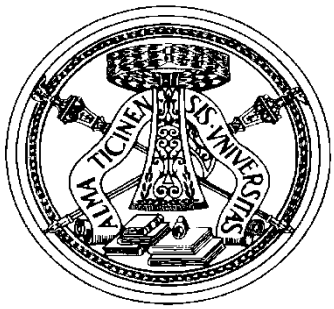


# UNIVERSITY OF PAVIA

## DEPARTMENT OF DRUG SCIENCE

**Ph.D. School in Chemical and Pharmaceutical Sciences XXXI Cycle**

**Executive Director Chiar.mo Prof. Mauro Freccero**


# «Technological strategies and pharmacoeconomic remarks in the prevention of photoinduced damages: product stability, efficacy and safety»


**Supervisor:**
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**Doctoral Thesis**


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This thesis has been submitted to the Graduate School in Chemical and Pharmaceutical Sciences at the University of Pavia

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# 1 INTRODUCTION

## 1.1 Skin anatomy and functions

The skin is the largest organ of the body, it represents about 15% of the total body weight with a surface area of 1.8 $m^2$. It is composed of three major structural layers: epidermis, dermis and subcutaneous tissue (Kanitakis J., 2002).

The epidermis is the outermost layer of the skin (**Figure 1**), its thickness range is from 0.05 mm to 1.5 mm (on the palms). It consists of many cell populations: melanocytes, synthetizing pigment (melanin); Langerhans cells, representing the skin immune system; Merkel cells, with not fully understand functions; keratinocytes, representing most cells. The epidermis has 5 layers of cells: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), stratum lucidum (SL) and stratum corneum (SC) and each layer represents a sequential differentiation stage of the keratinocytes.

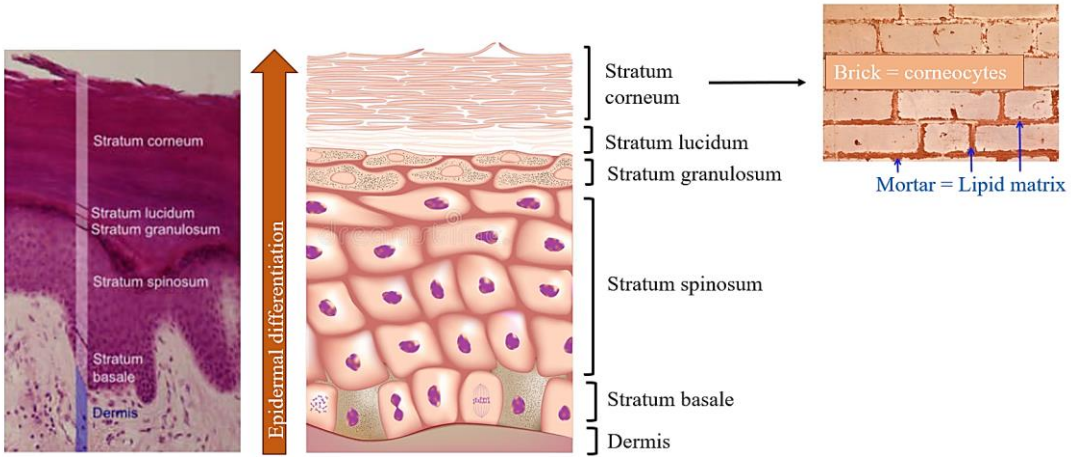



**Figure 1.** Representation of epidermis anatomy.

The SB or stratum germinativum is the deepest layer of the epidermis and is separated from the underlying dermis by a basement membrane. It consists of a single layer of column-shaped keratinocytes anchored to the basement membrane by means of junctions called desmosomes. It is composed mainly of proliferating and non-proliferating keratinocytes that go all the way up through SS, SG, SL and SC losing their nuclei and cytoplasm to became corneocytes: the SC cells. This process of maturation is known as terminal differentiation and it takes 14 days and the transit through the cornified layer to the outermost epidermis requires another 14 days (James W.D., 2006; Chu D.H., 2008). The SS is composed to 5-10 cells layers varying a lot, from zone to zone. Its cells derive from the basale layer cells which, moving upwards,

change their characteristics while still maintaining a certain mitotic capacity. The intercellular space is filled by desmosomes or gap junctions, junctional systems, able to provide mechanical and physical support and resistance. The SG is the last epidermal layer composed by living cells. It is made up of 1 to 4 cell layers and represents the transition zone the underlying epidermal layers and the superficially placed keratin material. Its cells are flat and very adhere between each other; their nuclei appear altered or absent, while in the cytoplasm there are keratohyaline granules responsible for the synthesis of interfibrillar matrix that holds keratin filaments together and the inner lining of the horny cells (Chu D.H., 2008).

SC is found in the richest regions of keratin, such as the palm and plantar, it consists of 10 to 20 layers of large, flat and polyhedral-shaped horny cells without nuclei. Epithelial cells structuring the epidermis derive from basal cells that progressively migrate towards the layers of the skin releasing glycolipids, and other elements, into the intracellular space, become flat and keratinize to form the SC. It supplies the prime line of defense against environmental threats being they mechanical, thermal, chemical, radiological or biological. They are rich of protein but with a low concentration of lipid content, therefore, they are surrounded by extracellular lipid matrix (Haake A.R., 1999; McLafferty E., 2013).

The regulation of epidermal proliferation and differentiation is essential, epidermis must maintain an internal equilibrium between cells growth and cells death and to do that, intrinsic processes are activated such as the cells apoptosis (programmed cell death). The terminal differentiation is an example of apoptosis process able to convert keratinocytes into protective corneocytes. This process is able to maintain the healthy structural and functional equilibrium of the epidermis, and also, to provide: cells renovation; defense against virus, microorganisms, infections, UV ray etc. The alteration of this dynamic balance could lead to diseases state such as eczema and psoriasis (Bovenschen H.J., 2005; Proksch E., 2006).

The dermis thickness varies depending on the location of the skin, usually it is between 0.3 mm (eyelids) to 3.00 mm (palms of hands). It is an integrated system of connective tissue (collagen and elastin), blood suppliers (the smallest blood vessels), hire follicle, lymph vessels, sweat and sebaceous gland and elements as fibroblasts, macrophages, and mast cells can enter the dermis in stimulus-induced situation. The dermis is composed of three types of tissues: collagen, elastic tissue and reticular fibers predictable in a depth-dependent manner. The biggest portion of collagen in our body

is type I collagen but collagen IV and VII also are found in some dermal spots (James W.D., 2006). Thin collagen fibers are in the upper dermis layer called papillary layer, and thick collagen fibers are in the lower dermis layer called reticular layer, extends for the base of the papillary layer to the subcutaneous tissue. Collagen is a very powerful stress-resistant material, determining the mechanical properties of the skin (Shen Z.L., 2008). Elastic tires are composed of elastin and fibrillin microfibrils and they are able to deform and storage energy and use it drive the structure back to a resting state (Kielty C.M., 2002). The aging process has deep effect on the structure and function of both collagen and elastic fiber.

Human skin development starts in utero, a complete finished SC is not available before 34 weeks and the barrier mutation is keep going on in relation to the gestational age (Harpin V.A., 1983; Evans N.J., 1986). At birth, SC and epidermal thickness are respectively 30% and 20% thinner, the corneocytes and keratinocytes are smaller, collagen fibers are less dense, less total lipids and less sebaceous lipids, lower concentration of melanin probably related to the rapid cell turnover happening during the first months of life, the concentration of NMF is lower and the functioning of acid mantle is missing (Li L., 2006; Stamatas G.N., 2010; Stamatas G.N., 2011). The reduced level of maturation/concentration of infant skin structure surface could compete to the not fully maturate skin barrier function, contributing to a height sensitivity to the harmful substances, environmental factors and loss of water.

The skin is in constantly displayed to potential hazards and, in order to maintain the homeostasis essential for health, skin needs to perform numerous functions. Among classical functions such as: i) protective barrier from environmental insults (mechanical and chemical), ii) regulation of the body temperature (regulation of the blood flow, releases of sweat followed by evaporation), iii) prevention water loss, iv) first line of defense against UV radiation (increased melanin production by melanocytes) and v) protection against environmental pathogenic microorganism (Kolarsick P.A.J, 2011), the skin has a cooperative attitude with other organs in various ways:

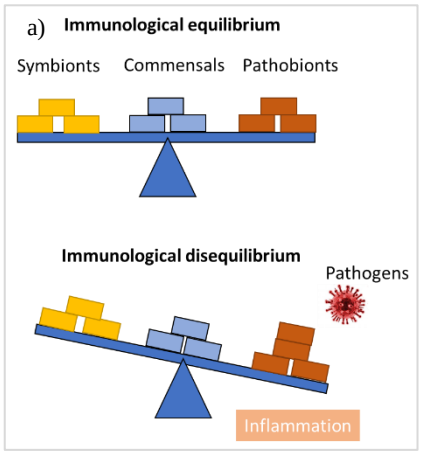
- The sensory function: tactile sensory touch, texture, temperature and pressure. The skin contains a huge quantity of receptors that respond to stimuli and transmit data about them to the brain.
- Vitamin D production: a precursor in our skin (7-dehydrocholesterol) reacts with UVB light and goes through a series of step to become activated Vitamin

D. The vitamin D is very important to reduce the risk of osteoporosis now and later in life, to maintain peak bone mass because it helps the absorption of calcium from the intestinal tract. Data show a correlation between good level of Vitamin D and reduced risk of hypertension and cardiovascular diseases and sunlight and darkness helps the circadian release of hormones from the brain.

- Immune function: immune responses are originated by cells and molecules from the innate or adaptive immune system. As soon as a pathogen passes the epidermis, the immune system is alerted and activated for a complement cascade which lead to a formation of the "membrane attack complex" where the photogenes are brought to cell lysis and death.
- Regulating adipose tissue function: the skin has a significant effect in regulating the body glucose metabolism. The link is, until now, uncertain but potentially related to skin endocrine function.

(Bangert C., 2011; Di Meglio P., 2011; Caton P.W., 2017)

The skin, also, is an eco-system with microbial communities that live in a range of physiologically and topographically distinct parts. Around $10^{12}$ resident bacteria/$m^2$ are sheltered on skin surfaces. Relative abundance of skin bacterial groups is related to microenvironment types: Propionibacteria species and Staphylococci species predominated in sebaceous sites Corynebacteria species predominated in moist sites, although Staphylococci species were also represented. A mixed population of bacteria resided in dry sites, with a greater prevalence of β-Proteobacteria and Flavobacteria. The collectivity of all those microbes living on the skin surface is called skin microbiota. There are three kinds of microbial cells: i) symbiotic, with health-promoting functions; ii) commensals, normally harmless; iii) pathobionts, with high potential to produce pathology. The skin microbiome and its interaction with the host is ruled by a delicate balance, when there is a disequilibrium between those parts, a potential disorder or infections can occur (**Figure 2**) (Grice E.A., 2011).




b)

| | Pathogen | Characteristics |
|---|---|---|
| **Bacterial diseases** | | |
| **Impetigo** | Staphylococcus Aureus | Superficial skin infection |
| **Erysipelas** | Streptococcus Pyogenes | Reddish patches, Fever |
| **Virus diseases** | | |
| **Herpes simples** | Herpes simplex | Vesicles |
| **Fungal diseases** | | |
| **Candidiasis** | Candida Albicans | Infection site |

**Figure 2.** a) skin microbiome balance, b) possible microbial diseases of the skin related.

### 1.1.1 Stratum corneum (SC)

The SC is the outermost layers of the epidermis, typically consists of ~ 10 to 20 piled-up layers of terminally differentiated not nucleated corneocytes embedded in an intercellular lamellar lipid matrix. The cells average thickness is related to the location, usually is ~10-25 μm over most body sites but it can reach of ~200-600 μm on the palm and sole. It has specific structures:

- Corneocytes: flat and anucleate cell composing the SC.
- Corneodesmosomes: functioning as the intercellular adhesive structures between corneocytes. They are go through a degradation process creating the desquamation of outermost. This process is severely controlled by proteases (kallikrein-related peptidases) and their inhibitors (lympho-epithelial Kazal-type related inhibitor) in order to maintain a healthy balance (Ishida-Yamamoto A., 2015).
- Intercellular lipids matrix: involving 13 species of lipids such as ceramides, cholesterol and free fatty acids. They fill the tortuous pathway between the corneocytes providing the permeability barrier.
- Lipolytic and proteolytic enzymes: taking part in the metabolism of pro-barrier lipids and of the SC.
- Other elements: such as, natural moisturizing factors (NMF).

(Boer M., 2016)

The "cornification" is the apoptosis process that bring to the formation of the cell death layers called SC, during this curse three are the key events: i) formation of the intracellular keratin network, ii) crosslinking of lipids and proteins and iii) formation

of a unique complex mixture of polar and non-polar lipids secreted from the lamellar body (LB). The LBs are organelles located in Golgi apparatus and contain phospholipids, glucosylceramides, sphingomyelin and cholesterol. During the cornification process, the contents of LBs is released into the extracellular area in the deep SC where there are different types of proteases, protease inhibitors and lipids. Their complex structural organization are responsible of the formation and maintenance of the skin barrier function (Madison K., 2003; Matsui T., 2015).

The term "skin barrier functions" include more than one defensive functions, and a big portion of them are co-localized and linked biochemically to the SC. The structure of the SC with corneocytes embedded in extracellular lipid matrix with a supramolecular organization in a series of lamellar bilayers, the hydrophobic profile, the lipids distribution, limit the passive water loss through skin. Just a small amount of water should be able to spread through the SC as function of keeping the SC hydrated as well as participating in the thermoregulation mechanisms. The limited permeability of SC to water reduces the flow in both directions, regulating the balance between inside and outside (Grice K.A., 1972). Some of the lipids synthetized in the SC, furthermore, have broad antimicrobial activity. The SC supply, furthermore, prevention of microbial pathogens ingress thanks to, first, mechanical barrier, and second, to some lipids synthesized in SC present broad antimicrobial activity such as free fatty acids (FFA), glucosylceramides, sphingosine and some other polar lipids (Miller S.J., 1988; Bibel D.J., 1992). They show antimicrobial activity for gram-positive and gram-negative bacteria such as S. aureus, S. pyogenes, S. epidermidis etc. (Bibel D.J., 1993). In the last decades, studies showed that also epithelial peptides have antimicrobial activity, the most studied are the defensins and cathelicidins which are synthetized by keratinocytes. Usually, they are produced in limited quantity but, when an infection is going on, their production increases near potential entry point such as follicles, skin damaged areas etc. (Imokawa G., 2001; Gallo R.L., 2002). When the SC undergo to a mechanical stress, the proliferating keratinocytes at the basement membrane increase in size and density, and the process of proliferation and differentiation of cells across the epidermis increases and subsequently the SC thickness. With a thicker SC the shear stress gradients are lower (Sanders J.E., 1995).

The main components of SC are ceramides (CERs), cholesterol (CHOL) and free fatty acid (FFAs) in approximately equimolar ratio (Weerheim A., 2001). FFAs are 1 and 2 carbon chain mainly saturated whereas CERs are 1 and 2 carbon chain mainly

unsaturated. Together these lipids form a unique spatial arrangement. The SC lipids can adopt three type of lateral packing arrangement which differ in their rotational and translational mobilities. In the healthy SC lipids are (**Figure 3**): densely packed lipids chains (orthorhombic phase) with no rotational or transactional mobility; less densely packed lipids chains (hexagonal phase) with some rotational mobility but no transactional mobility; low densely packed (liquid crystalline phase) which the lateral organization is completely lost, and they have full mobility (Van Smeden J., 2014).

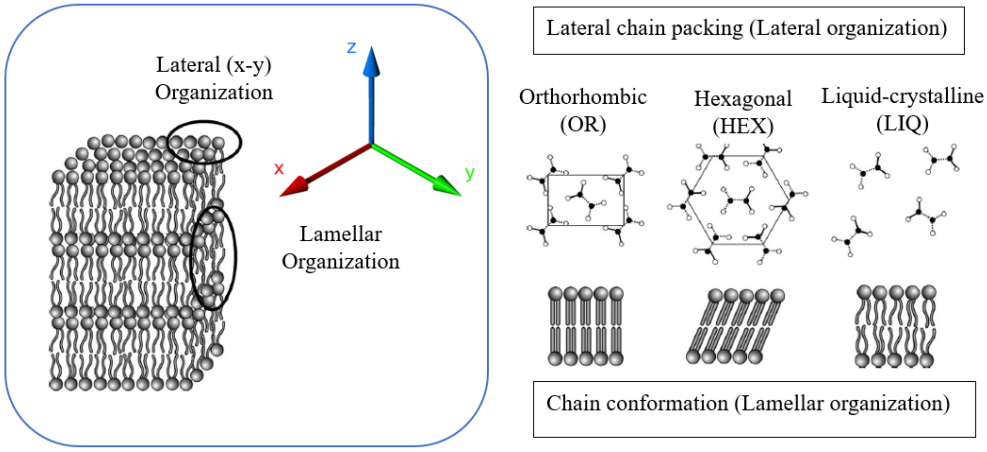



**Figure 3.** A scheme illustrating the structure of the lipid lamellae and a schematic show the lateral chain packing (top row) and the chain conformation (bottom row) in the orthorhombic (OR), hexagonal (HEX) and liquid-crystalline (LIQ) phases formed by the lipids.

Most of the experimental evidence show the validity of the "domain mosaic model" by Forslind (Forslind B., 1994) in which ordered lamellar crystalline structure are bordered by lipids in a liquid-crystalline arrangement. The organization of the lipids is not fully agreed, various modes to describe the organization have been proposed: "the sandwich model" by Bouwstra et al. (Bouwstra J.A., 2000), "the single gel phase model" by Norlèn (Norlèn L., 2001), "the armature reinforcement model" by Kiselev et al. (Kiselev M.A., 2005). The sandwich model presents a model 13 nm lamellar phase in which crystalline and liquids domains co-exist. It was observed the CERs and CHOL were essential in the formation of the lamellar phase and FFAs are able to increase the density of the structure, in particular, CER 1 was evaluated as crucial for the SC barrier function.

The disturbance of SC lipids specific structure and distribution, can cause several skin diseases (Sahle F.F., 2015). A disease in which the role of the lipids has been studied in relation to the skin barrier function is lamellar ichthyosis, a disorder that

appear in newborn and it is present throughout life presenting large scales all over the body. The patients present a reduced skin barrier function followed to trans-epidermal water loss (TEWL) values significantly high, unbalanced FFA/CHOL and FFA/CER ratios and changes in their lamellar lipid organization (Lavrijsen A.P., 1995). Also, in atopic dermatitis lesioned and non-lesioned skin, lipids abnormalities are observed: i) reduction in SC lipids level, ii) disequilibrium of CER/CHOL/FFA level, iii) reduced CER content, iv) abnormalities of enzymes involved in SC synthesis. Also, in patients with psoriasis, SC lipids (FFAs) were found in abnormal ration and conformation (Takahashi H., 2014). Some finding, also, suggest a relation between psoriasis and SC lipid abnormalities. Psoriasis is a chronical inflammation of the skin, presenting skin covered with thick, silvery scales. Therefore, information about SC lipids composition and structure are essential to understand how they are associated with changes in SC barrier function, protein expression, differentiation of keratinocytes and inflammation.

### 1.1.2 Melanin

Melanocyte can be found mainly into two the basal layer, they are responsible for the production of the pigment melanin and its transfer to keratinocytes. Melanin is synthesized by the enzyme tyrosinase which is able to convert tyrosine to dihydroxyphenylalanine (DOPA). There are two chemically distinct types of melanin: eumelanin (black-dark brown) and phaeomelanin (red-yellow), they are systemized following two distinct biosynthetic pathways regulated by the activity of the melanocortin receptor (MC1R). MC1R is express mostly by melanocytes and it is regulated positively by α-melanocyte stimulating hormone (αMSH) and negatively by agouti signal protein (ASIP). The melanogenesis is restricted to membrane-bound organelle called melanosomes (because the biosynthetic intermediates are toxic for the surrounding biosystem) via a series of receptor mediated reactions (Slominski A., 2004). The development of melanosomes involves four stages of differentiation: early matrix organization, matrix organization (without melanin production), melanin formation, melanosomes are filled with melanin. Every perturbation of this process could bring to diseases potentially dangerous for human health ex. skin melanoma (Bomirski A., 1988). The melanin is not retained in melanocytes but is transported along microtubes to be transferred to nearby keratinocytes in skin hair etc.

Historically, dark skin is related to more melanin production in each melanosome, larger size of melanosomes, the greater amount of dispersion of

melanosomes in keratinocytes, and the slower rate of melanosome degradation compared to light skin (Murphy G.F., 1997). This is because human skin has developed the ability to modify the melanin content in relation to the situation proposed, based on the subject's melanogenetic potential. Increased level of melanin during UVR exposure is related directly to the melanocytes response but it is also related to indirect hormonal factors. A complicate system of paracrine and autocrine factors (hormones, cytokines etc.) is influenced by UVR (Brenner M., 2008). The process of pigmentation is complex and not fully understood but, there is strong evidence that DNA photodamage stimulate the melanogenesis (Gilchrest B.A. and Eller M.S., 1999; Eller M.S., 2000).

The protective properties of human melanin against UV radiations is well documented, it absorbs in the range of 720-300 nm (Kollias N., 1991). Studies support the inverse correlation between skin pigmentation and incidence of skin cancer (Gilchrest B.A., 1999) but it has been valuated also the reactivity of melanin with DNA, its action as photosensitizer and relative reactive oxygen species (ROS). Pheomelanin is related to photodegradation after UVR exposure leading to UV-induced damages that, if not repaired, can produce mutation in melanocytes or other cells (Brenner M., 2008).

### 1.2 Skin penetration

The development of the skin is made up as a selective protective barrier, keeping harmful substances out but, especially in the last decades, it is considerate as a good pathway for drugs penetration or cosmetic actions. The skin must deal with daily application of topical products and three functions may be achieved. Introducing first point, it could be desirable to have the active staying on the skin surface, without penetrating the skin layers. It is the case of cosmetics products (skin decoration), skin disinfection and insect repellents. Secondly, some topical formulation could be developed to allow epidermal and dermal penetration of their actives in order to reach deeper regions of the human skin, but, without an absorption in systemic circulation. The last point, topical formulation designed to penetrate in the deepest layers of the human skin and reach the systemic circulation, aimed for topical therapy. This kind of selectivity is regulated by anatomical skin properties and formulation features (Trommer H., 2006).

Factors that may affect penetration are: molecular size, partition coefficient, compatibility with intercellular lipids, skin condition SC thickness, skin care routine, environmental factors etc. Skin condition as “dry skin” is directly related to intercellular depletion and fracture in SC layer, so, the topical product target could be modified in some way by this circumstance (Harding C.R., 2003). The particle size is also a key factor in topical product penetration, nanoparticles and microparticles are often used in percutaneous drug delivery. These devices can carry chemicals and, once applied on the skin surface, the actives usually unable to penetrate, can go through the skin barrier and reach the intradermal capillaries (Yokota J., 2018). However, drug penetration across the skin always been a challenge, skin and in specific SC offer a protective barrier with low permeability severely limits the transport of most pathogens, toxins and drug molecules (Karande P., 2009). In order to search the deepest layers of the skin, the main transport barrier, the SC, should be bypassed. To pass the SC, drugs has to navigate through the tortuous lipid pathways surrounding the keratin-rich cells, or repeatedly partition between the aqueous, keratin-rich phase and the lipid phase (El Maghraby G.M., 2008). The passive transport is available only for drugs with specific physicochemical properties such as molecular weight under 500 Da, high hydrophobicity, and adequate solubility in aqueous and non-aqueous solvents (Zhang J., 2010).

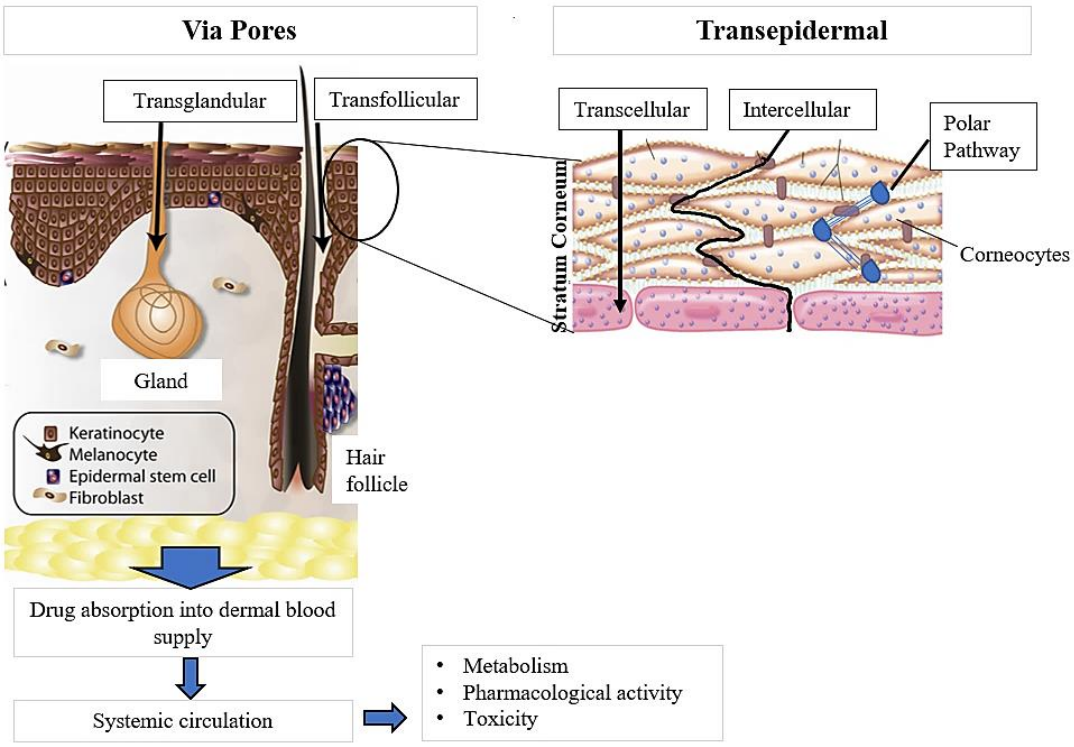


**Figure 4.** Schematic representation of the processes involved in drug penetration across the skin.

Several routes are available to pass SC and penetrate in deepest layer of the skin, they are shown in **Figure 4**:

- Transdermal route by transcellular route: the drug must cross the skin by directly passing through cytoplasm of corneocytes and SC lipid structures, it is the shorter way, but it is highly dependent upon its partition coefficient because they have to cross both lipophilic and hydrophilic structures.
- Transdermal route by intercellular route is a common way to penetrate the SC, the drug has to pass in between the corneocytes crossing the lipid bilayers. The corneocytes are not aligned between each other, so, a compound has to go through a windy way.
- Polar pathway: is composed to aqueous regions between the intercellular lipid as water microchannel. They have a high resistance versus lipophilic substances but a big affinity for hydrophilic compounds.
- Pores route: glands and hair follicle represent only 0.1 % of the total human skin surfaces, its contribution to the SC penetration is considered very small and the variation in follicle distribution related to the body location should be considered too.

(Dayan N., 2005; Trommer H., 2006)

The diffusion of a drug through the SC is a passive kinetic process that can be described by Fick's first law:

$$J = -AD\ \frac{(dC)}{(dx)} \quad (1)$$

Where: J represents the flux, A represents the unite area of the membrane, D represents diffusion coefficient, dC/dx represents the concentration gradient across the membrane. Equation (1) represent the diffusion coefficient of the drug into the membrane related to its solubility in the membrane. In the skin, the stratum corneum represents the rate-determining layer and the drug penetration is ordered by its diffusion coefficient inside the SC lipids and partition coefficient between drug and SC lipids.

Several strategies have been developed by scientist to deliver drugs to and through the skin, there are several compounds able to promote the substances pathway across the skin called chemical penetration enhancers (CPEs). They can act on: i) polar

headgroups of the lipids disturbing the SC lipids packing order; ii) water contentment, by hydration of the SC there is an increased diffusion of hydrophilic drugs; iii) interaction between lipophilic penetration enhancers and hydrocarbon chains of the bilayer lipids and consequent fluidization of the hydrocarbon chains; iv) an increased drug solubility, thanks to suitable vehicles or co-solvents, bringing to an improved partition coefficient; v) lipids extraction as the result of an interaction with chemical enhancers (Lane M.E., 2013). The treatment of the skin with solvent or CPEs modify the SC lipids composition, their unique molecular and structural arrangement, which leads to changes in skin permeability. The most citrated and common penetration enhancer include: alcohols, fatty acids, surfactant, amides, esters etc. Beyond chemicals (CPEs), physical factors such as temperature, environment factors (UV radiation) and water concentration could modify the lipid packing and subsequently alter the skin penetration behavior.

In vitro testing for skin absorption of chemicals are regulated by guidelines (OECD, 2004) (SCC, 2015) in order to presents general principles for measuring dermal absorption and delivery of a tested substance using excised skin.

### 1.3 Ultraviolet radiation and cutaneous response

Sunlight is composed of a continuous spectrum of electromagnetic radiation which is divided and named according to the range of wavelengths (λ): ultraviolet, visible and infrared. The UV radiation represents just the 5-10% of the solar radiation but they represent a potential hazard because some skin molecules can absorb UV radiation. UV energy can be divided into UV-A, -B and -C components based on the wavelength. The UVC (100-280 nm) light doesn't reach the earth because it is filtered by the atmosphere, whereas UVB (280-315 nm) light penetrates the upper layers of the skin and UVA (315-400 nm) can penetrated the deeper skin layer causing interaction with skin cells (**Figure 5**). Human first defense against UV light is the pigment melanin, which is able to absorb UV radiation, but when there is an excess of UV exposure or there is an unprotected exposure, damages such as DNA-damage could occur.

When there is an excess of UV exposure, UV radiations escape form melanin absorption and are absorbed by molecules called chromophores. DNA is the main epidermal chromophore. An excess of UV exposure can cause indirect DNA damages with consequence production of reactive oxygen species (ROS) that present a very high level of reactivity even with other molecules, and direct DNA damages. After

intense UV exposure the DNA undergo to damages which are repaired by internal biological system, but occasional mistakes during the fixing can happen such as the incorporation of wrong bases into the genetic material, cyclopiridine dimers formation etc. These types of mistakes often result in mutation or inappropriate expression of affected genes (Sinhaa R.P., 2002).

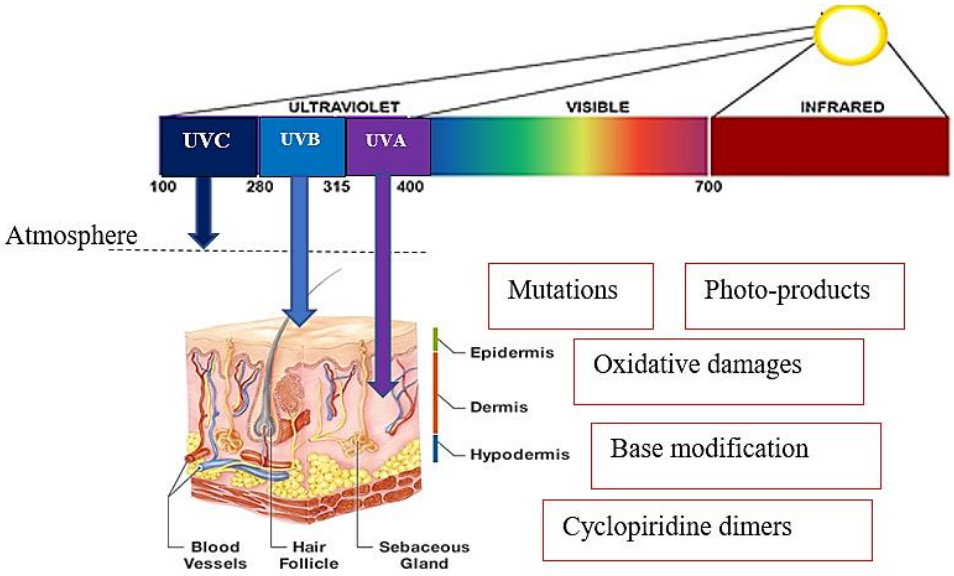



**Figure 5.** Sunlight spectrum, UV radiation penetration into the skin and possible damages.

The DNA damage is maybe the biggest delayed hazard, but it is not the only way in which UV light effects the skin physiology. UV induces, at first, a hyperkeratosis where keratin start to overgrow increasing the epidermal thickness, then UVB activate a cascade of cytokines, vasoactive and neuroactive mediators resulting in an inflammatory response called “sunburn” and, if the UV persist with a good level of intensity, keratinocytes activate apoptotic way and die. The damage persists also after hours of UV exposure (Coelho S.G., 2009; Scott T.L., 2012).

