Universidade de Lisboa

Faculdade de Farmácia

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Mariana Gonçalves Ferreira

Dissertation supervised by Professor Joana Marques Mato and co-supervised by Professor Helena Margarida Oliveira Marques Ribeiro.

Master in Regulation and Evaluation of Medicines and Health Products

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Resumo

Os produtos cosméticos estão sujeitos a controlos regulamentares em alguns territórios do mundo, de forma a garantir a sua segurança, qualidade e eficácia e evitar impacto negativo na saúde dos consumidores. No entanto, os quadros regulamentares diferem significativamente entre os diferentes mercados/países e estão longe de ser harmonizados. Estas diferenças, não só dificultam o comércio internacional como também não permitem que haja igualdade de oportunidades em todos os mercados. Todas estas consequências, não só restringem a inovação e reduzem o potencial de crescimento do mercado, como também dificultam o papel das autoridades regulamentares, que precisam de assegurar que todos os produtos cumprem a regulamentação aplicável no país em causa. Algumas das principais diferenças nos regulamentos em vigor encontram-se na categorização dos produtos, restrições ao uso de certos ingredientes e nos requisitos para o seu registo.

A definição de "produto cosmético", embora semelhante, difere entre mercados. De forma geral, baseia-se na função dos produtos, partes do corpo onde é aplicado, modo de ação, indicação de uso e reivindicações. No entanto, na prática, os produtos estão sujeitos a categorizações e, portanto, regulações distintas. Por exemplo, alguns produtos podem ser considerados cosméticos na União Europeia, e serem regulados como produtos de venda livre, etc., noutros mercados. Além disso, mesmo entre produtos classificados como cosméticos, existem diferenças na sua regulamentação, por exemplo, nos requisitos de embalagem e rotulagem (ex., nomenclatura dos ingredientes, informação necessária no rótulo, disposição da informação na embalagem) e nas diferentes restrições ao uso de certos ingredientes, o que pode levar a diferentes formulações para o mesmo produto. Tudo isto reduz a flexibilidade entre mercados, sem necessariamente aumentar a segurança dos produtos.

Relativamente aos ensaios de absorção cutânea, apesar de atualmente existirem várias metodologias para a sua avaliação, como métodos *in silício*, *in vitro*, *ex vivo* e *in vivo*, ainda não existe uma padronização ou harmonização global das mesmas. Nomeadamente, dentro da mesma metodologia existem diferenças de protocolo e condições experimentais. É o caso das células de Franz, um dos métodos *in vitro* mais utilizado no estudo da absorção cutânea. A absorção cutânea é um processo complexo e facilmente influenciado por vários fatores, nomeadamente, fatores fisiológicos (ex.,

idade, género, etnia, local anatómico, estado e hidratação da pele, fluxo sanguíneo, etc.), propriedades físico-químicas dos ingredientes (ex., estado físico, tamanho molecular, lipofilia, ionização, solubilidade) e características da formulação/veículo, que pode alterar o mecanismo de libertação e o fluxo de penetração. Deste modo, verifica-se que pequenas variações dos parâmetros experimentais (ex., dose aplicada, concentração do produto, tipo de membrana utilizada e a sua preparação) podem influenciar os resultados. Além disto, muitos dos documentos disponíveis foram escritos para uso geral e não para setores específicos, o que significa que as mesmas recomendações se aplicam a ingredientes cosméticos, pesticidas, químicos industriais e a sistemas transdérmicos. Isto pode ser um problema, uma vez que a avaliação da permeação cutânea é um processo crucial na avaliação da eficácia e segurança dos produtos cosméticos.

Por conseguinte, existem vários grupos de trabalho que têm como principal objetivo a harmonização de metodologias e protocolos relacionados com produtos cosméticos, nomeadamente o comité ISO/TC 217. Também a OECD tem vindo a publicar várias normas relacionadas com vários ensaios, como por exemplo os ensaios de absorção cutânea, aumentando assim a confiança na previsão dos resultados. No entanto, ainda há aspetos a serem discutidos que precisam de ser melhorados, nomeadamente, o facto destas normas estarem direcionadas para medicamentos e não para cosméticos e a dificuldade no doseamento dos mesmos, uma vez que, ao contrário dos medicamentos, os produtos cosméticos são compostos por vários ingredientes sem atividade terapêutica.

Esta dissertação incorpora uma análise crítica de algumas diferenças e semelhanças entre os principais quadros regulamentares existentes em todo o mundo, nomeadamente União Europeia, Estados Unidos da América, Canada, Japão, China e Brasil. Especificamente, discute as diferentes metodologias, regulações e *guidelines* disponíveis para o estudo da absorção cutânea de cosméticos, nomeadamente, as diferenças entre si e o seu impacto nos resultados finais e conclusões dos estudos.

Adicionalmente, foram realizados estudos *in vitro* de libertação e de permeação de um sérum de ácido hialurónico como formulação modelo, com o objetivo de estudar e avaliar a influência da quantidade de produto cosmético aplicado na absorção cutânea. Os resultados obtidos demonstraram que a quantidade de produto

aplicado influência a quantidade libertada e permeada. A aplicação de 0.1g de sérum libertou e permeou 92% e 4%, respetivamente, enquanto 0.3g de sérum libertou e permeou 31% e 2%, respetivamente e 1g de sérum libertou e permeou 18% e 0.5%, respetivamente.

Os principais mercados apresentam regulamentos e outros documentos que têm como objetivo principal garantir a segurança do consumidor. No entanto, ainda existem lacunas com vista à harmonização de metodologias experimentais de modo a garantir o cumprimento do quadro regulamentar. Assim, é necessário realizarem-se mais estudos, de forma a determinar o efeito dos diferentes parâmetros experimentais na absorção cutânea de cosméticos, de forma a desenvolverem-se metodologias e protocolos adequados, que permitam a obtenção de resultados reprodutíveis e que se aproximem das condições reais de uso.

Palavras-chave: cosmético; absorção dérmica; *in vitro*; regulamentação; harmonização

Abstract

Cosmetic products are subjected to regulatory controls all over the world in order to ensure their safety, quality and efficacy to avoid adverse impacts on consumers health. However, regulatory frameworks differ significantly between markets/countries and are far from being harmonized. As it's the case of *in vitro* dermal absorption studies. Nowadays, there are various methodologies to access and evaluate dermal absorption, such as *in silico, in vitro, in vivo* and *ex vivo* methods. However, there is no standardized protocol/methodologies. All of this impacts the industry, as it not only restricts innovation and reduces the market potential growth, but it also affects international trade and hinders the role of the regulatory authorities that need to ensure every product complies with the regulation applicable in the country in question. Because of this, several international organizations such as ISO, OECD, WHO, ICCR, etc., have been working towards the common goal of cosmetic regulatory harmonization, including the standardization of protocols relating to the study of dermal absorption in cosmetics, however there are still various elements that need to be improved as discussed.

This dissertation incorporates a review on some of the main differences and similarities between the existing regulatory frameworks around the world. In particular it discusses the different methodologies, regulations and guidelines available for the study of dermal absorption of cosmetics. Additionally, in order to study and to assess the influence of some parameters, such as the amount of cosmetic product used, *in vitro* release and permeation studies were carried out using a hyaluronic acid (HA) serum as a model formulation. The application of 0.1g of serum released and permeated 92% and 4% respectively, while 0.3g of serum released and permeated 31% and 2%, respectively and 1g of serum released and permeated 18% and 0.5%, respectively.

The main markets provide regulations and other guidance documents with the main goal of ensuring the consumer safety. However, there are still gaps in the harmonization of experimental methodologies to ensure the compliance of the regulatory framework.

Keywords: cosmetic; dermal absorption; *in vitro*; regulation; harmonization

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Symbols

% – Percentage

µg – Microgram

µL – Microliter

Ag – Silver

Au – Gold

C_5H_8 – Isoprene

cm – Centimeters

g – Gram

mg – Milligrams

nm – Nanometer

ºC – Centigrade Degree

rpm - Revolutions Per Minute

SiO_2 – Silicon Dioxide

TiO_2 – Titanium Dioxide

ZnO_2 – Zinc Oxide

Abbreviations

3D – Three Dimensional

AIC - Akaike Information Criterion

AUC – Area Under the Curve

CHSR – Cosmetics Hygiene Supervision Regulations

CIR - Cosmetic Ingredient Review

CLSM - Confocal Laser Scanning Microscopy

CMR - Carcinogenic, Mutagenic, or Toxic for Reproduction Substances

CNF - Cosmetic Notification Form

CPNP - Cosmetic Product Notification Portal

CPSC - Consumer Product Safety Commission

CRM - Confocal Raman Microspectorscopy

CSAR - Cosmetic Supervision and Administration Regulation

DE – Dissolution Efficiency

e.g. – For Example

EC – European Commission

ECETOC - European Centre for Ecotoxicology and Toxicology of Chemicals

EFSA - European Food Safety Agency

EMA - European Medicines Agency

EU – European Union

FD&C Act - Federal Food, Drug and Cosmetic Act

FDA – Food and Drug Administration

FPLA - Fair Packaging and Labelling Act

GAC - General Administration of Customs

GHCOS - Hygiene, Perfume, Cosmetics and Sanitizing Products Management

GMP - Good Manufacturing Practices

GQP - Good Quality Practice

GRAS – Generally Regarded Safe

GVP - Good Quality Practice

HA – Hyaluronic Acid

HC – Health Canada

ICCR - International Cooperation on Cosmetics Regulation

IECIC - Inventory of Existing Cosmetic Ingredients in China

INCI - International Nomenclature of Cosmetic Ingredients

IPCS - The International Programme on Chemical Safety

ISO - International Organization for Standardization

IVPT - *in vitro* Permeation Test

IVRT - *in vitro* Permeation Test

LPP - Lipid-Protein-Partitioning

LSE - Living Skin Equivalent Models

MHLW - Ministry of Health, Labour and Welfare

MSC - Model Selection Criterion

NHP – Natural Health Product

NLC – Nanostructured Lipid Carriers

NMPA - National Medical Products Administration

Nº - Number

O/W – Oil-in-Water

OECD - Organization for Economic Co-operation and Development

OTC – Over-the-Counter

PAL - Pharmaceutical Affair Law

PAMPA - Skin Parallel Artificial Membrane Permeation Assay

PBS - Phosphate Buffer Saline

PEG – Polyethylene Glycols

PIF - Product Information File

PMDL - Pharmaceutical and Medical Devices Law

QSAR - Quantitative Structure-Activity Relationship

RHE - Reconstructed Human Epidermis Models

RP – Responsible Person

SAMR - State Administration for Market Regulation

SC - Stratum Corneum

SCCNFP - Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers

SCCS - Scientific Committee on Consumer Safety

SD - Standard Deviation

SGAS - Cosmetic Automation System

SLN – Solid Lipid Nanoparticles

SPF – Sun Protection Factor

TG – Test Guideline

TPEFM - Two-Photon Excitation Fluorescence Microscopy

USA – United States of America

USEPA - United States Environmental Protection Agency

UV – Ultra-Violet

w/ - With

W/O – Water-in-Oil

WHO – World Health Organization

1 Objectives

The purpose of this dissertation is to review the different cosmetic regulatory frameworks around the world and identify the main differences and similarities between them. In particular, it aims to study and assess the various methodologies, regulations and guidelines available for the study of dermal absorption of cosmetics in order to comprehend what are the main differences between experimental conditions and their impact on these studies results and interpretation. The final goal is to draw a conclusion about the importance of the standardization and harmonization of experimental conditions and regulations regarding dermal absorption studies, more specifically to propose a methodology/protocol/guideline for the assessment of dermal absorption in cosmetics.

To achieve this, an experimental study will be carried out to access the influence of the amount of product used used in *in vitro* release and permeation studies, particularly the amount of cosmetic product used. The study will be conducted using a hyaluronic acid (HA) serum as a model formulation and the assessment will be performed on Tuffryn® and Strat-M® membranes using static Franz diffusion cells. Three different amounts of the formulation will be tested in order to evaluate if there are variations on the release and permeation values.

2 Overview of Cosmetic Regulatory Frameworks Around the World (EU, USA, Canada, Japan, China and Brazil)

Cosmetics are regulated by different entities around the world with a common goal of ensuring the safety, efficacy and quality of these products. However, the regulatory approaches taken by each country can differ significantly and impact the competitiveness and economic viability of the industry. In order to better understand the differences and similarities between the cosmetic frameworks around the world, as well as the associated impacts, some of the biggest markets worldwide – European Union (EU), United States of America (USA), Canada, Japan, China and Brazil - were selected and analyzed, with the main focus being the EU.

2.1 Regulatory Comparative Analysis Between the Six Markets

2.1.1 Definition and Categorization of Cosmetic Products

2.1.1.1 European Union

In the EU, the European Commission has the overall responsibility for cosmetic legislation and each member state designates a competent authority to enforce that same legislation. Nowadays, the cosmetic regulatory framework is provided by Regulation (EC) Nº 1223/2009 which replaced the previous Directive 76/768/EC, adopted in 1976. (1) Accordingly, the category "cosmetic product" has borders with a range of other categories including medicinal products, biocides and medical devices. However, as opposed to other countries, each product can only fall into one category. Regulation (EC) Nº 1223/2009 defines cosmetic product as:

> *"any substance or mixture intended to be placed in contact with the 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, protecting them, keeping them in good condition or correcting body odours"* (2)

2.1.1.2 United States of America

In the USA, the Food and Drug Administration (FDA) is responsible for the regulation of cosmetics under the authority of two laws: the Federal Food, Drug and Cosmetic Act (FD&C Act), from 1938, and the Fair Packaging and Labelling Act (FPLA), from 1966. (3) The FD&C Act defines two main categories of products:

i. Cosmetics, defined as: "*articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body...for cleansing, beautifying, promoting attractiveness, or altering the appearance*" (3); and
ii. Drugs, which includes a sub-category of over-the-counter (OTC) drugs that can be sold without prescription. (4)

It's possible for a product to be classified into both categories, which usually happens when it has two intended uses. One of the most well-known examples is the anti-dandruff shampoo which is considered a cosmetic, since it's indicated to clean the hair, but, because it contains anti-dandruff ingredients and it's also indicated for the treatment of dandruff, it's also considered a drug. In these cases, the product must meet the requirements of both regulations. (4,5)

2.1.1.3 Canada

In Canada, cosmetic products are regulated by Health Canada (HC) under the Food and Drugs Act and Cosmetic Regulation Act, from 1985, and are defined as:

> *"includes any substance or mixture of substances, manufactured, sold or represented for use in cleansing, improving or altering the complexion, skin, hair or teeth, and includes deodorants and perfumes."* (6)

Additionally, products can also be classified into two other categories: drugs - divided into prescription and non-prescription (OTC) drugs - and natural health products (NHP), which are considered a subset of "drugs". Each product can only be classified into one category.

2.1.1.4 Japan

In Japan, since 2014, cosmetic products are regulated under the Pharmaceutical and Medical Devices Law (PMDL) by their competent authority, the Ministry of Health, Labor and Welfare. The current regulation replaced the previous Pharmaceutical Affair Law (PAL), from 1960. (7) Accordingly, beauty products are divided into two categories, cosmetics and quasi-drugs. Cosmetics are defined as:

> *"articles with mild action on the human body, which are intended to be applied to the human body through rubbing, sprinkling or other methods, aiming to clean, beautify and increase the attractiveness, alter the appearance or to keep the skin or hair in good condition"* (7)

These are further classified into other six categories: perfume and eau de cologne, makeup products, skin care products, hair care products, special purpose cosmetics and cosmetic soaps; Whereas, quasi-drugs are defined as:

> "*articles for the purpose of preventing nausea and other discomfort, preventing heat rash, soreness, etc., encouraging hair growth or removing hair or exterminating and preventing mice, flies, mosquitos, fleas, etc.".* (7)

Table 1 - Examples of cosmetic and quasi-drug products in Japan (7,8)

Category		Examples
Cosmetics	Perfume and eau de cologne	Perfume, eau de Cologne, etc.
	Makeup products	Foundation creams, lipstick, etc.
	Skincare products	Skin lotion, essence, cleansing cream, etc.
	Haircare products	Shampoo, hair treatment, etc.
	Special-purpose cosmetic	Sunscreen, shaving cream, etc.
	Cosmetic Soaps	Soaps for cosmetics
Quasi-Drugs		Deodorants, hair growth treatment, depilatories, hair dyes, bath products, dentifrice, medicated cosmetics (anti-dandruff products; shaving products; anti-ace products)

2.1.1.5 China

China underwent a large institutional reform in 2018, by which currently there are three major competent authorities in the cosmetic sector: the State Administration for Market Regulation (SAMR), the National Medical Products Administration (NMPA) and the General Administration of Customs (GAC). The new cosmetic regulation, Cosmetic Supervision and Administration Regulation (CSAR), entered in force on January 1st, 2021, replacing the former Cosmetics Hygiene Supervision Regulations (CHSR), from 1990. As a follow up to this general regulation, on May 1st 2021, the NMPA released the Administrative Measures on Cosmetic Registration and Notification. (9)

In China cosmetics are defined as:

> *"daily chemical products intended to be applied on the external part of the human body (such as skin, hair, nails, lips, etc.) by spreading, spraying, or other similar ways for cleansing, protecting, beautifying, or grooming purposes"* (9)

Under the new CSAR, cosmetics can be divided into two categories, special cosmetics and general cosmetics. Special cosmetics include hair dyes, hair perming products, freckle-removing (whitening) products, sunscreen, anti-hair loss products and cosmetics with new efficacy, while the remaining products are classified as general cosmetics. It is possible for a product to fall under more than one category, as it is the example of hair growth products that are subdivided into three categories for supervision: general cosmetics (products that prevent hair breakage by improving hair quality), special cosmetics (products that prevent hair loss by improving the condition of the scalp) and drugs (products that promote hair growth by participating in human physiological activities). (9)

The NMPA specifies a coding system for the classification of cosmetics. The system has five layers: efficacy claim, application area, target user, dosage form and application method. Each layer is represented by a 2-digit number or letters and connected by "-" (Table 2). For example, the classification code of moisturizing masks may be 11-05-03-10-02 (9)

Table 2 – Coding system for the classification of cosmetic in China (9)

1st layer: Efficacy Claims	Includes 26 kinds of claims such as cleansing, makeup removing, moisturizing, sunscreen, deodorant, hair removal, etc.
2nd layer: Application Areas	Includes 10 areas such as hair, body skin, head, face, etc.
3rd layer: Target Users	Includes 3 types of target users, namely infants (between birth and the age of 3), children (aged between 3 and 12), and adults.
4rd layer: Dosage Forms	Includes 11 dosage forms such as creams and emulsions, liquids, gels, powders, pastes, aerosols, sprays, etc.
5th layer: Application Methods	Rinse off and leave on categories.

