a-1-Quantitative Structure Activity Relationships (QSAR)

The most intensively researched area involves QSAR that correlate experimental permeation data with physic-chemical properties, or molecular structural properties of the permeant. QSAR is also known as quantitative structure- permeability relationships (QSPR). Many of QSAR models available are based on chemical permeation dataset. The most used ones are Flynn's dataset and EDETOX. Flynn's dataset possessed 97 permeability coefficients for 94 compounds obtained in vitro through human skin [121]. The EDETOX database, born from a 3 years European project, contains a dataset of a total of 300 compounds permeation data [122–124].
It is important to note that these experimental results are obtained with saturated aqueous or ethanolic solutions of a permeant. The use of theses vehicles for building database can be a limitation. Ethanol is known for being a permeation enhancer and damaging SC barrier [96].
The most famous QSAR model is the one developed by Potts and Guy in 1992 [125]:

$$Log\ Kp\ (cm/s)\ =\ 0{,}71(Log\ Pow)\ -\ 0{,}0061(MW)\ -\ 6{,}3$$

Their approach focuses on the SC as the exclusive rate-limiting barrier. The weakness of this model is to significantly over-estimates Kp for highly lipophilic compounds [126,127]. Many ameliorations of this model have been proposed, however non of them have been validated by regulation agencies.
In the past decade, many QSAR models have been proposed [128–142]. As it is difficult to compare and assess all these available models, some studies compared QSAR models available in order to determine the most relevant ones. Moss observed that the main shortfalls in QSAR models is caused by the manner in which authors employ data

from a variety of sources and, in some cases, slightly different experimental protocols. Further, most current models are based on data generated from either aqueous or ethanolic solution, where each penetrant is present at its saturated solubility or a fraction of its saturated solubility [143]. Geinoz showed that some published models are based on non significant parameters and afford misleading mechanistic insight and will lead to over-interpretation of predictive data [144]. Bouwman selected four QSAR models over more than 30 available regarding their compliance to OECD recommendations. The predictivity was low for three of the four QSAR models selected, whereas one model gave reasonable predictions [145]. Brown tested the performance of both linear, QSAR, and nonlinear models, to predict the skin permeation of a series of 11 compounds. He observed that these models are not suitable for accurately predicting permeation the models but may be suitable for determining a rank order of permeation, which may help to select candidate molecules for in-vitro screening [146].

There are a number of principal technical problems associated with modeling dermal absorption in silico, which have so far limited the applicability of QSARs to estimate dermal absorption. One of the biggest challenges is that penetration is influenced not only by molecular and physicochemical properties of the chemical itself but also by the properties of the vehicle and the structure and properties of skin, along with their interactions. Another main limitation of QSAR models is that they help to the permeability coefficient Kp or the flux but not the percentage of dermal absorption of the applied substance or its cutaneous distribution.

a-2- Artificial neural network (ANN)

An artificial neural network (ANN) is inspired by the way biological nervous systems process information. ANN is a useful modeling technique for dataset with a non-linear relationship [113]. Some studies predicted Kp with molecules from Flynn database and added new parameters such as molecular orbital. The resulting ANN model was much superior to the conventional multiple linear regression model [147]. ANN analysis was used in a study by Degim for estimation of skin permeability of 40 compounds. In this study, ANN produced log Kp values that correlated well with the experimental data (R^2=0.997) [148]. Despite these promising experiments, regulatory agencies such as OECD haven't validated the use of ANN for prediction of skin absorption yet.

a-3- Quantitative retention permeability relationships (QRPR)

Another approach consists of the use of chromatographic retention factors, obtained under adequate experimental conditions, for the measurement of physicochemical parameters. Quantitative retention permeability relationships (QRPRs) relate chromatographic parameters (rather than molecular parameters as in QSAR) to permeability coefficients [149].

Immobilized artificial membranes (IAM) is a good example of QRPR. IAM were first developed to predict intestinal absorption. IAM column stationary phase undergoes a modification with the creation of covalent link with synthetic phospholipid analogues. After column modification, fast and accurate predictions of drug distribution in biological systems can be found estimated directly with HPLC retention time measurements. Molecules of interest are injected before and after the column modification and retention times are correlated to absorption data [150,151]. IAM column were used in several studies for predicting skin absorption of exogenous molecules. Alcohols and steroids skin absorption data were correlated to IAM [152]. Barbato correlated IAM retention a set of 12 drugs types to their Log Pow and permeability coefficient [153]. Coefficients of permeability through human skin did not correlate with either IAM results or Log Pow. Lazaro found that human skin permeation is more similar to C18 partition than to IAM partition [154]. To complete IAM model a new HPLC stationary phase was prepared by physical immobilization of keratin, a major skin protein, on silica support [155]. Retention parameters determined on the keratin column along with the retention parameters determined on IAM column can be combined chemometrically to predict skin permeability differences within individual classes of drugs. Turowsky also developed new stationary phases containing covalently bound collagen, an other skin protein [156] . A series of diversified test compounds was analyzed. Collagen phases were demonstrated to possess distinctive retention properties. However interactions with collagen did not appear to be relevant for predicting skin permeation.

None QRPR models have been validated yet by regulation agencies.

b- Mechanistic models

Mechanistic models predict permeation based on knowledge of skin compartments

and diffusion pathways. The three models considered by Russell and Guy are [114] (Figure 3):

1) the SC alone as a single finite membrane

2) the SC alone where it is assumed that the membrane is sufficiently thick and the exposure time short enough, such that it behaves as a semi-infinite membrane

3) both the SC and viable epidermis (VE) as a finite two-membrane composite, incorporating the partition, diffusivity and path length variables for both skin layers.

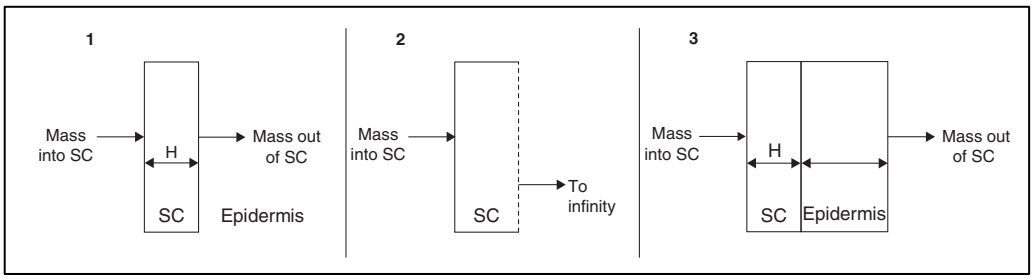


Figure 3: The three mechanistic models proposed by Cleek and Bunge: 1) Singe finite membrane 2) Semi-infinite membrane, 3) Finite two-membrane composite

A very few mechanistic models are available and capable to predict skin permeability directly. Most studies have been concerned with pathways of transdermal permeation, including the interaction with the heterogeneous structures of stratum corneum. The 'brick and mortar' model proposed by Michaels has been adopted by many researchers to estimate the diffusion length of solute in the lipid matrix [21].

Lian compared empirical and mechanistic models for the prediction of skin absorption. Lian correlated permeation results with predictive models and the best prediction found was this mechanistic model from Mitragotri, but with only a R^2 of 0,698 a little higher than the Potts and Guy model, R^2 = 0,676 [115]. Mitragotri model is defined as [157]:

$$Kp = 5{,}6\times10^{-6}\times K_{ow}^{0,7} exp(-0{,}46r^2)$$

where r is the molecular radius and Kow the partition coefficient.

According to Lian, significant progress was only made with the mechanistic approach

when Mitragotri presented a scaled particle theory on solute partition and diffusion across lipid bilayers [115,157]. This model assumes the lipidic matrix as the pathway of transdermal permeation. Its main limitation is that it under predicts skin permeability of hydrophilic compounds.

V- LIMITATIONS OF SKIN ABSORPTION STUDIES

Samaras summarized precisely all cause of variability encountered in skin absorption studies [139]. Current guidelines allow the use of animal or human skin samples and also the skin treatment prior the study (i.e. dermatome, full thickness etc.). These are sources of variability in the reported results. A good way to avoid this variability in skin procedure would be to use reconstructed epidermis for skin penetration studies, however they have not been validated yet for such studies. Their barrier allows a higher permeation compared to normal skin as the SC lipids of artificial skin are less densely packed compared tohuman SC [158].

Experimental procedures can also influence the results of the *in vitro* tests. For example, the choice of the vehicle for testing the permeant can significantly change the skin surface structure and act as a permeation enhancer (cf chapter 1). Solubility of the chemical in vehicles can significantly modify the skin absorption result. A vehicle can promote the penetration of a chemical by having low solubility, in this way a chemical will not be retained in the vehicle. In case in the vehicle there are components that can interact with the intercellular lipids of the SC then it is possible that permeation may be enhanced or suppressed. These sources of variability are discussed in chapter 3. Also, skin occlusion can enhance the kinetics of percutaneous permeation [159]. Inter- and intra-laboratory conditions can also be a source of results variation. Regarding mathematical modeling, recent studies showed for example that QSAR models are not suitable for accurately predicting permeation.

VI- CONCLUSION

Because of the limitation of the use of mathematical models, it could be interesting to develop a complementary criterion to Log Pow and MW in order to modulate percutaneous penetration estimation and to refine OECD predictions.

Chapitre 3

Assessing the Safety of Parabens: Percutaneous Penetration and Risk Analysis

Elsa Jungman, Cécile Laugel, Arlette Baillet-Guffroy

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Abstract

In cosmetics, parabens are widely used due to their low cost and efficacy. In recent years, however, some reports have claimed that these materials exhibit estrogenic activity, which has led to attempts to replace them in formulations. This article reviews penetration studies of parabens spanning the past 20 years to determine whether they pose a risk to human health.

Key words

Paraben, preservative, penetration, solvent, vehicle

I- INTRODUCTION

In order to assure a proper shelf life to cosmetics and to avoid microbial contamination, active antimicrobial ingredients such as parabens are added to formulations. They are widely used preservatives in cosmetics. The success of parabens as a cosmetic preservative is mostly due to easy use and low cost. In recent years, their presence in skin care products has been criticized, and many scientists worldwide have studied their effects on humans. Some studies reported an estrogenic activity of parabens and their presence in human breast tissues [160,161]. In response to a consumer fear of parabens, formulators have replaced these preservatives with new preservation systems and developed a new technology of preservative-free cosmetics named the "hurdle technology" [162–165]. These new products contain natural preservatives or cosmetic ingredients with antimicrobial properties that are not registered as preservatives. Despite the controversy surrounding parabens, they are still present in most of cosmetics. Assessing the skin delivery of parabens is crucial to estimate their potential risk as they cross the cutaneous barrier and enter into the systemic circulation. In this article, percutaneous penetration studies of parabens spanning the past 20 years are reviewed to determine paraben penetration as affected by a number of parameters and assess the preservative class' risk to humans as formulated in cosmetics.

II- PARABENS PROPERTIES

Parabens are a homologous series of hydroxybenzoic acid containing an ester group at the C-4 position (figure 1). Some popular examples include: methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP), isopropylparaben, isobutylparaben and benzylparaben, with most parabens in cosmetics being MP, EP, PP and BP. Their sodium salts are also used.

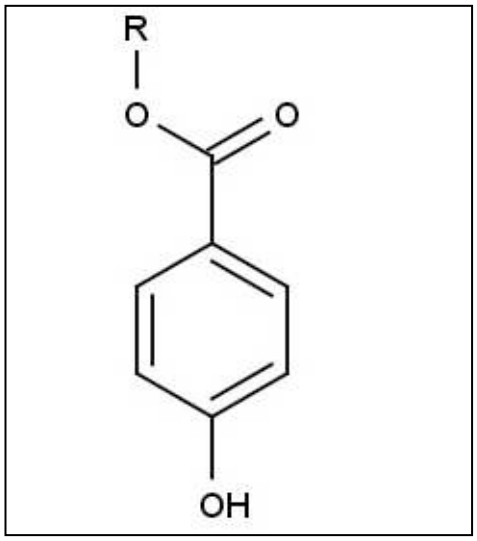

Figure 1: General chemical structure of parabens where R is an alkyl group

Parabens hydrophobicity and octanol-water partition coefficient (P_{ow}) increase with the ester chain length. For example, MP is more water soluble than BP. Their antimicrobial activity is related to the chain length of the ester group. Therefore, as their chain length increases, their antimicrobial activity increases as their water solubility decreases.

Parabens with longer alkyl chain such as PP and BP are most active against fungi. They have activity against Gram-positive bacteria, but are considered weak against Gram-negative bacteria. The amount of parabens dissolved in the water phase determines the preservative ability. Generally, the microbial replication occurs in the water phase [166,167]. Products with low water content are self-preserved, but many cosmetic products such as creams, lotions, shampoos, conditioners and liquid soaps have high water activity, and consequently, chemical preservation is necessary. The EU (EU Cosmetics Directive 76/768/EEC) and US Food and Drug Administration (FDA) allow a maximum of 0.4% of each paraben (expressed as p-hydroxy benzoic acid (HBA)) and a maximum of 0.8% of paraben mixtures in cosmetic products [168]. An industry survey from 2003 conducted by Personal Care Products Council, then the Cosmetic, Toiletry and Fragrance Associateion (CTFA), found that paraben were formulated in cosmetics at 0.0002% to 1% [169]. Their different solubilities allow them to be often used as a mixture. They are easy to formulate with because they have no odor, no color effect, are nearly pH neutral and have no effect on the product viscosity. Parabens are stable at high temperatures, so cosmetic products can be safely autoclaved without loss of antimicrobial activity [167].

III- PARABEN PERCUTANEOUS PENETRATION

1- Intrinsic paraben characteristics

Recent work by the Organization for Economic Co-operation and Development (OECD) claims that 100% dermal absorption may be assumed if the active molecular weight (M_W) is lower than 500g/mol and the Log P_{ow} is between -1 and +4 [116]. As the ester chain lengths increased, Log P_{ow} increased in the order of MP, EP, PP and BP. Table 1 shows Log P_{ow} and M_W paraben values.

Table 1: Physico-chemicals properties of parabens

Molecule	log P_{ow}	M_W (g/mol)
Methylparaben	1.93	152
Ethylparaben	2.27	166
Propylparaben	2.81	180
Butylparaben	3.53	194

Parabens with low Log P_{ow} have lower skin retention and permeate better through skin compared to high Log P_{ow} parabens. The permeation rate increases with the paraben's hydrophilicity. It is crucial to understand that the vehicle choice is important and, contrary to the OECD approach, it has to be taken into account for exploiting the penetration results. If one only follows the OECD rule, by referring to Table 1, the result would be 100% paraben penetration in the studies presented in this review. The OECD regulatory approach needs to be modulated.

Percutaneous penetration studies presented in this review were conducted ex vivo using a Franz cell device. The results varied from one author to another because of the skin type (human, minipig, mice, synthetic membrane, etc.), the vehicle choice (solvent, cosmetic formulation), the initial paraben concentration applied to the skin surface and the barrier condition during experiment.

2- Solvent vs. cosmetic formulation vehicle choice

Penetration results can vary between a cosmetic formulation and a solvent such as water, acetone or ethanol. The following studies highlight the dual importance of

paraben solubility in the solvent, and the solvent effect on the SC lipid integrity.

Dal Pozzo suggested that parabens with a high log P_{ow} became less soluble in water and more soluble in the stratum corneum (SC), allowing them to not cross the skin barrier, whereas low log P_{ow} parabens have less affinity for lipids and often cross the barrier.[7] In an aqueous vehicle, parabens with high P_{ow} (PP and BP) are more soluble in the SC lipids. Therefore, they are retained in the upper epidermis layer and do not penetrate as much as shorter chain parabens [166]. However, if lipophilic parabens are soluble in the vehicle (w/o emulsion), they can be retained in the vehicle and will not penetrate the SC. Dissolved in water, paraben penetration followed a negative correlation between their log P_{ow} and skin permeation. Low log P_{ow} parabens penetrate deeper [170].

Caon studied the effect of ethanol concentration on paraben skin permeation. A solution of ethanol-phosphate buffer (20:80 or 50:50) with a combination of parabens was dropped on pig ear skin for 6 and 7.5 hr, respectively. Lipophilic parabens have a lower permeation flux due to the strong interaction with the SC lipids. They are retained in the upper epidermis. At 20%, the presence of ethanol led to a higher paraben retention in the epidermis compared to the dermis, and at 50% ethanol, an increase in paraben permeation was observed and promoted a greater accumulation in the dermis [171].

Cross did not observe differences between ethanol and acetone used as a vehicle in paraben penetration [172]. These data highlight the dual importance of paraben solubility in the solvent, and the solvent effect on the SC lipid integrity.

Merle reported that the SC lipid cement can be destabilized ex vivo with emergence of pores in presence of ethanol [96]. The study revealed that paraben permeation through skin decreased with an increase of the partition coefficient.

Caon and Dal Pozzo suggested that lipophilic parabens are less soluble in hydrophilic vehicles but more soluble in stratum corneum lipids and that they are retained in the SC [170,171]. The dermis is more hydrophilic, and parabens have less difficulty to pass through it. The aqueous vehicle and the SC lipids act as a partition coefficient.

Moreover, the solvent can have an impact on the paraben metabolism. For example, MP metabolization in *p*-hydroxybenzoic acid (HBA), a less toxic metabolite is inhibited in the presence of ethanol. MP percutaneous penetration in the presence of ethanol was studied by Oh on Yucatan micropig skin [173]. Oh measured MP, EP and HBA concentration in the receptor phase in the presence or absence of ethanol with

PBS in the donor compartment. With no ethanol, MP and HBA concentration increased linearly with time in the receptor compartment. MP and HBA flux concentration decreased as ethanol concentration increased in the donor compartment. With 10% ethanol in the donor compartment, HBA flux decreased and a new EP flux appeared. The authors suggested that ethanol enhanced MP transesterification into EP and inhibited MP bioconversion in HBA.

Cross compared the influence of an ointment or solvents on parabens penetration. MP total penetration and flux were significantly smaller after ointment application compared to acetone or ethanol [172]. Dal Pozzo studied emulsion influence. Parabens in an o/w emulsion penetrate more than in a w/o emulsion. The contact with skin surface and water phase (containing the parabens) of the o/w emulsion caused lipophilic parabens more affinity for the apolar SC. In a w/o emulsion, the oily phase is in contact with the SC, allowing lipophilic parabens to have more affinity for the oily phase rather than the SC. Parabens skin permeation from o/w emulsion was higher than in an aqueous solution. There is a possibility that the presence of surfactants either increased the paraben concentration in the water phase or damaged the SC [170].

Esposito compared paraben diffusion in an aqueous solution, an o/w emulsion, a w/o emulsion and two hydrophilic gels: a carboxyvinyl polymer gel[a] and an acrylates/C_{10-30} alkyl acrylate crosspolymer[b] [174]. All formulations contained 0.05% of MP, EP and PP, and the permeation was tested through a synthetic membrane. The diffusion coefficient (flux/concentration) was much higher in the aqueous form compared to the viscous forms. PP permeation was smaller in water, w/o emulsion and o/w emulsion compared to MP and EP. Paraben diffusion coefficients from the carbomer gel were seven times higher than that of the acrylates/C_{10-30} alkyl acrylate crosspolymer gel. The acrylate with a C_{10-30} alkyl chain dissolves the parabens better in the gel matrix. Parabens are then more retained in the formulation compared to the carbomer gel. This result suggests that the use of acrylates/C_{10-30} alkyl acrylate crosspolymer could reduce paraben diffusion through skin. EP and PP diffusion coefficient was less in the o/w emulsion, whereas MP coefficient diffusion was higher, compared to the w/o emulsion. MP is mostly dissolved in the aqueous phase that diffuses faster in the receptor compartment whereas EP and PP are less soluble in water and may tend to be dissolved in the oil phase. These results are not comparable to Dal Pozzo experiments, as a synthetic membrane was used instead of real skin.

The influence of cyclodextrin on paraben penetration was examined by Tanaka et al [175]. with the use of 2-hydroxypropyl-b-cyclodextrin (HP-b-CyD) in vitro on hairless mouse skin. The investigators compared the cyclodextrin effect with the use of a nonionic surfactant, polyoxyethylene hydrogenated castor oil (HCO-60). MP cutaneous permeation rate was significantly decreased by the addition of cyclodextrin and its solubility was linearly increased as a function of HP-b-CyD concentration. The decrease was greater with cyclodextrin compared to the surfactant. The authors also studied the bioconversion of MP into p-hydroxybenzoic acid (HBA). They observed that the HBA concentration increased and MP concentration decreased significantly in the presence of cyclodextrin. In vitro, cyclodextrin significantly reduced MP absorption and promoted its bioconversion into HBA.

Kitagawa studied the permeability of a liposome suspension of MP, EP, PP and BP in PBS through pig skin [176]. He found that the permeability increased with the increase of log P_{ow}. The author observed a change of permeability when the SC lipids were depleted. The lipid depletion significantly increased the permeability for hydrophilic parabens in the order of MP, EP and PP. He proposed that parabens penetrate via non SC lamella.

These studies show that cosmetic formulation influences mainly paraben penetration through skin, so it is crucial for scientists to decide which paraben to add in function of the formulation. It is also important to examine if any ingredient(s) in the formulation enhance or retard paraben penetration. Kitawaga's results are the opposite of other penetration studies. It underlines that ex vivo absorption studies are difficult to generalize and shows that every formulation needs be tested.

3- Changing paraben solubility in a vehicle

Some ingredients decrease or increase paraben permeation by changing a paraben's solubility in the vehicle. Meanwhile, some ingredients fluidize SC lipids and, by disrupting the barrier, increased the skin permeability to parabens.

MP, EP, PP and BP transdermal permeation was tested in water or in nicotinamide on rabbit ear skin by Nicoli [177]. With nicotinamide in the donor compartment, the transdermal penetration significantly increased for PP and BP, was unchanged for MP and significantly decreased for EP. Glycol effects were tested, and when propylene glycol or polyethylene glycol 400 was added to water in the donor compartment, the

permeation decreased because these molecules increased paraben solubility when added to water. Glycols increase lipophilic paraben solubility in the vehicle compared to SC. Therefore, lipophilic parabens are retained in the solution and penetrate the SC less [170]. Similarly, when parabens were dissolved in liquid paraffin (a lipophilic vehicle) the permeation tended to decrease for parabens with a high log P_{ow}.

Romonchuk studied the effect of 4-cyanophenol (CP) on MP with human epidermis. With the presence of CP in a saturated solution of MP, MP permeation rates and SC uptake significantly increased by increasing MP solubility in the SC [178].

The effect on SC fluidization was observed with an addition of 1% l-menthol in 15% ethanol. It significantly increased MP (in a liposome suspension) permeation. It had no effect on PP and EP, but it decreased BP penetration. The same tendency, but weaker, was observed with just the addition of 15% ethanol. Kitawaga suggested that ethanol and l-menthol stimulate skin penetration of hydrophilic parabens in part due to SC lipids lamella fluidization. The decrease of BP permeation may be due to the increase of BP solubility in presence of ethanol in the vehicle, reducing BP partitioning between the skin and the vehicle. The effect of the N-dodecyl-2-pyrrolidone (ND2P) on paraben penetration was studied. ND2P stimulated the permeation of hydrophilic parabens in particular MP that have a log P_{ow} less than 2. ND2P has the same effect as ethanol on the SC lipid lamella; it pertubates the lipids by increasing their fluidization, thus enhancing hydrophilic paraben permeation [176].

4- Paraben combination

Caon compared the penetration of a single paraben to the penetration of a combination of parabens. Binary combination of PP with three others parabens reduced flux values, particularly when PP is combined with EP. Caon proposed it is due to physicochemical properties, since PP binary combination decreased solubility by 50% with EP and only by 10% with MP and BP in water. The flux decrease could be explained by the change of solubility that could modify the partition coefficient of parabens within the skin layers [171].

5- Reapplication and occlusion's effect on penetration

El Hussein investigated the potential effect of reapplying a product containing parabens during the course of a day [179]. The researchers applied body lotion containing MP, EP, PP and BP at 0, 12 and 24h. The concentration in the liquid receptor was measured before each reapplication at 12h, 24h and at 36h. The penetration order still followed the lipophilicity parameters, with the exception of PP, which had a higher concentration in the lotion than EP. The total parabens liberated in the liquid receptor were higher after reapplication of cosmetics containing parabens compared to a single application. Various paraben concentrations can modify the lipophilic order.

Occlusion is also a factor to account for in assessing paraben penetration, as clothes or accessories covering the skin can modulate the penetration. Occlusion was studied ex vivo by Cross with different paraben vehicles on human epidermis after the placement of a piece of high-density polyethylene. There was a significant change in the epidermal flux of parabens following occlusion. Increases were observed for acetone and ethanol vehicles, and a decrease was seen following occlusion for the ointment formulation. Changes in flux following occlusion appeared to result, for the ointment, from a significant decrease in parabens epidermal partitioning and from an increase in paraben epidermal diffusivity of the solvent vehicles. These studies show that paraben penetration is vehicle dependent with or without occlusion [172]. The choice of an ointment base can be advantageous to avoid paraben penetration for body care products that consumers apply under our clothes such as body lotion.

6- In vivo paraben pharmacokinetic studies

Janjua and Tanaka studied paraben urine concentration before and after application of a formulation containing parabens. Janjua focused on the systemic absorption of BP in 26 men volunteers [180,181]. An 2mg/cm^2 application of a cream without parabens was applied for a week (control week) and the next week a cream containing 2% BP was applied on the whole body everyday in the same conditions. Serum and urine samples were collected every 24h. Serum BP was undetectable during the control week but its concentration peak (130mg/l) was detected 3h after the first application of the BP cream, showing a rapid skin penetration and a systemic uptake. BP urine concentration remained constant during the treatment week (25mg/l). Every urine samples were

collected everyday and 24h excretion profiles were calculated. A mean flux of 32 $mg.cm^{-2}.h^{-1}$ was demonstrated. Reproductive and thyroid hormones levels were also analyzed in the blood sample, and no biologically significant effects were observed. BP urine concentration was under limit of detection during the control week. BP urine concentration increased a thousand times during the treatment week, but only 0.9% of the applied amount was recovered. A single paraben legal amount in a cosmetic is 0.4%, and Janjua applied a cream that was six times above this limit. The cream thickness on skin ($2mg/cm^2$) was higher than the amount typically applied by consumers [182]. It would be relevant to study in vivo parabens recovery with products available on the market and to let volunteers apply the product in accordance to their daily practices.

In vivo cyclodextrin still showed a great reduction of paraben absorption. A patch test containing radiolabelled methyl paraben [^{14}C], MP alone or with radiolabel cyclodextrin [^{14}C]HP-C-Cyd was applied on the dorsal skin of hairless mouse for 24hr. In presence of cyclodextrin, MP was significantly retained in the patch. Paraben amount excreted in urine, feces and respiration were significantly reduced by 66% in the presence of [^{14}C]HP-C-Cyd. Tanaka suggested that only the free fraction of MP in equilibrium with the complexed fraction was percutaneously absorbed [175].

IV- PARABEN RISK ANALYSIS

Parabens are generally considered safe preservatives because after penetrating the SC, they are converted by carboxylesterases into p-hydroxybenzoic acid (HBA), a less toxic metabolite. Carboxylesterase is present in the epidermis and dermis, and it hydrolyzes MP and EP into HBA more than others parabens [183,184]. Only 0.028% of MP was found to remain unmetabolized on the skin surface 12h hours after application [185]. It can, therefore, be concluded that MP and EP are less toxic than more lipophilic parabens after they penetrate the SC.

Consumers started to fear parabens when researchers demonstrated an estrogen activity for parabens that could be linked to various diseases such as breast cancer or have an impact on male reproductive system. Darbre found parabens in human breast tissues, but no control tissues were examined in the study, making the link between parabens and breast cancer unfouded [160].

Many studies report that parabens are weakly estrogenic. The estrogenic response increased with the alkyl group size: BP is 10,000 times less potent than 17b-estradiol; MP is 2,500,000 times less potent than 17b-estradiol; EP is 150,000 times less potent than 17b-estradiol; and PP is 30,000 times less potent that 17b-estradiol. HBA was found inactive [186]. Golden mentioned that if consumers use everyday cosmetics with BP, the total daily BP dose would be approximately 0.12–0.41 mg/kg/day. This corresponds to a BP daily intake between 1,500 and 5,000 times less than the minimal amount demonstrating estrogenic activity in an in vivo assay [187]. However BP and PP could increase estrogen activity by inhibiting estrogen sulfotransferases in skin, stimulating or prolonging estrogen signaling cascades. In this study, paraben concentration was equivalent to what could be achieved after 1% of paraben penetrated from a cosmetic formulation containing less than 1% paraben [161]. the Considering that BP and PP are less converted into HBA, it would be necessary to study this phenomena more.