Skin possesses different mechanisms to protect the process of damaging DNA transformations. p53 gene is one of the most intensively studied for its participation in to cell suicide process. It is considered to be the «guardian of the genome» that consists in sensing and reacting to DNA damage through the ATM/ATR and Chk1/Chk2 kinases, it is well defined that its mutant forms acquire pro-oncogenic activities. p53 mutation, after stress input (as extended exposure UV light), leads to an inability for the gene to block abnormal cellular growth so cells survivals and there is an uncontrolled cell division. Mutation in p53 activities frequently detected in many

tumor types. Squamous cell and basal cell carcinomas has correlations to mutation the p53 genes caused by UV light has been demonstrated (Benjamin C.L., 2007).

Geographic position and ambient exposure has a significative impact on UV induced damages. UV dose varies according to the amount of UV rays that pass through the atmosphere and reach the skin: ozone depletion, weather conditions, latitude and altitude are external factors influencing the potential risk. There are also intrinsic factors involved in development of photo induced damages: ethnic origin, relationship to personal exposure to the sun, personal sun protection, gene mutation as CDKN2A gene, intentional sun exposure etc.

### 1.3.1 Skin tumors

Skin cancer represent the most common type of malignant neoplasms in Caucasian population, over a million cases diagnosed each year (Rogers H.W., 2010). They account for nearly 15,000 deaths and more than three billion dollars each year in medical costs in the United States alone. The estimated number of new cases of skin melanoma in 2016 is 76,380, which represents 4.5% of all new cancer cases (Apalla Z., 2017; Apalla Z. and Nashan D., 2017).

The term “skin cancer” gathers two groups of pathologies, melanoma (MM) and non-melanoma (NMSK) skin cancer. MM and NMSK development regulated by multifactorial model included intrinsic and extrinsic factors. Intrinsic factors include random errors in DNA replication followed a genomic mutation. They represent ~10-30% of lifetime risk to cancer development, even though, there are significative intrinsic genomic alteration accumulation by endogenous processes, they are not enough to be relevant in skin cancer risks. Extrinsic factors are coming from the environmental, such as UV radiation. In literature, several epidemiologic and molecular linked skin MM and NMSK to UV exposure (Linos E., 2009). As previously mentioned, UV radiation escaping from the melanin, can be absorbed by skin cells or DNA producing genomic mutation or photo-products potentially toxic for our body, that could be deleterious with functional consequences. Therefore, the potential risk about extrinsic factors may related to the accumulation of mutation (Wu S., 2016).

There is different thinking about the origin of skin cancer. Until the last decade, the idea was the cancer was originated from mature tissue cell that underwent dedifferentiation in response to cancer progression (Sell S., 2004), today, cancers are proposed to originate from the malignant transformation of normal tissue progenitor

and stem cells (Reya T., 2001) even if the hypothesis is not fully agreed (Visvader J.E. 2011).

The risk of development skin cancer is regulated by genotypic, environmental and phenotypic factors. Sun-sensitivity, high number of melanocytic nevi, family history etc. are known as risk factors. Melanin is able to absorb UV radiation and survive to considerable genotoxic stresses, it is an intrinsic protection factor, therefore, the degree of pigmentation manifests the skin "phenotype" but it is also a significative indication of skin cancer risks (**Figure 6**) (Fitzpatrick T.B., 1988; Scherer D., 2010). Fair-skinned phenotype presents low levels of melanin resulting in less protection against UV-induced damages, they tending to burn rather than tan; black phenotype presents high level of melanin production resulting in high level of intrinsic protection against UV-induced damages. Lighter pigmentated skin (phenotype I) has a higher intrinsic risk factor for skin cancer compared to high pigmentated skin (phenotype VI) (Han J., 2006).

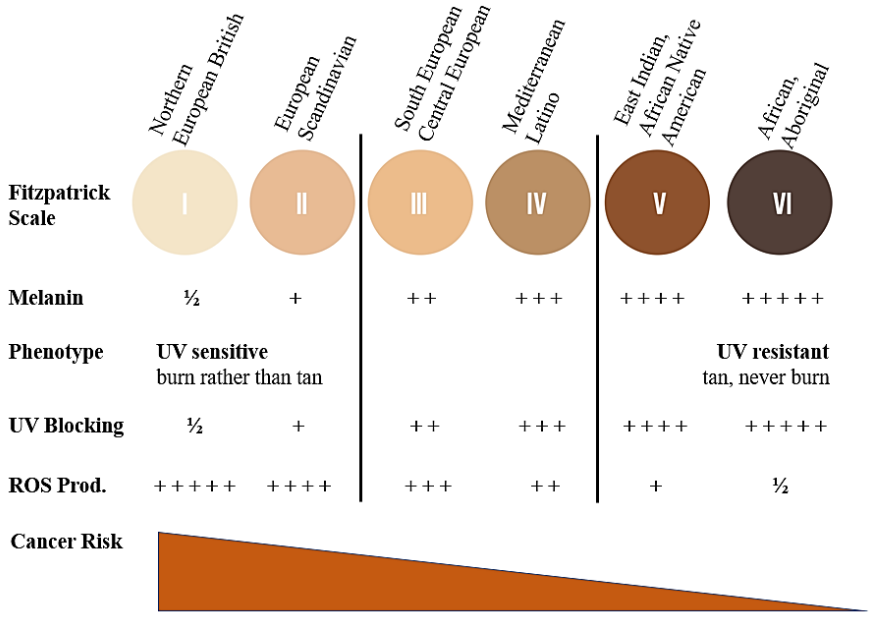



**Figure 6**. Influence of pigmentation on skin cancer risk.

### 1.3.2 Melanoma (MM)

More than 60,000 melanomas were diagnosed in 2010 in U.S.A. (U.S. Cancer Statistics Working Group, 2013), in recent epidemiologic studies, melanoma shows an annual incidence of 9.5%, (National Cancer Institute, 2016). The observed increases

could be related to several factors, including the tendency to go toward older population associated with a higher intrinsic risk.

MM tends to be an aggressive skin tumor classified by 4 main subclasses: i) superficial spreading, ii) nodular, iii) lentigo malign and iv) acral melanoma. Superficial melanoma is a form in which the malignant cells tend to stay "in-situ", without spreading into surrounding tissues, accounting approximately 70% of cases. When a portion of superficial spreading melanoma cross the epidermis and enter the dermis became a nodular melanoma (accounts for ~15% of melanomas). Lentigo malign melanoma is usually confined on face or neck with a low rate of transformation in invasive melanoma and slow grow (accounts for 13% of melanomas). Acral melanoma assault mainly palms and soles, and accounts for about 2-3% of all melanomas (Bradford P.T., 2009).

As previously discuss, UV radiation is a predominate cause in the skin cancer development, but, genetic predisposition also plays a significant role in individual's risk. Studies, in the past, have shown that ~10% of melanoma cases are related to a family history of melanoma. Two genes have been identified in high-risk families: CDKN2A and CDK4 (MacGeoch C., 1994; Gruis N.A., 1995). Both genes are important in controlling cell division, CDKN2A encodes for two proteins, p16-INK4A, involved in the retinoblastoma pathway and p14-ARF involved in the p53 pathway. They regulate the apoptosis pathway that inhibit progression of cancer cells, therefore, mutation in those genes can lead to abnormal cancerogenic cells growth (De Snoo F.A., 2005). Mutation in CDKN2A and CDK4 were found in 41% (p16: 38%, p14: 1.5% and CDK4: 1%) of a population sample of high-risk families (Zuo L., 1996; Soufir N., 1998). Also, melanocortin 1 receptor (MC1R) has a genetic implication in pigmentation and its association with melanoma has been investigated by meta-analyses and genome-wide association studies (Williams P.F., 2011; Pasquali E., 2015). MC1R is located on melanocytes surface and it has a key role in decreasing UV-mediated mutagenesis by enhancing genome maintenance pathways in melanocytes, mutations in MC1R polymorphisms are easily found in skin cancer-prone population (D'Orazio J., 2013).

The melanoma can be faced with surgery, targeted therapies and immunotherapies. Surgery is the privileged treatment of primary stages melanoma. In case of melanoma stage IA or primary melanomas (1 mm), the biopsy of the lesion or sentinel lymph node biopsy are selected, but in advance stage melanoma surgery

approach is selected only in case of radical intervention. The approach on advanced melanoma, in the last decades, has changed. The first step is represented by the assessment of mutational status, around 40-50% cases have a mutation in V600 of the BRAF gene (Davies H., 2002; Solit D.B., 2006). Those patients can use the targeted therapies, with combination of BRAF- and MEK-inhibitors that show an effective option with acceptable toxicity profile with regards about the application (sequential or cyclic) (Ho A.W., 2005; Chapman P.B., 2011; Flaherty K.T., 2012). Immunotherapies anti-CTLA-4 and anti-PD-monotherapy represented an innovative breakthrough in the treatment of metastatic melanoma. CLTA-4 is the receptor cytotoxic T-lymphocyte-associated antigen 4 and, data showed, long-term survival (10 years) in 20 % of the metastatic melanoma cases (Hodi F.S., 2010; Schadendorf D., 2015). PD-1 is a programmed cell death receptor expressed by T cells, one of its principal ligand PD-L1 was detected in significant high level in tumor cells. In tumor models was shown that antibodies blocking the interaction between PD-1 and PD-L1 may positively interfere with the tumor cells growth (Pardoll D.M., 2012). Moreover, anti-PD-monotherapy showed a better efficacy and lover toxicity profile compared to the anti-CTLA-4, in some cases both anti-PD and anti-CTLA-4 are used in combination but high risks of toxicity were potentially individuated (Fuchs C.S., 2014).

### 1.3.3 Non-Melanoma Skin Cancer (NMSC): basal cell carcinoma (BCC), and squamous cell carcinoma (SCC)

The term non-melanoma skin cancer (NMSC) includes two type of skin cancer: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). In 2006 an estimation of 3.5 million new cases of NMSC was made (Rogers H.W., 2010). The incidence of NMSC increases with population age, over 60 years of age and older present 80% of the cases and, usually, the incidence is higher in men then woman (Diffey B.L., 2005).

Both BCC and SCC are related to malignant transformation of keratinocytes and suppression of the cutaneous inflammatory response (Erb P., 2008). BCC appears as lesions with a slow growth, metastases are sporadic but undermining of surrounding structures can occur if untreated. SCC may present as ulcers or indurated keratinizing lesions on sun-exposed sites, actinic keratoses (AKs) and Bowen's disease are pre-malignant lesions and in 1 to 10% of the cases they are marker of invasive SCC (Madan

V., 2010). Here below, the main pathways related to NMSC development are presented. Mutation of:

- MC1R receptor, controlling cells division and regulating the production of p16/p14 proteins involved in apoptosis process.
- XPC protein, specific to genome repair pathway.
- CDKN2A and CDK4, both genes are important in controlling cell division.
- P53 gene, tumor suppressor gene.
- Cytochrome P450 (CYP), catalyze biotransformation of several xenobiotics.
- Telomerase enzyme, repeats at chromosome ends to compensate for telomere loss during cell division.

(Madan V., 2010)

For well-defined low-risk NMSCs (<2 cm diameter with a 4 mm margin), the surgery is the best standard with fair cosmetic results (Telfer N.R., 2008), but continuing search for non-invasive treatments has led to development of non-surgical therapy. They can be divided in: physical and chemical destruction and immunomodulation:

- Physical: radiotherapy, curettage, cautery and cryotherapy
- Chemical: topical photodynamic therapy (PDT), Topical fluorouracil or imiquimod
- Immunomodulation: imiquimod

(Griffin L.L., 2016)

### 1.4 Sun protection: Regulation

In the previous paragraphs, the attention was focused on the hazards side of the sun exposure, but, it is well known about the benefits coming from a moderate UVR exposure such as: i) increased production of vitamin D, reducing the risks of osteoporosis now and later in life; ii) increased absorption of calcium from the intestinal tract, maintaining the peak bone mass; iii) regulation of the circadian release of hormones in the brain, with the succession of also sunlight and darkness.

According to the Health World Organization, getting anywhere from 5 to 15 minutes of sunlight three times a week is enough to enjoy the benefits of sun exposure.

Even so, the authority identifies the UV exposure risks, therefore, there are European but also American recommendations that provided the general guidelines for a correct UV exposure in order to enjoy the beneficial sides of a healthy UV exposure and, at the same time protect our-self form the harmful sides:

- Limit time in the midday sun. The sunlight, during this time, is very strong and it is useful limit as much as possible the UV exposure.
- Watch for the UV index. It can give us information about the risks level of UV exposure.
- Protect yourself form direct UV exposure with umbrellas or protecting clothing.
- Use broad spectra sunscreen with SPF 15 or higher and reapply it every 2 hours or after working, swimming, outdoor physical activity**.**

The sunscreens are regulated and classified in different ways around the word, therefore, it is not easy to compare the different regulations available.

In European Union (EU) the sunscreens are considered as cosmetic products: *"any substance or mixture which is intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with the sole purpose of cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors"*. There are 2 different identification parameters: (i) destination, only superficial parts of the body; and (ii) scope, these products should have just cosmetic functions (cleansing, color, odor) and do not interact with systemic system. A sunscreen product is defined as *"any preparation (such as creams, oils, gels, sprays) intended to be placed in contact with the human skin with a view exclusively or mainly to protecting it from UV radiation by absorbing, scattering or reflecting radiation"*, so the protection of the skin against UV induced damages is referred to a cosmetic action. The current EU Regulation (3) EC/1223/09 (Regulation (EC) No 1223/2009) replaced the previous Directive 76/768, in order to provide some legal structure to all the member states, proposing a negative and positive list of ingredients. Same regulation was adopted in New Zealand, some of the Middle East/Arabic countries, Turkey and Association of South-East Asian Nations countries with relative minor changes. There are 30 allowed UV filters listed associated with relative concentration limits and specific warnings for the labelling of the products.

USA, by Food and Drug Administration (FDA), regulated sunscreen as "over the counter drugs (OTC)", allowing restrictive regulatory requirements for sunscreen (Food and Drug Administration, 2011). They have to succeed in all the approval processes and restrictive definition of labelling, therefore, some new UV filters already available in EU are rejected from FDA for lack of safety data. Some states (e.g. California) have a different regimentation and rules in order to supply specific needs. Canada has a regulatory system which is a mix of EU and US rules.

In Australia, sunscreens are classified as therapeutic or cosmetic sunscreens. Included in the first category are: i) primary sunscreens with SPF 4 or more, ii) secondary sunscreens, except those regulated as cosmetics, iii) primary or secondary sunscreens with SPF 4 or more that contain an insect repellent, iv) sunscreens that are exempt from being listed under the Act because they come within the exemption in Item 8(g) of Schedule 5 of the Regulations. All the products belonging to this category have to be listed in the Australian Register of Therapeutic Goods (ARTG). Included in the second category are sunscreen containing an ingredient with sunscreen activity, but the primary purpose of the product is neither to be a sunscreen or a therapeutic active. These products are regulated as cosmetics by the National Industrial Chemicals Notification & Assessment Scheme (NICNAS) (Australian Government, 2012).

Differentiating in term of regulation and classification, each country has a list of authorized ingredients with their maximum allowable concentrations in final products, in **Table 1** are listed UV filters approved in Australia (AUS), Europe (EU), and America (USA) with related concentration limits. In EU, annex VI of the Regulation (EC) No 1223/2009 of the European Parliament and Council (Regulation (EC) No 1223/2009) reports the 28 UV filters authorized and their concentration range in the final product. Only 10 are also approved by EU but with different use concentrations. Due to a more restrictive regulation only 16 UV filters are approved by the FDA. Considering the growing attention to sun products as an essential barrier in the protection against the UV rays, international harmonization of these regulations would be useful (Osterwalder U., 2014).

| | INCI (INCI abb.) | COLIPA | USAN | Trademark | Conc. Limits (%) AUS | EU | USA |
|---|---|---|---|---|---|---|---|
| Broad-Spectrum and UVAI (340-400nm) | Bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT) | S 81 | Bemotrizinol | Tinosorb® S | 10 | 10 | - |
| | Butyl methoxydibenzoylmethane (BMBM) | S 66 | Avobenzone | Parsol®1789 | 5 | 5 | 3 |
| | Diethylamino hydroxybenzoyl hexyl benzoate (DHHB) | S 83 | - | Uvinul® A Plus | 10 | 10 | - |
| | Disodium phenyl dibenzimidazole tetrasulfonate (DPDT) | S 80 | Bisdisulizole Disodium | NeoHeliopan® AP | 10 | 10 | - |
| | Drometrizole trisiloxane (DTS) | S 73 | Drometrizole Trisiloxane | Mexoryl® | 15 | 15 | - |
| | Menthyl anthranilate (MA) | - | Meradimate | - | 5 | - | 5 |
| | Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT) | S 79 | Bisoctrizole | Tinosorb® M | 10 | 10 | - |
| | Terephthalylidene dicamphor sulfonic acid (TDSA) | S 71 | Ecamsule | Mexoryl® SX | 10 | 10 | 10* |
| | Zinc oxide (ZnO) | S 76 | Zinc oxide | Z-Cote® HP1 | N.L. | 25 | 25 |
| UVB (290-320 nm) and UVAII (320-340 nm) | 4-Methylbenzylidene camphor (MBC) | S 60 | Enzacamene | Eusolex® 6300 | 4 | 4 | - |
| | Benzophenone-3 (BP-3) | S 38 | Oxybenzone | - | 10 | 6 | 6 |
| | Benzophenone-4 (BP-4) | S 40 | Sulisobenzone | Uvinul® MS40 | 10 | 5 | 10 |
| | Camphor benzalkonium methosulfate (CBM) | S 57 | - | Mexoryl® SK | - | 6 | - |
| | Polysilicone-15 (PS15) | S 74 | - | Parsol® SLX | 10 | 10 | - |
| | Polyacrylamidomethyl Benzylidene Camphor | S 61 | - | Mexoryl® SD | - | 6 | - |
| | Diethylhexyl butamido triazone (DBT) | S 78 | Iscotrizinol | Uvasorb® HEB | - | 10 | - |
| | Ethylhexyl dimethyl PABA (EHDP) | S 08 | Padimate O | Eusolex® 6007 | 8 | 8 | 8 |
| | Ethylhexyl methoxycinnamate (EHMC) | S 28 | Octinoxate | Uvinul® MC80 | 10 | 10 | 7.5 |
| | Ethylhexyl salicylate (EHS) | S 13 | Octisalate | NoHeliopan® OS | 5 | 5 | 5 |
| | Ethylhexyl triazone (EHT) | S 69 | Octyltriazone | Uvinul® T150 | 5 | 5 | - |
| | Ethoxylated ethyl-4-aminobenzoate (PEG-25 PABA) | S 3 | - | Uvinul®P | 10 | 10 | - |
| | Homomethyl salicylate (HMS) | S 12 | Homosalate | Eusolex® HMS | 15 | 10 | 15 |
| | Isoamyl p-methoxycinnamate (IMC) | S 27 | Amiloxate | NoHeliopan® E1000 | 10 | 10 | - |
| | Octocrylene (OCR) | S 32 | Octocrylene | Uvinul® N539 T | 10 | 10 | 10 |
| | Phenylbenzimidazole sulfonic acid (PBSA) | S 45 | Ensulizole | Eusolex® 232 | 4 | 8 | 4 |
| | Titanium dioxide (Ti02) | S 75 | Titanium dioxide | Eusolex® T2000 | 25 | 25 | 25 |
| | Tris biphenyl triazine (TBPT) | S 84 | - | Tinosorb® A28 | - | 10 | - |

**Table 1.** UV filters approved in Australia (AUS), Europe (EU), and America (USA). *Only as a component of certain approved sunscreen formulations approved under the new drug application (NDA), N.L. No Limit.

Commercially available sunscreen, usually, use only a restricted part of the allowed UV filters. This is because, sunscreen industry need four essential requisites to formulate a commercially available sunscreen formulation: i) efficacy, ii) safety, iii) registration, and iv) patent freedom. Most of the time, some allowed UV filters are not able to satisfy one of those needs, the principal reason is represented by their efficacy

such ah UV absorption capacity or compatibility with a specific sunscreen vehicle formulation.

### 1.5 UV filters

Sunscreens represent a practical approach to protect the skin from precancerous skin lesion (Darlington S., 2003), UV-immunosuppression (Roberts L.K., 1995), NMSK (Green A.C., 1999) and MM (Green A.C., 2011). They contain molecules or molecular complexes that are able to absorb (chemical UV filters) or reflect/scatter UV radiation (physical UV filter).

Inorganic sunscreen ingredients (or physical UV filters) reflect/scatter visible, UV and infrared radiation over a broad-spectrum. Scattering is a process where the UV photons, beating the physical UV filters, are refracted and diffused in all the directions and UV light reflection happens when the light is bounced off from the UV filters. No energy is generated, but there is a change in the spatial distribution of the energy (**Figure 7 a**). Reflection and scattering are established by intrinsic characteristics such as refractive index, particle size, dispersion into the vehicle etc. (Manaia E.B., 2013).

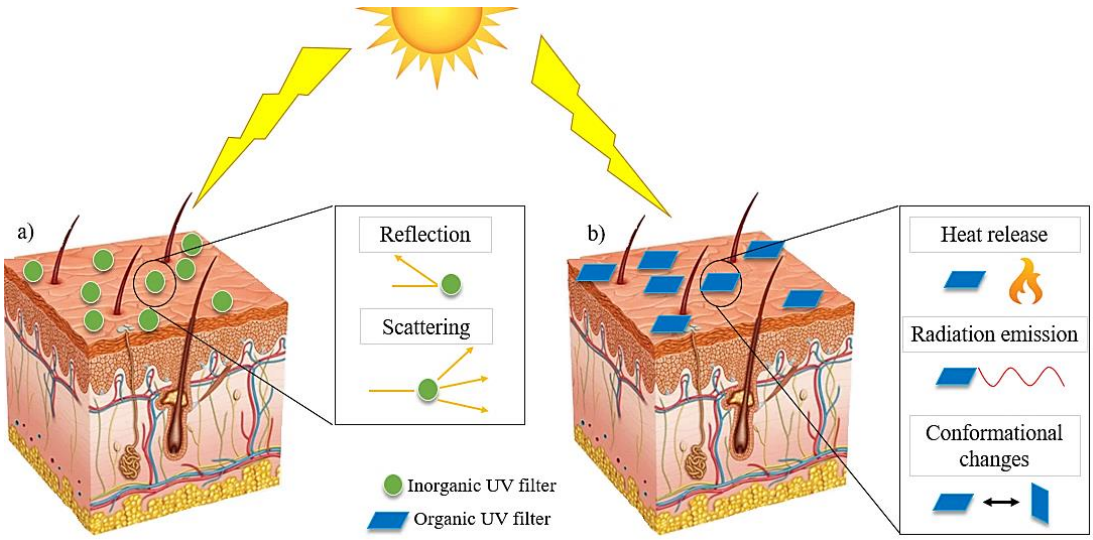



**Figure 7.** a) Action mode of inorganic and b) organic UV filters.

Zinc oxide (ZnO) and titanium dioxide ($TiO_2$) are mineral compounds, inorganic agents, permitted and commonly used in sunscreen (**Table 1**). They are biologically inert and have large application in protection of sensitive skins, since they provide a real physical barrier against UV radiation. They present a low reactivity versus organic

filters, indeed, often they have been used in combination with these even at high concentration. This union can create a synergic effect able to reach very high SPF values. Sunscreen products formulated with those UV filters, leave on the skin an undesirable whitish appearance and, because of aesthetic concerns, the acceptability of the product was negatively influenced until recently when particulate forms were introduced. Cosmetic acceptability has required inorganic filters with reduced particle size around 10-50 nm. They are made by micronization and so easily incorporated in to the sunscreen vehicle, transparent and with reflection/scattering maximized. These reduced particle size, however, have been criticized due to the potential cross through the skin and, consequently, its possible consequences for the human health (Serpone N., 2007).

The organic filters absorb UV radiations into specific wavelength ranges, as a function of their chemical structure. Usually they aromatic compounds with a carbonyl group capable of absorbing UV radiations and converting them into heat, vibrational and rotational energy (**Figure 7 b**). The energy absorbed by the filters is used by the electrons of the most external orbitals to perform electronic transactions, the molecule passes from a fundamental energetic state ($S_0$) to an excited state ($S_1$) in which it remains for a very short time and then returns to a fundamental state emitting a quantity of energy equal to the energy absorbed. The energy absorbed is dissipated in various ways: heat emission, fluorescence, photodegradation (modification of the chemical structure). In this photoreaction, structural changes occur, which can be reversible (isomerization) or irreversible. Irreversible photochemical reactions and its consequent photochemical products can compromise both the physical attributes of the UV filters (color, appearance, etc.) and their chemical properties. This instability, under UV radiations, could generate ROS, toxic derivative potential dangerous for human health, photoallergic contact dermatitis etc. (Gilbert E., 2013).

In order to prevent the potential UV-induced damages, the sunscreens have to meet specific criteria:

- Photostability: UV filters should dissipate the UV energy through physical and chemical ways without reactive intermediates that could lead to the production of ROS (photoproducts toxic for skin cells) or unknown products responsible for skin reaction (ex. allergic reaction). Also, the decomposition of the filter following irradiation reduces its protective efficacy. The development of

photostable UV filters is essential to obtain sunscreen formulation able to preserve their quality and efficacy when meeting skin (Gaspar L.R., 2006).

- Full sun protection: UVB rays are responsible for sunburn, erythema and UVA rays are responsible for tanning and early photoaging, both together have a synergic effect causing photoimmunodepression, DNA damage and skin cancer. Some of those effects are invisible and irreversible. Reason why, a sunscreen should protect over the entire range of UV radiation to offer a full sun protection. This extended range is difficult to reach, therefore, modern sunscreen contains a combination of several organic and inorganic UV filters to cover the entire spectrum.
- None penetration into the skin: UV filters are designed to stay on the superficial part of the skin in order to create a protective film able to reflect/absorb the UV radiation, without penetrate into the skin. With UV filters skin penetration, the photoprotection is lost and the skin is exposed to UV-induced damages. This absorption could create bioaccumulation, cytotoxic effect on the epidermal cells or can reach the blood vessels and the systemic circulation. This is not a crucial factor when there is the application of sunscreen for few months in a year during summer vacation but become incisive because, in the last decades, UV filters are incorporated in daily-routine products (cream, foundation etc.) so, the population is exposed to large quantity of chemical at relevant concentration (Hayden C.G.J., 2005).
- Well tolerated: potential skin reaction or endocrine consequences should be avoided.
- Good compatibility with other ingredients, good solubility in cosmetic emollients.
- Compatible with packaging materials.
- Biocompatible: Sunscreen products have been used for nearly 80 years, and in the past decades an increased use of sunscreen cosmetic products leading the introduction of new chemical compounds. There are around 45 UV chemical filters subjected to regulation in different countries, in addition to UV filters, sunscreen contains other ingredients such as preservatives, film forming agents, surfactants, viscosity controllers etc. Using those products, new chemicals went down household drains, made their way into rivers, lake and oceans. It has begun to raise concerns regarding marine pollution and its

consequences on flora and fauna. Some of those organic compounds show, already, toxic effects on the marine environment (Downs C. A., 2016). Until today, it is still a challenge and it seems to be an unreachable aim but in the next future, the resources should concentrate to study new and eco-compatible UV filters.

### 1.5.1 Avobenzone

Avobenzone (Buthyl Methoxydibenzoylmethane or 4-tert-butyl-4′-methoxydibenzoylmethane) is an oil-soluble UV filter that provide a protection in the UVA (340-400 nm). It is one of the most approved and common UV filters thanks to its great performances in the UVA range.

Avobenzone is an dibenzoylmethane derivative (called β-diketones), with an aromatic 1,3-diketo derivative of acetylacetone where both methyl groups in acetylacetone are substituted by phenyl groups. Its photochemistry is very complex due to the presence of two tautomer that exhibit different properties. **Figure 8** shows avobenzone tautomeric forms: the enol-tautomer (or enol form) and the keto-tautomer (or keto form) with intra-molecular hydrogen bonding (Zawadiak J., 2012). An essential requisite for an efficient sunscreen is the photostability, however, some UV filters such as avobenzone exhibits photo-reactivity after UV exposure leading to the formation of photo-products inactive, instable, toxic etc. Avobenzone photoinstability related to the solvent environment is well-known, it shows relatively stable in polar solvents and markedly photolabile in nonpolar media (Mturi G.J., 2008; Valleio J.J., 2011). This phenomenon has been attributed to enol-keto isomerization induced by irradiation.

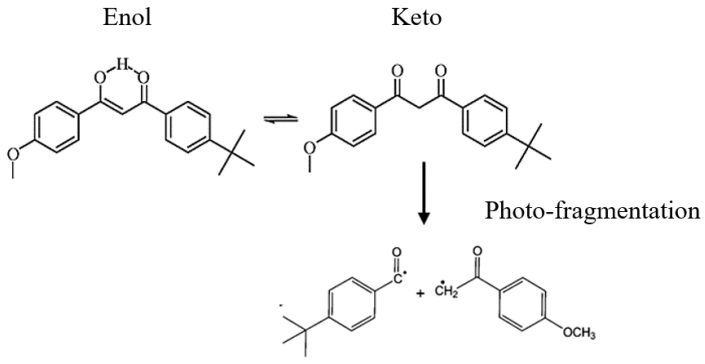



**Figure 8.** Enol form (right), keto form (left) and the photo-fragmentation of Avobenzone.

In its enol form it exhibits an excellent UVA absorption at 357 nm, but after irradiation, enol form photoisomerize in the keto form which absorbs in the UVC range and thereby ineffective as a UVA or UVB filter. Photochemical studies have shown the triplet excited state of the diketo form is responsible for the photodegradation (Paris C., 2009). This triplet derived is potentially reactive with skin components or biological substrates (Knowland J., 1993; Diamiani E., 2000).

There are several clinical reports about the potential photoallergy and phototoxic attributed to avobenzone (Wong T., 2011; Gaspar L.R., 2013), three class of avobenzone photo-products seems to be strong sensitizers: dibenzoylmethane, arylglyoxals and benzils (Karlsson I., 2009).

### 1.5.2 Octocrylene

Octocrylene is an approved and widely used UV absorber. It is an ester formed by condensation of diphenylcyanoacrylic acid with 2-ethylhexanol (**Figure 9**) and is considered to belong to the family of cinnamates. It provides a protection from 290 to 360 nm, covers mostly UVB wavelength but also short UVA wavelengths (UVAII), with a peak absorption at 303 nm.