2.1.1.6 Brazil

In Brazil, cosmetics are regulated by three regulatory authorities: the Ministry of Health, the Brazilian Health Regulatory Agency (Anvisa) and the Hygiene, Perfume, Cosmetics and Sanitizing Products Management (GHCOS), each with their own responsibilities. According with Anvisa, Personal Care Products, Cosmetics and Perfumes, fall into one category and are defined as:

> *"preparations made from natural or synthetic substances, for external use in various parts of the human body, skin, hair, nails, lips, external genitals, teeth and mucous membranes of the oral cavity, with the sole or principal purpose of cleaning, perfuming, altering and correcting bodily odors and / or protecting or keeping them in good condition."* (10)

These products can be classified into grade I and grade II according with their risk to consumers. (Table 3)

> *Grade I*: Include products with basic or elementary properties, that don't require detailed information on their labelling regarding their mode of use and their restrictions of use. (11)
>
> *Grade II:* Include products with specific indications that require proof of safety and/or efficacy, as well as more information on their labelling, on their mode of use and restrictions of use. (11)

Table 3 – Cosmetic Products Classification in Brazil, according with risk (11)

Grade I	Grade II
Face cleansing creams; Lotions; Gels and oils (except those for acne skin); Perfumes, lipsticks (without sunscreen); Fingernail polishes; Cleansing shampoos and hair conditioners; Eye and facial makeup preparations (without sunscreen).	Children's products; Sunscreen lotions and creams; Products for wrinkles; Antiseptic soap; Insect repellent products; Products for straightening, curling and/or dyeing hair.

2.1.1.7 Classification differences between the markets

In view of what was described above, it's perceptible that although similar the definitions for "cosmetic product" differ slightly between the six markets. For the most part, it's based on the functions of the product, parts of the body where it's applied, mode of application, indication of use, claims and consumers perspective. However, in practice, depending on the country, products have different regulations and classifications (Table 4). A major difference that directly arises from this divergence are the regulatory requirements. Products that are categorized as OTC, non-prescription, NHP or quasi-drugs can have significantly different requirements from those applicable to cosmetics. For example, they generally require pre-market approval and are subjected to limitations on composition and manufacturing processes. This reduces flexibility whilst not necessarily increasing safety. Two products that can illustrate these situations are soap and sunscreen:

Soap: Even though soap is considered a cosmetic in the EU, Japan, Canada, and Brazil, in the USA, soap is a product that needs special attention since the regulatory definition is different from the way in which people commonly use the word. The definition of soap in the FDA's regulations is based on three criteria: composition, intended use and what ingredients cause its cleaning action. If a product meets the regulatory definition of soap is considered a consumer product and regulated by the Consumer Product Safety Commission (CPSC), instead of the FDA, if not, it can be considered either a cosmetic, a drug, or both, depending on these criteria. (4,12) Furthermore, in China soaps are free of supervision by CSAR, except those that claim to have special cosmetic efficacy (e.g., whitening). (9)

*Sunscreen***:** In the EU, Japan, Brazil and China sunscreens are classified as cosmetics. However, in the USA, sunscreens are regulated as OTC drugs, as well as any product labelled with SPF values intended to protect consumers from the sun. This means that moisturizers, foundations or any kind of products with these types of claims are also categorized as OTC drug products by the FDA. (4,13) In addition, in Canada, similar to the USA, sunscreens include products intended to be applied to the face or skin, as makeup or some skincare, which also carry sunscreen claims. They can be classified as NHPs or non-prescription drugs, depending on the ingredients they contain. (14)

Table 4 - Examples of product categorization in the six markets (EU, USA, Canada, Japan, China and Brazil)

Product	EU	USA	Canada	Japan	China	Brazil
Soap	Cosmetic	Consumer product, Drug or Cosmetic	Cosmetic	Cosmetic	Cosmetic (w/exceptions)	Cosmetic
Lipstick	Cosmetic	Cosmetic	Cosmetic	Cosmetic	Cosmetic	Cosmetic
Sunscreen	Cosmetic	OTC Drug	NHP or OTC drug	Cosmetic	Cosmetic	Cosmetic
Anti-acne lotion	Medicinal Product	OTC drug	NHP or OTC drug	Quasi-drug	Cosmetic	Cosmetic
Anti-caries toothpaste	Cosmetic	OTC drug	NHP	Quasi-drug	Cosmetic	Cosmetic[1]
Anti-perspirant	Cosmetic	OTC drug	Cosmetic	Quasi-drug	Cosmetic	Cosmetic
Hair dye	Cosmetic	Cosmetic	Cosmetic	Quasi-drug	Cosmetic	Cosmetic
Antidandruff shampoo	Cosmetic	Drug and Cosmetic	NHP or OTC drug	Quasi-drug	Cosmetic	Cosmetic

[1] In China toothpastes are not defined as cosmetics but they are regulated with reference to general cosmetic regulations. Claims like 'anti-caries' are permitted, given that their efficacy was evaluated in accordance with national and industry standards.

2.1.2 Main Cosmetic Regulatory Differences and Similarities

2.1.2.1 Labelling/Claims

The cosmetic product label is what attracts the consumer and provides the information about the products content. Therefore, and in order to prevent misleading and misbranded labels, each country has implemented a set of regulations and requirements regarding, for example, the standard pieces of information that need to be included in the label, the label display, the listing and nomenclature of ingredients, as well as claims.

Generally speaking, there are broad similarities between the labelling requirements. For example, all the countries have identical label displays and information requirements and they all adopted the INCI (International Nomenclature of Cosmetic Ingredients) nomenclature in the labelling of ingredients (with some variations), which is very important not only to avoid language barriers but also to help free trade. The biggest differences rely on the labelling requirements and permitted claims for specific ingredients (e.g., SPF).

A cosmetic claim can be a text, image, symbol or any sign that is present on the product packaging, the brand website, in an advert, on social media, etc. and informs the consumer about the product's characteristics and benefits. They are used by cosmetic companies as marketing tools to differentiate their products from the competitors, thus stimulating innovation and competition between companies. Because of this, claims play a big role in advertising and thereby on the cosmetic industry. A lot needs to be taken into consideration when developing a marketing campaign, such as the market itself, the scientific progress, the consumers diversity and demands, etc. However, it all must be subjected to rules in order to protect the consumer from being misled.

In the EU, article 20(1) of the EU Regulation clearly states that:

> *"In the labelling, making available on the market and advertising of cosmetic products, text, names, trademarks, pictures and figurative or other signs shall not be used to imply that these products have characteristics or functions which they do not have."* (2)

Accordingly, in 2013, following the release of Regulation (EC) 1223/2009, the EU published a separate regulation – Commission Regulation (EU) No 655/2013 – with the purpose of establishing common criteria for the justification of claims used in relation to cosmetic products. In this regard, were established six common criteria – i) legal compliance; ii) truthfulness; iii) evidential support; iv) honesty; v) fairness; and vi) informed decision-making – with the main objective of not only ensuring consumers protection from misleading claims but also to establish a common approach at a Union level and enhance the cooperation between the national authorities of each Member State. (15) Additionally, in 2017, a sub-working group released a non-legally binding document – the Technical Document on Cosmetic Claims – to provide guidance for its application. The document contains in depth-descriptions and examples for the six criteria as well as two additional annexes, one on "free from" claims and other on "hypoallergenic" claims. (15) Even though there are standard principles that must be respected in the EU, the common criteria do not specify the wording that can be used for cosmetic product claims, therefore the responsible person needs to "*ensure that the wording of the message communicated is in compliance with the common criteria and is consistent with the documentation in his possession for supporting the claim*". (15)

It can be said, the existing European regulatory framework for claims and advertising of cosmetic products is very comprehensive and ensures a high level of consumer protection while enabling the industry, at the same time, to be competitive within the EU and the world. However, in countries like the USA and Canada the regulation is not as restrictive. Contrary to the EU, in the USA, the FDA does not have the authority to approve claims before the products are placed on the market. There is also no list of approved or accepted claims for cosmetics, the only requirement being that they must be truthful and not misleading. Therefore, to enforce control over these products, the FDA monitors cosmetics on the market and when any incompliance on the labels is found, it issues a warning letter to the company.(16) The most common incompliance is an unapproved drug claim on a cosmetic product. This happens because in the USA, it can be tricky to determine what type of claims are appropriate for cosmetics, rather than drugs, since some cosmetic products can alter the function of the body (e.g., cosmetics for aging, acne and rosacea). This is a problem because in the USA, the intended use of a product determines whether is classified as a drug and/or a cosmetic. So, if there is a cosmetic product with drug claims, then the product should

also be considered a drug and thus be approved by the FDA. For this reason, when describing the function of a cosmetic product, terms such as treat, cure and heal should be avoided. (17,18)

Another issue, due to the lack of legislation, is when companies use claims that don't apply to their products in order to appeal to consumers and increase their share in the market. For instance, hypoallergenic cosmetics are products that claim to produce fewer allergic reactions than other cosmetic products. In Europe, this claim is well defined, and there are strict requirements for the products that claim to be hypoallergenic. However, in the USA, there is no legal definition for this claim and the manufacturers are not required to submit any type of evidence to support this claim in order to include it on the label of the product. This means that consumers have no assurance that the product their buying does what it claims. (19) Another illustrative example of this issue are cruelty free claims. Nowadays, a lot of consumers want to buy cosmetic products that have not been tested on animals. But once again, because there are no legal definitions (unlike Europe) companies apply claims such as "cruelty free" or "not tested in animals" to promote their finished products even though they use ingredients tested on animals to assess their safety. (20)

Similar to the USA is the Canadian regulation applicable to cosmetic claims. Health Canada does not consider marketing terms to be related to health or safety and so they are not regulated by the authority. Instead, in Canada, cosmetic claims are regulated under the Consumer Packaging and Labelling Act and the Competition Act, overseen by Competition Bureau, which can take action in cases of false or misleading marketing claims. (21) Additionally, in Canada the classification of cosmetics is hugely based on product representation, including claims. Therefore, it is important to avoid drug-type claims such as "restores, repairs, prevents, heals, etc." to avoid the product being classified as a drug. For example, products that claim to have SPF, even if it's a secondary function, would be considered a drug in Canada, however if the product contains sunscreen ingredients but does not claim to have SPF, would be considered a cosmetic. Special attention also needs to be paid in respect to claims such as "fragrance free", "hypoallergenic" and "not tested on animals" which are also not as restrictive as in the EU. (22)

Additionally, in China, the new cosmetic regulation presents new requirements applicable to efficacy claims of cosmetics in view of enhancing consumers protection against misleading and false information. In accordance with the CSAR and the Standards for Cosmetic Efficacy Claim evaluation, efficacy claims of cosmetics need to be supported by scientific basis, which include literature documents, research data, test reports or efficacy evaluation. For that, the responsible person shall publish the summary of evidence on a website designated by NMPA for public supervision. (9)

2.1.2.2 Ingredients Regulation

All six markets have similar regulatory approaches, as the regulation of ingredients is mainly made through the establishment of positive and negative lists, the main difference being the amount and type of substances included in those lists. For example, contrary to the EU, the USA and Canada only have negatives lists, partly because some of the products positive listed in the EU (e.g., UV filters) are not regulated as cosmetics in those countries.

In the EU, cosmetic regulations are ingredients risk-based. The SCCS "Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation" include toxicological test procedures such as basic toxicity testing procedures to evaluate different human health-related toxicological endpoints. For SCCS safety evaluation, the systemic doses obtained (mostly) after oral administration are used. For local toxicity endpoints, normally only hazard identification is carried out. Safety evaluation is done for intact skin. In all, and for safety purposes, only validated non-animal methods is required for the evaluation of cosmetic. (23)

Regulation (EC) 1223/2009 establishes two negative lists, for prohibited and restricted substances, and three positive lists, for colorants, preservatives and UV filters. The safety of the Annex substances is evaluated by the SCCS while the safety of cosmetic products is evaluated by the industry. Thus, the Annex substances fall under the responsibility of the SCCS. All the ingredients in cosmetic products are the responsibility of the "Responsible Person, as defined by Regulation (EC) 1223/2009, through the safety assessor.

On this note, the SCCS regularly revises and updates the "Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation", which provides

guidance to public authorities and the cosmetic industry about the safety of cosmetic ingredients. Taking into consideration the opinions of the committee, is then up to the European Commission and the Member States to take the final decision on the addition or removal of substances from the lists. (24) Additionally, Article 15 of the Regulation prohibits the use of substances classified as CMR substances (substances classified as carcinogenic, mutagenic, or toxic for reproduction) of category 1A, 1B or 2 under Part 3 of Annex VI of Regulation (EC) No 1272/2008, which regulates the classification, packaging and labelling of hazardous substances in the EU. Nevertheless, a substance classified in category 2 may be used in cosmetic products if the substance has been evaluated by the SCCS and found safe for use. (2,25)

Similar to this approach are the regulations of Brazil, Japan and China who also establish negative and positive lists for the control of ingredients in cosmetic, that is lists for prohibited ingredients, restricted ingredients, as well as positive lists for preservatives, UV filters and colorants. However, China takes one step forward and also distinguishes cosmetic ingredients between "existing" and "new" ingredients. New cosmetic ingredients refer to natural or artificial ingredients used in a cosmetic for the first time in China. An ingredient is considered "new" if it's not included in the Inventory of Existing Cosmetic Ingredients in China (IECIC). They are also further divided into different risk levels. Those considered to be relatively high-risk (preservatives, UV filters, colorants, hair dyes and freckle removal or whitening agents) require a registration with NMPA to obtain approval while the others can be immediately used after notification to NMPA. (9, 26)

In respect to the FDA and Health Canada, in similarity to the EU, both have available some lists for the control of cosmetic ingredients, however, they are not as comprehensive as the European ones. Canada, has available, a Cosmetic Ingredient Hotlist, which is a document that is reviewed and updated periodically and lists the substances that are prohibited or restricted for use in cosmetics. (26) Likewise, the FDA as a similar approach as it only lists a small number of prohibited and restricted ingredients and the only substances that need to be approved are color additives, except coal-tar hair dyes. (27) Additionally, in support, the FDA takes the Cosmetic Ingredient Review (CIR) reports into consideration when evaluating cosmetic ingredients safety. CIR is an industry-funded panel of scientific and medical experts that review and assess

the safety of numerous ingredients used in cosmetics.(29) The FDA participates in CIR meetings but does not vote by which the final decision comes down to the panel. (28)

2.1.2.3 Pre-Market Approval and Notification of Products

In the EU, the responsible person (usually the manufacturer or the importer) has the responsibility to ensure the safety of the product before placing it on the market. For that it is necessary to notify some information through the cosmetic products notification profile (CPNP), making it available on the European market. (2, 30)

Additionally, the responsible person also needs to ensure that the cosmetic product safety report is up to date after the placing of the product on the market. The cosmetic product safety report is divided in two parts:

- *Part A: Cosmetic product safety information*: this part gathers all of the information necessary for the safety assessment of the product and consists of ten sections, 1. quantitative and qualitative composition; 2. physical/chemical characteristic and stability; 3. microbiological quality; 4. impurities, traces and information about the packaging material; 5. normal and reasonably foreseeable use; 6. exposure to the cosmetic product; 7. exposure to the substances; 8. toxicological profile of the substances; 9. undesirable effects and serious undesirable effects; 10. information on the cosmetic product. (2)

- *Part B: Cosmetic product safety assessment:* this part it's the cosmetic safety assessor's opinion on the safety of the product and consists of four sections, 1. assessment conclusion; 2. labelled warning and instruction of use; 3. reasoning; 4. assessor's credentials and approval of part B. (2)

