EFFECT OF INSECTICIDE DIETHYLTOLUAMIDE (DEET) AND CO-APPLIED SUNSCREENS ON PERCUTANEOUS ABSORPTION

By NATHALY CRISTINA MARTOS GIBAILE

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ABSTRACT OF THE THESIS

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Thesis Advisor: Bozena Michniak-Kohn, Ph.D.

The combination of sunscreens and insect repellents is widely used by the population, in all regions of the globe. Several published papers reported that the concomitant use of oxybenzone and N,N-diethyl-m-toluamide (DEET), common actives present in such products, can enhance the percutaneous permeation of each of the actives which is an undesirable outcome. In this study, we evaluated the effects of the insecticide DEET on the permeation of sunscreens octyl methoxycinnamate and octyl salicylate. Several combinations of the UV absorbers and the insect repellent were tested and percutaneous permeation of all actives was compared when they were co-applied on human skin, *in vitro.* The outcomes of these studies suggest that DEET did not enhance the skin permeability of octyl salicylate and octyl methoxycinnamate. However, the UV absorbers can be potential enhancers when mixed with DEET, because when the sunscreen actives were used in combination with DEET, the resulting skin permeation of the insect repellent was higher than the control.

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DEDICATION

I would like to dedicate this to my mom, Simone, for supporting my decisions to pursuit a better education so far away from home. Thank you mom, for having faith in me, for giving me all the right principles to be who I am today and for allowing me to follow my dreams. I would never have done it without you.

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INTRODUCTION

1.1 PURPOSE OF STUDY AND SIGNIFICANCE

Sunscreens are widely used to protect the skin against the ultraviolet rays (UVR) [1]. The active ingredients can be organic or inorganic and this will affect how the sunscreen will block the UV rays. Organic filters have been used for a long time and they absorb the rays by making conformational molecular changes on their structure and releasing heat. They are usually not very stable to light, and it is necessary to combine several filters to obtain a stable solution with broad spectrum activity. Inorganic filters are usually very photostable and they cause less skin sensitization than the organics. It is very common to find products that have both types/classes of actives in the composition [2, 3].

The quality of a sunscreen can be defined by the amount of skin permeation that is achieved. An ideal sunscreen stays on the outer layer of the skin, the epidermis, and does not permeate to the dermis or even to the blood. However, most of the studies showed that some organic actives are able to permeate the skin and can be detected in the urine after a few days following application [4, 5].

Because of the increasing necessity to protect the skin against UV rays and to avoid mosquito borne diseases, it is common to co-apply sunscreens with insect repellents. Some studies have reported that the combination of organic filters, such as oxybenzone and DEET (insect repellent) can result in an increase of the transdermal permeation of both actives [6, 7]. Besides, there

is a lot of discussion related to the fact that sunscreens can have a decreased efficacy when used with repellents [8, 9].

The UV rays are responsible for some types of skin cancers and because of the growing number of people that are affected, the use of sunscreens is being advocated to the population [10]. The study of the interaction between insect repellents and sunscreens is very relevant, especially to hot climates, where the combination of these products is common and used in all ages, including children.

The purpose of this this study was to help to elucidate the interaction between the sunscreen actives and insect repellents. The skin permeability of two commonly used organic filters was tested alone and in combination with the insect repellent DEET.

BACKGROUND

2.1 SKIN PHYSIOLOGY

Normal human skin is basically constituted of three layers: the dermis, which has connective tissue and blood vessels, the epidermis, which is avascular and is mainly composed of keratinocytes and the hypodermis that is responsible for the accumulation of fat. The *stratum corneum* is the upper layer of epidermis and is responsible for the skin impermeability because its lipid-rich matrix [11]. This outermost layer is 10-15μm thick with multilayers of corneocytes surrounded by hydrophobic lipids organized into lamellar structures, giving this layer a brick and mortar organization [12]. Besides that, it has touch, pain and pressure receptors, prevents extensive loss of water and keeps the thermoregulation of the body. The dermal-epidermal junction keeps the epidermis and dermis connected and increases the cohesion between them. It is also a barrier for large molecules, but not as important as the epidermis in diffusion studies [13].

The epidermis, the outermost layer, is a stratified squamous epithelium and it is histologically divided into sub-layers [14]. The structure and thickness of the epidermis can be different along the body and each layer is different from the other, being responsible for texture and humidity of the skin. The keratinocytes, melanocytes, Langerhans cells and Merkel cells are the most important cells that are found in the skin, each one having a different function and location. The first layer is located right above the basal membrane, which

connects the epidermis and dermis. The basal layer is responsible for the constant renew of the skin's cells (approximately 40 to 56 days, according to the age), because of its proliferating keratinocytes [15]. The spinous layer is mainly constituted of keratinocytes that differentiates producing lamellar bodies while they make its way to the *stratum corneum* (maximum of differentiation) and Langerhans cells that provide immunological protection. The granular layer contains enucleated keratinocytes that have only a granular cytoplasm and the extracellular space between the cells is filled with the lamellar bodies that were released through exocytosis [16]. When these cells reach the corneum layer, they have keratin all over the cytoplasm, forming a "brick wall" of corneocytes. The corneocytes are surrounded by the intercellular lipids, resulting in a protection barrier that avoid the penetration of pathogens or toxins and keep the skin hydrated. The *stratum corneum* is constituted of 40% protein, 40% water and 18-20% of lipids and beneath the epidermis, the dermis contains blood vessels that connect the tissue with the systemic circulation, so if a molecule penetrates the outermost layer (the *stratum corneum* diffusion is a rate limiting-step) [17], it can easily reach the dermis and enter the blood thorough passive diffusion. The dermis is divided into two layers, papillary dermis which is superficial and contains blood vessels that provides nutrition for the epidermis above and reticular dermis, which is thicker and contains all the sebaceous glands, sweat glands, hair follicles, blood vessels, lymph vessels and nerves.

The skin is designed to be a protective organ, blocking the entrance of unknown substances, like pathogens and toxins and limiting the penetration of large hydrophilic molecules, so the active has to be able to penetrate the skin considering these barrier properties [18]. In order to permeate the layers, the active can use the transcellular pathway (through the keratinocytes), paracelullar pathway (through the lipid matrix between the keratinocytes), and transappendageal pathway (across and along the shafts of hair follicles) [19, 20]. However, the active is only going to have a systemic action, if at first it can penetrate the *stratum corneum*, which behaves as a semi-permeable barrier, by passive diffusion [21].

2.2 PERMEABILITY OF THE HUMAN SKIN

The skin permeation of the substances is directly related to their size, oil solubility and partition coefficient [22]. The octanol-water partition coefficient ($K_{o/w}$) describes the transdermal permeability of many actives and it can be determined by doing an experiment in which the drug is mixed with water and a lipophilic solvent (n-octanol) that represents the oil phase of the skin [23]. The concentration of the drug in the oil phase divided by the concentration of the drug in the water phase gives the coefficient. The log P is a logarithm of this ratio and each substance has a known value that can also be considered a measure of lipophilicity. If the molecule is highly hydrophilic, its log P and its penetration will be low [24], at the same time, if a substance has a high log P, that means that it is lipophilic and it has a high skin permeation. The partition coefficient is an important indicator; since molecules with hydroxyl groups

(polar) will have a lower permeation and substances that are highly soluble in oil can stay in the stratum corneum, instead of penetrate into the skin with the vehicle. So, the ideal partition coefficient dose is between the affinity of the oil phase and the water phase [24].

The passive diffusion occurs when there is a difference of the concentration of a drug that is reduced by a spontaneous flux that intends to balance the difference (the molecules go from the most concentrate region to the less concentrate region) [25]. The transdermal permeation of molecules is usually studied using the Franz diffusion experiments [26]. The apparatus can be vertical or horizontal, but it is always consisted of two chambers separated by a membrane, which is usually human, animal skin or an artificial membrane. The active that is going to be tested is applied to the skin, located at the donor receptor, and samples are taken at determined intervals from the receptor compartment, which is filled with a buffer and kept at a constant temperature of 37°C. This experiment will determine the concentration of the active at each time point, showing how much of the active permeated the membrane.

The flux between the two compartments is proportional to the concentration gradient and can be defined by the Fick's First Law that describes the diffusion in a steady-state condition, which means that the difference does not change with time [27].

In sum, some actives can penetrate the skin through the stratum corneum, epidermis, dermis and finally will arrive into the blood stream. Other

factors that have an effect in the skin permeation of the active are its size, log P, transport pathway, ionized state and even the condition of the skin (normal or diseased). In addition, considering that the *stratum corneum* is permeable to the drug, its permeation through the skin can be described by Fick's First Law, the partition coefficient and the diffusion coefficient. However, a UV absorber, used in sunscreens, should not have the ability to permeate the skin. It has to stay on the top layer of the skin, in order to properly absorb the UV light and protect the cells against the UV damage.

2.3 EFFECTS OF UV LIGHT ON THE HUMAN SKIN

2.3.1 TYPES OF UV LIGHT

Solar radiation is consisted of UV rays, which are part of the electromagnetic spectrum and they can be divided into UVA, UVB and UVC. The UVC rays (200-290 nm) are toxic and can cause mutations on the cells, however, most of it is filtered by the ozone layer and does not present a health risk [28]. The UVA rays (320-400 nm) are correspondent to 95% of the ultraviolet spectrum and can also be divided into UVA1 (340-400) and UVA2 (320-340 nm). They penetrate into the deeper skin layers, like dermis, affecting blood vessels and connective tissues. UVA rays are not absorbed by nucleic acid as much as UVB rays [29] and since the dermis has a lower renew capacity, it is more affected than the epidermis. The exposure to sunlight leads to inflammation and the DNA can be indirectly damaged because of the increase of the production of reactive oxygen species (ROS). Besides that,

several molecular changes, including the degradation of collagen and elastin, lead to the development of premature ageing and wrinkles [30]. One of the defense mechanisms that occurs in order to prevent the skin damage is the photo-oxidation of melanin that is already present in the epidermis [31].

On the other hand, the UVB rays (290 – 320 nm) only penetrate in the upper layers and does not reach the dermis. Its exposure is dangerous and it is related to the most causes of skin cancer. The main mechanism of action is the formation of pre-mutagenic lesions on DNA (Cyclobutane Pyrimidine Dimer Mutations and 6.4 Photoproducts), which are repaired most of the times by the cell, however, sometimes the lesions are not repaired, the polymerases are inhibited and a mutation is originated [32]. Besides, the exposition to UVB causes a thickening of the epidermis and delayed tanning. This process happens after 72h after exposure because the melanocytes are stimulated by the UV light and the production of new melanin is increased [33].

2.3.2 TUMOR DEVELOPMENT

The exposition to UV light can lead to the development of skin cancer via the DNA mutations caused by the UVB and the productions of ROS activated by UVA [34]. There are two types of skin cancer: melanoma and non-melanoma. Non-melanoma cancers are the most common and they can also be divided into basal cell carcinoma or squamous cell carcinoma [35]. Both of them usually appear on areas that are exposed to sun, however, basal cell carcinoma usually grow slowly and it has a bigger chance of recurrence after

its removal. Squamous cell carcinoma is related to a chronic sun- exposition, it grows into the deeper layers of the skin and it can easily spread to other tissues [36]. The sun-exposure is not the only cause that leads to non-melanoma, some studies observed that genetic mutations can also be responsible for this cancer [37].