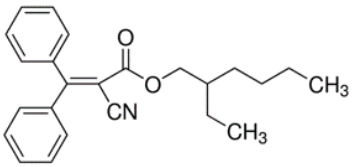



**Figure 9.** Molecule of Octocrylene

Due to its low efficacy as UVB filter, octocrylene very often is combined with other UV filters in order to reach high SPF values (Palm M.D., 2007). Very often, it used in association with photo-instable UV filters, such as avobenzone, because it is able to quench reactive species generated due to photofragmentation. It has a good compatibility with several cosmetic ingredients, such as cosmetic oils. However, octocrylene is expensive and its incorporation into the sunscreen vehicle has been a challenge (Palm M.D., 2007). Also, several studies showed the potential photoallergenic action of the UVB filter (Bryden A.M., 2006; (Karlsson I., 2009), photopatch testing presented the potential allergenic action of octocrylene (Avenel-Audran M., 2010).

### 1.5.3 Synergic combination UV filters

The combination of two or more UV filters with synergic actions is been introduced on the market several years ago. Strategies have been explored in order to improve photoinstability of certain UV filters or to obtain sunscreen formulation with broad-spectrum and high SPF. One well known combination is avobenzone-octocrylene using triplet–triplet quenchers (T–T) mechanism to improve avobenzone photostability (Lhiaubet-Vallet V., 2010). Molecules such as octocrylene are able to quench reactive species generated due to photofragmentation (Chaudhuri R.K., 2006) (**Figure 8**). Octocrylene is not the only one, in European Union it possible pick, also, between effective UVA filters: i) 4-methyl benzylidene camphor, ii) bis-ethylhexyloxy phenol methoxyphenyl triazone, iii) polysilicone-15 (Mendrok-Edinger C., 2009). There are several other molecules which are able to stabilize photoinstable molecules (mostly avobenzone) with this mechanism such as diethylhexyl naphthalate, diethylhexyl syringylidene malonate and polyester-8. They are registered as inactive ingredients without approval as ultraviolet filters by the governing bodies of their respective countries or organizations is unclear. (Chaudhuri R.K., 2006; Scalia S., 2010).

The singlet-singlet (S-S) quenching mechanism is another example of synergic effects related to UV filters combination. On example is the association between avobenzone and oxybenzone (benzophenone-3) or methoxycrylene. Oxybenzone and methoxycrylene are able to stabilize the single excited state of avobenzone (Bonda C., 2008).

In several commercial sunscreens, molecules that are endowed with antioxidant activity have appeared. They have the potential of quenching singlet oxygen and other reactive oxygen species (ROS), and studies show how this mechanism can contribute to the photostability of the UV filter (Chaudhuri R.K., 2005; Afonso S., 2014).

Very often those synergic combination of UV filters is combined with the encapsulation technology in order to obtain double advantages. The encapsulation allows to keep the synergic absorbing system in a closed environment incrementing the association action and providing a physical “protection”. Also, the encapsulation has significative advantages in the topical sunscreen application such as: decreasing of chemicals penetration thought the skin and none direct contact between skin and actives with consequence of less skin reaction (Puglia C., 2014; Cozzi A.C., 2018).

### 1.6 SPF and broad-spectrum

As already explained above, sunscreen products regulation is not uniform over the world but in all the countries sunscreen Sun Protection Factor (SPF) testing is mandatory. Sunscreens are made in a wide range of SPFs (Cole C., 2014). SPF value is based on in-vivo testing measuring the amount of UV radiation exposure it takes to cause sunburn when using a sunscreen compared to how much UV exposure it takes to cause a sunburn when not using a sunscreen. This value provides information about protection against sunburn or erythema induced primely by UVB, therefore, SPF values only indicate a sunscreen's UVB protection. A specific quantity of tested sunscreens is applied on the volunteers' backs (at least 10), rubs it in, waits 15/30 minutes for the sunscreen to absorb and then directs the solar simulator (Xenon lamp arc). The UV radiation source should be stable and uniform. Ideally, a "standard formulation control" should be formulated and use as control to verify the sunscreen tested, for example, FDA defined 8% Homosalate as standard. The sunscreen amount applied should be 2 mg/cm$^2$ (universally taken value) and water evaporation and loss of volatile components should be considerate (COLIPA, 2006).

SPF testing are made with in vivo testing using, thus, human subjects which are irradiated with a UV radiation amount potentially damaging. In recent years, due to increasing knowledge about UVA-induced skin damage, it has been much development on methods for determining UVA performance. Several methods are described in the literature to measure UVA or board-spectrum sunscreen: i) ex vivo on excited human or mouse skin (Sayre R., 1990; Marginean G., 1995), ii) in vitro (UVA:UVB absorbance ratio, UVA I:UV absorbance ratio, critical wavelength, UVA index) (Diffey B.L., 1994; Diffey B.L., 1996; Australian/New Zealand Standard, 1998; Wendel V., 2001; FDA, 2001). In 2011, the Food and Drug Administration (FDA), adopted in vitro critical wavelength (CW) measurements to assessing UVA or broad-spectrum protection. CW is defined as the wavelength at which 90% of the total area under the absorbance curve in the UV region. Specifically, the FDA has ruled that only products with CW ≥370 nm can be labeled as having "broad-spectrum" protection (Food Drug and Administration, 2011). In the nations regulated by the European Commission, all products must offer UVA protection that at least has to be a third as potent as the SPF (UVA PF/SPF ≥ 1:3) (European Commission, 2006). UVA protection is tested with in vitro testing on artificial surfaces, usually PMMA plates. The sunscreen formulation is spread on the artificial surface at an amount of 2 mg/cm$^2$

obtaining a 10 to 20 microns thickness of product. The surface of the support is produced with roughness from 2 to 6 microns in depth in order to mimic the topographical "roughness" of the in vivo skin. Ones the sunscreen formulation in spread on the support, the plate is irradiated (xenon lamp arc) simulating UV spectrum and, after exposure, the absorption curve is recorded with suitable technique (spectrophotometer measurements).

However, in vitro testing is often criticized for the inability to produced result reliable and repeatable (Rohr M., 2010). Substrate roughness, interaction between formulation and artificial support, formulation application on the support are incisive factors during in vitro SPF testing and each of them can have significative effects on the measurements (Ferrero L., 2006; Ferrero L., 2010). The UV protection depend on the uniform film forming all over the support surface after application and on the thickness of product applied that should be ~ 10 microns (density: 2 mg/cm$^2$). Each unfilled or with thickness lower than ~ 10 microns spot can affect the SPF values measured. There are huge amount sunscreen products commercially available (emulsion, W/O, O/W, emollients, etc.), and find a substrate able to be an equivalent of human skin it is been challenging and until now there is not a final answer (Garoli D., 2009).

### 1.7 Sunscreen application patters and vehicle type

To be effective against UV radiation, sunscreen need more than the right combination of UV filters. A sunscreen must coat the skin surface uniformly with a specific thickness, but it is easy to understand that areas of the body are hard-to-reach during sunscreen self-application (Sambandan D.R., 2011). The dose, the film-forming properties and the thickness are fundamental characteristics to reach the sunscreen efficacy, in fact, they are the key reason why product application is one of the primary sources of variability in SPF testing. Indeed, one needs to consider the topography of the skin. Macroscopically, the surface of the skin is made up of hills and valleys. A thin layer applied over such topography may result in uneven coverage where "valleys" are filled/covered, but "peaks" are not. The studies related to the skin aging, disease, effect sun exposure, dermatological and cosmetic treatments are several but just few studies implicated skin roughness in the appropriate sunscreen application (Korn V., 2016).

Sunscreen vehicle include emulsions, gels, creams, lipstick, spray etc. Studies showed how the type of the sunscreen vehicle may influence the amount of sunscreen applied, and with is the product efficacy and sunscreen durability. Oil in water (O/W) and water in oil (W/O) lotions, cream or emulsions are the most appreciate by the costumers and consist in a formulation composition that allow a good equilibrium between appreciation, durability and easy application on skin with minimum adverse effects. Water-based gels have a poor durability because easily washable by swimming, sweating but they are really appreciated by oily skin consumers. Sticks are able to cover limited area and sprays have a good appreciation form the consumers but doesn't allow a full covered body protection due to the spry applicator. The resistance against the water is essential of the sunscreen efficacy, FDA (Food Drug Administration) defined "water-resistant" a formulation with photoprotective properties after 20 minutes of exposure to the water and "water-proof" after 20 minutes of immersion into the water. Beyond specific cases, a sunscreen is composed by:

- UV filter or mix of UV filters.
- Oily phase (paraffin, fatty acids/alcohol/acid ester, silicon oils etc.).
- Aqueous phase (polymers, skin moisturizers etc.).
- Emulsifier O/W or W/O.
- Polymers improving the water resistance.
- Stabilizers (preservative, antioxidant etc.).
- Perfume.

### 1.8 Delivery systems for sunscreen agents

In the last decades, sunscreens are moving towards to the concept of new cosmetics formula, where the performance are not relying only on the physicochemical properties of the filters but also on the way used to carry them. Moreover, the development of new molecules and gaining the regulatory approval are long and expensive processes, so, sunscreen industries try to maximize the approved UV filters.

Delivery systems are carry agents used mainly for their ability to improve chemicals stability, chemicals incorporation into the formulation and to reduce the irritation potential between skin and actives. The encapsulation is a process in which the active ingredients (core material) is contained in a shell of different material (shell) permanently or temporarily (Benita S., 2005). In relation to their structure, they are classified as mononucleated or polynucleated. The actives in the core can be in solid,

liquid or gas phase or a blend of solid and liquid components. The core can be composed by active, mixture of actives, stabilizers, preservatives, solubilizers etc. The shell is, usually, a membrane composed by polymers with specific characteristics: i) high cohesivity with the core, ii) good compatibility with the core substances, iii) resistance, iv) flexibility, v) stability. Usually, the external membrane is composed of polymer plus additives in order to obtain those characteristics such as plasticizers (Estevinho B.N., 2013; Silva P., 2014).

The choice of the shell material is a critical point in the encapsulation process because it influences the efficacy and stability. Several types of encapsulated particles are used, which can be divided, according to their composition, into: inorganic, lipidic and polymeric. The inorganic encapsulated particles are made of non-biodegradable materials. The lipid nanoparticles are made of lipids, are biodegradable, and can therefore be used within the human body with a large margin of safety. The nanoparticles polymers are made of polymers, and depending on the polymer used, they may or may not be biodegradable. Material commonly used are: proteins (albumin, gelatine, collagen, casein), polysaccharides (starch, cellulose derivative, carrageenan, chitosan), polyesters (polylactic acid, polyglycolic acid) (Ai J., 2011; Ricles L.M., 2011).

### 1.8.1 Micro and nanoparticles

Micro (3-800 μm) and nanoparticles (1-100 nm) are colloidal systems that can be distinguished into two morphological classes: micro/nano capsules and micro/nano sphere. The first one is a system made up of a nucleus, in which the active principle is distributed uniformly, surrounded by a membrane, the second one is a matrix system with a less ordered structure in which the drug is physically and uniformly dispersed in the polymer matrix. The spheres are generally stiff and more resistant than the capsules and both can be positively or negatively charged. They can be prepared with both, natural (polysaccharides, proteins) and from synthetic polymers (polyesters, polyacrylates, etc.). A natural polymer is selected in order to obtain more biodegradable, biocompatible and non-toxic system and a synthetic polymer is preferred for its low price, stability, design and purity.

The most common techniques used to product micro-nano-particle systems are based on chemical-physical processes (e.g. coacervation, interfacial polymerization,

thermal gelation, solvent evaporation) and mechanical processes (spray drying, spray cooling, hoy melt coating) or on a combination of both (Harabagiu V., 2004).

### 1.8.2 Liposome

Liposome are closed vesicular system (25-5000 nm) composed by phospholipids organized as bilayer membranes highly organized and divided by aqueous cavity. They are system well-accepted in pharmaceutical and cosmetic field because they are able to encapsulate both hydrophobic and lipophilic molecules. The liposomes are constituted by phospholipids or phospholipids and cholesterol (either positively or negatively charged), natural components of all the cellular membrane, then metabolized by enzymatic way and therefore safe from the immunity point of view (mimic system). Commonly used lipid is phosphatidylcholine obtained from soy or egg yolk, while the presence of cholesterol tends to stabilize the structure of the liposome, increasing its rigidity (Benson H.A.E., 2005).

There are several methods to product liposomes (thin layer evaporation, sonication, extrusion, french press, LUV bubblesomes etc.) but 3 common phases can be identified: 1) lipid hydration, ii) liposome selection size-based, iii) non-encapsulated drug removal.

### 1.8.3 Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)

Some lipid nanoparticles (10-1000 nm) can be further divided into solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

SLNs are nanoparticles where the external polymeric membrane is substituted with a single lipid. The matrix consists of a solid lipid dispersed in an aqueous solution and stabilized by the presence of surfactants or polymers. These nanoparticles can be positively or negatively charged. The structural organization of their matrix may be a distorted crystalline lattice, an amorphous lipid mixture, or a solid lipid matrix that traps liquids in the lipid nano-compartments.

The NLCs, instead, are produced with a mixture of solid lipids and liquids. The structural organization of their matrix may be a distorted crystalline lattice, an amorphous lipid mixture, or a solid lipid matrix that traps liquids in the lipid nano-compartments. They can be positively or negatively charged. Both SLNs and NLCs are rigid particles (Müller R.H., 2007; Baroli. B., 2009).

### 1.8.4 Sol-gel silica glass microcapsule

The silica glass microcapsules are formed by a silica cage and a core of organic molecules (eg. UV filters). The molecules are entrapped in the superficial porosity of the silica shell which supply both physical and chemical stability. The silica glass microcapsules modeled by interfacial polycondensation sol-gel process. While high temperatures are normally necessary to produce glass, inorganic silica glass can be produced at room temperature by using a sol-gel process. This type of low temperature glass synthesis enables substances such as organic chemicals to be encapsulated within the glass by adding them to a reaction mixture. In a first step, water phase and oily phase with UV filters are emulsified by stirring, usually in presence of surfactant active ingredients, obtaining W/O or O/W emulsion. Ionic and non-ionic surfactants are mainly used (Nouria A., 2012). In a second step, an amorphous network of glassy material is prepared at room temperature by the hydrolysis of suitable monomers. The reaction proceeds to a condensation polymerization reaction, followed by subsequent formation of the sol to the gel and aerogel stages. Approximately 80% of the capsule's weight is made up of the selected organic molecules (Ciriminna R., 2011; Ashraf M.A., 2015). Thanks to the low temperature, the process is able to prevent the degradation of the compounds. Silica micro particles are able to show chemical stability even in corrosive environment.

## 1.9 Packaging and its functions

The packaging surrounds, enhance and protects the product (food, cosmetic, pharmaceutical) from degradation/contamination processing and manufacturing through handling and storage to the consumer till its use. It is essential distinguish between primary packaging and secondary or tertiary packaging. In the first case, it is the term used to define the layer of packaging in direct contact with the contained product, in other words, it is the first-level product packaging such as the bottle, can, jar, tube, etc. The secondary packaging contains many unites of primary packaging (e.g. cartons, boxes, etc,.), it is a physical distribution carrier, the tertiary packaging is made up of a several unites of secondary packages (e.g. stretch-wrapped pallet).

The functions of the packaging could be obvious as much as important.

- Containment: in order to be able to move the products form a place to another
- Protection: of the contained product form the outside environmental effects, such as vapor moisture, gases, UV light, microorganism, compressive forces

and, at the same time, to protect the environment form the product in case contains toxic chemicals.

The product can be liquid, solid, semi-solid, to nebulize, pellets etc. in all those cases the packaging has to adapt to its characteristics and requirements, also taking into account production costs at the industrial level. After the guarantee that the product reached a good quality and the containment function is carried out in a suitable way, it should be considerate, especially in recent years, that the packaging is a "silent seller" and so it has to be good-looking and express the right idea of the product (communicative function). The old idea of packaging, as just the container useful to transport the product, is surpassed, now picking a suitable packaging which is able to preserve the product from external contaminants and does not interfere with it, it is an integral and essential part of the product commercialization. The packaging is considerate as an effective advertising tool which should be attractive and explain the brand idea to promote and sale.

In pharmaceutical, cosmetic and food field the packaging can be made by many different materials such as glass, metal, paper, cardboard plastic. They should be convenient, safe, unexpansive and eco-compatible.

Glass provides superior protective qualities for its characteristics: very hard to undermine, inert, none degradation process, colorable (amber glass). However, it is inconvenient for its heavy weight and high cost. Metal and aluminum are principally used for the production of flexible tubes, cans for creams, flasks and the paper go to make paper envelopes (talc) and paper wraps (soap).

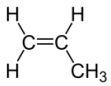
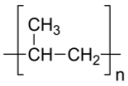
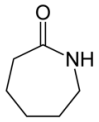
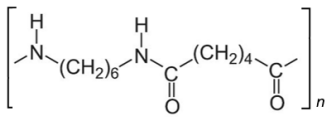
In the 1940s the plastic production exploded, replacing traditional materials (Lange J., 2003) and, up to 2012, more than 300 million tons per day were produced (The Worldwatch Institute, 2005). This boom of production is due to the plastic material characteristics: easily processable, inexpensive, colorability, resistance, flexibility, adaptable to the needs, electrical, mechanical and corrosion resistance as well as the water repellency. Also, they are unaffected by mold, fungi and bacteria. However, this material has significative backside: i) easily attacked by solvents (especially thermoplastics) and acids (in particular the thermosets); ii) poor resistance to high temperature; iii) usually high permeability to gasses and other molecules; iv) low degree of eco-compatibility.

## 1.10 Plastics

### 1.10.1 Chemical properties

The structure of the plastics (or polymers) is given by high molecular weight organic and semi-organic materials called macromolecules. Those macromolecules are the repetition of fundamental unites (monomers) that are linked together by strong bonds. The monomer unites are mostly carbon-based molecules, with elements such as oxygen, nitrogen, fluorine. Those elements give the basis for the determination of different plastic materials as showed in **Table 2** (Klein R., 2011).

**Table 2.** Chemical structure of some common plastics and their monomers.

| Monomer | | Polymer | |
|---|---|---|---|
| Ethylene |  | Polyethylene (PE) |  |
| Propylene |  | Polypropylene (PP) |  |
| Vinylchloride |  | Polyvinylchloride (PVC) |  |
| Caprolactame |  | Poly(E-Caprolactame) (PA-6) |  |

To be considerate a monomer, a molecule must possess functional groups that give it the ability to react with functional groups of other monomers to form more complex elements. Following specific temperature and pressure conditions, the functional groups of a monomer react with the functional groups of another monomer thus forming bonds. The reaction to form of a polymer is called polymerization and can take place in two different ways: i) polyaddition, ii) polycondensation. In the first case, the polymer is simply the sum of the molecular weights of the monomers present in the chain. Examples of addition polymers are polystyrene, polyethylene, polyacrylonitrile, polymethyl methacrylate and polyvinylchloride. In the second case, the polymer chain is obtained by condensation of monomeric unites followed by loss of small molecules such as water or alcohols. Examples of condensation polymers are polyamides (e.g., nylon 6,6), polyesters (e.g., polyethyleneterephthalate), urea-formaldehyde and phenol-formaldehyde resins, polysaccharides (starch, cellulose,

hyaluronic acid) and proteins (enzymes, cytochrome, hemoglobin, myoglobin, collagen, elastin, etc.).

If the monomers of a polymer are all the same it is called homopolymer, instead, if they are made up of different monomers it is called copolymer.

A polymer can be linear, if the growth takes place always and only in the same direction, branched or crosslinked, if constituent monomers have more than two functional groups.

Solid-state polymers can have two types of molecular macro-structure: crystalline and amorphous. In the first case, the molecular arrangement is irregular and disorganized, the polymer is characterized by random molecular disposition where the chains are able to move across each other when the polymer is pushed or pulled. Those class of polymers, mostly, have flexibility and elasticity. In the second case, the molecular arrangement is very ordered and highly organized. The chains are parallelly arranged between each other with regular distance and with constant trend. This organization gives them strength and rigidity. The polymers, usually, are classified and evaluated according to their degree of crystallinity which represents the percentage by weight of the crystalline zones compared to the total weight. This was done because, in crystalline polymers there is always some amorphous region consisting of a non-homogeneous trend, in fact they are also called semi-crystalline, where the crystalline structures are immersed in an amorphous matrix. The degree of crystallinity influences the polymer properties, increasing the crystallinity decreases the elongation at break, thermal coefficient expansion, permeability etc, and, at the same time, increases the density, yield strength, chemical resistance etc. Of course, during the manufacturing process, reach the suitable degree of crystallinity in order to obtain the material meeting the desirable performances is a significative advantage.

### 1.10.2 Physical properties

In relation to thermal properties and mechanical properties, plastic materials are distinguished into two classes. Depending on the polymeric material behavior in relation to the thermal energies, three types of polymers are designed: thermoplastics, thermosets and elastomer.

Thermoplastic polymer is linear or branched polymers that can be melted by energy input (thermal, mechanical or radiation) shaped and, after cooling, keep the imposed shape. This is possible because, increasing the temperature some

intermolecular bonds are broken and so the molecules have the possibility to move between each other, but, when the temperature decreases, the intermolecular bonds are restored, and the molecules are fixed in their new position. Indeed, they can be reversibly melted by heating and resolidified by cooling without significant changing of mechanical and optical properties. Since, the crystallization process required time, during which there is the formation of the highly organized crystalline structure, generally, thermoplastic polymers don't have a high crystallinity degree (Van de Velde K., 2001). **Figure 10** shows examples of amorphous and semi-crystalline thermoplastics.

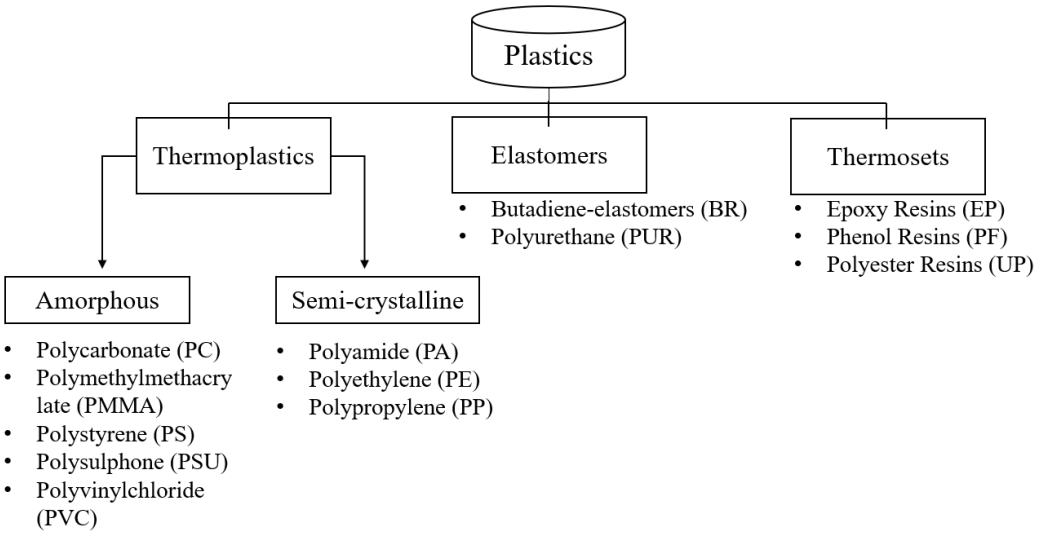



**Figure 10.** Classification of plastics.

Thermoset polymer contains polymers with narrow crosslinked chains. The cross-linking process allow to form irreversible chemical bond between polymers. Thanks to it thermosetting materials can be heated, processed and shaped only once, indeed, when re-heated, they become flexible but not liquid making thermosets ideal for high-heat application (ex. electronics) (Li C., 2015).

Elastomers are rubbery polymers with wide netlike crosslinking between the molecules, the polymer chains keep some freedom but not enough to move permanently. During the melting process, usually, they undergo to a degradation procedure of the molecule structure. They can be searched easily multiple times, without permanent changes.

Physical properties of polymers include all those properties that describe the behavior of a solid subject to a force. There are several mechanical tests and testing

instrumentation able to evaluate the physical properties of a plastic material. Some of them have been officially recognized as standardized (ex. ASTM standards). Few examples are: i) creep tests, ii) stess-relaxtation tests, iii) dynamic mechanical tests, iv) stress-strain tests. They can be classified according to the method of application of the force, in specific, mechanical traction static-testing allow to evaluate the tension and the elongation at break. The attention will be focused particularly on stress-strain tests.

Stress-strain curves are carry out when a standardized sample is placed between the loading cell and the moving system, which is able to move at constant and prefixed speed. Tensile tests measure the force required to break the sample (or specimen). Such tests produce stress-strain diagrams used to determine tensile modulus and record specific polymer parameters (**Figure 11**).

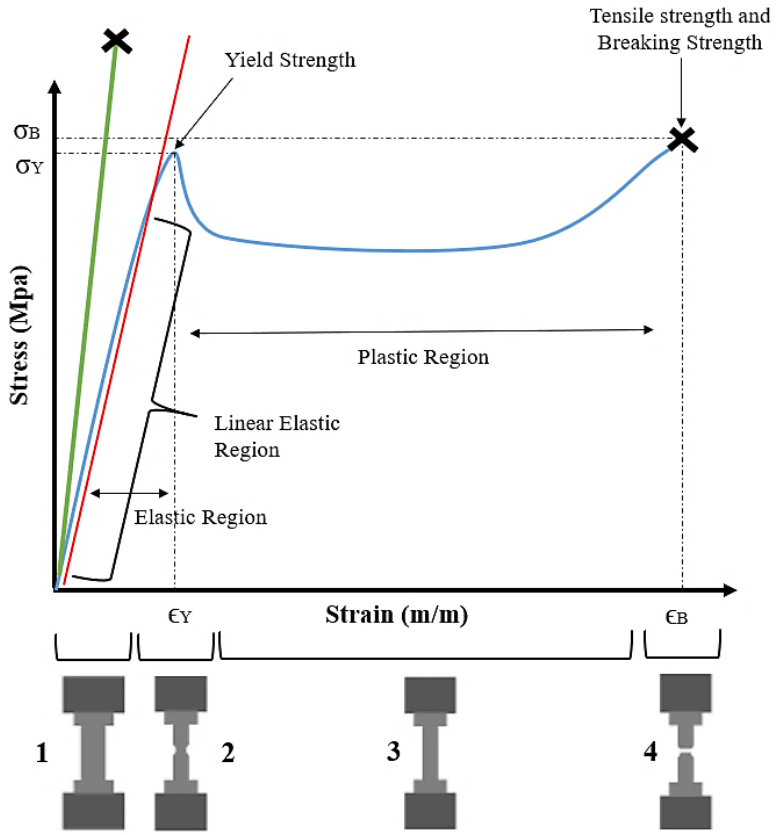



**Figure 11.** Typical stress-strain diagram for fragile (green line) and ductile (blue line) polymeric materials.

The engineering stress (σ) is defined as ratio between tensile force (*F*) and the cross-sectional area (*A*) of the gage section at starting point.

$$\sigma = \frac{F}{A}$$

The engineering strain ($\epsilon$) is defined as ratio between the change in gage length ($L$-$L_0$ = $\Delta L$) and the initial gage length ($L_0$).

$$\epsilon = \frac{L - L0}{L0} = \frac{\Delta L}{L0}$$

When force-elongation data are converted to engineering stress and strain, a stress-strain curve (Fig. 11) that is identical in shape to the force-elongation curve can be plotted. The elastic module or Young's modulus ($E$) is represented, for most material, in linear region of the first portion of the curve and it is defined as ratio between stress and strain.

$$E = \frac{\sigma}{\epsilon}$$

It depends directly and exclusively on the intermolecular bonding forces of the polymer and represents its ability to resist the applied small stresses by deforming elastically. Until this point, the sample is able to elastically return to its original dimension. This reversible deformation is called elastic deformation. When higher stresses are applied the deformation is not recovered when the stress is removed, so, the deformation become permanent. This irreversible deformation is called plastic defamation. Yield strength is the stress at which the curve has a pick and then becomes flat. It is well identified only in the case of thermoplastic polymers. Tensile strength is defined as the highest value of engineering stress. With ductile materials, the tensile strength corresponds to the point at which the deformation starts to localize, forming a neck. Less ductile materials fracture before they neck. Indeed, very brittle materials don't yield before fracture. Such materials have tensile strengths but not yield strengths (Seymour R.B., 1984; Landel R.F., 1993). Two different stress-strain curves can be observed for fragile or ductile polymeric material, they are showed in Figure 11. For fragile polymeric material, there is a linear trend (or almost linear) followed immediately by the break (Fig. 11, green line); in ductile materials behavior, the curve is characterized by three different zones: 1) an initial stretch defined as elastic (elastic

modulus); 2) and 3) an areas where the material undergoes plastic deformations (yielding); 4) a final breaking zone (breaking effort) (Fig. 11, blue line).

Several molecular characteristics can influence the physical characteristics, such as: molecular weight, crystallinity, linearity or ramification, chains orientation.

### 1.10.3 Polymers

8% of polymers all over the world are thermoplastic due to its adaptability. Some of the most commonly used plastic materials are: polyethylene (PE), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polypropylene (PP). They are petrochemical-based plastics, so the sustainability of these synthetic material is undoubtedly an issue that needs to be evaluated, increasing the recourses to develop material biocompatible presenting suitable characteristics in terms of efficacy and safety (Asghari F., 2017).

#### 1.10.3.1 Polyethylene (LDPE, HDPE, LLDPE)

Polyethylene (PE) is a thermoplastic semi-crystalline polyolefin obtained by direct polymerization of the olefin ethylene (**Figure 12**). The ethylene molecule consists of a double bond that connect the two carbon atoms and, the polymeric chain is composed of single carbon-carbon bonds. It presents itself as a transparent solid (amorphous state) or white solid (crystalline state), with excellent insulating properties and chemical stability, it is a very versatile material and one of the most economical.

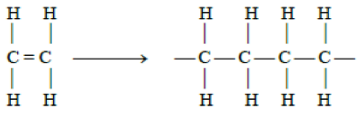



**Figure 12**. Polyethylene polymerization

It is a hydrophobic polymer, resistant to acid, basic solution, alcohol and saline solution; below 60 ºC is insoluble in organic solvents and the solubility increases rapidly with temperature. The resistance to water vapor or other gases is also very high and this characteristic is determined by the degree of crystallinity and density of the PE. High resistance to many chemicals with the exception of oxidizing acids, halogens (chlorides, bromides, etc.) and ketones; non-toxic and odorless, it can be re-used and

recycled. Polar liquids cause phenomena of embrittlement (environmental stress cracking) that decrease with increasing molecular weight and with the number of branches present in the chain. Their purposes: hollow containers, for example flasks, tubes, jars, but also capsules, dispensing systems, flexible films, etc. Molecular weight, crystallinity, structure and, consequently, their properties essentially depend on the polymerization system (Parvizi J., 2010).

Low Density Polyethylene (LDPE) obtained by high pressure process, in autoclave or tubular reactor, with an operating pressure ranging from 1000 to 3000 bar and temperature from 150 ºC to 300 ºC. It presents very low crystallinity because, in those conditions, the macromolecules are very branched with different length at low density (0,917-0,94 g/cm$^3$). LDPE has a low specific weight, extremely easy molding, high impact resistance, even at low temperatures, good electrical properties, low water vapor permeability and an attractive price. Because of its low density, however, it has a high permeability to gases in particular to $CO_2$ and is also represented by a poor resistance to UV rays.

High Density Polyethylene (HDPE) obtained by low pressure process, with operating pressure rating from 10 to 80 bars an temperature from 20 ºC to 300 ºC. The polymerization is performed with 3 types of catalyst (Ziegler/Natta, Cr/Mo oxide, Metallocene) following four different methods: in the low-pressure system in the gas phase, in solution or suspension and with a modified high-pressure method. Whit this process, a polymer with reduced ramifications is obtained presenting higher crystallinity and higher densities (0.95-0.96 g/cm$^3$) and consequently presents greater rigidity. It has high abrasion resistance, greater impact resistance even at low temperature, high UV resistance, however, its cost is usually high. HDPE has a poor barrier to essential oils, so the fragrances are easily dispersed in the environment.