This report can be found in the product information file (PIF) of the cosmetic, which content must also be kept updated. The PIF contains the following information: a description of the cosmetic product; the cosmetic product safety report; a description of the method of manufacturing and a statement on compliance with GMP; proof of the effect claimed for the cosmetic product and data on any animal testing performed by the manufacturer, his agents or suppliers, relating to the development or safety

assessment of the cosmetic product or its ingredients, including any animal testing performed to meet the legislative or regulatory requirements of third countries. (2)

The FDA does not require pre-market approval of cosmetics, with the exception of color additives (other than coloring materials used in coal-tar hair dyes) that need to be approved for the specific intended use. Therefore, the manufactures or distributors of the product have the responsibility of ensuring that the product is safe. Additionally, as opposed to the EU, product filling and establishment registrations are not mandatory. The manufactures or distributers may submit online information to the agency voluntarily under the Voluntary Cosmetic Registration Program. (31,32,33)

In Canada, the manufacturer has the responsibility of ensuring the safety of the cosmetic product. All cosmetics sold in Canada must be notified to Health Canada. Manufacturers also need to submit a Cosmetic Notification Form (CNF) for each product, within ten days after they first sell the product. The notification form is made online and includes information such as the address and contact of the manufacturer, the function and form of the cosmetic and the concentration of each ingredient. The notification does not constitute approval for sale or any type of agreement that the product complies with all legal requirements as that is the responsibility of the manufacturer. (34)

In Brazil, the registration procedure differs depending on the product. Some of the products classified as Grade II cosmetics and listed in Annex VIII of Resolution RDC 07/2015 are subjected to pre-market approval procedures. These procedures are valid for five years from the date of their publication in the Brazilian Official Gazette and can be renewed for equal and successive periods. The other cosmetic products, that are not included in Annex VIII of Resolution RDC 07/2015, are exempt from pre-market approval and only need to be notified to Anvisa. The notification procedure is carried out online, via the Cosmetic Automation System (SGAS System) and is valid for five years from the date when the online protocol is finalized. They can also be renewed for equal and successive periods. (35)

In Japan, to register a cosmetic product, it’s necessary to first obtain licenses such as a Cosmetic Manufacturing License and a Cosmetic Marketing License. Each has their own requirements, however Marketing License holders need to comply with two standards: the Good Quality Practice (GQP) standard, to maintain the quality of

products, and the Good Vigilance Practice (GVP) standard, to undertake appropriate actions for safety management. Subsequently, after obtaining the required licenses the manufacturers need to submit a cosmetic marketing notification to the same prefecture that granted the Cosmetic Marketing License. Lastly, after all the requirements mentioned prior have been implemented the product can be then placed on the market. (7,36)

Under China's new regulations, special cosmetics need to be register and approved by NMPA before production while general cosmetics can be directly put into the market after a notification. However, starting from January 1st, 2022, before registration or notification, the registrant or notifier shall perform a safety evaluation by themselves or by entrusting a professional agency and submit the product safety assessment documents during registration and notification. (9, 26)

2.1.2.4 Ban on Animal Testing

Nowadays the replacement of animal testing in cosmetics by alternative methods is a high priority for the industry and the list of countries with animal testing bans in force is growing, however a lot still needs to be done.

The EU leads this process with a ban on animal testing and marketing for both finished cosmetic products and cosmetic ingredients. The last ban entered into force on March 11, 2013 and extended the prohibition to repeated-dose toxicity, reproductive toxicity and toxicokinetic studies. (37)

In the USA, as of July 2021, seven states (California, Hawaii, Illinois, Maine, Maryland, Nevada and Virginia) have passed laws banning cosmetic animal testing. (38) As for the rest of the country, even though the FD&C Act does not specifically require the use of animals in safety cosmetic studies and while the FDA supports the use of alternative methods for the refinement, reduction and replacement of animal testing, it is the manufacturers responsibility to employ whatever test deem necessary to sustain the safety of their products. (39)

Currently, in Canada, there is no ban for animal testing on cosmetic products. In 2015, the Canada's Bill S-214 (the Cruelty-Free Cosmetic Act) was introduced in view of ending the use of animals for cosmetic testing as well as the sale of cosmetic

products developed using animal testing methods. However, as of today this bill has not yet been passed into law and therefore the use of such methods is still permitted. (40)

In Brazil, some states (Amazonas, Mato Grosso do Sul, Minas Gerais, Pará, Paraná, Pernambuco, Rio de Janeiro, Sao Paulo, Santa Catarina and Federal District) have already banned cosmetic tests on animals. Tests on animals are still recognized by Anvisa guidelines to assess the dangers of cosmetic products and their ingredients. (41)

Japan, is in the process of phasing out animal testing. Currently, there is no law that bans animal testing in products classified as cosmetics, but they are also not required or mandatory. However, for quasi-drugs, which include products classified as cosmetics in the EU, it is still mandatory to undergo animal testing. (42)

China's mandatory animal testing requirement for cosmetics registration has long-been a major obstacle for global trade between regions like the EU and other countries with “cruelty-free” testing policies. However, as many other countries are slowly introducing animal testing bans, China as also started to align its regulation. On May 1, 2021, China officially removed the mandatory animal testing for general cosmetics, whether imported or manufactured in China. However, this comes with some preconditions and exceptions. In particular, one precondition is to provide a GMP certification, issued by the cosmetic regulatory authority of the country or origin. This requirement is difficult to obtain since not many countries issue this kind of GMP certification. (9,42)

Table 5 - Comparison of Some Features of Cosmetic Regulations in the Six Markets (2,7,9,27,31,32,43,44,45,46,47)

		EU	USA	Canada	Japan	China	Brazil
Labelling	**Required Information**	- Name and address of the RP; - Nominal content; - Durability; - Batch Nº; - Product function; - List of ingredients.	- Name and place of business; - Nominal content; - Product identity; - Warnings.	- Name and address of the manufacturer; - Nominal content; - Product Identity; - List of ingredients - Warnings;	- Company name and address; - Durability; - Batch Nº; - Product Identity; - List of ingredients; - Warnings; - Other items specified by the MHLW	- Name and address of registrant/notifier and the production enterprise; - Nominal content; - Durability; - Product executive standard Nº; - Product Identity and special cosmetic registration licence Nº; - List of ingredients; - Warnings; - Application methods; - Other contents	- Address of the manufacturer; - Country of origin; - Nominal content; - Durability; - Batch Nº; - Product identity; - Brand; - List of ingredients - Warnings; - Indication of use;
	Ingredients Nomenclature: INCI	Yes	Yes (With variations)	Yes (With variations)	Yes (Translated to Japanese)	Yes (Chinese version)	Yes (With additional translation to Portuguese)
	Nominal Content: unit system	Metric is mandatory	Non-metric is mandatory (metric may be provided as a supplementary)	Metric is mandatory (non-metric may be provided as a supplementary)	---	---	Metric is mandatory

(To be continued)

		EU	USA	Canada	Japan	China	Brazil
Ingredients Regulation		Based on positive and negative lists: - Prohibited substances - Restricted substances - Colorants - Preservatives - UV filters	- Small list of prohibited and restricted substances. - Colour additives need to be approved by the FDA	List of prohibited and restricted ingredients (Hotlist)	Based on positive and negative lists: - Prohibited substances - Restricted substances - Colorants - Preservatives - UV filters	Based on positive and negative lists: - Prohibited substances - Restricted substances - Colorants - Preservatives - UV filters	Based on positive and negative lists: - Prohibited substances - Restricted substances - Colorants - Preservatives - UV filters
Pre-Market Requirements	**Pre-Market Approval**	Not required	Not required. (except for colour additives)	Not required	Not required	Special Cosmetics: Required General Cosmetics: Not required	Not required. (except for some Grade II products)
	Notification	Mandatory. Via the Cosmetic Product Notification Portal (CPNP)	Voluntary	Mandatory (Cosmetic Notification Form)	Mandatory. (Cosmetic Marketing Notification)	Mandatory	Mandatory. Via the SGAS system
Safety	**Responsible for safety**	Manufacturer	Manufacturer or Distributor	Manufacturer	Manufacturer	Registrant or Notifier	Manufacturer
	Market Surveillance	Yes	Yes	Yes	Yes	Yes	Yes
	Ban on Animal Testing	Yes	Partial	No	No	Partial	Partial

2.2 Consequences of Regulatory Differences Between the Six Markets

The cosmetic industry has grown over the past years and it is now a global sector where international trade plays a major role, contributing with significant benefits. However, the regulatory differences mentioned before, not only restrict innovation and reduce the market's potential growth, but also have implications for the industry, regulatory authorities and consumers.

For the regulatory authorities the main impact is in the need to ensure that the imported products comply with the applicable regulations, which means that each regulator has to carry out its own evaluation and assessment. For example, for an OTC product from the USA to be sold in the EU, the regulator has to check the ingredients - to ensure that they are permitted - and claims - to ensure that it meets the European definition of cosmetic. Additionally, if it contains a new cosmetic ingredient it might have to be assessed in terms of its safety, even though it has already been approved for use in another country. All of these modifications, as well as the need for new packaging, labelling and/or advertisement, increases the price of the products, to compensate the additional cost. Furthermore, the range of product available in the market differs from country to country which means that some consumers don't have access to more recent developed products. (5)

For these reasons, it is essential to find solutions that can lead to the alignment of cosmetics regulatory frameworks, to encourage innovation, enhance the market growth and eliminate restrictions to trade. In view of this, several international organizations have been working in this direction. It is the case of the International Cooperation on Cosmetics Regulation (ICCR), established in 2007, which is a voluntary group of cosmetic regulatory authorities from Brazil, Canada, Chinese Taipei, the European Union, Japan, the Republic of Korea, and the United States who meet on an annual basis to discuss several topics related to cosmetic safety and regulation (e.g., alternatives to animal testing, nanotechnology, microbiological limits, etc.). (49) Other examples include the OECD and ISO which have a key role in the mutual acceptance of testing methods guidelines as well as in the development of international standards on cosmetics. Accordingly, it is very important to keep the

ongoing dialogue and to strengthen and enhance the existing cooperation efforts between the different countries around the world.

3 Dermal Absorption Studies in Cosmetics

In reference to the previous section, *"Overview of Cosmetic Regulatory Frameworks Around the World",* dermal absorption studies present a challenge for cosmetic regulatory harmonization, since there is lack of a mandatory and standardized methodology or protocol that assures the achievement of robust and predictable results. This problem will be discussed in this section as it follows.

3.1 Dermal Absorption of Cosmetic Ingredients

3.1.1 Skin structure and function

The skin is the body's largest organ, accounting for approximately 16% of total body weight. It acts as a barrier to the exterior environment protecting other organs from external influences and preventing systemic exposure to allergens, pollutants, toxic chemicals and other types of organisms. It also plays an essential role on thermoregulation and takes part in immune and sensory functions. On top of that it also prevents loss of water and nutrients, as well as plays a role in metabolic processes. (50,51,52)

Histologically, the skin is divided into two distinct regions: the epidermis and the dermis. The epidermis, which is the outer region, can be divided in various cell layers depending of the anatomical site, but it's mostly composed by stratified squamous keratinized epithelium that undergoes a programmed proliferation and differentiation, resulting in the formation of the stratum corneum (SC). The SC consists of cells that have lost their nucleus and all capacity for metabolic activity. It is also, the outermost superficial epidermal layer in contact with the environment thus acting as the main barrier for permeation. The dermis, which is the inner region, provides flexibility, serves as a barrier to infection, and functions as a water storage organ. It's composed of connective tissue consisting mainly of collagen, elastin and reticular fibbers embedded in an amorphous ground substance. Dispersed in this layer is a network of blood vessels and connecting capillaries that nourish both the cells of the dermis and epidermis, as well as take part in the final stages of the dermal absorption of compounds. Other structures, such as nerves, lymphatics, sweat and sebaceous glands and hair follicles, can also be found in this layer. (50,52)

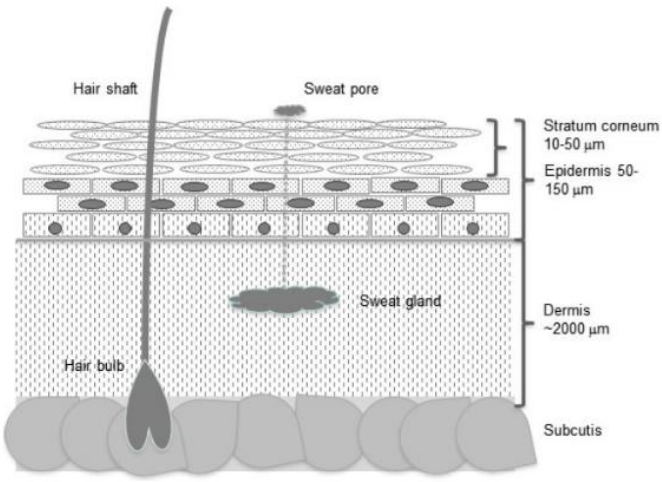


Figure 1 - Schematic Illustration of the Skin Structure. (Adapted from (53))

3.1.2 Skin Cosmetic Delivery

The skin is in constant contact with various substances that, depending on the circumstances, might be beneficial or harmful. Therefore, in some cases, the SC is perceived as an advantage and in others as an obstacle. For instance, a lot of the substances present in the environment are harmful to the skin and can cause irritation, rashes, burns or other health problems. In such cases, this layer has a beneficial purpose, since it acts as a barrier and prevents its penetration into the skin. On the other hand, other substances are deliberately applied on the skin for beneficial purposes, as it is the case of cosmetics. In this context, the SC might be viewed as an obstacle since is preventing the product from reaching the deeper layers of the skin. (54)

3.1.2.1 Dermal Absorption

Over the years, several international bodies have tried to define what is dermal or percutaneous absorption. Although there are some slight differences between those definitions, here is the one proposed by WHO:

"Dermal Absorption is a global term that describes the transport of chemicals from the outer surface of the skin both into the skin and the systemic circulation. This is often divided into:

- *Penetration: which is the entry of a substance into a particular layer of structure, such as the entrance of a compound into the stratum corneum;*
- *Permeation: which is the penetration through one layer into a second layer that is both functionally and structurally different from the first layer;*
- *Resorption: which is the uptake of a substance into the skin lymph and local vascular system and in most cases will lead to entry into the systemic circulation (systemic absorption).”* (51)

3.1.2.2 Skin Permeation Pathways

Understanding how cosmetic ingredients permeate the complex lipid matrix of the SC is the first step to understand cosmetic delivery. In view of this, there are three different pathways in the transport of substances through the skin (Figure 2):

1. *Transcellular (intracellular) pathway*: the substances permeate through the keratinocytes (low lipid content) and the lipid matrix;
2. *The intercellular pathway*: the substances are transported between the corneocytes, in the lipid-rich extracellular regions. The main barrier in this pathway is the SC.
3. *The transappendageal pathway*: the substances are transported through the ducts provided by the hair follicles, sweat glands, sebaceous glands and pores.

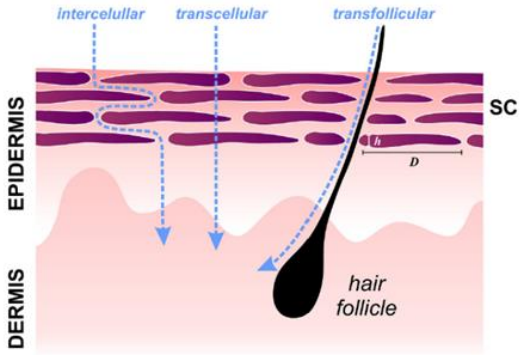


Figure 2 – Skin Permeation Pathways. (Adapted from (55))

Because appendages only comprise of about 0.1% of the total human skin, the transappendageal route is usually consider of little significance for permeation. In contrast, the transcellular pathway is the most direct route, as it allows a straight path through the SC into the lower layers. However, this is a route that offers significant resistance since the substance needs to be partitioned between lipophilic and hydrophilic structures. The most common route is therefore by intercellular passage between the corneocytes. (55,56)

3.1.3 Factors that Influence Dermal Absorption

As opposed to the pharmaceutical field, in which the skin is often the main barrier in drug delivery, in the cosmetic field the skin itself is the target. However, because of the high barrier function of the SC and because the skin is a very heterogeneous and complex organ, there are still a lot of challenges in the successful delivery of cosmetic ingredients to the skin. The degree of penetration and permeation depends not only on the ingredients physicochemical properties, such as molecular weight, solubility, ionization state, etc., but also on the vehicle and its interaction with the skin. Because this is a complex process, all of these factors need to be interpreted as a system instead of individual characteristic, as they are all connected and an alteration on one property can lead to a wide variety of effects. Below are some factors that can influence skin permeation:

Table 6 - Factors that Influence Dermal Absorption

Physiological Factors	Properties of the Substances/Ingredients	Properties and type of vehicle/formulation
Species Age, sex, ethnicity Anatomical site Temperature Blood and lymph flow Hydration Metabolism Integrity	Physical state Molecular weight Lipophilicity Ionization Solubility Partition Coefficient Diffusion	Liquid Dosage Forms Semi-solid Dosage Forms Solid Dosage Forms Nanoformulations

3.1.3.1 Physiological Factors

Skin permeability can be affected by a large number of physiological factors, including age, sex, ethnicity, anatomical site, skin disorders, temperature and hydration of the skin, metabolism and blood and lymph flow.

