Universidade de Lisboa

Faculdade de Farmácia

Formulation, characterization and stability studies of emulsions containing different vegetable oils and caffeine as active compound

Ana Margarida Colaço Dias de Lemos Dionísio

Trabalho de Campo orientado pela Professora Doutora Antonella Casiraghi, Professora Auxiliar, Università degli Studi di Milano, e coorientado pela Professora Doutora Joana Marto, Professora Auxiliar, Faculdade de Farmácia da Universidade de Lisboa.

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Resumo

A permeação eficaz de ingredientes ativos pela barreira cutânea é um dos principais pontos de discussão no que toca a Indústria Cosmética, que tem vindo a crescer de forma considerável nos últimos anos. Muito compostos não apresentam as características físico-químicas necessárias a uma permeação cutânea passiva em quantidades relevantes. De modo a tentar resolver este desafio, o uso de substâncias que facilitam a permeação, os chamados promotores de permeação cutânea, surge como potencial solução, tendo como foco primário a penetração do estrato córneo.

Do conjunto dos excipientes com estas características, os óleos vegetais, ricos em ácidos gordos como o ácido oleico, apresentam-se com possíveis candidatos. Para além de serem produtos naturais, o que acaba por ser a nova tendência no mundo da cosmética, e terem inúmeros benefícios para a pele, os óleos vegetais demonstram a capacidade de acelerar e aumentar a permeação de ativos pela pele. Ao alterarem o estado da bicamada fosfolipídica, destabilizando os ácidos gordos compactados e sendo integrados na própria membrana, permitem que outras substâncias atravessem a pele mais facilmente.

Esta monografia procura explorar a influencia de 3 óleos vegetais diferentes – azeite, óleo de amêndoas doces e óleo de semente de girassol – numa formulação de referência, avaliando os seus efeitos nos atributos físicos e sensoriais da emulsão, assim como na sua estabilidade ao longo do tempo. Foram desenvolvidos estudos de libertação e permeação cutânea usando membranas consideradas o standard para cada um dos testes. Foi utilizada como ativo a cafeína, um composto hidrofílico bem estabelecido e conhecido pelos seus benefícios para a pele, desde as suas propriedades antioxidantes à sua capacidade de aumentar a microcirculação.

Dos óleos vegetais testados, a formulação que continha azeite acabou por apresentar melhores resultados ao longo do tempo. Em comparação com a formulação de referência mostrou dados promissores, com uma permeação mais rápida e extensa da cafeína, apesar de serem necessários mais estudos para determinar se estes resultados são de facto de alguma significância estatística. Este estudo fornece uma base para futura investigação acerca das potenciais aplicações dos óleos vegetais em formulações de aplicação cutânea, nomeadamente enquanto promotores de permeação cutânea.

Palavras-chave: Permeação Cutânea; Óleos Vegetais; Ácidos Gordos; Promotores de Permeação Cutânea.

Abstract

The efficient permeation of active ingredients through the skin barrier is one of the main talking points when it comes to the Cosmetic Industry, which has experienced a considerable growth over the past few years. Numerous compounds lack the optimal physicochemical characteristics required for passive skin permeation in relevant amounts. To address this challenge, a strategy involving the use of substances that facilitate permeation, the so-called permeation enhancers, has emerged as a possible solution, with a primary focus on crossing the *stratum corneum*. Amongst potential excipients with these characteristics, vegetable oils, rich in fatty acids like oleic acid, have shown to be plausible contenders. Besides fitting the new trend of natural products being of cosmetic use and having multiple benefits to the skin, they demonstrate the ability to expedite and increase the permeation of active ingredients across the skin by disrupting the phospholipidic bilayer, destabilizing the densely packed fatty acids, and seamlessly integrating into the skin, thus allowing for other substances to be easily delivered transdermally.

This dissertation aimed to explore the influence of three different vegetable oils – olive oil, sweet almond oil and sunflower seed oil - on a reference formulation, assessing their effects on the physical and sensory attributes of the emulsion, as well as in its stability over time. Release and permeation studies were conducted using standard membranes for each study and employing caffeine, a well-established hydrophilic compound celebrated for its skin benefits, such as antioxidant properties and the ability of increasing microcirculation.

Out of the tested vegetable oils, the findings favored the formulation containing olive oil, which consistently exhibited superior performance over time. In comparison to the reference formulation, the results for this formulation were also promising, with faster and more extensive permeation of the caffeine being achieved, although further studies should be developed to ensure statistical significance. This study lays the groundwork for future research into the diverse potential applications of vegetable oils in skincare formulations, mainly as permeation enhancers.

Keywords: Skin Permeation; Vegetable Oils; Fatty Acids; Enhancer.

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List of Abbreviations

O/W – Oil-In-Water

W/O – Water-In-Oil

TEWL – Transepidermal Water Loss

PMR – Penetration modifier Ratio

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1 Introduction

1.1 The skin: structure and function

Accounting for 15% of the total weight of an adult and having a surface of around 2 m^2, the skin is known to be the largest organ of the human body. It functions as a barrier that protects from the hostile external environment (1,2).

The skin can be divided into three main layers: the epidermis, the dermis and subcutaneous tissue. The epidermis, the outermost layer of skin, is defined as a stratified squamous epithelium, primarily comprised of keratinocytes in progressively more complex stages of differentiation, but also melanocytes, langerhans cells and merkel cells. It is an avascular layer, completely dependent on the underlying layer for nutrients and irrigation. Its prime function is to act like a physical and biological barrier to the external environment, preventing, in its intact state, penetration by foreign substances and organisms (1).

The essential permeability properties that prevent loss of water and electrolytes are mainly located in the outer epidermal layer, the *stratum corneum*. It consists of corneocytes and keratinocytes that have undergone a level of terminal differentiation, surrounded by a lipid-enriched extracellular matrix (around 10% of this layer's weight). The mechanical strength of the skin is provided by the corneocytes, while the hydrophobic extracellular lipid matrix provides the barrier. This can be attributed to the lipids´ high hydrophobicity, their intercellular location, as well as their organization into enriched bilayers. The *stratum corneum* therefore acts as a barrier for hydrophilic drugs and macromolecules, whilst lipophilic drugs can penetrate the skin through partitioning into the intercellular lipids and using the transcellular route as well (2–4).

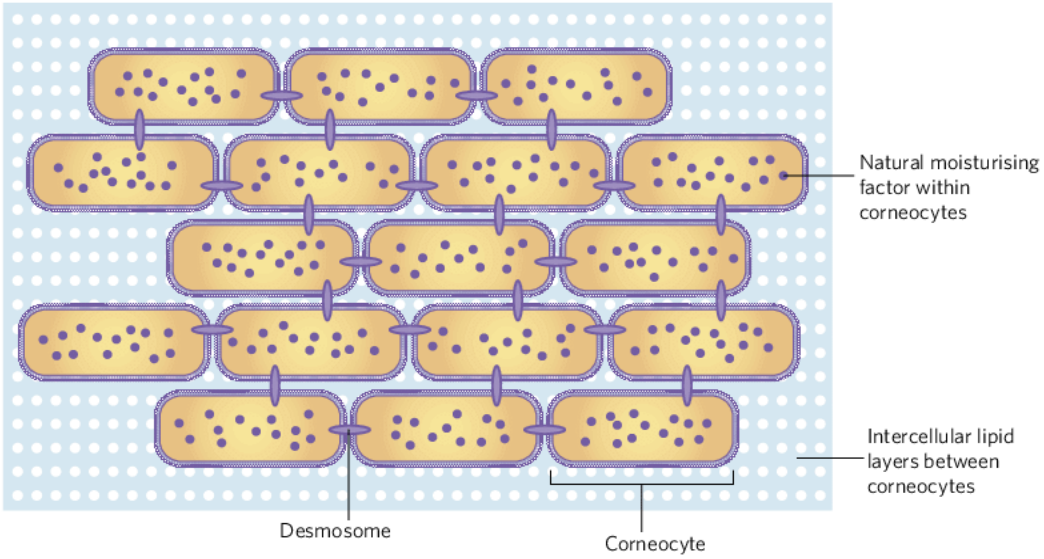


Fig. 1: *Stratum Corneum*'s general organization (5).