Linear Low Density Polyethylene (LLDPE) obtained by low and high pressure process. The system for high pressure production can be converted to produce a polymer that has intermediate characteristics between the products of the two processes at high or low pressure. The polymerization is performed with high-yield catalysts based on metal complexes following four different methods: in the low-pressure system in the gas phase, in solution or suspension and with a modified high-pressure method. The higher molecular weights of these poorly branched products result in better properties. It is a type of polyethylene with a structure similar to HDPE but having a sufficient number of short ramifications that prevent the polymer from

crystallizing as easily as the HDPE, so that the density (0.92-0.95 g/cm$^3$) is lower (similar to that of LDPE) (Pringer O.G., 2008).

### 1.10.3.2 Polyethylene terephthalate (PET)

Polyethylene terephthalate (PET) is a semi-crystalline polymer belonging to the family of aromatic polyesters characterized by ester groups in the principal chain (**Figure 13**).

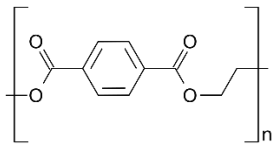


**Figure 13**. Structure of PET

It is obtained by polycondensation which involves the transesterification of the dimethylterephthalate (DMT) or terephthalic acid (TPA) with ethylene glycol (EG). PET synthesis occurs in two stages. In the first stage, it is conducted from 150 to 220 °C at atmospheric pressure, there is a direct esterification with waters molecules as waste products (TPA reaction) or by transesterification with production of methanol as waste products (DMT reaction). The second stage is conducted between 250 and 290 ° C at 0.1 mbar with the polycondensation taking place by the elimination of EG (Tomita K., 1977).

The advantages of those polymer are multiple: resistance to humidity, gases, oils and fats, wide range of mechanical properties obtainable by varying the molecular weight, the orientation level and the degree of crystallinity, resistance to commune chemical solvents, high degree of transparency etc. Furthermore, it presents an inertia to the attack of saprogenic bacteria, fungi, molds, and it is not physiologically active, indeed, it is commonly used to produce prosthesis. The fields of use of PET films include photographic films, bases for magnetic, video and computer cassettes, electrical insulators, membrane switches, containers (especially in metallized and printed form), bag-in-box containers for wine and decorative products.

### 1.10.3 Polyvinyl chloride (PVC)

Polyvinyl chloride (PVC) is produced by polymerization of the vinyl chloride monomer (VCM), it has an amorphous structure with polar chlorine atoms in the molecular structure (**Figure 14**).

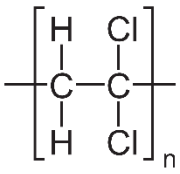



**Figure 14.** Structure of PVC.

PVC pure is a hard and rigid material and so fragile. It is produced in two forms: unplasticized polymer and flexible plastic. The second one is produced with the addition of plasticizer to make it flexible. Those plasticizers usually are polycarboxylic acid esters, in fact the most common plasticizers are esters of the phthalic acid of adipic acid with alcohols of variable length. The bond PVC-plasticizer is characterized by a physical bond but, since it is not chemical bond, the plasticizer could gradually migrate from the PVC to the product. Once the plasticizers have been added, the applications are countless because they can be molded by hot molding. It can be reduced to film or liquid with which fabrics or coated surfaces, tanks, valves, faucets, tanks and artificial textile fibers are coated. It is chemically resistant to alcohols and acids, but it is soluble in esters ketones, it is also resistant to fats and oils. It is stable and safe under room temperature, but, at high temperature thanks to the chloride molecule in its structure, it is able to release hydrochloric acid, dioxin or vinyl chloride monomer. It is essential have specific areas, during the manufacturing process, for its production.
This plastic material is being criticized due to the formation of hazardous molecules during the production process and the issue about the plasticizer migration for soft PVC films, indeed, it is continually being replaced by other plastics such as polypropylene (PP) (Titow W.V., 1990).

### 1.11 Packaging regulation

The packaging regulation is defined by different organisms all over the world.

The cosmetics sector is regulated by Regulation (EC) 1223/2009 of the European Parliament and of the Council. One of the subject introduced by this regulation is that the manufacturing must have a conformity with the rules of good manufacturing and it is necessary to study both the chemical-physical and microbiological stability but also on the compatibility of the product with the primary packaging in order to compile the framework on the protection of the European consumer. All that important information about product identity, safety and quality, become an integral part of the

PIF (Product Information File). All the subjects of the cosmetic industry scenario must contribute a PIF for each product that is placed on the European market. Several information are required from PIF, one of them is "Impurities, traces and information on the packaging material ", therefore the objective of this section of the cosmetic product safety assessment report is to assess whether the cosmetic product contains substances that have not been intentionally added to the formulation, and which could affect its safety. However, there is a lack of information about the execution of the various chemical assessment studies. The European Commission gave explanatory acts in order to fill this lack: it may be useful to use as a reference the Regulation (EU) 1935/2004 which refers to the materials in contact with food and Regulation (EU) 10/2011 specific for food-contact plastic materials. In the Regulation (EU) 1935/2004 specific requirements are established, packaging material do not: i) release their component into the food in quantities potentially hazardous for human health, ii) induce significative changes in food taste, smell or composition; and the regulatory includes: i) special rules for active packaging (where the packaging is not designed to be inactive), ii) possibility of adopting additional EU measures for specific materials (eg. plastics), iii) 17 groups of different materials are defined (Regulation (EC) No 1935/2004). The Regulation (EU) No 10/2011 give specific guidelines about food in contact with plastic material:

- List of authorized compounds (Annex I) for the production of plastic layer. This list covers monomer, additives, starting substances etc.
- Specific migration limits (SML): “the maximum permitted amount of a given substance released from a material or article into food or food simulants”. It represents the limit amount of substances coming from the plastic material that can be transferred into the food stuff (Annex I). They are expressed in mg/kg with a generic SML of 60 mg/kg.
- Overall migration limits (OML): “the maximum permitted amount of non-volatile substances released from a material or article into food simulants”
- Total specific migration limit (SML(T)): “the maximum permitted sum of particular substances released in food or food simulants expressed as total of moiety of the substances indicated”.
- Compliance testing requirements: food simulant (FS). The FS is “a test medium imitating food; in its behavior the food simulant mimics migration from food contact materials”

- Declaration of compliance (DoC) requirement. A written declaration of finished plastic material.

  (Regulation (EU) No 10/2011)

However, in cosmetic products, the formulation matrix is very complex and, often, very different from food. Requirements such as FS are not representative in the cosmetic industry.

In USA, Food and Drug Administration regulate the materials that are susceptible to food contact. These materials are regulated by the Federal Food, Drug and Cosmetic Act which should be convinced that a specific packaging will preserve the drug contained, in term of efficacy, purity, identity, strength and quality, for its entire shelf-life. However, a lack of specification or standards for packaging evaluation is been observed. Attention is put on plastic materials, regulated by C.F.R. (Code of Federal Regulation). Devices intended for human use in contact with food must go through a process called "Premarket Notification" (PN) which is the process through which the FDA authorizes the use of indirect food additives, provided that their concentration in the diet is less than 50 ppb. All the substances intentionally added to food is defined as food additives and indeed subject to PN followed to FDA approval, unless the substances are in the list of substances "Generally Recognized As Safe" (GRAS). They are substances that have shown safe behaviors under certain conditions. FDA regulation states "containers, closures and other component parts of drug packages, to be suitable for their intended use, must not be reactive, additive or absorptive to an extent that the identity, strength, quality or purity of the drug will be affected". New drug application (NDA) is an informative file formally approved by FDA when new pharmaceuticals are introduced in the markets. It represents the complete history of the drug such as container and packaging components in contact with the pharmaceutical products. If the NDA results positively approved by FDA means that the drug is safe and the packaging adequate.

### 1.12 Interaction packaging-contained: permeation, migration and absorption

Product-packaging interactions could be defined as mass or energy interplay between contained, packaging and external setting which produces effects on the product and/or packaging.

Packaging and contained products should be considerate two different entities with a very close relation in terms of efficacy and safety during the shelf-life. Primary

packaging is in direct contact with the contained product for all its shelf-life, undergo to short/long periods in stressful conditions. Interactions between the products and/or ingredients and their packaging systems can affect the quality of the product, or less frequently, the quality of the packaging systems themselves. These interactions are either: additive (release of substances from the packaging to the product), reductive (release of ingredients from the product to the packaging) and transformative (transformation of the product, ex. loss of stability). Indeed, in order to characterize the packaging-contained interaction, studies to determinate the extractables and leachables are carry out. Extractables are organic and inorganic chemicals that can be released into an appropriate solvent when packaging is subjected to extreme conditions such as high temperature, prolonged contact time, etc. Depending on the analytical method considered, conditions can be accelerated or exaggerated. The extractables, therefore, have the potential to pass into the cosmetic product under normal conditions of use. Leachables are organic and inorganic chemical entities that have the ability to migrate from a packaging system to the cosmetic product under normal conditions of use and storage or under conditions of accelerated stability. Generally, leachables are a subset of extractables. Also, there are the disposals from the product to the packaging, all those constituents of the formulation that are transferred from the product and that have the ability to migrate through the packaging. Deep analyses should be done when additives are added to the polymeric material to improve the aesthetic and/or functional modification capabilities of the packaging in order to respond to market needs and marketing strategies, and some attention should be put when in the contained product special class of chemical are added (UV filters, preservative). Some of these substances may interact with the product compromising safety and functionality of the primary container.

Mass transfer processes product-packaging and packaging-product are named as: permeation, migration and absorption (**Figure 15 b**). The penetration is a double way process in which the molecule crosses the polymeric barrier form the product to the external environment or form the external environment to the product (Koros W.J. 1990). The chemicals that usually penetrate are: oxygen, water vapor, carbon dioxide and other gasses with adverse consequences such as: oxidation, microbial growth, mold growth etc. Chemical additives that are added into the polymeric fuse during the manufacturing process, could be released into the product under specific stressed condition (e.g. high temperature, UV radiation etc.). This migration process is the type

of interaction product-packaging that it could concerned directly human health because involve monomers and additives potentially toxic. The absorption are chemicals originally contained in the product that are absorbed into the polymeric film. Usually they are aroma compounds, fats, organic acids, pigments with consequent loss of perfume intensity and damages to the packaging (swelling, cracking) (Cirillo G., 2015).

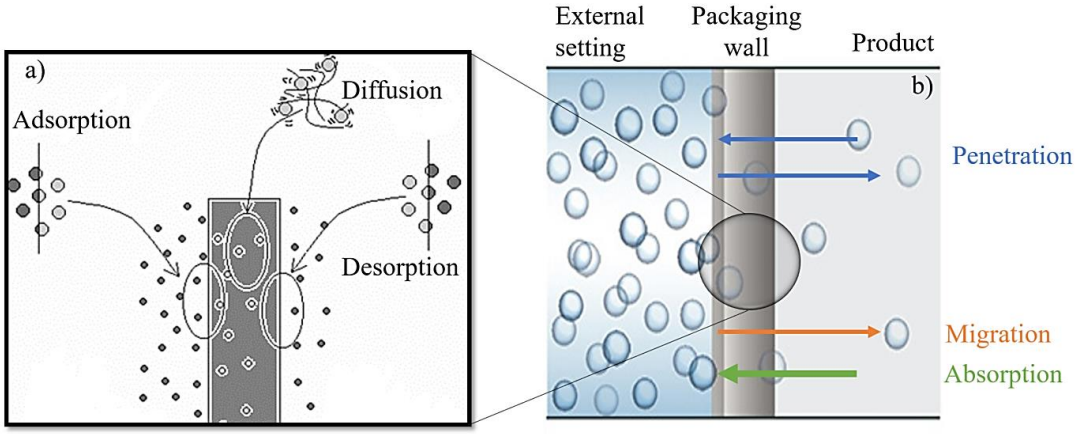



**Figure 15.** a) Mass transport of molecules and b) possible interaction between external setting-packaging-product.

Low molecular weight substances permeate through the polymer matrix, undergoing to a concentration gradient following 3 steps: adsorption, diffusion and desorption (**Figure 15 a**) (Hansen C.M., 2004). The molecules, in the high concentration region, collide with the packaging surface, then they dissolve and absorb into the polymeric matrix. There is a random diffusion in the chains segments of the polymer from the side in contact with high concentration area to the side with low concentration and then the molecules desorb and evaporate from the polymeric matrix (Lagaron J.M., 2004).

Diffusion, penetration and desorption are related to the permeants characteristics, as the molecular size increases, diffusion coefficient decreases, and solubility coefficient increases regulated by the following equation:

$$P = D(C) * S$$

where

$$C = Sp$$

Where *P* describes the permeability coefficient, *D(C)* describes the diffusion coefficient, *S* describes the solubility coefficient and *p* is the partial pressure of the penetrant.

Plastic material, compared to most traditional packaging materials such as metal and glass, have several limitations related to their permeability. The key to understand this is the chemistry of the polymer. Small variation in the chemistry of the macromolecule lead to big variation in barrier properties. In recent studies it has been seen that the transport of gas in semi-crystalline polymers, such as PE, is determined by the absorption and diffusion of the molecules through the amorphous regions of the semi-crystalline polymers but remaining excluded from the crystalline zones. These parts, therefore, seem to show themselves as non-absorbent and impermeable barriers. Other impact factor regulating the barrier properties it the free volume such as the microcavities in the polymeric wall determinate by the polymer characteristics and the polymerization process. In this case the transport properties are related to the number of microcavities and their size. HDPE has a higher crystallinity compared to LLDP and LDPE, moreover, homogeneous or heterogeneous character of the incorporation of branches along the polymer chains has a large impact on properties, including barrier properties (Lagaron J.M., 2000).

Of course, the chemical structure, size and shape of the migrant are also essential factors in the migrant mobility across the polymeric material. In particular, their interaction with the surrounding material. Alcohols and short chained ester have a high partition coefficient in hydrophilic polymers compared to the hydrophilic polymer. Aldehydes have a long carbon non-polar chains which brings to a lower partition coefficient un oil/polymer than in the water/polymer. Increasing the ethanol concentration decreases the partition coefficient of all volatile compounds.

### 1.12.1 Organic compounds (degradation products and UV filters)

Several production methods are available to fabricate thermoplastic such as: extrusion, injection molding, blow molding, thermoforming. During all those procedure, high temperature and extreme mechanical stresses are used to produce packaging. The oxygen, present in the manufacturing process, could cause the degradation of polymers (Hodgson S.C., 1998). The thermooxidative degradation produces volatile organic compound (VOCs) such as, hydrocarbons, alcohols, aldehydes, ketones and carboxylic acid (Villberg K., 2001). Those elements could migrate from the plastic material to the

formulation contained compromising the aspect, odor, performances, safety, quality etc. of the product. The entities of those interactions are related to the temperature, contact time, concentration, solubility, diffusion coefficient and molecular weight (Clough R.L., 1996). To assure the non-toxicity of those compounds their identification and quantification should be carry out and in order to do that basic knowledge about plastic structure and products components are necessary to make estimation of migration phenomenon (Ezquerro O., 2003).

A big portion of the UV filters is represented by organic and lipophilic molecules such as avobenzone and octocrylene. Polymer form the class of polyolefins and others also are lipophilic material and so, they are able to retain large amounts of compounds with the same nature. In previous study, the potential migration of organic UV filters was evaluated showing the potential absorption of molecules coming from the sunscreen formulation into the packaging material after stress condition resulting in a reduction of the protective effect of the product when applied in the skin (Briasco B., 2017).

### 1.12.2 Plastic additives

Commonly, plastic additives such as antioxidants, stabilizers and plasticizers etc. are added to the polymer in order to improve chemical and physical properties of the packaging such as color, opacity flexibility, resistance to heat/light/air etc. influencing the polymer molecular proprieties and its shelf-life (Haider N., 1999). They are used at levels of 0.1% to 1% (Dilettato D., 1991) and dispersed in the polymer matrix. The interaction between additives and polymer is related, mostly, to the polymer molecular mass: chain scission causes decrees of molecular mass and a loss of toughness, while cross-linking increases molecular mass and toughness in the early stage. Since, the most used additives have low molecular weights, in the food industry, it been already correlated to migration process. Moreover, in polymeric matrices it is possible to find monomers and oligomers that have not reacted in the polymerization reactions (Wang F.C., 2000). In food, pharmaceutical and medical industry, the use of additives is well regulated under severe legislation and environment rules, however, in the cosmetic industry a lack is been observed. The most relevant classes are: antifogging, antistatic, antioxidant, colorant, filler, lubricant, plasticizer, stabilizer, UV absorber.

### 1.13 Pharmacoeconomic

The pharmacoeconomic is a set of evaluation models used to identify the value (convenience) and the economic impact of a specific intervention. These economic evaluations help to make decisions following the more convenient, efficient and productive way to act. Their advantage is that the result is obtained by applying known and validated models, therefore, the basis of decisions is well-known and supported (Townsend R.J., 1987).

With the increasing skin cancer cases and the constant pressure to reduce medication costs, it has been fundamental and challenging to go beyond the valuation just of the estimated value of healthcare goods and services, but also evaluate the association of providing quality patient care with the best and efficient use of resources. The clinicians are focused on providing a high-quality patient care in the best (unexpansive) way, but sometime this correspond to offer the cheapest solution rather than the alternative that represents the best value for the money. Most of the time, this attitude doesn't correspond with the best solution for patients, health care systems, and institutions. The quality of the patient treatment shouldn't be affected by the try to limit the costs, instead, they should prove pharmacoeconomic benefits, namely, a collection of economic, humanistic and clinics benefits.

The methods of pharmacoeconomic can be divided into two categories: economic and humanistic evaluation techniques. The economic part includes:

- Cost-benefit analysis (CBA). It is an analytical tool needed to identify, measure and compare the economic benefits and costs of a program or treatment alternative in monetary terms (equivalent dollars in the year in which they will occur) to be easily compared. The benefits acquired from a program or treatment alternative are compared with the costs necessary to obtained it. Costs and benefits are stated as a ratio (a benefit-to-cost ratio), a net benefit, or a net cost. The benefits, sometime, are hard to convert in economic terms, they need a personal judgment, therefore, it may overlook intangible benefits (Freund D.A., 1992) (Bootman J.L., 2005).
- Cost-effectiveness analysis (CEA). It is a series of analytical and mathematical methods able to help the decision-maker in choosing the preferred solution among possible alternatives. It recaps the benefits, resources and treatment alternative by comparing them with different safety and efficacy profiles. The costs are weighed in monetary terms (dollars) and outcomes in non-dollar unites such as lives saved,

life expectancy etc. CEA, most of the time, is based on pre-existing data available in medical literature, however, it could be useful evaluate CEA during the clinical trial. The result of CEA is expressed as incremental cost-effectiveness (*ICEA*) or as average of cost effectiveness (*ACER*), determinate as follows:

$$ICEA = \frac{Cost\ A - Cost\ B}{Benefits\ \ A - Benefits\ B}$$

$$ACER = \frac{Health\ Care\ Cost}{Clinical\ Outcome}$$

(Detsky A.S., 1990; Sanchez L.A., 1994; Thwaits R., 1998)

- Cost-minimization analysis (CMA). It is a method used to determinate the least expensive alternative between two or more treatment alternatives with demonstrate equal efficacy, safety and tolerability. First, it should be guarantee the equivalence for the compared therapies, then the cost can be quantified and compared in monetary unites (dollars). When the therapeutic equivalence is not demonstrated, then a more comprehensive method such as cost-effectiveness analysis should be employed. CMA presents the "cost-saving" of one treatment over another, therefore, it is relatively simple as method (Sanchez L.A., 1994).
- Cost-utility analysis (CUA). It is a method that is able to compare treatments alternative including the measure of patients' preferences and the health-related quality of life (HRQOL). HRQOL can be defined as the effects of treatment on some aspects of the patient's health and life (physical, psychological and social dimensions of health), seen as distinct areas that are influenced by personal experiences, beliefs, perceptions and expectations, and then measured completely through the perspective of patient himself. It is subjective and multidimensional (physical, mental and social). CUA can compare cost, quality, and the quantity of patient-years. Cost is measured in monetary value (dollars) and therapeutic outcome is measured in patient-weighted utilities rather than in physical units. Very often, the utility measurement used in CUA analysis is a quality-adjusted life year (QALY). The QALY is a unit of measure of health status used that combines morbidity and mortality data. Another way used to express CUA results is the cost-utility ratio (C:U ratio) (Hepler C.D., 1990; Pathak D.S., 1995).

### 1.14 The socio-economic impact of skin cancers

In 2016 in Italy, 369,000 new cases of malignant tumor were planned, and about 13,800 are melanoma. Data showed an increasing incidence for both sex in geographic areas with predominance of Caucasian phenotype population in the northern part of Italy (16,1), compared to the population of south Italy with darker phenotype (8,3). With regard to age groups, melanoma represents 9% of juvenile tumors in men (second most frequent neoplasia), 3% and 2% in age groups 50-69 and 70+.The risk of developing cutaneous melanoma is high in men (1 out of 66) in women (1 in 84): in men the risk is somewhat lower in young people while in women the risk remains constant in all three age groups. In 2013, 1948 were the deaths for cutaneous melanoma, equal to 1% of tumor deaths, however, the survival incidence is statistically higher than the European average (85.4% vs 83.2%) but lower than Northern Europe (87.7%) (AIOM, 2016).

In USA from 2011 to 2015, 372,335 new cases of melanomas were reported. In California age-adjusted rate of new melanomas was 22.1 per 100,000 people with 43,878 melanomas recorded, in Florida the numbers are very similar, it presents a rate of new melanomas of 23.2 per 100,000 people with 29,913 melanoma cases recorded. The situation is very different in country such as North Dakota and Alaska where age-adjusted rate of new melanomas was, respectively, 13,6 and 23,2 per 100,000 people with 452 and 902 melanomas recorded. On 372,335 new cases of melanomas reported 45,982 people died (U.S. Cancer Statistics Working Group, 2013).

Those are statistics about pathology with a high impact on familiar, social and economic life of people suffering. Each pathology involves several subjects, starting from the patients directing involved to his relational network, therefore, the significant size of economy and society consequences are clear. Because of this, it is essential to extend the attention to health aspects and costs of treatment and prevention but also to socio-economic complex of factors involved, such as impact on working life and the ability to produce income, up to psychological and human intangible costs which it concerns patients but also people who have relationships and/or care for patients. The assessment of those costs is helpful to concentrate resources in strategies. Health, prevention and treatment costs are easily tractable using official channels, but socio-economic costs, sometime, require ad hoc investigation on representative population.

Four types of costs are detected: i) direct cost or medical expenses, ii) direct nonmedical costs, iii) indirect cost or extra-medical expenses and iv) intangible cost.

The first one includes all the resources associated with prevention, treatment and healthcare such as drugs, medical visits (family doctor, specialist), other therapies (rehabilitation, long-term care, psychotherapies etc.), instrumental diagnostics, hospitalizations etc. Direct nonmedical costs are any cost that it is related to the illness but do not involve purchasing medical services such as the transportation, food, family care home aides. Indirect costs cover all the resources that haven't been produced due to the disease for both, patients affected and parents: day-off for treatment and health care, for temporary disability or for family members in order to offer patients care. Intangible costs are psychological and physical suffering costs caused by the disease (The Economist Intelligence Unit, 2009).

By 2020 237,912 new cases of melanoma are predicted in the world, with an increment of +12% compared to 2009; it is clear that this trend is followed to an increase in terms of additional costs, which it is inevitably destined to produce (The Economist Intelligence Unit, 2009). In this picture, it is essential to have well-defined in mind the resources available and the appropriated way to use them, to make rational choices and implement health policies aimed at ensuring greater quality of care.

### 1.15 Prevention

The prevention prevents or reduce the risk of unwanted events such as skin cancer.
It includes actions of preventive actions and early detection of disease to reduce mortality, morbidity. The prevention tool is part of the broader "health protection" activity, in their various application fields (medical, nursing, obstetric, psychological etc.). There are three levels of prevention, which refer to different acts and stages: i) primary prevention, ii) secondary prevention and iii) tertiary prevention. **Figure 16** shows prevention timeline (Rothman K.J., 1981).

The fist main form of prevention is the primary prevention, focused on actions and interventions on healthy population to avoid the development of diseases now and later in the future. For example, sun-safe habits are learned easily during the childhood rather than later in the life and, study show that, effective sun-protective behavior early in the life reduce significative the risk or skin cancer (Stern R.S., 1986). Also, several demographic changes are happening in recent years, the population is becoming increasingly older, therefore, the primary prevention of diseases is of outstanding significance to our society. Frequently, primary prevention is based on behavioral or psychosocial actions (health education, psychological interventions and

psychoeducational changes in behavior, attitudes or representations). Example: sunscreen and anti-smoke campaigns.

Secondary prevention is directed to the early diagnosis of a disease, allowing it to intervene early on the same, but not avoiding or reducing its appearance. An early intervention increases therapeutic opportunities reducing negative effects. The early identification of diseases is made through screening interventions. Example: pap test and mammography in the healthy female population.

Tertiary prevention is directed to the prevention of the progression and complications of the disease. It consists in a complex of action able to improve the patents quality life and reduce the negative side of the diseases.

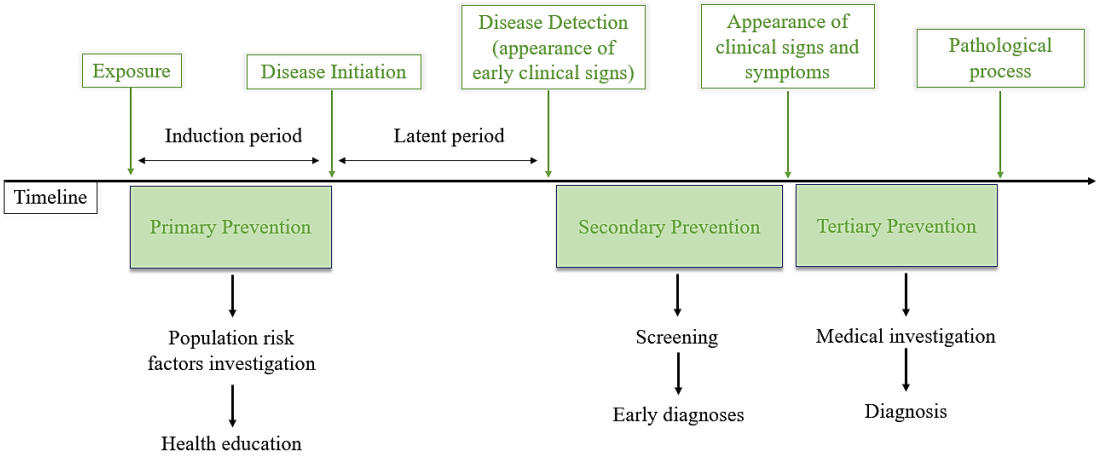



**Figure 16.** Prevention timeline.

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## 2 SCOPE OF WORK

The scope of this work of thesis is to investigate the effects of UV radiations on the skin and to study the actual interactions of UV-filters with the skin and the corresponding efficacy, stability and safety of these compounds with a focus about their impact on the health-care system.

Part of this work focuses on the effects of physical perturbations (solar UVR irradiation) on the skin in order to assess the impact of UVR on the cutaneous permeability barrier and enhance percutaneous absorption. Also, innovative and suitable tools for in-vitro studies on SC lipids are evaluated to detect techniques able to investigate the impact of different environment stresses (UVR, Ozone, PM, pollution, etc.,) on the skin barrier function. Other main objective of the study is the investigation of UV-filters chemical stability and performances along with their interaction with skin, evaluating conventional forms and innovative technological strategies able to improve the efficacy and safety of products. Also, this study intends to investigate the possible mass transfer processes that could happen between packaging and products which can affect the safety and quality of the product or the quality of the packaging systems themselves. The results and understandings from this investigation is made with the goal of assist formulators in creating better sunscreen products, in measuring their efficacy over a broader range of conditions and to be able to study and improve the knowledge about the influence of packaging on sunscreen product safety.

Even after decades of human use with healthy benefits closely related, sunscreen long-term utility has been criticized and questioned. Therefore, the study aim, also, is to investigate and clarify this discrepancy between “ideal” and “real” efficacy. Furthermore, this work intends to provide a first approach for a pharmacoeconomic evaluation about sunscreen and its preventive action against UV-induced damages.

The specific aims of the study are:

- Evaluation of a new technological strategy, such as the UV filters encapsulation (Chapter I). It discusses about the tasks of human safety, product quality and efficacy assurance of a synergic combination of UV filters (avobenzone-octocrylene), based on in-vitro approaches. Using FTIR Spectroscopy and UV/Vis measurements, the behavior of sunscreen based on free form UV filters was compared to sunscreen based on encapsulated

form UV filters in terms of skin penetration, retention on the skin surface and photostability.

- Study of photoinduced changes in the lipidic structure of the skin (Chapter II). In order to improve our knowledge of the effects of UVR solar irradiation on the stratum corneum intercellular lipids and visualize these alterations by FTIR spectroscopy and imaging techniques. Isolated stratum corneum was exposed to short and prolonged UVR dose, FTIR Spectroscopy and ATR-FTIR Spectroscopy Imaging were both used to analyze (a) the photoinduced modifications in the stratum corneum lipid organization and (b) visualize the impact on the skin barrier function.

- Explain how the apparent long-term inefficacy could depend to erroneous patterns of use crating inaccurate expectation and the discrepancy between the in-real life and the "ideal" sunscreen employment and define the relevance role of public education initiative and investing in research to obtain safer and more effective preventive products was underlined (Chapter III).

- Provide a scientific tool that uses a reproducible and transparent approach to summarize the results of individual clinal trial about the effectiveness of sunscreen as preventive tool, creating a starting point for a pharmacoeconomic evaluation (Chapter IV).

- Analyze the interaction packaging-product contained and the possible mass transfer process of organic and lipophilic compounds (such as UV filters) which can affect the safety and quality of the product or the quality of the packaging systems themselves (Chapter V and VI).