Sex, Age and Ethnicity: with the increase of age, there are structural changes on the skin that might influence penetration - as for example reduction in sebaceous glands activity, decrease of elasticity and increased of dryness. However, overall, despite differences in sex, age and ethnicity the barrier properties of the skin are reasonably similar. (51,53)

Anatomical Site: there is a considerable variability in penetration between different anatomical sites within and between individuals depending, for example, on the thickness and nature of the SC or on the density of skin appendages. However, there isn't a defined pattern that accounts for all substances. (51,57)

Temperature and Blood Flow: the increase of the skin temperature can affect the blood flow and diffusion within the skin leading to an increase of the rate of penetration. It can also affect the structure of the SC resulting in a higher permeability. (51)

Hydration: when hydrated, the amount of water present in the SC can increase significantly and affect the permeability of the skin, resulting in an increase of absorption. This often happens in occlusive conditions, however, it is important to clarify that this is not always the case, an increase of hydration, due to occlusion, not always increases penetration rates. (51)

Metabolism: even though low, in comparison with the hepatic activity, the skin has metabolic capacity. It contains enzymes that degrade the penetrating substances by increasing their polarity and producing more water-soluble products that are easily eliminated from the body. The purpose of this mechanism is to detoxify potentially reactive chemicals, however, in some cases, the enzymes can also activate molecules to more toxic metabolites, leading to enhance local and/or systemic toxicity. (51,57)

3.1.3.2 Properties of the Substances/Ingredients

As mentioned already, the most common skin permeation pathway is the intercellular route between the corneocytes. As such, the rate of permeation is largely dependent on the physicochemical characteristics of the ingredients - such as physical state, molecular weight, lipophilicity, ionization, solubility, etc. If a substance is intended to penetrate the skin it requires solubility in both lipophilic and more aqueous environments. The reason is that the skin changes from an avascular and lipophilic structure (SC) to a more aqueous layer (lower epidermis and dermis). Therefore, if a substance is highly lipophilic it will easily cross the SC but will have trouble reaching the hydrophilic layers, as a consequence, the substance accumulates in the skin (reservoir effect). The opposite happens for highly hydrophilic compounds. Additionally, although it might not be the limiting factor, smaller molecules with low molecular weight tend to penetrate easier into the skin. The biggest the size the more complex the chemical structure becomes, which leads to changes in the portioning behavior and results in a low penetration rate. (57,58,59)

Another three major variables that influence skin permeation are the concentration of permeant applied, the partition coefficient between the skin and the formulation and the diffusivity of the substance. (60)

Partition is the term applied to the distribution of a substance between two adjacent but different phases at equilibrium. When a cosmetic is applied onto the skin, the SC is the first contact the product has with the skin. In order to be absorbed into that layer, portioning between the SC and the formulation must happen. (56) This process is described by the partition coefficient (Km), which is defined as:

$$\textbf{\textit{Equation 1}}: \qquad K_m = \frac{C_{penetrant} in\ stratum\ corneum}{C_{penetrant}\ in\ formulation}$$

where $C_{penetrant}$ is a measure of the solvated amount of the particles of the penetrating ingredient. In pharmaceutics, the octanol/water partition coefficient ($K_{oct/water}$), typically expressed in logarithmic form ($logK_{oct/water}$), is more commonly used, as it represents

the ratio of concentrations of a compound between two phases, one being octanol and the other water (e.g., creams). (56)

Equation 1 shows that the partitioning of a substance into the SC is enhanced when it has a higher affinity for the SC than for the formulation. Therefore, the most common way, to enhance the portioning of a substance, is to decrease its solubility in the formulation. (56)

Partition is also influenced by diffusion and so both factors are important in determining skin permeation. Diffusion is the random movement of molecules in a solution in the presence of a concentration gradient. (61) The diffusive flux is related to the diffusion coefficient (D) - a constant related to the inherent properties of a given molecule in a given formulation - and can be described by Fick's First Law of Diffusion:

$$\textbf{Equation 2}: \qquad J = -D\frac{\partial C}{\partial x}$$

where J is the flux of the permeant (mass per cm^2), D is the diffusion coefficient and ∂C and ∂x is the differential concentration and distance, respectively. (60)

Fick's first law relates the diffusive molecular flux to the concentration. It says that the flux goes from regions of high concentrations to regions of low concentrations with a magnitude that is proportional to the concentration gradient. However, diffusion causes the concentration to change with time, therefore Fick's second law can be used to predict this process:

$$\textbf{Equation 3}: \qquad Fick's\ Second\ Law\ of\ Diffusion = \frac{\partial c}{\partial t} = D\frac{\delta^2 c}{\delta x^2}$$

3.1.3.3 Properties and Type of Vehicle/Formulation

One of the most important factors for a successful cosmetic delivery system relies on the design of an appropriate formulation, since the vehicle can not only alter the skin barrier properties but also deeply influence the release mechanism, which in turn can affect the extent and rate of penetration of the ingredients into the skin.

Accordingly, topical formulations can be differentiated into two general categories, those applied for local action and those for systemic effects - transdermal formulations. Topical formulations include those designed to be delivered to the epidermis and/or dermis and those designed to remain on the skin surface. Cosmetics include a wide range of products, by which the most conventional are topical dosage forms that "*contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs)".* (2) They can be classified into three categories, according to the physical state: liquid dosage forms (e.g., solutions, suspensions, lotions), semi-solid dosage forms (e.g., ointments, creams, pastes, gels) and solid dosage forms (e.g., powders).

3.1.3.3.1 Liquid Dosage Forms

Topical Solutions: topical solutions are liquid preparations composed of one or more solutes dissolved in an aqueous, nonaqueous or hydroalcoholic solvent intended for topical application. (62)

Suspensions: suspensions are two-phase systems consisting of a finely, insoluble substance dispersed in a liquid in a concentration up to 20%. They are liquid heterogenous preparations that typically contain an aqueous or alcoholic vehicle. Because the suspended particles have a tendency to coalesce and separate, shaking the preparation before application may be required. (63,64)

Lotions: the term "lotion" and "suspension" are often associated. Lotions are liquid and somewhat viscous preparations in which a finely divided insoluble substance is suspended or dispersed. They generally contain an aqueous vehicle and >50% water and volatiles. These formulations have occlusive agents that retard the water loss by restoring moisture in the surface of the skin. (62,64)

3.1.3.3.2 Semi-Solid Dosage Forms

Ointments: ointments are semi-solid, homogenous and viscous preparations. They are composed by a hydrophilic petrolatum-based vehicle, capable of providing occlusion, hydration and lubrification, making them effective at enhancing permeability. (63,64) The vehicle of an ointment is known as the ointment base and they can be classified into four categories: i) hydrocarbon bases, ii) absorption bases, iii) water-removable bases and iv) water-soluble bases.

i. *Hydrocarbon Bases:* hydrocarbon bases, also called oleaginous bases, are composed of a mixture of hydrocarbons of varying molecular weights and are often referred to as emollients, because of their occlusive properties. They are generally stable and do not contain preservatives. Additionally, because they are composed by lipophilic materials, they cannot absorb water, and thus water-soluble drugs are difficult to incorporate into these bases. Examples include white petrolatum and white ointment. (63,65)

ii. *Absorption Bases:* absorption bases can be classified into two subgroups: anhydrous absorption bases and water-in-oil emulsions:

- Anhydrous absorption bases do not contain water. They are composed by hydrophilic substances which allows the absorption of water-soluble ingredients. They are lubricating, hydrophilic and function well as emollients and protectants. Examples include hydrophilic petrolatum and anhydrous lanolin. (63,66)
- Water-in-Oil bases are commonly referred to as creams and are composed of a system in which water is dispersed in an oil continuous phase. They have moderately good protective, occlusive and emollient properties, being effective at preventing water loss. (67)

iii. *Water-Removable Bases (Oil-in-Water Bases):* water-removable bases are also commonly referred to as creams. They are oil-in-water (o/w) emulsions – composed of oil droplets dispersed in an aqueous phase – which makes them less emollient and less occlusive than hydrocarbon and absorption bases. They are also easily washed from the skin. (67)

iv. *Water-Soluble Bases:* most of water-soluble bases consist primarily or completely of PEGs (Polyethylene Glycols). These formulations are water soluble and do not require preservatives. Additionally, because they do not contain water, they have a low percutaneous absorption, making it useful for scenarios desired to have a high surface concentration and low absorption of the ingredients. Gels are made from these types of bases. (62,67)

Creams: creams are semisolid emulsion formulations of water-in-oil (w/o) or oil-in-water (o/w) bases, with an active agent dispersed between the oil and water phases. They have moistening and emollient properties and, in comparison with ointments, are less greasy, viscous and more spreadable, but also less hydrating due to their less occlusive effect. (64)

Pastes: pastes are semisolid preparations containing a high concentration of a finely, insoluble dispersed solid (up to 50%) into an ointment such as a hydrocarbon base or a water-in-oil emulsion. There are two types of pastes: non-greasy pastes, made from a single-phase aqueous gel, and fatty pastes, made from a thick ointment. These formulations are good protective barriers duo to their water-impermeable function. (62,64,66)

Gels: gels are semisolid preparations that consist of a solution or dispersion and a gelling agent to provide stiffness. (64) They can be divided into single-phase systems - which contain soluble, organic macromolecules distributed in a liquid - and two-phase systems - that consist of a suspension made of small inorganic and insoluble particles. In the latter, if the dispersed particles are relatively large, the gel might be referred to as magma. (62,65)

3.1.3.3.3 Solid Dosage Forms

Powders: powders are solid or mixtures of solids in a dry, finely divided state. (62) They absorb moisture and decrease friction and are mainly used for cosmetic and hygienic purposes since they adhere poorly to the skin. (64)

3.1.3.3.4 Nanoformulations and Nanomaterials

With the globalization of cosmetics, the demand for innovative and personalized products increased and led to the emergence of new technologies, particularly nanotechnology. Nanotechnology has been in use in the cosmetic industry for more than 30 years as the sector was among the first industries to consider nanotechnology-based products. Hence, nowadays the cosmetic industry is a global leader in the incorporation of these technologies in the development of new products. These products can be developed in the form of a delivery system, in order to encapsulate active ingredients (nanoformulations), or by the introduction of nanomaterials as an active ingredient or as a carrier in the final formulation. (68,69)

Nanomaterials are made from a variety of chemical compounds and have been used in the cosmetic industry for a long time as they can be applied to a large range of cosmetic categories (e.g., skin care, hair care, cleansers, make up and toothpastes). However, there is still no ambiguous global definition for nanomaterials as cosmetic ingredients, meaning that each country follows its own definition and legislation. (68,69) In the EU, according with Regulation (EC) 1223/2009, a nanomaterial is defined as "an insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100nm". (2) Additionally, as with any other material, nanomaterials can present some unwanted risks to the consumers health and also the environment. The main concern for a potential health risk is related to the route of exposure, as there as still uncertainties regarding the penetration of nanomaterials through the skin into viable layers, where toxicological concerns may arise. Moreover, there is a possibility of oral exposure (e.g., toothpastes) or inhalation (e.g., sprays and aerosols) of these nanoparticles. Therefore, there's a need for a standardized safety evaluation for nanomaterials skin formulations, including long-term toxicological studies. (68,69)

Similar to nanomaterials, the use of nanoformulations or nanosystems by the cosmetic industry aims for the improvement of intrinsic properties of cosmetic ingredients such as color (e.g., in lipsticks and nail polishes), transparency (e.g., in sunscreens), stability and long-lasting effects (e.g., in makeup) as well as for a more efficient penetration of ingredients into the skin. (68) Skin permeation can be enhanced through a sustained release, increased physicochemical stability, reduced irritability,

improved textural quality and improved dispersion/spreading properties of the active cosmetic ingredients. However, it should be noted that some nanosystems claim to benefit the epidermis (e.g., nourishment and hydration) while others claim to penetrate into the dermis (e.g., repairment of damaged cells). This should be taken into consideration, since, according with the definition of cosmetic product in the EU, cosmetic ingredients should only penetrate to the epidermis, without reaching the dermis or systemic circulation. Additionally, some nanosystems may be incompatible with other nanosystems or ingredients which can result, for example, in an alteration of the mechanism of action of surfactants/emulsifiers and gelling agents, leading to a decrease of stability of the final formulation. (69)

According to their chemical composition, nanosystems used in cosmetic products fall within the following categories: i) lipid nanosystems, ii) polymeric nanoparticles, iii) inorganic nanoparticles, and iv) carbon-based nanoparticles. (Table 7)

Table 7 - Most commonly used nanosystems in cosmetics. (68,69,70,71,72,73)

Type			Features	Products examples	Properties/Advantages
Lipid Nanosystems	**Lipid nanoparticles: SLN, NLC**		Solid (SLN) or solid/liquid (NLC) matrix-based nanoparticles stabilized with surfactants	Sunscreens and creams	Occlusive properties (skin hydration); Stability; UV resistant properties; Close contact with the SC increases penetration
	Vesicles	**Liposomes**	Vesicular structures with an aqueous core which is enclosed by a hydrophobic lipid bilayer composed of natural or synthetic phospholipids which are GRAS (generally regarded as safe) products.	Skin care and hair care products	Suitable for delivery of both hydrophobic and hydrophilic compounds; Biocompatible and biodegradable; Ease of penetration in dermal layer
		Niosomes	Bilayer structure vesicles made up of hydrated nonionic surfactants, with or without incorporation of cholesterol or their lipids	Antiwrinkle creams, skin whitening and moisturizing cream, hair repairing shampoo	Suitable for delivery of both hydrophobic and hydrophilic compounds; Stability; Increase dermal penetration
	Nanoemulsions		Oil-in-water (O/W) or water-in-oil (W/O) colloidal dispersions, which contain nanoscale droplets	Deodorants, sunscreens, shampoos, lotions, nail enamels, conditioners, and hair serums	Suitable for delivery of both hydrophobic and hydrophilic compound; Skin Occlusion; Increase rate of absorption
	Cubosomes		Amphiphilic lipid-based 3D honeycomb-like structures	Skincare, hair care, antiperspirants	Ability to encapsulate hydrophilic; hydrophobic, and amphiphilic substances; Controlled and targeted release; Stable
Polymeric Nanoparticles	**Nanospheres**		Spherical particles which exhibit a core-shell structure	Skin care products (e.g, antiwrinkle creams, moisturizing and antiacne creams)	Controlled release; Easy penetration in cells and tissues
	Nanocapsules		Polymeric capsules that are surrounded by an oily or water phase	Deodorants	Protection of ingredients; Decreasing of chemical odours; Resolution of incompatibilities between formulation components

(To be continued)

	Type	Features	Products examples	Properties/Advantages
	Dendrimers	Spherical structure composed of a core from which symmetric units are built	Shampoos and deodorants, sunscreen	Monodispersity; Polyvalence; Stability
Inorganic Nanoparticles	**Metal: Ag, Au**		Ag: antidandruff shampoos, antiacne creams Au: skin wound disinfectants, anti-aging creams	Antimicrobial (Ag); Antioxidant (Au); Nanopigments
	Metal Oxides: ZnO, TiO_2		Sunscreens	Used in sunscreens for: transparent properties; high sun protection factor (SPF)
	Nano-Hydroxyapatite	Nano particles of hydroxyapatite which is the main mineral component of vertebrate bone and tooth.	Oral care like toothpastes and mouthwashes	Teeth remineralization and desensitization properties
	Silica (SiO2)	Stabilized nanodispersions with a range size of 5 to 100nm	Products for hair, skin, lips, face and nails	Suitable for delivery of both hydrophobic and hydrophilic compounds; Enhances shelf-life; Improves appearance and distribution of pigments (e.g., lipsticks)
Carbon-based nanoparticles	**Fullerenes**	C70, C76, C84, C90 e C36 but mainly C60 buckyballs	Skin care: anti-age and skin repair products	Antioxidants properties; Antimicrobial properties
	Carbon nanotubes	Seamless cylindrical hollow fibers, comprised of walls formed by graphene as hexagonal lattice of carbon, which are rolled at specific and discrete “chiral” angles are rolled at specific and discrete “chiral” angles	Hair colouring	High compound payload; Prolonged release

3.1.4 Permeation Enhancement

One of the main challenges in cosmetic delivery is to design a formulation in which the cosmetic ingredients can be successfully delivered to the epidermis. In order to overcome this limitation, over the years, research has been carried out in this area and a number of techniques to enhance the delivery of ingredients has been developed. These methods can be mainly divided into chemical and physical approaches. Physical enhancement techniques involve the use of external energy to reduce the barrier function of the SC, thereby enhancing de permeability of the compounds. Examples include iontophoresis, electroporation, sonophoresis, microneedles, magnetophoresis, photomechanical waves, etc. (74) On the other hand, chemical approaches aim to modify the SC through an optimization of the formulation. All of these methods can be used separately or in combination as they all present their own advantages and disadvantages. The principles of various methods under the chemical approaches are briefly discusses as follows.