For the skin surface to be considered healthy, an acidic pH of 4-6 is optimal. pH plays a fundamental role in the skin's barrier (acid mantle) and assists in regulating the skin's microbiome (the resident bacteria). The acidic pH has an additional role: ensuring optimal *stratum corneum* cohesion and barrier function, being that its formation largely relies on pH-dependent enzymes. Both the processing of lipids secreted by lamellar bodies, as well as the formation of these structures, require an acidic environment. Moreover, free fatty acids in the extracellular space form lamellar liquid crystals at pH values of 4.5–6 through partial ionization (6).

1.2 Topical pharmaceutical forms: Emulsions

Even though the most common routes for drug delivery are the oral and parental route, topical and transdermal systems have shown to be an attractive, non-invasive alternative for the administration of active substances. The last few years have seen relevant growth to skin formulation development, with the global dermatological drug market project to reach around 40 billion USD by 2030 (7,8).

The therapeutic efficiency of drugs is characterized by the rate and extent at which the active substance reaches the desired site of action. Being that the human skin presents itself as

a remarkably good barrier to the permeation of substances, most topical drug systems only deliver a small portion of the total dose applied, resulting in low bioavailability. In spite of this, the pharmacokinetic profile for dermatological drugs is more uniform with less accentuated peaks, therefore minimizing the risk of toxic side effects (7,8).

The drug firstly penetrates through the *stratum corneum*, the hardest to permeate, and only then does it go through epidermis and dermis, becoming available for systemic absorption *via* dermal microcirculation, if desired (7).

Topical drug delivery has also proven to be the preferred method for delivering substances locally, confining the drug´s pharmacological activity to the desired site in the skin´s surface. Despite foams, sprays, powders, solutions and adhesive systems being in use, semi-solid preparations still dominate the field of topical delivery, namely emulsions like creams and gels (9,10).

Emulsions consist of dispersions of one liquid into another in which it is immiscible. This happens in the form of liquid droplets, the dispersed phase, being surrounded by the liquid that represents the continuous phase. The size of the droplets is usually from 100 nm to 100 μm. Depending on the composition of the continuous and dispersed phases, emulsions can be classified as either oil-in-water (o/w) or water-in-oil (w/o) (11).

Emulsions are broadly used in our day-to-day life, with examples ranging from mayonnaise and salad dressings to cosmetic creams and lotions, as well as some types of paint. But in the context of pharmaceutical applications, they are widely used as carrier and encapsulation systems for hydrophilic or lipophilic compounds, providing protection against deterioration and enabling controlled release (11).

Emulsified systems prove to be challenging in terms of the preservation of their physical stability, regardless of type or target use. They are thermodynamically unstable systems and tend to “split” or “break” during storage *via* a variety of instability mechanisms: gravitational separation, droplet flocculation, Ostwald ripening or droplet coalescence. This makes it imperative that topical formulation provides a stable chemical environment and a suitable dispensing container (10,11).

1.3 Permeation Enhancers

The *stratum corneum* is an effective barrier that limits skin penetration and therefore its potential for drug delivery. Many compounds do not possess the ideal physicochemical features (low molecular weight, sufficient lipophilicity and a low melting point) for passively permeating the skin in therapeutic or even relevant quantities, which was limiting the topical market (12).

Researchers have therefore been devoted to developing technologies that enhance the delivery of substances into the skin for more than fifty years. These technologies encompass both passive approaches involving the use of formulation excipients and chemical penetration enhancers, and active or physical methods which employ an external force. We can also consider a subdivision within physical methods, between the so-called indirect methods, that involve the application of electrical, acoustic, laser and magnetic energy, and the direct methods, or minimally invasive methods, which involve creating a pore or hole in the *stratum corneum* barrier, using thermal, mechanical or pressure-based technologies (12).

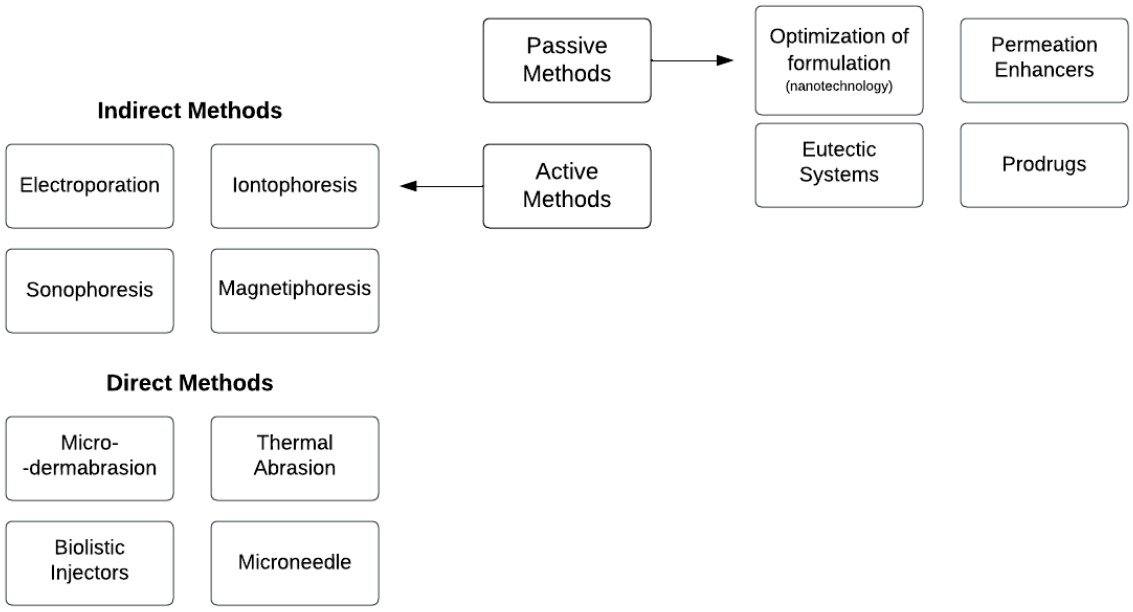


Fig. 2: Examples of Topical Drug Delivery Technologies (7,12).

One of the most widely used passive approaches to enhance drug permeation into the skin is the use of chemical penetration enhancers. These enhancers facilitate drug partitioning into the barrier presented by the *stratum corneum* without causing long-term damage to the skin. They employ various mechanisms of action, such as increasing the fluidity of the *stratum corneum* lipid bilayer *via* its disruption or altering the solubility of the skin for the permeant. The former influences the diffusion process of the permeant through the skin altering the diffusion coefficient D, and the latter results in the change of the partition coefficient K between the skin and vehicle. Other more complex mechanisms include interacting with intercellular proteins, disrupting or extracting intercellular lipids, increasing the drug's thermodynamic activity and enhancing *stratum corneum* hydration (13,14).

Penetration enhancers need to meet specific criteria to be effective, such as being pharmacologically inert, non-toxic, non-irritating, non-allergic, odorless, tasteless, colorless, compatible with most drugs and excipients, affordable and possessing good solvent characteristics. Over the past two decades, a variety of penetration enhancers have been developed, including alcohols and polyols (e.g., ethanol, propylene glycol), surfactants (e.g., Tween, Span, SLS), fatty acids (e.g., oleic acid), amines and amides (e.g., Azone, N-methylpyrrolidone), terpenes (e.g., limonene), sulfoxides (e.g., dimethylsulfoxide) and esters (e.g., isopropylmyristate) (9).