# Chapter I

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## Comparative behavior between sunscreens based on free or encapsulated UV filters in term of skin penetration, retention and photo-stability

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**Comparative behavior between sunscreens based on free or encapsulated UV filters in term of skin penetration, retention and photo-stability**

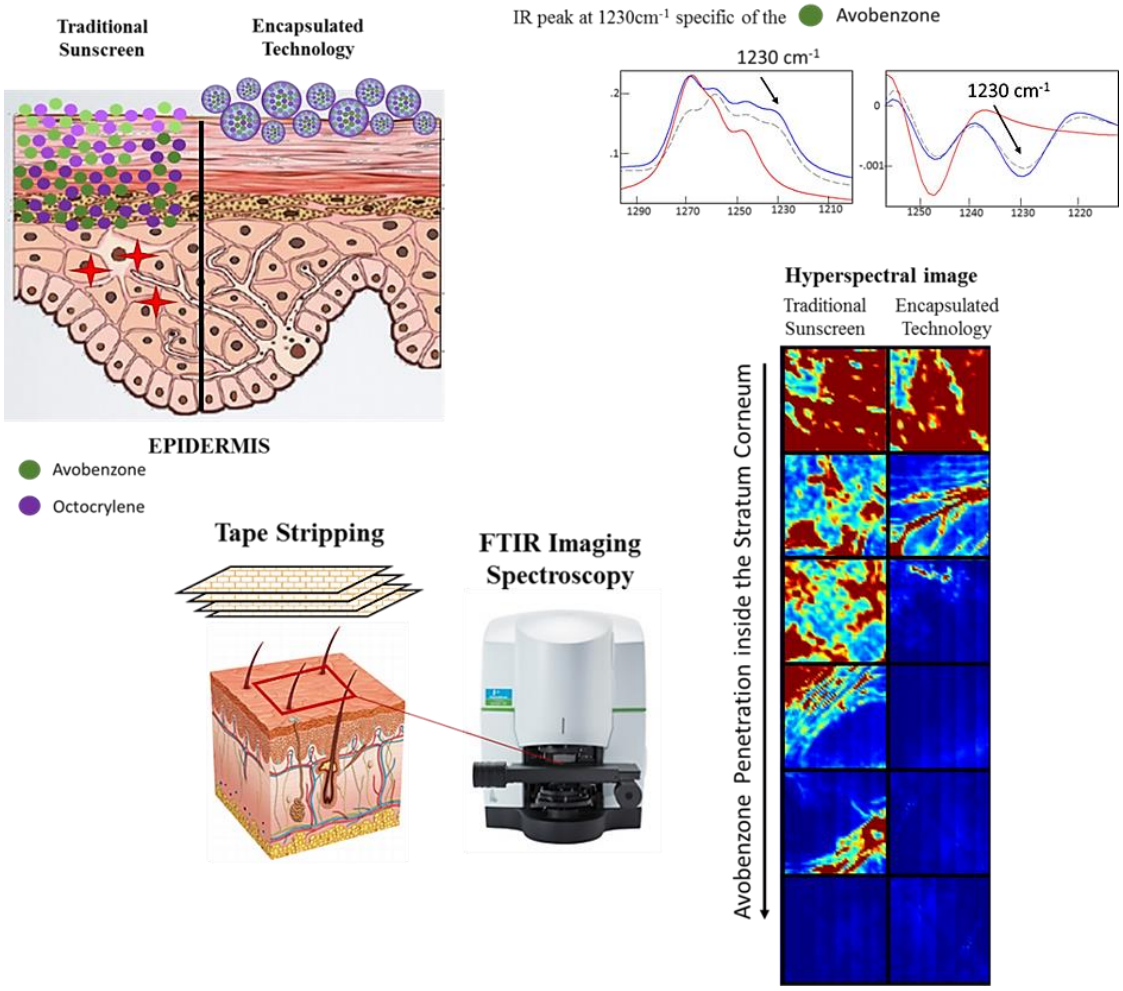
**Abstract**

Background: The growing incidence of photodamaging effects caused by UV radiation (e.g. sunburn, skin cancer) has increased the attention from health authorities which recommend the topical application of sunscreens to prevent these skin damages. The economic stakes for those companies involved in this international market are to develop new UV filters and innovative technologies to provide the most efficient, flexible and robust sunscreen products. Today the development of innovative and competitive sunscreen products is a complex formulation challenge. Indeed, the current sunscreens must protect against skin damages, while also being safe for the skin and being sensory and visually pleasant for the customers when applied on the skin. Organic UV filters, while proposing great advantages, also present the risk to penetrate the stratum corneum and diffuse into underlying structures with unknown consequences; moreover, their photo-stability are noted thorny outcomes in sunscreen development and subsequent performance. In recent years, the evaluation of the interaction between skin and sunscreen in terms of penetration after topical application has been considered from European authority but still its testing as their photo-stability assessment are not mandatory in most countries. Objective: This study, based on in-vitro approaches, was performed to evaluate and compare the retention and the penetration of organic UV filters in free or encapsulated form inside the skin as well as their respective photostability. Methods: Sunscreen formulation with a combination of Avobenzone and Octocrylene in "free form" and a formulation using the same UV filters but encapsulated in a sol-gel silica capsule, were analyzed and compared by FTIR Imaging Spectroscopy. Tape stripping method was used to investigate the penetration of these UV filters inside the stratum corneum. Their photo-stabilities were evaluated by spectroscopic measurements (FTIR, UV/ Vis) and standard measurements were calculated: AUC (Area Under the Curve) and SPF (Sun Protection Factor). Result: With traditional formulation, the organic UV filters penetrated significantly into the stratum corneum while the same UV filters combined with encapsulation technology remained on the skin surface. The encapsulation technology also improved significantly their stability. Conclusion: Encapsulation technology is a promising strategy to improve the efficacy of sunscreen product using organic UV

filters and to reduce safety problem. On the other hand, this study highlighted the pertinence of the FTIR Spectroscopy to test, compare and investigate sunscreen formulations.

**Keywords:** Encapsulated UV filter, skin penetration, Avobenzone, FTIR imaging Spectroscopy, sunscreen efficacy evaluation.

## Graphical Abstract



## 1 Introduction

UV radiations represents only 5-10% of solar radiations but constitute a major hazardous risk for human health with acute adverse outcomes like sunburns and chronic ones like skin cancer (IARC, 2012). Our first and main defense line against the UV radiation is our skin but this barrier does not provide a total protection and a significant part of these UV radiations penetrates our body. UVB penetrates the upper layers of the skin while UVA penetrates the deepest skin layer and interacts with DNA. When skin cell DNA absorbs UV radiation, crosslinking of pyrimidine bases can occur (Setlow, 1966); when damaged DNA dimers are not repaired, mutations are created and can ultimately lead to skin cancer (Holick, 2004; Halliday, 2014; Brash et al., 1991). To reduce the effects of overexposure to UV radiation the international health authorities have recommended the use of sunscreen products which are currently commonly apply all over the globe. To respond to this international market need, the companies develop new UV filters and innovative technologies to provide the most efficient and flexible sunscreen products. The development of efficient and innovative sunscreens is a complex formulation challenge as more and more UV filters are incorporated into day-to-day products such as moisturizers, creams, lip sticks and other skin care products. The current sunscreens must protect against skin damages associated to sun exposure, while also being safe for the skin and being sensory and visually pleasant for the customers when applied on the skin.

UV filters are the key ingredients of sunscreen, providing the essential protection against skin photodamages. In order to guarantee skin protection, the ideal sunscreen product should create a stable protective film on the outermost layer of the skin to absorb or reflect the UV radiations (Jiang et al., 1997; Lu, 1999) during the entire period of UV exposure (Nash and Tanner, 2014). The reality is different especially for organic UV filters. While organic UV filters offer significant cosmetic advantages compared with inorganic UV filters (Mancebo et al., 2014), they have been challenged for their poor photostability (Afonso et al., 2014; Gonzenbach et al., 1992; Schwack and Rudolph, 1995) and for their safety (Gonzalez, 2010; Gonzalez et al., 2006). Indeed, the photo instability of these UV filters can lead to photochemical reactions which compromise both their physical (color, appearance) and chemical properties (efficacy). Chemical alterations can cause undesirable reactions as the production of inactive sunscreen products or highly reactive molecules that can react with the skin (Damiani et al., 2010; Vallejo et al., 2011). Regarding safety, some studies have shown

that these organic UV filters could penetrate the stratum corneum SC (Hayden et al., 2005) and diffuse into underlying skin structures. Few studies highlighted their potential systemic absorption as they were detected in human plasma and urine (Janjua et al., 2008) and in 85% of Swiss human milk samples (Schlump et al., 2010). New formulations of organic UV filters-based sunscreens are necessary to reduce their skin penetration and diminish at once their toxicological risks and efficacy.

Avobenzone (Butyl Methoxydibenzoylmethane) is one of the most common organic UV filters for its strong UVA protection and its versatility (Shaath, 2010; Cabrera et al., 2014). However, it has been reported to have a significant photo instability (Afonso et al., 2014; Gonzenbach et al., 1992; Schwack and Rudolph, 1995) and to lead to photo-allergies (Schauder and Ippen, 1986; Motley and Reynolds, 1989). To increase avobenzone photo-stability and efficacy, it is typically combined with a variety of photo-stabilizers, including other UV filters such as octocrylene (Cantrell and JMcGarvey, 2001). Innovative technologies have also been developed to improve the efficacy and the safety of these actives. Encapsulation is becoming a technique widely explored by the pharmaceutical and chemical industries and its incorporation in cosmetics and personal care products has shown great expansion. Microencapsulation is a process of encapsulating an active ingredient into a shell permanently or temporarily. The result is capsules having diameter between 1 to few micrometers providing a large surface area that could be available for sites of adsorption and desorption, chemical reactions, light scattering, etc. (Benita, 2005; Jyothi et al., 2010; Kaur and Sharma, 2013). Using this technique, encapsulated UV filters, do not have direct contact with the skin which concomitantly prevents their potential toxicological risks.

In the present study, we investigated and compared the behavior of sunscreen formulations based on the same combination of organic UV Filters, Avobenzone and Octocrylene (Eusolex OCR), in free or encapsulated form in terms of skin penetration, retention on the skin surface and photo-stability. For the encapsulated form, the UV filters were entrapped inside sol-gel silica glass microcapsules sufficiently small to be transparent when applied to the skin and provide a pleasant skin feeling. FTIR imaging spectroscopy and ATR-FTIR Spectroscopy techniques were used to investigate the penetration of the UV filters into the stratum corneum and their retention overtime on the skin surface. The photo-stability of the sunscreen formulations was evaluate using

two criteria: (i) Area Under the curve (AUC) and (ii) SPF values calculated in-vitro following the COLIPA method.

## 2 Materials and method

### 2.1 Chemicals

Avobenzone (INCI: Butyl Methoxydibenzoylmethane, Eusolex 9020), Octocrylene (Eusolex OCR), Eusolex® UV-Pearls™ B-O X (INCI: Aqua, Octocrylene, Sorbitol, Butyl Methoxydibenzoylmethane, Silica, PVP) from Merck®; Xanthan gum and Glycerin from Sigma-Aldrich®; Tegosoft TN (C12-15 Alkyl Benzoate) and Abil XL80 (Bis-PEG/PPG-20/5 PEG/PPG-20/5 Dimethicone (and) Methoxy PEG/PPG-25/4 Dimethicone (and) Caprylic/Capric Triglyceride) from Evonik®; Euxyl 9010 (INCI: Phenoxyethanol and Ethylhexylglycerin) from SchÜlke®.

### 2.2 Morphological evaluation by scanning electron microscopy (SEM)

Morphological evaluations of the encapsulated UV filter were performed using scanning electron microscopy (SEM). The SEM used was a FEI XL30 FEG-SEM equipped with an EVEX EDS. The samples were coated with iridium by a sputter coater (Leica EM ACE600). The SEM pictures were recorded at 5KeV with a working distance of ~15mm. Low magnification (2500x) and high magnification (6000x) were used.

### 2.3 Skin Samples

The skin samples were obtained from the belly of pig. They were flash frozen with liquid nitrogen and stored at -40°C wrapped in aluminum foil until the use. Before to start the experiments, the skin samples were defrosted at room temperature for 20 min. Skin pieces 2x2cm or 2x7cm were cut and cleaned to remove dirt and sebum.

### 2.4 Formulation tested

Free and encapsulated UV filters were incorporated in a cold lotion water-based. F1 represents the formulation without actives; F2 is the formulation containing UV filters (Butyl Methoxydibenzoylmethane and Octocrylene) in free form and the formulation F3 contains encapsulated UV filters (Butyl Methoxydibenzoylmethane and Octocrylene). The detailed composition of those formulations is shown **Table 1**. The formulation tested were made to obtain sunscreen with moderate SPF. BASF

Sunscreen Simulator software was used to evaluate the theoretical performances of the formulations regarding SPF (Herzog and Osterwalder, 2015).

**Table 1**. Detailed composition of each formulation tested in this study.

| Phase | Ingredient | INCI | F1 | F2 | F3 |
|---|---|---|---|---|---|
| A | Water | Water | 83.1 | 71.1 | 53.1 |
| | Xanthan Gum | Xanthan Gum | 0.9 | 0.9 | 0.9 |
| | Glycerin | Glycerin | 2 | 2 | 2 |
| | UV Pearls | Water, octocrylene, sorbitol, butyl methoxydibenzoylmethane, silica, PVP | - | - | 30 |
| B | Tegosoft TN | C12-15 alkyl benzoate | 10 | 10 | 10 |
| | Abil XL 80 | Bis-PEG/PPG-20/5 PEG/PPG-20/5 dimethicone (and) methoxy PEG/PPG-25/4 dimethicone (and) caprylic/capric triglyceride | 3 | 3 | 3 |
| | Eusolex 9020 | Butyl methoxydibenzoylmethane | - | 3 | - |
| | Eusolex OCR | Octocrylene | - | 9 | - |
| C | Euxyl 9010 | Phenoxyethanol and ethylhexylglycerin | 1 | 1 | 1 |

Phase A and phase B are stirred separately. Phase B was addicted to phase A under stirrer for 5 minutes at 1000 rpm following by 2 min at 200 rpm by Apolytron PT 10-35 (Kinematica). At the end, phase C was added to the formulation. Formulation were stored at 25 °C for 14h before direct stability characterization. A multisampling analytical centrifuge Hettich® Universal 320R D-78532 PRO Scientific Inc. USA was used in two phases to assess the stability of these formulations: 10 minutes, 3000 rcf, 25°C and right after 30 minutes, 5000 rcf, 25°C.

### 2.5 Skin treatment

Cleaned skin samples were treated with 2 mg/cm$^2$ of sunscreen formulations applied topically with 1 min of massage to cover the entire skin surface uniformly and mounted in diffusion cells (PermeGear, Inc. USA) 15 mm jacketed Franz Cell system with 12 mL receptor chamber filled by phosphate buffer solution pH 7.2 (Fluka Analytical). The diffusion cells were connected to heated bath circulators to maintain the

temperature constant at 32 °C. The skin samples were maintained in this condition for 2 h for the penetration measurement and during 4 h for the retention measurement on the skin surface.

### 2.6 Skin penetration measurement

#### 2.6.1 Tape stripping

At the end of the 2 hours treatment, the skin samples were removed from the diffusion cells and the sunscreen remaining on the skin surface was gently removed with three spatula movements before analysis. Tape stripping technique is a well-established method to investigate the skin penetration of topically applied substances inside the stratum corneum (Lademann et al., 2009). This non-invasive procedure (Klang et al., 2012) sequentially removes layers of stratum corneum from exvivo or in-vivo skin samples. Commercial adhesive tape (Scotch™) was used. The adhesive tapes were applied onto the skin surface, followed by gentile pressure to guarantee a good contact between the most superficial SC layer and the adhesive tape and progressively removed. The pressure, velocity of removal and the type of tape are factors influencing the amount of Stratum Corneum removed per each strip. To standardize the procedure, the same operator applied with the finger a constant pressure on the tape and the same velocity to remove the tape strips. After every removal the skin samples were scanned by FTIR imaging.

#### 2.6.2 FTIR Imaging analysis

All the FTIR images were acquired with a Spotlight 400 Imaging System (Perkin Elmer Instruments, USA) using a MCT (mercury-cadmium- telluride) focal plane array detector. FTIR images were collected in reflective mode with an ATR imaging accessory at a spectral resolution of 4 cm−1 in the mid-infrared (MIR) region between 4000 and 850 cm−1 with a spatial resolution of 6.25×6.25 μm and sample size of 300×300 μm. The ATR imaging accessory used a germanium crystal placed directly in contact with the skin samples. The FTIR Imaging System records hyperspectral images that can provide maps showing the co-localization of specific molecular components or spectroscopic parameters. These images are generated with false colors where the red represent highest values and blue lowest values for each parameter investigated. By scanning skin samples after sequential tape strips, an FTIR

spectroscopic "mapping" inside the stratum corneum can be obtained. These maps were used to visualize the penetration of the UV filters inside the stratum corneum.

### 2.7 Retention measurement on the skin surface

#### 2.7.1 ATR-FTIR Spectroscopy

The retention of UV filters on the skin surface was investigated by ATR-FTIR spectroscopy (Nicolet 6700-Thermo Scientific) after topical application of the sunscreen formulations at different time points; 30 min, 1 h, 2 h and 4 h. After every time point the surface of skin samples were scanned by ATR-FTIR Spectroscopy. ATR-FTIR spectra were recorded in the mid-IR region range from 400 to 750 $cm^{-1}$ with a spectral resolution of 4 $cm^{-1}$ and 64 scans accumulation.

### 2.8. Spectroscopic data processing

FTIR spectra and FTIR images presented in this work were processed using GRAMS/AI (Thermo Fisher Scientific) and ISys software from Spectral Dimensions (Olney, MD) respectively. Using these software spectroscopic parameters were defined to investigate and follow specifically the UV filters tested in this study inside the skin samples. In order to have an optimal data interpretation pre-processing technique were used. Its aim is increase the interpretability and accuracy of the data correcting issues associated with spectral data acquisition (Rinnan et al., 2009). All the FTIR spectra were baseline corrected. In previous study, the Amide I and II band shape and position were evaluated and a similarity between the mean spectra of Amide I and II in different deep was found. The contribution to absorbance in the Amide I and Amide II region are essentially constant in relation to the deep (Zhang et al., 2006). All the FTIR spectra were normalized using the Amide I peak (1710–1590 $cm^{-1}$).

### 2.9 Photo-stability evaluations

Sunscreen samples were irradiated by Xenon Lamp with QSun Xenon Test Chamber 3100. This system is able to reproduce the damage caused by full-spectrum sunlight, the sample was exposed to irradiance: 0.55 ± 2 $W/m^2$ with a temperature: 40 ± 1 °C and humidity: 45 ± 1%. Photo-stability of the sunscreen formulations were evaluated by the Area Under the curve (AUC) (Gonzalez et al., 2007). The Area Under the Curve (AUC) for both UVB (290–320 nm) and UVA (320–400 nm) were calculated for each

sunscreen on PMMA plate before and after UV exposure. The AUC were calculated following the equation:

$$AUC\ UVR = \int_{290}^{400} A\lambda\ d\lambda$$

$$AUC\ UVB = \frac{1}{2}A320 + \int_{290}^{319} A\lambda\ d\lambda$$

$$AUC\ UVA = \frac{1}{2}A320 + \int_{321}^{400} A\lambda\ d\lambda$$

Where A is Absorption and *d* the wavelength.

To compare the photo-stability for each sunscreen before and after exposure the areas under the curve were compared using Student's t-test ($p<0,05$). The AUCI (Area Under the Curve Index) was calculated following the equation:

$$AUCI = \frac{AUC\ after}{AUC\ before}$$

If the AUCI was ≥0.8 the sunscreen was considered photo-instable.

The ratio between the irradiated and non-irradiated samples curve areas was calculated following the equation:

$$Ratio = \frac{AUC\ after}{AUC\ before} * 100$$

The results were reported in percentage.

The SPF in vitro was calculated with the well-established method by the International Sun Protection Factor Test Method COLIPA (Colipa, 2011). COLIPA (the European Cosmetic Products Trade Association defined equation for the estimation of SPF in vitro:

$$SPF\ in\ vitro = \frac{\int_{\lambda=290nm}^{\lambda=400nm} E\lambda\ I\lambda\ d\lambda}{\int_{\lambda=290nm}^{\lambda=400nm} E\lambda\ I\lambda\ 10^{-A0\lambda}\ d\lambda}$$

Where: E λ = Erythema action spectrum (CIE-1987), I λ = Spectral irradiance of the UV Source (SSR for SPF testing), $A_0$ λ = Mean monochromatic absorbance

measurements per plate of the test product layer before UV exposure, d λ = Wavelength step (1nm).

### 2.9.1 Spectrophotometric measurements

Substrate and product application were carried out in according to the COLIPA Method (Cosmetics Europe, 2011). 1.3 mg/cm$^2$ of sunscreens products were spread on roughened PMMA plate (SUNPLATES PMMA plates (5 cm×5 cm) roughness 4.5 to 5.5 μm Lot. XT 1601-1 by HelioScience® Sun Technology). The formulation was applied as a large number (approximate 12) of small drops (approximate equal volume) over the whole surface of the PMMA plate. After application, the formulation was spread using a fingertip "pre-saturated" with the formulation. Spreading is two phases process: (i) product distribution with quickly movements but without pressure (30 s.), (ii) circulate movements using pressure (30 s.) The samples were left 30 min in the dark 25 °C to facilitate the formation of standard product film. Each formulation was spread onto three PMMA plates and each plate was measured in five different sites to ensure a total area of 5 cm$^2$.

The absorption curve before and after irradiation was recorded with an UV/Vis/NIR Spectrophotometer equipped with a 150mm integrating sphere (Lambda 1050 from PerkinElmer). The plates were placed into the spectrophotometer transmittance port facing the light emission source. A 100% transmission reference sample was prepared by spreading 15 μl of Glycerin on the roughened side of the PMMA plate. UV Measurement method: set the scan range from 400 nm to 290 nm, ordinate mode of T%, data Interval 1 nm, bandpass (PMT fixed) of 2 nm, Integration time of 0.2 nm. All obtained transmittance values were converted to absorbance according with the equation (Cosmetics Europe, 2011):

$$A\lambda = -\log(T\lambda)$$

Where A λ = Mean monochromatic absorbance measurements and T λ = Fraction of incident transmitted by the sunscreen film.

## 3 Results and discussion

### 3.1 Morphological evaluation of encapsulated UV filters

The encapsulated UV filter evaluated in this study used micro-encapsulation technology that entraps organic chemicals in sol-gel silica glass. This process produces aqueous dispersion of capsules with approx. 37% (w/w) of UV absorber. Those

capsules were prepared by polycondensation reaction at room temperature. This type of low temperature glass synthesis enables substances such as organic UV filters to be encapsulated within the glass by adding them to a reaction mixture. Approximately 80% of the capsule's weight is made up of the UV absorber. **Figure 1** shows scanning electron microscopy (SEM) images of obtained sol-gel silica-shell. The capsules were formed as sphere with calculated average diameters of ~ 1-2 µm. The capsules were sufficiently small to be transparent when applied to the skin and provide a pleasant skin feeling.

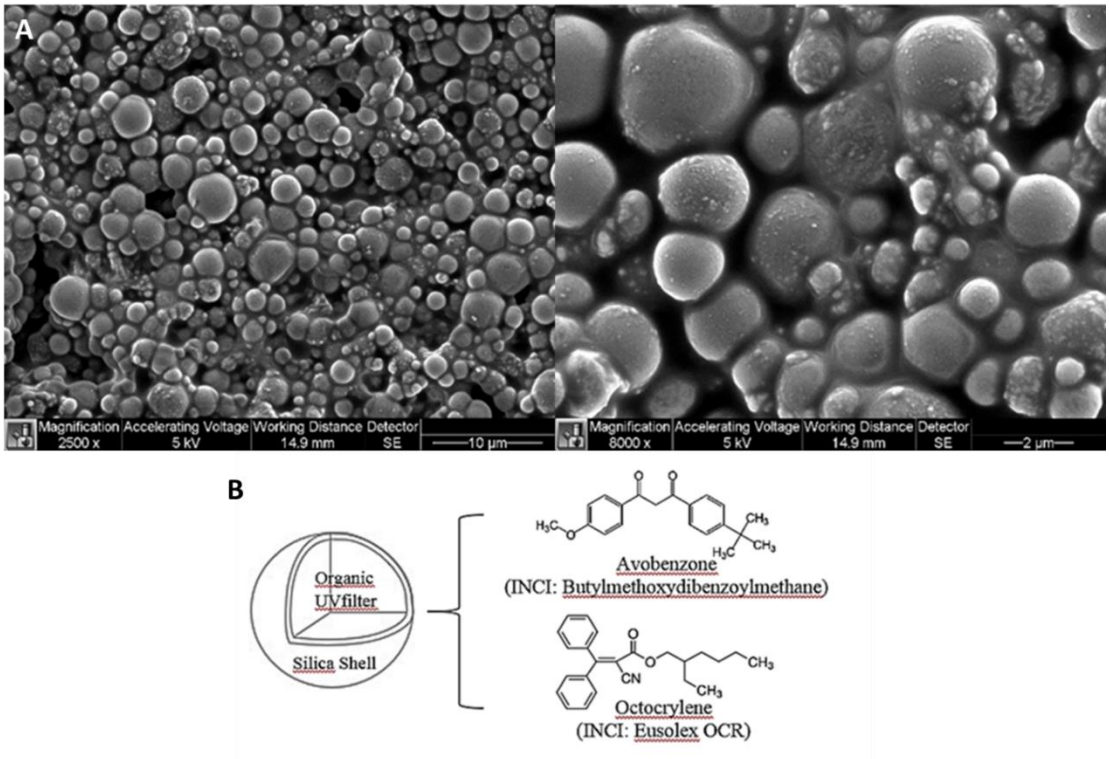



**Figure 1**. a) SEM pictures recorded on Eusolex UVPearls (Merck). The SEM pictures present the capsules loaded with Avobenzone and octocrylene at low magnification (2500x on the left) and high resolution (6000x on the right) b) illustration of Avobenzone/Octocrylene silica shell capsules.

### 3.2 IR markets used to follow the UV filters

All the formulations were shown to be stable. Formulations F1, F2 and F3 were scanned by FTIR Spectroscopy. The resulting FTIR spectra and second-derivative spectra of the formulation F1, F2 and F3 were used to define the most relevant IR marker to investigate the UV filters used in this study. Average spectra for each formulation F1, F2 and F3 were calculated from several IR spectra recorded on each formulation and the second-derivative spectra were obtained from these average

spectra. In **Figure 2**, significant differences were observed between these 3 FTIR spectra, the most prominent being the band at 1231 $cm^{-1}$ (Fig. 2b). The band at wavenumber 1231 $cm^{-1}$ can be observed in the spectra recorded on the formulation F2 and F3 but this contribution is absent in the spectrum recorded on the based formulation F1 without the UV filters. Deepest investigations were made in order to validate the IR peak at 1231 $cm^{-1}$ as an IR marker of the UV filters. Second derivative spectra of formulation F1, F2 and F3 were obtained (Fig. 2c) and clearly the IR contribution at 1231 cm−1 was observed only in the formulation containing the UV filters; F2 and F3.

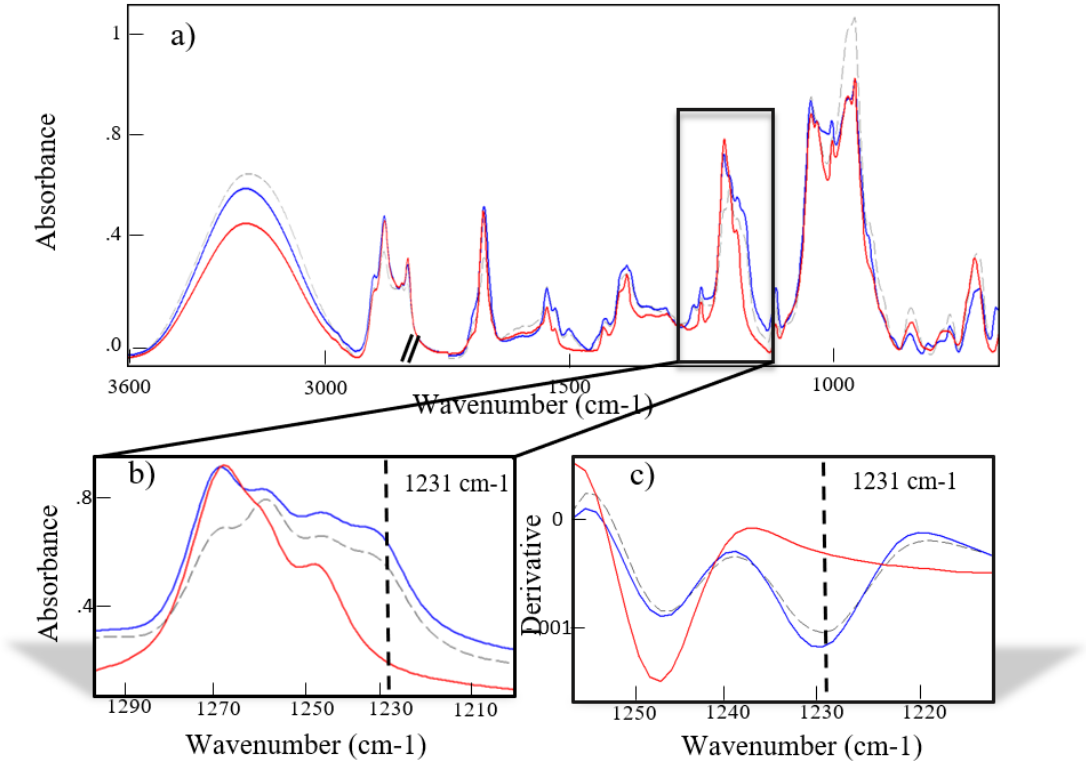



**Figure 2.** a) mean FTIR spectra between 3600-850 $cm^{-1}$ region b) enlargement in the 1290-1210 $cm^{-1}$ region and c) second derivative spectra recorded on formulation F1 (red line), F2 (dashed grey line) and F3 (solid blue line).

The FTIR spectra recorded on Avobenzone and Octocrylene (raw material) are presented in **Figure 3**. The IR contribution detected around wavenumber 1231 $cm^{-1}$ in the formulations F2 and F3 is consistent with the IR band observed around 1226 $cm^{-1}$ in the spectrum of the Avobenzone (Fig. 3b) validating the assignment of the band at 1231 $cm^{-1}$ to the avobenzone contribution. The shift from 1226 cm−1 to 1231 $cm^{-1}$ can be explained by the modification that the Avobenzone powder underwent

when it was added into the formulation. No Octocrylene contribution was observed in this area (Fig. 3b, C). All together, these data shown the Avobenzone content can be monitored by the IR band at 1231 $cm^{-1}$. Avobenzone is the most important organic UVA absorber that has been globally approved, Octocrylene is used mostly to stabilized and solubilized the Avobenzone implementing the level of primary photoprotection (Lionetti and Rigano, 2017; Afonso et al., 2014) (Wang et al., 2010). In the presented study, Avobenzone was considered as the principal UV filter in order to evaluate the penetration and the retention of organic UV filter into the stratum corneum.

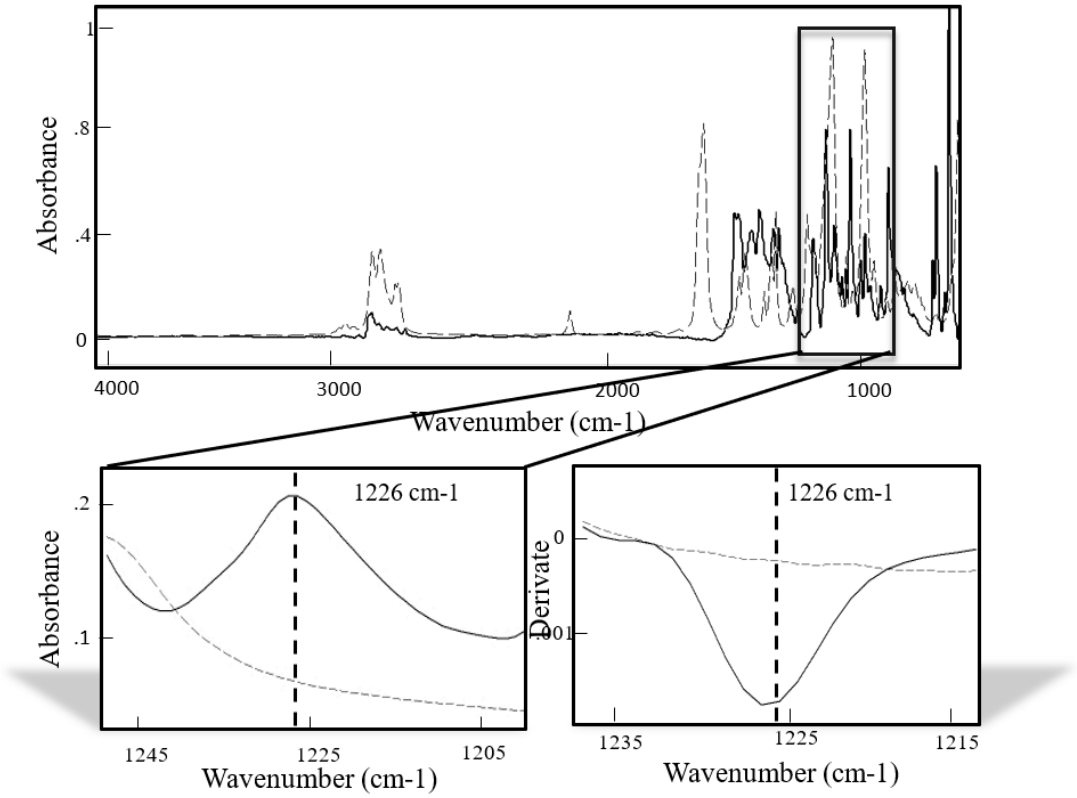



**Figure 3**. a) Mean FTIR spectra recorded between 4000-850 $cm^{-1}$, b) enlargement in the 1280-1200 $cm^{-1}$ region and c) second derivative of Avobenzone powder (solid line) and Octocrylene (dashed grey line).