Contact allergy to parabens is relatively rare. Seventy years of paraben use have shown that they are not irritating preservatives [188]. However, Fisher highlighted the "paraben paradox," which finds that when a paraben sensitive skin is at a normal state, the patient does not react to the application of a cosmetic containing parabens, but if the skin is traumatized or eczematous it, then easily reacts to parabens. So generally, these preservatives are well tolerate by normal skin even if the skin is sensitive to parabens [189].

V- PARABEN ALTERNATIVES

After the communication of the potential estrogenic effect of parabens, consumers have started to fear preservatives, and marketers have listened to them. The current trend is to remove parabens from cosmetics and to promote self-preserved products that claim to be "paraben-free" or "preservative-free." The fear for parabens has influenced industries to replace parabens with natural preservatives or cosmetic ingredients with antimicrobial properties that are not registered as preservatives in order to satisfy the consumer. The development of preservative-free cosmetics is named the "hurdle technology" defined first by Leistner for food application [190]. It accounts for the manufacturing process, the packaging, the emulsion form, the water

activity, the pH control and the multifunctional antimicrobial ingredients. For example, cosmetics have to be manufactured in strictly aseptic conditions. Protective packaging such as a tube (preferably a metal tube) is preferred over a wide mouth jar. This shows the microbial challenges from multiple finger contact. The metal tube does not allow for air to be drawn into the tube after dispensing the product. Similiarly, a pump also limits finger contamination. A preferable packaging would be an air-less pump for the same reasons as the metal tube; however, cost considerations must be considered. Manufacturers must ensured that consumers will be ready to pay more money the same cosmetic for a different packaging. The most secure packaging is the single application pack or single shot capsule, as it is a perfect aseptic environment, but not ecological at all. The consumer will have to throw away the packaging after every use.
A beneficial method to avoid microbial contamination is to lower the water activity of the product with water binding substances and favor o/w emulsions. Also pH<4 or >10 reduces microbial growth, but can cause skin irritation [162,163].
Antimicrobial ingredients not registered as preservatives such as caprylyl glycol and glyceryl caprylate are only effective in combination with other ingredients. For example, natural ingredients like lonicera extracts seem promising for developing self-preserved products, but they need the addition of glyceryl caprylate, levulinic acid, p-anisic acid and ethanol (5% w/w). Organic acids need to be combined with diazolidinyl urea [164].
Self-preserved formulations need high microbiological and stability testing to ensure their effectiveness because the utilization of such alternative or natural substances does not ensure complete elimination of adverse events, irritating effects or sensitization. To replace parabens with natural preservatives is not necessarily preferable. Natural ingredients are not suitable for all consumers because they can contain impurities, causing allergic reactions [165].

VI- CONCLUSION

This review highlighted two key points on paraben percutaneous penetration factors. First, the more parabens are soluble in the formulation, the more they are retained and penetrate less. Secondly, lipophilic parabens are retained by the SC lipids, and it is difficult for them to cross this barrier, whereas more hydrophilic parabens can cross

this the upper epidermis but are mostly bioconverted into HBA.

It is difficult to generalize the diffusion properties of just one or a paraben combination, as the whole formulation needs to be taken into account. Penetration results from ex vivo studies depend on operative conditions, skin origin, skin treatment (dermatomed, heat separated, etc.) and cutaneous barrier status. In vivo experiments, such as the studies by Janjua and Tanaka, could be a solution, but they are no longer viable trends because of a strict regulation and the banning of animal testing [180,181]. Every formulation would need to be tested, but it is complicated to do so and published mathematical models (often polynomial function) are not sufficient. An interesting alternative would be to establish physico-chemical factors that would lead to a complete mathematical penetration model. This model would account for the formulation and the skin origin and not just the Log P_{ow} and M_w.

Preservatives presence in cosmetic formulations is controversial. The “ideal case” that will replace traditional/chemical preservatives and will be absolutely safe, effective and compatible for all applications has not been found yet. Consumers that avoid preservatives should be educated about cosmetic contamination especially for house fabric cosmetics. The only secure way to avoid microbiological contamination is the use of well-known preservatives. Consumers should be aware that new "natural" preservatives might not have been tested to the extent of parabens and penetration data probably does not exist for those alternative preservatives.

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Travaux Personnels

Chapitre 1 : Développement d'un critère prédictif de l'absorption cutanée par chromatographie d'affinité sur colonne de carbone graphite poreux.

Introduction

L'une des méthodes utilisée pour prédire l'absorption gastro intestinale de molécules exogènes consiste à calculer des facteurs de rétention chromatographique. Des chercheurs ont développé des protocoles d'étude à partir de phases stationnaires de colonnes chromatographiques sur lesquelles sont greffées des phospholipides par liaisons covalentes. Ces colonnes sont connues sous le nom de colonne « Immobilized Artificial Membrane » (IAM). Avant et après le greffage des phospholipides dans la colonne, les temps de rétention de molécules d'intérêt sont mesurés permettant ainsi le calcul des facteurs de rétention chromatographique. Cette technique est rapide et économique pour prédire l'absorption gastro intestinale. Certaines études ont tenté de prédire l'absorption cutanée de molécules exogènes via des colonnes IAM, bien que la matrice lipidique du SC ne possède pas phospholipides. Les facteurs chromatographiques calculés étaient peu corrélés aux données expérimentales de pénétration cutanée. Il a alors été proposé des modèles avec un greffage de la phase stationnaire de la kératine ou du collagène. Ces modèles n'ont pas non plus permis de prédire l'absorption des molécules à usage topique.

Etant donné que les lipides intercornéocytaires du SC sont constitués en majorité de céramides (de l'ordre de 50%) et que ces lipides jouent un rôle crucial dans la barrière et la pénétration cutanées, nous nous sommes intéressés à la modification de la phase stationnaire de carbone graphite poreux (PGC) avec différents céramides afin de proposer un nouveau critère pour la prédiction de l'absorption cutanée.

Notre travail a constitué premièrement à développer un protocole d'imprégnation de la colonne PGC avec un céramide-like 2 (céramide synthétique ayant des propriétés organisationnelles proches du céramide 2 naturel). Différentes concentrations de

céramides ont été testées et l'imprégnation de la colonne a été suivie grâce à un détecteur évaporatif à diffusion de la lumière (DEDL). Avant et après l'imprégnation de la phase stationnaire avec un céramide, des molécules d'intérêts cosmétiques et pharmaceutiques ont été injectées et leur temps de rétention étudié. Dans la seconde partie de notre travail le protocole développé a été appliqué à une dizaine de molécules d'intérêts, un critère chromatographique de prédiction de l'absorption cutanée (α) a été défini. Ce critère a ensuite été comparé aux résultats de pénétration cutanée obtenus en cellules de Franz.

Ceramides immobilized on porous graphit carbon column as a new stationary phase for chromatographic modeling of percutaneous penetration

I- INTRODUCTION

Phospholipid bilayer is the main barrier for drugs absorption in the gastrointestinal tract. A predictive method of intestinal absorption was launched twenty years ago by affinity chromatography with the development of Immobilized artificial membranes (IAM). IAMs are chromatographic columns where the stationary phase undergoes a modification with the creation of covalent link with synthetic phospholipid analogues. After column modification, fast and accurate predictions of drug distribution in biological systems can be found estimated directly with HPLC retention time measurements. Molecules of interest are injected before and after the column modification and retention times are correlated to permeability data [1,2].

With the ban of animal testing in cosmetic industries, alternative methods development for skin absorption prediction has been a major priority in research and development. Stratum corneum, the upper skin layer, is composed of three major lipids: ceramides, fatty acids and cholesterol. Because ceramides play a crucial role in barrier function and against penetration of exogenous molecules we found interesting to build a column modified with ceramides. In this study we aimed to mimic ceramides and exogenous molecules interaction in order to predict better skin permeation. A Porous Graphit Column (PGC) stationary phase was modified with a synthetic ceramide, named ceramides-like 2 (CER), a saturated ceramide detaining similarity with natural ceramide 2 (figure 1).

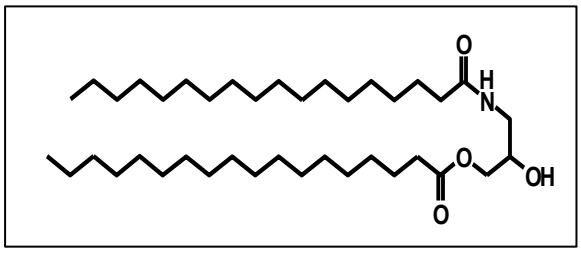

Figure 1: Ceramide-like 2, the ceramide used for modifying the PGC column

The PGC loading with CER was followed with a DEDL detector. Before and after CER loading inside PGC, molecules of interest were injected and detected by UV. Their retention times were recorded and compared. The charging of the column was tested with different CER concentration at 10^{-5}, 10^{-6} and 10^{-7} M. Five dermocosmetic molecules were used for method development (table 1). Due to the photosensibility of retinol, after method development, this molecule was dropped from the study and three parabens were added to it. Chromatographic results were correlated to classic skin absorption experiments with a Franz cells device.

Table 1: Physicochemical properties of molecules studied

Molecules	Abbreviations	Properties	Log Pow	Mw (g/mol)
Caffeine	CAF	Cellulite reducer	-0,07	196
Prednisolone	PRED	Corticoid	1,62	360
Benzophenone 3	BP3	UV filter	3,79	228
Octocrylene	OC	UV filter	6,88	361
Retinol	RET	Anti wrinkle	7,62	286

II- MATERIAL AND METHODS

1- Affinity Chromatography

Solvents: HPLC grade acetonitrinile, methanol, ethanol and chloroform were purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France).
Ceramides: ceramide-like 2 from Solabia (France).
Molecules : benzophenone 3, methyl, ethyl and propyl parabens and retinol were purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Octocrylene (Parsol 40®) at DSM (Courbevoie, France). Caffeine was purchased at Alfa Aesar (Schiltigheim, France) and the prednisolone at Molekula (Dorset, United-Kingdom).

Chromatographic experiments were run on a porous graphit carbon column (PCG) (supplied by ThermoScientific, Hypercarb, 10x3mm, 5µm, Villebon sur Yvette, France). The column was thermostated at 35°C. The chromatographic system was composed of an isocratic pump (Kontron instruments, nBA 01005, HPLC PUMP 422,

Toulon, France). The detection system for the column impregnation was a detector Corona® (supplied by Thermo Scientific, Villebon sur Yvette, France). A UV was also connected in order to detect the exogenous molecule retention times. Results were integrated with the computer software Kroma system 2000 KONTRON PC – Integrator, version 3.90 was used for the determination of the retention times. Debit was set at 0,7 ml/min for the molecule injection.

- Solvents: ACN was used in order to solubilize the molecules at the concentration 5.10^{-4}M and then at 2.10-5M in order to improve the chromatographic detection with a better solubility.
- Impregnation solutions: A ceramide-like 2 solution was diluted in MeOH with 1% of ($CHCl_3$) at 10^{-5}, 10^{-6}, 10^{-7} M (99 % MeOH+1% CHCl3).
- Rinses: After the column impregnation, the system was washed out with a mobile phase containing 100% of CHCl3 to eliminate all the adsorbed ceramide. The duration of the washing with the CHCl3 solution depended on the concentration of the impregnation solution. The washing was stopped once the molecule retention time was identical as the retention before the PGC impregnation with ceramides. After that a second washing solution was programmed for 3 hours with pure ethanol. The PGC column is then conditioned with pure ACN.

2- Franz cells

Fat was removed from fresh skin from abdominal plastic surgery. Skin was stored at -25°C and full thickness skin was used for the experiments. Before the experiment the skin was immersed in physiologic serum (0,85% NaCl) for 20 min and then cut in circle shapes to fit the glass diffusion cells. Each skin was carefully washed with Millipore Q water and dried with paper before to be mounted on the Franz cell. Franz cells had a diameter of 2 cm^2 and a volume of liquid receptor of 11,5 ml (Lara Spirals, Couternon, France). A magnetic stirrer bar was added in the donor compartment. The liquid receptor was filled with distilled water containing 0,85% NaCl and 0,01% of bovine serum albumin. Air bubbles in the donor compartment were removed. The system was thermostated at 37°C above a magnetic stirrer to ensure the homogeneity of the liquid receptor during the experiment. After 30 min of stabilization, 200 µl of a solution containing one of the exogenous molecules diluted in EA at 10^{-2} M was

dropped on the skin surface. During the experiment Franz cells were not occluded left at open air.

After 22 hours, the liquid receptor and the skin were collected. The skin surface was washed with a cotton swab on which 200 µl of liquid receptor (distilled water containing 0,85% NaCl and 0,01% of bovine serum albumin) was dropped. After washing, the skin surface was tape-stripped 3 times with D-squame tape (Cuderm, Dallas, USA) at the same constant pressure. The 3 tapes were pooled in a 20 ml vial and stored at -20°C until analysis. The liquid receptor was filtrated and stored at -20°C until analysis. The epidermis was separated from the dermis by heating and then cut into small pieces with a scalpel. The epidermis and the dermis were stored at -20°C until analysis. The exogenous molecule was extracted from the tapes by adding 2 ml of toluene in the vial over night. The next day, tapes were removed from the vial and 1 ml of the solution recovered was diluted in methanol and filtrated (Sterile filters, 0,22mm, Roth, Lauterbourg, France) until the solution was completely limpid. The dermis and epidermis were grounded with an adapted mixer and tubes (ULTRA-TURRAX® Tube Drive and BMT-20-S tubes, IKA, Staufen, Germany) in 5 ml of methanol. The methanolic recuperated was filtrated. All solutions from collected samples were stored at -20°C until analysis.

All samples (tapes, epidermis, dermis and liquid receptor) were quantified by HPLC analysis coupled to a UV detector. A sample of 20 µl was injected. For each analysis, standard samples were prepared ranging from 10^{-6} M to 10^{-4} M. Operatory conditions can be found in our work [3].

3- Chemometrics

Principal component analysis (PCA) was performed with MATLAB (MathWorks, Meudon, France).

III- RESULTS AND DISCUSSION

The methodology to calculate the ceramide-molecule interaction consisted of:
1- Measuring exogenous molecules retention in the PGC column before impregnation.
2- Impregnating the column with a mobile phase containing CER. The impregnation was followed with the corona detector, in the same conditions as before impregnation.
3-When a plate was observed, the molecules of interest were then chromatographied using MeOH as mobile phase and their retention time was recorded using the UV detector.
4-PGC column was rinsed and the washing was followed also on the corona detector.
5-After cleaning the PGC from CER, molecules were injected again in order to make sure the column for emptied of CER.

1- Preparation of the modified stationary phase

a- Impregnation procedure

The stationary phase was modified using one of the solution of ceramide-like 2 in MeOH/ $CHCl_3$ (99:1) at a defined concentration (cf part *d*). The flow rate was set at 0,5mL/min until the equilibrium was obtained (cf part *d*). The impregnation was followed using the Corona detector.

b- Choice of Ceramide

In preliminary studies, we studied the impact of saturation (0, 1 or 2 double bonds) and chain length on ceramide-modified PGC column stability. The ceramide with the most stable organization was a synthetic saturated ceramide (C36) similar to natural saturated ceramide 2, the ceramide-like 2 (CER). Despite of the polar head different from natural ceramides, ceramide-like-2 was preferred to natural ceramide 2 because it presents no microheterogeneity of the chain length and it is available in large quantity.

c- Solvent choice

For CER mobile phase it was necessary to add 1% $CHCl_3$ to assume a good solubilization of the CER, particularly at 10^{-4} M concentration.

ACN was chosen for the retention time measurements of interest molecules by chromatography. The advantage of ACN, is that first, this solvent well solubilized the molecules and second, it does not take off the absorbed ceramide-like 2. So we were able to obtain a good reproducibility of the retention times.

$CHCl_3$ was chosen for washing the CER modified-stationary phase. $CHCl_3$ has the property to dissolve ceramides and in consequence to remove them from the PGC column.

d- Ceramide concentration for the impregnation.

The Corona detector permits to follow the column impregnation with CER. Once a plateau is reached, it indicates that the equilibrium is reached and column is charged with CER (figure 2). After impregnation is over, the modified PGC stationary phase was conditioned with ACN mobile phase and the signal variation is followed with the corona detector (figure 3). The change of mobile phase stopped CER flux and in consequence the intensity dropped. Exogenous molecules were then injected in order to observe their retention times.

Different concentrations of CER were tested for the impregnation of the PGC stationary phase.

CER concentrations of 10^{-5}, 10^{-6} and 10^{-7} M were tested to find the most appropriate CER concentration for the impregnation protocol.

We compared regarding the different concentrations, the time for CER impregnation and washing. To charge the column at CER 10^{-5} M, 10 hours were necessary to reach the plateau, with CER 10^{-6} M, 18 hours and at 10^{-7} M 24hours (figure 2). The more the CER solution is concentrated, the less time is required to have a fully charge PGC column of CER. However, the contrary is observed for the washing: 63 and 120 hours were necessary to wash the column of CER at 10^{-6} and 10^{-5}M.

Another important point is the signal intensity height indicating the impregnation level of the stationary phase. If the height stay at the same level, it means that there is no need to use a superior CER concentration.

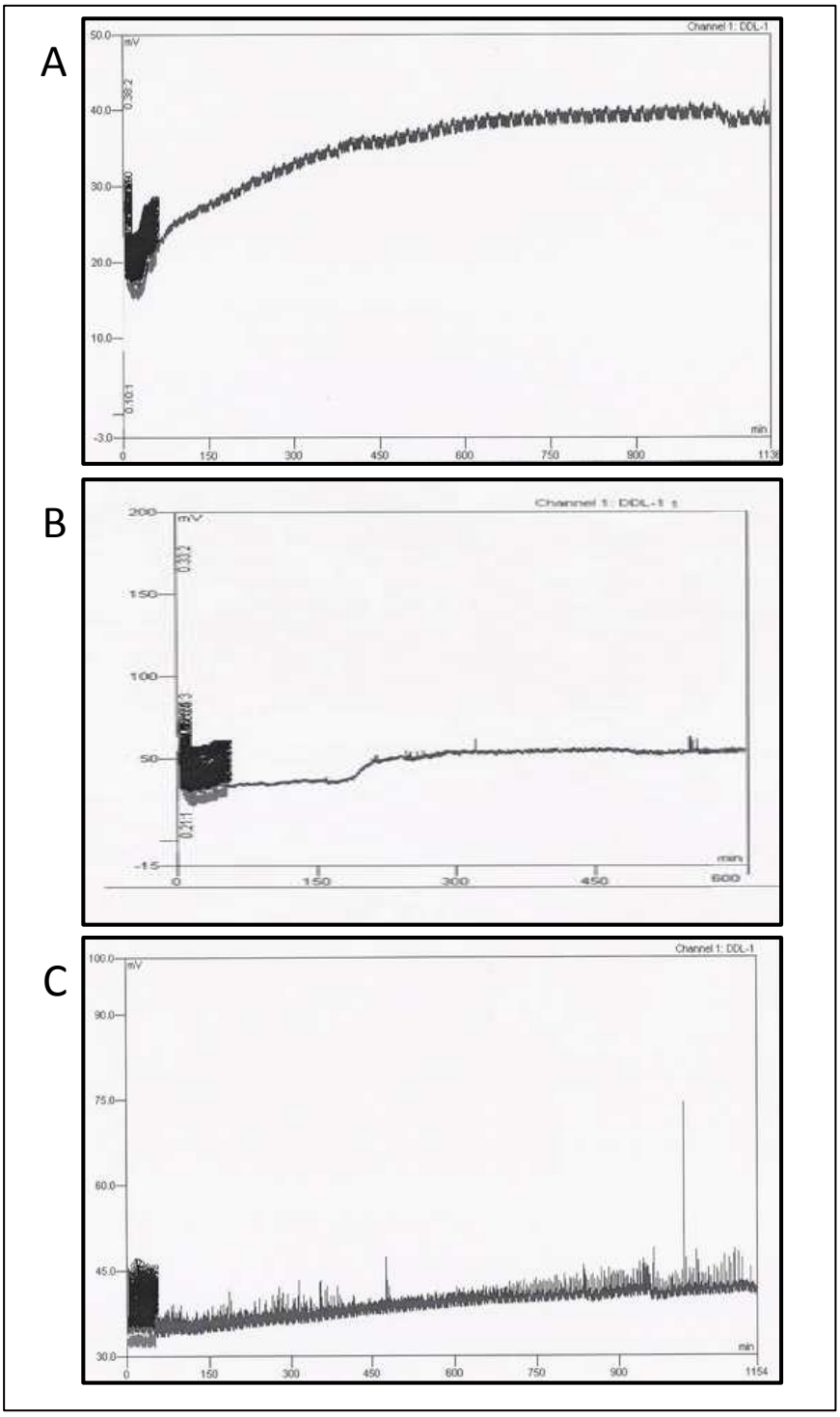

Figure 2: Chromatograms from CORONA detector. The signal intensity in related to CER quantity impregnated within PGC column. With a solution of CER 10^{-5} M, a plate was observed after 10 hours, meaning that PGC column is charged with CER (A). For CER 10^{-6} M, 18 hours were necessary (B) and 24 hours for CER 10^{-7} M (C).

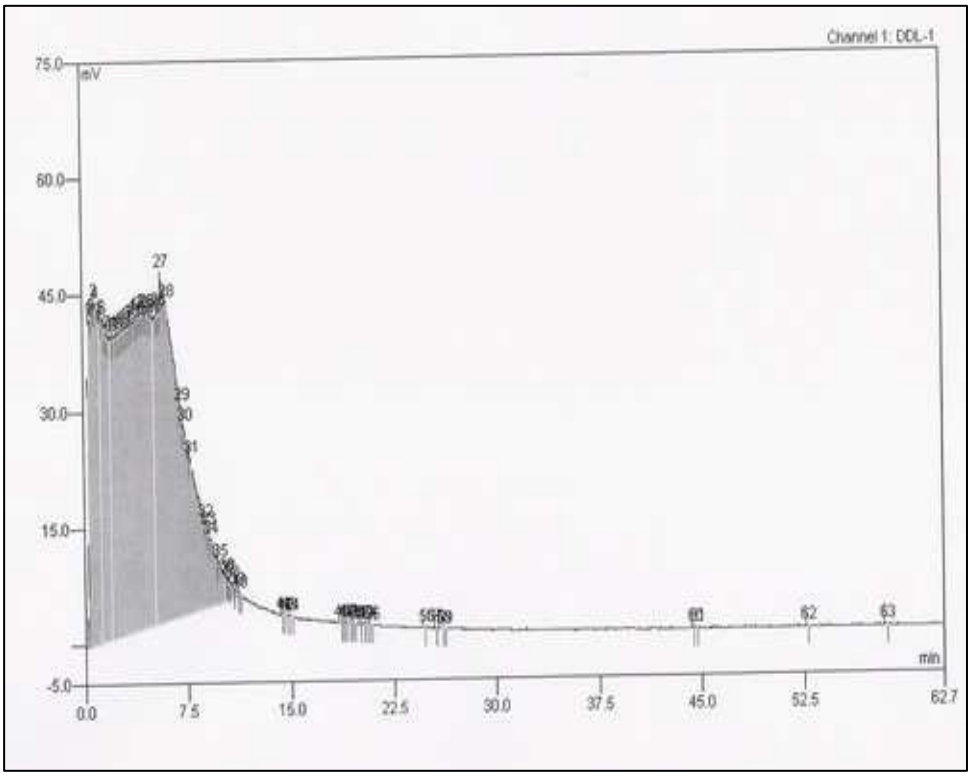

Figure 3: Intensity signal decrease with ACN as a mobile phase, showing that this mobile phase keeps CER fixed on PGC column.

b- Impregnation final protocol

To make sure that a maximum CER are impregnated on the PGC column stationary phase, we selected the protocol with a CER concentration of 10^{-5}M. The protocol we developed was:

1- ACN is programmed as the mobile phase. Molecules of interest are dissolved into ACN and injected into the PGC column. Their retention time is detected with a UV detector.

2- PGC column is charged with a 10^{-5} M CER solution in MeOH with 1% $CHCl_3$ at a flow rate of 0,5 ml/min for 10 hours. The CER impregnation if followed with Corona detector. After 10 hours a plateau is observed.

3- ACN mobile phase was then used on the CER modified PGC column. Tested molecules are dissolved and injected within an ACN mobile phase. Their retention times are recorded.

In order to rinse properly the column, the procedure below was followed after step 3-:

1- After injection of all tested molecules, the modified PGC column is then rinsed with a $CHCl_3$ mobile phase for 120 hours in order to wash out the column of CER.

2- Molecules of interested are injected again into ACN and their retention time is recorded.

2- Study of molecule retention of the modified PGC column stationary phase with CER

a- Retention times

Molecules retention times in function of the concentration of CER for impregnation are illustrated in table 2 and the variation of their retention compared to the empty column in table 3. Retention times decreased when increasing the CER impregnation compared to the empty PGC column.

Molecules retention on PGC is essentially explained by adsorption and presents a particular behavior toward polar compounds [4].

CAF the most hydrophilic molecule, was the more retained on the PGC column modified with CER and OC, the most lipophilic one, was the less retained. All variation of retention time were between 30 to 40% with an impregnation of CER 10^{-5} M. BP3 was the molecule that had the highest variation and RET, OC and CAF the smallest.

Table 2: Retention time (min) of molecules PGC column modified with 10^{-7} M, 10^{-6} M or 10^{-5} M CER concentration and the variation of their retention times between the empty PGC and modified with 10^{-5} M of CER.

	OC	**PRED**	**RET**	**BP3**	**CAF**
PGC	4,4	7,3	20,1	22,9	23,1
PGC + CER 10^{-7}	4	6,8	19,6	17,4	22,7
PGC + CER 10^{-6}	3,8	5,7	17,7	16,3	21,1
PGC + CER 10^{-5}	3	4,4	13,7	12,2	15,7

Table 3: Variation of molecules retention times in % between the empty PGC and modified with 10^{-7}, 10^{-6} and 10^{-5} M of CER.

	OC	**PRED**	**RET**	**BP3**	**CAF**
PGC + CER 10^{-7}	9	7	2	24	2
PGC + CER 10^{-6}	14	22	12	29	9
PGC + CER 10^{-5}	32	40	32	47	32

Contrary to IAM columns, PGC column modified with CER is reversible. The links created are not covalent and PGC can be clean of CER with $CHCl_3$. A decrease of retention times is observed when molecules were injected on PGC stationary phase modified with CER. In IAM columns, an increase of retention times with phospholipids on IAM columns was observed. It can be explained by a stronger interaction of the molecules of interest with stationary phase phospholipids [1]. Here, in our impregnation protocol, CER are not attached to the column with covalent bonds, contrary to IAM column. The fact that retention time decreases with CER may be explained by the hypothesis that if a molecule has good affinity for CER, it is able to remove CER from the column. In consequence, instead of being retained by CER, as it is the case with phospholipids in IAM columns, labile molecules-CER complex formation resulted in a decrease of retention. From this hypothesis, more the interaction between molecules and CER is high, more the decrease in retention times should be important. However, the % of decrease appeared to be quite similar for all the tested molecules. Thus, the interaction between CER and molecules should be here a complex mechanism that we didn't succeed to highlight.

b- Constants determination

In order to calculate a retention criterion, we used the same approach as the one developed in previous studies for IAM column interaction with molecules [2,5].

In order to determinate the dead time t_0, pure urea in ACN was injected. This molecule is not retained by the PGC and is detectable by the UV detector retention time was, $t_0 = 0,84$ min.

IAM chromatography is used to mimic the gastrointestinal absorption. In order to reflect the molecule distribution between the stationary and mobile phase,

Log k_{IAM} is shown as a strong indicator of the lipophilic properties represented by Log Pow. In order to mimic any potential interaction between molecules and PGC stationary phase charged with CER, we calculated the retention factors k'_{PGC} for PGC column before impregnation and $k'_{PGC\text{-}CER}$ after modification of PGC column with CER.

Retention factors were calculated for all molecules before PGC impregnation with CER and after modification of the column with different [CER] tested. All results are showed in table 4. No correlations were found between retention factors and Log Pow.

Table 4: Retention factor k'PGC and Log k'PGC was calculated for molecules with retention time before PGC modification and after impregnation with different concentration of CER.