3.1.4.1 Chemical Enhancement Approaches

Chemical enhancers are pharmacologically inactive compounds like water, alcohols, glycols, surfactants, fatty acids, urea, sulfoxides, etc. that can temporarily modify the SC, by disturbing the packing orders of lipids. A simple explanation to how chemical enhancers affect skin permeability is the Lipid-Protein-Partitioning (LPP) theory by Barry.B.W. The LPP theory assumes the chemical enhancers affect permeation via one or more of three main mechanisms: i) disruption of the intercellular lipid domains, ii) interaction with the intracellular protein domains and iii) increasing the partitioning of an ingredient and/or fluidize the lipid chains, creating a more permeable domain. (74,75)

3.1.4.1.1 Water/Hydration

Water is the safest and one of the most common used penetration enhancers in cosmetic formulations. An increase in water can have two effects: i) it can alter the solubility of an ingredient in the formulation, thereby modifying the partition coefficient, (56) or ii) since water has a polar nature, it can interact with the polar head

groups of the lipid bilayer and disrupt de lipid packing which increases the fluidity of the barrier leading to a higher permeability of both hydrophilic and lipophilic compounds. (76)

3.1.4.1.2 Fatty Acids

Fatty acids consist of an aliphatic hydrocarbon chain along with a terminal carboxylic acid group and are often used as permeation enhancers. They present several benefits such as a non-irritational effect on the skin, no-toxicity, a wide range of compatibility and a very high skin flux. (77) One of the most common used fatty acids is the oleic acid. This acid acts though the fluidization or delipidization of the SC, which affects the permeability of the pores therefore reducing the resistance towards the permeation of polar molecules. Other examples include lauric acid, myristic acid and capric acid. (76)

3.1.4.1.3 Alcohols

Different type of alcohols can enhance skin permeation through a variety of mechanisms, however one of the most used in cosmetic formulations is ethanol. Ethanol is usually good to increase the permeation of hydrophilic compounds as it modifies the polar head regions of the lipid bilayer and increases the fluidity of the intercellular lipid matrix. (76)

3.1.4.1.4 Terpenes

Terpenes are natural compounds and consists of a number of repeated isoprene (C_5H_8). They can present a high enhancement effect and are generally regarded to be safer than other synthetic permeation enhancers, reason why they are receiving attention on cosmetic formulations. Examples include menthol, limonene, camphor, etc. (78) Additionally, they can improve permeation of both lipophilic and hydrophilic compounds. In the case of hydrophilic compounds, they can enhance permeation through the disruption of the lipid structure of the SC and the increase of the ingredient's diffusivity. For lipophilic compounds, in addition to increasing the ingredient's

diffusivity they can also increase the partitioning of the ingredients into the SC. Moreover, the higher solubility of a lipophilic substance in the enhancer also results in its higher permeability to the SC. (79)

3.1.4.1.5 Surfactants

Surfactants are amphiphilic molecules composed of a lipophilic allylic chain (their tail) connected to a hydrophilic head, which means they contain both a water-insoluble and a water-soluble component. Surfactants are usually used as solubilizers - as they have the potential to solubilize lipids within the SC and as permeation enhancers – by disrupting the packing of membrane lipids, forming structural defects that reduce the membrane integrity. However, the effect on skin permeation depends on the concentration and type of surfactant – anionic, cationic, nonionic or amphoteric surfactant. (76,79)

3.1.4.1.6 Urea

Urea acts as a permeation enhancer by facilitating hydration of the SC and forming hydrophilic diffusion channels within the barrier. Cyclic urea is commonly used as an enhancer as it is biodegradable and nontoxic. It consists of a polar parent moiety and long chain alkyl ester group acting through both hydrophilic activity and lipid disruption mechanisms. (77,80)

3.1.4.1.7 Formulation enhancement

Other approaches to increase permeation rely on the chemical modification of the poorly penetrating ingredients. Examples include:

Eutectic Systems: eutectic systems consist of a mixture of two components in a certain ratio. The combination originates a new compound with a lower melting point than each of the constituents. This has a direct effect on its solubility through the skin, thus increasing skin permeability. (56,74)

Ions Pairs: ions pairs are formed by electrostatic attraction between ions with opposite charges. This leads to the formation of a neutral charged lipophilic molecule that more easily permeates thought the SC than a charged one. When the ion-pair reaches the aqueous microenvironment of the epidermis it dissociates, releasing the parent charged substance that will diffuse into the remaining layers. (74,81)

3.2 Methods to Evaluate Dermal Absorption *in vitro*

The assessment of skin permeation is a critical process in the cosmetic field. However, these studies present a complex challenge for the industry and the existence of a robust and global accepted model is yet to be achieved. The reason behind this is that, although there are many quantitative and qualitative methods available, the different techniques are not fully equivalent and each model has associated advantages and disadvantages, making it difficult to define an ideal model. Moreover, in some cases, even within the same model there are small variations that can affect the final data, as it is the case of Franz diffusion cells, in which the study parameters can differ between experimental procedures. Most of the guidelines and guidance documents for the conduct of these studies were written for a general use and not specific sectors or conditions. This means that the same recommendations can be applied to cosmetic ingredients, pesticides, industrial chemicals or dermal and transdermal drugs.

For a long time, the use of animal models was the main approach in the assessment of skin permeability. However, in the past years, there has been an increasing concern for animals well-being. Therefore, and with some countries having animal testing bans in force, efforts to develop alternative methods without the use of laboratory animals are being made. Nowadays, there are a number of *in vitro* models to access percutaneous absorption such as Franz diffusion cells, tape stripping, Skin Parallel Artificial Membrane Permeation Assay (PAMPA), a variety of skin models, confocal fluorescence microscopy techniques, mass spectrometry imaging technology, Confocal Raman Microspectorscopy (CRM), mathematical models, etc. With the focus being mainly on Franz diffusion cells, since they are the most widely used method globally, a brief overview of some of these models is discussed as follows.

3.2.1 Franz Diffusion Cells

Franz diffusion cells have been one of the most used methodologies to evaluate and study *in vitro* permeation. In this system, the skin (or synthetic membrane) is fixed in between two chambers: the donor compartment, containing the test product, and the receptor compartment, filled with medium. The temperature of the system needs to be regulated, either by a thermostatically controlled water jacket that surrounds the chamber or by a heating block or water bath. (82) In the last case, for a homogeneous temperature distribution, a magnetic stirring bar is placed in the receptor medium. Then, to measure the diffusion of the substance across the membrane, samples of the receptor medium are collected, at predetermined time points, and further analyzed. According with the EMA document "Draft Guideline on Quality and Equivalence of Topical Products", these experiments can be divided into two categories: *in vitro* permeation tests (IVPT) and *in vitro* release tests (IVRT). In permeation studies, biological membranes are used to show the flux profile of the formulation, in order to establish the characteristic permeation profile of the product. On the other hand, release studies use synthetic membranes or skin to evaluate the rate and extent of release of an active substance in the proposed formulation. (83)

In this regard, franz diffusion cells are available in a range of shapes and sizes and can be divided into two categories: static cells and flow-through cells. The objective of the study should be taken into consideration when choosing the design.

Static cells: static cells can be further divided into vertical or horizontal (side by side) cells, nevertheless both have a very simple design. As mentioned before, they consist of a donor and receptor chamber separated by a skin sample, with the stratum corneum facing the first. The receptor fluid is kept homogenous in concentration and in temperature by a magnetic stirring bar. (53,57)

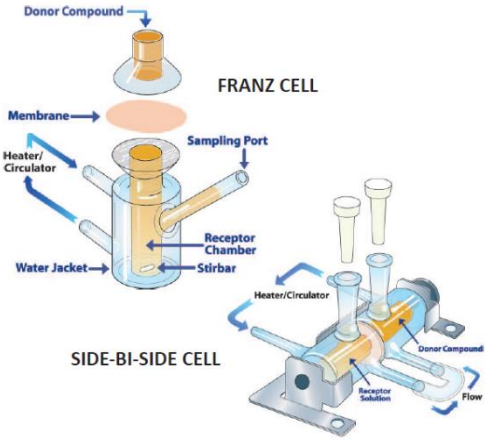


Figure 3 - Illustration of a static diffusion cell. (Adapted from (84))

Flow-through cells: as opposed to the static cell system, consisting of only two compartments, the flow-through system consists of multiple cells in which the receptor fluid is continuously replaced. This allows to mimic *in vivo* conditions as the continuous flow in the receptor chamber acts as the blood circulation in the skin and removes the substance once is has permeated the skin. (53,57)

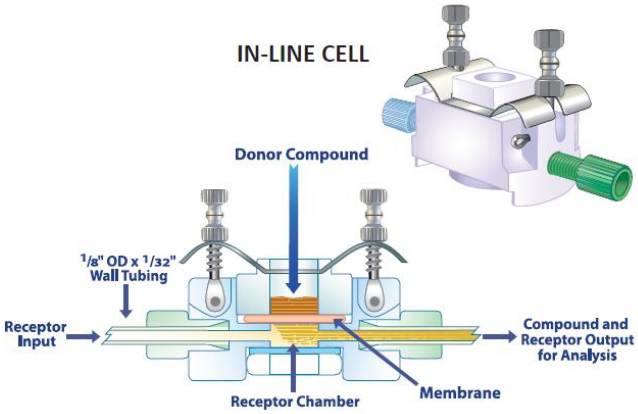


Figure 4 - Illustration of a flow-through diffusion cell. (Adapted from (84))

In a general manner, static diffusion cells are more advantageous in comparison to the flow-through system. To begin with, they have a much simpler design with no technical features, which eliminates many of the technical problems that can occur. They also have a lower cost and a larger area of absorption which makes the absorption indicator and mass balance assessment better. However, the flow-through system has some advantages of its own, the most important one being that it provides an environment similar to the real physiological conditions of the systemic network. Another benefit regards the sink capacity, which is influenced by the solubility of the substance and is therefore of great importance when it comes to choosing the right sampling frequency and receptor chamber dimension. Accordingly, flow-through cells can be of advantage, specifically in long-term studies, since the continuous replacing of the fluid maximizes the sink conditions. As opposed to the static-cell design where the concentration of the substance in the receptor chamber slowly increases to a certain degree of saturation. (51, 53,57)

3.2.1.1 Test Conditions

3.2.1.1.1 Receptor Chamber

An ideal receptor medium, for an *in vitro* permeability test, should mimic the *in vivo* situation and maintain a physiological environment. It's very important to select the solution of the receptor chamber according with the nature of the test product and the type of diffusion cell used, so it does not limit the extent of penetration of the compound. Therefore, for hydrophilic and moderately lipophilic compounds its common to use aqueous receptors, usually phosphate buffer saline (PBS, pH7.4) if the compound is ionizable, if not, a solubilizer should de added. In the case of lipophilic compounds or compounds with low aqueous solubility an additional solubilizing agent such as surfactants, protein (bovine serum albumin) or organic solvents (ethanol/water systems) should be added. However, their effect on the compound penetration needs to be considered as they may cause damage to the barrier and alter the permeability results. (84) Another element that should be taken into consideration is the use of preservatives, which in long duration experiments may be necessary to prevent microbial growth. These substances must not interfere with the barriers function of the skin nor the assessment of the compound under study. (82)

3.2.1.1.2 Dose

Two dose regimens are commonly used is this type of studies, finite and infinite dosing. They are useful in different situations so the choice depends upon the aim of the study. For infinite doses, throughout the experiment, there is no change in the permeant concentration within the formulation of the donor phase. This is achieved through a regular replenish of the donor solution or by applying a large amount of the formulation to prevent any significant reduction of the donor phase during the course of the experiment. Alternatively, for finite doses, the amount and concentration of the formulation is smaller, which means the permeant concentration in the formulation changes during the experiment, duo to its penetration, permeation and evaporation. These conditions are more representative of the "in-use" scenario. (85)

The criteria for the dosage of formulations varies between guidelines and guidance documents, reason why is unclear which is the most appropriate amount for which situations and in which conditions. This is a problem, since the amount of formulation used can affect dermal absorption and, therefore, the study outcomes.

3.2.1.1.3 Membranes

One of the main variants in the design of *in vitro* skin permeation studies is the nature of the skin membrane. The golden standard and most reliable model would be human skin (obtained, for example, from autopsies or cosmetic surgery), however, it can be expensive and difficult to obtain. Another option, is the use of animal tissue from porcine, primates, rodents, rabbits, etc., as they have a lower cost and are easy to obtain. Nonetheless, both options are associated with several difficulties and limitations, such as the complex nature of the tissue and preparation process and the inter- and intra-variability of the skin. Moreover, they can also present ethical and legal issues, especially in Europe with the ban of animal testing on the cosmetic field. Therefore, in an attempt to overcome these limitations, artificial and reconstructed skin models appeared as an alternative and substitute.

The artificial skin models range from simple polymeric materials, like silicone membranes (e.g., Silatos™, Silastic™ and Start-M®), to more complex lipid-based models, such as the parallel artificial membrane-permeability assay (PAMPA). The

PAMPA is composed by an artificial liquid membrane that separates the donor compartment, filled with a buffer solution, from the receptor compartment, that contains the product, in 96-well plates. (86)

Moreover, in the past years, several reconstructed skin models have been developed. They are cultured-based, composed by layers of human cells in culture laid down over a polymeric matrix, and can be further divided into reconstructed human epidermis models (RHE) and living skin equivalent models (LSE). RHEs simulate the epidermis and are developed by proliferation of normal human keratinocytes into the multilayer epidermis (e.g., EpiSkin®, SkinEthic®, EpiDerm®) while LSEs simulate the full human skin, comprising both the epidermal and dermal layers, (e.g., GraftSkin®, EpiDermFT®, Pheninon®). Some of these models have been suggested as suitable candidates for *in vivo* and *ex vivo* skin models in the evaluation of skin penetration of cosmetic products, however, none is currently approved by the OECD. (86,87)

3.2.1.1.4 Temperature

The absorption process is highly influenced by the temperature. Therefore, the temperature of the receptor phase must be controlled either by immersing the cells in a water bath, heating block or by using jacketed cells perfused with water at the selected temperature. Normally, the experiments are conducted with a skin temperature between 35˚- 37˚C.

3.2.2 Tape Stripping

Tape stripping is one of the most traditional used methods in *in vivo* skin penetration studies, but can also be used *in vitro*. It is an inexpensive and minimally invasive method that consists of a repeated application and removal of adhesive tape to the same skin site, in which a topically applied test substance (often radioactively labelled) has been applied. The aim is to remove microscopic layers of the SC to later be analyzed using an appropriate analytical method. This allows to determine the absorption profile and potential temporary deposit of the substance within the upper SC. (82,88)

3.2.3 Confocal Raman Microspectorscopy (CRM)

CRM is a technique that has increasingly being used to determine penetration profiles in the skin due to its potential for non-invasive studies with skin samples *in vivo* and *in vitro* and its minimal need for sample preparation. CRM results from the combination of confocal microscopy with Raman spectroscopy and it is based on discovering the characteristic vibrational energy levels of a molecule excited by a laser ray. This can give information about the molecular structure of tissue components without the use of fluorescent labels or chemical stains. (88,89)

3.2.4 Confocal Fluorescence Microscopy Techniques

Two major confocal fluorescence microscopy techniques that have been used in skin related experiments are Confocal Laser Scanning Microscopy (CLSM) and Two-photon excitation fluorescence microscopy (TPEFM). Both methods are fully non-invasive and can provide detailed information about the layers studied. (53)

- *Confocal Laser Scanning Microscopy (CLSM):* is a non-invasive method developed from fluorescence microscopy. It can be used both *in vivo* and *in vitro* and it consists on the visualization of fluorescent model compounds in the skin which allows to identify the penetration profiles of these fluorescent markers across the skin or skin appendages. The images are obtained at a higher resolution with depth selectivity compared to conventional optical microscopy or fluorescence microscopy. (90) This method is specially used to study the mechanism and transport of nanoparticles and nanoformulations into the skin. (88)

- *Two-photon Excitation Fluorescence Microscopy (TPEFM):* this technique represents a relatively young technology as it involves the simultaneous absorption of two infrared photons whose combined energy promotes a molecule to an excited electronic state 2PEF. The imaging of this method is particularly suitable for studying deep tissue, as it achieves cellular-level visualization of exogenous fluorescent molecules. (91)

3.2.5 Mathematical Models

A number of mathematical models have been proposed for predicting skin permeability however one of the most used is the quantitative structure-activity relationship (QSAR) model. This is model reveals the relationship between biological activities and the structural properties of the chemical compound. It statistically combines structural or physicochemical parameters of a substance (e.g., molecular weight, atomic charge number, van der Waals force, electron density) with percutaneous permeability to quantitatively predict the percutaneous absorption of a compound based on its structure. (56, 92)

3.3 General Guidelines for Dermal Absorption Studies

In the last 20 years, due to the growing interest in the study of dermal absorption, a number of guidance documents on the conduct and interpretation of these studies have been published. Some international organizations such as The Organization for Economic Cooperation and Development (OECD) and The World Health Organization Programme on Chemical Safety (WHO/IPCS) have issued several documents to encourage harmonization of methodology. For example, the OECD Guidance Notes on Dermal Absorption (No.156), the OECD Test Guidelines 427 and 428, the OECD Guidance Document for the Conduct of Skin Absorption Studies and the WHO/IPCS Environmental Health Criteria 235. Furthermore, there are some other documents that present opinions and descriptions on how to perform dermal adsorption assays, like the United States Environmental Protection Agency (USEPA) report on dermal exposure assessment - from the USA - and the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Monograph 20, the European Food Safety Agency (EFSA) Guidance on Dermal Absorption for plant protection products, and the Scientific Committee on Consumer Safety (SCCS) Basic Criteria for *in vitro* assessment of the dermal absorption of cosmetics ingredients - from Europe.