### 3.3 Deposition and penetration of the UV filter

To investigate the impact of the formulation on the penetration behavior of the UV filters inside the stratum corneum, 3 different sunscreen formulations (F1, F2 and F3) were applied topically on skin samples. Skin from many mammalian species, including pig and humans, can be used to evaluate the penetration of cosmetic products into the

skin as the permeability properties are maintained after excision (Lademann et al., 2009). Indeed, the integrity of the stratum corneum, the main component for the skin barrier function, is not altered. Skin samples were scanned by ATR-FTIR Imaging Spectroscopy in association with a tape stripping procedure to provide a penetration profile of exogenous UV filters into the stratum corneum. Four skin samples were analyzed and compared: skin treated with formulation F1, F2 and F3 were compared with untreated skin. For each sample FTIR images were scanned before (control), after topical treatment (deposition) and after the tape strip were removed. The FTIR images were concatenated to produce the FTIR Images shown in **Figure 4**. These FTIR images were generated to follow specifically the UV filters in the skin samples by using the wavenumber 1231 cm−1 to Amide I intensity ratio. This ratio allows to visualize specifically the Avobenzone inside the skin samples and in consequence was used to compare the penetration of this active in function of the different sunscreen formulations.

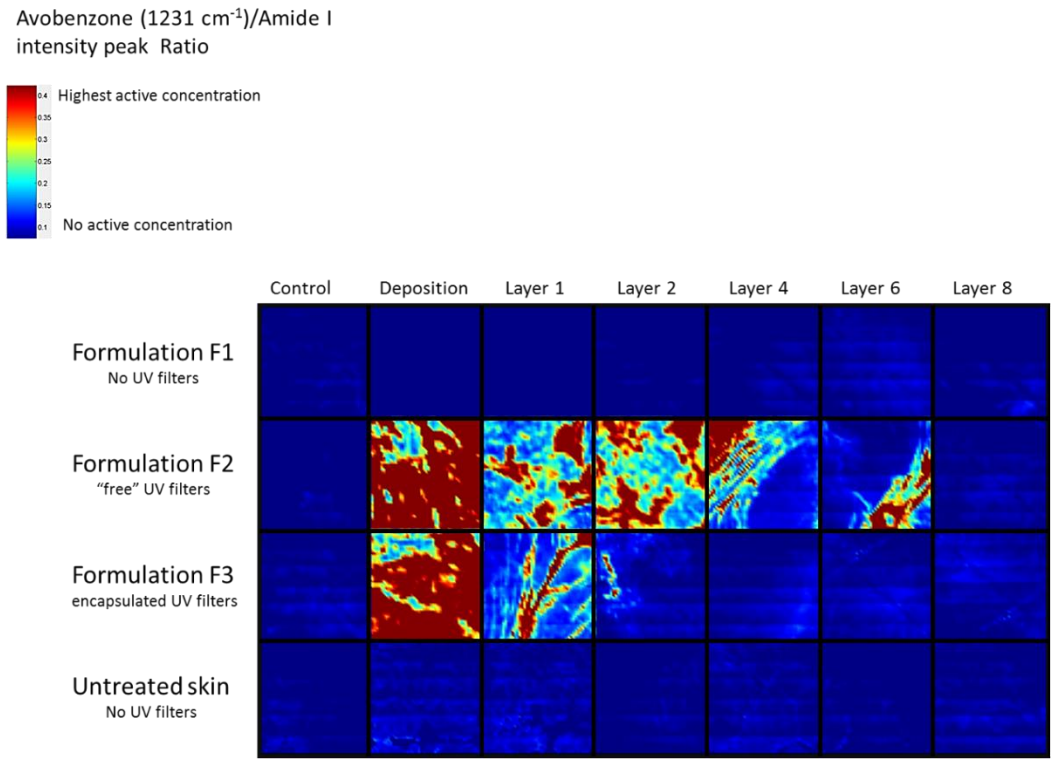


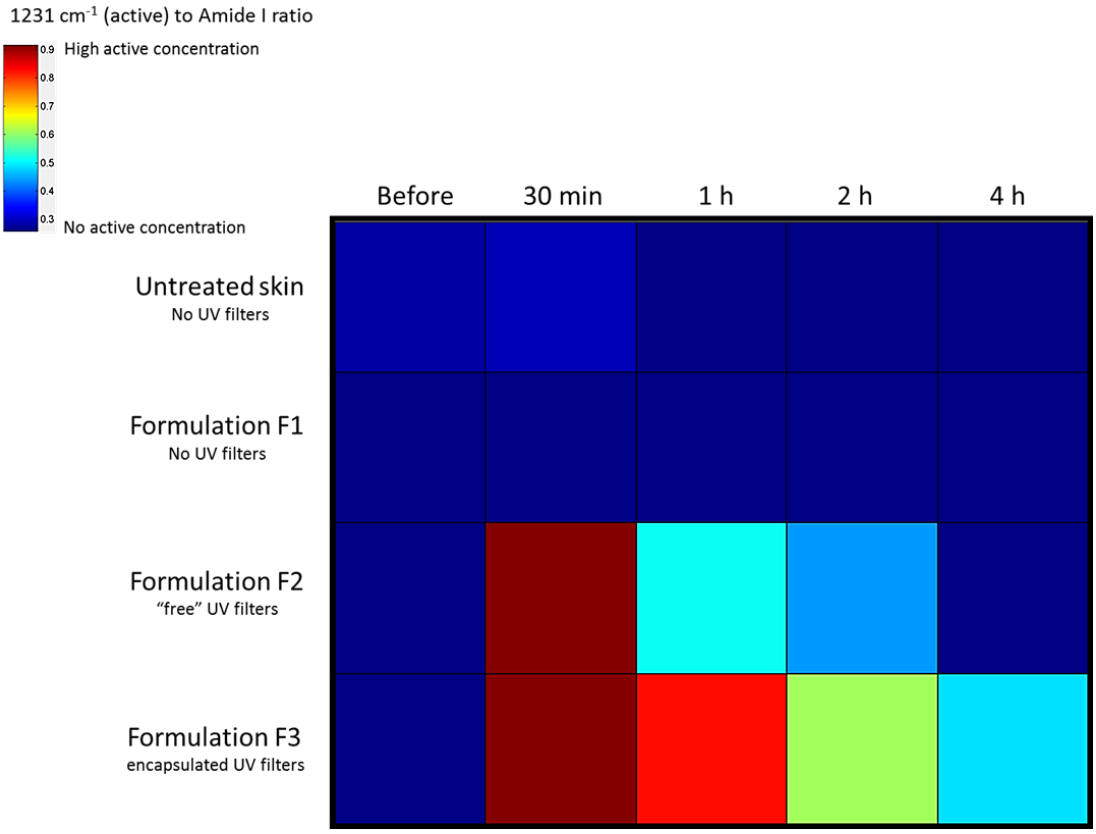

**Figure 4**. FTIR images generated by calculating the intensity peak ratio between 1231cm$^{-1}$ (Avobenzone) and the Amide I. These FTIR Images allow to visualize and compare the avobenzone penetration inside the stratum corneum for different skin samples: skin samples treated with formulation F1, F2 and F3 compared to untreated skin. For each sample the FTIR images were scanned before (control), after topical application on the sunscreen formulation and after 8 sequential tape strips.

As expected the Avobenzone was not detected in all the skin samples before treatment, in the untreated skin and in the sample treated with formulation F1. High deposition of UV filter on the skin surface was recorded on skin samples treated with formulation F2 and F3. As discussed previously, sunscreen should have a high affinity for stratum corneum to stay and adhere on the superficial layer of the skin to create a protective and stable film. The high active concentration observed on the skin surfaces after the treatment (deposition) demonstrated the formulation F2 and F3 have created a uniform protective film on the skin surface. The FTIR Imaging technique coupled with a tape stripping procedure allows to visualize and compare the UV filter penetration into the stratum corneum related to a specific sunscreen formulation. In this work we detected, a different penetration behavior for the Avobenzone between the traditional sunscreen formulation and the sunscreen formulation based on encapsulation technology. With the regular formulation, the UV filters presented as expected a high concentration on the skin surface but also a significant concentration deep inside the stratum corneum indicating the Avobenzone under "free" formulation did not remain on the skin surface but penetrated deep inside the skin. Indeed, the UV filters were detected up to the layer 6 under free formulation after just one single topical application. On the other hand, the same UV filter combined with encapsulation technology were observed on the skin surface and almost no penetration was detected inside the stratum corneum. Indeed, the encapsulated avobenzone was not detected after the layer 1 clearly indicating that the encapsulation technology allowed to keep the UV filters at the surface of the skin where they will the most efficiently exert their purpose.

The current results are in accordance with earlier investigation (Scalia et al., 2011) which studied the effect of encapsulation technology on the penetration of Ethylhexyl Methoxycinnamate (EHMC) and Butyl Methoxydibenzoylmethane (Avobenzone) in human skin. The study demonstrated that sunscreen loaded in lipid microparticles penetrated less depth into the stratum corneum compared to the UV filters "free". The main fraction of the sunscreen which penetrated the skin was localized only in the upper layers of the skin. More recently (Puglia et al., 2014) another group evaluated the nanostructured lipid carriers (NLC) to optimize the topical application of organic UV filters. In agreement with our study, this previous report showed that this different NLC encapsulation technology limits skin penetration of UV filters that remained primarily on the surface of the skin.

### 3.4. Retention overtime

To validate the previous data and assess their impacts on the efficacy of these different sunscreen formulation technologies we analyzed the retention of the Avobenzone overtime on the skin surface. This test provided relevant data concerning the UV filters incorporated in the formulations in term of deposition on the skin surface and how long these UV filters stay on the surface in function of the sunscreen technologies used to elaborate the formulations. The FTIR images presented in **Figure 5** were generated using the same intensity ratio between the Avobenzone (1231 $cm^{-1}$ region) and the Amide I. These FTIR images confirmed the previous data concerning the UV filter concentration on the skin surface. The FTIR images were generated with false color. Redder was the image, higher was the Avobenzone concentration on the skin surface.




| Sample | Time | | | | |
|---|---|---|---|---|---|
| | **Before** | **30 min** | **1 h** | **2 h** | **4h** |
| **Untreated** | 0.28 | 0.29 | 0.25 | 0.26 | 0.19 |
| **Formulation F1** | 0.26 | 0.21 | 0.26 | 0.20 | 0.21 |
| **Formulation F2** | 0.23 | 1.48 | 0.51 | 0.44 | 0.24 |
| **Formulation F3** | 0.18 | 1.27 | 0.83 | 0.61 | 0.48 |

**Figure 5**. FTIR images were generated by calculating the 1231cm$^{-1}$ to Amid I intensity peak ratio from skin samples treated with formulation F1, F2 and F3 before (control), after (deposition) up to layer 8, compared to untreated skin.

Skin samples treated with formulation F2 and F3 presented the highest concentration of UV filter after 30 min confirming the ability of these formulations to create a protective film on the superficial areas of the skin. This uniform coating provided the skin protection against the UV radiation. The presence of this coating overtime and as well as its stability will determine the efficacy of the sunscreen formulation. Sample treated with formulation F2 showed a significantly lower concentration of UV filter on the skin surface after exposure for an hour. This tendency is confirmed and amplified after 2 h and even more after 4 h. Indeed, after 4 h the IR ratio calculated on the sample was similar to the one recorded before treatment indicating that after 4 h no more UV filter was present on the skin which was treated with the sunscreen formulation F2. These data provided information regarding the inclination for the organic UV filters to go across the stratum corneum especially in regular sunscreen formulation where the UV filters are "free". To limit this penetration and improve the efficiency of the sunscreen, encapsulation technology is a relevant option to formulate UV filters in sunscreen products. Indeed, samples treated with formulation F3 based on encapsulation technology presented a similar UV filter concentration level than the one observed in the skin treated with the formulation F2 after 30 min. In contrast, after 2 h and 4 h, a significantly higher amount of Avobenzone was still detectable on the skin surface after topical application of the formulation F3 compared with the formulation F2 highlighting the ability of the encapsulation technology to reduce the penetration of the UV filter in the skin and in consequence to improve the efficacy of the sunscreen product.

### 3.5. Photo-stability evaluation after exposure

Photo-stability of sunscreens is a key parameter that must be taken into consideration during their development and to assess their performance especially when they incorporate organic UV filters. Organic UV filters were designed and used to efficiently absorb the UV radiation during a given time period. This absorption can induce photochemical reactions in these molecules which result in some degradation of these UV filters and in consequence decrease the efficiency of the sunscreen products (Nash and Tanner, 2014; Kockler et al., 2012). To strengthen the previous

data and to confirm the benefit of the encapsulation technology in term of sunscreen efficacy, we analyzed and compared the photo-stability of these sunscreen formulations. The first parameter used to evaluate the photo-stability was the area under the curves (AUC). The AUC calculated versus wavelength (290–320 nm for UVB and 320–400 nm for UVA) before and after irradiation are presented in **Figure 6**.

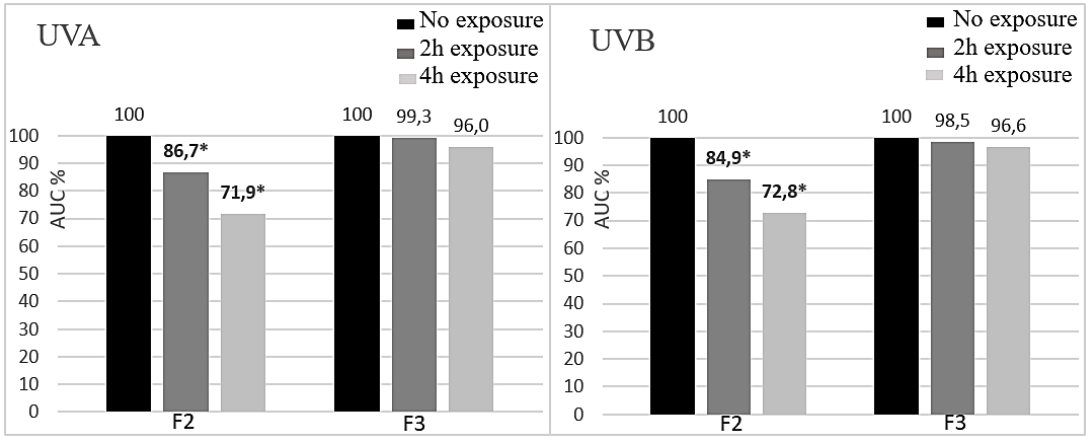


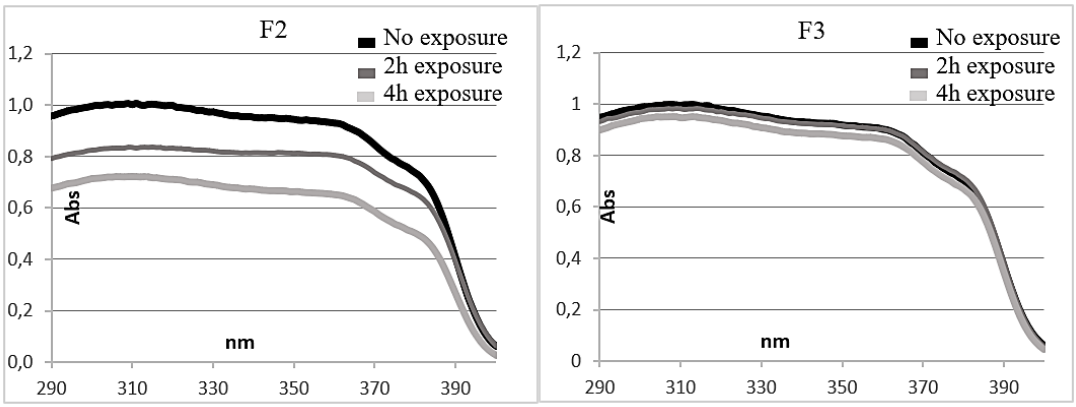

**Figure 6**. Area under the absorption curve % (AUC) before, after 2h and 4h of UV exposure recorded on formulation F2 (free) and F3 (encapsulated) for the UVA (320-400nm) and the UVB (290-320nm). The areas were compared using Student's t-test ($p<0,05$), The results statistically different ($p<0,05$) were marked with *.

The sunscreen protection provided by the formulation F2 decreased significantly overtime under UV exposure. Formulation F2 designed with "free" UV filters showed a reduction of 13% in the UVA protection and 15% in the UVB protection abilities after two hours of UV exposure. This degradation was confirmed and amplified after 4 h of UV exposure. Indeed, the formulation F2 showed a reduction of 28% in the UVA protection and 27% in the UVB protection after 4 h of UV exposure. The AUCI for formulation F2 after 2 h of exposure was 0.86 and 0.72 after 4 h. F2 presented unstable behaviors when exposed to UV radiation. Formulation F3 did not present a statistically significant reduction in their UVB or UVA absorption after irradiation. The AUCI value for F3 after 2 h of exposure was 0.99 and 0.96 after 4 h indicating a photostable behavior for F3. Data have shown the encapsulation technology associated with a combination Avobenzone/Octocrylene could prevent efficiently the photo-degradation of the sunscreen products formulated with organic UV filters. These data can be compared with a previous study (Yang et al., 2008) which investigated the influence of hydroxypropyl- beta-cyclodextrin (HPCD) complexation on the

photodegradation of Avobenzone. The complexation was shown to significantly reduce the photodegradation of Avobenzone after UV irradiation for 16, 40 and 80 min.

The effect of the temperature on the PMMA plate could create some interference in the absorbance curve determination. In order to eliminate the interference of the temperature after 2 h and 4 h of UV exposure, PMMA plates without formulation were studied in the same testing condition but without UV exposure. The analysis shown thermal stability of the PMMA plate (data not presented).

The second parameter used to evaluate the photo-stability of formulation tested is the SPF (Sun Protection Factor). Absorbance spectra for formulations F2 and F3 as well as percent variance of SPF values before and after 2, 4 h of UV exposure are shown in **Figure 7**.




| Sample | Variation % SPF in vitro | |
|---|---|---|
| | **2 h exposure** | **4 h exposure** |
| **F2** | 31.5 | 50.7 |
| **F3** | 3.9 | 8.4 |

**Figure 7**. UV absorbance spectra of formulation F2 and F3 and percent variance of SPF values calculated in-vitro before and after 2 and 4 h of UV exposure.

By evaluating the spectra of sunscreen formulation F2 before and after exposure, an absorption decreases in UVA and UVB region was observed. Formulation F2, with free form of UV filters, exhibited a decrease of 31% in SPF value after 2 h of exposure and 50% after 4 h of UV exposure. A significant reduction in SPF values can be associated with a decrease in photoprotection effectiveness of the sunscreen

formulation. An insignificant reduction in SPF values were detected for formulation F3 after 2 and 4 h. These results confirm that encapsulation technology can maintain "the in-vitro SPF values of UV filters" and consequently be a good strategy to improve the photo-stability of organic UV filters.

## 4 Conclusions

Extended exposure to ultraviolet radiation plays a prevalent role in skin damages like photo-carcinogenesis or photo-aging. Sunscreens are currently leading products to protect our skin and avoid these alterations. With the continuous increase in air pollution levels and global warming, sunscreens will become even more essential products in our day-to-day life. While no regulations are yet established in terms of sunscreen skin penetration and stability, a growing customer safety concern should be taken seriously into consideration when designing future sunscreen products. To respond to these new international market needs, companies must develop new UV filters and innovative technologies to provide safe, more efficient and flexible sunscreen products not only protecting against skin damages, but also providing a pleasant application experience and visual finish for cosmetic perspective. In the present study, we show that FTIR spectroscopy and FTIR imaging techniques are efficient methods to investigate and visualize several essential parameters of new sunscreen formulations, such as their penetration profile inside the skin and their retention on the skin surface. Notably, we show that sunscreens based on encapsulation technology can reduce the penetration of the organic UV filters inside the skin improving thereby their overall safety. Performance is also increased by this process knowing that encapsulated organic UV filters showed a significantly extended photostability. In conclusion, this work highlights the potential of innovative strategies such as micro-encapsulation technology, to become a relevant plan of action to produce superiorly efficacious organic UV filters- based sunscreen products with limited toxicological risks.

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# Chapter II

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**The Impact of solar exposure on the Stratum Corneum investigated by FTIR Spectroscopy and imaging**

(Submitted to European Journal of Dermatology)

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**The impact of solar exposure on the stratum corneum investigated by FTIR spectroscopy and imaging**

**Abstract**

Background: The Stratum Corneum (SC), the most superficial layer of the epidermis, is our first protection from external stresses such as UV exposure or exogenous components. The skin barrier function is related to the unique lipid composition of the SC and their complex structural organization; changes to these lipids may result in a significant modification of the skin barrier function and ultimately, in a deficient protective function of the skin. Objective: The purpose of this study is to improve our knowledge of the effects of UVR solar irradiation on the stratum corneum intercellular lipids and to use FTIR spectroscopy and imaging techniques in order to visualize the alterations. Methods: Isolated SC was exposed to short and prolonged UVR dose. Both FTIR Spectroscopy and ATR-FTIR Spectroscopy Imaging were used to analyze (a) the photo-induced modifications in the stratum corneum lipid organization and (b) visualize the impact on the skin barrier function. Result: The lipid organization inside the SC was strongly disorganized by this exposure. Moreover, the lipid composition itself was altered by the solar exposition. The main part of these modifications appeared after short-term exposure to solar radiation. Conclusion: The solar UV radiation compromised the skin's fundamental barrier function thereby reducing its natural ability to protect us. The lipid organization inside the SC, as well as its composition, were strongly impacted by the solar UV exposure. Together these modifications could dramatically alter the cutaneous permeation of exogenous components. Moreover, this study highlighted the pertinence of the FTIR Spectroscopy to investigate, assess and visualize the status of the skin barrier function.

**Keywords:** FTIR Spectroscopy and Imaging, Intercellular Lipids, Lipid Organization, Solar UV Exposure, Skin Barrier Function, Stratum Corneum.

## 1 Introduction

The skin is the largest organ of the body and the only one which is external. Acting as a barrier between our body and the external environment, the skin supports many vital functions including; (a) regulating the body temperature, (b) maintaining water and electrolyte in our body and (c) protecting us from environmental insults. The skin is composed of three major structural layers; the hypodermis (fatty subcutaneous layer), the dermis (vascularized layer with a rich supply of capillaries, nerves, hair follicles etc.) and the epidermis. The epidermis is the outermost layer of the skin and its principal cells are the keratinocytes. It comprises four layers of cell and each layer represents a sequential differentiation stage of the keratinocytes. These different layers from inside to outside are: stratum basal (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). The unique lipid composition of the SC and their complex structural organization are responsible of the formation and maintenance of the skin barrier function (Madison K., 2003). Typically, the SC consists of ∼ 10 to 20 sub-layers of terminally differentiated, non-nucleated corneocytes that are embedded in an intercellular lamellar lipid matrix (Kanitakis J., 2002). The intercellular lipids in the SC are made of unique complex mixture of polar and non-polar lipids. The main components are ceramides (CERs), cholesterol (CHOL) and free fatty acid (FFAs) in approximately equimolar ratio (Weerheim A.P., 2001). There are 6 subclasses of human ceramides, with straight and saturated hydrophobic chains enabling them to be resistant to oxidative damages and to be impermeable to water. The ceramides have a large range of chain lengths; the amide-linked fatty acids varied between 14 and 30 carbon atoms whilst the sphingoid base was mainly16–20 carbon atoms long (Wertz P.W., 1985). Later, Robson in 1994 (Robson K.J., 1994) and Ponec in 2003 (Ponec M., 2003), introduced the $7^{th}$, $8^{th}$ and $9^{th}$ CER subclass, using TLC/NMR. The introduction of LC/MS led to the introduction of 3 additional subclasses by Masukawa in 2008 (Masukawa Y., 2008). These advancements have led to the discovery of a total of 12 CER subclasses that are present in the human SC lipid matrix. Four of these subclasses have a long ω-hydroxy fatty acid chain ester linked to the linoleic acid, referred to as CER EO. The significance of CHOL on the skin barrier was investigated and it was reported that CHOL was essential in maintaining the structure, fluidity and orientation of the lipids and as a result necessary for a proper skin barrier function (Kucerka N., 2010; Feingold K. R., 2014). In previous studies it has been suggest that CHOL plays a key role for the

formation of lamellar phases and the highly dense orthorhombic lateral packing (Mojumdar E.H., 2015). A wide distribution of FFA chain lengths has been observed in the past years, the most abundant are 22, 24 and 26 carbon atoms, but in the human SC these are mainly saturated with a range in chain length from C14 to C34 (Norlen L., 1998).

Together, the SC lipids form a unique spatial arrangement. Most of the experimental evidence shows the validity of the "domain mosaic model" by Forslind (Forslind B., 1994) in which some shorter chain conformational disordered with a liquid-crystalline arrangement are embedded in ordered lamellar crystalline structure. The organization of the lipids is not fully agreed, various models describing the organization have been proposed; "the sandwich model" by Bouwstra et al. (Bouwstra J.A., 2000), "the single gel phase model" by Norlen (Norlén L., 2001), and "the armature reinforcement model" by Kiselev et al. (Kiselev M.A., 2005). The SC lipids are able to adopt three type of lateral packing arrangement that differ in their rotational and translational mobility. In healthy SC, lipids are present as (i) densely packed lipids chains (orthorhombic phase) with no rotational or transactional mobility; (ii) less densely packed lipids chains (hexagonal phase) with some rotational mobility but no transactional mobility; (iii) low densely packed (liquid crystalline phase) where the lateral organization is completely lost, and they have full mobility (Van Smeden J., 2014). Alterations in the composition of the SC lipids or their organization can be directly correlated with alternated skin barrier functions. Skin barrier disruption plays a role in the pathogenesis of atopic dermatitis, psoriasis, ichthyoses etc. (Harding C.R., 2007). Moreover, these modifications could impact the skin permeation of foreign substances (Jain A., 2017).

Due to the high cost of human skin, biological and synthetic skin substitutes have been found useful since earliest centuries in both medical and drug sciences (Halim A.S., 2010). Pig skin was largely used as a good model for human skin because its characteristics were extremely similar (Vardaxis N.J., 1997; Jacobi U., 2007). The skin SC lamellar organization is closed to the human one and, even if, the lateral packaging is quite different from the human, pig skin SC is considered a valid model to investigate the skin (Jacobi U., 2007).

The SC is constantly exposed to solar UV irradiation that can cause photodamage to different levels. The SC molecules, especially the not saturated lipids, are sensitive to UV exposure. The effects of UV irradiation have been previously

studied, and three major consequences were found; a) β scission (fragmentation of the carbon chain), (b) hydrogenation of the double bond of unsaturated compounds and (c) formation of oxygenated entities from unsaturated lipids (Shibamoto T., 2006). Alterations in the SC may result in significant modification of the skin barrier function. Furthermore, changes to the thermal profile, from lower to higher wavenumber, reflect an increase in disorder of lateral packing in the SC lipids thereby resulting in an impaired barrier function. Studies have correlated the structural alteration of SC to more permeable barrier function with some disease(s). Atopic dermatitis (AD) is a multifactorial, skin disorder that affects up to 20% of children in Western counties (Laughter D., 2000), where it has been hypothesized that the permeability barrier abnormality could drive disease activity (Ghadially R., 1996). An alternated lipid composition in the intercellular matrix of the stratum corneum has also been shown in lamellar ichthyosis patients. They presented an altered ceramide profile and the amount of free fatty acid is decreased compared with a healthy skin. The levels of transepidermal water loss (TEWL), for the pathology, are usually towards higher values indicative of an altered barrier function (Pilgram G.S., 2001).

This work using FTIR spectroscopy and ATR-FTIR spectroscopy Imaging, investigates the changes in the supramolecular organization/conformation of the SC lipids after UV exposure. Initially, asymmetric and symmetric $CH_2$ stretching bands (2800-2950 $cm^{-1}$) were studied to evaluate the supramolecular order of SC lipids before and after progressive UV exposure. Thermotropic studies were carried out to observe the variation of $CH_2$ asymmetric and symmetric stretching bands describing the mobility associated with SC lipids domains after UV exposure. Secondly, FTIR spectroscopy Imaging experiments were carry out on isolated SC to visualize the modifications in function of progressive UV exposure.

## 2 Materials and methods

### 2.1 Isolated stratum corneum

The skin samples from the belly of white Yucatan pig were purchased from Sinclair Research Center Inc., USA. The pig skin was flash frozen with liquid nitrogen, wrapped in aluminum foil and then stored at -40°C, until the use. The skin samples were defrosted at room temperature for 20 min. Then the skin samples (skin pieces 2x2cm) were cut and cleaned to remove dirt and sebum. The SC was separated using a trypsin procedure (Kligman A.M., 1963). Briefly, the skin samples were placed with

the SC side up in 0.5% wt/vol trypsin (Sigma Chemical, purified porcine pancreas, Type IX) in phosphate buffer solution pH 7.2 (Fluka Analytical). Then, the SC was physically separated, rinsed in distilled water and left overnight to dry.

### 2.2 Solar Irradiation

SC samples were irradiated by Xenon Lamp in Q-Sun Xenon Test Chamber 3100. It reproduces the damage caused by full-spectrum sunlight (UV and visible light). The samples were exposed with irradiance 0.55±1 W/m$^2$ at 45±1 °C and 40±1 % of relative humidity. During the exposition, the SC samples were placed on a customized support as showed in **Figure 1.** This platform was designed to preserve the SC during the testing and prevent the SC from sticking.


**Figure 1.** Illustration of the support used to place the SC during the irradiation in the Q-Sun Xenon Test Chamber.

### 2.3 FTIR Spectroscopy measurement of conformational order in skin lipids

The spectra were obtained using a Fourier Transform IR (FTIR) spectrometer (Thermo Scientific Nicolet 6700) equipped with a temperature-controlled transmission cell. The accessory is able to create a gradual heating of the isolated SC samples for FTIR analysis. The whole instrument was constantly purged with nitrogen in order to remove water vapor. In all experiments, the FTIR spectra were acquired at 2 to 3 C° intervals from 5 °C to 95 °C. All the spectra were collected with a spectral resolution of 2 cm$^{-1}$ and they represented an average of 64 interferograms. During data collection the temperature of the samples remained constant within +/-1 °C. Each experiment was run in triplicated.

### 2.4 FTIR Imaging Spectroscopy measurement of stratum corneum after solar UV exposure

ATR-FTIR images were recorded with a Spotlight 400 System (Perkin Elmer Instruments, Shelton, Conn., USA), consisting of a FTIR spectrometer with a mercury-cadmium-telluride (MCT) focal plane array detector placed at the image focal plane of an IR microscope. ATR-FTIR images were collected in reflective mode at a spectral

resolution of 4 $cm^{-1}$ in the mid-infrared (MIR) region between 4000 and 850 $cm^{-1}$ with a spatial resolution of 6.25 x 6.25 µm at room temperature (24 °C). The ATR imaging accessory used a germanium crystal placed directly in contact with the skin samples. The FTIR Imaging System recorded hyperspectral images which provide maps showing the co-localization of specific molecular components or spectroscopic parameters. These images were generated with false colors where the red represents highest values and the blue the lowest values for each parameter investigated.