	CAF	BP3	OC	PRED	RET
Log Pow	-0,1	3,8	6,9	1,6	7,6
k' PGC	26,5	26,3	4,2	7,7	22,8
Log k' PGC	1,4	1,4	0,6	0,9	1,4
k' PGC-CER 10^{-5}	17,7	13,6	2,6	4,3	15,3
Log k' PGC-CER 10^{-5}	1,2	1,1	0,4	0,6	1,2
k' PGC-CER 10^{-6}	24,2	18,4	3,5	5,8	20,1
Log k' PGC-CER 10^{-6}	1,4	1,3	0,5	0,8	1,3
k` PGC-CER 10^{-7}	26,0	19,7	3,8	7,0	22,3
Log k` PGC-CER 10^{-7}	1,4	1,3	0,6	0,8	1,3

c- Criterion development and validation

After developing by affinity chromatography a method to modify PGC column with different concentrations of CER. We tested a group of molecules of interest with various Log Pow and MW (table 5). In parallel penetration of these molecules was also studied by classic franz cells experiments. Penetration results from franz cells experiments were compared to the predictive method developed by chromatography.

Table 5: Physicochemical properties of molecules studied

Molecules	Abbreviations	Properties	Log Pow	Mw (g/mol)
Caffeine	CAF	Cellulite reducer	-0,07	196
Prednisolone	PRED	Corticoid	1,62	360
Methyparaben	MP	Preservative	1,93	152
Ethylparaben	EP	Preservative	2,27	166
Propylparaben	PP	Preservative	2,81	180
Benzophenone 3	BP3	UV filter	3,79	228
Octocrylene	OC	UV filter	6,88	361

Molecules were injected before PGC impregnation with CER and after PGC modification with a concentration of 10^{-5} M of CER. Capacity factors were calculated and α ratio was calculated with the equation:

$$\alpha = \frac{k'PGC}{k'(PGC - CER)}$$

Capacity factors are presented in table 6. α is considered as our chromatographic criterion and will be compared to franz cells data.

Table 6: Capacity factors K'PGC and K'PGC-CER were calculated before and after PGC modification with CER. Ratio α was calculated.

	CAF	**OC**	**BP3**	**PRED**	**MP**	**EP**	**PP**	**BP**
k' PGC	26,5	4,2	26,3	7,7	6,1	6,3	8,4	11,8
k'PGC-CER	19,9	3,1	18,3	5,4	3,4	3,7	5,0	7,0
α	1,3	1,4	1,4	1,4	1,8	1,7	1,7	1,7

3- Comparison with Franz cells experiments

Molecules quantity detected in different compartments are shown in table 7. Molecules quantity profiles detected in skin compartments don't seem to be related to their Log Pow and MW.

Table 7: Molecules quantities (nmol) in the skin different compartments (TS, EP and DE) and LR.

	Log Pow	**TS**	**EP**	**DE**	**LR**
CAF	-0,07	85 ± 6	51 ± 14	102 ± 32	91 ± 62
PRED	1,62	369 ± 148	534 ± 99	207 ± 91	1 ± 2
MP	1,93	380 ± 40	88 ± 12	135 ± 35	52 ± 8
EP	2,27	40 ± 40	82 ± 43	146 ± 70	<LOD
PP	2,81	5 ± 3	46 ± 29	94 ± 94	2 ± 3
BP3	3,79	381 ± 30	311 ± 74	128 ± 52	<LOD
OC	6,88	440 ± 173	336 ± 36	77 ± 27	<LOD

In order to compare our results from classic Franz cells skin absorption experiment and the predictive method developed by affinity chromatography, we performed chemometric tests. It was not possible to perform a Linear Multiple Regression analysis (LMR) due to a poor molecules number. We performed instead a principal component analysis (PCA) in order to highlight variability sources of the

percutaneous penetration profiles of the molecules studied in Franz cells (figure 8). The ACP shows that in the Franz cells experiments, CAF, PP and EP tended to be more present in DE and LR whereas UV filters (BP3 and OC) and PRED tended to be more retained in SC and EP. Especially for PRED that has a good epidermis representation. We observed that PRED, BP3 and OC have the same ratio α value at 1,4. It shows that these 3 molecules may interact the same way with ceramides in the SC. However it was not possible to find a linear correlation between molecules distribution and α. The limitation to this method development is that we were not able to understand the retention mechanism of CER with molecules within the stationary phase.

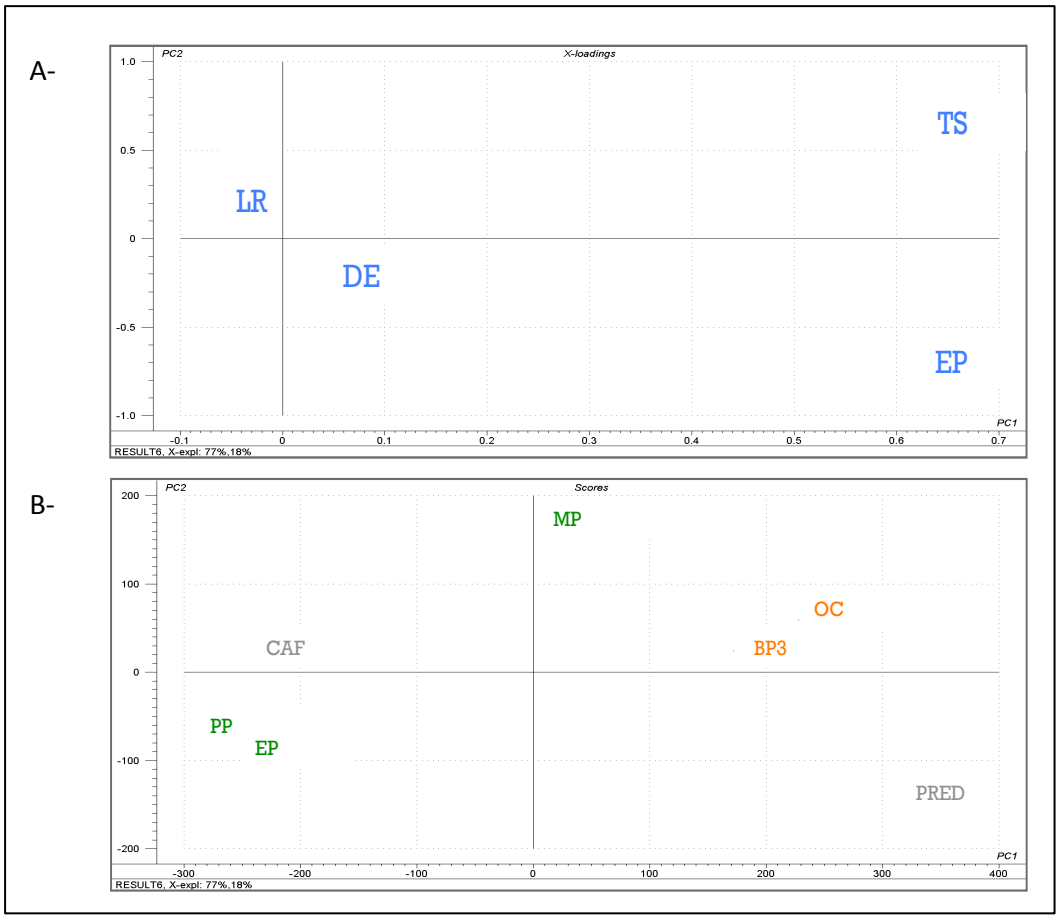

Figure 8: ACP results with A representing X-Loadings and B Scores, X expl=77%, 18%. BP3, OC and PRED tended to be more retained in EP, PP, EP and CAF in DE/LR.

IV- CONCLUSION

To Predict percutaneous penetration Log Pow and MW are not enough relevant parameters. That is why our studied introduced an additive parameter that focused on ceramides-molecules interaction. This investigation was a feasibility study that provided new trends and next these experiments are going to be reproduced with a much higher molecules number in order to realize PLS. Even if α seems to be an interesting complementary criterion for prediction of percutaneous penetration, we did not manage to understand the retention mechanism of our exogenous molecules within the modified PGC with CER. In consequence we had to drop this method for a criterion development until the mechanism is not clearly understood. Chromatographic models are much easier to use compared to ex vivo experiments as that the protocol can be easily standardized and that the results are very reproducible. Because IAM columns showed a good correlation for predicting gastrointestinal absorption [1], it would interesting in the future to understand better the retention mechanism of CER within PGC columns in order to find a better way to develop a criterion or to use a different column for the impregnation.

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Conclusion

Ce travail par chromatographie d'affinité nous a permis d'établir un protocole d'étude du rôle rétentif des céramides dans le SC. Une méthode d'imprégnation d'une colonne carbone graphite poreux (PGC) avec un céramide synthétique ayant un comportement proche du céramide 2 naturel a été développée. La concentration de CER à 10^{-5} M nous a paru la plus probante, permettant d'obtenir une colonne modifiée après 10h d'impregnation. La particularité de cette méthode est que, contrairement aux colonnes IAM, le greffage des céramides ne se fait pas par liaison covalente mais par adsorption réversible.

Une fois la colonne PGC modifiée, il a été possible d'étudier l'interaction de molécules exogènes ayant des Log Pow et MW différents, avec les céramides de la phase stationnaire. Les temps de rétention des différentes molécules ont été enregistrés avant et après la modification de la colonne. A partir de ces données, le critère représentatif de l'interaction céramide-molécules, α, a été calculé. Nous avons observé que ce critère α et les variations des temps de rétention calculés avant et après greffage des céramides sont indépendants des propriétés physicochimiques des molécules. Ce critère apporte donc des informations différentes du Log Pow et MW.
La valeur de α a été comparée aux données de référence de pénétration percutanée réalisées en cellules de Franz. Une analyse par composante principale des données de cellules de Franz a mis en avant que certaines molécules (PRED, BP3 et OC) étaient retenues principalement au niveau de l'épiderme. Nous avons observé que ces trois molécules possèdent le même critère α. Il peut donc être supposé que ces trois molécules ont la même interaction particulière avec les céramides, ce qui explique leur distribution au niveau de l'épiderme contrairement aux autres molécules.

Comme nous n'avons pas pu expliquer clairement la nature de l'interaction des molécules avec les céramides dans la colonne, nous avons préféré ne pas continuer l'exploitation de cette méthode. En effet, dans les colonnes IAM, lorsqu'il y a une interaction entre les molécules d'intérêt et les phospholipides greffés, le temps de rétention des molécules augmente alors que dans notre méthode le temps de rétention diminue. Une compréhension plus approfondie du mécanisme d'interaction des

molécules avec les céramides de la phase stationnaire permettrait de poursuivre le développement de cette technique et de valider ainsi le critère α pour améliorer la prédiction de l'analyse du risque d'ingrédients cosmétiques.

Chapitre 2 : Etude de l'interaction céramide-molécules exogènes par spectroscopie de fluorescence pour le développement d'un modèle prédictif de la pénétration percutanée

Introduction

Comme expliqué précédemment, les estimations de l'absorption cutanée sont basées principalement sur le Log Pow et le MW des molécules. Etant donné que les céramides sont des lipides essentiels à la protection barrière de la peau et que la voie de pénétration des molécules se fait principalement via le ciment lipidique intercornéocytaire, nous nous sommes intéressés au développement d'un critère prédictif de la pénétration cutanée basée sur l'interaction céramides-molécules exogènes. L'interaction entre ces principaux lipides du SC, les céramides, et des actifs de nature cosmétique ou pharmaceutique (filtres UV, conservateurs etc..) a été étudiée. Dans cette partie, le but de notre travail a été d'étudier l'effet de cette interaction sur la rétention des molécules dans la peau par spectroscopie de fluorescence à l'aide de sondes lipophiles environnementales et de proposer ainsi un critère d'évaluation de la pénétration cutanée, complémentaire du Log Pow et du MW.

De précédents travaux réalisés au sein de l'EA4041, ont mis au point des protocoles d'étude des céramides par spectroscopie de fluorescence avec l'utilisation de sondes lipidiques comme le *m*-THPP ou le DPH, les lipides n'émettant pas intrinsèquement de fluorescence. Ces sondes utilisées émettent un signal de fluorescence seulement si elles intéragissent avec des lipides. Plus la sonde a d'affinité pour les lipides plus le signal est fort. S'il n'y a pas d'interaction, le signal est quasiment nul.

A partir de ces études antérieures, nous avons développé un modèle in vitro de caractérisation de l'interaction entre les céramides et des molécules exogènes par spectroscopie de fluorescence. Le principe de notre étude a été de mélanger une solution de céramide avec une solution contenant la sonde choisie afin d'obtenir une interaction sonde/céramide optimale et donc une intensité de fluorescence maximale.

Une molécule exogène est alors ajoutée au milieu. Si la molécule interagit avec le céramide, il y a alors une compétition entre la molécule et la sonde pour le céramide. La sonde est alors déplacée de la chaine lipidique et une baisse de fluorescence est observée. Un critère représentatif de cette interaction, ΔI, a été défini et calculé.

Tout d'abord, notre travail a constitué à choisir le lipide et la sonde la plus appropriée pour mettre en évidence une interaction lipides cutanés/molécule. Puis, nous avons développé la méthode prédictive pour laquelle nous avons établi un protocole précis et définit le critère de fluorescence ΔI, caractérisant cette interaction. ΔI a été calculé pour différentes molécules tests d'intérêt cosmétique et comparé à des données de référence de pénétration percutanée de ces mêmes molécules réalisées en cellule de Franz couplées à HPLC.

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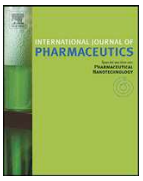

Study of the potential of stratum corneum lipids and exogenous molecules interaction by fluorescence spectroscopy for the estimation of percutaneous penetration

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ABSTRACT

Considering that the skin barrier properties are closely linked to the ceramides composition and conformation within the SC, our work focused on developing a new evaluation criterion in complement of the Log Pow and MW: lipids retentive role within the SC. We developed an in vitro model to study exogenous molecules (Mol) and SC lipids interaction by fluorescence spectroscopy. As ceramides do not fluoresce, fluorescence probes that emit a fluorescence signal in contact with lipidic chains were selected for the study. A protocol was developed based on the exogenous molecule (cosmetic actives) affinity for the SC lipids. A fluorescence criterion (ΔI) was calculated from our results and compared to ex vivo skin penetration measurements realized with a Franz cell device. Our results indicated that polarity seems to be very representative of the ceramide and exogenous molecule interaction for most of the molecules tested. However, the ΔI calculated highlighted the particular interaction of some exogenous molecules with ceramides and their skin distribution. This particular behavior was not initially possible to estimate with the Log Pow and MW. This work aimed to develop a new alternative method to enhance the percutaneous penetration estimation of exogenous molecules for the risk analysis.

I- INTRODUCTION

The main function of the skin is to protect the human body against water loss, ultraviolet light and chemical absorption. The stratum corneum (SC), the outermost epidermal layer, plays a central role to achieve this function. It is composed of dead cells, corneocytes, embedded in a unique lipidic matrix. The SC lipidic matrix is mainly composed of ceramides, cholesterol and long-chain free fatty acids.

Ceramides are known to play a key role in structuring the SC, they consist of a sphingoid base N-acylated by a long chain of fatty acid. Recently, eleven classes of CER have been determined [1]. Their structural variety results from possible combination between 4 types of sphingoid base (sphingosine, phytosphingosine, 6-hydroxysphingosine or dihydrosphingosine) and 3 types fatty acids (non-hydroxyacids, α or ω hydroxyacids) [2,3]. The acyl chain length is variable from 18 to 32 carbons. The long hydrophobic ceramides chains are essential to maintain the barrier properties. Short chains ceramides are able to increase significantly skin permeability to drugs [4,5]. Also a change in their composition can be associated with a change in the lamellar lipid organization in atopic eczema patients that altered then their SC properties [6].

With the banned of animal testing in the cosmetic industries, there is a need to develop efficient alternative methods to predict the percutaneous penetration of cosmetics ingredients. Many in vitro and in vivo methods are referred to study the absorption of chemicals through the skin [7] . Without in vivo methods validated yet the current in vitro guidelines for percutaneous penetrations are referenced by the OECD and SCCS [8,9]. The issue with in vitro tests is their limitations due to the lack of homogeneity between the experiments i.e. skin type (animals or human) or skin treatment (full thickness or dermatomed). In a recent draft, the OECD recommended that the prediction of percutaneous penetration should be based on structural characteristics of these molecules i.e. Log Pow (polarity) and MW (molecular mass) [10]. If the MW of a molecule is ≤ 500 g/mol and the Log Pow is between -1 and +4, a 100% percutaneous penetration can be considered. Outside these criteria, it is considered at 10%. Studies tried to improve the OECD prediction using models including more parameters such as the vehicle and formulation [11,12].

Many mathematical models can be found in the literature. Most of the models predict skin permeability from two parameters, Log Pow and MW i.e. Potts and Guy model [13].

Potts and Guy mathematical model:
Log Kp (cm/s) = 0,71Log Pow – 0,0061MW – 6,3

Lian correlated permeation results with predictive models and the best prediction found was from Mitragotri mathematical model, but with only a R^2 of 0,698 a little higher than the Potts and Guy model, $R^2 = 0,676$ [14]. These empirical models fit mostly for molecules with a Log Pow comprised between 0 and 4. The Potts and Guy model cannot predict the permeation for molecules with a Log Pow above +6.
Considering that the skin barrier properties are closely linked to the lipidic composition and conformation within the SC, our work focused on developing a new evaluation criterion for the prediction of percutaneous penetration in complement of the Log Pow and MW: lipids retentive role within the SC.
We developed an in vitro model to study exogenous molecules (Mol) and SC lipids interaction by fluorescence spectroscopy. As ceramides do not fluoresce, fluorescence probes that fluoresce in contact with lipidic chains were selected for the study. The protocol was developed based on exogenous molecules (cosmetic actives) affinity for the SC lipids.
A probe in solution was first mixed with a lipidic solution (ceramides). The interaction between the probe and lipids increased the fluorescence emission of the probe. Then an exogenous molecule was added to the probe-lipid solution. If the molecule had affinity for the lipid, the molecule-lipid interaction removed the probe from the lipidic chain and decreased in consequence the fluorescence intensity (I_{fluo}).
Three equilibrium were implied:
[Probe]+[Lipid]⇌[Probe-Lipid] (k_1)
[Lipid]+[Mol]⇌[Lipid-Mol] (k_2)
[Probe]+[Mol]⇌[Probe-Mol] (k_3)
To establish a simple predictive model of SC lipids and exogenous molecules interaction, our work was conducted according the following experimental design. The study included various exogenous molecules (UV filters, preservatives, corticoid and cosmetic actives) i.e. Log Pow ranging between -0,07 and 6,88 and MW between

152 and 361 g/mol (table 1). In preliminary studies, operating conditions were set in two steps: First, the appropriate fluorescent probe was chosen between the 1,6-diphenyl-1,3,5-hexatriene (DPH) and 5,10,15,20-tetrakis (3-hydroxyphenyl)-21H,23H-porphirine (m-THPP) (figure 1), according to the stability of the probe-lipid complex fluorescence. These probes have no fluorescence in a polar environment but strongly fluoresced in a hydrophobic media. Previous study revealed that in contact with lipidic chains they emitted a fluorescence signal as a result of non-covalent interaction [15–17]. In a second step, different synthetic ceramides (figure 2), (saturated: Ceramide 2 (Cer 2) or unsaturated: Ceramide IIIa (Cer IIIa)) and SC lipids extracted from human skin were tested with a constant probe concentration and various molecules-concentration. A lipid was selected according to the intensity and the stability of the [Probe-Lipid] fluorescence.

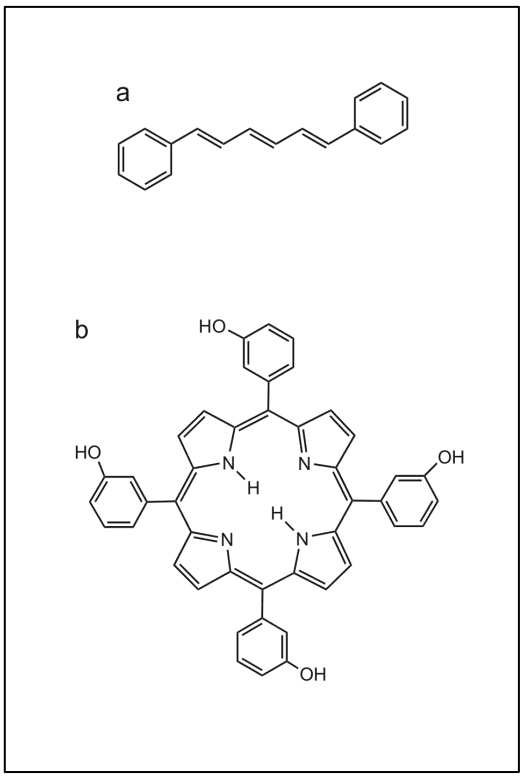

Figure 1: The two fluorescence probes tested for the study: (a) 1,6-diphenyl-1,3,5-hexatriene (DPH), (b) 5,10,15,20-tetrakis(3-hydroxyphenyl)-21H,23H-porphine (m-THPP).

The second part of our study was to develop the predictive approach. We tested the selected lipid with the molecules at a constant concentration and the probe at various concentrations. A SC lipids and exogenous molecule interaction fluorescence criterion, ΔI, was calculated from our results. ΔI was validated by comparing its values with ex vivo skin penetration measurements realized with a Franz cell device coupled to HPLC analysis. This work aimed to develop a new alternative method to enhance the percutaneous penetration estimation of exogenous molecules for the risk analysis.

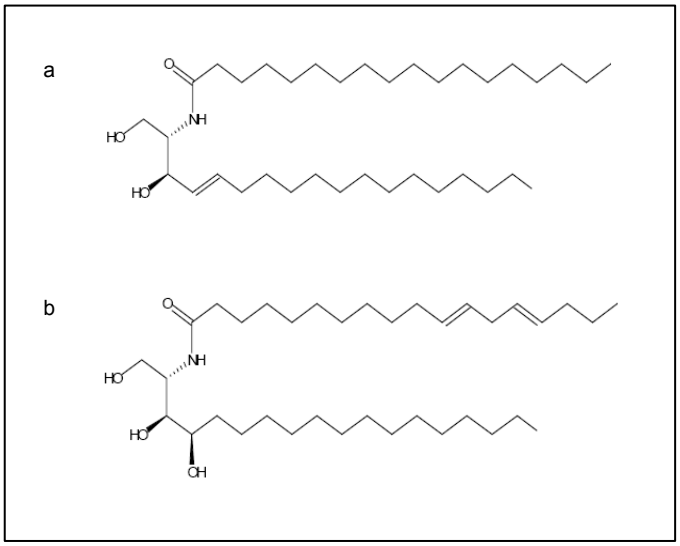

Figure 2: Synthetic lipids tested. (a) Ceramide 2 (Cer 2) and (b) Ceramide IIIa (Cer IIIa)

II. MATERIAL AND METHODS

1- Chemicals and Material

Exogenous molecules: benzophenone 3, methyl, ethyl, propyl and butyl parabens, octinoxate and octisalate were purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Octocrylene (Parsol 40®) at DSM (Courbevoie, France). Caffeine was purchased at Alfa Aesar (Schiltigheim, France) and the prednisolone at Molekula (Dorset, United-Kingdom).

Synthetic ceramides: Cer 2 was purchased at Sederma (Le Perray, France) and Cer IIIa at Evonik Industries (Essen, Germany).

Stratum Corneum lipids (LIP) extraction: Squares of 3 x 3 cm^2 were designed on skin surface from abdominal biopsies of human volunteers. Four cotton swabs drenched in

a mixture of EA/MeOH (20:80 v/v) were used for each square. Each cotton swab was rubbed 10 times on each square and plunged into 2 ml of a mixture of chloroform/methanol (CHCl3/MeOH) (2:1, v/v) during a few minutes. After removing the cotton swabs, solutions were centrifuged at 4000 rpm for 10 minutes to eliminate some cotton particles and skin cells elements. The homogeneous phase containing the lipids was recuperated and evaporated. Residues were recuperated in MeOH and diluted at a concentration of 2.10^{-5}M approximately; calculated from an average lipid molecular mass of 400 g/mol.

Fluorescent probes: DPH was purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France) and the *m*-THPP at Frontier Scientific (Logan, Utah, USA).

Bovine serum albumin was purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Sodium chloride salts was purchased at Carlo Erba Réactifs-Sds (Val de Reuil, France).

HPLC grade methanol was purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Acetic acid was purchased at Prolabo (VWR, Fontenay Sous Bois, Bois).

Fluorescence measurements were performed with a spectrofluorimeter equipped with a red sensitive photomultiplier (Perkin-Elmer, LS50B, Courtaboeuf, France). Excitation and emission wavelengths were set at 419 and 648 nm, respectively, for m-THPP and at 357 and 428 nm for DPH. Data acquisition was performed with FL WinLab Software (Perkin-Elmer, Courtaboeuf, France).

The HPLC was composed of an autosampler (HPLC 565 Autosampler), a column thermostat (HPLC 582 column thermostast), a diode array detector (HPLC 545V diode array detector), a pump (System 525) and a degasser (3493 Degasser). All these elements were purchased at Kontron (Toulon, France). Two C18 columns (150 X 4,6mm and 250 X 4,6mm, Kromasil, AIT, Houilles, France) and C18 guard columns (Kromasil C18, 5mm Guard Column Cartridges, Supelco, Bellefonte, PA, USA) were used. Mobile phase composition was improved for each exogenous molecule to achieve a good selectivity from skin endogenous compounds. Data acquisition was performed with Kroma system 2000 software (Kontron Instruments, Italy). A specific detection wavelength was set for each molecule. a Cary Bio 300 spectro-photometer (Varian, USA).

Table 1: Exogenous molecules studied, ranged by increasing Log Pow

Molecules		Properties	MW	Log Pow
Caffeine	CAF	Cellulite reduction	196	-0,07
Prednisolone	PRE	Corticosteroid	360	1,62
Methyl Paraben	MP	Preservative	152	1,93
Ethyl Paraben	EP	Preservative	166	2,27
Propyl Paraben	PP	Preservative	180	2,81
Butyl Paraben	BP	Preservative	194	3,53
Benzophenone 3	BP3	UV filter	228	3,79
Octisalate	OS	UV filter	250	5,77
Octinoxate	OX	UV filter	290	5,8
Octocrylene	OC	UV filter	361	6,88

2- Preparation of the solutions

a- Fluorescence Spectroscopy

Ceramides: Methanolic solutions of lipids (Cer 2 and Cer IIIa) were conserved at a concentration of 10^{-4} M. Stock solutions were diluted in series in methanol (MeOH) to reach 2.10^{-5} M. Lipids solutions (Cer 2, Cer IIIa and LIP) were conserved at -20 °C.
Probes: Stock solutions of DPH and m-THPP were prepared respectively in MeOH at 4.10^{-4} M and 7.10^{-5} M. These stock solutions were diluted in series daily at a concentration of 2.10^{-7} M in Millipore Q water. All solutions were hidden from light and conserved at -20 °C.
Exogenous molecules were diluted in MeOH at 10^{-2} M, 10^{-4} M and 10^{-5} M and conserved at -20°C before use.

b- Percutaneous Penetration

Exogenous molecules were diluted in ethyl acetate (EA) at 10^{-2} M and conserved at -20°C before use.

3- Protocols

a- Fluorescence Spectroscopy

a-1 Preliminary study

Choice of the probe: First, a methanolic solution of Cer 2 at 2.10^{-5} M was mixed with an aqueous solution of DPH or m-THPP at 2.10^{-7} M with a ratio of 85/15 (v/v). Fluorescence intensity was then recorded during 60 min. Then, the stability of lipids solution (Cer 2, Cer IIIa and LIP) at 2.10^{-5} M mixed with DPH at 2.10^{-7} M was followed during 60 min by fluorescence. At last, the variation of fluorescence intensity with the DPH probe was recorded with variable Cer 2 concentrations. ranging from 10^{-6} M to 10^{-4} M.