Nevertheless, these excipients might face limitations in efficiently enhancing the delivery of molecules required in high doses to result in noticeable activity. Their efficacy in enhancing the permeability of barriers to macromolecules is also limited. Consequently, there is ongoing interest in exploring chemicals that can safely enhance drug permeability, making it an interesting area of research in transdermal drug delivery (4).

In literature, the term "penetration modifier" has been used instead of "penetration enhancer" due to a study presented by Michniak-Kohn at the AAPS meeting in 2007 (San Diego, CA, U.S.A), which demonstrated that the effects of penetration enhancers and penetration retardants vary depending on the vehicle used. Different vehicles, such as water, ethanol, propylene glycol, and polyethylene glycol, were employed to incorporate known penetration enhancers (e.g., Azone and S,S-dimethyl-N-(4-bromobenzoyl) iminosulphurane) and penetration retardants (e.g., Azone analogue N-0915 and S,S-dimethyl-N-(2-methoxycarbonylbenzenesulphonyl) iminosulphurane). The literature describes the enhancing

and retardant effects of these compounds. However, the extent of penetration enhancement or retardation depends on the specific vehicle used. As a result, the term "penetration modifier" seems more appropriate since enhancement or retardation can occur due to the influence of the vehicle (13).

1.4 The effects of vegetable oils on skin permeation

The popularity of skincare and cosmetics formulated with natural products is increasing day-by-day, being considered more ecological, ethical, safer for use and even sometimes more effective than their synthetic equivalents. As so, vegetable oils, produced by plants for obtaining energy, are recently attracting a lot of attention in the dermatological field for being sustainable renewable-sourced products with multiple health benefits, namely for the skin. In spite of this, their first use probably dates to China, around the 2nd Century BC, and many other ancient civilizations took advantage of their use. For example, the Egyptian Pharaohs applied olive oil to moisturize and nourish their skin, and the Romans used the oil´s healing, anti-inflammatory and antioxidant properties for treating wounds (15–17).

Their effects on the skin can be mainly attributed to the similarity in lipidic composition to the *stratum corneum*. Vegetable oils are composed by triacylglycerols, as well as diacylglycerols and monoacylglycerols in smaller amounts. They also contain phospholipids, free sterols, tocopherols and tocotrienols, triterpene alcohols, hydrocarbons and fat-soluble vitamins (15,18).

Fatty acids, which are naturally present in the *epidermis* of the skin, can be classified according to the presence or absence of double bounds as saturated (without double bonds), monosaturated (with one double bond) or poly-unsaturated fatty acids (with more than two double bonds). Free fatty acids in the *stratum corneum* are mostly saturated, with chains of up to 36 carbon atoms. Monounsaturated atoms represent about 20% of the total proportion. The only unsaturated fatty acids detected unbound in the *stratum corneum* are oleic (C18:1) and linoleic (C18:2) acids, accounting for 6 and 2%, respectively. The chain length and degree of unsaturation can have a great influence on the chemical biological properties of these compounds (15,19,20).

As it is shown in Table 1, when it comes to vegetable oils, coconut oil contains mainly saturated fatty acids, while other oils, such as olive oil, almond oil or sunflower oil, largely contain unsaturated fatty acids (oleic acid, linoleic acid, and linolenic acid) (17).

Table 1: Commonly used vegetable oils, their fatty acid composition and unsaponifiable content, in percentage (19).

	Saturated Fatty Acids (%)	*Monounsaturated Fatty Acids (%)*	*Poly-unsaturated Fatty Acids (%)*	*Oleic Acid (%)*	*Linoleic Acid (%)*	*Linolenic Acid (%)*	*Unsaponifiable Content (%)*
Coconut Oil	78-93	5-6	1-2	5-6	1-2	-	0.02-1.5
Olive Oil	14	74	9	73-78	7-9	1	0.6-3
Almond Oil	5	75	20	53-78	13-26	<0,7	0,5-1
Sunflower Oil	10	31	57	30	55	2	0.6-1.5
Argan Oil	20	45	35	45	35	-	0.7-1
Marula Oil	22-26	67-70	6	65-78	4-9	<0,7	0.7-3
Avocado Oil	16	67	15	47-60	13-14	1	0.4-12.2
Canola Oil	5	65	29	46-63	19-26	10	0.5-5

- : typically not present.

The main dermal property of vegetable oils is the emollience of triglycerides, which results in improved hydration and decreased transepidermal water loss (TEWL). They can be classified as occlusive moisturizers, preventing water evaporation to the environment by creating a hydrophobic barrier over the skin surface, that water cannot physically penetrate. This forces water to move from the lower viable epidermal and dermal layers to the *stratum corneum*, replenishing its moisture without being able to further escape from the skin. The most commonly use skin occlusives are petrolatum derivates, such as solid petroleum jelly or liquid paraffin, but vegetable oils have shown to be a sustainable alternative in skincare formulations, not providing such rapid effects when it comes to skin occlusion but performing comparably in a 6-hour time course (21,22).

Other more specific effects such as antimicrobial, anti-inflammatory and antioxidative can be mostly attributed to the unsaponifiable fraction of these oils (Table 2) (17,19).

Table 2: Most common unsaponifiable compounds and their respective function (19).

Unsaponifiable Compound	***Function***
Phytol	Cytotoxic, autophagy- and apoptosis-inducing, anti-inflammatory, immune-modulating, antioxidative, antimicrobial
Squalene	Antitumor, anti-inflammatory, wound healing, antioxidative
Triterpene Alcohols	Antitumor, anti-inflammatory, antibacterial
Phytosterols	Antitumor, anti-inflammatory, wound healing, antioxidative, angiogenic
Carotenoids	Antitumor, anti-inflammatory, antioxidative
Tocopherols and tocotrienols	Antioxidative
Flavonoids	Anti-inflammatory, antimicrobial, antioxidative
Ferulic acid	Antimelanogenesis, antioxidative, wound healing
Waxes	Anti-inflammatory, antibacterial, antioxidative
Gamma Oryzanol	Anti-inflammatory, antioxidative
Phospholipids	Wound healing

Dermally applied free fatty acids have also been shown to penetrate the *stratum corneum* as well as act as penetration enhancers for other substances (19).

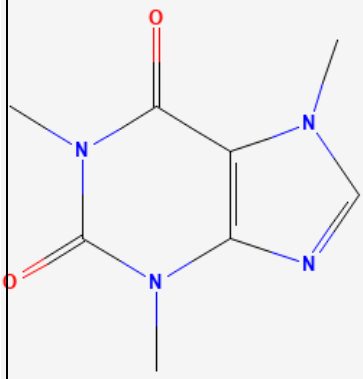
In fact, the epidermis primarily has a defensive barrier role; so, to increase the percutaneous diffusion, topical and transdermal formulations can be integrated with stimulators of skin penetration. For example, it has been reported that oleic acid increases epidermal permeability through a mechanism that involves the perturbation of the phospholipidic bilayer, reducing the tight packing of fatty acids inside the skin, resulting in increased lipidic mobility and forming gaps that allow it to get incorporated into the skin. This makes it easier for drugs to be delivered transdermally when in formulations containing vegetable oils (23).

1.5 Bioactive Ingredient: Caffeine

Numerous natural products provide novel possibilities for the treatment of oxidative stress-mediated skin diseases, acting as powerful antioxidants that act through several mechanisms, such as the inhibition of the peroxidation process or by scavenging free radicals (24,25).

Caffeine is a compound of natural origin that can be considered a purine alkaloid. It is mostly consumed by humans from tea leaves, coffee and cocoa beans. Structurally, it is related to adenosine and acts primarily as an adenosine receptor antagonist with known psychotropic and anti-inflammatory activities (26–28).

Table 3: Chemical Structure and Properties of caffeine. Adapted from (29).