Melanoma is the most fatal and aggressive type of cancer and it originates in the cells that produce the melanin, the melanocytes [38, 39]. People with fair skin have a higher chance to develop this form of cancer when exposed to UV light. There is also a genetic predisposition that can lead to the disease, but the effects caused by UV rays are directly linked to the immunosuppression and mutagenic effects on melanocytes [40].

2.4 SUNSCREENS

Sunscreens are an efficient way to prevent the effects of UV rays. The minimum sun protection factor (SPF) recommended by the FDA is 15 and the sunscreen needs to be reapplied every 2 hours. Products cannot be labelled as water-proof or sweat-proof, and if it states on the label that a sunscreen is water-resistant, it is important to inform for how long the protection is effective [41]. Besides, the SPF has to be tested *in vivo* in order to establish the protection of the product. The SPF is the measurement of UVB protection and it is basically the amount of light that makes a protected skin red/ the amount of light that makes an unprotected skin red [42].

An ideal sunscreen usually stays on the top of the skin either absorbing or reflecting the UV rays. Organic (chemical) filters are aromatic molecules with a carbonyl group, they protect the skin by absorbing the UV rays and releasing low-energy rays [43]. This mechanism can cause molecular changes in the structure, making it photo-unstable. For instance, a very commonly used UV absorber is avobenzone, which has a broad-spectrum, but it is extremely unstable, so it always has to be combined with other organic or inorganic filters, in order to properly protect the skin when exposed to light [44].

Inorganic (physical) filters, such as ZnO and TiO_2, are useful against UVA and UVB. They are very stable, non-irritant and the permeation of the actives is very low. These type of filter protects the skin by reflecting the UV light, without any conformational changes in the molecule [45]. It is very common for a sunscreen to have both types of filters in its composition, because the organic filters are easier to use in formulations, however, most of them protect only against UVB, so inorganic filters are added to stabilize the emulsion and to enhance the protection against UVA. The problem about inorganic filters is that when used in high concentrations, they form a thick, white and difficult to spread emulsion. Because of that, nanotechnology was used to try to improve the cosmetic feel of inorganic filters and even increase the reflective properties [46]. On the other hand, some studies showed the concern of a possible skin permeation of nanosunscreens, because of their size. Until now, it was not observed any penetration of the actives [47, 48], but it was observed that they can have toxic and mutagenic effects on keratinocytes

[49]. Besides, nanoparticles can bind to proteins, leading to autoimmune diseases [50], so it is still not completely understood the safety of nanosunscreens.

So far, only 17 sunscreens actives are allowed by the FDA and only some of it is still being widely used on products: oxybenzone, avobenzone, octinoxate, octisalate homosalate, octrocrylene, Titanium Dioxide and Zinc Oxide. Several compounds that are approved in Europe, Asia or South America are not approved in the U.S and that makes the development of new sunscreens very limited.

2.5 INSECT REPELLENTS

The use of insect repellents is extremely important especially in tropical regions, because they not only protect against mosquito bites, but also against vector-borne diseases. The most used active is DEET (*N*,*N*-diethyl-3-methylbenzamide), which has been on the market for over 60 years [51]. The mechanism of action of this insect repellent is the creation of a vapor barrier that activates an odorant receptor, providing a bad odor and taste on insects [52, 53]. Despite of being widely used, DEET has a strong odor and it can be irritant to the skin. Besides, its skin permeation is considerably high and several studies reported that in human and rats this active can be found in the urine [54-56], blood and blood cord of pregnant women [57, 58]. The concern about DEET is that it can be toxic when used in high concentrations. It was observed that it can induce central nervous symptoms [59], cardiovascular symptoms and

allergic symptoms and it can be fatal if ingested [53]. There is a warning on the FDA website recommending that people should never reapply a sunscreen that contains DEET, because several applications can cause serious toxic effects, and also, products containing DEET should never be used on children with less than 2 years old. Since there are so many evidence about the possible toxicity of DEET and the importance of minimizing its penetration, this active was chosen to be used on this study to observe its interaction with some organic sunscreens.

One of the options to replace DEET is the use of Picaridin. This active is widely used on Europe and Asia because its odorless, non-greasy and it has less irritant effects than DEET [53].

2.6 CO-APPLICATION OF SUNSCREENS AND INSECT REPELLENTS

The co-application of insect repellents and sunscreens has become more prevalent over the years and as a consequence, the interaction between the actives is being studied. It is important to know that both products have to stay on outer layers of skin and do not permeate to the dermis in order to be completely efficient. As mentioned before, DEET is known to easily permeate the skin barriers and also it can be considered a permeation enhancer [60]. Besides, one of the most used UV absorber, oxybenzone (benzophenone-3), can also permeate easily the skin, causing irritation and contact dermatitis [61]. Human studies found this active in urine [62] and endocrine disruption was also

observed in animal studies caused by oxybenzone and its metabolite benzophenone-1, because both have affinity to estrogen receptors [63-65].

It is also known that some UV absorbers can interact with each other, enhancing their skin permeation [66], so the same could happen when different products are co-applied. Since many products on the market have DEET and oxybenzone, several studies tried to elucidate if there is an interaction between them and if their efficacy can be affected when they are co-applied. First, it was observed that DEET can decrease the efficacy of the sunscreen [8] by 33%, but the properties of the insect repellent were not affected [9]. After that, it was observed that the permeation of DEET and oxybenzone were increased when they were used concomitantly in artificial membranes, piglet skin and human skin, especially when dissolved in ethanol [6, 67-69]. It was also observed that the droplet size and type of emulsion can have influence on the transdermal permeation of the actives. When a thickening agent was used in the formulation of a sunscreen, the final droplet size of the emulsion was reduced and that caused an increase on the permeation of oxybenzone and DEET [70]. The effects of Picaridin when mixed with a sunscreen were also observed and this insect repellent did not have an increase on its permeation and when co-applied with oxybenzone, the UV absorber had a decrease on its skin permeation. This results were the completely opposite to when DEET was used instead of Picaridin, indicating that it is not all the insect repellents that affect absorption of sunscreens [7]. In most of this experiments, only emulsions containing the

actives or even products that are already available on the market were utilized, but the active alone was not tested.

Since many studies elucidated that DEET and oxybenzone can act as enhancers to each other, on this study we planned to test different UV absorbers, in order to determine if this interaction is valid for all organic sunscreens. Besides, the actives were used in their standard state in the experiments and not dissolved on emulsions or lotions, in order to eliminate any external influence on the percutaneous permeation, like the addition of thickening agents or other emollients and surfactants.

The UV absorbers octyl methoxycinnamate and octyl salicylate were used in several combinations with DEET to evaluate if the skin permeation of any of them was affected when they were co-applied onto human skin, *in vitro*. There are no studies, until this day, that compare the transdermal permeation of octyl methoxycinnamate and octyl salicylate with DEET, so the results are not based on any previous information. However, in a previous study, octyl methoxycinnamate had a significant skin permeation that was observed *in vivo* and it could also be detected in plasma and urine [71]. There is also a lack of information about the effects of octyl salicylate on the skin, but it was observed that it has a low permeation through human skin *in vitro*.

METHODS

The sunscreen actives octyl methoxycinnamate (OM) and octyl salicylate (OS) were a gift from Polytherapeutics, Inc. (Lakewood, NJ, USA). Diethyltoluamide (DEET) and polyoxyethylene 20-oleyl ether (Brij® 98) were purchased from *Sigma*-Aldrich (St. Louis, MO, USA). Ethanol, methanol (HPLC grade), potassium phosphate monobasic, water (HPLC grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.1 HUMAN SKIN PREPARATION

Human skin obtained from The New York Firefighters Skin Bank (New York, NY), dermatomed to 400-1500 μm thickness was kept at -80°C and then moved to –20 °C the night before the experiment. At the beginning of the study, the skin was cut into pieces using sterilized scissors and was soaked in filtered pH 7.4 phosphate buffered saline (PBS) for 15 minutes to prevent the dehydration and allow it to thaw.

3.2 DIFFUSION STUDY

The permeability experiment was prepared using amber jacketed Franz diffusion cells with 5 mL receptor volume, 1 mL donor compartment and a donor area of 2.01 cm^2 (PermeGear, Hellertown, PA, USA). They were previously washed and rinsed with deionized water. The Franz cells were connected to a circulating water bath (PermeGear, Hellertown, PA, USA) and a magnetic stir bar was placed in each receptor compartment of the cells. Approximately 2 cm^2

of the skin was mounted between the receptor compartment and the donor compartment and with a metal clamp. The receptor compartment was filled with 5 mL of PBS (pH 7.4) with 4% Brij® 98 (w/v), the surfactant allowing the hydrophobic actives to solubilize [69]. The cells with the mounted skin were left for 10 minutes at 300 rpm to reach 37 °C before the application of the actives on the skin. The donor compartment was occluded with Parafilm after the application of 1 mL of the sample, keeping the skin under infinite drug dosing during the study. The concentrations of actives used were 150 mg/ mL for DEET, 5 mg/ mL for octyl methoxycinnamate and 7.5 mg/ mL for octyl salicylate applied either individually or in combination. The actives were weighed, dissolved in 50:50 v/v of ethanol/water and the concentrations used were equal to the maximum allowed in commercially available products in the U.S.

An aliquot from the receptor of the Franz cell (300 µL) was collected every hour for 10 h and the same volume of PBS was replenished at each hour. Six replicates were made for each experiment and the concentrations of the actives in receptor samples were analyzed using a previously validated HPLC method.

3.3 HPLC ASSAY DEVELOPMENT

The HPLC assay for the detection of all the actives was based in previous studies [7, 72], but were adapted and modified in order to optimize them for this study. The system used was a HP Agilent 1100 HPLC System

(Agilent, Waldbronn, Germany) together with a Symmetry® C18 column (3.9 x 150 mm, 5 µm) (Waters, Milford, MA, USA).

The mobile phase A was methanol 88% v/v and mobile phase B was water 12% v/v, delivered at a flow rate of 1.0 mL/min [73]. The detection wavelength was 310 nm for octyl methoxycinnamate [74] and 238 nm for octyl salicylate and DEET [73], analyzed at 20 °C. This absorbance value was established after analysis of the full absorbance spectrum of each compound using a Cary 60 UV-Vis Spectrophotometer (Agilent, Waldbronn, Germany). Figure 1 shows the absorption of octyl methoxycinnamate in eight concentrations from 1 µg/ml to 1000 µg/ml. The average retention time on the HPLC column was 1.4 min for DEET, 5.6 min for octyl methoxycinnamate and 7.3 min for octyl salicylate respectively.

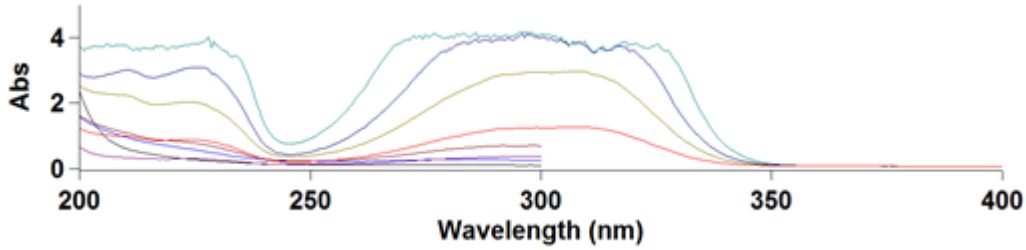

Figure 1 - Octyl methoxycinnamate absorbance (200-400 nm). – The highest absorbance was observed in 310 nm.