Choice of the lipidic model and experimental parameters: An aqueous DPH solution at 2.10^{-7} M was mixed with a 2.10^{-5} M methanolic lipid (Cer 2, Cer IIIa or LIP) solution with a ratio of 85/15 (v/v) respectively. The molecule of interest was added at different increasing concentrations to the [probe-lipid] solution, ranging from 5.10^{-7} M to 4.10^{-5} M final concentration. A DPH-MeOH (85/15) (v/v) solution was used as a blank. After 10 min, the fluorescence was measured for each molecule concentration. In parallel, to check any background noise, the same molecules of interest concentration were added directly to the 2.10^{-7} M DPH aqueous solution without lipids. Experiments were realized in triplicate.

a-2 Predictive approach: Determination of the [Cer IIIa – Molecule] interaction criterion

Variation of the probe concentration: First, DPH was diluted into water at a concentration ranging from 10^{-9} to 2.10^{-6} M. The aqueous DPH solution was mixed with Cer IIIa at 2.10^{-5} M in methanol with a ratio of 85/15 (v/v) respectively. The molecule of interest was added at a 10^{-5} M final concentration to the DPH-Cer IIIa solution. The fluorescence was measured 10 minutes after the addition of the molecule. In parallel, an appropriate DPH solution without lipids was prepared, the ratio 85/15 (v/v) was kept with pure methanol instead of a lipid methanolic solution. Its fluorescence intensity was systematically subtracted from the fluorescence

intensity of the probed lipid solution with and without the addition of the exogenous molecule.

b- Percutaneous Penetration Studies

b-1 Preparation of the human skin and Franz cells

Fat was removed from fresh skin from abdominal plastic surgery. Skin was stored at -25°C and full thickness skin was used for the experiments. Before the experiment the skin was immersed in physiologic serum (0,85% NaCl) for 20 min and then cut in circle shapes to fit the glass diffusion cells. Each skin was carefully washed with Millipore Q water and dried with paper before to be mounted on the franz cell. Franz cells had a diameter of 2 cm^2 and a volume of liquid receptor of 11,5 ml (Lara Spirals, Couternon, France). A magnetic stirrer bar was added in the donor compartment. The liquid receptor was filled with distilled water containing 0,85% NaCl and 0,01% of bovine serum albumin. Air bubbles in the donor compartment were removed. The system was thermostated at 37°C above a magnetic stirrer to ensure the homogeneity of the liquid receptor during the experiment. After 30 min of stabilization, 200 µl of a solution containing one of the exogenous molecules diluted in EA at 10^{-2} M was dropped on the skin surface. During the experiment Franz cells were not occluded left at open air.

b-2 Preparation of collected samples

After 22 hours, the liquid receptor and the skin were collected. The skin surface was washed with a cotton swab on which 200 µl of liquid receptor (distilled water containing 0,85% NaCl and 0,01% of bovine serum albumin) was dropped. After washing, the skin surface was tape-stripped 3 times with D-squame tape (Cuderm, Dallas, USA) at the same constant pressure. The 3 tapes were pooled in a 20 ml vial and stored at -20°C until analysis. The liquid receptor was filtrated and stored at -20°C until analysis. The epidermis was separated from the dermis by heating and then cut into small pieces with a scalpel. The epidermis and the dermis were stored at -20°C until analysis. The exogenous molecule was extracted from the tapes by adding 2 ml of toluene in the vial over night. The next day, tapes were removed from the vial

and 1 ml of the solution recovered was diluted in methanol and filtrated (Sterile filters, 0,22µm, Roth, Lauterbourg, France) until the solution was completely limpid. The dermis and epidermis were grounded with a an adapted mixer and tubes (ULTRA-TURRAX® Tube Drive and BMT-20-S tubes, IKA, Staufen, Germany) in 5 ml of methanol. The methanolic recuperated was filtrated. All solutions from collected samples were stored at -20°C until analysis.

b-3 HPLC Analysis

All samples (tapes, epidermis, dermis and liquid receptor) were quantified by HPLC analysis coupled to a UV detector. A sample of 20 µl was injected. For each analysis, standard samples were prepared ranging from 10^{-6} M to 10^{-4} M. The mobile phase and the UV wavelength used for the experiments are summarized in table 2.

Table 2: Schedule of operating conditions for chromatographic analyses with methanol (MeOH), acetic acid (AA) and water (H_2O). LOD represents the limit of detection and LOQ the limit of quantification.

Molecule		Wavelength (nm)	Column	Mobile Phase	Linearity (R^2)	Accuracy (%)	Repetability (%)	LOD (µM)	LOQ (µM)
Butyl Paraben	BP	254	C18, 5 mm; 150 mm x 4,6 mm	50 % H_2O (1 % AA) / 69 % MeOH	0,99	5	1,1	0,45	1,5
Benzophenone 3	BP3	291	C18, 5 mm; 150 mm x 4,6 mm	31 % H_2O (1 % AA) / 69 % MeOH	0,99	3,6	0,1	0,02	0,05
Octisalate	OS	250	C18, 5 mm; 150 mm x 4,6 mm	5 % H_2O (1 % AA) / 95 % MeOH	0,99	2,8	2,4	0,78	2,6
Octinoxate	OX	290	C18, 5 mm; 150 mm x 4,6 mm	5 % H_2O (1 % AA) / 95 % MeOH	0,99	2,6	1,2	0,38	1,3
Octocrylene	OC	307	C18, 5 mm; 150 mm x 4,6 mm	21 % H_2O (1 % AA) / 79 % MeOH	0,98	7,5	0,1	0,01	0,05

III- RESULTS AND DISCUSSION

1- Preliminary Study

a- Probe choice

The probe was chosen regarding the fluorescence intensity and the stability of the complex [probe-lipid]. Experiments were conducted to choose the most appropriate fluorescent probe between DPH and *m*-THPP for the predictive. In a previous work, DPH and *m*-THPP showed high fluorescence intensity when associated to organized lipidic edifices such as micelles. *m*-THPP was shown to induce a higher response than

DPH for lipids (Ibrahim et. al, 2011). The interest of *m*-THPP in comparison to the other probes used for lipid detection is, from a spectroscopic point of view, to present an emission at 600–800 nm (maximum around 650 nm) and so potentially a better selectivity. Ibrahim determined the most appropriate probe-lipid ratio for the fluorescence lipidic detection at 85/15 water/methanol mixture (v/v) with either DPH or *m*-THPP. It was found that 15% of methanol (v/v) avoided precipitation of phospholipids species and resulted in the maximum difference in fluorescence emission in the presence and in the absence of lipids (Ibrahim et. al, 2011). The same ratio was selected for our protocol.

In our experiments, [m-THPP-Cer 2] fluorescence intensity was higher than [DPH-Cer 2] after 10 min of contact. It reaches 350 AUF compared to 150 AUF for the DPH. We noticed that [*m*-THPP-Cer 2] fluorescence decreased continually during the 60 min of observation. After 60 min, *m*-THPP lost 100 AUF whereas [DPH-Cer 2] fluorescence increased during the 10 first minutes of the measurement and then staid stable during the observation.

DPH was then considered the most appropriate probe for our study due to a more stable association with the ceramide carbonyl chains. This stability is a key point to determine the interaction between ceramides and exogenous molecules without being trouble by the variation of fluorescence intensity of the complex [Probe-Lipid].

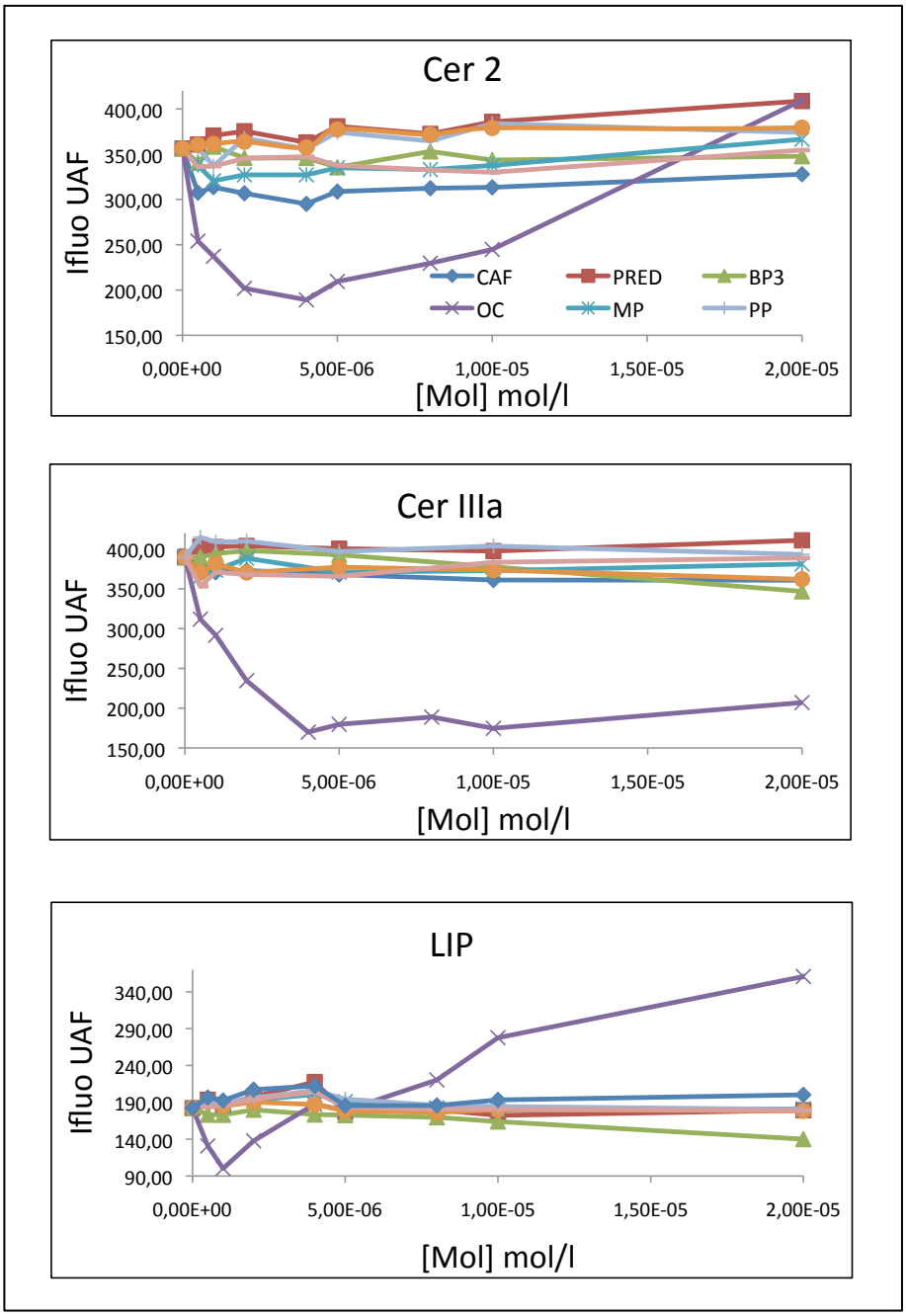

Figure 3: Fluorescence curves obtained with a constant DPH concentration and eaxh of the three lipidic models tested separateley, Cer 2 (a), Cer IIIa (b) or cutaneous lipids (LIP) (c) at constant concentrations. Mol were added at different increasing concentration into the solution [Lipid-DPH]. In the three models tested, only OC decreased significantly the fluorescence intensity. After a certain concentration, the fluorescence increased with Cer 2 and LIP. For Cer IIIa, the fluorescence remained low with the different OC concentrations tested.

b- Choice of the lipidic model and experimental parameters

The lipidic model was chosen first according to the fluorescence intensity and stability of the complex [probe-lipid] with the 3 lipidic models (Cer 2, Cer IIIA and LIP). Second in function of I_{fluo} variation after addition of the molecule at various concentrations into the complex [probe-lipid] and the stability of the equilibrium regarding the molecule concentrations tested.

DPH was tested with the different lipid models. DPH fluorescence emission at 427 nm recorded a stable value after 10 min after mixing with Cer 2, Cer IIIa or LIP and remained stable for the next 60 min. Fluorescence intensity was around 360 UA for Cer 2, 400 for Cer IIIa and 190 for LIP.

Fluorescence intensity did not vary significantly with the three lipidic models after the addition of exogenous molecules at different concentration into the solution [DPH-lipid] except for OC (figure 3). Two parameters were considered for OC: I_{fluo} decrease and the dynamic concentration range in which I_{fluo} decrease was noted. We observed two parts in the OC fluorescence curve after its addition into [DPH-lipid] solution.

1) After the addition of the lowest OC concentration (5.10^{-7} M) into the [Probe-Lipid] solution, I_{fluo} immediately decreased and kept decreasing until the addition of a particular OC, named the threshold concentration. For low [OC], the fluorescence intensity decrease was related to the reaction:

[Probe-Lipid] + Mol $\rightleftharpoons$ [Mol-Lipid] + Probe (k) with $k = k_2 / k_1$

2) After this threshold concentration reached, I_{fluo} remained constant (Cer IIIa) or increased significantly (Cer 2 and LIP). The increase was not observed with [Probe-Cer IIIa], probably according to a stronger interaction between Mol and Cer IIIa.

The fluorescence intensity increase (for Cer 2 and LIP) was related to the reaction:

[Probe-Lipid] + Mol $\rightleftharpoons$ [Probe-Mol] + Lipid (k') with $k' = k_3 / k_1$

We compared I_{fluo} variation between [OC] = 0 M and the threshold concentration for each lipidic model. For Cer 2 and Cer IIIa, I_{fluo} lost a maximum of 167 and 220 AUF respectively after the addition of a concentration of [OC] = 4.10^{-6} M into the [DPH-Lip] solution. For LIP, I_{fluo} lost a maximum of 82 AUF after the addition of 10^{-6} M of OC. After these threshold values reached, 4.10^{-6} M and 10^{-6} M respectively for Cer 2 and LIP, I_{fluo} increased. OC was able to induce a lipophilic environment after reaching

the threshold concentration to interact with DPH and increased the fluorescence intensity in consequence with the Cer 2 and LIP. For Cer IIIa, the I_{fluo} did not vary significantly between 4.10^{-6} M and 2.10^{-5} M, I_{fluo} variation remained at the lowest value, showing that OC had a stronger interaction for the Cer IIIa rather than with the DPH. Moreover, OC showed the highest fluorescence variation with the Cer IIIa.

The lack of significant variation of I_{fluo} for the other molecules may be explained by their inability to "remove" the [Probe-Lipid] complex. It may have been caused in part by the DPH polarity (Log Pow = 5,6) compared to the polarity of the other molecules. The difference of Log Pow prevented the exogenous molecules, except OC that had the highest Log Pow (Log Pow = 6,88), to enter in competition with the DPH for interacting with the lipidic chains.

As DPH had the highest I_{fluo} in solution with Cer IIIa and highest I_{fluo} variation after addition of OC, this lipid was selected for the predictive approach protocol.
For the predictive approach, two UV filters with a Log Pow closer to DPH, OS (Log Pow = 5,77) and OX (Log Pow = 5,8), were added to the study and more hydrophilic molecules removed (CAF, PRED, MP and EP). The molecule concentration was set at 10^{-5} M as at this concentration is high so molecules will in sufficient quantity to enter in competition with the DPH and at this concentration OC remained in interaction with the ceramides chains.

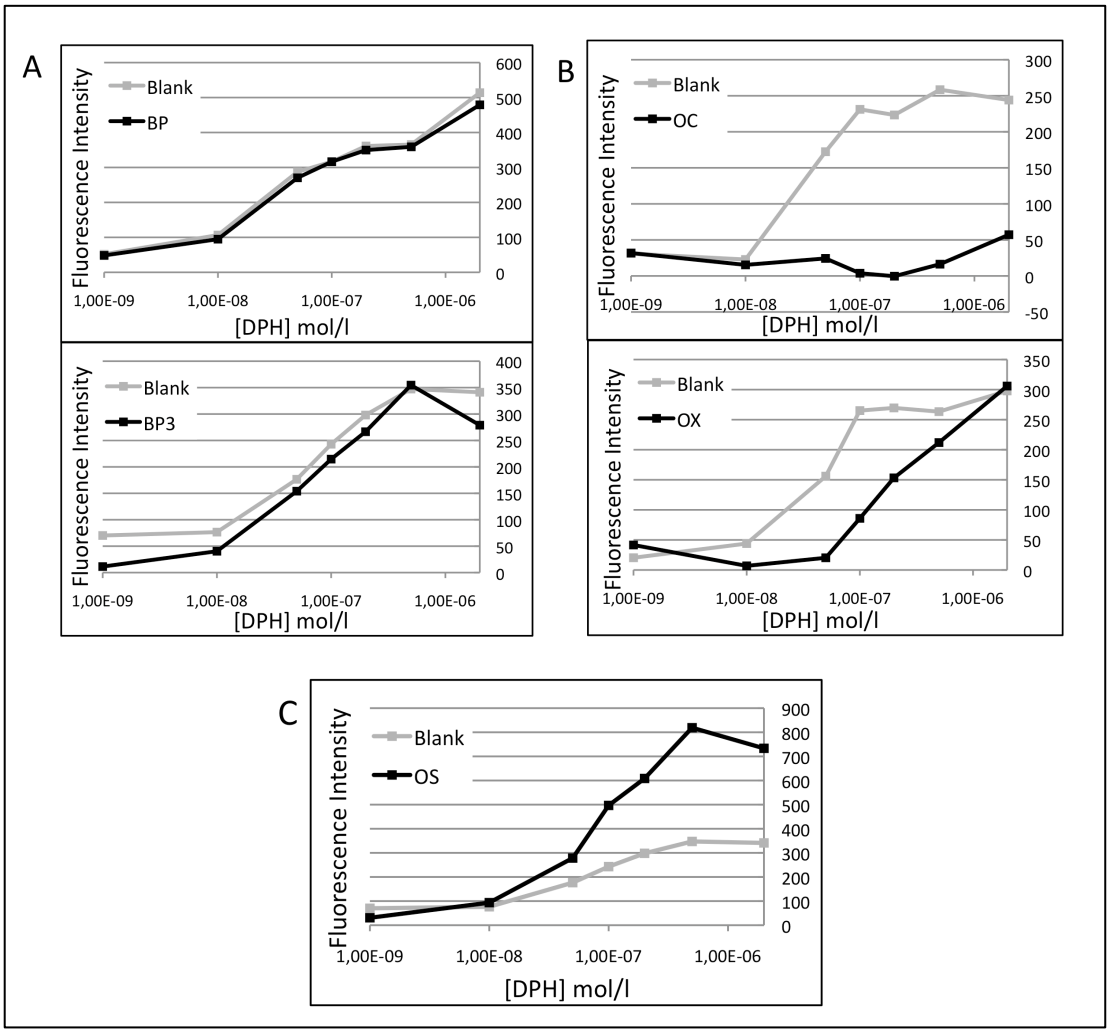

Figure 4: Variation of fluorescence intensity in the predictive approach with a variation of DPH concentration and a constant exogenous molecules (Mol) and Cer IIIa concentrations. Mol were butyl paraben (BP), benzophenone 3 (BP3), octinoxate (OX), octisalate (OS) and octocrylene (OC). Each Mol was added separately to the [DPH-Cer IIIa] solution and the variation of fluorescence intensity variation was recorded after 10 min. Three profiles were observed A, B and C. The white curve represents the fluorescence curve with [Cer IIIa-DPH], I_{fluo}(Molfree), and the black the fluorescence curve with Mol added to [Cer IIIa-DPH], I_{fluo}(Mol).

2- Predictive approach: Determination of the Cer IIIa and Mol interaction criterion.

For the predictive approach, a new protocol was developed with a constant Cer IIIa and Mol concentration and a variation of DPH concentration. The concentration of Cer IIIa was set at 2.10^{-5} M and Mol concentration at 10^{-5} M. Increasing concentration

of DPH were tested from 10^{-9} M to 2.10^{-6} M. The DPH concentration variation was based on a protocol initiated in a previous study with phospholipids [18].

The fluorescence intensity of [DPH-Cer IIIa] solution was represented as I_{fluo} (MolFree) without molecules and as I_{fluo} (Mol) with Mol into the solution. We observed 3 different curves profiles (figure 4).

$I_{fluo}\ (Mol) = I_{fluo}\ (MolFree)$ (A)

For BP3 and BP, the I_{fluo}(MolFree) and I_{fluo}(Mol) were not notably different. There was no competition between BP3, BP and DPH for the Cer IIIa. No specific interactions were detected for these two molecules with the Cer IIIa. This observation showed the unability of the model to highlight specific interaction between molecule and Cer IIIa when the molecules polarity are quite lower than the probe polarity.

$I_{fluo}\ (Mol) < I_{fluo}\ (MolFree)$ (B)

For OX and OC, the I_{fluo}(Mol) was lower than I_{fluo}(MolFree). This observation highlights that an interaction between these molecules and Cer IIIa occured. I_{fluo}(OC) stayed closed to zero overall the DPH concentration range and showed a stronger interaction [OC-Cer IIIa] than [OX-Cer IIIa]. OC interacted with Cer IIIa and not with the probe. I_{fluo}(OX) was close to zero until the addition of a DPH concentration of 8.10^{-8} M, after this threshold I_{fluo}(OX) increased to reach the same intensity as I_{fluo}(MolFree) for the highest concentration. The I_{fluo} increase may be due to OX removal from the lipidic chain and to its interaction with DPH. OC had a stronger interaction with Cer IIIa compared to OX.

$I_{fluo}\ (Mol) > I_{fluo}\ (MolFree)$ (C)

I_{fluo} (OS) was higher than I_{fluo}(MolFree) after the addition of 7.10^{-8} M of OS. The complex [OS-DPH] fluoresced more than the complex [Cer-DPH]. OS may have had more affinity for the DPH than for the Cer IIIa. OS was able to induce a lipophilic environment for the DPH to fluoresce complexed to OS.

From these results, we proposed a fluorescence criterion to evaluate Mol-Cer interaction as a model to highlight a potential interaction between Mol and the SC lipids. The fluorescence criterion (ΔI) was the difference of fluorescence intensity at

[DPH]=10^{-7} M between I_{fluo}(MolFree) and I_{fluo}(Mol): The 10^{-7} M DPH concentration was chosen as this concentration was within the dynamic zone of response.

$\Delta I = I_{fluo}$ (MolFree) – I_{fluo} (Mol)

Mol were classified in function of their interaction with the Cer IIIa, represented by the fluorescence criterion, ΔI that showed their potential interaction with SC ceramides (Table 3). ΔI = 0 meant no interaction between Mol and Cer IIIa occured, ΔI > 0 meant that Mol interacted with Cer IIIa and ΔI < 0 meant that Mol interacted more with DPH than with the Cer IIIa.

Table 3: Exogenous molecules ranged in function of their fluorescence criterion ΔI.

	ΔI	**Log Pow**	**MW**
OS	-253,8	5,77	250
BP	0	3,53	194
BP3	28,25	3,79	228
OX	179,24	5,8	290
OC	227,41	6,88	351

3.3. Percutaneous Penetration Results

The molecules of interest were detected in each compartment 22h after the deposit except in the liquid receptor. The average amount (n=3) of molecules quantity distribution within tape stripping, epidermis, dermis and liquid receptor was summarized in table 4. The ratio EP_{tot}/DE was calculated in order the show the proportion of molecule present in the total epidermis compared to the quantity detected in the dermis. The higher was the ratio EP_{tot}/DE, the more the molecule was retained in the total epidermis compared to the dermis.

BP (EP_{tot}/DE = 6,9) and BP3 (EP_{tot}/DE = 6,4) had the lower Log Pow and EP_{tot}/DE ratio within the five tested molecules. They tended to be more present in the dermis rather than the epidermis compared to the other molecules tested. OC (EP_{tot}/DE = 10,0) and OX (EP_{tot}/DE = 15,1) were proportionally the most retained n the epidermis. OX that had the same polarity as OS (EP_{tot}/DE = 8,3) was proportionally much more distributed into the epidermis than the dermis. OC that was the most apolar molecule had a smaller ratio at 10,0 compared to OX meaning that OC was less retained in the epidermis than OX. The ratio value (EPtot/DE) of these three molecules (OC, OX and OS) was not completely related to their Log Pow or MW.

Table 4: Molecule quantity (nmol/cm^2) within the 3 tapes stripping (TS), epidermis (EP), total epidermis (EPtot = TS + EP), dermis (DE) and the ratio EP_{tot}/DE.

	TS	EP	DE	LR	EP_{tot}	EP_{tot}/DE
BP	250,0 ± 106,0	88,1 ± 37,6	49,6 ± 5,4	≤ LOD	338,1	6,8
BP3	161,5 ± 12,7	131,9 ± 31,3	54,1 ± 22,1	≤ LOD	293,3	5,4
OS	187,9 ± 30,2	107,7 ± 19,8	35,4 ± 10,3	≤ LOD	295,6	8,3
OX	173,3 ± 29,9	150,1 ± 65,6	21,4 ± 7,8	≤ LOD	323,3	15,1
OC	186,5 ± 73,5	142,4 ± 15,2	32,8 ± 11,5	≤ LOD	329,0	10

3.4. Comparison of the percutaneous penetration results with the fluorescence criterion and Log Pow

The fluorescence intensity curves in comparison of the molecules distribution profile provided relevant information. For example, BP3 and BP had the same fluorescence curve profile, they didn't enter in competition with the DPH for the Cer IIIa and they had the lower EP_{tot}/DE ratio. These observations meant that BP and BP3 were proportionally less retained in the epidermis and had no specific interaction with the SC lipids compared to the other molecules. The fluorescence criterion revealed that OC and OX had affinity for the Cer IIIa and their distribution showed that these molecules were proportionally more retained in the epidermis compared to the dermis. Despite their different Log Pow, OX and OC seemed to have similar interaction with SC lipids within the epidermis. OS that have the same polarity as OX, had a different affinity for the ceramide in fluorescence and was proportionally much less retained in the epidermis compared to OX. ΔI was more related to the distribution profile of the molecules within the skin.

To confirm these observations a correlation was performed between the fluorescence criterion ΔI and the Log Pow of the molecules tested. The results are illustrated in figure 5. It was difficult to correlate ΔI (OS) (ΔI < 0) with the other ΔI (ΔI > 0) ΔI had opposite value. In consequences, correlations were calculated with and without OS. ΔI and Log Pow were very much correlated (R^2 = 0,99) without OS but not correlated at all with OS (R^2 = 0,08). This correlation indicates that polarity seems to be very representative of the ceramide and exogenous molecule interaction in this study, meaning that this interaction may be mainly due to the lipophily. For most of the molecules, Log Pow was sufficient to predict their skin distribution profile, but if the

molecule did not enter into the correlation, such as OS, it means that this molecule had a lower retention that was represented by its fluorescence criterion ΔI.

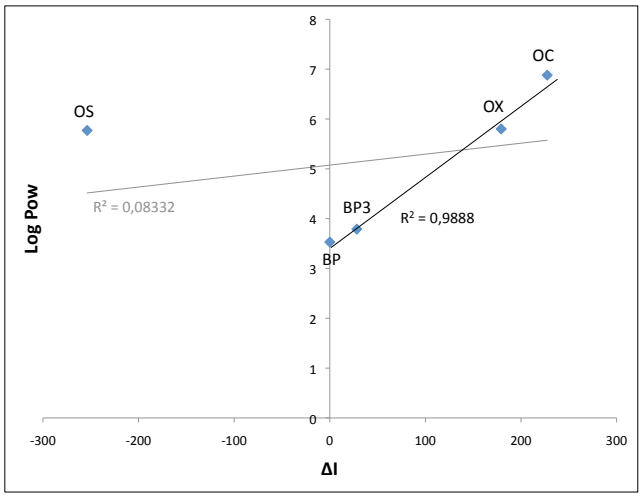

Figure 5: Ratio EPtot/DE expressed in function of the fluorescence criterion (ΔI).

In parallel we expressed our results according to the SCCS recommendations [9]. The SCCS claims that both the epidermis (except for the stratum corneum) and dermis are considered as a sink. The amounts found in these compartments are considered as absorbable and are added to those found in the receptor fluid. The amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed, and thus they are not expected to contribute to the systemic dose**.** We expressed the epidermal and dermal absorption (EP+DE) in absolute amount of skin surface (µg/cm^2) in Table 5.

Table 5: Molecule quantity (µg/cm^2) within the 3 tapes stripping (TS), epidermis (EP), dermis (DE) and EP+DE.

	TS	EP	DE	LR	EP + DE
BP	48,5 ± 20,6	17,1 ± 7,3	49,6 ± 1,0	≤ LOD	26,7
BP3	36,8 ± 2,9	30,1 ± 7,1	54,1 ± 5,0	≤ LOD	42,4
OS	47,0 ± 8	26,9 ± 5	35,4 ± 3	≤ LOD	35,8
OX	54,5 ± 8,7	31,2 ± 19,0	21,4 ± 2,3	≤ LOD	41,5
OC	65,5 ± 25,8	50,0 ± 5,3	32,8 ± 4,0	≤ LOD	61,5

We did not consider the amount in the LR as the quantity detected was less than the limit of detection. There might be SC left in EP as the 3 tapes stripping on human biopsies did not remove the entire SC. We expressed EP+DE molecule quantity detected in function of the ΔI and Log Pow (Figure 6). A strong correlation was not observed neither for Log Pow nor ΔI with EP+DE (R^2 = 0,53 and 0,38 respectively). The four molecules, BP, BP3, OX and OC epidermal and dermal distribution were quite related to the ΔI and Log Pow (R^2 = 0,67 and 0,71 respectively). However, Log Pow did not highlight the particular behavior of OS, this was only possible with the fluorescence criterion (ΔI < 0).