	Molecular formula:	$C_8H_{10}N_4O_2$
	Molecular Weight:	194.19 g/mol
	Boiling Point:	178ºC
	Melting Point:	236ºC
	Density:	2.13 g/cm3
	Solubility:	2.17 g/ 100 mL, in water
	LogP:	- 0.07
	pH:	6.9 (1% solution)
	Physical Description:	Odorless, white powder, bitter taste

The use of caffeine in topical formulations is no longer a novelty. Given its any known favorable effects on the skin, it has become widely used in pharmaceutical and cosmetic preparations, namely creams and lotions (30,31).

In addition to its potent antioxidant properties, characteristic of its class, it has been successfully used for many other purposes. It is often included in cosmetics for dry and sensitive skin, as well as in topical treatments for swollen and dark under eyes (the so called 'puffy eyes'). It actively functions in cellular dehydration and blood vessel constriction, stimulating cutaneous microcirculation. The use of caffeine has shown to reduce hair loss in hair care products, especially for men, through the inhibition of 5-α-reductase activity. Moreover, this compound is a protector against the effects of UVB radiation, therefore acting as a sunscreen, and also acts as an anti-cellulite product, preventing excessive accumulation of fat in cells by promoting lipolysis and inhibiting phosphodiesterase, as well as activating the triglyceride lipase enzyme and breaking triglycerides down into free acids and glycerol (26,27,31).

2 Aim and Hypothesis

This work aims to develop and characterize semi-solid formulations containing vegetable oils as an alternative to traditional oil phase excipients like mineral oils, as well as assessing the impact that these changes have on the permeability of the chosen active substance, caffeine.

3 Materials and Methods

3.1 Materials

Cetomacrogol 1000, cetostearyl alcohol and white soft paraffin BP-USP were purchased from A.C.E.F (Fiorenzuola d'Arda, Italy). Paraffin oil was obtained from Carlo Erba Reagents (Italy). Virgin Olive Oil was obtained from Fontana (Italy). Almond oil and Sunflower oil were obtained from the Faculty of Pharmacy of the University of Milan (Italy). NaCl (Sodium Chloride) and caffeine were obtained from ITW Reagents, S.R. L. (Monza, Italy). Acetic Acid and Acetonitrile were obtained from Sigma Aldrich (Milan, Italy). Purified water was obtained by reverse osmosis and electrodeionization (Milli-Q Gradient A10).

3.2 Methods

3.2.1 Formulation Development

A standard cream formulation from the Italian Pharmacopeia (Monografia Preparazioni semisolide per applicazione cutanea (0132)) was used as a control formulation. Different vegetable oils were used in each attempted formulation, and the impact of these changes was measured on the quality of the final product. After optimization of the formulation for each of the vegetable oils that were used (olive oil, almond oil and sunflower seed oil), caffeine was introduced to all of them in the aqueous phase. Table 4 presents the qualitative and quantitative composition of all four final formulations.

Table 4: Qualitative and quantitative composition of the formulations containing caffeine: Control (FC1.3), FC8.3, FC9 and FC10.

			Quantitative composition (%, (w\w))			
Commercial Name	Excipient (INCI Name)	Function	**FC1.3**	**FC8.3**	**FC9**	**FC10**
Aqueous Phase						
Water	Water (Aqua)	Solvent	69.3	69.3	69.3	69.3
Caffeine	Caffeine	Bioactive Ingredient	1	1	1	1
Oil Phase						
Cetomacrogol 1000	Ceteareth-20	Emulsifier	1.78	1.78	1.78	1.78
Cetostearyl Alcohol	Cetearyl alcohol	Viscosity Enhancer	7.13	7.13	7.13	7.13
White Soft Paraffin	Petrolatum	Emollient	14.85	-	-	-
Liquid Paraffin	Paraffinum Liquidum	Emollient	5.94	5.94	5.94	5.94
Olive Oil	Olea Europaea (Olive) Fruit Oil	Emollient	-	14.85	-	-
Sunflower Seed Oil	Helianthus Annuus (Sunflower) Seed Oil	Emollient	-	-	14.85	-
Sweet Almond Oil	Prunus Amygdalus Dulcis (Sweet Almond) Oil	Emollient	-	-	-	14.85

3.2.1 Formulation Production

All formulations were developed using a manual method. Both the aqueous and the oil phase were heated in a water bath until 65ºC. Afterwards, the aqueous phase was poured into the oil phase becker, with continuous stirring using a glass rod until reaching room temperature. The volume of water lost during the heating process was calculated and then replenished. In total, 40 grams of each formulation were produced.

Each formulation was then stored. 3 portions of 5 g were stored separately for specific stability testing and the remaining 25g were stored for further analysis.

3.2.2 Validation and Exclusion Methods

After selecting the oils, several initial formulations were developed and their physicochemical properties were studied for a period of 1 month, to understand their effect in the desired product quality. A Validation and Exclusion method was performed to select the combination of excipients that allowed for better stability and from there move on to the release studies. This process was developed as shown in Figure 3.

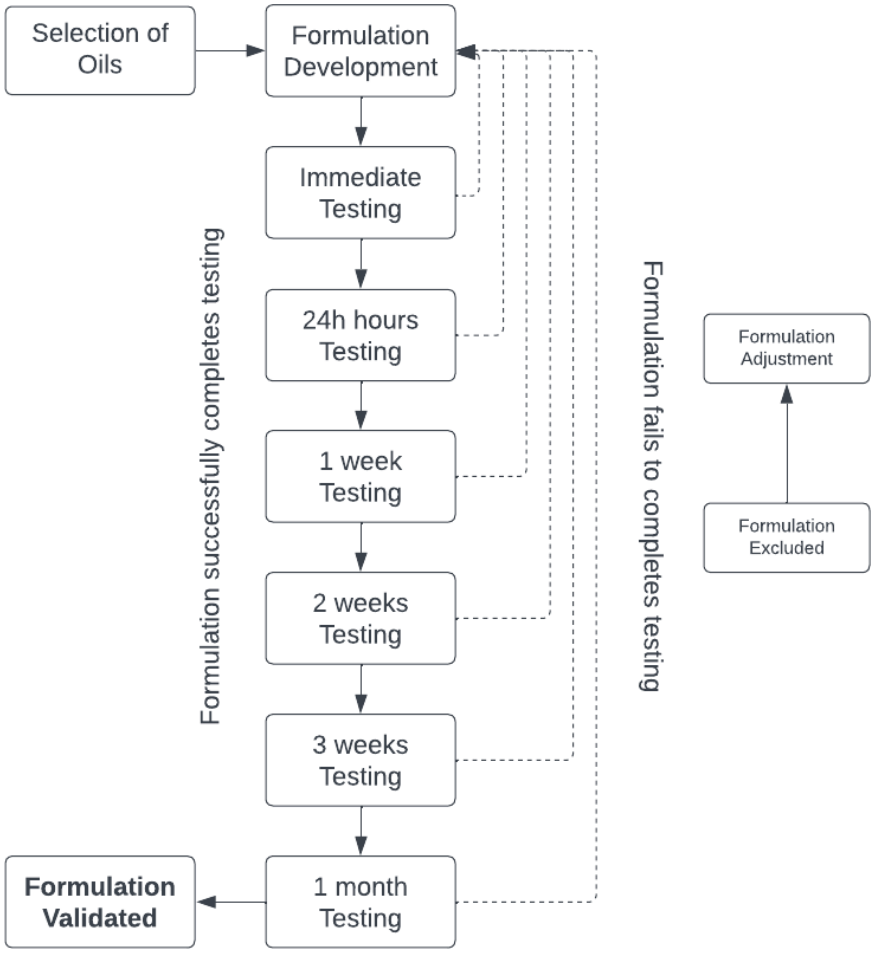


Fig. 3: Validation and exclusion method.

After performing each round of testing, the resulting data was analyzed and the formulations were deemed either to successfully complete testing or to fail testing, which led to the exclusion and readjustment of the formulation. Failing testing meant not matching pre-established conditions related mainly to pH (in immediate testing) and stability. When it came to immediate testing, macroscopic appearance was a priority over pH. A cut-off of between 4-6 was established for the pH value.