3.4 PREPARATION OF STANDARDS SOLUTIONS

The stock solutions were prepared in 25 mL volumetric flasks, each active was weighed and the volume was completed with methanol, resulting in a 1 mg/mL solution. Serial dilutions were made using methanol, resulting in the standards: 1, 2.5, 5, 10, 25, 50 and 100 µg/mL.

3.5 HPLC METHOD VALIDATION

Linearity

Linearity verified if the area under the peak obtained by the chromatographic evaluation was linearly proportional to the concentration of each sample.

The calibration curve was determined using seven standard solutions ranging from 1 to 100 µg/ml. They were analyzed three times for DEET and four times for octyl methoxycinnamate and octyl salicylate. The acceptability of linearity was based on the correlation coefficient of >0.999 [75].

Linearity for DEET was observed with a correlation coefficient (R^2) value of 0.9999 and the equation obtained was y = 25,148x - 2,2238 – Table 1 - Figure 2.

Table 1 - Standard concentrations, average of the peak, standard deviations and the percentage of relative standard deviation (%RSD) of DEET.

Concentration (µg/ml)	Day 1 (mAu*S)	Day 2 (mAu*S)	Day 3 (mAu*S)	Average (mAu*S)	Standard Deviation	%RSD
1	25.58	25.47	25.25	25.40	0.22	0.87
2.5	64.07	64.30	65.35	64.57	0.68	1.06
5	123.81	124.70	125.55	124.69	0.87	0.70
10	245.89	243.13	244.56	244.56	1.38	0.56
25	607.93	615.03	605.65	609.54	4.89	0.80
50	1262.88	1278.30	1281.65	1274.28	10.01	0.79
100	2476.41	2511,63	2534.65	2507.56	29.33	1.17

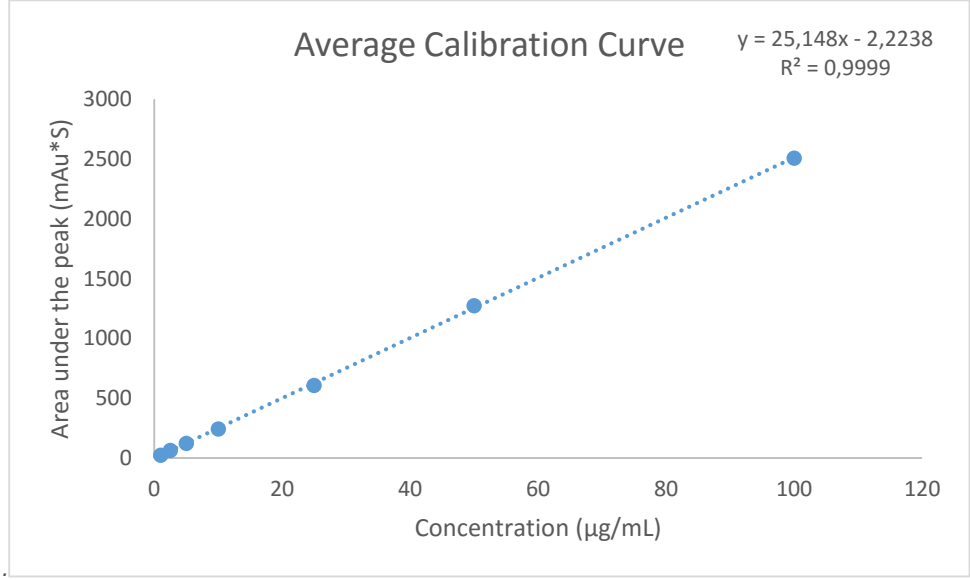

*Figure 2 - Calibration curve of absorbance (mAu*S) x concentration (µg/mL) of DEET.*

Linearity for octyl salicylate was observed with an R^2 value of 0.9997 and the equation obtained was y = 44,1x - 33,318 – Table 2 and Figure 3.

Table 2 - Standard concentrations, average of the peak, standard deviation and the percentage of relative standard deviation (%RSD) of octyl salicylate.

Concentration (µg/ml)	Day 1 (mAu*S)	Day 2 (mAu*S)	Day 3 (mAu*S)	Day 4 (mAu*S)	Average (mAu*S)	Standard Deviation	%RSD
1	77.00	77.07	78.67	76.70	77.36	0.89	0.99
2.5	179.99	181.08	181.47	181.00	180.88	0.63	0.30
5	378.74	379.46	383.97	380.80	380.74	2.31	0.53
10	767.51	772.79	790.40	766.60	774.33	11.06	1.24
25	1925.98	1941.64	1983.60	1991.10	1960.58	31.71	1.40
50	3858.46	3846.43	3836.53	3839.00	3845.11	9.84	0.26
100	7973.17	7960.12	7980.33	8003.00	7979.16	17.96	0.23

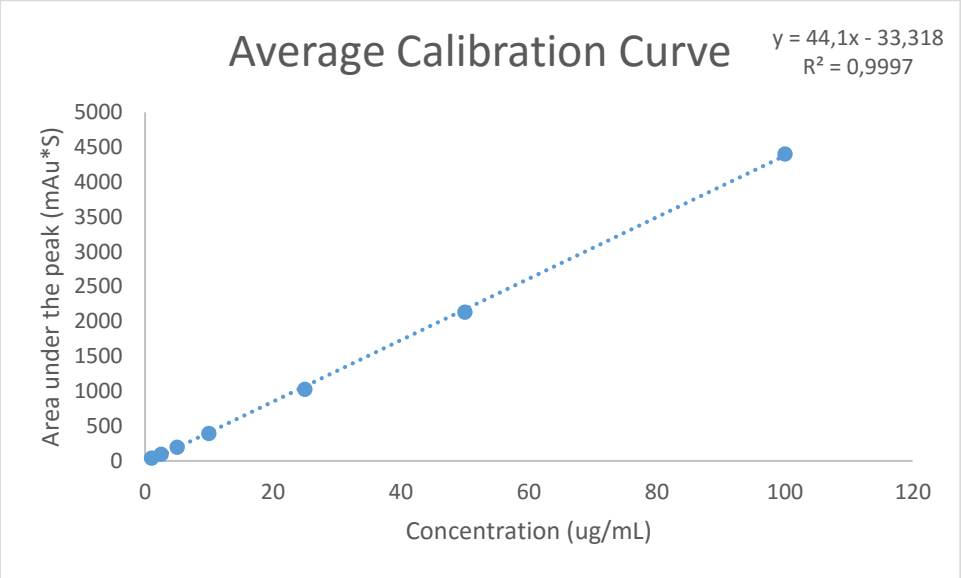

*Figure 3 - Calibration curve of absorbance (mAu*S) x concentration (µg/mL) of octyl salicylate.*

Linearity for octyl salicylate was observed with an R^2 value of 0,9997 and the equation obtained was y = 79,567x - 28,286 – Table 3 and Figure 4.

Table 3 - Standard concentrations, average of the peak, standard deviation and the percentage of relative standard deviation (%RSD) of octyl methoxycinnamate.

Concentration (µg/ml)	Day 1 (mAu*S)	Day 2 (mAu*S)	Day 3 (mAu*S)	Day 4 (mAu*S)	Average (mAu*S)	Standard Deviation	%RSD
1	77.00	77.07	78.67	76.70	77.36	0.89	1.15
2.5	179.99	181.08	181.47	181.00	180.88	0.63	0.35
5	378.74	379.46	383.97	380.80	380.74	2.31	0.61
10	767.51	772.79	790.40	766.60	774.33	11.06	1.43
25	1925.98	1941.64	1983.60	1991.10	1960.58	31.71	1.62
50	3858.46	3846.43	3836.53	3839.00	3845.11	9.84	0.26
100	7973.17	7960.12	7980.33	8003.00	7979.16	17.96	0.23

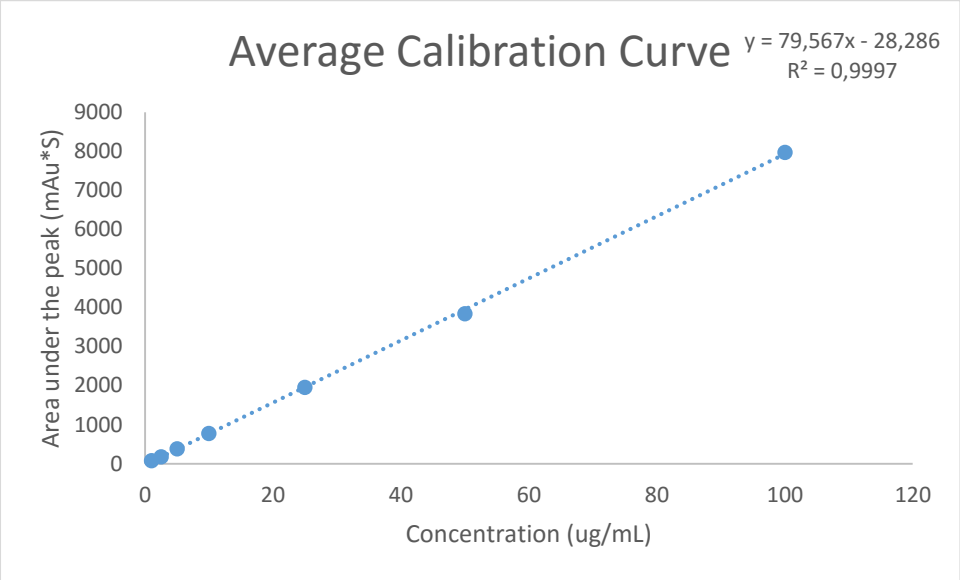

*Figure 4 - Calibration curve of absorbance (mAu*S) x concentration (µg/mL) of octyl methoxycinnamate.*

Precision

The method is precise when the test is applied repeatedly to the same sample and the results are reproducible. The repeatability of this method was obtained by %RSD of eight replicates at concentrations of 10, 50 and 100 µg/ml. All the results had a %RSD lower than the required 2% - Table 4.

Table 4 - Analytical performance of the method: precision values of DEET, octyl methoxycinnamate and octyl salicylate. SD is standard deviation and %RSD is relative standard deviation.

	DEET			OM			OS		
Concentration (µg/ml)	Measured value (mean)	SD	%RSD	Measured value (mean)	SD	%RSD	Measured value (mean)	SD	%RSD
10	245.57	1.18	0.48	777.67	1.62	0.21	398.20	2.16	0.54
50	1258.13	3.35	0.27	3879.80	22.77	0.59	2132.07	3.32	0.16
100	2513.63	5.09	0.20	7992.77	49.05	0.61	4431.60	21.43	0.48

The intermediate precision is described according to the variability of the intra and inter-day precisions. Inter-day precision was based on the comparison of three days curves and the intra-day precision was obtained comparing three

curves from the same day. The same concentrations were utilized in this analysis – Table 5 and 6.

Table 5 - Analytical performance of the method: intraday precision of DEET, octyl methoxycinnamate and octyl salicylate. SD is standard deviation and %RSD is relative standard deviation.