The ratio EP_{tot}/DE seems more relevant than EP+DE in this work to compare the fluorescence criterion calculated with the franz cells data. The ratio calculated reflected here the fast kinetic of OS at 22h. This observation was not possible to determine with EP+DE.

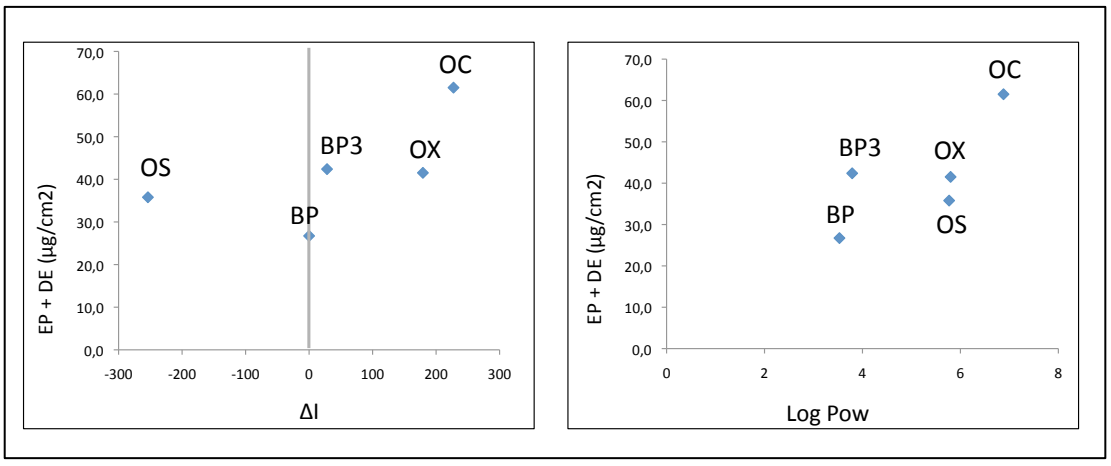

Figure 6: Correlation between exogenous molecules fluorescence criterion (ΔI) and their polarity (Log Pow). The correlation was calculated with OS (R^2 = 0,08332) and without OS (R^2 = 0,9888)

IV- CONCLUSION

Our study helped to understand the exogenous molecule and ceramide specific interaction with a fluorimetric indicator. Exogenous molecules were able to displace the [probe-lipid] equilibrium and this capacity was correlated to our percutaneous penetration data. Molecules with affinity for the Cer IIIa (OC and OX) were found to be proportionally more distributed into the epidermis. Molecules that were not able to

remove the probe from the lipid (BP and BP3) were found to be the less proportionally distributed into the epidermis. These results revealed that the nature of the ceramide and exogenous molecules interaction is mainly of a lipophilic nature but the fluorescence criterion defined provided complementary information. The fluorescence criterion (ΔI) highlighted the particular interaction of some molecules with the ceramide and their skin distribution. This particular behavior was not initially possible to estimate with the Log Pow and MW. It was not possible to test molecules with a too high difference of polarity with the probe: molecules with a much lower Log Pow were not able to enter in competition with the probe. The protocol of the predictive approach can now be transposed to more investigations with other fluorescence probes and cutaneous lipids.

This study showed the interest of a new criterion, a fluorescence criterion, which characterized the interaction between cutaneous ceramides and exogenous molecules. Because of the limitation of ex vivo protocols by franz cells for the study of percutaneous penetration and their lack of homogeneity, there is a real need to improve predictive models with a constant protocol for the risk analysis of new compounds. This new criterion could help to develop more improved mathematical models in complement of the current coefficient used.

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Conclusion

La première partie de notre travail a permis de choisir la sonde de fluorescence et le céramide le plus approprié pour notre protocole de caractérisation de l'interaction de molécules exogènes avec des céramides du SC. La sonde DPH et le céramide IIIa ont fournis les résultats les plus intéressants considérant la stabilité de l'interaction et l'intensité de la fluorescence. Les tests préliminaires ont montré qu'il n'était en revanche pas possible d'étudier l'interaction ceramide-molécule avec des molécules donc le Log Pow était très inférieur à celui de la sonde. En effet, ces molécules n'ont pas la capacité d'entrer en compétition avec la sonde de nature lipophile (Log Pow=5,8) vis-à-vis du céramide sélectionné. C'est pourquoi la deuxième partie de notre étude s'est focalisée sur des molécules avec une lipophilie proche ou supérieure à celle du DPH et a eu pour objectif de développer et de valider un critère prédictif de la pénétration percutanée par spectroscopie de fluorescence.

Les expériences ont montré trois profils de courbes de fluorescence représentatives de l'interaction céramide-molécule en fonction des molécules testées. Pour certaines molécules, aucune compétition n'a été observée avec la sonde supposant que ces molécules n'ont pas d'interactions spécifiques avec le céramide (BP et BP3). Dans un deuxième cas, les molécules se sont révélées être de bonnes compétitrices vis-à-vis de la sonde pour le céramide (OX et OC) et une baisse de fluorescence a été observée. Cette baisse de fluorescence témoigne de l'interaction entre ces molécules et le céramide IIIa. Un troisième profil de courbe a montré, une augmentation du signal de fluorescence lors de l'ajout de la molécule dans le milieu (OS). Ce résultat suggère une plus grande affinité de la molécule pour la sonde que pour le céramide IIIa.

A partir de ces courbes, le critère d'évaluation de l'interaction entre les molécules exogènes et le céramide, ΔI, a été calculé. Les molécules ont été classées en fonction de leur ΔI, c'est à dire de leur capacité ou non à déplacer la sonde des céramides. $\Delta I = 0$ suggère qu'il n'y a pas d'interaction entre la molécule et le céramide, $\Delta I > 0$ suggère que la molécule a interagi avec le céramide et a déplacé la sonde, $\Delta I < 0$ suggère que la molécule a plus d'affinité pour la sonde que pour le céramide.

La comparaison des valeurs de ΔI avec les données de référence en cellules de Franz a validé ce critère et permis de mettre en évidence une relation entre les données de pénétration percutanée et la valeur du ΔI. BP et BP3, qui ont un ΔI nul, étaient les moins retenus au niveau de l'épiderme sur cellule de Franz. Cela montre que ces deux molécules n'ont probablement pas d'affinité particulière pour les céramides et sont en conséquence peu retenues dans le SC. OC et OX qui ont un ΔI positif sont les molécules les plus retenues au niveau de l'épiderme total d'après les données de pénétration percutanée. En revanche OS, qui présente le même caractère lipophile que OX, a un ΔI<0 ce qui suggère que cette molécule n'a pas interagi avec le céramide mais avec la sonde. A partir des données de pénétration percutanée, on remarque que OS est deux fois moins retenu que OX au niveau de l'épiderme. Le critère ΔI reflète donc bien le profil de distribution des molécules à travers la peau.

ΔI et Log Pow sont très corrélés sans OS ($R^2 = 0,99$), la polarité semble donc être très représentative de l'interaction céramide-molécules mais si une molécule a peu d'affinité pour les céramides comme c'était le cas pour OS, seul le ΔI a montré cette particularité qui pourrait être très utile pour l'analyse à priori du risque toxique potentiel.

Cette étude a donc permis, grâce à la spectroscopie de fluorescence, de caractériser l'interaction entre des molécules exogènes et un céramide représentatif des lipides du SC. Ces données montrent que l'évaluation de cette interaction in vitro par fluorescence apporte des informations complémentaires aux paramètres physicochimiques de la molécule (LogPow et MW) et met en évidence des interactions particulières. Ce nouveau critère de fluorescence ΔI pourrait également être utilisé comme critère complémentaire dans le développement de nouveaux modèles mathématiques prédictifs de la pénétration percutanée, le modèle mathématique de Potts and Guy ne permettant de prédire l'absorption de molécules avec un Log Pow>6.

La limite de cette étude est qu'elle n'est pas applicable aux molécules ayant une polarité inférieure à celle de la sonde DPH, d'où la nécessité d'utiliser des sondes de lipophilie différentes. Nous avons ainsi en parallèle tenté d'appliquer ce protocole à

une nouvelle sonde moins lipophile, l'acide cis-parinarique. Le manque de stabilité de la sonde en solution nous a contraint à arrêter les travaux à leur stade préliminaire.

Chapitre 3 : Développement d'un critère prédictif de la pénétration percutanée par microspectroscopie FTIR couplée à une source synchrotron

Introduction

La spectroscopie infrarouge à transformée de Fourier (FTIR) est un outil utilisé depuis de nombreuses années pour l'investigation de la barrière cutanée. La microspectroscopie FTIR s'est révélée être une méthode très utile pour étudier le SC à l'échelle moléculaire et supramoléculaire ainsi que la composition des entités chimiques présentes dans la peau en fonction du site anatomique ou de la profondeur cutanée. La microspectroscopie FTIR permet de suivre l'état de la barrière cutanée grâce aux vibrations des groupements CH_2 des lipides du SC (νCH_2) que l'on peut observer entre 2847 et 2856 cm^{-1} sur leur spectre infra-rouge. Ce signal est représentatif de l'organisation des lipides (état orthorhombique, hexagonal ou désordonné). Récemment, grâce à la puissance du signal Synchrotron, l'utilisation de la microspectroscopie FTIR couplée à cette source a permis de détecter la pénétration de molécules exogènes à travers la peau, en suivant simultanément l'état de la barrière cutanée. Le rayonnement synchrotron permet le suivi de la plupart des molécules à travers la peau qui émettent dans le domaine infrarouge distinctif des éléments de la peau, quelles que soient leurs propriétés physicochimiques (Log Pow et MW) ou la profondeur de la peau.

Dans le but de développer une méthode prédictive plus précise de l'absorption cutanée, notre travail s'est focalisé ici sur le développement d'un nouveau critère par microspectroscopie FTIR avec une source synchrotron pour affiner la prédiction de la pénétration cutanée.

La distribution cutanée d'une quinzaine de molécules avec différents Log Pow et MW a été étudiée. Nous avons tout d'abord réalisé des tests de pénétration percutanée sur cellules de Franz avec des biopsies humaines abdominales. Après 22h, les biopsies

ont été conservées, microtomées puis fixées sur lame afin d'analyser la pénétration des molécules par FTIR avec une source synchrotron. Ou bien, après 22h, les biopsies ont été tape-strippées et les molécules d'intérêt ont été dosées dans les différents compartiments cutanés et tape strips par HPLC couplée à une détection spectroscopique UV. Les données HPLC ont servi de référence. Les résultats de distribution cutanée déterminés par FTIR ont été comparés aux données HPLC.

Dans le but de déterminer un critère prédictif de la pénétration cutanée à partir des données FTIR, une analyse chimiométrique par multi-block, connue sous le nom d'analyse co-dimensionnelle (ComDim) a été réalisée. A partir de ces résultats, le critère prédictif S_{index} a été déterminé pour chacune des molécules étudiées puis une cartographie S_{index}/LogPow, prédictive de la pénétration percutanée, a été réalisée et comparée à celle LogPow/MW de l'OCDE.

Development of a new evaluation criterion by FTIR microspectroscopy with synchrotron source for prediction of percutaneous penetration

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Abstract

This work focused on developing a new evaluation criterion of percutaneous penetration, in complement to Log Pow and MW, based on FTIR microspectroscopy data with a synchrotron source. Skin distribution of fifteen exogenous molecules with various Log Pow and MW were selected. Classic Franz cell experiments were run and after 22h molecule distribution in skin was determined either by HPLC or by FTIR microspectroscopy using a synchrotron source. HPLC data served as reference. HPLC and FTIR results were compared and a new predictive criterion based on the FTIR results, named S_{index}, was determined using a multi-block data analysis technique (ComDim). A predictive cartography of the distribution of molecules in the skin was built and compared to OECD predictive cartography. This new criterion S_{index} and the cartography using FTIR/HPLC results provide relevant information for risk analysis regarding prediction of percutaneous penetration and could be used to build a new mathematical model.

Key words

FTIR microscpectroscopy, synchrotron, Percutaneous penetration, Predictive model.

INTRODUCTION

The main function of skin is to protect the human body against water loss, ultraviolet light and chemical absorption. The stratum corneum (SC), the outermost epidermal layer, plays a central role to achieve this function. It is composed of dead cells, corneocytes, embedded in a unique lipidic matrix. SC lipidic matrix is mainly composed of ceramides, cholesterol and long-chain free fatty acids [1]. Ceramides are known to play a key role in structuring SC, they consist of a sphingoid base N-acylated by a long chain fatty acid. They play a major role in skin permeability, their organization is essential to the maintenance of the barrier status.

With the banning of animal testing in cosmetic industries, there is a need to develop efficient alternative methods to predict percutaneous penetration of cosmetics actives. Many in vitro and in vivo methods exist to study chemicals absorption through skin [2]. While no in vivo methods are as yet validated, current in vitro guidelines for percutaneous penetration are given by the OECD and SCCS [3,4]. Issues with in vitro tests are their limitations due to the lack of homogeneity between experiments i.e. skin type (animals or human) or skin treatment (full thickness or dermatome). In a recent draft, the OECD recommended that prediction of percutaneous penetration should be based on structural characteristics of these molecules i.e. Log Pow (polarity) and MW (molecular mass) [5]. If the MW of a molecule is $\leq$ 500 g/mol and Log Pow is between -1 and +4, 100% percutaneous penetration can be considered. Outside these values, it is considered to be at 10%. In order to develop more precise predictive tools, our work focused on evaluating a new predictive criterion of percutaneous penetration, in complement to Log Pow and MW, based on skin distribution data from FTIR microspectroscopy with a synchrotron source and a reference method by HPLC.

FTIR is a spectroscopic tool that has been used to investigate the cutaneous barrier. It can rapidly and non invasively quantify uptake of a chemical into SC with the tape-stripping method [6,7]. Infrared spectroscopy is an interesting method to study SC molecular components. It is very useful for example for looking at molecular variations, as a function of anatomic site or depth, in order to understand skin biology and physiology [8]. In SC, vibration of CH_2 (νCH_2) from ceramides and other skin lipids give an infrared signal around 2850 cm^{-1} that is specific of lipids organization and correlated to cutaneous barrier status. Thus, barrier status can be monitored by

FTIR and the variation of the νCH_2 stretching peaks can help to follow solvent or penetration enhancer effects on skin lipids [9,10].

Recently, synchrotron FTIR microspectroscopy has been used for further cutaneous studies such as skin penetration. The advantage of using a synchrotron source is that, thanks to its high quality light, it is possible to follow simultaneously skin composition and structure and molecule penetration [11]. Synchrotron light allows a much higher resolution in microspectroscopy and imaging of biological tissues, individual cells, and subcellular components. A small number of studies have used a synchrotron source to study the effect of penetration enhancer on SC lipidic structure and to follow exogenous molecule penetration into the skin [7,12]. In fact, synchrotron light has potential to follow molecules within skin regardless of their infrared signal, physicochemical properties (i.e. Log Pow and MW) and skin depth.

In order to develop a new predictive approach of percutaneous penetration we worked on the feasibility to develop a new evaluation criterion in complement to Log Pow and MW by synchrotron FTIR microspectroscopy. Skin distribution of fifteen exogenous molecules, mostly UV filters and preservatives, with various Log Pow and MW, were studied (table 1). First, classic Franz cells experiments were run for 22h. After 22h the distribution of molecules in the skin was determined either by HPLC after tape stripping and epidermis-dermis heat separation or skin was kept intact and mounted on CaF_2 lames. HPLC analysis was the reference method. The distribution of molecules was then analyzed by FTIR microspectroscopy with a synchrotron source. FTIR results were compared to Franz cells experiments. To determine the predictive criterion S_{index}, a multi-block data analysis technique (ComDim) was carried to relate HPLC and FTIR data and a predictive cartography (S_{index}/Log Pow) of these molecules was built and compared to the OECD map (Log Pow/MW).

Table 1: Exogenous molecules used for the study. Molecules are ranged by increasing Log Pow.

Molecules	Abbreviations	Properties	Log Pow	Mw (g/mol)
Caffeine	CAF	Cellulite reducer	-0,07	196
Prednisolone	PRED	Corticoid	1,62	360
Methyparaben	MP	Preservative	1,93	152
Ethylparaben	EP	Preservative	2,27	166
Propylparaben	PP	Preservative	2,81	180
Choloroxylenol	CL	Preservative	3,37	157
Bisphenol A	BPA	Material coating	3,40	228
Butylparaben	BP	Preservative	3,53	194
Benzophenone 3	BP3	UV filter	3,79	228
Dioxybenzone	DB	UV filter	3,93	244
Irgansan	IR	Preservative	4,80	289
Avobenzone	AB	UV filter	5,00	310
Octisalate	OS	UV filter	5,77	250
Octinoxate	OX	UV filter	5,80	290
Octocrylene	OC	UV filter	6,88	361

II- MATERIAL AND METHODS

1- Chemicals and materials

Exogenous molecules: benzophenone 3 (BP), methyl (MP), ethyl (EP), propyl (PP) and butyl (BP) parabens, octinoxate (OX), octisalate (OS), dioxybenzone (DB), chloroxylenol (CL), bisphenol A (BPA), avobenzone (AB) and irgasan (IR) were purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Octocrylene (OC) (Parsol 40®) at DSM (Courbevoie, France). Caffeine (CAF) was purchased at Alfa Aesar (Schiltigheim, France) and the prednisolone (PRED) at Molekula (Dorset, United-Kingdom).

Bovine serum albumin was purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Sodium chloride salts was purchased at Carlo Erba Réactifs-Sds (Val de Reuil, France).

HPLC grade methanol was purchased at Sigma-Aldrich (Saint-Quentin Fallavier, France). Acetic acid was purchased at Prolabo (VWR, Fontenay Sous Bois, France).

The HPLC system was composed of an autosampler (HPLC 565), a column thermostat (HPLC 582), a diode array detector (HPLC 545V), a pump (System 525) and a degasser (3493). All these elements were purchased at Kontron (Toulon, France). C18 columns (150 X 4,6mm or 250 X 4,6mm, Kromasil, AIT, Houilles, France) and C18 guard columns (Kromasil C18, 5mm Guard Column Cartridges, Supelco, Bellefonte, PA, USA) were used. Mobile phase composition was adjusted for each exogenous molecule to have a good separation from endogenous skin compounds. A Cary Bio 300 spectrophotometer (Varian, USA) was coupled to the HPLC system. A specific detection wavelength was set for each molecule. Data acquisition was performed with Kroma system 2000 software (Kontron Instruments, Italy).

Synchrotron FTIR microspectroscopy experiments were performed on the SMIS beam-line at SOLEIL (Gif sur Yvette, FRANCE) with a Thermo Nicolet Continuum XL microscope (Thermo Fisher, Courtaboeuf, France) equipped with a 50x50 µm² liquid nitrogen cooled MCT/A detector, 32X/NA 0.65 and 15X/NA0.6 Schwarzschild objectives, a Prior XYZ motorized stage, and coupled to a Nicolet 5700 spectrometer equipped with a Michelson interferometer, and a KBr beamsplitter. The spectra were recorded at a resolution of 4 cm^{-1} between 4000 and 700 cm^{-1} with 32 accumulations and a pixel size of 3µm x 3µm to study the SC lipid organization, and a pixel size of 6µm x 6µm for the penetration study, to have bigger images in a short time. The background was recorded with a resolution of 4 cm^{-1} with 256 scans.

2- Percutaneous Penetration Studies

a- Preparation of the human skin and Franz cells

Fat was removed from fresh human skin from abdominal plastic surgery. Skin was stored at -25°C and full thickness skin was used for the experiments. Before experiments, skin was immersed in physiologic serum (0.85% NaCl) for 20 min and then cut in circle shapes to fit glass diffusion cells. Each skin was carefully washed with Millipore Q water and dried with paper before being mounted on the Franz cell. Franz cells had a diameter of 2 cm^2 and a volume of liquid receptor (LR) of 11.5 ml (Lara Spirals, Couternon, France). A magnetic stirrer bar was added in the donor compartment. Liquid receptor was filled with distilled water containing 0.85% NaCl

and 0.01% of bovine serum albumin. Air bubbles in donor compartment were removed. The system was thermostated at 37°C above a magnetic stirrer to ensure homogeneity of liquid receptor during experiment. After 30 min of stabilization, 200 µl of a solution containing one of the exogenous molecules diluted in EA at 10^{-2} M was dropped onto the skin surface. During the experiment, Franz cells were not occluded but left open to the air. Each molecule was tested in triplicate.

b- Preparation of collected samples

After 22 hours, LR and skin were collected. Skin surface was washed with a cotton swab on which 200 µl of liquid receptor (distilled water containing 0.85% NaCl and 0.01% of bovine serum albumin) was dropped. After washing, skin surface was tape-stripped (TS) 3 times with D-squame tape (Cuderm, Dallas, USA) at same constant pressure. These 3 tapes were pooled in a 20 ml vial and stored at -20°C until analysis. LR was filtered and stored at -20°C until analysis. Epidermis (EP) was separated from dermis (DE) by heating and then cut into small pieces with a scalpel. EP and DE were stored at -20°C until analysis. To extract exogenous molecules from tapes, 2 ml of toluene was put in the vial containing the 3 tapes over night. The next day, tapes were removed from the vial and 1 ml of the solution recovered was diluted in methanol and filtered (Sterile filters, 0.22µm, Roth, Lauterbourg, France) until the solution was completely limpid. DE and EP were ground with an adapted mixer and tubes (ULTRA-TURRAX® Tube Drive and BMT-20-S tubes, IKA, Staufen, Germany) in 5 ml of methanol. The recovered methanolic solution was filtered. All solutions from collected samples were stored at -20°C until analysis.

c- HPLC analysis

All samples (TS, EP, DE and LR) were quantified by HPLC system coupled to a UV detector. A sample of 20 µl was injected. For each analysis, standard samples were prepared ranging from 10^{-6} M to 10^{-4} M. HPLC conditions used for some of the molecules studied here are to be found in a previous study [13].

3- FTIR microspectroscopy with synchrotron source

a- Preparation of the samples

Human abdominal skin biopsies studied by FTIR were treated using the same preparation procedure as skin in the Franz cell experiments (cf 2.a). 200 µl of each solution with a concentration of 10^{-1} M of the molecule to be studied in EA was dropped onto the skin surface. A sample skin with pure EA was also tested to serve as blank. After 22h, skin samples were collected and kept intact. Each skin surface was washed with a cotton swab on which 200µl of liquid receptor (distilled water containing 0.85% NaCl and 0.01% of bovine serum albumin) was dropped. The skin was then frozen at -20°C and then -80°C. Biopsies were cryo-tomed at -20°C with a thickness of 10µm and mounted on CaF_2 windows a couple of days before the FTIR measurements.

b- IR measurements

FTIR microspectroscopy was performed on Synchrotron (SMIS beam−line at Soleil). The distribution of molecules was examined at 10 µm (SC), 40, 60, 80, 120 µm (epidermis). 8 spectra were measured at each skin depth. Lipidic barrier status (νCH2) was observed between 2849-2854 cm^{-1}.
All spectra were automatically smoothed with OMNIC software from Thermo Fisher Scientific (Waltham, US).

3- Chemometrics

A multiblock statistical data analysis technique, abbreviated as ComDim, was applied to relate Franz cells results and Synchrotron FTIR spectroscopic data. ComDim, was performed with MATLAB software (MathWorks, Meudon, France).

III- RESULTS AND DISCUSSION

1- FTIR spectral data treatment and selection of the specific band for following distribution of molecules in the skin

The distribution of molecules was examined at different skin depths: 10, 40, 60, 80 and 120 µm (figure 1). 10 µm represented molecule distribution within SC. At 40, 60, 80 µm, it represented molecule distribution within epidermis, from upper to lower levels. At 120 µm it represented molecule distribution at the epidermis-dermis interface.

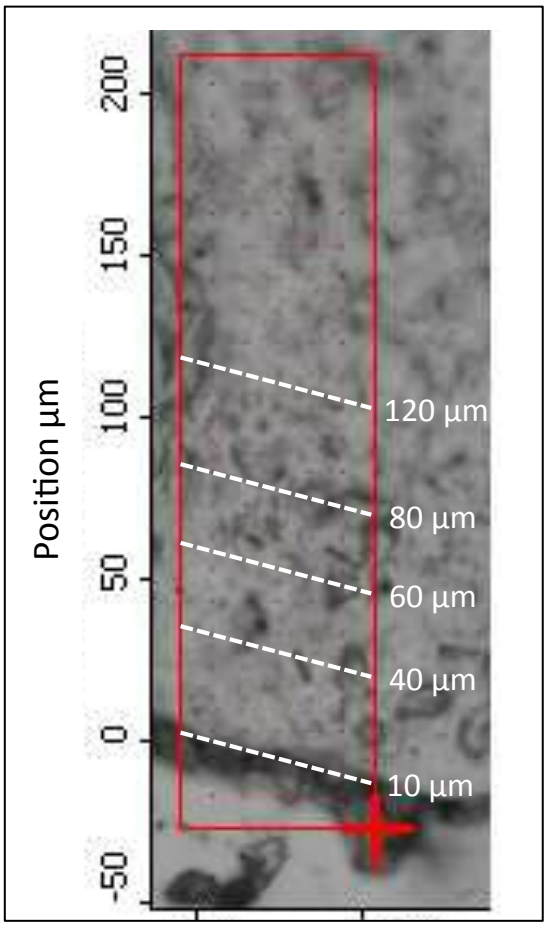

Figure 1: Picture of skin sample on CaF2 window during FTIR acquisition. Molecule distribution was studied at 10, 40, 60, 80 and 120 µm by taking into account skin orientation on the window.

For our all study, to follow molecule distribution within skin, the same method was applied in the following order:

1- 8 spectra were recorded at each of these skin levels (10, 40, 60, 80 and 120 µm). The average spectrum of these 8 spectra at each level was calculated. To improve the resolution of the average spectra, spectra were preprocessed by calculating second derivative, using a third-order Savitzky-Golay function with nine smoothing points [14].

2- To follow molecule skin distribution, a specific band present in the second derivative spectrum of the pure molecule and the treated skin spectrum (after step 1 calculation) was selected. For each skin treated with a different molecule, it was verified that the specific molecule band was not observed in the skin blank at any level.

Specific bands were selected for all the molecules except for OC and CL, where no specific band was found. As a consequence, these two molecules were left out of the study. Table 2 presents the specific band wavenumbers selected for each molecule.

3- A reference band was selected at 1335 cm^{-1} on all spectra. This specific band was present in all blanks and treated skins at every level.

d- The intensity ratio *specific band / reference band* (S/R) was calculated at each level for each molecule in order to limit the effect of skin thickness on signal intensity.

e- In order to compare the skin penetration profile of each molecule, the ratio S/R was expressed in % using the following relation, where x(n) is the intensity ratio S/R at the skin depth n (µm):

$$\frac{S}{R}(\%) = \frac{100 \times x(n)}{\sum[x(10), x(40), x(60), x(80), x(120)]}$$

Ratio S/R (%) was used during our all study.

For example with BPA, a specific band was selected at 1177 cm^{-1} on the second derivative average spectrum (figure 2). This band wavenumber was not detected in blank skin at any depth. We calculated the intensity ratio S/R of this specific band at 1177 cm^{-1} on the intensity of the reference band at 1335 cm^{-1}. The ratio S/R (%) was calculated at each depth (10, 40, 60, 80 and 120 µm).

Table 2: Specific wavenumber selected for each molecule in FTIR microspectroscopy for following their distribution within the skin. These specific bands were not observed in blank skin at any depth. For OC and CL no specific bands could be found.