The best formulation containing each one of the vegetable oils that were used was then subject to a release study.

3.2.3 Physiochemical Characterization of the Formulation

The macroscopic appearance of each formulation was visually analyzed and used as the first stability indicator. The formulations were evaluated in terms of appearance, color, smell and feel immediately after preparation. Measurements of pH were determined and controlled using a digital pH-Meter with a glass electrode (SevenCompact™ by Mettler Toledo) 24 hours after preparation. Three measurements were performed, and an average result was calculated.

3.2.4 Stability Testing

A centrifuge test was performed to each formulation 24 hours after its preparation using a Hermle Z326K refrigerated universal centrifuge (HERMLE Labortechnik, Germany). An amount of 5 g of each formulation was centrifuged at 20ºC for 60 minutes, at 3500 rotations per minute. The resultant product was then inspected for signs of creaming or separation of phases.

Each formulation was also subject to temperature variation testing. Two samples of 5 g were collected and placed in an oven, at 40ºC and 70% humidity, and in a fridge, at 4º C. The samples were then observed at 24 hours, 1 week, 2 weeks, 3 weeks and 1 month, and any changes to physical appearance were registered.

Light exposure testing was performed by placing 5 g of each formulation in direct sunlight inside a transparent container and registering changes in appearance at 24 hours, 1 week, 2 weeks, 3 weeks and 1 month.

Viscosity measurements were performed at 24h, 1 week, 2 weeks, 3 weeks and 1 month using an Ika Rotavisc me-vi Complete viscometer (IKA, China) employing the VOL-SP-7.1 spindle (IKA, China), at 2 rpm. Three measurements were performed, and an average result was calculated.

The remainder of each cream was stored in an opaque container, in a closed cabinet, at 20±5ºC, as control.

3.2.5 *In vitro* Release Studies

The release studies were performed using a single-use hydrophilic cellulose acetate membrane obtained from the Faculty of Pharmacy of the University of Milan (Italy), and conducted in 3 independent vertical Franz cells per formulation with a nominal volume of the acceptor compartment of 3 mL and a diffusion area of 0.636 cm^2. For the donor compartment, an amount of 0.1 g of each formulation (1% *w*/*w* caffeine) was initially set. The experiments were carried out at 32 °C and 1500 rpm for 6 h. The receptor media (0.9% w/v saline solution) was filtered through a 0.22 μm nylon membrane filter before use.

At 1, 2, 4 and 6 h, 0.2 mL of the receptor media was withdrawn and replaced with an equal volume of fresh media. The sample solutions were assayed by HPLC for drug content.

3.2.6 *In vitro* Permeation Studies

The permeation study was performed by using pig ear, recovered by a local slaughterhouse (Lodi, Italy). Skin samples were obtained from the ears of the pig, removed, and cut by means of a dermatome. Each skin section was cut into circles of approximately 2.5 cm in diameter, sealed in aluminium foil and frozen at -20°C. Prior to preparation, the skin was thawed to room temperature and the thickness of each membrane used for the experiments was measured. The dermatomed skin was used as a membrane placed between the two chambers of the Franz cell. The skin was carefully mounted on the lower half of the Franz cell with the *dermis* facing downwards and the *stratum corneum* side in contact with the formulation. The upper (donor compartment) and lower (receptor compartment) parts of the Franz cell were sealed with Parafilm® and fastened together by means of a clamp. The studies were performed using 3 independent vertical Franz cells per formulation with a nominal volume of the acceptor compartment of 3 mL and a diffusion area of 0.636 cm2. For the donor compartment, an amount of 0.1 g of each formulation (1% *w*/*w* caffeine) was initially set. The experiments were carried out at 32 °C and 1500 rpm for 24 h. The receptor media (0.9% p/v saline solution) was filtered through a 0.22 μm nylon membrane filter before use.

At 1, 3, 5, 7 and 24h, 0.2 mL of the receptor media was withdrawn and replaced with an equal volume of fresh media. The sample solutions were assayed by HPLC for drug content.

3.2.7 High-Performance Liquid Chromatography (HPLC) Analysis

Samples were analyzed for caffeine using an Agilent 1100 Series HPLC-UV system. The stationary reverse phase used was a Phenomenex Luna® 5 µm C18 column (150x4,6 mm). A mobile phase of water with acetic acid at pH3 and acetonitrile at a 90:10 ratio (v/v) was filtered using a 0,45 µm filter paper. The flow rate was 1.2 mL/min and detection was performed at 272 nm. Samples of 20 µl were injected in to the HPLC system where a sharp peak was obtained. Chromato-graphic peaks were identified by comparing the retention times of samples with those of the standard compound. The concentration of caffeine was calculated through the respective calibration curve. The calibration curve for quantitative analysis was linear up to 1 mg/mL.

4 Results and Discussion

4.1 Formulation Development

In order to explore which was the best formulation for the preparation of the emulsion containing each of the vegetable oils that was used, changes to the control cream base formulation were introduced in an empiric way. Caffeine was only added to the formulation in a later stage. Firstly, the paraffin oil was exchanged for each of the vegetable oils, which resulted in formulations that showed good results in terms of immediate testing but were slightly non-homogenous. After 24 hours, both the samples in the fridge and in the oven changed in appearance. The formulation in the fridge formed solid grains of the lipidic phase, and phase separation occurred in the formulation in the oven (Fig. 4). The results regarding the viscosity also drastically diminuished over time, which according to the Validation method meant these formulations were to be excluded.

Fig. 4: Formulation where paraffin oil was replaced with Olive Oil (F3) after 24h in the fridge and in the oven, respectively.

As so, white soft paraffin was then the excipient that was replaced with the vegetable oils, keeping the paraffin oil at its original concentration. This resulted in homogenous formulations with a slight yellowish coloration and a mild scent of the respective vegetable oil.

Each of these formulations was prepared twice, not only to ensure reproducibility but also to improve on some of the critical processes of the manual method. For example, it was proven that performing the mixing of the aqueous and oil phase in a room that was not climatized to at least 20ºC had an impact on the way the formulation cooled down and the droplets formed, with a faster cooling process leading to less homogenous formulations.

Vegetables oils (namely olive oil) are polar substances. They contain a mixture of polar and nonpolar components, but the polar components, such as the hydroxyl (-OH) groups in their fatty acids and glycerol backbone, contribute to their overall low-polarity. Even though both the liquid paraffin (a nonpolar alkane) and the white soft paraffin (a nonpolar hydrocarbon) are highly insoluble in water, they both still represent an ideal solvent for fats and oils. It is to be noted that paraffin oil, being a highly refined mineral oil, is less prone to oxidation than other options (32–34).

After the development, the creams obtained were characterized by measuring their pH, performing centrifuge and viscosity testing as described before, allowing for the validation and exclusion method to be performed parallelly.

Firstly, the pH of all formulations without caffeine was measured. Afterwards, the same formulations but with caffeine had their pH measured, which allowed for the conclusion that the addition of caffeine had no relevant impact in the pH. All measures were performed 24 hours after preparation.

The average pH value for the control formulation was of 6.0. When it came to the formulations containing the vegetable oils, they all present more acidic pHs, with formulation FC8.3 having an average of 4.5, and formulations FC9 and FC10 showing pHs of 5.0 and 5.1, respectively.

All the results fit within the 4-6 range of pH considered to be optimal for the skin. However, it is noticeable that the inclusion of all three oils in the formulation resulted in a lower pH result, with the formulation containing olive oil showing the most acidic result. This may be due to a higher free fatty acid content percentage. These free fatty acids, mainly oleic and linoleic acid, are a natural byproduct of the olive fruit and are released when the olives undergo the crushing and extraction process. For cooking, the lower the acidity level, the higher the quality of the oil. However, studies have shown that highly acidic olive oil is a more efficient

skin permeation enhancer vehicle than less acidic ones and can be efficiently used in formulation of cutaneous drug delivery systems (6,35).