Concentration (µg/ml)	Intraday Precision					
	DEET		OM		OS	
	Measured value Mean ± SD	%RSD	Measured value Mean ± SD	%RSD	Measured value Mean ± SD	%RSD
10 µg/ml	245.89±0.77	0.31	767.51±6.20	0.81	398.46±1.23	0.31
50 µg/ml	1262.88±5.82	0.46	3858.46±11.75	0.30	2146.19±3.07	0.14
100 µg/ml	2476.41±32.27	1.30	7973.17±113	1.42	4368.81±70.25	1.61

Table 6 - Analytical performance of the method: interday precision of DEET, octyl methoxycinnamate and octyl salicylate. SD is standard deviation and %RSD is relative standard deviation.

Concentration (µg/ml)	Interday Precision					
	DEET		OM		OS	
	Measured value Mean ± SD	%RSD	Measured value Mean ± SD	%RSD	Measured value Mean ± SD	%RSD
10 µg/ml	244.56±1.38	0.56	776.90±11.99	1.54	398.3±0.22	0.05
50 µg/ml	1274.28±10.01	0.79	3847.14±10.98	0.29	1023.09±6.40	0.30
100 µg/ml	2507.56±29.33	1.17	7971.21±10.25	0.13	4401.11±38.06	0.86

Stability

The stability of the actives in PBS with 4% Brij® 98 (w/v), methanol and ethanol/water (50:50 v/v) were compared. Two samples of each were analyzed at 0 and 48 hours. The concentration was 100 µg/mL and the data on Table 7, 7 and 8 indicates that differences between the concentration (µg/ml) of the actives were not statistically different ($p<0.05$). So, in this study, the actives were stable at least for 48 hours and degradation was not a concern during the diffusion experiment.

Table 7 - Analytical performance of the method: stability of DEET.

Time (hours)	DEET		
	PBS Mean(SD)	Methanol Mean(SD)	Ethanol/water Mean(SD)
0	3992.7±146.65	6894.95±77.15	4051.65±216,45
48	4549.25±98.64	7020.60±90.37	3355.0±97.58
P value	0.0593	0.2763	0.0956

Table 8 - Analytical performance of the method: stability of octyl methoxycinnamate.

Time (hours)	OM		
	PBS Mean(SD)	Methanol Mean(SD)	Ethanol/water Mean(SD)
0	38417.7 ± 1836.21	27979.15 ± 111.51	12064.15 ± 848.17
48	73446.55 ± 2389.1	28388.4 ± 277.89	12625.3 ± 1024.46
P value	0.1129	0.2548	0.2431

Table 9 - Analytical performance of the method: stability of octyl salicylate.

Time (hours)	OS		
	PBS Mean(SD)	Methanol Mean(SD)	Ethanol/water Mean(SD)
0	9056.9 ± 775.27	8257.2 ± 248.76	9056.9 ± 775.27
48	11855.9 ± 695.23	8381.6 ± 82.87	6354.6 ± 239.28
P value	0.0639	0.6066	0.5213

3.6 DATA ANALYSIS

The cumulative mass of the active that permeated through the skin (µg/cm2) was measured for 10 hours and the results were plotted on a graph (time x concentration). The flux at the steady state was obtained using the slope of the curve and the concentration of the active that permeated the skin after 10 hours (Q_{10}) was obtained using the final hour concentration.

The results are presented as mean ± standard deviation. The data was analyzed to determine if the difference between groups was significant. Student- t test and ANOVA were performed (GraphPad Prism 6.0), and a p-value<0.05 was considered significant.

RESULTS

Diffusion experiments were performed to obtain the transdermal permeation of the actives: octyl methoxycinnamate (OM), octyl salicylate (OS) and DEET.

Each active was tested individually or in combination in order to obtain all the possible outcomes. The intention was to determine if the order of the application can interfere with the final cumulative permeation of the active and its flux.

4.1 DETERMINATION OF CONTROL AND TEST GROUPS

For each control and test group, two experiments were performed on different days to test the reproducibility of the data. The means together with the standard deviations of groups (n=6 each) were used for final comparisons between the groups.

4.1.1 OCTYL METHOXYCINNAMATE

Two groups that had only octyl methoxycinnamate (7.5% v/v) applied onto the skin were compared. The objective was to use the mean of the results and consider all the possible differences and the variabilities. The flux and the cumulative permeation of the groups OM A and OM B, n=6 each, after 10 hours were compared (Table 10). There was no significant difference between the groups (p value = 0.07 for the flux (J) and p value = 0.42 for the cumulative permeation (Q_{10}) (Figure 5).

Table 10 - Flux (J) and Cumulative permeation (Q_{10}) of OM A, OM B and OM control.

	Flux(*J*) (µg/cm²/hr) **Mean (SD)**	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** **Mean (SD)**
OM A	0.13 ± 0.045	2.02 ± 0.39
OM B	0.17 ± 0.033	2.18 ± 0.26
OM Control	0.14 ± 0.031	2.10 ± 0.32

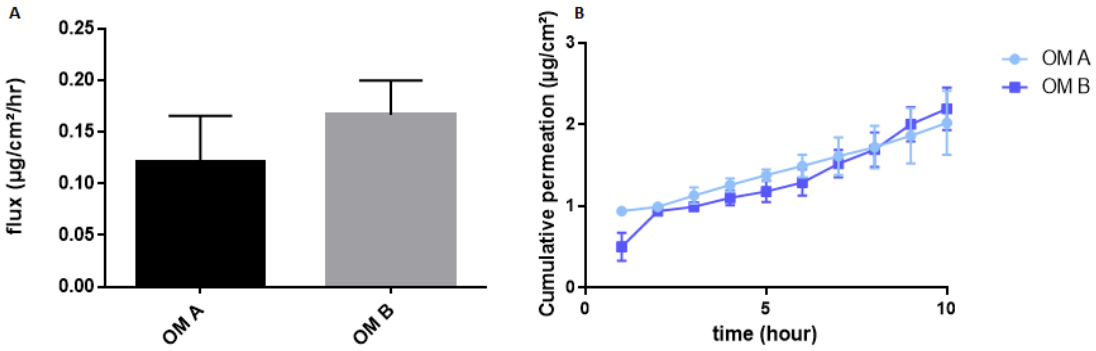

Figure 5 - A. Flux of Octyl Methoxycinnamate across human skin over 10h B (n=6). Cumulative permeation of Octyl Methoxycinnamate across human skin over 10h.

For the second group, the skin was previously treated with DEET at 15% v/v for 1h and then exposed to the sunscreen active at 7.5% v/v. Two experiments (OM pretreated with DEET A and OM pretreated with DEET B; n=6 each) were performed and the mean of both (OM pretreated with DEET) was used for comparison (Table 11). The results presented in Figure 6 correspond to the active Octyl Methoxycinnamate at 7.5% v/v. The difference between the flux of both groups was not statistically different (p value = 0.48), however, the cumulative permeation after 10h was significantly higher in group A (p value = 0.0003). This difference was expected since there is high biological variability between skin samples obtained from human donors. . The mean of both groups was used as the group “OM pretreated with DEET”.

Table 11 - Flux (J) and Cumulative permeation (Q_{10}) of OM pretreated with DEET A, OM pretreated with DEET B and OM pretreated with DEET.

	Flux(*J*) (µg/cm²/hr) Mean (SD)	Cumulative permeation over 10h (Q_{10}) (µg/cm²) Mean (SD)
OM pretreated with DEET A	0.22 ± 0.01778	3.48 ± 0.23
OM pretreated with DEET B	0.22 ± 0.007927	2.76 ± 0.08
OM pretreated with DEET	0.22 ± 0.009062	3.12 ± 0.41

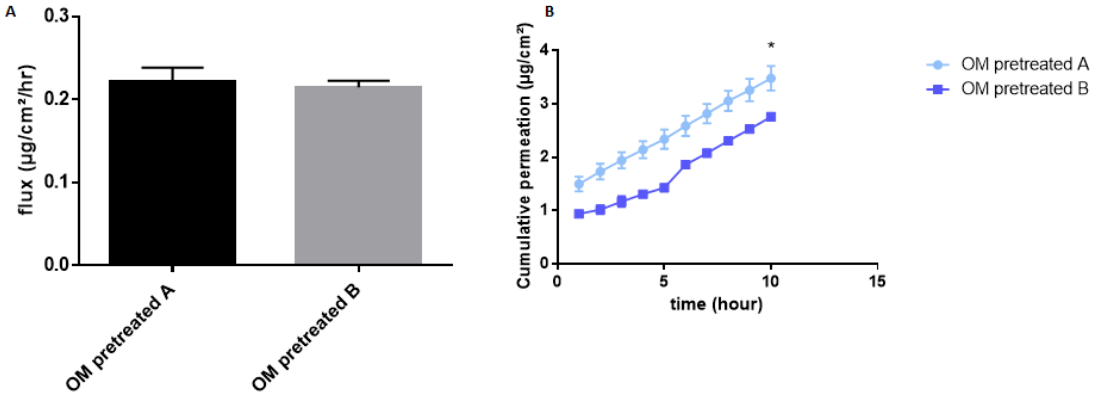

Figure 6 - A. Flux of Octyl Methoxycinnamate pretreated with DEET across human skin over 10h (n=6). B. Cumulative permeation of Octyl Methoxycinnamate pretreated with DEET across human skin over 10h.

Finally, the last experiment octyl methoxycinnamate was mixed with the sunscreen absorber at 7.5% v/v with DEET at 15% v/v. The mean of the flux and permeation of the groups OM mixed with DEET A and OM mixed with DEET B, n=6 each, were used for the further comparisons (Table 12). The flux of the actives between the two experiments was statistically different (p value = 0.0149). This difference was not considered an issue since as mentioned above, it represents the variability inherent in different types of skin. The cumulative permeation was significantly similar after 10h (p value = 0.23) (Figure 7).

Table 12 - Flux (J) and Cumulative permeation (Q_{10}) of OM mixed with DEET A, OM mixed with DEET B and OM mixed with DEET.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	Cumulative permeation over 10h (Q_{10}) (µg/cm²) Mean (SD)
OM mixed with DEET A	0.2084 ± 0.05	3.073 ± 0.7057
OM mixed with DEET B	0.1367± 0.03	2.385 ± 0.1834
OM mixed with DEET	0.1700 ± 0.03	2.729 ± 0.6090

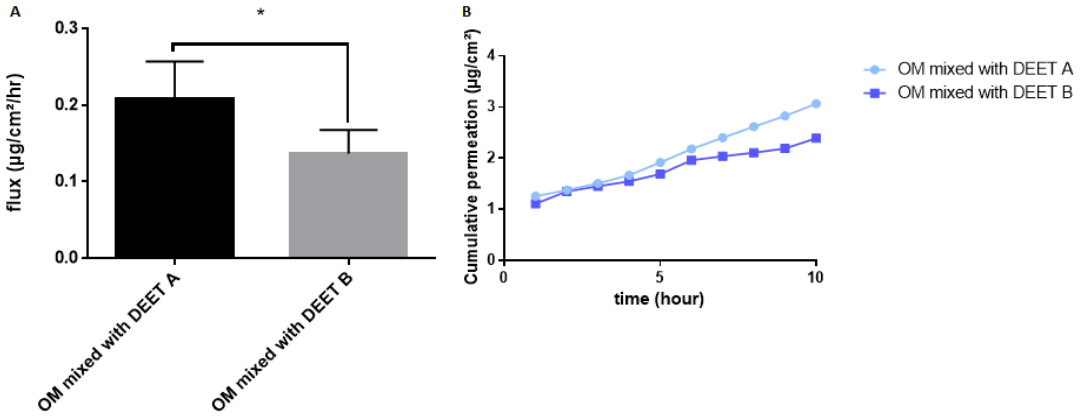

Figure 7 - A. Flux of Octyl Methoxycinnamate mixed with DEET across human skin over 10h (n= 6). B. Cumulative permeation of Octyl Methoxycinnamate mixed with DEET across human skin over 10h.