Molecules	Molecular signature: specific wavenumber selected (cm^{-1})
BP	1063
BP3	1023
OS	1645
OX	1023
AB	1595
DB	1590
IR	1068
BPA	1177
PRED	1391
MP	1127
EP	1445
PP	1390
CAF	1580

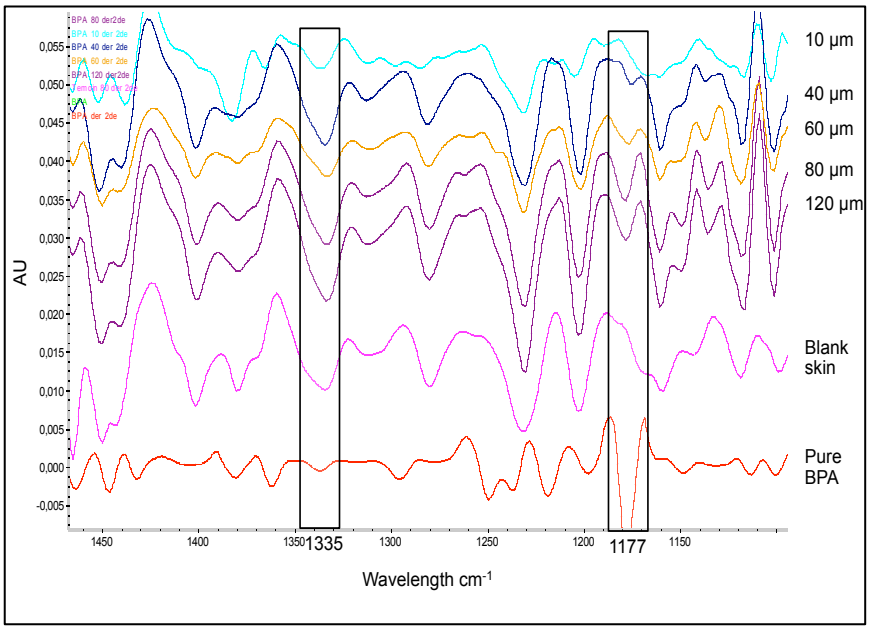

Figure 2: Average second derivative spectra of skin treated with BPA, at 10, 40, 60, 80 and 120 µm, spectrum of blank BPA-free skin and spectrum of pure BPA molecule. A reference band was selected at 1335 cm^{-1}, this band was detected in all skins treated with the molecule or blank and served as a reference to correct for skin thickness. A specific band was selected at 1177 cm^{-1} to follow BPA diffusion into the skin. This specific band was found in pure BPA spectrum but was absent in blank skins.

2- Cutaneous barrier status

A main feature of FTIR with a synchrotron source is the possibility to follow simultaneously cutaneous barrier status and molecule penetration. Cutaneous lipid status can be described by infrared descriptors. The νCH_2 stretching mode in skin is situated between 2847 and 2856 cm^{-1} ,depending on the material and vary regarding the material studied, and describes an orthorhombic, hexagonal or disorganized packing of cutaneous lipids [15]. An orthorhombic lipid packing reflects a compact organization related a normal barrier status. If lipids are closer to a disorganized state, the barrier is defective.

This organizational descriptor can be followed in different materials: lipidic films, stratum corneum sheets, biopsies or in vivo skin. In lipidic films and non-treated human skin biopsies, the compact state gives a peak between 2849 cm^{-1} and 2850 cm^{-1} [9,16]. However in untreated dermatome abdominal porcine skin, pig ear skin and human heat separated skin, wavenumbers of 2853, 2851 and 2853 cm^{-1} are respectively observed [17].

Monitoring the lipidic status in this study is very relevant as skin biopsies are being used. The impact of the solvent, EA, on barrier function was examined. EA contact time was around 30 minutes. EA had the same affect on lipidic status as water, both perturbed moderately the cutaneous barrier, νCH_2 was at 2851 cm^{-1}. EA had less effect on barrier than other solvents such as ethanol, as demonstrated by Merle [9]. EA was a relevant solvent choice because it solubilizes a large panel of molecules and does not affect SC. We observed νCH_2 variation regarding the molecules tested, 2852 ± 1,9 cm^{-1}. This variation was probably due to the barrier heterogeneity of the abdominal biopsy rather than the effect of the molecules on the stratum corneum lipids.

3- Characterization of the percutaneous distribution of exogenous molecules

a- Maximum ratio distribution

In order to compare the distributions of molecules, we represented skin levels where (?) the maximum ratio S/R (%) was detected for each molecule - either at 10, 40, 60, 80 or 120 µm (table 3). Molecules were classified according to their Log Pow. Different molecule groups were observed regarding their highest S/R ratio (%) distribution: molecules with their maximum S/R (%)ratio in the SC at 10 µm, in the upper epidermis at 40 µm, in the medium epidermis at 60 µm or in the lower epidermis at 80 µm. No maximum S/R (%)ratio was found at 120 µm. From this observation we can say that 22h is a relevant time for assessing the percutaneous penetration of these molecules because no maximum ratio was found at 120µm showing that the molecules are distributed mostly within the skin.

We observed that molecules with their maximum ratio in SC had lipophilic Log Pow and lipophily decreased for molecules with their maximum at 40 µm. Log Pow was very high for molecules with their maximum at 80 µm. However, Log Pow was not representative of maximum ratio distribution. Many molecules with similar Log Pow and MW had a different distribution such as OX-OS, IR-AB and BPA-BP. These observations confirmed that for a same lipophily, molecules could have a very different distribution despite a similar cutaneous barrier status. The Log Pow and MW seemed not to be sufficient parameters to evaluate the distribution of molecules within skin.

Table 3: Schematic representations of the distribution of molecules within the skin 22h after deposit on skin. Colored squares represent at which skin depth the maximum intensity S/R ratio calculated from the FTIR spectra was observed. For example, in skin treated with CAF, maximum S/R ratio was found at 80 µm, meaning that CAF was detected at a maximum at 80 µm in skin.

Molecules	Log Pow	10 µm	40 µm	60 µm	80 µm	120 µm
CAF	-0,07					
PRED	1,62					
MP	1,93					
EP	2,27					
PP	2,81					
BPA	3,4					
BP	3,53					
BP3	3,79					
DB	3,93					
IR	4,8					
AB	5					
OS	5,77					
OX	5,8					

b- Kinetic profiles

The skin distribution profile was determined for each molecule from FTIR results. Profiles obtained were classified into three groups based on the molecule cutaneous distribution in figure 3. Molecule presence at different epidermis levels provides percutaneous penetration kinetics information. It links molecule penetration profile to a contact time of 22 hours.

The first group represented distribution profiles where maximum S/R(%)ratio was detected in the SC at 10 µm. There are BP3, OX, AB and BP. The second group, had their highest ratio located in middle epidermis, between 40 and 80 µm. There are MP, EP, PP, DB and PRED. The third group, CAF, IR, BPA and OS, had a maximum ratio in lower epidermis at 80 µm.

Molecules from the first group were considered as the most retained within the SC and with lowest percutaneous penetration. Their diffusion kinetic in the skin was slowest compared to the others molecules. BP, BP3, OX and AB have Log Pow at 3,53, 3,79, 5,8 and 5, respectively. They are quite lipophilic molecules, so their SC retention can be explained in part by their lipophily. However some of lipophilic molecules tested here, such as OS, IR and DB, were not retained in the SC.

The second group molecules were retained more in the epidermis. They are more hydrophilic molecules compared to the first group molecules with a Log Pow comprised between 1.93 for MP to 3.93 for DB. DB and BP are lipophilic and PRED has high MW, but it is surprising to find MP retained in the upper epidermis as this molecule is quite hydrophilic. In this group, we observed that Log Pow was not sufficient to predict their kinetic profile and percutaneous penetration. These molecules also had a slow kinetic but penetrated faster into the epidermis than the first group.

The third group contained molecules with the fastest kinetics. BPA for example had a maximum S/R (%)ratio at 80µm. meaning that BPA diffused faster and was not retained in the upper epidermis. IR and OS had a quite low ratio through the whole distribution and we did not detect any particularly high variation of ratio values. This particular profile showed that these molecules diffused quite fast through the epidermis. Even though BPA, OS and IR have lipophilic Log Pow of respectively of 3.4, 3.93 and 4.8, these three molecules have the fastest skin diffusion as well as CAF (low Log Pow and MW).

From these observations, FTIR microspectroscopy data helped to classify the molecules into three categories according to their kinetic profile. Log Pow was not very related to these three diffusions profiles. These profiles can help to assess a potential risk for new molecules. Molecules from the second and particularly the third group may present a toxicological concern. From our results, we showed that Log Pow failed to detect particular kinetic profile such as IR and OS ones.

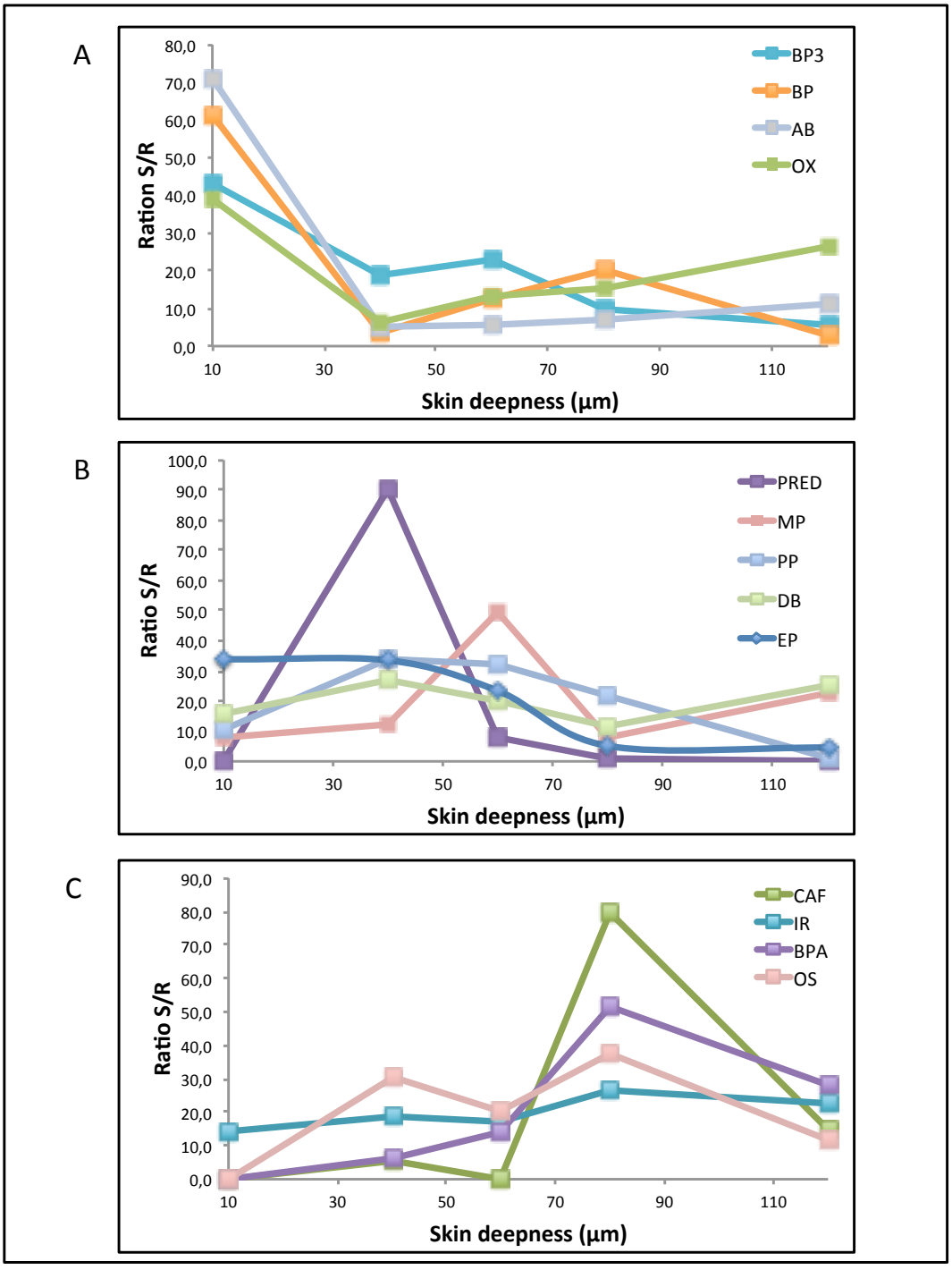

Figure 3: FTIR molecule distribution profiles in skin. Normalized intensity ratio S/R is expressed as a function of skin depth. (A) group represents molecules with maximum ratio in SC, (B) group represents molecules with maximum ratio in epidermis, between 40 and 60 µm, (C) group represents molecules with maximum ratio in lower epidermis at 80 µm.

5- Comparison of FTIR data with Franz cells results

Molecules of interest were detected after Franz cells experiments and HPLC analysis in each compartment 22h after deposit. General time to study the penetration profile of a molecule ex vivo is around 22h-24h, which is the time the product usually stays on skin. After 24h, the skin starts to deteriorate. The quantity of molecules detected within TS, EP, DE and LR is summarized in table 4. Ratio EPtot/DE was calculated in order the show the proportion of molecule present in total epidermis (EPtot, EPtot=TS+EP) compared to the quantity detected in DE. The lower the ratio EPtot/DE, the faster the molecule diffused within skin. A molecule with a high EPtot/DE ratio will be more retained and as a consequence may not have a systemic effect.

Table 4: Molecule quantity (nmol/cm^2) within the 3 tapes stripping (TS), epidermis (EP), total epidermis (EPtot = TS + EP), dermis (DE) and the ratio EPtot/DE 22H after skin deposit.

	BP	**BP3**	**OX**	**OS**	**AB**	**IR**	**DB**	**BPA**	**PRED**	**MP**	**EP**	**PP**	**CAF**
TS	250,0	161,5	173,3	187,9	267,8	168,3	262,3	93,1	156,1	161,0	16,9	2,2	36,1
EP	88,1	131,9	150,1	107,7	124,2	122,9	112,4	246,5	226,3	37,3	34,9	19,4	21,5
DE	49,6	54,1	21,4	35,4	22,8	51,4	68,5	86,0	87,7	57,1	61,7	39,8	43,4
LR	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0,6	22,2	<LOD	1,0	38,7
EPtot	338,1	293,3	323,3	295,6	392,0	291,2	374,8	339,6	382,4	198,3	51,8	21,6	57,7
EPtot/DE	**6,8**	**5,4**	**15,1**	**8,3**	**17,2**	**5,7**	**5,5**	**3,9**	**4,4**	**3,5**	**0,8**	**0,5**	**1,3**

We observed similar penetration behaviors between Franz cells and FTIR data. For example CAF was found to be distributed in lower epidermis by FTIR and had a low EPtot/DE ratio in Franz cells. Also particular behavior of molecules with similar Log Pow and different skin distributions observed by FTIR was also found in Franz cells. For example, Franz cells showed that OX was more retained in EPtot compared to OS. The same observation was made in FTIR as OX has its maximum in the SC whereas OS was not detected in the SC and has it maximum S/R (%)ratio at 80 µm. Previous work from our laboratory showed by fluorescence spectroscopy that OS had no affinity for SC ceramides compared to OX [13]. We confirm here again that OS may have no interactions with the ceramides and as a consequence diffuses faster than OX. The same observation was made for molecules with similar Log Pow for whom skin

distribution was found to be different both by FTIR and Franz cells. This was the case for example for EP-PP and IR-AB. These results validated the correlation between synchrotron maximum S/R (%)ratio signal and Franz cells EPtot/DE ratio.

6- Criterion determination by chemometrics

a- ComDim Analysis

Recent advances in chemometric methods for the interpretation of multi-block data have provided new methodologies for data treatment. Multi-block methods facilitate the comparison of different blocks of variables describing the same samples, highlighting similarities and differences among the blocks and also among the variables within each block. The Common Components and Specific Weight Analysis multi-block approach is abbreviated as ComDim [18].

ComDim looks for directions of dispersion of the samples that are common to all the data tables. Each data table has a specific weight, or salience, which quantifies its contribution to each common dimension. Samples can be projected onto the common space to determine sample distributions which are common to all data tables.

ComDim analysis was performed between FTIR data (S/R (%) ratio at each depth 10, 40, 60, 80 and 120 µm) and Franz cells data (quantity detected in TS, EP and DE in µmol/cm^2) for all molecules.

The common component N°1 (CC1) was the most interesting component to examine (figure 4). Scores values were attributed to the distribution of molecules within skin. Some molecules had a positive score (≥ 0), from the highest to the lowest: AB, OX, BP, DB and BP3. Other molecules had a negative score (< 0) from less negative to more negative: BPA, MP, OS, CAF, PP, PRED, IR and EP.

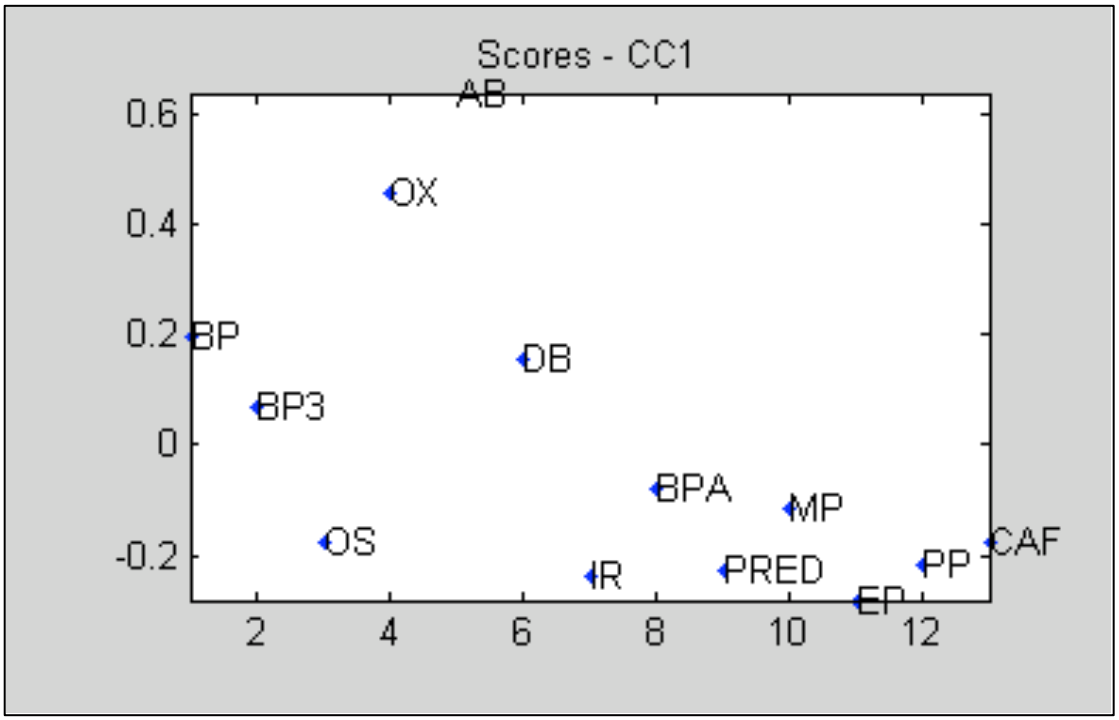

Figure 4: Distribution of molecules based on their scores obtain from common dimension analysis (ComDim) from the HPLC/FTIR data. AB, OX, BP, DB and BP3 had positive scores and BPA, MP, OS, IR, PRED PP, CAF and EP negative scores.

b- Evaluation of scores as a predictive criterion : S_{index}

In order to evaluate ComDim scores as a percutaneous penetration predictive criterion, molecules scores were correlated to the EPtot/DE ratio from Franz cells (figure 5). Scores and EPtot/DE were well correlated with a R^2=0.74. Correlation Log Pow and EPtot/DE was lower with R^2=0.54. Scores and Log Pow were not correlated with R^2=0.3.

From these correlations, it can be seen that FTIR/HPLC data represented by scores from ComDim analysis could predict skin distribution of exogenous molecules, better than Log Pow. What is very interesting here is that this criterion, that we named S_{index}, provides information different from Log Pow as these two criteria are not correlated at all (R^2=0.3). S_{index} has as a consequence potential to be a complementary criterion to molecules physicochemical parameters for the prediction of percutaneous penetration.

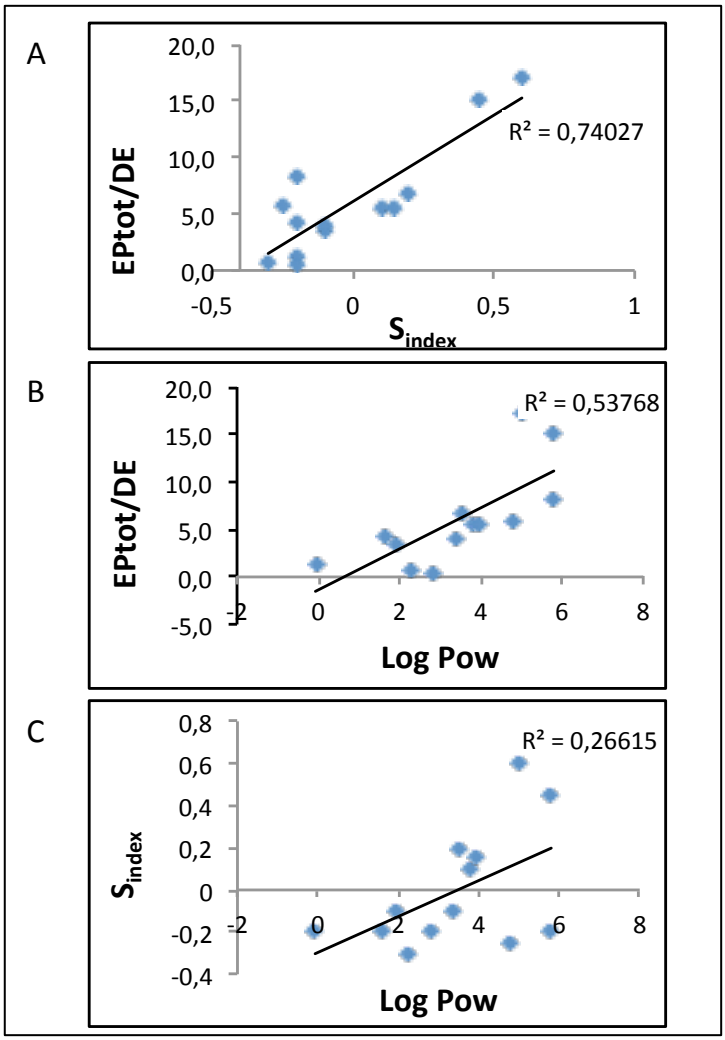

Figure 5: (A) S_{index} correlated to EPtot/DE, (B) Log Pow correlated to EPtot/DE, (C) Log Pow correlated to S_{index}. S_{index} correlated better to Franz cells results than to Log Pow. S_{index} provides complementary information to Log Pow as these two criteria are not correlated.

c- Construction of a new predictive mapping

Our last objective was to determine a predictive mapping from the S_{index} calculated above. We referred to the OECD proposed predictive mapping by drawing our molecule maps with MW vs Log Pow (figure 6B) [5], called here the OECD table. Then we draw up our molecule mapping containing S_{index} vs Log Pow, S_{index} (figure 6A), called here the FTIR table. Both of these predictive maps are compared to the Franz cells data.

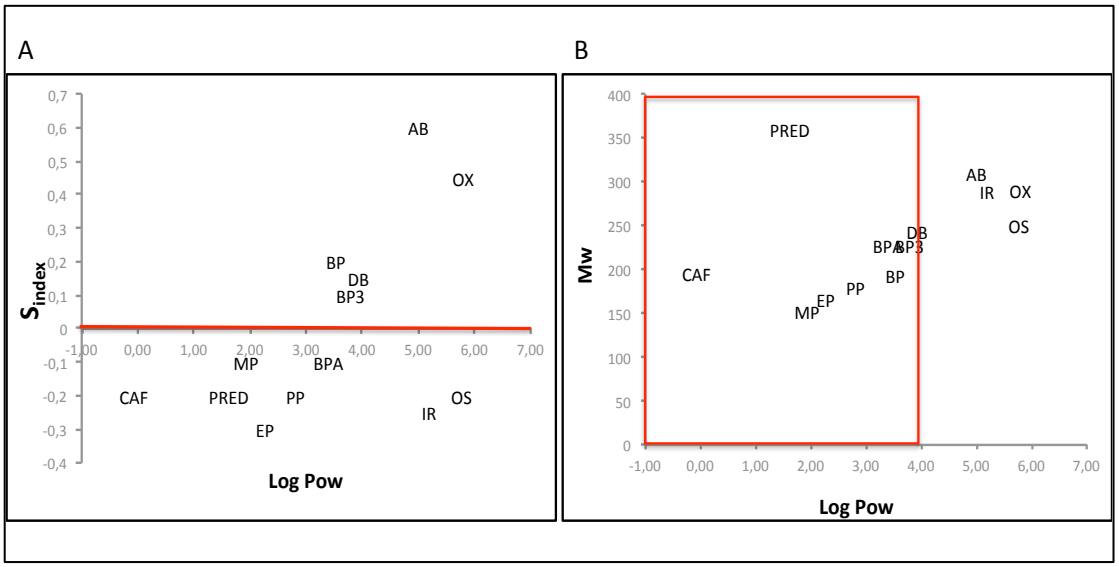

Figure 6: Predictive maps, (A) S_{index} vs Log Pow and (B) MW vs Log Pow. Predictive map A is created from the FTIR/HPLC results and B from current OECD regulations. The red line in A separated molecules with positive S_{index} from molecules with negative S_{index}. This line provides information regarding skin distribution of molecules. In map B, the red square represents OECD recommendations for molecules with Log Pow between -1 and +4 and MW<500g/mol.

When examining OS and OX cutaneous distribution that have similar Log Pow, the OS EPtot/DE ratio is half that of OX, meaning that OS tends to be less retained in epidermis despite its high Log Pow. S_{index}/Log Pow table is in agreement with this observation with OX in the positive S_{index} zone and OS in the negative one. On the other hand, the OECD table was not in agreement with this observation, as OS and OX are very close. The same observation is made for AB and IR that have similar Log Pow and but very different epidermal distributions. S_{index} also gave additional information regarding skin distribution for molecules with similar physicochemical parameters. BP, DB and BP3 have similar Log Pow and similar cutaneous distribution in Franz cells. In S_{index}/Log Pow table these 3 molecules were grouped. We compared BPA to these 3 molecules that have a similar polarity but a lower ratio EP/DEtot. BPA tends to be less retained in the epidermis and more present in the dermis. Its negative S_{index} represented well its cutaneous distribution as shown in S_{index}/Log Pow table. BPA is in the negative zone whereas the 3 other molecules are in the positive zone. PP and EP were almost not retained at all in the epidermis as shown by their EPtot/DE ratio. They both had negative S_{index}. MP and PRED have close Log Pow and

EPtot/DE ratio also had both negative S_{index}. CAF was not retained in the upper epidermis and its negative S_{index} reflects this.

The OECD table did not provide relevant information regarding skin distribution for molecules with similar Log Pow whereas the S_{index}/Log Pow table did. For example, AB, IR, OX and OS were all in the same OECD table space whereas these molecules have a different skin distribution. For risk assessment it is very interesting to understand that molecules with similar physicochemical properties can be distributed very differently into the skin. This new criterion represented by S_{index} provides relevant complementary information. Molecules with negative S_{index} tend to be less retained in epidermis and to be as a consequence a higher potential risk for the consumer.

IV- CONCLUSION

FTIR with synchrotron source is a relevant analytical tool for studying and predicting percutaneous penetration of exogenous molecules. This study brings a new approach in order to map the prediction of exogenous molecules cutaneous distribution profiles regardless of their Log Pow or MW. This requires setting up and following a very detailed operating procedure.

This work provided a new evaluation criterion for percutaneous penetration, S_{index}, based on FTIR/HPLC data. S_{index} provides complementary information to molecule physicochemical properties. The main advantage of using the present approach for predicting percutaneous penetration compared to other methodologies such as the artificial membranes or fluorescence is that it is carried out in a much more complex environment taking into account all skin parameters. Also it is able to look at the distribution of molecules at selected depths or wideness. The measurements presented here were limited to 120 µm in depth but it is possible to go further into the dermis.

This work constitutes a feasibility study. The next step will be to complete the S_{index}/Log Pow table with more molecules in order to have more widespread predictive information. Moreover, a new mathematical model for the prediction of percutaneous penetration could be determined including S_{index}.

Aknoledgments:

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Conclusion

Ce travail a permis de définir un protocole d'étude de la pénétration percutanée par microspectroscopie FTIR avec une source synchrotron et de développer un critère prédictif de la pénétration, S_{index}, complémentaire du Log Pow et MW.