The first stability test to be performed, 24 hours after producing the formulation, was a centrifuge test, which all formulations excelled. The dispersed phase of an oil-in-water emulsion tends to separate and rise to the top of the emulsion forming a layer of oil droplets. This phenomenon is called creaming and it represents one of the first signs of impending emulsion instability (11). However, this didn´t appear to happen to any of the formulations.

When it came to light and temperature variation testing, the most noticeable differences to the original state of each formulation and to the control formulation where regarding minor sensorial questions. Formulation FC1.3 showed lesser variation when it came to smell when compared to, for example, formulation FC8.3, which displayed a progressive increase in olive oil scent. Formulations FC9 and FC10 did not show as much variation in this regard. All formulations became paler in color in all three conditions (light, fridge, oven). It is also to be noted that the Control Formulation, FC1.3, gained a sticky feeling on the skin with time in all the conditions.

Light causes a breakdown in chlorophyll, the compound responsible for olive oil's distinctive colour. This photo-oxidation process also alters the taste and aromatic components of the oil, which can explain the macroscopic changes to the formulation containing olive oil. It has also been proven that the percentage of free fatty acids (and therefore the acidity) of olive oil increases over a period of as long as 30 months of storage in standard conditions. This means the % oleic acid increases, possibly having an impact on its success as a permeation enhancer (36). The results regarding viscosity are presented below in Table 5.

Table 5: Viscosity (mPa.s) of the formulations containing Caffeine (1% w/w): Control (FC1.3), FC8.3, FC9 and FC10.

	Viscosity of formulations (mPa.s)			
Time	FC1.3	FC8.3	FC9	FC10
24 hours	18750	16950	21550	21350
1 week	16425	15075	21825	20325
2 weeks	15650	15075	17200	17550
3 weeks	12975	15250	15825	14525
1 month	12325	14400	15050	14225

As noticeable, the emulsion that proved to be the most stable in terms of viscosity is formulation FC8.3, the formulation containing olive oil, even though the values related to the standard deviation of the three measurements proved to be quite high in all formulations except the Control.

The differences in the stability of the formulations can be due to many factors. When comparing the results of the formulation with olive oil with the ones containing almond and sunflower oils, the higher consistency in the measurements can be due to the composition of the oils themselves. Olive oil contains a higher percentage of monounsaturated fatty acids, namely oleic acid, which tends to be more stable and less prone to oxidation than the polyunsaturated fatty acids found in almond and sunflower oils. Olive oil also contains a higher percentage of natural antioxidants, such as tocopherols and polyphenols, which protect the oil from oxidation and contribute to the overall stability of the emulsion. The quality of the oils themselves can affect their composition and percentage of impurities (37,38).

Only one measurement was performed which does not allow for an accurate interpretation.

4.2 *In vitro* Release Studies

An *in vitro* release study was performed for each of the four formulations, which each contained 1% of caffeine. The experiments were carried out on two separate dates, due to a limitation in the availability of enough Franz cells needed to perform them parallelly. On one day, the release study for the control formulation (FC1.3) and olive oil (FC8.3) was performed. The study for almond oil (FC9) and sunflower oil (FC10) was carried out the following day. The same calibration curve and conditions were used for both sets of samples. New receptor media was prepared for each set.

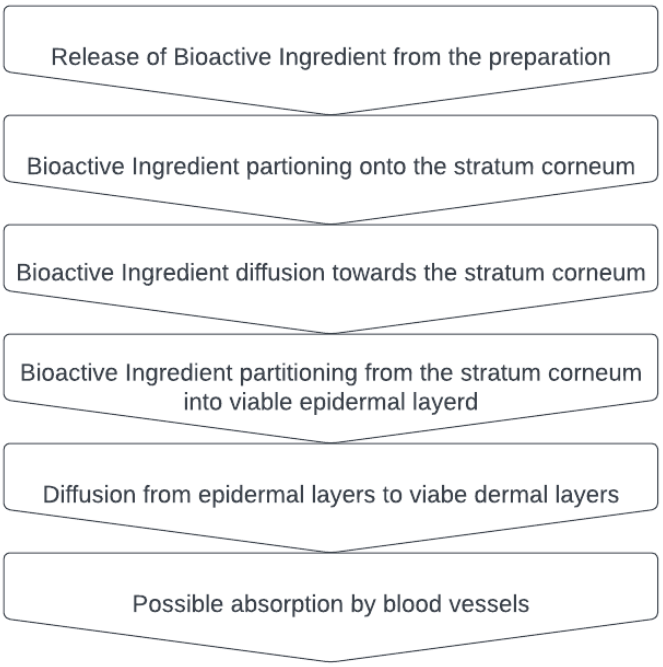


Fig. 5: Bioactive transportation across the skin. Adapted from (39).

In vitro techniques offer a high level of experimental control through simpler protocols. Nevertheless, it's important to note that these assays may not capture the full complexity of biological systems. Therefore, it is advisable to complement *in vitro* assessments with *in vivo* evaluations to validate findings and, if feasible, establish a meaningful correlation between *in vivo* and *in vitro* results. Despite this, *in vitro* assessments remain indispensable tools in the

development and initial screening of formulations, helping to predict the cutaneous absorption potential in *in vivo* settings (40).

A skin release study assesses how a bioactive ingredient is released from a topical formulation (such as a cream) when applied to the skin's surface. It focuses on the drug's release onto the external environment or the skin's outermost layers. It therefore presents some limitations as it does not accurately represent how the formulation will permeate the skin (only covering the first step presented in Fig. 5). Especially in this case, where the properties of the vegetable oil that would potentially deem them enhancers of permeability are related to the skin´s composition, the relevancy of this study is mainly related to making sure the bioactive ingredient, caffeine, is in fact being released from the formulation onto the membrane.

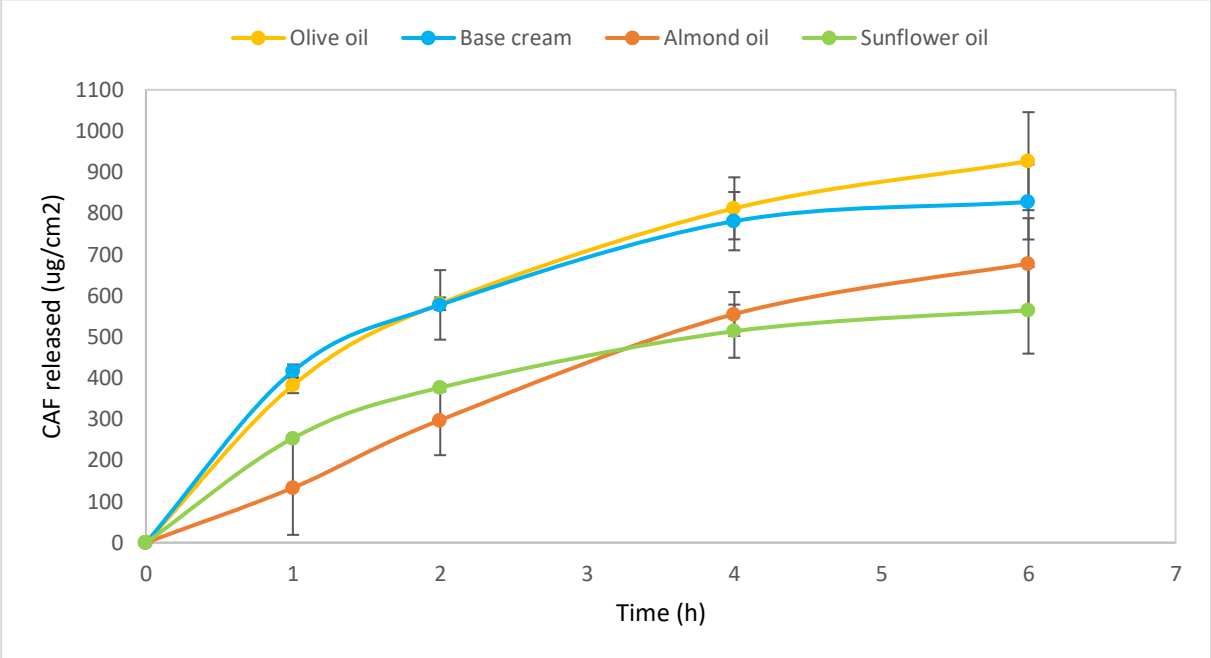


Fig. 6: Results of the release studies performed on the Formulations containing Caffeine (1% w/w): Control/ Base Cream (FC1.3), Olive Oil (FC8.3), Almond Oil (FC9) and Sunflower Oil (FC10), in amount of caffeine released (µg/cm²).