All the data presented in this work were processed (baseline correction, second derivate spectra, peak position, generation of spectroscopic parameters) using GRAMS/AI (Thermo Fisher Scientific) or ISys software from Spectral Dimensions (Olney, MD).

## 3 Result and discussion

### 3.1 Thermotropic studies: $CH_2$ asymmetric and symmetric stretching peak

In the FTIR spectrum recorded on the stratum corneum, the vibration of $CH_2$ group could be used to evaluate the lipid hydrocarbon chain-melting phase transitions. The $CH_2$ asymmetric stretching peak around 2920 $cm^{-1}$ and $CH_2$ symmetric stretching peak around 2850 $cm^{-1}$ are the most widely used (Lewis R.N., 2007). They are sensitive indicators for the chain conformational order in the lipid chains and the mobility associated with SC lipids domains; they can also provide information on the strength of intermolecular hydrogen bonding and the strength of bonds between lipid head groups (Naik A., 1995; Nair V.B., 2003). Changes in the intensity or the peak position of these two bands reflect a modification in hydrocarbon chain conformational disorder and mobility. The $CH_2$ stretching mode (symmetric and asymmetric) frequencies were determined to obtain information about the conformational order and the lateral packing of lipids in the SC.

In **Figure 2** by studying the peak position of $CH_2$ asymmetric and symmetric as a function of temperature, we can observe a phase transition (Tm) in the SC lipids from a high density ordered to a low-density disordered conformation. The peak position of $CH_2$ asymmetric and symmetric in isolated pig skin SC was plotted over the range 5-95 °C.

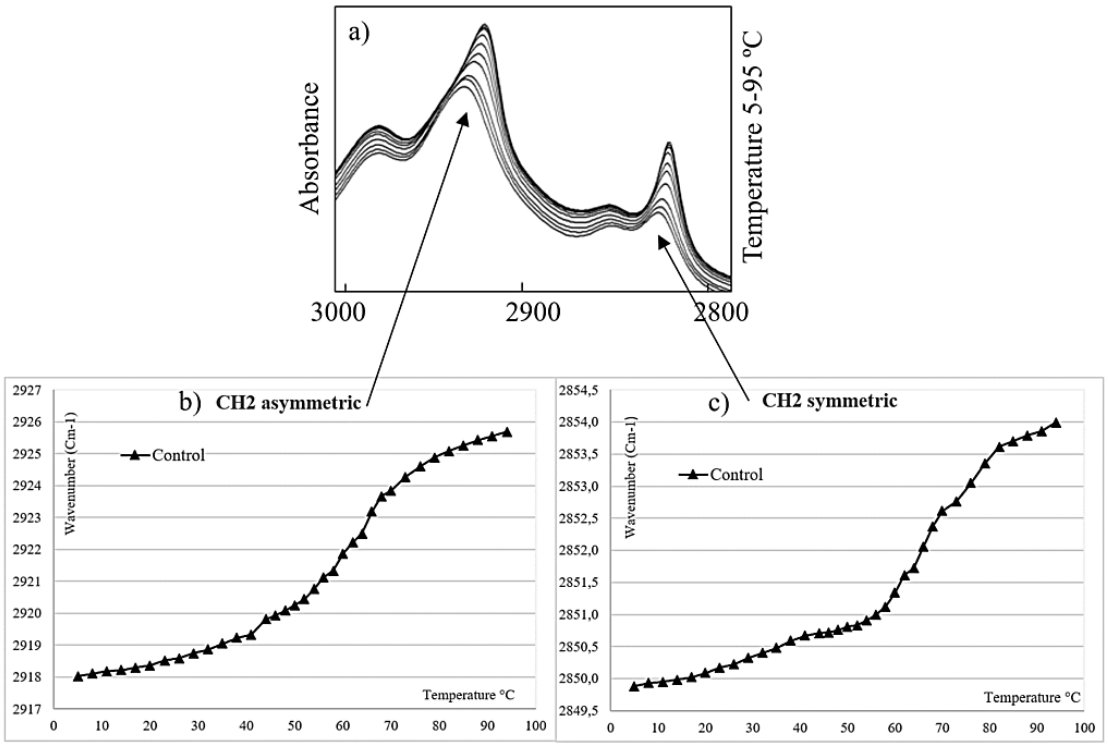


**Figure 2.** a) IR spectra of $CH_2$ stretching modes in a temperature range of 5 and 95 °C. Temperature-induced changes in the b) $CH_2$ asymmetric and c) $CH_2$ symmetric stretching mode displaying transitions of lipid chain conformational order and packing in isolated SC.

The $CH_2$ asymmetric vibrations around 2918 $cm^{-1}$ and the $CH_2$ symmetric around 2850 $cm^{-1}$ indicate highly ordered hydrocarbon chains. The SC intercellular lipids are considered in a rigid crystalline order with an orthorhombic lateral chain packing structure below 20 °C. Upon heating, a vibrational shift toward higher wavenumber was observed (Fig. 2 b, 2c). Two transitions can be identified; the first is observed between 30 °C and 50 °C, a small frequency increase of ~0.5 $cm^{-1}$ ($CH_2$ symmetric) and suggests a solid–solid phase interconversion, the orthorhombic hydrocarbon chain packing change in to a hexagonal hydrocarbon chain packing. The second one present a stronger shift happening above 54 °C finishing above 90 °C. The hexagonal packing change to a liquid crystalline phase so it is indicative of a transition from ordered hexagonal packing to a liquid disordered phase (Golden G.M., 1986; Moore D.J., 1999). Transition temperatures were studied in previous publications, but they are slightly different because of the different sample composition. In several articles, lipid mixture models were mainly used in order to evaluate the SC lipids properties and understand the specific role of each species in this unique conformation (Gooris G.S., 2007; Uchiyama M., 2016).

### 3.2 Low UV exposure effects on SC Lipid

Thermotropic response of the $CH_2$ asymmetric and symmetric stretching frequency after 10 and 20 hours of solar UV exposure compared with untreated sample are shown in **Figure 3.**

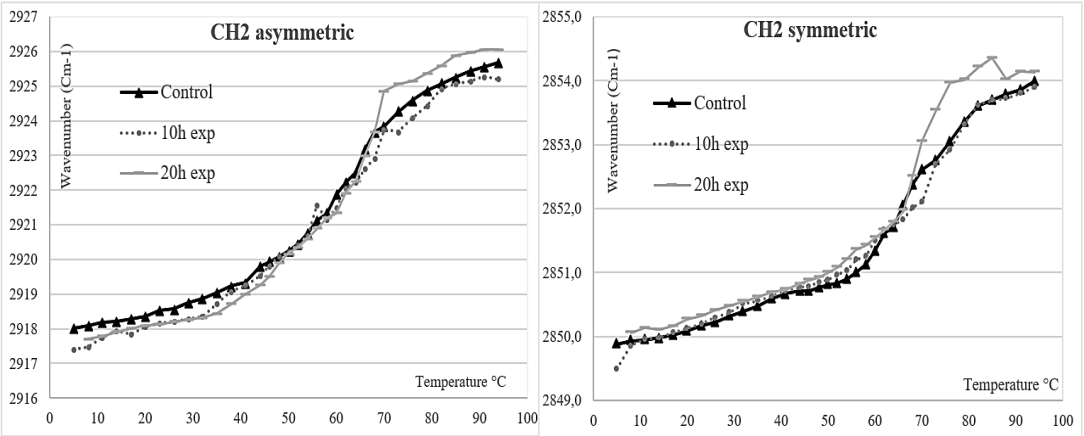


**Figure 3.** The thermotropic response of the $CH_2$ asymmetric and symmetric stretching mode of isolated porcine SC untreated, after 10h and 20h of solar UV exposure.

The untreated sample shows the two transitions. Firstly between 30 °C and 50 °C which corresponds to the solid–solid phase interconversion; here the weak transition in the porcine SC presents less orthorhombic lateral packing than human SC; the majority of the SC lipids are packed in hexagonal lattice (Caussin J., 2008). The second transition presents a stronger shift above 54 °C which represents the transition of ordered lipids into a disordered state. No significant changes were observed in the thermal profile of SC expose during 10 hours in the Q-sun chamber. In the samples exposed for 20 hours, the $CH_2$ asymmetric and symmetric stretching vibration showed the first transition appears at the same temperature as the untreated sample. For the second transition, the untreated and exposed samples with 20 hours exhibited similar wavenumbers until they reach ~68 °C. Above this temperature the $CH_2$ band position (asymmetric and symmetric) for the exposed sample (20 hours) shifts to higher wavenumber and the transition appears at lower temperature. These modifications indicate that the lipid disorder in isolated SC was stronger and appeared at lower temperature after 20 hours of solar UV exposure.

### 3.3 Intense UV exposure effects on SC Lipids

In **Figure 4** the thermotropic response of the $CH_2$ asymmetric and symmetric stretching mode from isolated SC exposed to 50 and 100 hours of solar UV exposure were compared with untreated sample**.**

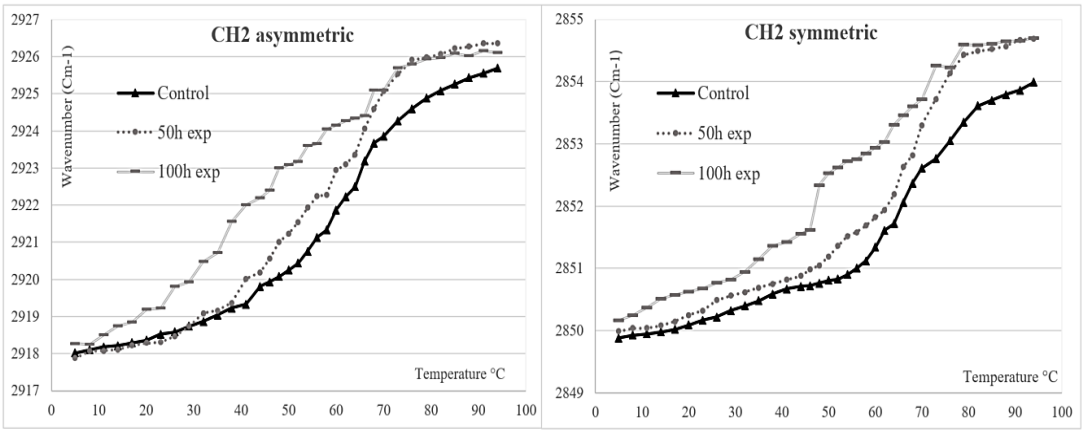


**Figure 4.** The thermotropic response of the $CH_2$ asymmetric and symmetric stretching mode of isolated porcine SC untreated and after 50h and 100h of solar UV exposure.

The untreated sample shows the same thermal transitions. The solid–solid phase interconversion around 38 °C and the transition from ordered lipids into a disordered state organization above 54 °C. In the isolated SC exposed for 50 hours, the $CH_2$ asymmetric stretching vibration showed a transition phase to a gel chain conformation at the same temperature as the untreated sample. In $CH_2$ asymmetric samples untreated and exposed for 50 hours exhibited similar wavenumbers until ~38 °C where after the exposed SC sample showed a shift to higher wavenumber. The transition from the gel chain conformation to a liquid-crystalline (liquid) conformation was observed at lower temperature and the final disorder status was higher than in the untreated sample. After 100 hours of solar exposure these modifications were amplified. In the isolated SC exposed for 100 hours the $CH_2$ asymmetric stretching mode do not show clear transitions. The disordered transition was observed at lower temperature compared with the untreated sample. In the isolated SC exposed for 50 hours the $CH_2$ symmetric stretching mode showed a similar solid-solid transition. The transition from the gel chain conformation to a liquid–crystalline conformation was observed at lower temperature. The final disorder status was higher than in the untreated sample. In the isolated SC exposed during 100 hours the $CH_2$ symmetric stretching mode present a higher wavenumbers position at ~5 °C indicating the sample presents less ordered

lipids at the beginning of the experiment. The transition from the gel chain conformation to a liquid-crystalline (liquid) conformation was observed at lower temperature. Both exposed SC samples exhibited a shift to higher wavenumber. These data reflect an increasing disorder of the SC lipids conformation in samples exposed to solar UV light. **Table 1** shows the peaks position of $CH_2$ asymmetric and symmetric stretching mode in isolated SC for all the sample at 58°C. Each value was extracted from average spectra calculated from three experiments.

The results clearly show that the solar UV exposure increased SC lipid disorganization by disturbing the SC lipid arrangement. This modification impacts directly the skin barrier function and its capability to protect us against environmental conditions.

### 3.4 Visualize supramolecular alterations of the lipids by FTIR Spectroscopy Imaging

The $CH_2$ asymmetric and symmetric stretching mode (~2920 and 2850$cm^{-1}$) and the $CH_3$ scissoring mode were used to study the apolar chain organization inside the SC by vibrational spectroscopy (Lewis R.N., 2007).

**Figure 5** shows FTIR images recorded on isolated SC before and after solar UV exposure. Specific spectral parameters were used to visualize the lipid order inside these samples. Fig. 5a and 5b show, respectively, FTIR spectra and second derivative of SC in the $CH_2$ stretching region (2800-2950 $cm^{-1}$) before and after exposure. In the untreated simple, the SC lipids are in orthorhombic/hexagonal order (2849.99 $cm^{-1}$/ 2918 $cm^{-1}$) but, after progressive irradiation, we observe a gradual shift toward higher wavenumber values (+1.5 units/ +7.94 units) where the SC lipids organization tends to lose their ordered organization.

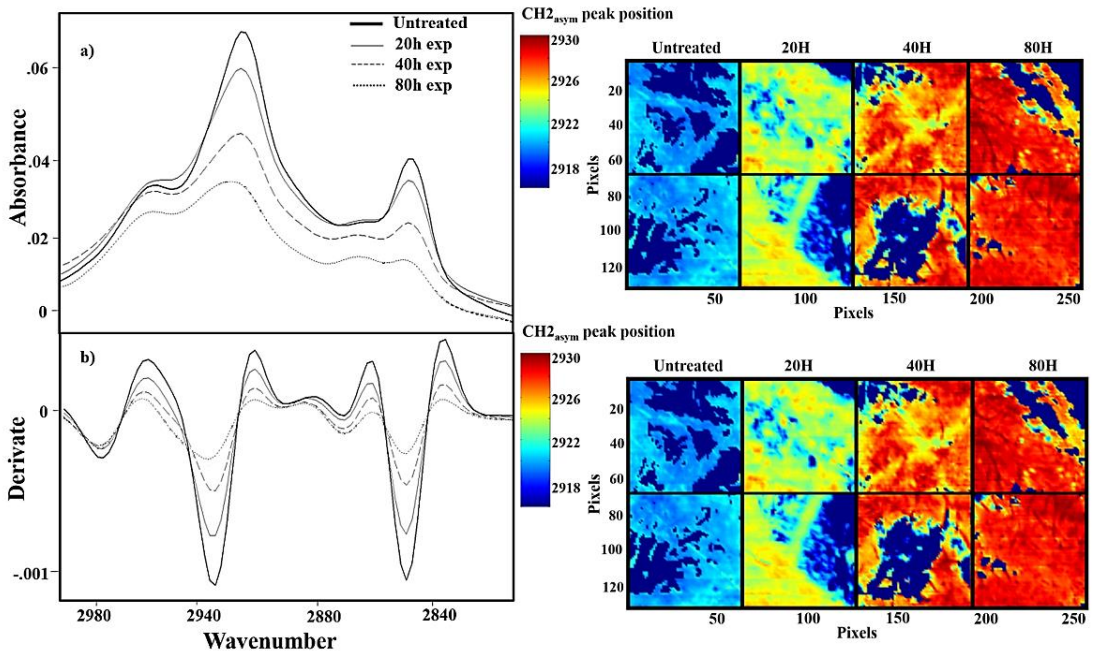


**Figure 5**. Average FTIR spectra (a) and second derivative spectra (b) in the CH stretching region (2800-2950 $cm^{-1}$) for untreated and exposed isolated SC; (c and d) FTIR images generated by calculating the $CH_2$ symmetric and asymmetric peak position for each SC samples.

The FTIR images of untreated and exposed SC samples were concatenated to produce the FTIR Images shown in **Figure 5 c** and **d**. The FTIR images were generated following the shift position of $CH_2$ asymmetric (Fig 5c) and symmetric (Fig 5d) modes. As expected the peak position of the $CH_2$ symmetric and asymmetric is shifted to higher frequencies (red color) with an exposure-dependent behavior.

The higher frequencies observed for exposed SC samples can be associated with a loss of ordered lipid organization. This is finding agrees with studies in the literature (Jiang S.J., 2006; Merle C., 2009; Merle C., 2010). Merle C. et al. in 2010 (Merle C., 2010), where a significant increase towards higher frequency in $CH_2$ symmetric mode after exposing cutaneous lipids (CER IIIa and IIIb) with UV light. By APCI mass spectra new molecules and new arrangements were found for both ceramides after irradiation. The irradiation of both CER IIIa and IIIb leads to the formation of oxidative entities, the formation of epoxide was observed for both molecules while the formation of hydroperoxide occurs in CER IIIa. The new oxygenated molecules increase the space area between the polar headgroup and then indirectly decreases the carbon chain packaging. By increasing the UV exposure, the SC ceramides are losing their organization. The reduction of $CH_2$ symmetric and asymmetric peak intensities and the broadening of these peaks (Fig. 5) can be used associated with the lipids losing

organization in the stratum corneum related to environmental conditions (UV, pollution, etc.).

**Figure 6** shows the general lipid content inside the SC before and after solar UV exposure. FTIR images were generated by calculating the $CH_2$ symmetric and $CH_2$ asymmetric to Amide I area peak ratio. The FTIR Images use false scale color where the red represents the highest lipid content and blue lowest lipid content.

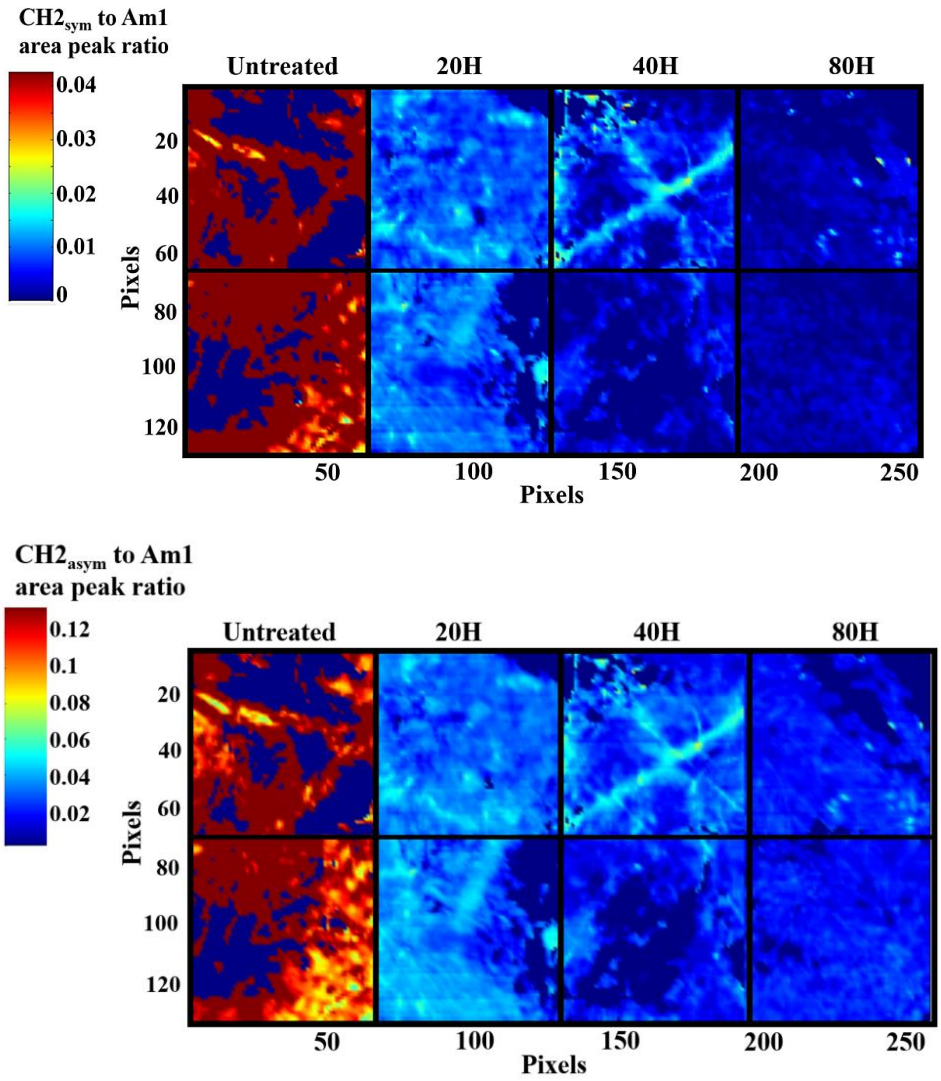


**Figure 6.** FTIR images were generated by calculating the peak area ratio between the $CH_2$ symmetric and $CH_2$ asymmetric peaks and the Amide I band for each sample.

The general content of lipids inside the SC decreased significantly after solar UV exposure. After a short exposure (20 hours) a significant decrease was observed. The lipid content decrease proceeds with an exposure-dependent behavior.

The instability of SC lipids under UV irradiation and the degradation of specific lipids were already investigated with the study of carbon chain of long fatty moiety

(Merle C., 2008). In our study, the degradation of the fatty acid hydrocarbon chain was investigated by following the modification in the intensity ratio between $CH_3$ asymmetric to $CH_2$ asymmetric mode. **Figure 7** shows FTIR spectra and second derivative spectra for untreated and exposed samples in the CH stretching region (3000-2800 $cm^{-1}$). The $CH_3$ asymmetric mode is not affected by the solar UV exposure while the $CH_2$ asymmetric mode is strongly affected by the exposure. In Fig. 7 (c) FTIR image was generated by calculating the intensity peak ration between $CH_3$ asymmetric band and the $CH_2$ asymmetric band. Clearly the solar UV exposure strongly impacts the lipid content. The breaking of the fatty acid moiety can be assessed and visualized by spectroscopy imaging. The main degradations appeared in the first 20 hours of exposure.

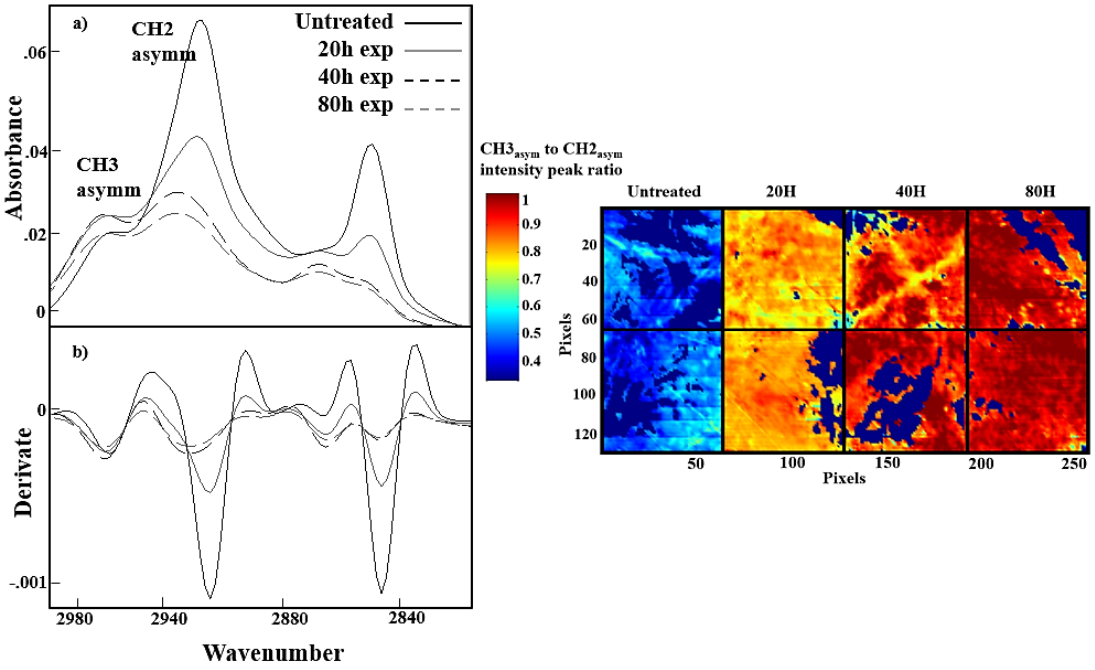


**Figure 7.** Average FTIR spectra (a) and second derivative spectra (b) in the $CH_2$ and $CH_3$ region 3000-2800 $cm^{-1}$. The FTIR image (c) was generated by calculating the intensity peak ratio between $CH_3$ asymmetric to $CH_2$ asymmetric for each sample.

## 4 Conclusion

In this work, the effects of physical perturbations (solar UV irradiation) on the SC lipids organization have been studied. The results show that solar exposure is responsible for a disorganization of the SC lipids, mainly after short-term exposure.

The radiation of the stratum corneum by UV resulted in the shift of the $CH_2$ asymmetric and symmetric peak position to higher wavenumber following an

exposure-dependent modification. These translations in the stretching frequencies are related to an increased freedom of SC lipids hydrocarbon chains, producing an increase of fluidity in the skin barrier. Moreover, a modification of the supramolecular order of the lipids was observed over time after the irradiation, showing that the SC lipids are losing their organization/arrangement and justifying the presence of broader peaks. Even the content of SC lipids decreased significantly already after 20 hours of exposure, and the decrease proceed with an exposure-dependent behavior.

Together these modifications of the SC lipids could dramatically alter the cutaneous permeability barrier and enhance percutaneous absorption. The abnormal stratum corneum lipid profiles can also be associated with common skin diseases (e.g. actinic dermatosis, psoriasis) presenting faulty permeability barrier function.

Moreover, FTIR spectroscopy and IR imaging micro-spectroscopy were found to be suitable tools for in vitro studies to assess and visualize the lipid modification related to solar UV exposure. The same experiments could be performed to assess the impact of different environment stresses not just UV but also pollution (Ozone, PM etc.) on the skin barrier function. As the vibrational spectroscopy can now be applied in-vivo (REMSPEC or in-vivo system for confocal Raman spectroscopy), these methods could be used to assess the performance of skin product or drug delivery to prevent environmental damages and restore the initial lipid composition and organization.

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# Chapter III

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**Sun-Protection Behaviors: Sunscreen**

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**Sun-Protection Behaviors: Sunscreen**

**Abstract**

Avoiding extended exposure to direct sunlight and topical application of sunscreen when exposed, protects the skin from sunburn, photoaging and potential hazardous UV damages such as melanoma and non-melanoma skin cancer. Despite decades of human use with health benefits closely related, sunscreen use and efficacy collect dissenting opinion. Sunscreen thickness, application and the sun protection factor are key elements for the sunscreen activity and they should undergo to specific conditions to obtain the claimed activity and safety. Erroneous patterns of use can happen to make inaccurate expectations. In the past decades, it has been suggesting that sunscreen has little long-term benefits but, in this paper, the "apparent long-term inefficacy" was evaluated and so the sun protection behaviors associated. It is possilbe there is a gap between the ideal sunscreen employment and consumer habits in real-life condition. Indeed, public health education campaigns have a key part. Educational programs could assess knowledge on sun exposure, with focus on sun exposure behaviors. In this way, it should grow the awareness of the damages we are going through and the way we can protect our self, making an informed decision.

**Keywords:** melanoma, sun-protection behaviors, sunscreen, UV-damages.

## 1 Introduction

UV radiation is a hazardous risk for the human health in our natural environment. In relation to the wavelength the UV light can be classified as UVA, UVB and UVC. The UVC light does not reach the earth, because it is efficiently absorbed by the ozone layer, whereas UVB and UVA radiation do reach the earth surface. The skin is continually exposed to the UV radiation indeed is well-adapted to condition of UV stress. In response to direct sunlight exposure the skin: increases the upper dead cell layers (stratum corneum), in order to reflect and refract the radiation; increases the production of melanin, which it is able to absorb the UV radiation. But, when the skin receive an excess of UV exposure, interaction with skin's cellular DNA can happen producing direct or indirect damages that if not repaired are involved in mutagenic events that can lead to skin cancer (Setlow R.B., 1966; Brash D.E., 1991; Halliday G.M., 2014; Holick M.F., 2016). The incidence rate of skin cancer is closely related to environmental factors but also to epidemiology and etiology factors. Ozone depletion, estimated ambient solar UV, weather conditions, latitude, altitude, ethnic origin, relationship to personal exposure to the sun, personal sun protection, gene mutation as CDKN2A gene etc. are all involved in the development of skin cancer. Ozone layer is a region of the Earth's stratosphere that it is able to absorb some of sun UVR radiations, its depletion lead to region overexposed to UVR (De Fabo E.C., 2005). Ozone depletion is most evident in polar regions. Studies have related close correlations between an increase of the skin cancer incidence in Caucasians living near those regions (Schaart F.M., 1993). Data from the California Cancer Registry (United States) were analyzed and the melanoma incidence rate was calculated for Hispanic, Asian and Afro American and compared with non-Hispanic. Average, annual, age-adjusted incidence rates per 100,000 population were: 28.5 for non-Hispanic, 5.8 for Hispanic, 1.7 for Asian and 1.7 for Afro American. In 2009 a pooled analysis of 5700 cases and 7216 controls showed how the patters of sun exposure, when the exposure is intentional, are significant indicator of melanoma at all the latitudes (Chang Y.M., 2009). Those studies strongly support that the skin cancer incidence depends on multifactor evidences. Therefore, the incidence is higher (i) in sun-sensitive rather than darker skinned, (ii) at low latitudes rather than high latitudes, (iii) after intentional exposure rather than occasional exposure, (iv) in sun exposed parts of the body rather than least exposed, (v) part of the world with ozone depletion etc. (Armstrong B.K., 1993; Armstrong B.K., 2001).

Clinical benefit of regular use of sunscreen for preventing non-melanoma and melanoma skin cancer has been demonstrated in recent years (Van Der Pols J.C., 2006; Ulrich C., 2008; Green A.C., 2011), subsequently, several investigators have confirmed the potential UV damages protective action (Olsen C.M., 2015; Ghiasvand R., 2016;). International health authorithies promote some recommendations in order to enjoy the beneficial effects of sunlight, as the production of vitamin D, and prevent the negative side: (i) avoiding sun exposure during the hottest hours, (ii) watch for the UV Index, (iii) use shade wisely (umbrella, protecting clothing), (iv) apply sunscreen SPF 15 or higher 30 min before UV exposure and thereafter every two hours (Gilbert E., 2013; Skin Cancer Foundation, 2017; WHO, 2017). The efficacy and safety of a sunscreen is defined by specific conditions as amount and frequency of susncreen applyed, film forming, thickness, houors of exposure and constumers intrinsic carateristics. Erroneous patterns of use can happen making inaccurate expectation.