4.1.2 OCTYL SALICYLATE

Two groups that had only Octyl Salicylate (5% v/v) applied onto the skin were compared. The flux and the cumulative permeation of the groups OS A and OS B, n=6 each, after 10 hours are described in Table 13. There was no significant difference between the groups (p value = 0.31) for the flux (*J*) and for the cumulative permeation (Q_{10}) (p value = 0.29) (Figure 8).

Table 13 - Flux (J) and Cumulative permeation (Q_{10}) of OS A, OS B and OS control.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	Cumulative permeation over 10h (Q_{10}) (µg/cm²) Mean (SD)
OS A	0.37 ± 0.04	7.31 ± 0.56
OS B	0.47 ± 0.21	6.39 ± 1.87
OS Control	0.42 ± 0.11	6.85 ± 1.40

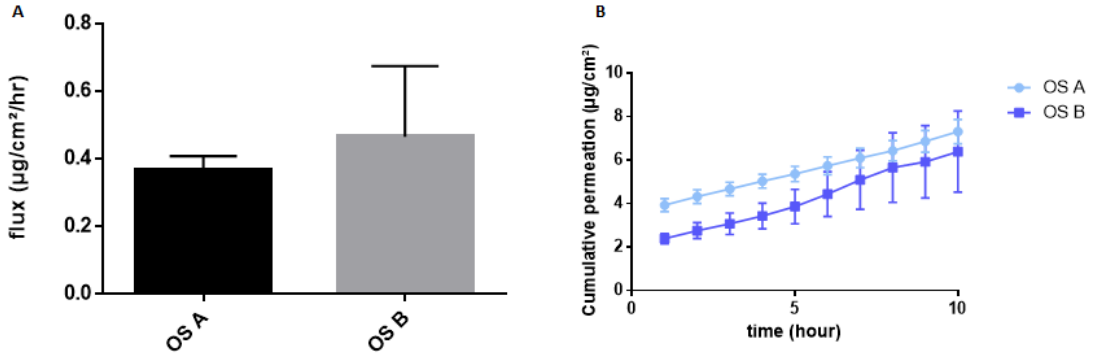

Figure 8 - A. Flux of Octyl Salicylate across human skin over 10h B (n=6). Cumulative permeation of Octyl Salicylate across human skin over 10h.

The results for the skin pretreated with DEET for 1h before the application of Octyl Salicylate (OS pretreated with DEET A, OS pretreated with DEET, n=6 each) are described in Table 14. The mean of both (OS pretreated with DEET) was used as the pretreated group for the active OS. The flux and the permeation between the groups were not significantly different after 10 hours (p value = 0.24 and p value = 0.85, respectively) (Figure 9).

Table 14 - Flux (J) and Cumulative permeation (Q_{10}) of OS pretreated with DEET A, OS pretreated with DEET B and OS pretreated with DEET.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	Cumulative permeation over 10h (Q_{10}) (µg/cm²) Mean (SD)
OS pretreated with DEET A	0.41 ± 0.067	7.36 ± 1.09
OS pretreated with DEET B	0.34 ± 0.12	7.47 ± 0.97
OS pretreated with DEET	0.38 ± 0.07	7.41 ± 0.98

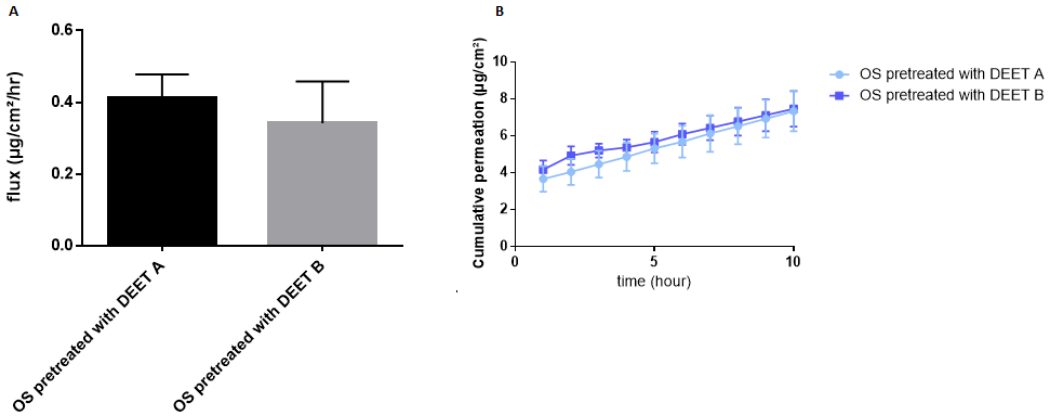

Figure 9 - A. Flux of octyl salicylate pretreated with DEET across human skin over 10h (n=6). B. Cumulative permeation of octyl salicylate pretreated with DEET across human skin over 10h.

The mix of octyl salicylate at 5% v/v and DEET at 15% v/v is presented in Table 15. The flux between the groups OS mixed with DEET A and OS mixed with DEET B, n=6 each, was statistically different (p value = 0.0042) and the concentration that permeated after 10h was also significantly different (p value = 0.0003) (Figure 10). These differences were not considered an issue, since as mentioned above they represent the high variability that is found in the skin

Table 15 - Flux (J) and cumulative permeation (Q_{10}) of OS mixed with DEET A, OS mixed with DEET B and OS mixed with DEET.

	Flux (*J*) (µg/cm²/hr)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)**
	Mean (SD)	**Mean (SD)**
OS mixed with DEET A	0.31 ± 0.028	5.74 ± 0.39
OS mixed with DEET B	0.74 ± 0.21	10.84 ± 1.55
OS mixed with DEET	0.53 ± 0.10	8.29 ± 2.87

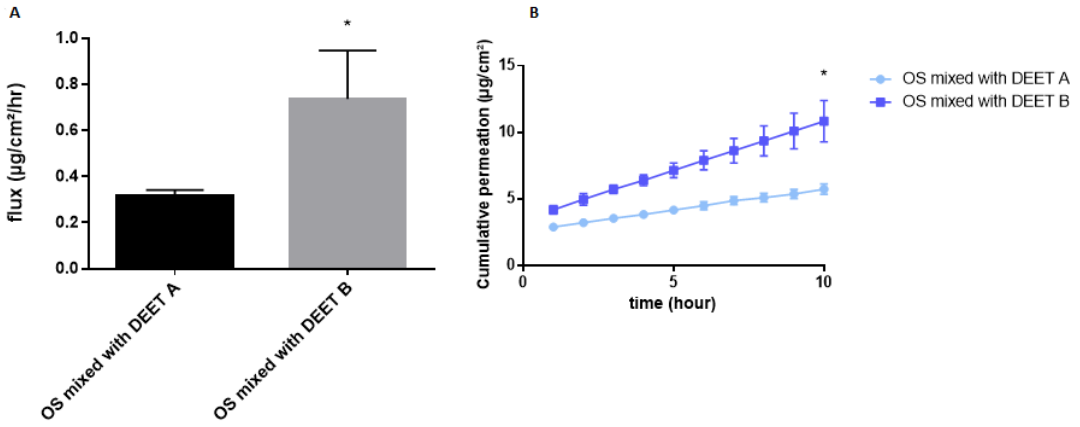

Figure 10 - A. Flux of octyl salicylate mixed with DEET across human skin over 10h (n=6). B. Cumulative permeation of octyl salicylate mixed with DEET across human skin over 10h.

4.1.3 DEET

Two groups that had only DEET (15% v/v) applied onto the skin were compared. The flux and the cumulative permeation of the groups DEET A and DEET B, n=6 each, after 10 hours is presented in Table 16. There was no significant difference between the groups (p value = 0.52) for the flux (*J*) and for the cumulative permeation (Q_{10}) (p value = 0.53) (Figure 11).

Table 16 - Flux (J) and cumulative permeation (Q_{10}) of DEET A, DEET B and DEET control.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** Mean (SD)
DEET A	28.90 ± 13.72	252.1 ± 132.2
DEET B	33.29 ± 7.880	292.2 ± 67.69
DEET Control	31.10 ± 8.759	272.1 ± 102.32

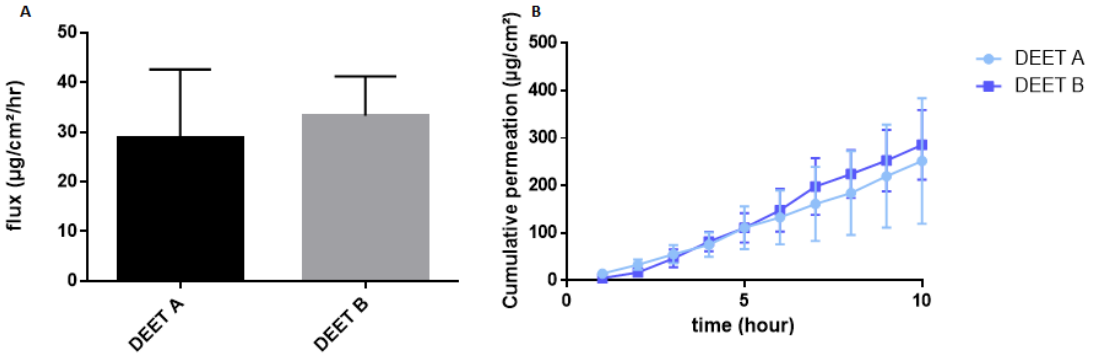

Figure 11 - A. Flux of DEET across human skin over 10h (n=6). B. Cumulative permeation of DEET across human skin over 10h.

DEET at 15% v/v was used to pretreat the skin for 1h before the application of the sunscreen actives. The results for octyl methoxycinnamate and octyl salicylate are describe in Table 17. As mentioned before, the mean of the groups was used as the final flux and cumulative permeation. There was no difference on the flux and the final concentration after 10h (Q10) between the groups Pretreatment of DEET before OM A and pretreatment of DEET before OM B (p value = 0.20)(Figure 12). The groups exposed to octyl salicylate also did not exhibit any difference between the flux (p value = 0.85) and the final concentration (p value = 0.73) (Figure 13).

Table 17 - Flux (J) and cumulative permeation (Q_{10}) of Pretreatment of DEET before OM (A, B and control) and Pretreatment of DEET before OS (A, B and control).