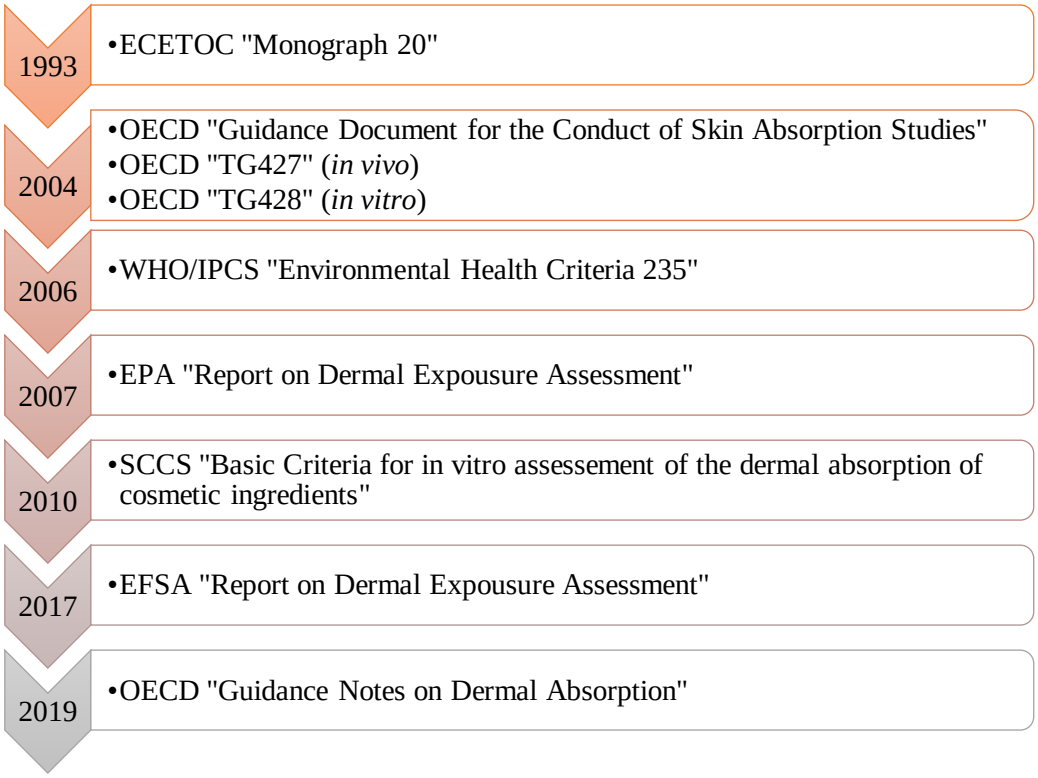


Figure 5 – Guidance Documents for the study of dermal absorption

Annex I displays a comparison table of a few parameters and requirements specified in some of the most relevant test documents and guidelines to evaluate dermal absorption *in vitro.*

3.4 *In vitro* Release and Permeation Studies

In order to study and assess the influence of some parameters, such as the amount of cosmetic product used, an *in vitro* release and permeation study was carried out using a hyaluronic acid (HA) serum as a model formulation.

3.4.1 Materials

The L'Oréal Paris Revitalift Anti-Wrinkle Filler Serum were kindly provided by LÓréal Portugal (batch: TGS70E). Rodhamine B (purity degree ≥ 95%) and Phosphate buffer saline (PBS) tablets 7.4 were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Purified water was obtained by reverse osmosis and electrodeionization (Millipore).

3.4.2 Methods

Both the IVRT and IVPT were performed using vertical Franz diffusion cells. Because it was not possible to quantify the HA in the samples, a fluorescent probe based on rhodamine B was use for its assay. For both the release and permeation studies, Rhodamine B (model molecule) was incorporated in the serum at the recommended concentration of 125mg/g, and the serum was kept in the dark at 25°C. Three amounts of the same formulation were tested – 0.1g, 0.3g and 1g - to represent the insufficient (F0.1), "standard" (F0.3) and excessive (F1.0) quantity of application, respectively. Six replicated cells were performed in the release study and three in the permeation study.

3.4.2.1 *In Vitro* Release Test (IVRT)

The *in vitro* skin release test (IVRT) was conducted using a static vertical Franz diffusion cell with a diffusion area of 1 cm^2 and a receptor compartment of 4mL. A Tuffryn® membrane was placed between the donor and receptor chambers after being submersed into phosphate buffer solution (PBS) for 30 min. The receptor medium, PBS solution, was appropriately screened based on Rhodamine B solubility studies to ensure the sink conditions during the experiment (results not shown). The formulation samples, previously prepared, were applied on the membrane surface, in the donor compartment,

and sealed with Parafilm® to prevent water evaporation and maintain occlusive conditions. The release media was continuously stirred with a magnetic bar (200 rpm) and maintained at 35 ± 2ºC by a thermostatic water pump, assuring a temperature of 32ºC at the membrane surface (to mimic skin conditions). Samples of 200µl were collected from the receptor fluid at pre-determined time points: 0.15, 0.30, 0.45, 1, 1.30, 2, 3, 5 e 6 hours and replaced with an equivalent amount of receptor medium.

The absorbances of the samples were measured in a Fluostar Omega microplate reader (BMG Labetch, Ortenberg, Germany) at 580 nm (maximum emission wavelength of Rhodamine B). The percentage of Rhodamine B released into the medium was calculated using the following equation:

$$\textbf{\textit{Equation 4}}: \qquad \text{Cumulative release percentage} = \sum_{t=0}^{t} \frac{M_t}{M_0} \times 100$$

where Mt is the cumulative amount of Rhodamine B released at each sampling time point, t is time, and M_0 is the initial weight of the Rhodamine B in the formulations.

The release studies were performed six times, and mean values of cumulative release (%) were plotted against time.

The data obtained from IVRT was computed using DDSolver, an Excel-plugin module. Taking the formulation in consideration, three kinetic models were selected and evaluated for data fitting. The selected models are displayed in Table 8 in which *F* is the fraction of Rhodamine B release at the time (*t*). The selection of the most fitting model was made through comparison of three parameters – the adjusted criteria of determination ($R^2_{adjusted}$), the Akaike Information Criterion (AIC) and the Model Selection Criterion (MSC).

Table 8 – Models selected for fitting Rhodamine B release data on DDSolver

Model	Equation	Parameters
Higuchi	$F = k_H \times t^{0.5}$	(K_H) Higuchi release constant
Korsmeyer-Peppas	$F = k_{kp} \times t^n$	(K_{kp}) Release constant (n) Diffusional exponent indicating the ingredient release mechanism
Weibull	$F = 100 \times \left[1 - e^{-\frac{(t-T_i)^\beta}{\alpha}}\right]$	(α) Scale parameter which defines the time scale of the process (β) Shape parameter which characterizes the curve (T_i) Location parameter which represents the lag time before the onset of the dissolution or release process

3.4.2.2 *In vitro* Permeation Test (IVPT)

The experimental setup for the *in vitro* skin permeation test (IVPT) was the same as described in the *in vitro* release study (section 3.4.2.1) with the difference that the membrane used was a Strat-M® membrane and the time points of sample collection were 1, 2, 3, 4, 6, 8 and 24 hours. The Rhodamine B quantification was performed using the same method described in section 3.4.2.1. The cumulative amount of permeated Rhodamine B (Qt) through Strat-M® membranes was plotted as function of time and determined based on the following equation:

$$\textbf{Equation 5}: \qquad Qt = \frac{Vr \times Ct + \sum_{t=0}^{t-1} Vs \times Ci}{S}$$

where, Ct is the drug concentration of the receiver solution at each sampling time, Ci is the drug concentration of the sample applied on the donor compartment, and Vr and Vs are the volumes of the receiver solution and the sample, respectively. S represents the skin surface area (1 cm^2).

According to Fick's first law of diffusion, the steady-state flux (Jss, μg/cm^2/h) can be expressed by the following equation:

$$\textbf{Equation 6}: \qquad J_{ss} = DC_0P/h = C_0K_p$$

where D is the diffusion coefficient of the drug in the stratum corneum, C_0 represents the drug concentration in the donor compartment, P is the partition coefficient between the vehicle and the skin, h is the diffusional path length, and K_p stands for the permeability coefficient.

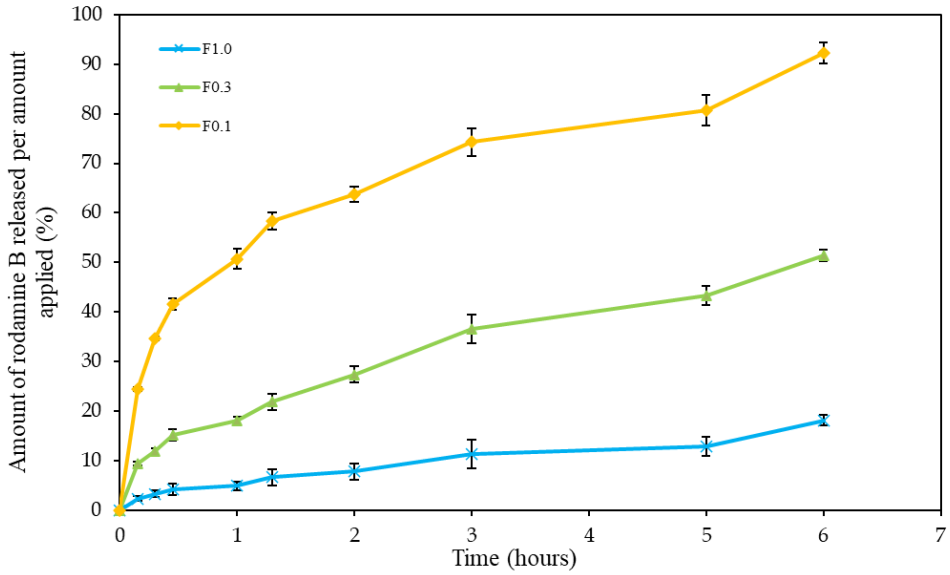
The flux and K_p of the formulations were measured and compared accordingly. The J_{ss} and K_p of the yielded formulations were calculated and compared. The permeation lag time, a parameter related to the required time to achieve the steady-state flux of a molecule through the skin, was also considered for analysis.

3.4.3 Results and Discussion

3.4.3.1 *In vitro* Release Test (IVRT)

The Rhodamine B release profiles from the serum, as a function of time, during 6h, through the Tuffryn® membrane are shown in Figure 6.

(A)



(B)

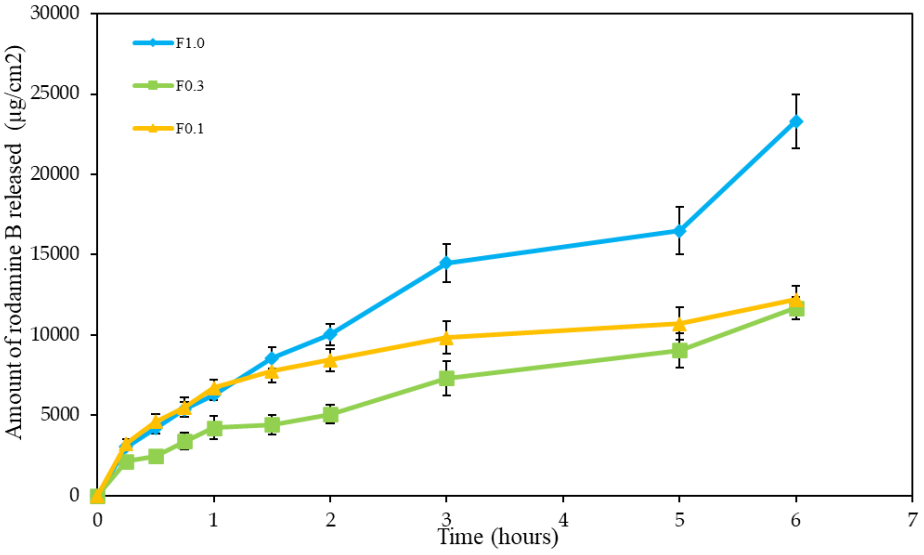


Figure 6 - Release profile of Rhodamine B (mean±SD, n=6). (A) Cumulative amount of Rhodamine B released per amount applied (%). (B) Cumulative Amount of Rhodamine B released (µg/cm^2).

Figure 6A shows that the release profiles of the three formulations are significantly different and that an increase of quantity leads to a decrease of release. Within 6 hours, the released amount of Rodhamine B with the F0.1 (0.1g) was almost complete (92.22±4.86%) whereas with the F0.3 (0.3g) and F1.0 (1g) the release was of 30.62±2.12% and 18.13±1.40%, per amount applied, respectively.

However, the same is not observed in Figure 6B. Some quantities express a higher release percentage than others but express a lower value in terms of released amount. For example, the F0.1 had the highest release (Table 9), but in respect of quantity, the F1.0 released more (23311.91±1686.67 µg/cm^2), while the F0.3 and F0.1 released a similar amount of 11680.75±710.65 µg/cm^2 and 12222.57±818.27 µg/cm^2, respectively (Figure 6B). The similarity factor (f2) was also obtained. The result between 0.1g and 0.3g was f2=18.2%, between 0.1g and 1g was f2=15.1% and between 0.3g and 1g was f2=57.9%, which is in line with the results.

Table 9 – Values obtained from the *in vitro* release studies after 6h (mean±SD, n=6)

Samples	$Release_{6H}$(%)	DE_{6H}(%)	AUC
F0.1	92.22±4.86	0.68	407.05
F0.3	30.62±2.12	0.18	106.36
F1.0	18.13±1.40	0.10	59.56

$Release_{6H}$ is the released amount of Rhodamine-B after 6h; DE_{6H} is the dissolution efficiency after 6h; AUC is the area under the curve.

The kinetics and release mechanisms of the Rhodamine B were studied using three different kinetic models: Higuchi, Korsmeyer-Peppas and Weibull (Table 10). Further, the $R^2_{adjusted}$ and the AIC were estimated for each model and used in the choosing of the best-fitting model. That is, the one that presents the highest $R^2_{adjusted}$ values and the lowest AIC values.

Table 10 – Regression coefficients obtained by fitting Higuchi, Korsmeyer-Peppas and Weibull mathematical models to the release data (mean±SD, n=6)

Models		Samples		
		F0.1	**F0.3**	**F1.0**
Higuchi	K_H	41.6±2.5	11.3±1.2	6.3±0.5
	$R^2_{adjusted}$	0.867±0.04	0.961±0.02	0.952±0.01
	AIC	69.9±2.9	34.7±3.8	27.5±2.8
	MSC	1.4±0.4	2.9±0.5	2.6±0.2
Korsmeyer-Peppas	K_{KP}	51.2±3.0	10.9±1.4	5.6±0.3
	n	0.3±0.0	0.5±0.0	0.6±0.0
	$R^2_{adjusted}$	0.988±0.01	0.958±0.02	0.962±0.01
	AIC	45.9±4.5	36.3±3.5	25.8±2.3
	MSC	3.8±0.6	2.7±0.5	2.8±0.3
Weibull	α	1.3±0.1	11.7±6.0	18.0±1.4
	β	0.53±0.06	0.69±0.19	0.65±0.05
	T_i	0.00±0.00	-0.23±0.38	-0.03±0.03
	R^2	0.983±0.01	0.950±0.02	0.955±0.01
	AIC	50.7±3.3	39.0±2.5	28.2±2.3
	MSC	3.3±0.3	2.5±0.4	2.6±0.3

K is the release rate constant; $R^2_{adjusted}$ is the adjusted criteria of determination; AIC is the Akaike Information Criterion; MSC is the Model Selection Criterion; n is the diffusional exponent; α is the scale parameter; β is the shape parameter; T_i is the location parameter.

The results show that the model with the best fitting values was the Korsmeyer-Peppas model, with the highest $R^2_{adjusted}$ and MSC values between 0.958-0.988 and 2.7-3.8, respectively, as well as the lowest AIC values between 45.9 and 25.8. This model is highly informative because the value *n* (diffusion exponent) indicates the type of mechanism involved (Table 11). There are four possibilities for the value *n*: (i) n=0.5 the formulation follows a Fickian diffusion mechanism, (ii) 0.5<n<1.0 corresponds to a non-Fickian diffusion process, (iii) n=1.0 follows a case-II transport or zero-order kinetic and (iv) n>1.0 corresponds to a super case-II transport.

Accordingly, the results of this study show that the diffusion exponent (*n*) values range between 0.3 and 0.6 (Table 10). The standard and insufficient amounts present a n=0.5 and n=0.3, respectively, which means they undoubtedly follow a Fickian type of diffusion, which occurs by a gradient of chemical potential, that is diffusion. However,

with the insufficient amount the *n* value fits into a non-Fickian diffusion (n=0.6). A non-Fickian diffusion means that the release mechanisms is a combination of diffusion and swelling. However, because the *n* value is very close to 0.5, and since the formulation is the same, it could be presumed that the release is closer to a diffusion-based release.

Table 11 – Release mechanism in agreement with Korsmeyer-Peppas model

Diffusion exponent (n)	Release mechanism
$n \leq 0.5$	Fickian diffusion
$0.5 < n < 1.0$	Anomalous (non-Fickian) diffusion
$n = 1.0$	Case-II transport or zero-order kinetic
$n > 1.0$	Super case-II transport

Considering that the Rhodamine B is released by diffusion, one possible explanation for the inverse relationship between dosage and release percentage might me related to the surface area/volume ratio. In other words, when there is more volume (higher dose), and less surface area, diffusion takes longer.