The results presented above are divided into two sets, which accurately represent the two sets of studies that were performed. This is not ideal and can be due to errors in the execution of the experimental protocol in one of the days. To minimize these differences, it would have

been preferable to perform the four studies parallelly at the same time. They do, however, as intended, show that caffeine was released from all four formulations.

4.3 *In vitro* Permeation Studies

Looking at all the previous results, out of the formulations containing vegetable oils, the one with olive oil (formulation FC8.3) has continuously shown the best results. Besides that, olive oil has the most similar composition to the skin in terms of fatty acids and the highest percentage of oleic acid, which acts a skin softener, meaning that its impact as a permeation enhancer might be of larger and more significant impact than the other alternative vegetable oils (41). As so, and due to some limitations in time, the permeability studies were only performed comparing the control formulation (FC1.3) and the formulation containing olive oil (FC8.3). The results were as follows in Fig. 7 and 8.

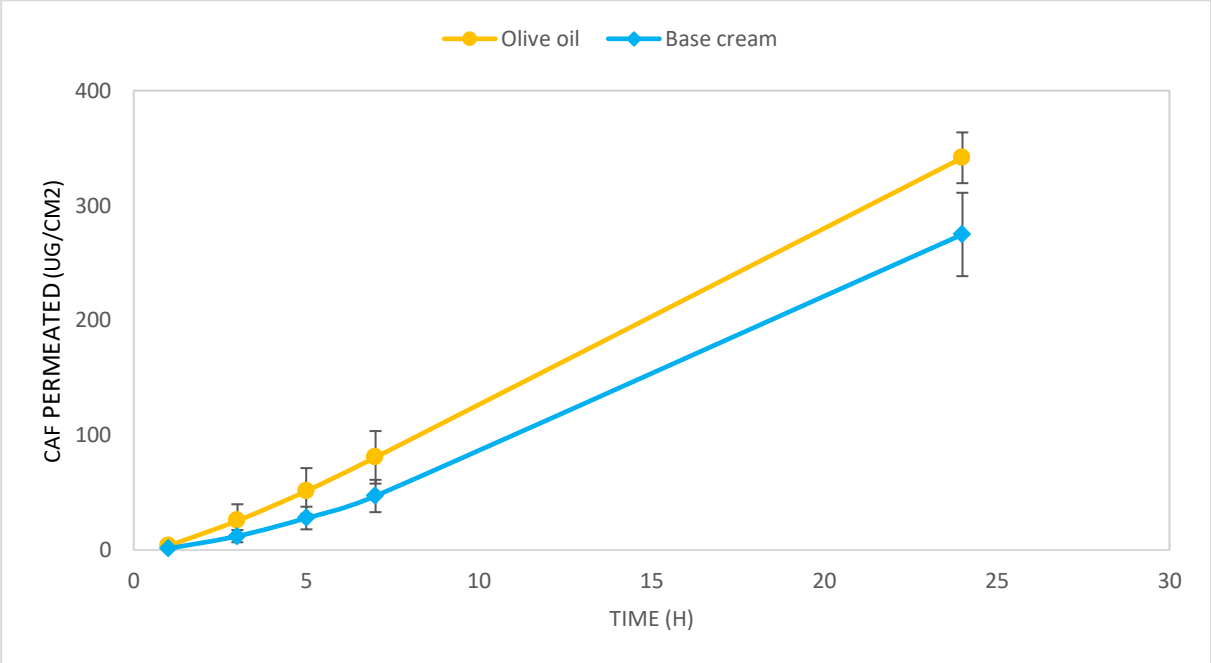


Fig. 7: Results of the permeability studies performed on the formulations containing Caffeine (1% w/w): Control/ Base Cream (FC1.3) and Olive Oil (FC8.3, in amount of caffeine permeated (µg/cm²).

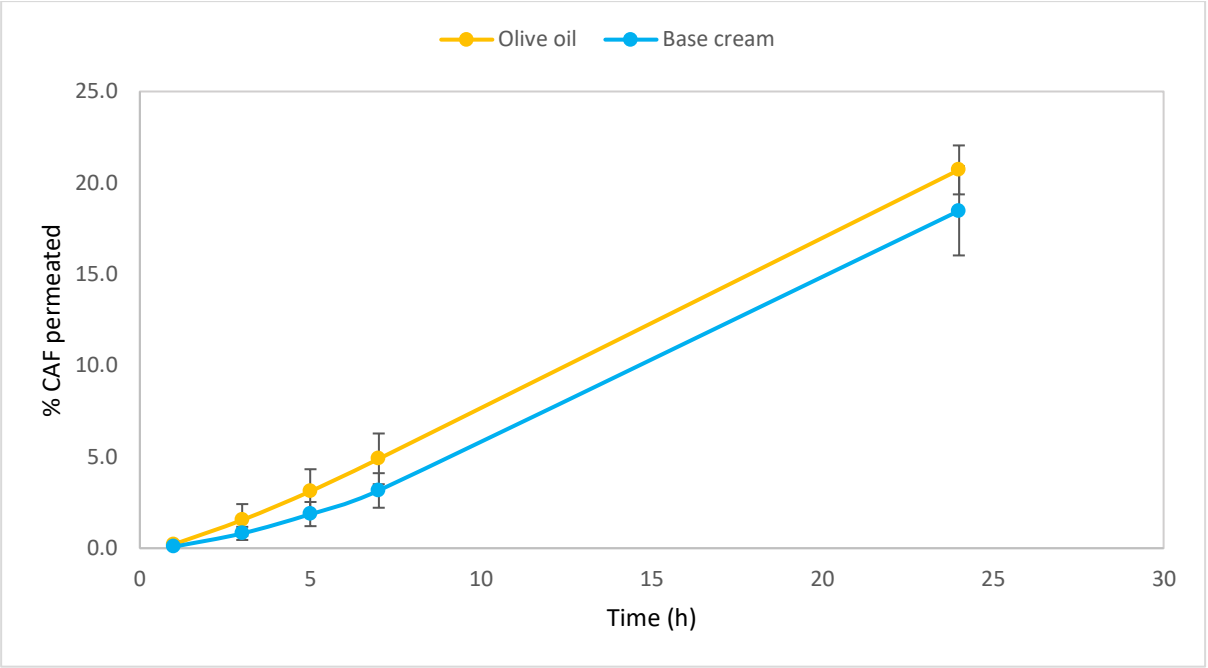


Fig. 8: Results of the permeability studies performed on the formulations containing Caffeine (1% w/w): Control/ Base Cream (FC1.3) and Olive Oil (FC8.3, in percentage of total caffeine permeated (%).

The results show that the formulation containing olive oil did in fact permeate caffeine more efficiently than the control/ base cream. However, taking the standard deviation into account, it can be debatable whether these results are of statistical significance or not. While in Fig. 6, where the results are shown in amount of caffeine released (µg/cm²), the standard deviation values of both formulations do not cross, the same cannot be said for Fig. 7, where the results are presented in percentage.