	Flux (*J*) (µg/cm²/hr) Mean (SD)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** Mean (SD)
Pretreatment of DEET before OM A	35.81 ± 8.62	506.50 ± 170.70
Pretreatment of DEET before OM B	42.76 ± 8.72	462.10 ± 126.40
Pretreatment of DEET before OM	39.29 ± 6.16	484.30 ± 145.10
Pretreatment of DEET before OS A	26.48 ± 17.65	263.50 ± 217.30
Pretreatment of DEET before OS B	25.02 ± 4.90	296.60 ± 50.43
Pretreatment of DEET before OS	25.75 ± 8.76	280.00 ± 151.40

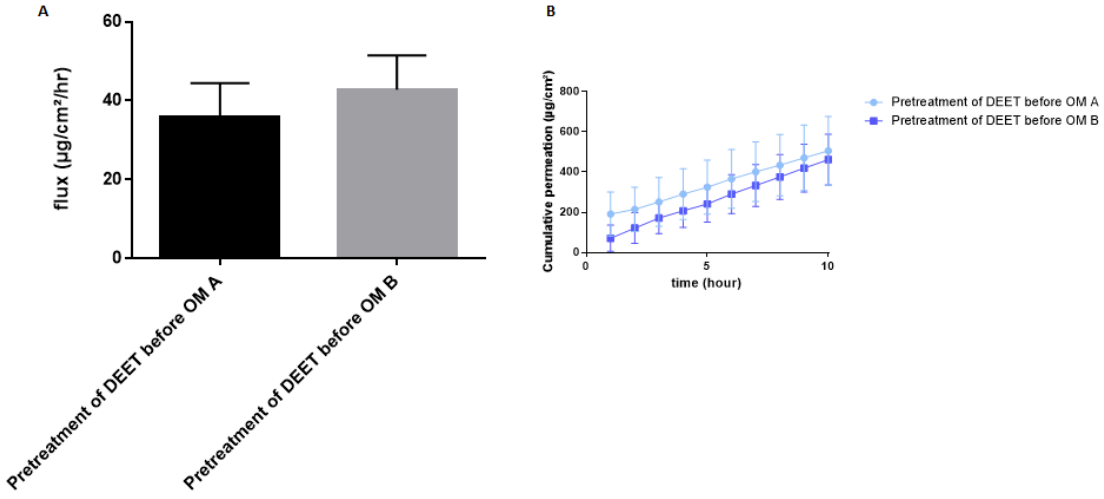

Figure 12 - Flux of DEET when used to pretreated the skin before the application of octyl methoxycinnamate across human skin over 10h (n=6). B. Cumulative permeation of DEET when used to pretreated the skin before the application of octyl methoxycinnamate across human skin over 10h.

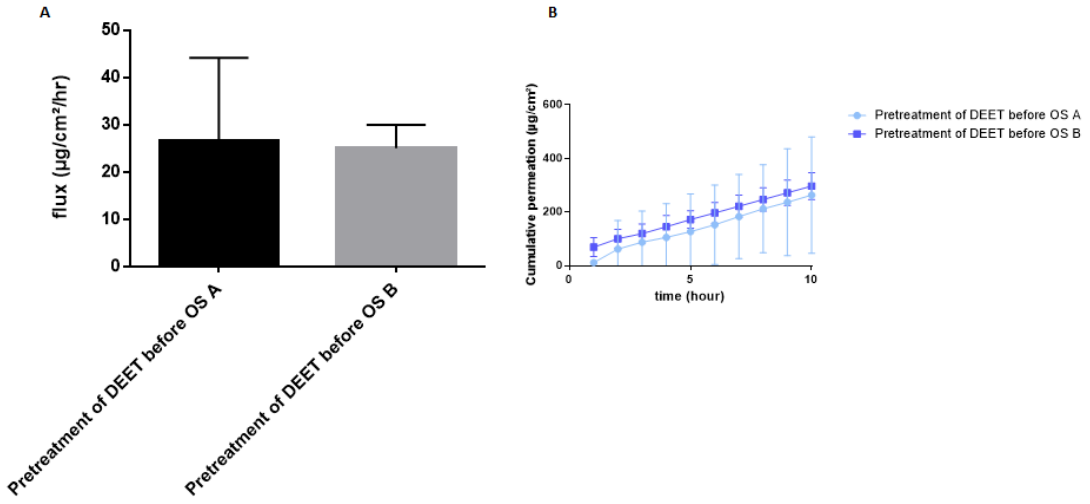

Figure 13 - Flux of DEET when used to pretreated the skin before the application of octyl salicylate across human skin after 10h (n=6). B. Cumulative permeation of DEET when used to pretreated the skin before the application of octyl salicylate across human skin over 10h.

The results of DEET mixed with octyl salicylate and octyl methoxycinnamate are illustrated in Table 18. The flux between the groups DEET mixed with OM C and DEET mixed with OM D, n=6 each, was not statistically different (p value = 0.08), however, the concentration after 10h was statistically different (p value = 0.02) (Figure 14). The difference between the concentrations was not significant to the study, since the mean of both groups (DEET mixed with OM) was used as the final result. The flux and the concentration after 10h between the groups DEET mixed with OS C and DEET mixed with OS D were also statistically different (p value = 0.0027 and p value = 0.0004, respectively) (Figure 15). The mean of both (DEET mixed with OS) was used as the final result.

Table 18 - Flux (J) and cumulative permeation (Q_{10}) of OM mixed with DEET (C, D and control) and OS mixed with DEET (C, D and control).

	Flux (*J*) (µg/cm²/hr) Mean (SD)	Cumulative permeation over 10h (Q_{10}) (µg/cm²) Mean (SD)
DEET mixed with OM C	72.86 ± 15.53	809.70 ± 195.70
DEET mixed with OM D	58.25 ± 8.205	554.00 ± 79.46
		681.90 ± 195.20
DEET mixed with OM	65.55 ± 10.12	
DEET mixed with OS C	36.52 ± 16.60	348.20 ± 153.40
DEET mixed with OS D	73.85 ± 16.13	665.70 ± 140.30
DEET mixed with OS	55.19 ± 10.85	507.00 ± 217.10

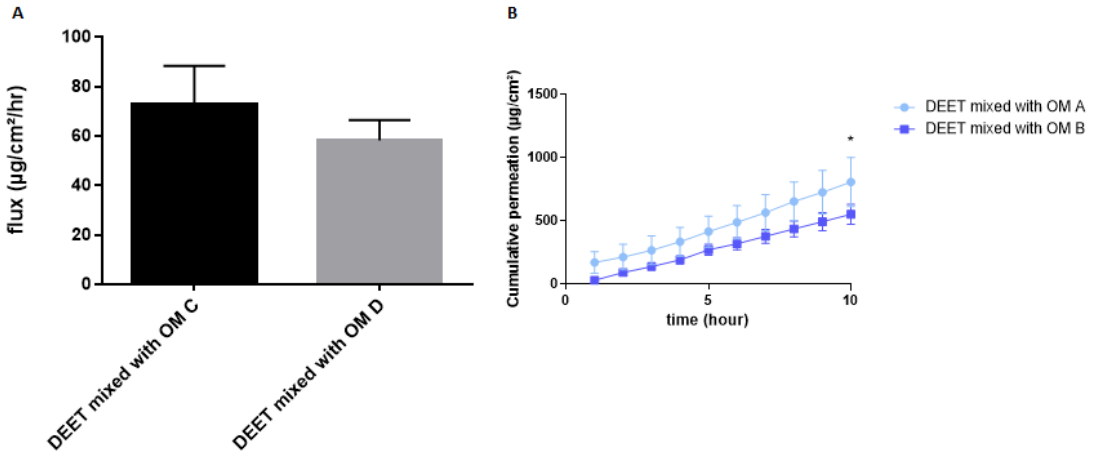

Figure 14 - A. Flux of DEET mixed with OM across human skin over 10h (n=6). B. Cumulative permeation of DEET mixed with OM across human skin over 10h.

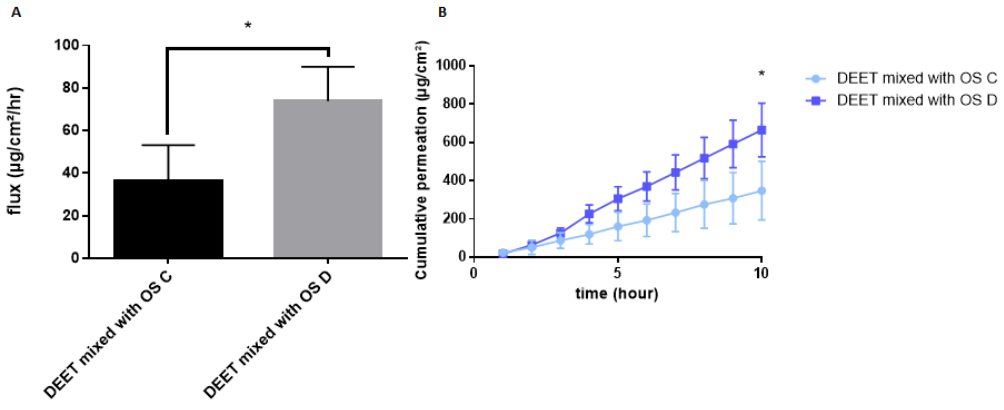

Figure 15 - A. Flux of DEET mixed with OS across human skin over 10h (n=6). B. Cumulative permeation of DEET mixed with OS across human skin over 10h.

4.2 PERMEATION OF OCTYL METHOXYCINNAMATE CO-APPLIED WITH DEET THROUGH HUMAN SKIN

The purpose of this experiment was to mimic the application of a sunscreen after the use of an insect repellent. The skin was pretreated with an infinite dose of DEET at 15% v/v for 1h and before the application of octyl methoxycinnamate at 7.5% v/v, the skin was cleaned with a cotton swab, in order to remove all the residues of DEET. The other experiment was designed to mimic the concomitant application of a sunscreen and an insect repellent, so DEET at 15% v/v was previously mixed with OM at 7.5% v/v and the resulting solution was applied onto the skin for 10h.

The control, containing the results of the application of octyl methoxycinnamate alone on the human skin was compared to the results of the skin pretreated for 1h with DEET followed by exposure to the sunscreen active and with the results of the sunscreen mixed with DEET, n=12 each (Table 19). The flux and the Q_{10} between the control and the group mixed with DEET were not significantly different (Figure 16), however, the group that was pretreated with DEET before the application of OM had a higher cumulative permeation when compared to the other groups (p value = 0.0003), indicating that DEET does not affect directly this UV absorber when co-applied with it, but when this insect repellent is used to pretreat the skin, it can increase the flux significantly (Figure 17). When the final concentration of OM is compared after 10h, it is observed that both groups that were exposed to DEET had a higher cumulative permeation at the end of the experiment, when compared to the control,

indicating that a higher concentration of the active penetrates the skin when used in combination (p value < 0.0001). Also, the difference between the groups exposed to DEET is not significantly different.

Table 19 - Flux (J) and cumulative permeation (Q_{10}) of OM Control, OM pretreated with DEET and OM mixed with DEET.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** Mean (SD)
OM Control	0.14 ± 0.04	2.10 ± 0.32
OM pretreated with DEET	0.22 ± 0.01	3.12 ± 0.41
OM mixed with DEET	0.17 ± 0.03	2.73 ± 0.61

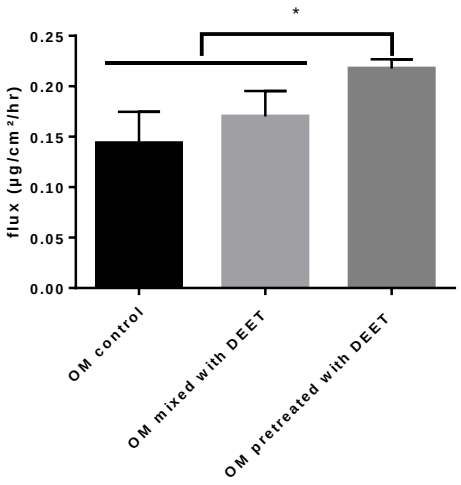

Figure 16 - Flux of octyl methoxycinnamate co-applied with DEET across human skin over 10h (n=12). The group pretreated with DEET has a higher flux when compared to the control and the group mixed with DEET (p value = 0.0003).