Accordingly, when comparing the F0.3 and F0.1 amount results, the last had a higher release percentage, but in terms of quantity the results were similar. This might have happened because the F0.3 amount, which has a bigger volume, had an inferior release rate. The F1.0, which had the lowest release percentage, had the highest released amount - the double, in comparison with the other two dosages, which is proportional to the amount of sample.

3.4.3.2 *In vitro* Permeation Test (IVPT)

The Rhodamine B permeation profiles from the serum, as a function of time, during 24h, through the Strat-M® membrane are shown in Figure 7.

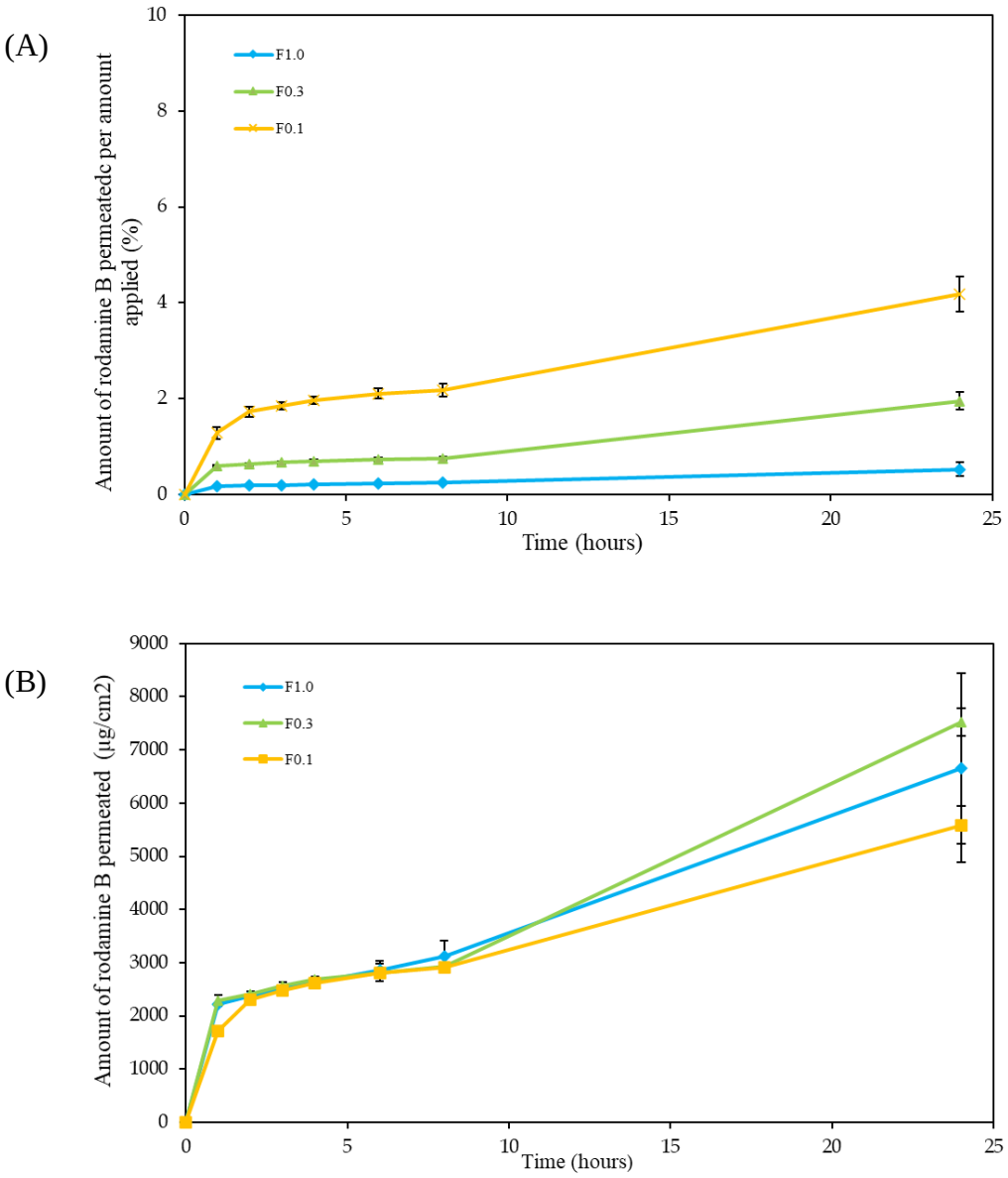


Figure 7 – Permeation profile of Rhodamine B (mean±SD, n=3). (A) Cumulative amount of Rhodamine B per amount applied (%). (B) Cumulative amount of Rhodamine B permeated (µg/cm^2)

The permeation profiles showed on Figure 7A are very similar with the release study, an increase of quantity leads to a decrease of permeation, which can be substantiated by the J_{ss} and K_p values presented on Table 12.

Whitin 24 hours, the permeated amount (%) for F0.1 (0.1g) was 4.18±0.36%, for F0.3 (0.3g) was 1.96±0.18% and for F1.0 (1g) was 0.53±0.14%. In terms of quantity, the results were also similar. F0.1 released 5584.80±354.81 µg/cm^2, F0.3 released 7524.26±258.87 µg/cm^2 and F1.0 released 6661.99±1774.69 µg/cm^2.

This inverse relationship between amount of sample and permeation suggests that applying an excessive amount of product to the skin might have caused saturation of the membrane leading to a decrease of the absorption rate. Additionally, when comparing the results from the IVRT study to the ones from the IVPT study, it can be noted that the percentage of Rhodamine B that permeated the membrane it is very low comparing with the amount that was released. For instance, with 0.1g the release was almost complete (92.22%), but the amount permeated was only 4.18%. This might be explained by the fact that Rhodamine does not permeate well through the Strat-M® membrane (lipophilic membrane) since it is a water-soluble macromolecule.

Therefore, it can be deduced that the main reason why the permeation of Rhodamine B was inferior to the release, was due to the barrier function of the SC, which is a very lipophilic structure and the main barrier to the permeation of most molecules.

Table 12 - Permeation parameters according to experimental permeation data of serums (mean ± SD, n = 3).

Samples	J_{ss} (µg/cm^2/h)	K_p (cm/h)	Q_{8h} (µg/cm^2)	Q_{24h} (µg/cm^2)	Lag time (h)
F0.1	154.8 ± 15.3	0.12 ± 0.01	2910 ± 64	5584 ± 354	12.1 ± 1.4
F0.3	231.4 ± 13.8	0.18 ± 0.01	2922 ± 20	7524 ± 258	7.5 ± 0.9
F1.0.	195.0 ± 80.9	0.16 ± 0.01	3128 ± 279	6662±1775	11.8 ± 8.0

J_{ss} is the flux; K_p is the permeability coefficient; Q_{8h} is the permeated amount of Rhodamine B after 8h; Q_{24h} is the permeated amount of Rhodamine B after 24h

In short, both the IVPT and the IVRT results suggested an inverse relationship between dosage and permeation/release, respectively. The permeation study results between the three doses were similar and the Rhodamine B had a small permeation (0.5-4%), possibly due to the physicochemical properties of the Rhodamine B. Both the studies also had the limitation that only one product and three doses were tested with a short number of repetitions. Additionally, a lot of questions were left unanswered. Therefore, a more thoroughly evaluation would be required, in order to reach more robust conclusions. For instance, retention studies would be valuable to conclude if the Rhodamine B was retained on the membrane, as well as to quantify it. Other studies such as stability studies - to evaluate if certain intrinsic factors (e.g., the formulation and ingredients) can affect and modify the products characteristics - or more analytical methods, to quantify the concentration of Rhodamine B in different skin compartments according to physicochemical properties, would also be of benefit. However, this leads to the conclusion that the standardization and harmonization of the experimental conditions is crucial for the efficacy and safety assessment of cosmetic products.

An interesting approach included on the SCCS "Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation" suggests the calculation of dermal exposure that not only takes into account the product category and the concentration of the substance in the product, but also the amount of product applied and the retention factor. This approach reflects the in-use real conditions of the products and takes into considerations parameters that lack in other guidance documents, such as the superficial area of exposure, which allows a realistic risk assessment and safety evaluation of cosmetic ingredients.

It is clear that dermal absorption is a complex process that can be affected by many variables and parameters, as it can be seen in this study with the variation of only one parameter. Hence, it becomes clear the importance of the standardization and harmonization of protocols. There is an extensive database available with numerous *in vitro* experiments aimed to improve and better understand topical delivery, however, a lot of the data is directed towards drug delivery instead of cosmetic delivery. There is also a lack of studies that evaluate the effect of specific parameters on reproducibility, both individually and in association. All of these, combined with the great amount of variability that these studies present, pose a challenge to method standardization.

Accordingly, further research is needed. The industry needs to carry out more studies that target cosmetic products and explore the different experimental parameters that can affect dermal absorption and the reproducibility of the results.

It would also be important for regulatory authorities to enforce the regulation of these studies. In view of this, it would be interesting to introduce an additional annex to Regulation (EC) 1223/2009 regarding the conduct of permeation studies. This would allow a better control and a more harmonized approach over these studies, which are extremely important to evaluate the safety and efficacy of the product. Another proposal would be to add a permeation report in the PIF of the cosmetic product, for a more complete report on the products safety.

4 Conclusions

The main markets provide regulations and other guidance documents on cosmetic products, with the main goal of ensuring the consumer safety. Additionally, several international organizations such as the OECD, WHO and ISO have also issued several guidance documents, aiming for the global standardization of regulations and protocols related to cosmetic products. However, there are still gaps in the harmonization of experimental methodologies/protocols that hinder this goal, in particular regarding dermal absorption studies. Although there are several methodologies available do assess dermal absorption, such as *in silico*, *in vitro*, *in vivo* and *ex vivo* methods, none represents a robust and standardized model. This is a concern since permeation studies are a critical process in the efficacy and safety assessment of these products. Hence the need for the existence of a robust and global accepted model, that enables the reproducibility of the results.

In this study, an *in vitro* release and permeation study were carried out using Franz diffusion cells. The results obtained in this research work suggested that the amount of product applied affected permeation. This led to the conclusion that small change in an experimental parameter, in this case the dosage, can impact the final results, reinforcing that the standardization and harmonization of the experimental conditions is crucial for the efficacy and safety assessment of cosmetic products. Accordingly, further research is needed to determine the effects and relationships between the different parameters that can affect dermal absorption of cosmetics, in order to develop appropriate methodologies and protocols that can reproduce results that come close to the real in-use conditions.

Bibliography

1. European Commission. Legislation . 2021. Available from: https://ec.europa.eu/growth/sectors/cosmetics/legislation_en
2. Regulation (EC) No 1223/2009
3. U.S. Food & Drug Administration. FDA Authority Over Cosmetics: How Cosmetics Are Not FDA-Approved, but Are FDA-Regulated . 2021. Available from: https://www.fda.gov/cosmetics/cosmetics-laws-regulations/fda-authority-over-cosmetics-how-cosmetics-are-not-fda-approved-are-fda-regulated
4. U.S. Food & Drug Administration. Is It a Cosmetic, a Drug, or Both? (Or Is It Soap?) . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-laws-regulations/it-cosmetic-drug-or-both-or-it-soap
5. Risk & Policy Analysis Limited. Comparative Study on Cosmetics Legislation in the EU and Other Principal Markets with Special Attention to so-called Borderline Products. 2004.
6. Food and Drugs Act (R.S.C., 1985, c. F-27)
7. Hu A. Japan Cosmetic Regulation . ChemLinked | Cosmetic Comlpiance Intelligence & Solutions. 2021. Available from: https://cosmetic.chemlinked.com/cosmepedia/japan-cosmetic-regulation#C0
8. RANNOU E. Guidebook for Exporting/Importing Cosmetics to Japan . 2015. Available from: https://www.eu-japan.eu/publications/guidebook-exportingimporting-cosmetics-japan
9. ChemLinked. China Mainland Cosmetic Regulation . ChemLinked | Cosmetic Compliance Intelligence & Solutions. 2021. Available from: https://cosmetic.chemlinked.com/cosmepedia/china-mainland-cosmetic-regulation
10. Agência Nacional de Vigilância Sanitária - Anvisa. Personal Hygiene Products, Cosmetics and Fragrances — Português (Brasil) .2020. Available from: https://www.gov.br/anvisa/pt-br/english/regulation-of-products/personal-hygiene-products-cosmetics-and-fragrances

11. Agência Nacional de Vigilância Sanitária - Anvisa. Conceitos e definições . 2020. Available from: https://www.gov.br/anvisa/pt-br/acessoainformacao/perguntasfrequentes/cosmeticos/conceitos-e-definicoes

12. U.S. Food & Drug Administration. Frequently Asked Questions on Soap . 2020. Available from: https://www.fda.gov/cosmetics/cosmetic-products/frequently-asked-questions-soap

13. U.S. Food & Drug Administration. Questions and Answers: FDA announces new requirements for over-the-counter (OTC) sunscreen products marketed in the U.S.. 2014. Available from: https://www.fda.gov/drugs/understanding-over-counter-medicines/questions-and-answers-fda-announces-new-requirements-over-counter-otc-sunscreen-products-marketed-us#Question7

14. Health Canada. Draft: Guidance Document, Sunscreen Monograph

15. Commission Regulation (EU) No 655/2013

16. U.S. Food & Drug Administration. Cosmetics Labeling Claims . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-labeling/cosmetics-labeling-claims

17. Soap Queen. Understanding FDA Cosmetic vs. Drug Claims . 2015. Available from: https://www.soapqueen.com/business/understanding-fda-cosmetic-vs-drug-claims/

18. U.S. Food & Drug Administration. Wrinkle Treatments and Other Anti-aging Products. 2020. Available from: https://www.fda.gov/cosmetics/cosmetic-products/wrinkle-treatments-and-other-anti-aging-products

19. U.S. Food & Drug Administration. “Hypoallergenic” Cosmetics . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-labeling-claims/hypoallergenic-cosmetics

20. U.S. Food & Drug Administration. “Cruelty Free”/"Not Tested on Animals". 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-labeling-claims/cruelty-freenot-tested-animals

21. Canada.ca. Cosmetic advertising, labelling and ingredients . 2017. Available from: https://www.canada.ca/en/health-canada/services/cosmetics/cosmetic-

advertising-labelling-ingredients.html

22. Cosmetics Bussiness. How to make claims for cosmetics sold in Canada . 2020. Available from: https://cosmeticsbusiness.com/news/article_page/How_to_make_claims_for_cosmetics_sold_in_Canada/166030

23. SCCS (Scientific Committee on Consumer Safety) Notes of Guidance for The Testing of Cosmetic Ingredients and Their Safety Evaluation.

24. European Commission. Scientific and technical assessment . 2021. Available from: https://ec.europa.eu/growth/sectors/cosmetics/scientific-and-technical-assessment_en

25. European Commission. CMR substances . 2021. Available from: https://ec.europa.eu/growth/sectors/cosmetics/cosmetic-products-specific-topics/cmr-substances_en

26. Su Z, Luo FY, Pei XR, Zhang FL, Xing SX, Wang GL. Final Publication of the "Regulations on the Supervision and Administration of Cosmetics" and New Prospectives of Cosmetic Ccience in China. Cosmetics . 2020;7(98):1–17.