L'exploitation des données de la pénétration percutanée par FTIR a nécessité de suivre certaines étapes de traitement des spectres afin d'homogénéiser au maximum les résultats. Lors de la microspectroscopie une dizaine de spectres étaient enregistrés à chaque profondeur de peau étudiée sur la coupe. Ainsi, la moyenne des spectres a été calculée à chaque profondeur puis traitée en dérivée seconde. Le signal spécifique de la molécule étudiée (S) a été choisi en sélectionnant un signal présent dans le spectre de la molécule seule et dans le spectre moyen obtenu sur la biopsie cutanée traitée, mais absent dans le spectre de la biopsie de peau témoin. Un signal de référence à 1335 nm, présent dans les spectres de peaux traitées et non traitées a été sélectionné également (R). Afin de pouvoir quantifier la distribution de la molécule sans être perturbé par les variations d'épaisseur des coupes de peau, l'intensité du signal spécifique de la molécule (S) a été divisée par la valeur de l'intensité du signal de référence (R). La valeur normalisée du rapport S/R nous a ainsi servi à comparer la distribution des molécules à chaque profondeur. Seulement deux molécules, CL et OC n'ont pas pu être détectées car aucun signal spécifique FTIR n'a pas été trouvé. Les données de référence en cellules de Franz couplées à l'HPLC, ont validé nos données FTIR.

En plus de nous permettre de définir la distribution cutanée d'une molécule, la microspectroscopie FTIR nous a permis également de suivre l'état de la barrière lipidique en même temps que la pénétration des molécules. Le solvant utilisé pour mettre en solution les molécules, l'acétate d'éthyle, n'a désorganisé que de manière minime la barrière cutanée. L'utilisation de ce solvant a permis de solubiliser l'ensemble des molécules sélectionnées et donc d'étudier l'absorption des molécules dans les mêmes conditions de dépôt.

Un traitement par chimiométrie avec un test d'analyse codimensionelle (ComDim) des données de pénétration obtenues par FTIR et HPLC, nous a permis de développer un nouveau critère prédictif de la pénétration percutanée, appelé S_{index}. Cette analyse a la particularité de pouvoir comparer deux matrices de données différentes, ici les données FTIR et les données de référence en HPLC, et d'en définir les connexions spécifiques. A partir des résultats de ce test, le critère S_{index} a été défini pour chaque molécule. Comparativement au Log Pow, S_{index} a montré une meilleure corrélation avec les données de pénétration cutanée sur cellule de Franz couplées à l'HPLC.De plus, S_{index} et Log Pow ne sont pas corrélés. S_{index} apporte ainsi une information complémentaire aux données physicochimiques des molécules.

Afin de confirmer l'intérêt de ce nouveau critère, nous avons tracé la cartographie prédictive de la pénétration cutanée des molécules en fonction de leur S_{index} et Log Pow. En parallèle, nous avons également tracé la cartographie prédictive des mêmes molécules selon leur Log Pow et MW (cartographie OCDE). La cartographie de l'OCDE n'a pas apporté de prédiction significative pour les molécules comportant un Log Pow et MW proche. Certaines molécules comme OS et OX ont des propriétés physicochimiques similaires mais une distribution cutanée différente. La cartographie utilisant le S_{index} a bien montré cette différence mais pas celle de l'OCDE. Les molécules avec un S_{index} négatif peuvent représenter un risque pour le consommateur car elles sont moins retenues au niveau de l'épiderme que les molécules avec un S_{index} positif.

Le critère S_{index} défini dans ce travail a donc permis de créer une nouvelle cartographie prédictive de la pénétration percutanée quelles que soient les propriétés physicochimiques des molécules. L'intérêt de cette carte est principalement pour l'analyse du risque toxique car il est très intéressant de pouvoir préciser les différences de distribution cutanée pour des molécules présentant une lipophilie similaire. L'avantage de notre approche est que, contrairement à d'autres méthodes prédictives comme les membranes artificielles (ex PAMPA) ou la spectroscopie de fluorescence, on se trouve dans un environnement bien plus complexe prenant en compte tous les paramètres de la peau, même si la standardisation d'une biopsie est difficile. La technique d'échantillonage choisie en FTIR nous a permis également d'étudier la pénétration des molécules en choisissant la profondeur étudiée. Nous

avions choisi d'étudier la distribution des molécules à 10, 40, 60 80 et 120 µm mais il est possible de l'étudier plus profondément ou à des profondeurs plus rapprochées.

Ce travail est une étude de faisabilité. Il serait désormais intéressant de construire un protocole standardisé pour établir les valeurs de S_{index} pour un ensemble beaucoup plus large de molécules, en particulier celles pour lesquelles il est déjà connu que l'approche actuelle par le Log Pow et MW est insuffisante, et d'obtenir ainsi une cartographie plus précise. De plus, ce nouveau critère pourrait permettre d'établir un nouveau modèle mathématique en incluant S_{index} comme paramètre complémentaire Log Pow et MW.

Conclusion générale

Le but de ce travail a été de développer un critère prédictif de la pénétration percutanée, complémentaire du Log Pow et MW, par différentes approches chromatographiques et spectroscopiques. Afin d'améliorer l'analyse prédictive du risque des actifs cosmétiques, il nous a paru intéressant de développer un critère se référant à la pénétration cutanée des molécules. Aujourd'hui, les critères développés se réfèrent principalement aux propriétés physicochimiques des molécules (Log Pow, MW) ou à leur perméabilité (Kp) mais non à leur distribution cutanée. Nous avons donc voulu développer un critère qui permettrait d'estimer si une molécule aura tendance à être retenue ou non au niveau du SC et donc à diffuser ou non dans le derme.

Suite au récent intérêt du rôle des céramides dans la pénétration cutanée, il a donc semblé intéressant de se focaliser sur l'interaction de molécules exogènes avec des céramides afin de définir un nouveau critère prédictif. Cette interaction a été étudiée in vitro, par chromatographie d'affinité sur phase stationnaire de carbone graphite poreux modifié avec un céramide et en spectroscopie de fluorescence avec l'étude de l'interaction de molécules exogènes et de céramides en présence de sondes de fluorescence.

Une troisième méthode prédictive ex vivo en microspectrospie FTIR avec une source synchrotron nous a permis de définir un critère de la prédiction de la pénétration cutanée prenant en compte l'ensemble du tissu cutané et pas uniquement les céramides du SC.

Afin de valider les trois approches développées, la pénétration percutanée des molécules d'intérêt, principalement des filtres UV et conservateurs (cf annexe 1), a été évaluée avec une méthode de référence ex vivo sur des biopsies humaines sur cellules Franz couplée à des mesures en HPLC. Nous avons préféré établir nos propres données plutôt que de reprendre celles de la littérature afin d'homogénéiser nos conditions expérimentales et faciliter ainsi la comparaison. Le même véhicule, l'acétate d'éthyle, a été utilisé pour toutes les molécules étudiées afin d'éliminer la contribution de la formulation avec les résultats de pénétration percutanée.

La première étude en chromatographie d'affinité a permis d'établir un protocole d'étude du rôle rétentif des céramides dans le SC. Nous avons développé une méthode d'imprégnation d'une colonne carbone graphite poreux (PGC) avec un céramide synthétique ayant des propriétés organisationnelles similaires au céramide 2 naturel. Une fois la colonne modifiée, il a été possible d'étudier l'interaction de molécules cosmétiques ou dermopharmaceutiques présentant différents Log Pow et MW avec le céramide fixé de manière labile sur la phase stationnaire.

Le critère de rétention α a été calculé à partir des temps de rétention des molécules avant et après modification de la colonne. La valeur du critère calculé et la variation des temps de rétention semblent indépendantes de la lipophilie des molécules étudiées. Le critère α a ensuite été comparé aux données de pénétration percutanée réalisées avec la méthode référence par cellules de Franz couplées à HPLC. Les données de référence ont été traitées par analyse en composantes principales. Les molécules les plus retenues au niveau du SC (BP3, PRED et OC) avaient toutes les trois le même critère α calculé en chromatographie, ceci suppose donc que ces molécules pourraient interagir de la même manière avec les céramides du SC.

La limitation de cette méthode est qu'il n'a pas été possible de comprendre clairement le mécanisme de rétention des molécules sur la phase de carbone graphite poreux modifiée par le céramide. Dans les colonnes IAM, une interaction entre les molécules d'intérêt et les phospholipides greffés, augmente le temps de rétention des molécules alors qu'ici le temps de rétention diminue après adsorption des céramides sur la phase stationnaire PGC. Une compréhension plus approfondie du mécanisme d'interaction des céramides et molécules dans la colonne pourrait permettre dans le futur de continuer le développement de cette technique pour l'analyse du risque d'ingrédients cosmétiques.

La deuxième partie de notre travail a été réalisée en spectroscopie de fluorescence. Nous avons mis en place un protocole d'étude de l'interaction entre le céramide IIIa (CER IIIa) et des molécules exogènes à l'aide de la sonde de fluorescence 1,6-diphenyl-1,3,5-hexatriene (DPH). La fluorescence nous a permis de suivre l'affinité des molécules pour ce céramide test. A partir des résultats de fluorescence, un critère d'évaluation de l'interaction entre les molécules et CER IIIa, ΔI a été calculé. La valeur du ΔI est représentative de l'intensité de l'interaction entre les molécules et CER IIIa. Plus une molécule a de l'affinité pour le céramide plus le ΔI est élevé.

La comparaison du ΔI avec les données de pénétration cutanée réalisées avec la méthode de référence par cellules de Franz, a validé le critère ΔI et a permis de mettre en évidence une relation entre la distribution cutanée des molécules et leur valeur du ΔI. Le critère ΔI a principalement mis en évidence les différences de distribution cutanée pour des molécules ayant des propriétés physico-chimiques proches ; c'était le cas notamment pour OS et OX. Même si la polarité semble contribuer de façon très représentative à l'interaction céramide-molécules, seul le ΔI a permis de mettre en avant le comportement à risque de certaines molécules, comme pour OS qui malgré sa lipophilie n'était pas retenu par les céramides du SC. En effet OS a tendance à être moins distribué au niveau de l'épiderme que OX et présente donc un risque supérieur à diffuser dans l'organisme. En fluorescence, le ΔI qui était très négatif pour OS a bien montré que OS n'avait pas d'affinité pour CER IIIa, ce qui explique le fait qu'il n'ait pas été retenu au niveau du SC comme l'a été OX, qui lui a un ΔI élevé.
Ce nouveau critère de fluorescence ΔI pourrait être utilisé comme critère complémentaire dans le développement de nouveaux modèles mathématiques.

La dernière partie de notre travail a été de développer un critère prédictif de la pénétration percutanée par microspectroscopie FTIR avec une source synchrotron. La pénétration cutanée d'une quinzaine de molécules de différents Log Pow et MW a été étudiée par la méthode de référence en cellules de Franz. La distribution cutanée des molécules a été réalisée par chromatographie, servant de données de référence, ou par une méthode FTIR/Synchrotron que nous avons développée. La concentration utilisée pour les molécules exogènes a été choisie volontairement élevée (10^{-1}M) afin d'améliorer la détectabilité d'un signal spécifique des molécules utilisées en FTIR/Synchrotron.
Les résultats de la méthode de référence par HPLC ont validé nos données FTIR. Une analyse chimiométrique par multi-block (ComDim) entre les données HPLC et FTIR, a permis, de définir un critère prédictif, S_{index}. A partir des données de la composante commune 1, S_{index} a montré avoir une importante corrélation avec les données de pénétration de la méthode de référence. S_{index} n'étant pas corrélé au Log Pow, ce critère apporte donc une information complémentaire aux données physicochimiques des molécules.
La cartographie prédictive définie avec S_{index}/Log Pow a apporté une prédiction plus fine de la distribution cutanée des molécules que celle de l'OCDE MW/LogPow, en

particulier pour les molécules avec des propriétés physicochimiques similaires. Dans notre étude, les molécules avec un S_{index} négatif ont le potentiel de représenter un risque pour le consommateur car ces molécules sont moins retenues au niveau de l'épiderme que les molécules avec un S_{index} positif. Dans le futur, il pourrait être intéressant de développer ce protocole in vivo en microspectroscopie Raman après validation en FTIR ex vivo.

L'objectif principal du développement de ces méthodologies pour définir de nouveaux critères de prédiction de la pénétration percutanée, était de ne pas suivre une approche par modèle mathématique mais d'améliorer l'approche simplifiée proposée par l'OCDE. Nous voulions ainsi proposer un ou plusieurs critères qui permettent, en complément des propriétés physicochimiques des molécules, d'établir une base de données additionnelle pour affiner l'estimation de la pénétration percutanée.

La recherche d'un critère chromatographique d'interaction molécules-céramide s'est appuyée sur l'efficacité des méthodes chromatographiques développées avec les colonnes IAM pour étudier l'absorption gastro-intestinale ; il nous a paru très intéressant de la transposer pour l'absorption cutanée. Malheureusement le mécanisme d'interaction entre les molécules étudiées et la phase stationnaire de carbone graphite poreux modifié par un céramide n'a pas été élucidé. Même si le critère de rétention α semble apporter des résultats intéressants il nous a paru plus prudent de ne pas poursuivre cette approche tant que le mécanisme intrinsèque de rétention ne sera pas compris.

La spectroscopie de fluorescence a apporté une piste très intéressante, le critère ΔI permettant notamment de souligner des différences de pénétration cutanée pour des molécules de Log Pow similaire. En revanche, la limite de cette méthode est le fait qu'elle ne permet pas de caractériser une interaction ceramide-molécule pour des molécules ayant un Log Pow inférieur à celui de la sonde utilisée, d'où la nécessité d'utiliser plusieurs sondes. Cependant, le critère ΔI, est pertinent dans l'estimation de la distribution cutanée de molécules dans un domaine de lipophilie pour lequel le modèle mathématique de Potts & Guy n'est pas opérationnel (modèle limité aux molécules ayant un Log Pow inférieur 6). ΔI pourrait ainsi aider à affiner la prédiction

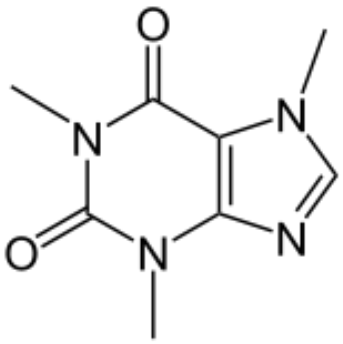
par la cartographie OCDE MW/LogPow qui se limite à estimer la pénétration percutanée à 10% pour les molécules avec un Log Pow supérieur à 4.
Le critère S_{index} développé en FTIR avec une source synchrotron et HPLC apporte les données les plus intéressantes car toutes les molécules peuvent être étudiées quelque soit leur Log Pow ou MW. Ce critère pourrait permettre d'établir une base de données en appliquant un protocole très précis et standardisé. En complément, la valeur du νCH_2 des lipides du SC permettra de valider la qualité des biopsies cutanées mise en œuvre pour l'élaboration de la base de données des S_{index}.

Ce travail est une contribution au développement de modèles prédictifs de la pénétration percutanée. Il a permis de proposer différents critères prédictifs selon trois approches différentes et d'en établir les limites ou le potentiel. Il a été réalisé sur un nombre restreint de molécules principalement d'intérêt cosmétique. Les meilleures perspectives sont offertes par les critères ΔI et S_{index} sous réserve de les calculer sur un nombre beaucoup plus large de molécules en application topique. Ces deux critères, en complément de LogPow et MW, pourraient soit s'intégrer dans l'approche prédictive simplifiée de cartographie OCDE, soit être pris en compte dans des modèles mathématiques, ceci en vue d'affiner l'estimation des molécules à usage topique pour l'analyse du risque en amont de la mise au point d'une formulation cosmétique ou dermopharmaceutique.

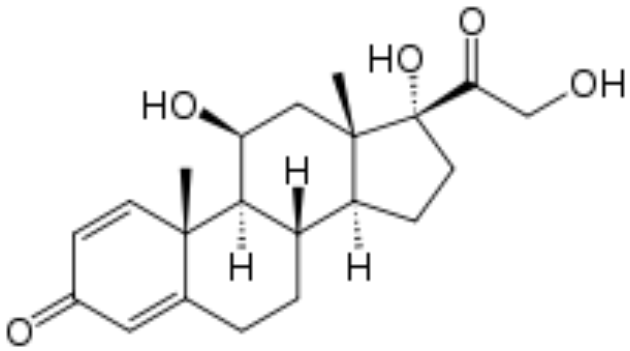
Annexes

Annexe 1 : Molécules exogènes étudiées

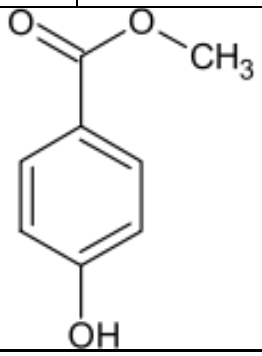
Molécules	Abréviation	Propriétés	Log Pow	Mw (g/mol)
Caféine	CAF	Amincissant	-0,07	196

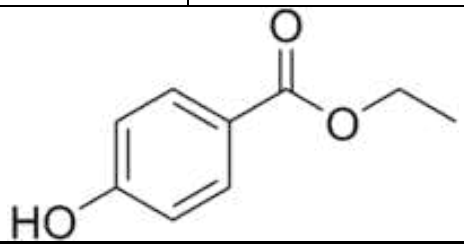
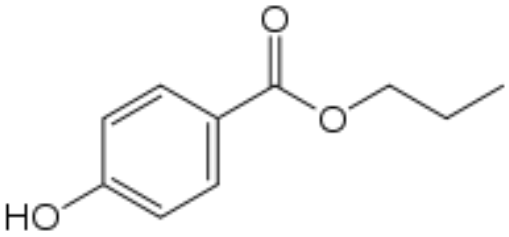
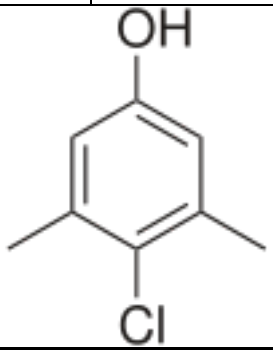
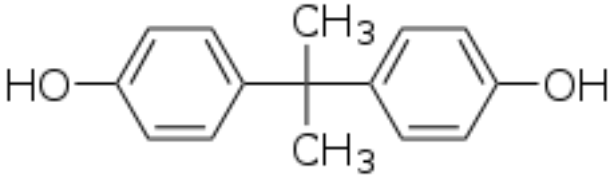


Prédnisolone	PRED	Corticoïde	1,62	360

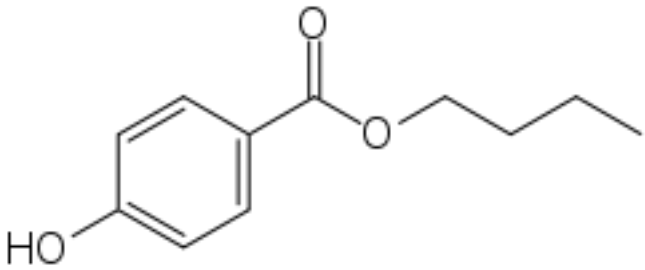


Méthyl parabène	MP	Conservateur	1,93	152

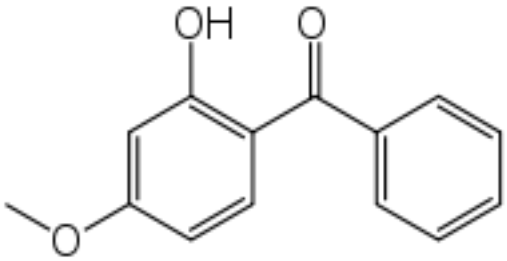


Molécules	Abréviation	Propriétés	Log Pow	Mw (g/mol)
Ethyl parabène	EP	Conservateur	2,27	166
				
Propyl parabène	PP	Conservateur	2,81	180
				
Choloroxylenol	CL	Conservateur	3,37	157
				
Bisphénol A	BPA	Material coating	3,4	228
				

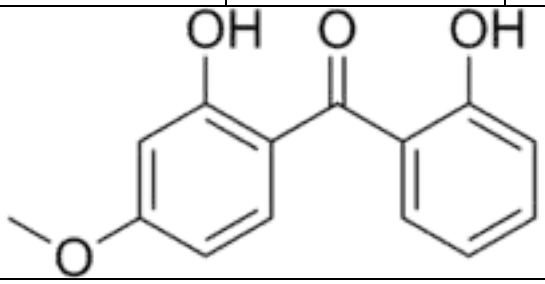
Molécules	Abréviations	Propriétés	Log Pow	Mw (g/mol)
Butyl parabène	BP	Conservateur	3,53	194



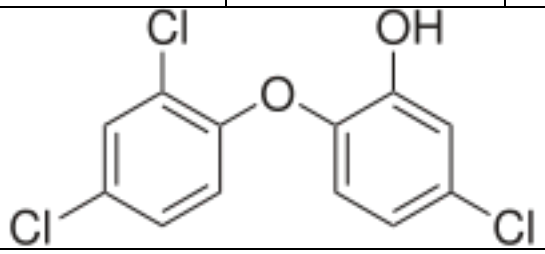
Benzophénone 3	BP3	UV filter	3,79	228



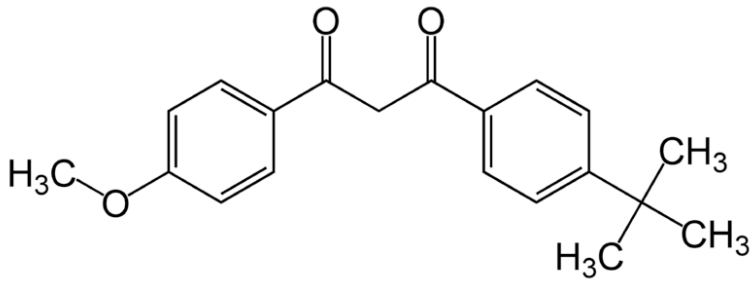
Dioxybenzone	DB	UV filter	3,93	244



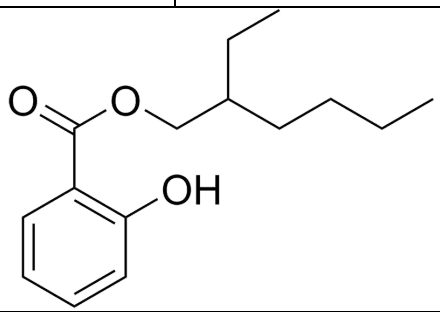
Irgasan	IR	Conservateur	4,8	289



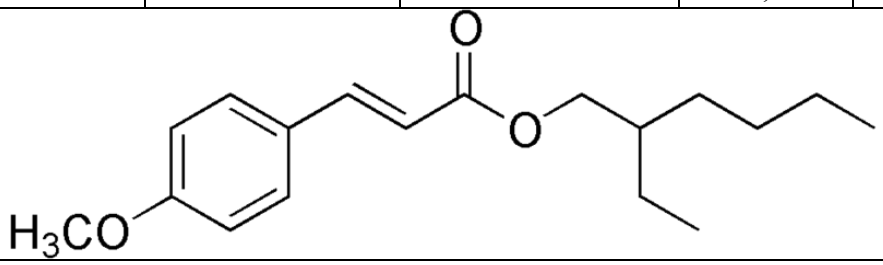
Molécules	Abréviations	Propriétés	Log Pow	Mw (g/mol)
Avobenzone	AB	UV filter	5	310



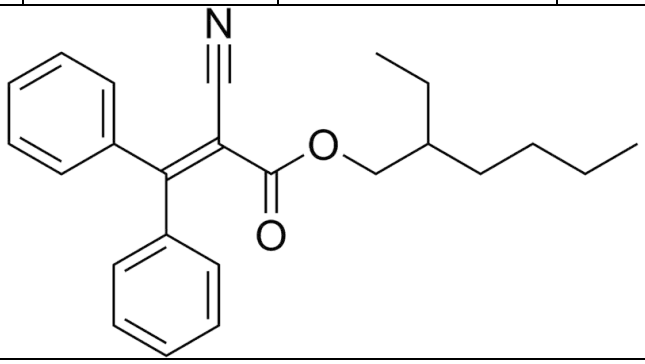
Octisalate	OS	UV filter	5,77	250



Octinoxate	OX	UV filter	5,8	290



Octocrylène	OC	UV filter	6,88	361



Molécule		Longueur d'onde (nm)	Colonne	Phase mobile	Linéarité (R^2)	Précision (%)	Répétabilité (%)	LOD (µM)	LOQ (µM)
Caféine	CAF	272	C18 5mm; 250mm x 4,6mm	75% H_2O (1%AA) / 25% MeOH	0,99	4,2	0,2	0,15	0,5
Prednisolone	PRED	242	C18 5mm; 150mm x 4,6mm	60% H_2O / 40% MeOH	0,99	1,1	1,0	0,01	0,32
Méthyl parabène	MP	254	C18 5mm; 150mm x 4,6mm	50% H_2O / 50% MeOH	0,99	4,6	0,5	0,34	1,14
Ethyl parabène	EP	254	C18 5mm; 150mm x 4,6mm	50% H_2O / 50% MeOH	0,99	2,0	0,1	0,22	0,75
Propyl parabène	PP	254	C18 5mm; 150mm x 4,6mm	50% H_2O / 50% MeOH	0,98	7	0,5	0,55	1,2
Chloroxylénol	CL	281	C18 5mm; 150mm x 4,6mm	30% H_2O / 70% MeOH	0,99	1,8	2,2	0,44	1,47
Bisphénol A	BPA	278	C18 5mm; 150mm x 4,6mm	50% H_2O / 50% MeOH	0,98	6	0,7	0,46	1,3
Butyl Parabène	BP	254	C18 5mm; 150mm x 4,6mm	50% H_2O (1 % AA) / 50 % MeOH	0,99	5	1,1	0,45	1,5
Benzophénone 3	BP3	291	C18 5mm; 150mm x 4,6mm	31% H_2O (1%AA) / 69% MeOH	0,99	3,6	0,1	0,02	0,05
Dioxybenzone	DB	300	C18 5mm; 150mm x 4,6mm	40% H_2O / 60% MeOH	0,99	4,2	0,7	0,21	0,69
Irgasan	IR	281	C18 5mm; 150mm x 4,6mm	20% H_2O / 80% MeOH	0,99	1,9	0,8	0,5	1,7
Avobenzone	AB	310	C18 5mm; 150mm x 4,6mm	5% H_2O / 95% MeOH	0,99	3,3	1,6	0,44	1,47
Octisalate	OS	250	C18 5mm; 150mm x 4,6mm	5% H_2O (1 % AA) / 95% MeOH	0,99	2,8	2,4	0,78	2,6
Octinoxate	OX	290	C18 5mm; 150mm x 4,6mm	5% H_2O (1 % AA) / 95% MeOH	0,99	2,6	1,2	0,38	1,3
Octocrylène	OC	307	C18 5mm; 150mm x 4,6mm	21% H_2O (1%AA) / 79% MeOH	0,98	7,5	0,1	0,01	0,05

Annexe 3 : Publications, Communications et Prix

Publications

Jungman E, Laugel C, Routledge DN, Dumas P, Baillet-Guffroy A
Development of a new evaluation criterion by FTIR microspectroscopy with synchrotron source for prediction of percutaneous penetration
International Journal of Pharmaceutics, Submitted (Research article)

Jungman E, Laugel C, Kasselouri A, Baillet-Guffroy A
Study of the potential of stratum corneum lipids and exogenous molecules interaction by fluorescence spectroscopy for the estimation of percutaneous penetration.
International Journal of Pharmaceutics, 2012, 434(1-2):183-190 (Research article)

Jungman E, Laugel C, Baillet-Guffroy A
Assessing the Safety of Parabens: Percutaneous Penetration and Risk Analysis
Cosmetics and Toiletries, 2011, 126(11):816-22 (Review)

Communications orales

Jungman E, Laugel C, Routledge DN, Dumas P, Baillet-Guffroy A
"Development of a new evaluation criterion by FTIR microspectroscopy with synchrotron source for prediction of percutaneous penetration."
Stratum Corneum conférence, 10-12 septembre 2012 (Cardiff, Royaume-Uni)

Jungman E, Laugel C, Kasselouri A, Baillet-Guffroy A
"A new criterion of stratum corneum lipids and exogenous molecules interaction for the prediction of percutaneous penetration."
Perspective in Percutaneous Penetration conférence, 10-14 avril 2012 (La Grande-Motte, France)

Jungman E, Laugel C, Perez N, Khoury S, Kasselouri A, Chaminade P, Baillet-Guffroy A
"Etude de l'Interaction Céramides-Molécules Exogènes pour la Prédiction de la Pénetration Percutanée"
Congrès de la Société de Pharmaco-Toxicologie Cellulaire, 19-20 mai 2011 (Toulouse, France)

Jungman E, Laugel C, Khoury S, Baillet-Guffroy A
"New investigation Tools to Predict Percutaneous Penetration"
Skin Forum 12th Annual Meeting, Penetrating the Stratum Corneum, 28-29 mars 2011 (Frankfort, Allemagne)

Communications par poster

Jungman E, Laugel C, Kasselouri A, Baillet-Guffroy A
"A new criterion of stratum corneum lipids and exogenous molecules interaction for the prediction of percutaneous penetration."
Perspective in Percutaneous Penetration conference, 10-14 Avril 2012 (La Grande-Motte, France)

Jungman E, Laugel C, Baillet-Guffroy A
"Development of a New Model to Predict Percutaneous Penetration"
21th International Federation of Societies of Cosmetic Chemists Conference (IFSCC), 31 Oct-2 Nov 2011 (Bangkok, Thaïlande)

Jungman E, Laugel C, Perez N, Khoury S, Kasselouri A, Chaminade P, Baillet-Guffroy A
"Potential Impact of Exogenous Molecules and Stratum Corneum Ceramides Interaction to Predict Percutaneous Penetration"
Congrès de la Société de Pharmaco-Toxicologie Cellulaire, 19-20 Mai 2011 (Toulouse, France)

Jungman E, Laugel C, Khoury S, Baillet-Guffroy A
"New investigation Tools to Predict Percutaneous Penetration"
Skin Forum 12th Annual Meeting, Penetrating the Stratum Corneum, 28-29 Mars 2011 (Frankfort, Allemagne)

Prix et distinctions

- **Skin Forum Annual Bursary Awards** (Mars 2011)
Award delivered by the Skin Forum for the best abstract submitted for an oral communication.