The fact that the difference in permeation for both formulations is not as significant as initially expected can be due to many different factors. Firstly, the study was only performed once, and only using three cells per formulation. If it would have had the chance of being performed in a larger number of cells and multiple times, the results that were obtained would be more reliable and their reproducibility could also be assessed.

Even though most *in vitro* permeation studies are performed using Franz diffusion cells, which is a classic yet still the most relevant technique for quantifying the degree of

enhancement/ retardation of percutaneous diffusion of active compounds, many factors related to the protocol that was followed can also have an impact (42).

The most applicable model for assessing the *in vitro* skin absorption of bioactive ingredients involves utilizing human skin samples sourced from cadavers or obtained during plastic surgeries. However, the availability of human skin is restricted, prompting the use of animal models. Animal skin serves as a highly recommended substitute, particularly in the initial assessment of new formulations. Commonly employed animal models to mimic human skin include domestic pigs, rats, mice, guinea pigs, and snakes. Notably, porcine ear skin has exhibited results comparable to those observed with normal human skin. Investigations into skin layer thickness have revealed similarities between pig ear skin and human skin, including the *stratum corneum* and *epidermis*. Furthermore, the pig ear skin closely resembles human skin in terms of its follicular structure, vascular anatomy, collagen fiber arrangement in the dermis, as well as the presence of glycosphingolipids and ceramides (40). However, pig skin is not human skin, and it does show some differences in composition that can have a slight impact.

Caffeine serves as a common hydrophilic compound employed in studies related to transdermal permeation. The ability of polar molecules, such as caffeine, to permeate the skin is influenced by the heterogeneous characteristics of the skin, including variations in lipid content within the *stratum corneum*, the presence of pores or imperfections in the *stratum corneum*, and the arrangement of skin features like hair follicles and sweat and sebaceous glands. Within the skin, intracellular lipids provide binding sites for polar molecules, facilitating their permeation. Given that oleic acid enhances permeability by disrupting the integrity of the phospholipid bilayer, therefore reducing the tight arrangement of fatty acids within the skin and creating openings that enable its integration into the skin, it can be said that this mechanism perfectly complements caffeine´s way of permeating the skin. However, for polar molecules that tend to show saturable binding to the polar heads of skin lipids, the permeation will be sensitive to the variation of lipid content across the skin used in the donor compartment. Therefore, inhomogeneity in the skin will result in a higher standard deviation of permeation data (23,43). This means that errors in the preparation of the skin or the measurement of its thickness might be relevant.

Furthermore, as previously mentioned, literature has deemed the term “permeation enhancers” as incorrect, mentioning that “permeation modifiers” is a much more accurate way

of describing the type of properties these substances present. This is mainly due to the fact that the activity of a dermal permeation modifier can be that of an enhancer or a retardant depending on factors such as its shape, H-bonding potential, polarity, chemical structure and the accompanying formulation. The latter can play a major role in this case, as highly occlusive substances like liquid paraffin were included in the formulation, in high concentration. Multiple studies have shown that dissolving permeation modifiers in various vehicles has a tremendous impact on the behavior of the substance when it comes to either enhancing or retarding the permeation of the active across the skin. It can therefore be proposed that for an excipient to be considered a true enhancer, it should prove to always depict enhancement, no matter which formulation it is incorporated in (42).

A parameter that might me deemed interesting to access is the Penetration Modifier Ratio (PMR), which can be assessed and compared to references in literature for other compounds (42). The equation for its calculation is as presented below:

$$PMR = \frac{Amount\ of\ penetrated\ agent\ in\ presence\ of\ penetration\ modifier}{Amount\ of\ penetrated\ agent\ in\ absence\ of\ penetration\ modifier}$$

Table 6: PMR calculation results per withdrawal time and average PMR.

	Time					Average PMR
	1h	3h	5h	7h	24h	
PMR	2.77±0.52	2.11±0.49	1.85±0.18	1.72±0.03	1.24±0.18	1.93±0.56

With the results presented in Table 6 in mind, it can be hypothesized that the impact the fatty acids in the olive oil had on the permeation of caffeine was greater in the first few hours and decreased proportionally over time. This most likely means that the onset for permeation for the formulation with olive oil was much earlier than for the control formulation, which is a

big advantage when you take into account that cream formulations usually don’t remain on the skin for long. This way, a larger amount of bioactive ingredient is absorbed in a shorter amount of time.

5 Conclusions

Given the enormous growth of the Cosmetic Industry in recent years, one of the main concerns is ensuring active substances are permeating through the demanding barrier that is the skin to the best of their abilities, thus leading to enhancing their action at lower concentrations. As so, one of the strategies that is being widely explored is the use of substances that will support permeation, with their mechanisms of action mainly targeting the penetration of the *stratum corneum*, considered the toughest layer for bioactive ingredients to cross.

Vegetable oils, rich in fatty acids, have emerged as a strong contender in terms of their ability to provide a faster and more extensive permeation for active substances across the skin, by disruption of the phospholipidic bilayer, perturbating the tight packing of fatty acids and getting incorporated into the skin, facilitating the dermal delivery of substances included in the formulation. One of the fatty acids for which this has been majorly reported is oleic acid, a main components of olive oil.

The work presented in this dissertation aimed to study how the inclusion of different vegetable oils in a reference formulation influenced its physical and sensory characteristics, as well as its stability over time. Release and permeation studies were also performed to try and understand the true impact these oils can have on permeation, in this case using a common hydrophilic compound that is traditionally used in this type of studies and has proven benefits to the skin: caffeine.

Three vegetables oils (olive oil, almond oil and sunflower oil) were included in a base cream formulation taken from the Italian Pharmacopeia by swapping out one of the mineral oils that was originally present. Firstly, paraffin oil was traded with the oils, but after discouraging results, taking out the white soft paraffin for the vegetable oils proved to be a better option. The caffeine was then added to these formulations at 1%, and they were then studied for the period of one month, with weekly assessments as for macroscopic characteristics (in different stress conditions) and viscosity. These evaluations allowed for the understanding that the formulation with olive oil proved to be the best over time. It would have been interesting to try and include other excipients to the formulation that would help with some of the problems that were faced.

For example, perfumes for masking the unpleasant scent of the vegetable oils, often associated to food, or preservatives to improve shelf life and microbiological stability.

The release study was mainly performed to ensure the caffeine was being released from the formulation, which proved to be the case, even if the results themselves were not ideal, and were divided into the two sets of experiments that were performed. These should have been repeated and, if possible, performed for the four formulations all at once to minimize the errors that caused the discrepancies in the results. As for the permeation study, only the olive oil (as it showed the most promising results in all other fields) was assessed against the control formulation. It would have been ideal to perform this study for all four formulations to get a better assessment of how vegetable oils as a whole can work as permeation enhancers. In a future study it would be interesting to include even more different options for vegetable oils to get a true reading on which is the best option. The results of the permeation study did in fact show better penetration of the skin for the formulation with the olive oil, even though it was not a very differentiated result. This might be due to the other excipients in the formulation, which have previously proven to be able to make substances with permeation enhancing potential into permeation inhibitors. Testing of how different excipients influence the oil's permeation enhancing abilities might be a good study to perform, as to understand how to maximize its potential and even manipulate this feature for the intended purpose. As showed, the permeation not only was improved but also showed an earlier onset, which might be interesting when it comes to active ingredients that usually have a difficult time permeating the skin, unlike caffeine.

In conclusion, the use of vegetable oils in topical formulations can represent a relatively sustainable manner of improving the way some active ingredients permeate the barrier the skin imposes. The inclusion of these oils mostly improved the characteristics of what is considered a traditional base cream formulation, with olive oil presenting itself as the best option out of the three that were evaluated. The results presented in this study might present a baseline for future analysis of their potential in different applications.

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