27. U.S. Food & Drug Administration. Prohibited & Restricted Ingredients in Cosmetics. 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-laws-regulations/prohibited-restricted-ingredients-cosmetics

28. Cosmetic Ingredient Review (CIR). About the Cosmetic Ingredient Review . 2016. Available from: https://www.cir-safety.org/about

29. U.S. Food & Drug Administration. Product Testing of Cosmetics . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-science-research/product-testing-cosmetics

30. European Commission. Cosmetic product notification portal. 2021. Available from: https://ec.europa.eu/growth/sectors/cosmetics/cosmetic-product-notification-portal_en

31. U.S. Food & Drug Administration. Does FDA approve cosmetics before they go on the market?. 2016. Available from: https://www.fda.gov/industry/fda-basics-industry/does-fda-approve-cosmetics-they-go-market

32. U.S. Food & Drug Administration. Voluntary Cosmetic Registration Program . 2020. Available from: https://www.fda.gov/cosmetics/voluntary-cosmetic-registration-program

33. U.S. Food & Drug Administration. Product Testing of Cosmetics . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-science-research/product-testing-cosmetics

34. Canada.ca. Notification of Cosmetics. 2017]. Available from: https://www.canada.ca/en/health-canada/services/consumer-product-safety/cosmetics/notification-cosmetics.html

35. Agência Nacional de Vigilância Sanitária - Anvisa. Personal Hygiene Products, Cosmetics and Fragrances. 2020. Available from: https://www.gov.br/anvisa/pt-br/english/regulation-of-products/personal-hygiene-products-cosmetics-and-fragrances

36. Trial Expert. Cosmetics Registration Process In Japan . Credevo Articles. Available from: https://credevo.com/articles/2020/12/15/japan-cosmetics-registration-and-market-approval-process/

37. European Commision. Ban on animal testing. Available from: https://ec.europa.eu/growth/sectors/cosmetics/ban-animal-testing_pt

38. The Humane Society of the United States. Cosmetics testing FAQ . 2021. Available from: https://www.humanesociety.org/resources/cosmetics-testing-faq

39. U.S. Food & Drug Administration. Animal Testing & Cosmetics . 2020. Available from: https://www.fda.gov/cosmetics/product-testing-cosmetics/animal-testing-cosmetics

40. Toronto Humane Society. Cosmetic Testing - a Cruel and Deadly Practice . 2021. Available from: https://www.torontohumanesociety.com/cosmetic-testing-cruel-deadly-animal-practice/

41. Humane Society International. Brazilian Supreme Court confirms Rio de Janeiro state ban on animal testing for cosmetics . 2021. Available from: https://www.hsi.org/news-media/brazilian-supreme-court-confirms-rio-de-

janeiro-state-ban-on-animal-testing-for-cosmetics/

42. Grum T. Global ban on animal testing: where are we in 2019? . Cosmetics Design Europe. 2019. Available from: https://www.cosmeticsdesign-europe.com/Article/2019/03/05/Global-ban-on-animal-testing-where-are-we-in-2019

43. Grum T. Global animal testing ban for cosmetics closer to EU Parliament 2023 goal . Cosmetics Design Europe. 2021. Available from: https://www.cosmeticsdesign-europe.com/Article/2021/01/29/Global-animal-testing-ban-for-cosmetics-closer-to-EU-Parliament-2023-goal

44. Agência Nacional de Vigilância Sanitária - Anvisa. Cosméticos. Available from: https://www.gov.br/anvisa/pt-br/assuntos/cosmeticos

45. U.S. Food & Drug Administration.. Cosmetics Labeling Guide . 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-labeling-regulations/cosmetics-labeling-guide

46. U.S. Food & Drug Administration. Cosmetic Ingredient Names. 2020. Available from: https://www.fda.gov/cosmetics/cosmetics-labeling/cosmetic-ingredient-names

47. Canada.ca. Industry Guide for the labelling of cosmetics . 2006. Available from: https://www.canada.ca/en/health-canada/services/consumer-product-safety/reports-publications/industry-professionals/labelling-cosmetics.html#one4

48. Canada.ca. Safety of Cosmetic Ingredients. 2020. Available from: https://www.canada.ca/en/health-canada/services/consumer-product-safety/cosmetics/labelling/safety-ingredients.html

49. International Cooperation on Cosmetics Regulation (ICCR). About ICCR . 2021. Available from: https://www.iccr-cosmetics.org/about-us

50. Wickett RR, Visscher MO. Structure and function of the epidermal barrier. Am J Infect Control . 2006;34(10 SUPPL.):98–110.

51. WHO. Dermal Absorption. Environmental Health Criteria

52. Abdo JM, Sopko NA, Milner SM. The applied anatomy of human skin: A model

for regeneration. Wound Med . 2020;28(100179):2213–9095.

53. Holmgaard R, Benfeldt E, B. Nielsen J. Percutaneous Penetration - Methodological Considerations. Basic Clin Pharmacol Toxicol. 2014;115:101–9.

54. Dąbrowska AK, Spano F, Derler S, Adlhart C, Spencer ND, Rossi RM. The relationship between skin function, barrier properties, and body-dependent factors. Ski Res Technol. 2018;24(2):165–74.

55. Mercuri M, Rivas DF. Challenges and opportunities for small volumes delivery into the skin. Biomicrofluidics . 2021;15(011301):1–30.

56. Förster M, Bolzinger MA, Fessi H, Briançon S. Topical delivery of cosmetics and drugs. Molecular aspects of percutaneous absorption and delivery. Eur J Dermatology. 2009;19(4):309–23.

57. Holmgaard R, Nielsen JB. Dermal absorption of pesticides – evaluation of variability and prevention . Danish Environmental Protection Agency. 2009.

58. Hu X, He H. A review of cosmetic skin delivery. J Cosmet Dermatol. 2021;00:01–11.

59. Supe S, Takudage P. Methods for evaluating penetration of drug into the skin: A review. Ski Res Technol. 2021;27:299–308.

60. Morganti P, Ruocco E, Wolf R, Ruocco V. Percutaneous absorption and delivery systems. In: Clinics in Dermatology. 2001. p. 489–501.

61. Brodin B, Steffansen B, Uhd Nielsen C. Passife diffusion of drug substances: the concepts of flux and permeability. In: Molecular Biopharmaceutics. p. 135–52.

62. Ueda CT, Shah VP, Derdzinski K, Ewing G, Flynn G, Maibach H, et al. Topical and Transdermal Drug Products. Pharmacopeial Forum. 2009;35(3):750–64.

63. Souza A De, Strober BE. Principles of Topical Therapy. In: Fitzpatrick's Dermatology in General Medicine. 2012. p. 2643–51.

64. Mayba JN, Gooderham MJ. A Guide to Topical Vehicle Formulations. J Cutan Med Surg. 2018;22(2):207–12.

65. Garg T, Rath G, K. Goyal A. Comprehensive review on additives of topical

dosage forms for drug delivery. Drug Deliv. 2014;22(8):969–87.

66. Sharadha M, Gowda D V., Vishal Gupta N, Akhila AR. An overview on topical drug delivery system – Updated review. Int J Res Pharm Sci. 2020;11(1):368–85.

67. de Villiers M. Ointment Bases. In: A Practical Guide to Contemporary Pharmacy Practice. 2017. p. 277–90.

68. Fytianos G, Rahdar A, Kyzas GZ. Nanomaterials in Cosmetics: Recent Updates. Nanomaterials. 2020;10(5):1–16.

69. Ferraris C, Rimicci C, Garelli S, Ugazio E, Battaglia L. Nanosystems in Cosmetic Products: A Brief Overview of Functional, Market, Regulatory and Safety Concerns. Pharmaceutics. 2021;13(9).

70. Salvioni L, Morelli L, Ochoa E, Labra M, Fiandra L, Palugan L, et al. The emerging role of nanotechnology in skincare. Adv Colloid Interface Sci . 2021;293:102437.

71. Kaul S, Gulati N, Verma D, Mukherjee S, Nagaich U. Role of Nanotechnology in Cosmeceuticals: A Review of Recent Advances. J Pharm. 2018;1–19.

72. Coelho CC, Grenho L, Gomes PS, Quadros PA, Fernandes MH. Nano-hydroxyapatite in oral care cosmetics: characterization and cytotoxicity assessment. Sci Rep . 2019;9(11050):1–10.

73. Raj S, U.S. S, Jose S, Sabitha M. Nanotechnology in cosmetics: Opportunities and challenges. J Pharm Bioallied Sci . 2012;4(3):186–93.

74. Vitorino C, Sousa J, Pais A. Overcoming the Skin Permeation Barrier: Challenges and Opportunities. Curr Pharm Des. 2015;21(20):2698–712.

75. Pereira R, Silva SG, Pinheiro M, Reis S, Luísa Do Vale M. Current status of amino acid-based permeation enhancers in transdermal drug delivery. Membranes (Basel). 2021;11(5).

76. Kim B, Cho H-E, Moon SH, Ahn H-J, Bae S, Cho H-D, et al. Transdermal delivery systems in cosmetics. Biomed Dermatology. 2020;4(10):1–12.

77. Das S, Sen Gupta K. A Comprehensive Review on Natural Products as Chemical

Penetration Enhancer. J Drug Deliv Ther. 2021;11(5-S):176–87.

78. Chen J, Jiang QD, Chai YP, Zhang H, Peng P, Yang XX. Natural terpenes as penetration enhancers for transdermal drug delivery. Molecules. 2016;21(12):1–22.

79. Vasyuchenko EP, Orekhov PS, Armeev GA, Bozdaganyan ME. Cpe-db: An open database of chemical penetration enhancers. Pharmaceutics. 2021;13(66):1–14.

80. Pathan IB, Setty CM. Chemical penetration enhancers for transdermal drug delivery systems. Trop J Pharm Res. 2009;8(2):173–9.

81. Suresh P, Paul S. Ion-paired Drug Delivery: An Avenue for Bioavailability Improvement. Sierra Leone J Biomed Res. 2011;3(2):70–6.

82. Finnin B, Walters KA, Franz TJ. In vitro Skin Permeation Methodology. In: A..E. Benson H, C. Watkinson A, editors. Transdermal and Topical Drug Delivery: Principles and Practise . 1st ed. John Wiley & Sons, Inc.; 2012. p. 85–108.

83. European Medicines Agency. Draft Guideline on quality and equivalence of topical products.

84. PermeGear Inc. Diffusion Testing Fundamentals . Permegear. 2014. p. 1–8.

85. Man Lau W, Wooi Ng K. Finite and Infinite Dosing. In: Percutaneous Penetration Enhancers Drug Penetration Into/Through the Skin. 2017. p. 35–44.

86. Neupane R, Boddu SHS, Renukuntla J, Babu RJ, Tiwari AK. Alternatives to Biological Skin in Permeation Studies: Current Trends and Possibilities. Pharmaceutics. 2020;12(152):1–25.

87. Abd E, Yousef SA, Pastore MN, Telaprolu K, Mohammed YH, Namjoshi S, et al. Skin models for the testing of transdermal drugs. Clin Pharmacol Adv Appl. 2016;8:163–76.

88. Zsikó S, Csányi E, Kovács A, Budai-Szűcs M, Gácsi A, Berkó S. Methods to evaluate skin penetration In Vitro. Sci Pharm. 2019;87(19):1–21.

89. Lunter DJ. How confocal is confocal raman microspectroscopy on the skin?

Impact of microscope configuration and sample preparation on penetration depth profiles. Skin Pharmacol Physiol. 2016;29(2):92–101.

90. Zhang LW, Monteiro-Riviere NA. Use of confocal microscopy for nanoparticle drug delivery through skin. J Biomed Opt. 2012;18(6):061214.

91. Pena AM, Chen X, Pence IJ, Bornschlögl T, Jeong S, Grégoire S, et al. Imaging and quantifying drug delivery in skin – Part 2: Fluorescence andvibrational spectroscopic imaging methods. Adv Drug Deliv Rev. 2020;153:147–68.

92. Kwon S, Bae H, Jo J, Yoon S. Comprehensive ensemble in QSAR prediction for drug discovery. BMC Bioinformatics. 2019;20(521):1–12.

93. OECD Guideline for the Testing of Chemicals Skin Absorption: in vitro method.

94. OECD Guidance Notes on Dermal Absorption No.156

95. EFSA Guidance on Dermal Absorption for plant protection products and their residues.

96. SCCS Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients.

97. ECETOC. Monograph No. 20 on Percutaneous Absorption.

Annexes

A1. Table 13 - Comparison of a few parameters and requirements specified in some of the most relevant test documents and guidelines to evaluate dermal absorption *in vitro* (OECD, SCCS, EFSA, WHO/IPCS and ECETOC) (51,93,94,95,96,97)

Parameter		**OECD**	**SCCS**	**EFSA**	**WHO/ IPCS**	**ECETOC**
Aimed At		Pesticides and Biocides	Cosmetic Ingredients	Pesticides	General	General
Diffusion Cell		Static or Flow-through	Static or Flow-through	---	Static or Flow-through	Static or Flow-through
Skin/ Membranes	**Cultured or Reconstructed Human Skin Models**	Not Recommended	Not Recommended	Not Recommended	Not Recommended	Not Recommended
	Species	- Human (preferred) - Rat	- Human (preferred) - Pig - Rat is not recommended	- Human - Rat	- Human (preferred) - Pig (cosmetics)	- Human - Pig - Monkey - Mouse
	Anatomical Location	<u>Human</u> Abdomen, back, breast or upper leg <u>Rat</u> Abdomen or back	<u>Human:</u> Abdomen, breast or leg <u>Pig:</u> Abdomen, back, breast, flanks or ears	<u>Human:</u> Abdomen, back, breast or upper leg <u>Rat:</u> Abdomen or back	<u>Human:</u> Abdomen or breast	---

(To be continued)

Parameter	OECD	SCCS	EFSA	WHO/ IPCS	ECETOC
Thickness + Preparation Techniques	- Split thickness 200-400/500µm; dermatomed *[Most appropriate]* - Isolated epidermis with SC Separation by heat or enzymatically - Full-thickness SC + epidermis + dermis up to 1000 µm *[when justified]*	**Human:** - Split thickness 200 – 500 µm *[Most appropriate]* **Pig:** - Full thickness 500–1000 µm --- - Dermatomed skin is often used - *[when justified] e*pidermal membranes can be used	**Human:** - Split thickness 200 – 400 µm dermatomed **Rat:** - Split thickness 200-400 µm --- - Epidermal membranes can be used - Full thickness can be used	- Split thickness 200 – 400/500 µm; dermatomed - Epidermal membranes SC + epidermis separation by heat, chemically or enzymatically - Full thickness SC + epidermis + dermis *[when justified]*	**Human, pig, monkey**: - Split thickness **Mouse** - Full thickness 500 µm --- - Dermatomed skin is often used on the preparation of split thickness - Epidermal membranes can be used (mechanical, thermal or chemical separation)
Viability	- Non-viable but intact skin - Metabolically active (viable) skin	- Freshly excised, viable skin	---	- Viable skin (preferred) - Non-viable skin	- Freshly excised skin - Cadaver skin
Integrity evaluation methods	- Trans-epidermal water loss - Trans-epidermal resistance - Reference substance penetration	- Trans-epidermal water loss - Trans-cutaneous electrical resistance - Measuring the penetration of a marker molecule	- Trans-epidermal water loss - Trans-epidermal resistance - Reference substance penetration	- Trans-epidermal water loss - Trans-ctaneous electrical resistance - Tritium method	---

(To be continued)

Parameter		OECD	SCCS	EFSA	WHO/ IPCS	ECETOC
Test Conditions	**Receptor Fluid Composition**	<u>Hydrophilic compounds</u> Normal saline <u>Lipophilic compounds</u> Solvent mixtures such as: - ethanol and water (50% aqueous ethanol); - <6% polyoxyethelene (20) oleyl ether in water; - 5% bovine serum albumin	<u>Hydrophilic compounds</u> Saline or buffered saline solutions <u>Lipophilic compounds</u> Serum albumin or addition of appropriate solubilisers/ emulsifiers	<u>Hydrophilic compounds</u> Normal saline <u>Lipophilic compounds</u>: Depending on the expected concentration, mixtures containing solvents (e.g. ethanol/water) might be required	<u>Hydrophilic compounds</u> Normal saline or an isotonic buffered saline solution <u>Lipophilic compounds</u> Solvent mixtures such as: - ethanol:water; - <6% polyoxyethylene (20) oleyl ether in water; - 5% bovine serum albumin	<u>Hydrophilic compounds</u> Physiological saline buffered to pH 7,4 <u>Lipophilic compounds</u> Addition of non-ionic surfactants like - polyenthylene glycol oleyl-ether (6% in saline); - methanol; - ethanol; - olyethyleneglycol; - serum; - albumin
	State of Occlusion	- Depends on the properties of the test substance and exposure scenario - Non-occlusion is more likely to mimic the majority of pesticides, cosmetics and industrial chemical scenarios	---	---	- According with the objectives of the study - Non-occlusion, usually, simulates the exposure situation more accurately	---
	Temperature	32 ± 1˚C	32 ± 1˚C	---	32 ± 1˚C	30 ± 1˚C
	Dose	- Finite (Preferred) - Infinite	---	Finite	- Finite (preferred for cosmetics) - Infinite	Finite (most often used)

(To be continued)

Parameter		OECD	SCCS	EFSA	WHO/ IPCS	ECETOC
	Concentration of test substance	**Finite dose:** - Solids: 1-5mg/cm^2 - Liquids: up to 10µl/cm^2 **Infinite dose:** - Larger volumes	- Solids / semi-solids: 2-5 mg/cm^2 - Liquids: up to 10µl/cm^2 - Oxidative hair dye formulations: 20 mg/cm^2	---	**Finite dose:** - Solids: 1-5mg/cm^2 - Liquids: up to 10µl/cm^2 **Infinite dose:** - Solids: >10 mg/cm^2 - Liquids: >100 µl/cm^2	---
	Test Duration /Sampling Period	24 Hours	24 Hours	At least 24 Hours	Minimum of 24 Hours	Minimum of 24 Hours
	Exposure Period	At least 6-10 Hours	At least 30 minutes	6-10 Hours	- A few minutes for rinse-off products - Maximum of 24h for leave-on products - 6-8h for industrial products	---
	Sampling Frequency	6-12 Sampling points	At least 6 post-application time points	---	---	---
Analysis	**Mass Balance Recovery**	95-100%	85-115%	± 5%	90-100% (OECD) or 85-115% (SCCNFP)	---
	Tape Stripping	- Up to 10-5 tape strips - Exclude the amount that was found in the first upper two tape strips	---	- The first two tape strips are excluded - All the tape strips are excluded: only if the permeation is essentially complete at the end of the study	---	---

"---" means no information available; OECD includes the TG428 and "Guidance Notes on Dermal Absorption (No.156)"; SCCS is the "Basic Criteria for in vitro Assessment of the Dermal Absorption of Cosmetic Ingredients; EFSA is the "Guidance on Dermal Absorption for plant protection products"; WHO/IPCA is the "Environmental Health Criteria 235"; ECETOC is the "Monograph 20";