- **Tony Naylor Memorial Bursary Fund** délivrée par the Society of Cosmetic Scientists (Septembre 2011)
The Society of Cosmetic Scientists has established the Tony Naylor Memorial Bursary Fund with the objective of encouraging research in cosmetic science and related fields by supporting scientists wishing to present their findings at the annual congress /conference of the International Federation of the Societies of Cosmetic Chemists (IFSCC).

- **Prix du meilleur poster** délivré la Société Française de Pharmacologie et Toxicologie Cellulaire (Mai 2011)

Annexe 4 : Compte-rendus de conférences

IFSCC Presents the Effectiveness, Economic and Ecological Aspects of Cosmetics

Contributing author Elsa Jungman is a doctoral candidate with the faculty of pharmacy, University Paris–Sud.

BANGKOK—After sudden flooding in Thailand postponed the initial 2012 International Federation of Societies of Cosmetic Chemists (IFSCC) Conference date, the Society of Cosmetic Chemists in Thailand (SCCT) organized a successful event at Bangkok's Dusit Thani Hotel on Dec. 12–14, 2011. The conference opened with a preconference workshop focused on skin whitening. Kiyoshi Sato of Shiseido presented the mechanisms of skin-whitening products; many attendees enjoyed the scientific level of the physiological aspects described.

Panvipa Krisdaphong, PhD, president of the SCCT, initiated the opening ceremony with a warm welcome and discussed the difficulties encountered by the people of Thailand during the flooding. The whole praesidium was then invited to join Krisdaphong on stage, and the opening ceremony was followed by a traditional dance performance. An impressive fashion show also was presented to attendees, in which models were clad in designs inspired by Thai plants such as rice and silk that are currently used as cosmetic actives. A reception wrapped up the opening ceremony on the top floor of the hotel, providing attendees with an incredible view of Bangkok.

Keynote Presentations

The morning of Dec. 13, 2012, was devoted to the keynote speakers, including: R. Randall Wickett, PhD; Chika Katagiri, PhD; Krisada Duangurai, MD; Dominique Moyal, PhD; Ubonthip Nimmannit, PhD; and Bang-on Kiethankorn.

Wickett, a professor of pharmaceutical science at the University of Cincinnati, presented the concept of natural cosmeceuticals and the different paths to strategically test them.

Katagiri, a senior research scientist at Shiseido, identified a new molecule to regulate barrier function. This molecule, the protein serpin B3, is responsible for the inhibition of the denucleation of keratinocytes in the cornification process, which results in the presence of nuclei in the cornified layers of the skir and the subsequent disruptio of the barrier function. I response, Shiseido develope a new active, 1-piperidine-pr pionic acid (1PP), that inhibi the production of serpin B3.

Duangurai of Pramongk Hospital then described cosn ceuticals as "cosmetics that work as a drug," meaning they are used for and are as safe as cosmetic products (topical application) but often function as drugs. Cosmeceuticals do not need clinical trials, according to Duangurai, but require

During the opening ceremony, a fashion show featured designs inspired by plants used as cosmetic actives.

Krisdaphong, left, presents the IFSCC Conference Award to Yukiko Mastunga.

Please submit news, events, photos or any other IFSCC information to:
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A dance show was presented during dinner at the closing ceremony.

safety testing as regular cosmetics do. He noted that cosmeceuticals should have no side effects and should not alter the skin function. He concluded that cosmeceuticals function as drugs due to advances in delivery systems.

Moyal of L'Oréal discussed ways to reduce the concentration of UV filters used in sunscreens while maintaining good photoprotection. She showed how Mexoplex, the company's combination of UV filters, synergistically enhances UV protection, versus one filter alone.

Finally, Nimmannit from the National Nanotechnology Center presented different natural whitening cosmetics, and Kiethankorn from Thai-China Flavours and Fragrances Industry Co., Ltd., rounded out the morning with a presentation on certified organic cosmetics.

Delivery and Formulation

After a buffet-style Thai lunch with a view of the hotel's garden and fountain, attendees gathered for the afternoon sessions. Gabriele Blume from Sopharcos introduced the innovative delivery system "ophi-Hydro-Tops," consisting of nanovesicles that are suitable for the encapsulation of hydrophilic and amphiphilic drugs. Compared to liposomes, these vesicles have a higher encapsulation efficacy of amphiphilic substances and acid derivatives.

Alicia Roso from Seppic presented a sensorial evaluation method to measure the soaping effect of cosmetics, i.e., their whitening upon application to skin, to understand the cause of this whitening. Among the tested emulsifiers, sucrose stearate and hydroxystearyl alcohol (and) hydroxystearyl glucoside exhibited suitable non-soaping profiles. While sucrose stearate should be combined with a suitable co-emulsifier or consistency agent to optimize its stability, hydroxystearyl alcohol (and) hydroxystearyl glucoside can be used as the sole emulsifier.

In vitro Cell Studies

Alain Deguercy, PhD, from Bioalternatives described the new SEBO662 sebocyte line for the in vitro evaluation of sebocyte physiology and the screening of compounds to control disorders linked to these cells. A 3D model was developed using this cell line showing properties such as the organization of cells and the expression of specific sebocyte differentiation markers. This model could be used to test sebaceous function regulators.

Also discussing cells, Ken Inomata, PhD, from Kosé Corp., showed that epidermis stem cells maintained epidermal homeostasis in a low-oxygen cell culture. The medium oxygen tension had a great impact on the cell culture in vitro. These findings indicate that basal keratinocytes are well-organized and can maintain their capacity in a hypoxic or antioxidant environment, which could be key for the development of anti-aging technologies.

Pierre-Yves Morvan of Codif International then presented a marine exopolysaccharide produced and secreted by a marine plankton micro-organism that can activate fibroblast contraction. It tightens the collagen fiber network, resulting in a tensing effect and reducing wrinkles just 15 min after application.

Hair and Skin Aging

The second day of the event included sessions on skin and hair aging. Paul Mouser, PhD, of Ashland/ISP emphasized the importance of protecting hair from aging, as he noted, "When you look at a person, you look

The IFSCC's praesidium was invited on stage during the opening ceremony.

at skin first, then hair." The principal consequences of hair follicle aging are graying and decreases in hair growth, density, metabolism and keratin. Therefore, it is important to keep hair roots healthy. The scalp biopsies used in Mouser's study enabled the observation of damage induced by UV irradiation, which led his team to develop a biofunctional antioxidant compound to protect the scalp and hair follicle. This compound was also found to help prevent oxidative-related pigment loss in the hair bulb.

David Boudier from Silab presented his clinical and cellular investigation of the effect of menopause on skin due to hormonal changes such as estrogen deficiency. The study showed abrupt changes in skin quality, in particular on the face. Wrinkles were more pronounced and biomechanical properties such as elasticity deteriorated. In addition, epidermal cell renewal capacity decreased as a result of reduced cell metabolism. This new data could provide the basis for the development of topical skin care products specifically adapted to the needs of women with respect to their hormonal status.

Surfactants and Skin

Two morning presenters discussed the effect of surfactants on skin moisturization. Kaori Yanase from Kracie studied the loss of natural moisturizing factor (NMF) due to surfactant use. Skin cleansing experiments were performed on human subjects as well as structural changes of the stratum corneum (SC) in surfactant solution analyzed using X-ray diffraction. A correlation was shown between the loss of NMF and increase in transepidermal water loss (TEWL). Also, cleansing specifically affected the spacing between keratinocytes, and adding N-acetylglucosamine to a surfactant inhibited the elution of NMF and reduced skin roughness due to cleansing.

Shuliang Zhang, PhD, of Unilever discussed a tape stripping method to quantitatively assess the damaging effects of surfactants in cleansers. The objective was to determine how each layer of the SC barrier responds to cleansers under different washing conditions, how the barrier responds to moisturization with lotion and how the NMF content changes concurrently. TEWL was read after tape stripping, and the tape stripping protein content was analyzed after exaggerated washing and repeated normal washing. Results revealed that cleansing with harsh surfactants can have a damaging effect on barrier quality throughout the SC, therefore the optimal skin care regimen should include mild cleansing to avoid barrier damage and maintain skin health.

Actives and Delivery

Actives were the topic in the afternoon on the second day. One presentation introduced hexapeptides to decrease skin inflammation while another focused on increasing the absorption of actives. Cristina Carreño, PhD, of Diverdrugs, presented a new set of hexapeptides with the ability to inhibit PAR-2 activity. The skin receptor PAR-2 plays a pivotal role as a sensor, and its activation increases skin inflammation. This receptor is considered a target in cosmetics and dermatology for the treatment of inflammatory and/or pruritogenic skin conditions, disorders or pathologies. The efficacy of the peptides to inhibit PAR-2 activity was validated in Carreño's studies.

Yukiko Matsunga of Shiseido then spoke about self-dissolving microneedles (MN) containing hyaluronic acid. This nonsulfated glycosaminoglycan is composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid, and plays a key role in water retention in the extracellular matrix. The molecule size of hyaluronic acid prevents this molecule from penetrating the skin; therefore, self-dissolving MNs were used to deliver it into the subcutaneous tissue via a cosmetic mask. This appears to have upregulated hyaluronic acid production by the cutaneous cells, leading to a significant improvement in the appearance of wrinkles. Self-dissolving MNs may therefore have potential as novel cosmetics to increase transdermal absorption of active agents and to improve the appearance of wrinkles.

Closing Ceremony

During the closing ceremony, a number of awards were presented. Mastunga was given the IFSCC Conference Award for her presentation on dissolving MNs. Pinky Purohit of Lubrizol received the Maison G. de Navarre Young Scientist Prize, and Theeraya Krisdaphong won the Henri Maso Award for her research of natural anti-acne treatments.

The event closed with a dinner, dance and puppet show, as well as a fundraiser to support the Thai population affected by the floods; thoughts of attendees went out to the flood victims. The IFSCC and SCCT worked diligently to make this conference possible, and now the cosmetic industry will now prepare for the 2012 IFSCC Congress in South Africa, which will take place on Oct. 15-18, and will feature beauty and diversity.

Author's note: *Rarely have I met such kind, welcoming and courageous people as the Thai, and I wish them a bright 2012. I also wish to thank the Tony Naylor Memorial Bursary Fund committee and Gem Bektas from the Society of Cosmetic Scientists for generously supporting my attendance at the event.*

SOCIETY OF COSMETIC SCIENTISTS
NEWSLETTER
MARCH 2012

Report on the IFSCC Conference 2011

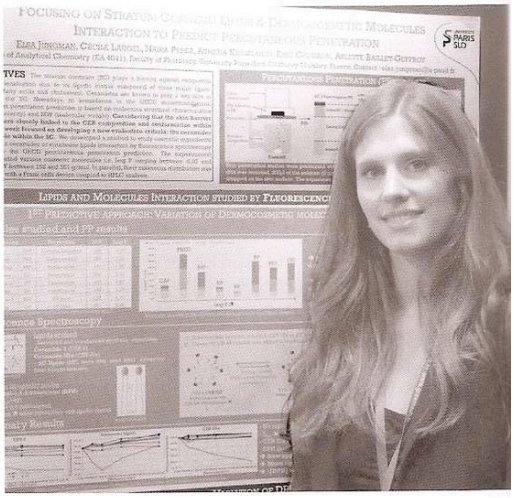
12-14 December 2011
Bangkok, Thailand

Reported by
Elsa Jungman, final year PhD student at the Faculty of Pharmacy of the University Paris-Sud.

This report is the writer's interpretation of the event. It is not intended as a verbatim account and should not be read as such.

POSTER PRESENTED AT THE RE-SCHEDULED IFSCC CONFERENCE 2011

Due to the terrible flood which occurred in Thailand in October 2011, the IFSCC conference was postponed from October 2011 to December 2011. The need to re-schedule this event thus meant a lot of extra hard work to organise the re-scheduling by the Thai Society of Cosmetic Scientists which they coped with admirably and made the event a reasonable success given the circumstances.



Elsa Jungman

Thailand and more specifically Bangkok, is an amazing place where the cosmetic industry is expanding very fast. With the support of the Tony Naylor Memorial Bursary Fund, I was given the wonderful opportunity to travel to Bangkok to present a poster on some of my PhD studies at the IFSCC conference. My research deals with the development of a new method to predict the percutaneous penetration of cosmetic ingredients. The poster I presented at the conference concerned the study of the interaction between stratum corneum lipids and cosmetic actives by fluorescence spectroscopy in order to better understand the mechanism of retention of molecules within the skin.

...with the support of the Tony Naylor Memorial Bursary Fund, I was given the wonderful opportunity to travel to Bangkok to present a poster on some of my PhD studies at the IFSCC conference...

The theme of the IFSCC 2011 was "3E: Effective, Economic & Ecological". I attended many of the presentations and poster sessions and learned about the research area of many of the companies who were present. The keynote presentations were on the topics of cosmeceuticals "a cosmetic that functions as a drug", sunscreen technology aimed at reducing the concentration of UV filters, the discovery of a new protein that regulates the barrier function, organic cosmetics, and skin whitening. I also learned about topics such as the latest trends in cosmetology, skin and hair aging, the effect of menopause on women's skin, the anti-aging protection of a low oxygen medium for skin basal cells, the immediate skin tensing effect of a marine extract, and a new biofunctional compound that can act as an antioxidative protector for scalp and hair follicle.

I also learned the new phenomenon to me of the 'soaping effect' of a formulation which is an undesired foaming (and whitening) phenomenon observed in skin creams formulated with soap based emulsifiers. Interesting ways of preventing this effect were presented.

Another point of particular interest for me was a new protocol presented for assessing the effect of surfactant and washing on skin moisturisation by tape-stripping. My favorite talk was given by a young scientist from Shiseido, Yukiko Matsunga. She presented a technology involving 'dissolving microneedles' that can deliver hyaluronic acid into the subcutaneous tissue.

This new technology, I believe, can be the future for such delivery and a good alternative to many anti-aging products and devices. Indeed, the paper was so good that Yukiko won the IFSCC conference award of best podium paper.

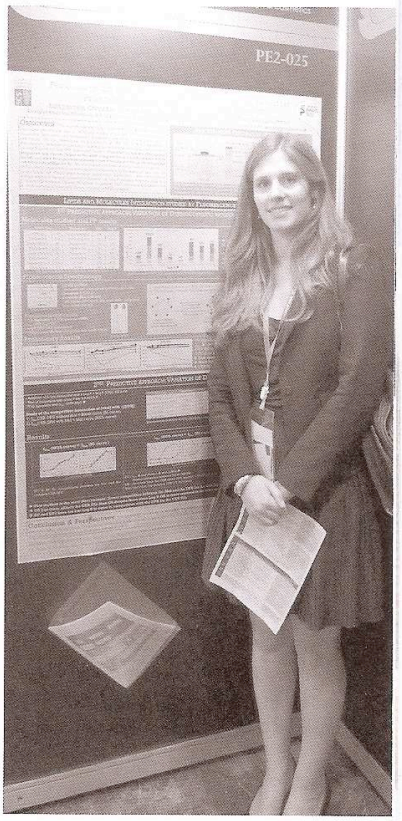
I discovered during my stay a somewhat different environment in the cosmetics arena from the one we are used to in Europe. For example, skin whitening is the major advertising theme for most of the cosmetic products. Even sunscreens contain whitening agents! I was also very impressed by the local Thai brands that range from from value-brands to luxury products such as Pranali. The opening and closing ceremonies were amazing and the Thai gastronomy so delicious. We were treated to an amazing fashion show where the models dressed up in 10 different Thai plants used in cosmetics. I also really enjoyed the puppet show of the closing ceremony, it was a really unforgettable moment.

The Tony Naylor Memorial Bursary Fund helped me to achieve one of my scientific dreams: to present a paper on my work at a prestigious cosmetic science conference and meet world renowned researchers from well known global skin care companies. I've always had the ambition to work as a scientist in a cosmetic company but with my academic education and my PhD research in a public laboratory it is not always easy to really come into contact with the private sector and to attend to such conferences. The SCS kindly offered me this unique opportunity for which I am very grateful. After my PhD graduation, I have the ambition to work for a skin care company outside Europe, and South-East Asia is amongst my top choices!

The Thai and IFSCC societies did amazing work to make this conference possible. During the conference all our thoughts went to the flood victims. A fundraiser was organized during the closing ceremony to support the Thai population. I have very rarely met in my life so kind, welcoming and courageous people as is the Thai population. I wish them the very best and a bright future for this new year.

I would like to thank very much the Tony Naylor Memorial Bursary Fund committee and Gem Bektas from the Society of Cosmetic Scientists for giving me the chance to both attend and present at my first IFSCC conference. I hope it will be the first of many!

Elsa Jungman and the Thai Society

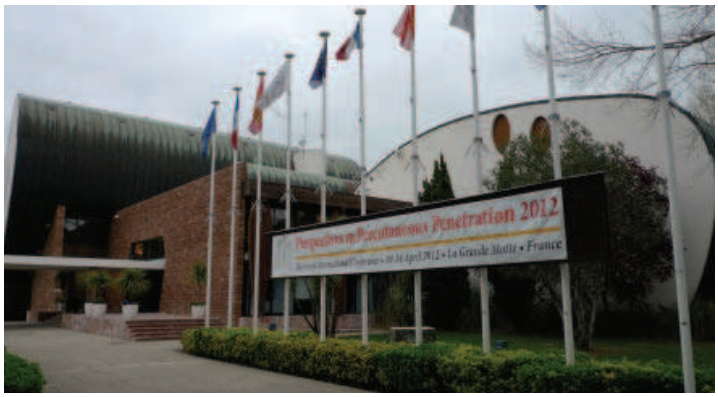


Elsa Jungman with her Poster at the IFSCC Conference

...it is not always easy to really come into contact with the private sector and to attend to such conferences. The SCS kindly offered me this unique opportunity...

Exploring the Depths of Percutaneous Penetration

The Perspectives in Percutaneous Penetration (PPP) conference was held at the Palais des Congrès de La Grande Motte on April 10–14, 2012. For more than two decades, the conference has provided a unique forum for the interchange of ideas on percutaneous penetration among the academic, industrial and regulatory sectors. The event provides an optimized combination of plenary lectures, oral contributions, poster sessions and debates combined with an appropriate experience of local culture. Following are some highlights most relevant to the cosmetics and personal care industry.



Conference entrance, Palais des Congrès of the Grande Motte

Pre-conference Fundamentals

The event opened with a full day pre-conference course on the fundamentals of percutaneous penetration. Michael Roberts, of the University of Queensland, Australia, presented a general overview of skin structure and function. This was followed by a discussion of skin transport mechanisms and permeation enhancement, by Kenneth Walters, PhD, of An-eX Analytical Services Ltd. Various penetration enhancement strategies were presented; from pyrrolidones, anionic and non-ionic surfactants, fatty acids and alcohols, to terpenes, a synthetic enhancer: azone, and sunscreens.

Darren M. Green, PhD, also of An-eX, highlighted methods for measuring percutaneous penetration, which for cosmetics, is important for risk assessments and claims substantiation. Green explained there are major limitations of mathematical models because physiological and formulation effects are not taken into account. Further, the theoretical values from the Potts and Guy model do not help to predict permeability coefficients.

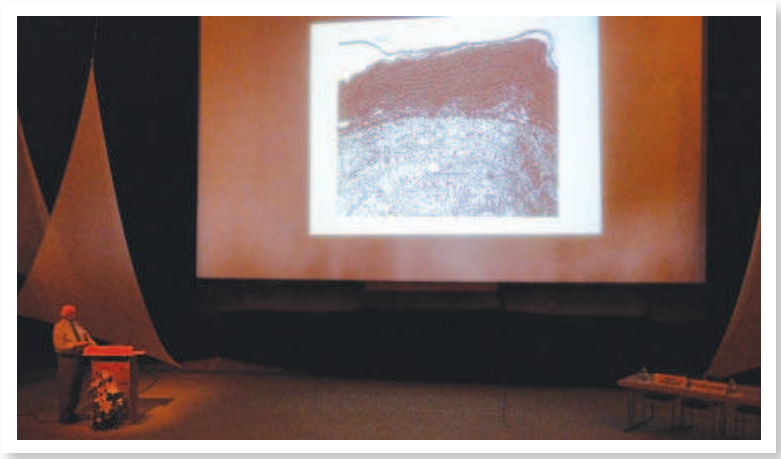
Joke Bowstra, PhD, of the Leiden/Amsterdam Center for Drug Research, showed the importance of skin barrier in drug transport and more specifically, the role of epidermal lipids. Finally, Heinz Ahlers of the US Department of Health and Human Services presented on the use of skin notation in the prevention of occupational skin disease.

The first day ended with a welcome reception including a typical French buffet with a nice glass of wine.

Paul Matts, PhD, of P&G spoke on aqueous BP creams, noting that the routine prescription of this preparation for atopic dermatitis is questionable.

Digging Deeper

The official conference opened with a presentation from Tom Franz, the inventor of Franz cells, who discussed the use of in vitro permeation as a bioequivalence tool. He talked about the correlation between in vivo and in vitro data and, from the literature, showed that data from studies conducted with fully harmonized protocols could be totally correlated to in vivo data. Pauls Matts, PhD, of Procter & Gamble, then spoke on the effects of an aqueous British Pharmacoepia-registered (BP) cream on eczema. Such an aqueous cream (AQC) is a light, paraffin-based emulsion that is categorized by the British National Formulary as non-proprietary emollient preparation. It contains the anionic surfactant sodium lauryl



Robert Scheuplein, PhD, of Keller and Heckman presented on the concept of barrier, skin penetration and beyond.

sulfate (SLS). However, the recommendation for using AQC as an emollient in atopic skin is surprising, as SLS is a known skin irritant.

Matts described an experiment wherein AQC was applied for one month on the volar forearm of skin. Results showed an increase in desquamatory and inflammatory protease activity, and changes in corneocyte maturity and size, which are also indicative of accelerated skin turnover induced by chronic application of this emollient. These findings question the routine prescription of this preparation as a moisturizer in patients with atopic dermatitis.

Johanna Brandner, PhD, of the University of Hamburg, explained the role of tight junction assembly in the barrier function. Tight junctions, cell-cell junctions localized in the stratum granulosum, are a barrier for ions, water and macromolecules, especially claudine-1. Brandner showed that a decrease of this protein would increase the TEWL, and that an alteration of the tight junction in the epidermis leads to skin diseases such as psoriasis, ichthyosis and atopic dermatitis. The next step would be to study the role of these proteins in exogenous molecule penetration.

Peter Caspers, PhD, of Erasmus MC & River Diagnosis, presented a Raman spectroscopy method to rapidly determine the natural moisturizing factor (NMF) in the stratum corneum of adults and young babies. The new protocols presented offer a direct and non-invasive method to determine early in infants whether there is a deficiency of NMF that could lead to atopic skin.

Cosmeceuticals

The second day opened with two talks on cosmeceuticals. Randy Wickett, PhD, of the University of Cincinnati, and Chris Gummer, PhD, of Cider Solutions Ltd., presented their viewpoints on cosmeceuticals. For Wickett, cosmeceuticals have no legal definition. *Cosmeceutical* is a term used to describe active cosmetics that have a positive effect on skin beyond cleaning and moisturizing. According to Wickett, the most common cosmeceuticals are retinol, antioxidants, niaciamide, natural anti-aging peptides, etc. All of these compounds aim to decrease fine lines and wrinkles.

For Gummer, cosmeceuticals are an "itch that won't go away." As he explained, for him, a product can be either a cosmetic or a medicine; there is no middle ground. Cosmeceuticals are borderline products that are not clear. Gummer explained that the US Food and Drug Administration, and UK and Australia guidelines do not recognize the term *cosmeceuticals;* only Japan recognizes them as "quasi-drugs." Some products claim, for example, to work against hair loss with no side effects. However, according to Gummer, this is not possible because if a product works, there are effects; if not, it does not work. So for Gummer, "Cosmeceuticals exist in the advertising world," because they cannot be defined and regulations need to fast-track these borderlines products. He concluded that it would be in the industry's best interest "to make cosmeceuticals go away."

Historical Perspective

A special lecture was presented on the concept of barrier, skin penetration and beyond with a look at concepts presented from 1850–1980 by Robert Scheuplein, PhD, of Keller and Heckman. Scheuplein is an international expert on risk assessment who worked for nearly 20 years at the US FDA. His mentors were Irvin Blank and Albert Kligman, who introduced him to skin permeation and medical/biological research on skin.

Scheuplein explained that between 1850 and the 1860s, the fact that there was a barrier in the epidermis was discovered but it took around one hundred years to locate it in the stratum corneum. Kligman showed that the stratum corneum was stiff by placing it on the top of a bottle full of water, and even with the bottle turned upside-down, the water was contained. In this way, Kligman demonstrated that the stratum corneum was impermeable to water.

Blank demonstrated that water could remain in the stratum through the use of tape-strips. After eight strips, he found transepidermal water loss (TEWL) to increase significantly. The tape-stripping technique was introduced by Wolf, and showed precisely the location of the barrier zone in the stratum corneum.

Two decades of work determined that the principal barrier layer of the skin is the bulk of the stratum corneum.

Studies also showed that the skin does not behave like a passive diffusion medium; it was demonstrated that the stratum corneum/epidermis or full thickness skin has the same permeation rate as whole skin. However, this was not the case for the permeation rate of exogenous chemicals. The effect of the vehicle was also a great discovery. Diffusion was tested and an increase in the transepidermal flux with, for example, water or methanol as a vehicle was observed. However, lipophilic solvents tended to decrease the flux.

Scheuplein stated, "To increase penetration, [use] a solvent with the opposite lipophilicity." He proposed in 1971 a stratum corneum diffusion model that would be related mainly to transcellular diffusion with parallel pathways. Bound water in the keratinocytes provides the primary diffusion resistance. It was not until the 1990s that the first semi-empirical predictive model appeared by Potts and Guy, based on the molecular weight and partition coefficient of the molecule.

The day's sessions ended with a pleasant dinner and a closing speech by Keith Brain, University of Cardiff.

Nailing Down Nails

On the final day, the morning was focused on nail permeation. Stuart Jones, King's College, London, explained how the nail is a rich keratin barrier and that 80% of the keratin found in the nail is a hard, hair type of keratin; the remaining 20% is a soft, skin type keratin. This combination gives a rigid structure that is difficult to penetrate, including with drugs, to treat nail diseases. The penetration lag time can last hours but one approach to improve this penetration rate is to manipulate the keratin structure.

The nail environment is different from the skin in that the nail has the ability to be charged, and charged molecules make molecular interactions possible. Four categories of interactions can be developed for improving the drug permeation through the nail. First are solvent-barrier interactions, for example an alcohol-water mixture, which can enhance diffusion; a change of viscosity does not affect water flux. Second are drug-solvent interactions, as supramolecular structures can modify ungual diffusion. Third are drug ion pairs, ions and complexes, which can modify diffusion. Finally, drug-drug interactions; dimer forms have the potential to modify ungual diffusion. This could be a key function; however, it is only in the early stages of development.

—Elsa Jungman, Université Paris-Sud