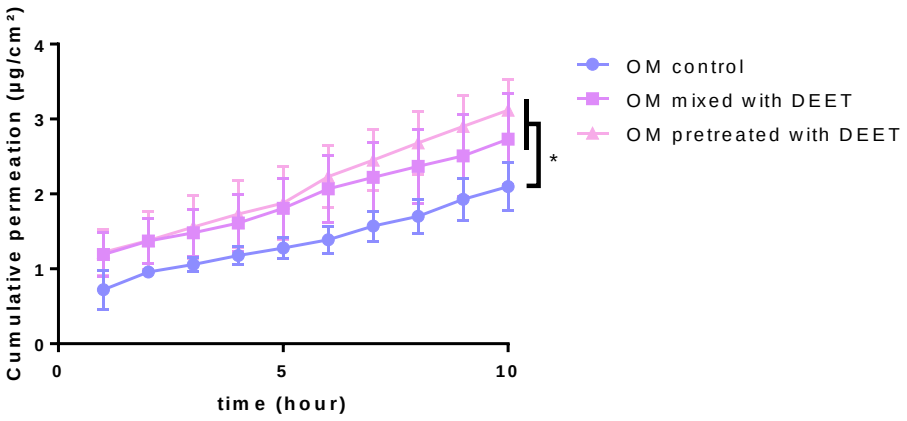

Figure 17 - Cumulative permeation of octyl methoxycinnamate co-applied with DEET across human skin over 10h (n=12). The groups exposed to DEET had a higher concentration when compared to the control (p value < 0.0001).

4.3 PERMEATION OF OCTYL SALICYLATE CO-APPLIED WITH DEET THROUGH HUMAN SKIN

The control, consisting of the application of octyl salicylate (5% v/v) alone on the human skin was compared to the results of the skin pretreated for 1h with DEET (15% v/v) and then exposed to the sunscreen active and with the active mixed with DEET, n=12 each (Table 20). The flux of the group mixed with DEET was higher than the group pretreated with the insect repellent after 10 hours, but both groups exposed to DEET were not significantly different from the control (p value = 0.04) (Figure 18). The concentration of octyl salicylate found in the receptor compartment was similar in all three experiments, indicating that DEET does not affect the final cumulative permeation of the OS (p value = 0.1981) (Figure 19).

Table 20 - Flux (J) and cumulative permeation (Q_{10}) of OS Control, OM pretreated with DEET and OM mixed with DEET.

	Flux (*J*) (µg/cm²/hr) **Mean (SD)**	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** **Mean (SD)**
OS Control	0.42 ± 0.11	6.85 ± 1.40
OS pretreated with DEET	0.38 ± 0.07	7.41 ± 0.98
OS mixed with DEET	0.53 ± 0.10	8.29 ± 2.87

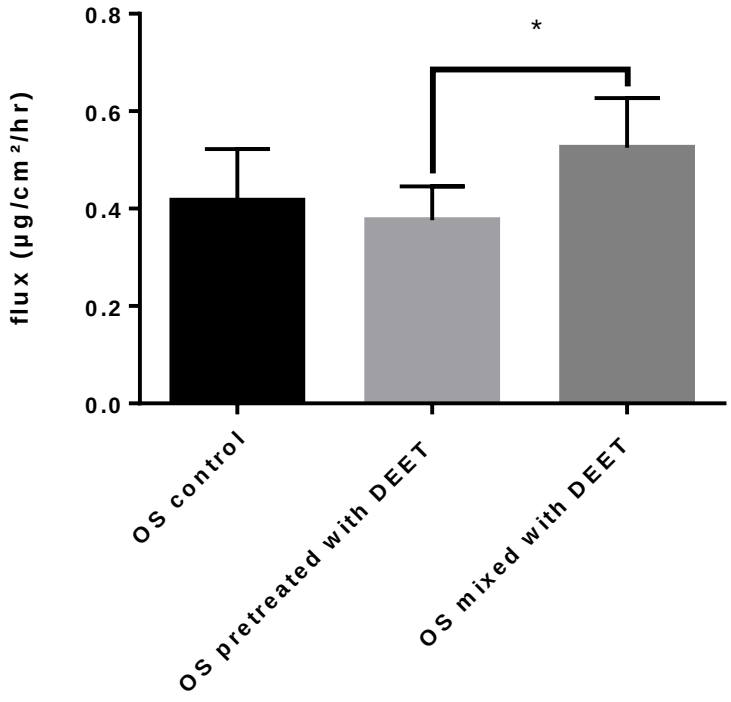

Figure 18 - Flux of octyl salicylate co-applied with DEET across human skin after 10h (n=12). The group mixed with DEET had a higher flux when compared to the pretreated group (p value = 0.0401).

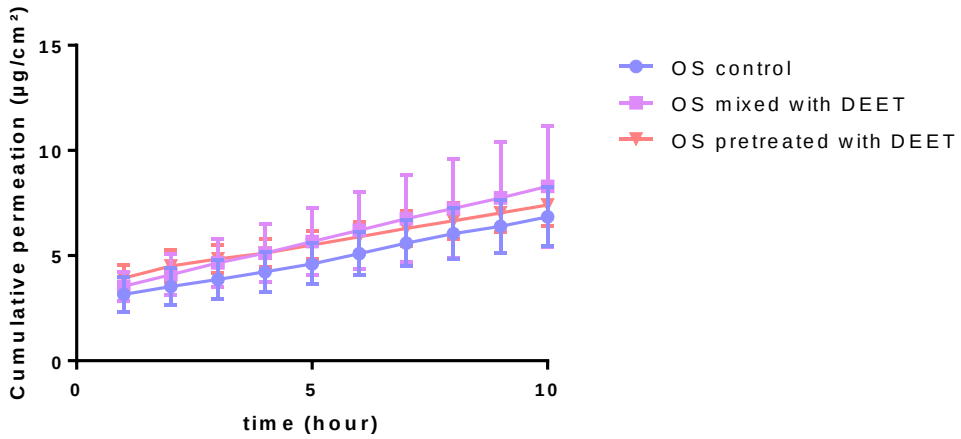

Figure 19 - Cumulative permeation of octyl salicylate co-applied with DEET across human skin over 10h. (n=12). No difference between the groups was observed (p value = 0.1981).

4.4 PERMEATION OF DEET CO-APPLIED WITH OCTYL METHOXYCINNAMATE THROUGH HUMAN SKIN

The control was compared to the flux and the cumulative permeation after 10h of three different groups, DEET mixed with OM, pretreatment of the skin with DEET for 1h before the application of OM and pretreatment of the skin with OM for 2 hours before the exposure to the insect repellent. The results of each experiment are presented in Table 21. The flux of the group that was exposed to the mix of DEET and OM has higher, when compared to all the other groups (p value <0.0001) (Figure 20). All the groups exposed to octyl methoxycinnamate had a higher concentration at the end of the experiment when compared to the control, however, they were not statistically different from each other (Figure 21) (p value =0.0004).

Table 21 - Flux (J) and cumulative permeation (Q_{10}) of DEET Control, Pretreatment of DEET before OM, DEET mixed with OM and DEET pretreated with OM.

	Flux (*J*) (µg/cm²/hr)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)**
	Mean (SD)	**Mean (SD)**
DEET Control	31.10 ± 8.759	272.1 ± 102.32
Pretreatment of DEET before OM	39.29 ± 6.160	484.3 ± 145.10
DEET mixed with OM	65.55 ± 10.12	681.9 ± 195.20
DEET pretreated with OM	31.58 ± 7.505	524.3 ± 168.0

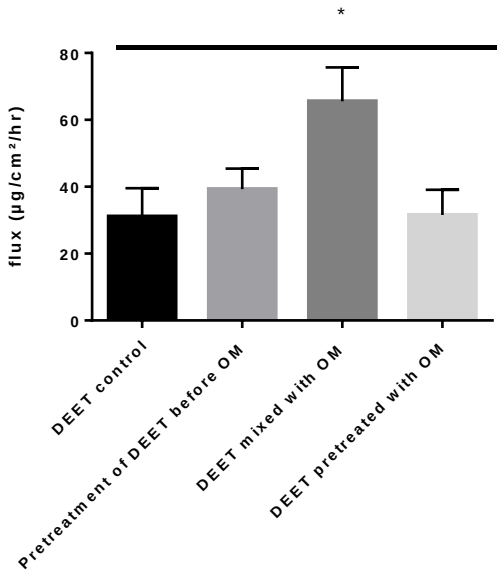

Figure 20 - Flux of DEET across human skin over 10h (n=12). The flux of DEET mixed with OM was higher than the control and all the other groups (p<0.0001).

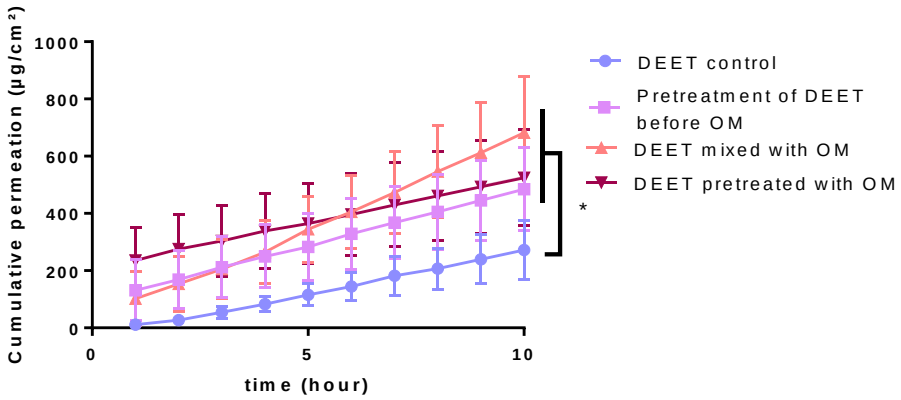

Figure 21 - Cumulative permeation of DEET across human skin over 10h. (n=12). All the groups exposed to OM have a higher transdermal permeation than the control after 10h (p = 0.0004).

4.5 PERMEATION OF DEET CO-APPLIED WITH OCTYL SALICYLATE THROUGH HUMAN SKIN

The data for DEET (15% v/v) with and without octyl salicylate (5% v/v) are illustrated in Table 22. The control, containing only DEET alone on the human skin was compared to the results of the skin pretreated for 1h with DEET and then exposed to the sunscreen active and the skin pretreated with OS for 2 hours before the application of DEET, n=12 each. The flux of DEET when it was mixed with OS was higher than for all the other groups, including the control (p value = 0.0001) (Figure 22). The cumulative concentration that permeated after 10 hours was higher in all groups exposed to OS when compared with control (p value = 0.002) (Figure 23).

Table 22 - Flux (J) and cumulative permeation (Q_{10}) of DEET Control, Pretreatment of DEET before OS, DEET mixed with OS and DEET pretreated with OS.

	Flux (*J*) (µg/cm²/hr) Mean (SD)	**Cumulative permeation over 10h (Q_{10}) (µg/cm²)** Mean (SD)
DEET Control	31.10 ± 8.76	272.1 ± 102.32
Pretreatment of DEET before OS	25.75 ± 8.76	280.0 ± 151.4
DEET mixed with OS	55.19 ± 10.85	507.0 ± 217.1
DEET pretreated with OS	38.11 ± 8.766	384.0 ± 91.19

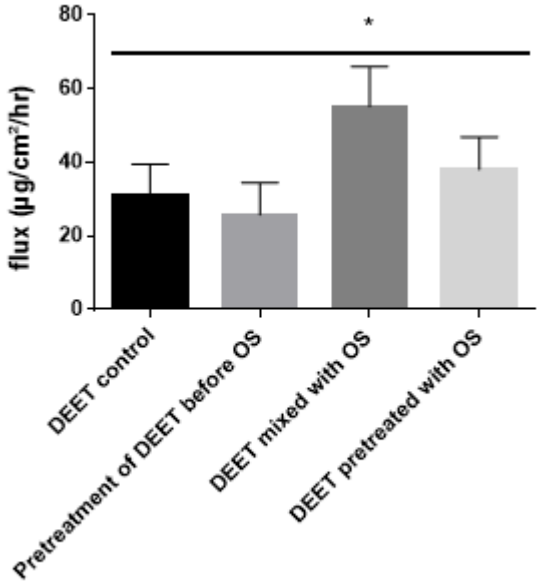

Figure 22 - Flux of DEET across human skin over 10h (n=12). The flux of DEET mixed with OS was higher than the control and all the other groups (p=0.0001).

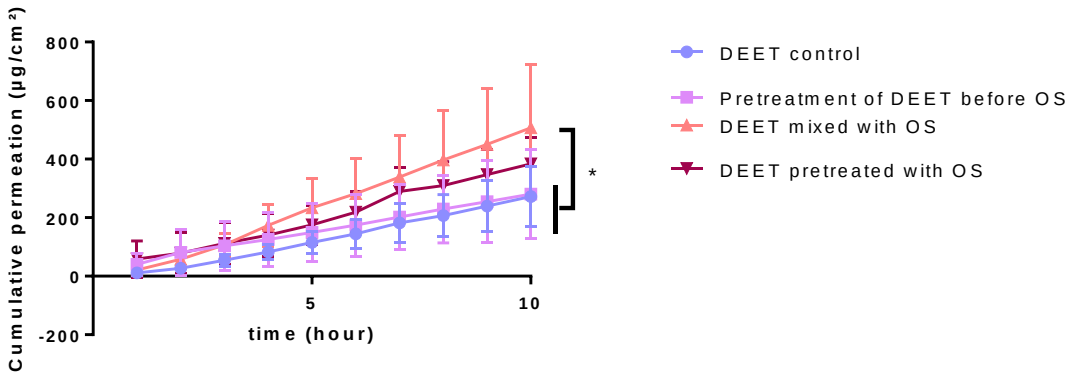

Figure 23 - Cumulative permeation of DEET across human skin over 10h. (n=12). The concentration over 10h of DEET when mixed with OS was higher than the control group and the group pretreated with DEET (p value = 0.0021).

4.6 PERCUTANEOUS PERMEATION OF OCTYL METHOXYCINNAMATE, OCTYL SALICYLATE AND DEET

All experiments were compared to evaluate which combination had the highest flux and cumulative permeation after 10 hours (Q_{10}). The fluxes of octyl salicylate and octyl methoxycinnamate when applied alone onto the skin are not statistically different when compared to all the other groups. The same was observed when these sunscreen actives were mixed with DEET or even when the skin was previously pretreated with the insect repellent before the application of OM or OS. The fluxes corresponding to DEET control, DEET when it was applied to the skin after the pretreatment with OM and OS and DEET when it pretreated the skin before the addition of OM and OS were higher than the results of OM and OS (mentioned above) and lower than the two final groups. DEET mixed with OS (55.19 ± 10.85 µg/cm²/hr) and DEET mixed with OM (65.55 ± 10.12 µg/cm²/hr) resulted in the highest flux of all the groups. The

lowest flux found was for OM control (0.1437 ± 0.03089 µg/cm²/hr) and the difference between the lowest and the highest concentration observed was significantly different (p value < 0.0001) (Figure 24). The highest cumulative concentration permeating the skin after 10 hours was recorded for DEET when it was mixed and co-applied with OM (681.9 ± 195.2 µg/cm²) and the difference between this group and DEET pretreated with OM was not significantly different. The lowest concentration was recorded for OM control (2.103 ± 0.3243) and again the difference between these two groups was significantly different (p value < 0.0001) (Figure 25).

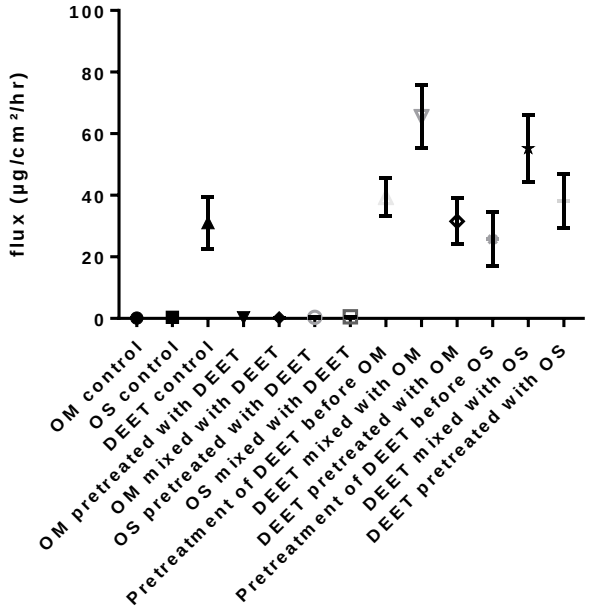

Figure 24 - Flux of octyl methoxycinnamate, octyl salicylate and DEET across human skin over 10h (n=12). The fluxes of DEET mixed with OS and DEET mixed with OM were higher than for all the other groups (p<0.0001).

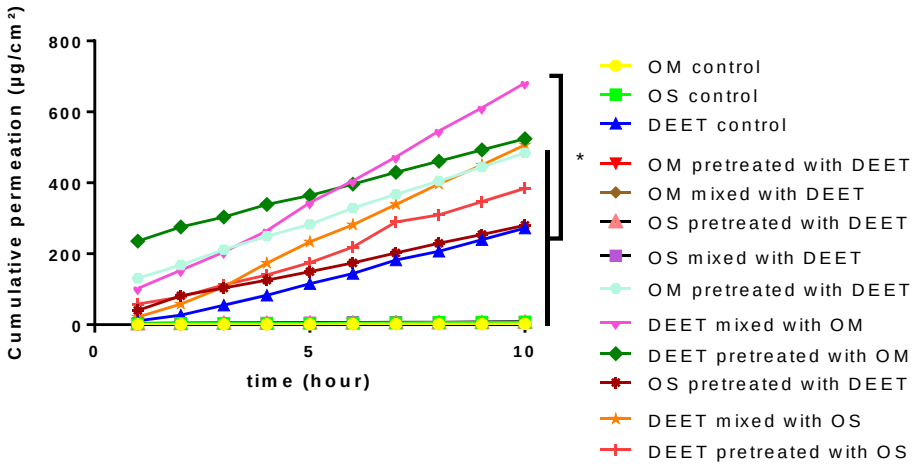

Figure 25 - Cumulative permeation of octyl methoxycinnamate, octyl salicylate and DEET across human skin after 10h (n=12). The concentration over 10h of DEET when mixed with OM was higher than for all the other groups - p value < 0.0001).

DISCUSSION

Previous studies demonstrated that sunscreens actives and DEET have an ability to permeate the skin, even when applied alone [4]. When these actives are used in combination, it was observed that the insect repellent can decrease the efficacy of the sunscreen [9] and they can act as enhancers to each other [67, 68, 76]. The most used active to test this interaction is oxybenzone, and all *in vitro* and *in vivo* studies observed that the percutaneous permeation of both actives increased when they were co-applied [4, 77]. However, all the results showed that the ability to permeate of DEET is higher than the sunscreen and even when other factor is responsible for the increased permeation, the penetration of the insect repellent is always more affected [67, 68].

The interactions associated with the permeation of sunscreens in the presence of insect repellents are still not completely understood and more studies are needed to elucidate the skin transport effects between these two commonly used actives. In this study, it was observed that even when these actives are applied individually onto the human skin in vitro, there are some clear differences that were recorded. The sunscreen actives octyl methoxycinnamate and octyl salicylate have similar fluxes in human skin in vitro and the concentrations that permeate the skin are also not significantly different from each other. However, the permeation of DEET is higher even when applied alone, when compared to the sunscreens. Since DEET is applied by a large population globally over large surfaces of the body and in also in children,

many scientists have been concerned about the outcomes. Since it is also known to act as a skin “penetration enhancer” which means that it is able to facilitate the skin transport of co-administered actives into the skin [78], the concern is magnified (potentially) when such insecticides are applied onto skin with products containing sunscreens. Little work is reported in this area however, the FDA warns against using such combinations. In this study we tested some commonly used FDA approved sunscreens to test out what actually happens with these combinations in human skin *in vitro* and whether the concerns are justified or not.

It was found that with octyl salicylate a low permeation through skin was observed even when it was co-applied with DEET. The differences found between the fluxes of the group that had OS mixed with DEET and the control was insignificant and the final concentration found in the receptor compartment was not different from that for all the other groups. The same was observed for octyl methoxycinnamate, which had a little difference between the group mixed with DEET and the control. These findings indicate that for some sunscreen actives, even the co-application with an enhancer, like DEET, does not affect the ability of the sunscreen molecule to permeate the skin. It can be higher, but when compared to the control, the differences are not particularly high and *in vivo* would probably be insignificant.

However, when permeation of DEET was studied, several interesting points were observed. When DEET was co-applied with octyl salicylate, the flux observed was higher than for the control or the other treatment groups and the

concentration that permeated after 10 hours was almost double when compared to control. This is an indication that OS can act as a weak enhancer of DEET *in vitro*. On the other hand, experiments with the mixture of DEET and octyl methoxycinnamate produced even more interesting data. The flux for the mix of DEET plus OM was almost two times the flux of the control group and the cumulative permeation recorded after 10 hours was almost three times the Q_{10} of control. Therefore, the concomitant application of OM and DEET, *in vitro*, did show significant enhancement, and based on these data, the use of DEET and OM together cannot be recommended, since OM will enhance the penetration of the insect repellent into the skin. Since the over-exposition to DEET is connected to many toxic effects, this co-application, based on the results of this study, should not be recommended [53, 59].

In summary, this thesis presents an overview of some of the possible interactions between two of the most commonly used sunscreen absorbers in the U.S. and the most popular insect repellent. It was clear that the ability to permeate the skin is very different between the two UV absorbers utilized in this study, especially when all the results obtained were compared. DEET mixed with OM and DEET mixed with OS produced the highest transdermal permeation *in vitro* and the lowest was obtained with OM applied alone onto the skin.

CONCLUSION

In conclusion, DEET does not act as an enhancer in skin *in vitro* when used in combination with octyl salicylate and octyl methoxycinnamate. The skin permeation of these sunscreens actives were not significantly affected by the insecticide. Furthermore, it is possible to acknowledge that the UV absorbers used on this study can be potential enhancers when co-applied with DEET. When both sunscreen actives were mixed with DEET, the resulting skin permeation was higher than the control, however, the differences found *in vitro* can be insignificant *in vivo*, so more studies are necessary to determine if this interaction can be considered dangerous to the population and what can be done to minimize the possible effects.

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