Sunscreens are products combining several ingredients and actives to prevent the UV radiation harmful effects. UV filters are the actives ingredients in sunscreen which block or absorb UV radiation, making the sunscreen protective against skin photodamages (Palm M.D., 2007). There are 2 classes of UV filters: inorganic (or physical filters) and organic (chemical filters). The inorganic UV filters ($TiO_2$ and ZnO) are able to reflect or disperse UV radiations over the whole UVA/UVB range (290-400 nm), they have large application in the protection of sensitive skins, however, tends to be opaque on the skin and consequently it is barely suitable for cosmetic use. The organic filters absorb UV radiations into specific wavelength ranges, as a function of their chemical structure. They are most of the time used in combination because no active agent alone, used at levels currently allowed, provides high enough SPF (sun protection factor) protection or broad-spectrum absorption and, in the last years, it is well known their high potential of producing irritant reactions (Serpone N., 2007). Stay stable during the entire period of UV exposure, or at least during the 2 hours defined by the health authorities, just on the superficial part of the skin, in order to create a UV radiation protective film, are essential requirements for a safe and efficient sunscreen (Nash J.F., 2014; Skin Cancer Foundation, 2017; WHO, 2017). However, studies showed that some organic UV filters are photochemically unstable which leads to loss in efficacy increasing the risk of skin photodamages (Gonzenbach H., 1992; Schwack W., 1995; Afonso S., 2014). The UV exposure of photo-unstable sunscreen filters can lead to photochemical reactions that can compromise both their physical (color,

appearance) and chemical properties leading to undesirable reactions as the production of inactive products or high reactive molecules which can penetrate and react inside the skin (Damiani E., 2010). Unlike inorganic UV filters, several studies (Hayden C.G., 2005) have shown organic UV filters could penetrate the SC and potentially reach the dermis where they may be absorbed by systemic circulation. UV filters have been detected in human plasma, urine (Janjua N.R., 2008) and in human milk samples (Schlumpf M., 2010). The toxicity of sunscreens is poorly understood making the potential risks unknown. Increasingly, UV filters are being incorporated into day-to-day products such as moisturizers, creams, lotions and other skin care products therefore a noted skin penetration profile and toxicological profile are essential requirements. Sunscreen products are applied topically, effectiveness implies that sunscreen filters adhere to skin like a protective film, remain on the uppermost layers of the skin and assumes that the penetration through the skin is extremely limited (Jiang R., 1997; Lu Z., 1999).

There are no common rules concerning sunscreens; they are regulated and classified in very different ways around the word. European Union (EU), New Zealand, some of the Middle East/Arabic countries, Turkey and ASEAN countries, the sunscreens are considered as a cosmetic product. The current EU Regulation (EC) No 1223/2009 (Regulation (EC) No 1223/2009, 2009), replacing the previous Directive 76/768, aimed to harmonies the rules in the Community about cosmetic products with the intention to protect the human health. The UV filters are considerate as *"substances which are exclusively or mainly intended to protect the skin against certain UV radiation by absorbing, reflecting or scattering UV radiation"*, therefore, the protection of skin from photodamage is referred to as a cosmetic action. Also, Commission Recommendations (Commission Recommendation (2006/647/EC), 2006) defined the testing and labelling of sunscreens. In United State of America, the sunscreens are regulated by the Food and Drug Administration (FDA) and classified as "over the counter drugs" (OTC) which is a class where it is necessary to show that the active ingredients are both effective and safe and fulfill all the conditions that are stated in the final monograph. In Australia, mostly m, sunscreens are classified as therapeutic goods, otherwise, equivalent to medical products, they have to be listed in the Australian Register of Therapeutic Goods (ARTG) (Raj R.K., 2017). Differentiating in term of regulation and classification, each country has a list of authorized ingredients with their maximum allowable concentrations in final products.

Considering the growing attention to sun products as an essential barrier in the protection against the UV rays, international harmonization of these regulations would be useful (Osterwalder U., 2014).

## 2 Sun-protective behaviors and sunscreen application patterns

### 2.1 Sunscreen thickness and SPF

Sunscreens are made in a wide range of SPFs. SPF value is based on in-vivo testing measuring the amount of UV radiation exposure it takes to cause sunburn when using a sunscreen compared to how much UV exposure it takes to cause a sunburn when not using a sunscreen. This value provides information about protection against sunburn or erythema induced primely by UVB, so SPF values only indicate a sunscreen's UVB protection. In 2011, the US Food and Drug Administration (FDA), adopted in vitro critical wavelength (CW) measurements to assessing UVA or broad-spectrum protection. CW is defined as the wavelength at which 90% of the total area under the absorbance curve in the UV region. Specifically, the FDA has ruled that only products with CW ≥370 nm can be labeled as having "broad-spectrum" protection. In the nations regulated by the European Commission, all products must offer UVA protection that at least has to be a third as potent as the SPF (UVA PF/SPF ≥ 1:3) (Commission of the European Communities, 2006). Laboratory tests of 20 common US sunscreen (broad spectrum, SPF 15-100) underlined the differences in the FDA and European requirement for sunscreen labelled "broad spectrum". 19 of 20 sunscreen met the US require requirement of CW >370 nm, but just 11 of 20 sunscreen met the EU desired ratio of UVA PF/SPF > 1:3 (Wang S.Q., 2017).

Many standard methods to SPF and UVA testing were developed in USA, UK, Europe, Japan, Australia. First in 1994 (COLIPA, 1994), with validated in vivo SPF test method and then in 2007 (COLIPA, 2007), with validated in vitro UVA test method, COLIPA formed the basis for the current ISO24443 in vitro UVA test method (ISO 24443, 2012). Guidelines from the FDA (Food and Drug Administration, 2011) and the international Organization for Standardization (ISO 24444, 2010) agreed the amount of sunscreen applied for testing SPF should be 2 mg/cm$^2$. This data is based on studies showing the best reproducibility and the lowest variation of test results (Bimczok R., 2007) and the method employed to determine SPF is from calculated UV transmittance based on experimental film thickness and thickness distribution, and concentration and spectral properties of the UV filters. A good quantity of studies were

made in order to define the relationship between the sunscreen thickness and SPF (Faurschou A., 2007; Kim SM., 2010; Teramura T., 2012) but, until now, the link is not fully determinate. Even so, it is clear the necessity to have a minimum amount of sunscreen able to supply the protection against the UV radiations.

To achieve the labeled SPF the recommended amount of sunscreen should be applied but studies fully show that sometimes it does not correspond to the real amount applied by consumers. In the recent years, I. M. Heerfordt et al., made an observational study examined the trend from the 1990s to 2016 of sunscreen use. The amount of sunscreen applied during a sunny day was 0.48 mg/cm$^2$ in 1992 to 0.57 mg/cm$^2$ in 2016. An increase of sunscreen quantity used was observed from 90s to today, maybe for a deeper education about the negative side of UV exposure, but still the sunscreen applied is much lower than recommended quantity (Heerfordt I.M., 2017). Similar data can be observed in many publications (Neale R., 2002; Diaz A., 2012; Petersen B., 2013).

Several factors influenced sunscreen application quantity and thickness, some studies have shown how the type of the sunscreen application method may influence the amount of sunscreen used. R. Novick in 2015, studied the amount of sunscreen applied to skin by appling lotion, spry and stick sunscreen. Fifty-two volonteers applied sunscreen and the means for the application of spray, lotion, and stick sunscreens were 1.6, 1.1, and 0.35 mg/cm$^2$, respectively. Different amounts of sunscreen were calculated depending on the application method (Novick R., 2015). Spray application data have showed that ablout 25% of the spray exited the bottle is lost. The stick application resulted the most unsatisfying and the lotion was evaluated as the more compatible and practical application method but still the raccomanded sunscreen amount wasn't reach. Application method was shown to affect sunscreen amount. On this line A. Diaz et al., made a crossover trial investigating about children's sunscreen application thickness and the influence of the 3 sunscreen dispenser: pump, squeeze bottle, or roll-on. Significant more sunscreen was applied when using the pump (0.75 mg/cm$^2$) and the squeeze bottle (0.57 mg/cm$^2$) compared with the roll-on (0.22 mg/cm$^2$). The sunscreen dispenser type was shown as an effective factor for sunscreen amount used (Diaz A., 2012).

**2.2 Sunscreen application and re-application**

Appling the recommended quantity of sunscreen is not a guarantee for proper protection against UV radiation. The sunscreen application and the relative body coverage after application are premises for a full activated protection. Standardized UV photographs, evaluated by Image Analysis, were conducted before and after single whole-body product application to evaluate relative body coverage of fifty-two healthy volunteers following their usual sunscreen application routine to assess sunscreen usage habits. The front side was significantly more covered than the backside showing how the usual sunscreen application routine may never provide complete body coverage (Jovanovic Z., 2017). Sunscreen applied on skin surface needs to create a protective film in order to reflect or scatter the UV radiations, but it is easy to understand that areas of the body are hard-to-reach during sunscreen self-application. The possible relationship between lack of sunscreen application and melanoma distribution incidence was investigated by Poljšak et al. in 2016. The "missing area" after sunscreen application were detected on 25 volunteers in the age interval 19-35 years and data were compared in order to evaluate a possible correlation between the observed sunscreen lack and melanoma incidence. The upper part of the back was observed as the most difficult spot to reach during sunscreen application, carrying an inconsistent coverage and data based on literature review showed closed relationship between those missed areas (Poljšak B., 2016). The frequency of application and re-application became critical factors to reach for a full covered bady. The international authorities recommend the re-application of sunscreen every two hours, or after working, swimming, playing or exercising outdoors. I. M. Heerfordt et al. in 2017, determinate the amount of sunscreen used during a first and second sunscreen application and the relation between time spent on sunscreen application and the amount of sunscreen applied. In a laboraty study, volonteers applied a mean quantity of 0.71 mg/cm$^2$ during the first application and 1.27 mg/cm$^2$ after second application and sunscreen applied increased linearly and significantly with time spent on application (Heerfordt I.M., 2018). Under real-life conditions, an re-application could be well-accepted as practice to improve the protection rather than an incresing of suncreen amount for a single application time. In support of this steatment, authors have exposed themselves (Pruim B., 1999; Diffey B., 2001; De Villa D., 2010).

**3 Age-related changes in skin phisiology and topography**

Human skin development starts in utero, a complete finished SC is not available before 34 weeks and the barrier mutation is keep going on in relation to the gestational age (Harpin V.A., 1983; Evans N.J., 1986). At birth, SC and epidermal thickness are respectively 30% and 20% thinner, the corneocytes and keratinocytes are smaller, collagen fibers are less dense, less total lipids and less sebaceous lipids, lower concentration of melanin probably related to the rapid cell turnover happening during the first months of life, the concentration of NMF is lower and the functioning of acid mantle is missing (Li L., 2006; Stamatas G.N., 2010; Stamatas G.N., 2011). The reduced level of maturation/concentration of infant skin structure surface could compete to the not fully maturate skin barrier function, contributing to a height sensitivity to the harmful substances, environmental factors and loss of water. In the basal layer, the stem cells are more exposed to UV radiation promoting the initiation step of non-melanocytic skin, the dermal papillae is more exposed, without the acid mantle the transepidermal water loss is not regulated, condition that could lead to dehydration (Hoath S.B., 2004; Volkmer B., 2011). Even if the sunscreen is universally recommended, there are controversial recommendation about the sun protection for infant. The US Food and Drug Administration with the Skin Cancer Foundation recommend keeping babies of the direct sun exposure for the first six months and even then, apply sunscreen on the smallest exposed skin areas possible if appropriate clothing and shade are not available (American Academy of Pediatrics, 1999). Because of the specific anatomic structure of infant's skin, presenting thin stratum corneum, sunscreen chemicals might penetrate deeper, exposing the newborn to possible allergies, dermatitis and unknowns' risks for the health (Gilaberte Y., 2014).

The dose, the film-forming properties and the thickness are fundamental characteristics to reach the sunscreen efficacy. In fact, film formation/thickness is likely the key reason that product application is one of the primary sources of variability in SPF testing. Indeed, one needs to consider the topography of the skin. Macroscopically, the surface of the skin is made up of hills and valleys. A thin layer applied over such topography may result in uneven coverage where "valleys" are filled/covered, but "peaks" are not. The studies related to the skin aging, disease, effect sun exposure, dermatological and cosmetic treatments are several but just few studies implicated skin roughness in the appropriate sunscreen application. In recent years,

V. Korn et al. highlighted the variation of skin surface roughness related to the site-age combinations. Areas as wrinkles and furrows may represent potentially high concentrated areas of the skin surface and other areas potentially uncovered. The exanimated study shows how for aged skin, with increased roughness, a large amount of sunscreen may be recommended (Korn K., 2016).

Preadolescents and adolescents are also thorny categories: firstly, because the sunscreen application depends on the parents and the self-application generally is not incisive; secondly, as we mentioned before, they present thinner skin and lower melanin concentration presenting as subjects more vulnerable to the exposure. The skin thickness increases with the age, initially the skin is very thin showing a maximum value just around 30-50 years, followed to a significative decree with the age (Dąbrowska A.K., 2018). A decline in the pigment of the skin is observed when passing through adolescence. During the puberty there is a fall in the melanocyte stimulating hormones, exposing the young adults to greater health risks during the UV exposure (Kalla A.K., 1973). It is possible that those classes request more than the recommended dose of to guarantee an effective UV induced damages protection.

**4 Sunscreen vs sun exposure (sunscreen abuse)**

Until the 1930s, UV radiation had been promoted as for their beneficial effects in Vitamin D metabolism, so the sunlamps and sunbathing became extremely popular and, historically, the tanning was fashionable. Only after the publication of several studies about the closed link between the UV lamp/sunlight and skin tumors and the publication of guideline for the approval of UV lamps and the appropriate therapeutic uses (es. Phototerapy) made by the American Medical Association, there was significative changes in the consideration of UV rays connected to a massive growth of sunscreen chemicals industry (Autier P., 2009). On the market few UV filters were available since the century's start, but they were not of intensive interest until the explosion in popularity.

Controlled trial from 1987 to 1990 made by Mark F., at first and other researchers later, through laboratory experiments and in-real-life condition studies have showed the sunscreen ability to reduce UV induced damages such as solar keratoses, squamous cell carcinoma and melanoma (Naylor M.F., 1995; Ulrich C., 2009; Green A.C., 2011). However, few studies found still a connection between the use of sunscreen and non-significative long-term benefits from UV induced damages.

A systematic review made by Autier (Autier P., 2007), evaluated the evidence linking sunscreen use versus sun exposure time, bringing data to support the idea that sunscreen use leads to longer duration of sun exposure. In a double blind randomized trial 58 volunteers (18-24 years old) were tested with sunscreen SPF 10 and 30 recording the daily sunbathing duration. Different number of sunbathing hours, daily sunbathing duration, and daily UVB exposure were observed in two groups. The highest number of hours in sunbathing activities were spent in sunscreen SPF 30 group showing that sunscreens used tends to increase the duration of exposures (Autier P., 2000). In all those studies, it was observed the tendency to a longer exposure when protected with high SPF sunscreen just when the sun exposure was intentional, with the desire to acquire a tan. What we observe is: i) two different type of sun exposure patterns came up: non-intentional sun exposure (NISE) and intentional sun exposure (ISE); ii) the SPF concept is not clear. The non-intentional sun exposure type doesn't have an interest to acquire a tan, the exposure is related to the daily life activities, the intentional sun exposure type stays under the sun a big number of hours per day with uncover skin with the porpoise to acquire tan. Information on sunscreen should reflect the current knowledge of potential health hazards associated during intentional sun exposure. The information on the container about SPF (Sun Protection Factor) should be clarify, SPF is a measure of how well a sunscreen will protect skin from UVB rays and not it is not meant to help you determine duration of exposure.

It should be avoided the conception of "safe tanning" not just for sunscreen and outdoor tanning but also for indoor tanning. In the late 1970s, sunlamps emitted a spectrum of radiation from UVC to infrared, in the early 1980s was suggest that UVA radiations were safer. In recent years several studies show the connection between sunbed use and skin cancer risk (Boniol M., 2012), implementing the regulation about the use and the claims related (World Health Organization, 2017; Wright C.Y., 2017). Labelling and advertising should bear message on the UV induced damages associated with intentional exposure, without misunderstanding that could lead to a false sense of security and an idea of more protection allowing a longer UV exposure.

If we look deeper, the studies should considerer the typical sunscreen users. International authorities recommend sun avoiding and sunscreen application for all the age group and especially for those high-risk groups with light skin color, frequent sunburn after unprotected exposure. Skin pigmentation is related to the melanin amount into the skin. Melanin plays a key role mediating the UV radiation, when

uncovered skin is under UV light the first defense against UV is the production of melanin that is able to dissipate UV radiations. When UV radiations escaping to melanin absorption, UV rays are absorbed by molecules called chromophores, which absorb the light energy, DNA is the main epidermal chromophore. This event can induce irreversible DNA damages (Setlow R.B., 1966). In a cluster prevalence survey, total nevus counts were associated with heavy facial freckling, time spent outdoors on weekends in summer, and Caucasian ethnicity showing how the phenotype can play a role. Darker skin and ability to tan was associated with low nervous counts showing how our pigmentation can be a good defense against the UV damages (Whiteman D.C., 2005). Low pigmentation could represent a lower innate defense. Therefore, the sunscreen users generally are subject more sun sensitive, phenotype I/II/III, with low pigmentation and easy sunburns. Then the increased risk of melanoma in sunscreen user could be just the reflection of their inherently bigger risk of melanoma (Stanton W.R., 2004).

**5 Barrier functions of compromised skin**

The skin is an excellent and efficient barrier against the environment when it is intact, however, its barrier functions can be affected by numerous small impacts during every-day life actions and/or pathology. Personal care and pharmaceutical products dermal absorption is determinate, almost exclusively, using in vitro techniques following the in vitro OECD 428 testing guideline using normal intact barrier properties (OECD, 2004), current regulations use industry-specific protocols for dermal penetration, each of these guideline documents assume testing with healthily intact human skin. However, intact human skin percutaneous absorption may be deeply different from compromised skin. Limited previously published studies, underlined this difference. Diseases as psoriasis, atopic dermatitis, eczema, mycosis, keratinization disorders etc. are related to a compromised barrier function. There are insufficient experimental data available to deeply understand the chemical penetration behavior in damaged skin, a more complete physiochemical spectrum is needed but what it is clear is additional experiment should carry out and more representative safety factor may be established (Chiang A., 2012; Davies D.J., 2017). A compromised skin condition is showed also after mechanical interaction. A potential stress factor is a commune cosmetic procedure widely used in our society: removal of body hair. The skin can be freed from hair by methods of depilation (dry and wet shaving, depilatory cream) as well as

methods of epilation (electric epilation, waxing). Studies showed, how the skin barrier function can be alternated and destructed by removal of body hair (Bhaktaviziam C., 1963; Marti V.P., 2003; Jung S., 2016) the visible irritation that comes right after is related to the skin barrier damages (Holbrook K.A., 1974). In the past years, some UV filters have been questioned for issues connected to percutaneous permeation of sunscreen chemicals into the circulatory system (Janjua N.R., 2008; Schlumpf M., 2010). These observations have encouraged researches to study more percutaneous absorption of chemicals in topical products applied on not-intact skin (Krishnaiah Y.S., 2004; Senzui M., 2010; Lin L.L., 2011; Lucová M., 2013). Furthermore, the literature showed a clear need for further investigations regarding the extent of skin barrier damage that may be caused by hair removal and relative consequences with larger clinical study.

**6 Impact of sunscreen on skin microbiota**

Human are born from a sterile environmental, but after birth, they face the external environment a quickly become colonized by a diverse milieu of microorganisms (Hrncir T., 2008). The microbial taxa associated with humans is called "microbiota" and the entire collection of all the genomic elements of a specific microbiota is called "microbiome" (Turnbaugh P.J., 2007; Peterson J., 2009). Human have two genomes, one inherited from our parents and the other acquired during lifetime (Dominguez-Bello M.G., 2010), the microbiome is extremely dynamic and can be influenced by several factors, among which, age, diet, hormonal cycles, travel, therapies, and illness. Initial reports have focused on the gastrointestinal microbiota (Gibson G.R., 2004; Penders J., 2006; Palmer C., 2007) and in the past years it is increasing the attention also on skin microbiota. The SC represents the first line of protection against environment assaults, supports the innate antioxidant system, production of antimicrobial peptides, activation of the host innates immune system. Grace et al. in 2009 studied the relative abundance of the most copious bacterial relative to three microenvironment types: i) sebaceous, Propionibacteria and Staphylococci species; (ii) moist, Corynebacteria and Staphylococci species; (iii) dry, mixed population of bacteria with a greater prevalence of β-Proteobacteria and Flavobacteriale (Grice E.A., 2009). Knong et al. in 2012 showed how a temporal shift in the skin microbiota could be associated with disease flares. This study looks down at the genes level showing a dramatic increase of Staphylococci species in the skin of patient with atopic dermatosis

(Kong H.H., 2012). Microbiome showed a significative role in the psoriasis development too, Langan in 2017 highlighted the importance to investigate the microbiome in psoriasis could be an efficient way to know more about disease pathogenesis and treatment selection (Langan E.A., 2018). Some cosmetic products have been proved to impact the progression of the cutaneous bacteria (Taylor D., 2003; Mijouin L., 2013) but just few detailed analyses were made about the impact chemicals used in sunscreen on the human skin microflora and its possible implication with skin disease. Rowenczyk in 2017, studied the impact of coated TiO2-nanoparticles on Staphylococcus aureus and Pseudomonas specie putting emphasis on further works are needed to fully understand the different phenomena involved (Rowenczyk L., 2017). These studies are of interest not just to understand the possible effects of sunscreen has on the human skin microflora, but also, to evaluate the possible contamination of the product coming from the human skin microbiota. Another study made by Baek in 2018, showed a significant antibacterial growth inhibition in silica-coated TiO2-nanoparticles compared to the uncoated one (Baek S., 2018). Zinc Oxide (ZnO) is used as UV filter for its good optical properties but, studies show also its good antimicrobial activity of pharmaceutical and cosmetic formulation (Pasquet J., 2014; Pasquet J., 2015). There is a delicate balance between host cells and bacteria population, disturbing this balance with a drastic reduction of skin bacterial growth could result in an unbalanced microbial state resulting in inflammatory skin diseases.

**7 Sunscreen and vitamin D deficiency**

Vitamin D3 is made in the skin from 7-dehydrocholesterol under the influence of UV light with smaller contributions from dietary sources. An excessive decrees of UV light exposure could decrease drastically the vitamin D level. Severe vitamin D deficiency is associated with cardiomyopathy, insulin resistance, tuberculosis, osteoporosis and fractures (Chowdhury R., 2014). Updated reviews in literature show controversies about the role of Vitamin D production and sunscreen such as protection against UV radiations. Recent randomized control trial made by Faurschou et al., 37 volunteers were randomized to different thickness layers of sunscreen SPF 8 (0.5 mg/cm$^2$, 1 mg/ cm$^2$, 1.5 mg/ cm$^2$ or 2 mg/ cm$^2$) and irradiated with UVB dose 20 min after sunscreen supplication, repeated four times. Blood level of pre-vitamin D were collected. The study showed vitamin D production have had a significative increase when thinner sunscreen layers are applied (Faurschou A., 2012) and lower levels of vitamin D were

showed with recommended quantity of sunscreen. Sunscreen applied at 2 gm/cm$^2$ may reduce vitamin D synthesis however, sun protective methods can result inadequate to obtain suitable Vitamin D levels. E.

Linos et al. and N. Jayaratne et al. studied the photo-protective behaviors such as sun avoidance, stay under the shade or clothing would be at risk for vitamin D deficiency (Linos E., 2011; Jayaratne N., 2012). New strategies are now growing able to maximize vitamin D production and maintaining its sun protection for reducing sun burn and erythema. D. Kockott et al. in 2016 describes a calculation method for optimizing a sunscreen supporting both the production of pre-vitamin D3 and the protection against UV radiation. The new developed sunscreen SPF 15 was compared to a commercial available sunscreens SPF 15, in vitro studies showed 50% more pre-vitamin D compared to the commercial one (Kockott D., 2016).

**8 DNA UV-induced damages**

DNA has been considered as the main target of UVR by direct (cross linking of pyrimidine bases, thymine and cytosine) and indirect (production of very reactive specs ex. ROS) DNA damages (Setlow R.B. 1966). Helix-distorting photoproducts, as well as oxidative damage to DNA bases, are among the key DNA lesions associated with photoaging and tumorigenesis. Recent investigations have shown how some elements such as antioxidant, xenogeneic DNA repair enzymes or DNA repair liposome reduce the UV-induced DNA damages (Chen L., 2012; Stingele J., 2015). M. Carducci et al. and E. Emanuele et al. have presented two comparative studies of sunscreen alone and sunscreen plus antioxidant or DNA repair enzymes. In both studies the improved sunscreen showed better performances reducing the UV-induced DNA damages (Emanuele E., 2014; Carducci M., 2015). Direct absorption of solar UV photons by DNA may induce the production of highly genotoxic dipyrimidine photoproducts, named cis–syn cyclobutane pyrimidine dimers (CPDs) as well as oxidative damage to DNA bases, including the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8OHdG) (Rochette P.J., 2003; Valavanidis A., 2009) and reactive oxygen species may promote the production of protein carbonylation (PC) usually refers to a process that forms reactive ketones or aldehyde. In the first investigation by Carducci et al, 6-month randomized clinical study with 28 patients was conducted. Patients were assigned to topically application of traditional sunscreen SPF 40 or sunscreen plus DNA repair enzymes (SPF 50 plus 1% photolyase form Anacystis

nidulans and 1% endonuclease form Micrococcus Iuteus) with a density of 2 mg/cm$^2$. Hyper-keratosis, field cancerization by fluorescence, levels of CPDs by biopsies were measured. The study showed a positive effect on hyperkeratosis, a reduction of field cancerization and CPDs was observed for the sunscreen plus DNA repair enzyme group. The second one by E. Enzo, a complex topical product TPF50 (consisting in: physical sunscreen SPF50, liposome-encapsulated DNA repair enzymes complex and antioxidant complex) was compared vs DNA repair enzyme and antioxidant topical products, a standard application thickness of 2 mg/cm$^2$ was used for each product on 60 volunteers. PC, CPDs and 8OHdG levels were detected form skin biopsy specimens. This work showed that the combination of an sunscreen, an AO complex and a DNA repair complex in the TPF50 product was able to achieve a multiplicative effect in terms of reduction of CPD and PC, and an additive for 8OHdG showing a more effective than existing products for reducing UVR-induced DNA and protein damages.

**9 Skin cancer detection and prevention: educational programs**

A key aspect in preventing the skin cancer is primary care skin cancer detection efforts that in collaboration with dermatologists many detection opportunities can be created. Skin cancer often starts with changes in the skin color, they usually are a mix of color (brown, light brown, pick, light red etc.), some of them are able to spread quickly but those signals are not enough to detect a skin cancer. The identification process is carry out in various stage, in relation to the technique used. Initially, an image of the interested area is acquired with a digital camera and the proper interpretation of these images can lead to increased clinical diagnostic accuracy. Mainly, the methods adopted is the standard computer-aided diagnosis (CAD) made up of 5 steps: i) imaging acquisition, ii) processing, iii) segmentation, iv) feature extraction, v) detection, vi) post-processing. After image acquisition, there is the processing to improve the image quality, then the segmentation witch it is able to separate or making a group of different parts. The segmented image is given to the feature extraction block which consists of lesion region analysis for its geometrical features and ABCD features (Asymmetry, Border, Color, Dermoscopic structures). The extracted features are classified as skin lesion cancerous or normal by comparing its feature parameters with the predefined thresholds (Rao N.D., 2016). Skin cancer detection and imaging digital processing are very challenging but, in the years, several techniques are

developed in relation to the spot and condition of the skin cancer. Ganster in 2001(Ganster H., 2001) developed system for the computerized analysis of images obtained by several segmentation algorithm with fusion technique, Przystalski in 2010 (Przystalski K., 2010) made a system for a fast image processing and feature extraction/classification using an semantic artificial neutral network (database of dermoscopic images), Deshpande in 2016 (Deshpande A. S., 2016) used filter for removing noise and Fuzzy C-Means (FCM) during the segmentation, Grey Level Co-Occurrence Matrix (GLCM) for the textural feature extraction and Support Vector Machine (SVM) for classification.

The early detection and the prevention strategies of skin cancer are the most effective goals to reach in our society. The American Cancer Society promoted the "Slip! Slop! Slap! And Wrap" slogan, to emphasize the key steps: to slip on a shirt, to slop on sunscreen, to slap on a hat and to wrap on sunglasses. However, even with sufficient prevention methods, a lack of education and promotion of a practice will not lead to favorable results. International organizations recommend the use of sunscreen as one of the most efficient prevention action against UV radiation damages. Adequate sunscreen application habits are essential to providing adapt sunscreen protection and previously in this paper, it was showed how bed costumers behaviors could lead sunscreen efficacy. Comprehensive health education on the correct way to protect our self is fundamental. Standardized method to apply sunscreen and educational programs were evaluated in the past years. Jeanmougin M. et al. in 2014 validated a sunscreen application technique for adults and children evaluating the amount of sunscreen used, homogeneity of sunscreen application and volunteers' appreciation of the new technique. Statistically significant results were showed: an increase of the products amount applied, the body areas covered and the good appreciation (Jeanmougin M., 2014). In other studies, knowledge outcomes in sunscreen use were improved by video-based online education (Armstrong A.W., 2011), books, swim shirts, weekly text-message reminders (Ho B.K., 2016), encouraging supportive sun protective attitudes and beliefs (Hawkes A.L., 2012), smartphone sun-safety mobile application (Buller D.B., 2015) ect. The studies showed an improvement of sun protection behaviors, included a higher sunscreen amount use. The educational programs should especially be directed to preadolescents and adolescents it showed an excessive sun exposure in childhood increases the lifetime risk of melanomas and other forms of skin cancer (Whiteman D.C., 2001) and their families. Relying on scientific literature,

Glanz K. et al. in 2001 according with specialist, presents guidelines for school to implement new approach to preventing skin cancer (Glanz K., 2001). Those kinds of task force could be proposals very important to decrease the rising skin cancer incidence in the next future, mutually with providing: (i) environments that support the sun safety, (ii) health services, organizations making all the essential information available to the public, (iii) promotion of sun protection, (iv) age-appropriate information, easy to understand.

The category "young adults" is also affected to a higher skin cancer risk. Mainly, they are motivated by the perceived appearance-enhancing benefits of the sun exposure, as tanned skin resulting a better look, without thoughts about the risky consequences. Heike et al. in 2005 (Mahler H.I., 2005), studied the effect of information about appearance, as photoaging (e.g. wrinkles), on sun exposure and sun protection behaviors of young adults, showing an intervention significantly stronger in sun protection intentions relative to controls. This is an evidence about the importance of an effective intervention that uses evidence-based individualized plans to be incisive.

**10 Cost-Effective approach**

Recent peer-reviewed national incidence estimates 3 507 069 non-melanoma skin cancer cases in a year, with 2 152 500 treated cases (Rogers H.W., 2010). Gary in 2015 (Guy G.P., 2015) examined the treated prevalence and treatment costs of non-melanoma and melanoma skin cancer from 2002 to 2011. The skin cancer increased from 3.4 million to 4.9 million and this was followed to a substantial increase of the average annual total cost for skin cancer from $3.6 billion to $8.1 billion. The skin cancer cost is not just for the cure, but it is a combined costs of cancer diagnosis, treatment, loss of productivity, care cost, drugs, therapies etc. Hanly (Hanly P., 2015) estimated, using the human capital approach, the lost productivity costs due to premature mortality. In Europe in 2008 were €75 billion and the melanoma had the highest cost per death estimated at €312,798. In literature, published data demonstrate the convenience in cost for non-surgical extraction instead of surgery extraction. The cost of treatments for low-risk non-melanoma skin cancer, as curettage and electrodesiccation, are 50% to 60% less expensive than more invasive technique, as standard surgical excision and Mohs surgery (Cook J., 1998; Ravitskiy L., 2012). Following a study made by Lim in 2017 (Lim H.W., 2017), the total population

medical costs for non-melanoma skin cancer is $ 4 585 and for melanoma skin cancer is $ 1 467. Estimating the cost of the skin cancer is advantageous not just for comprehend the entity of the expenses but also to measure the potential cost saving from skin cancer prevention, behind the social benefit (Hall P.S., 2009). Cost-effectiveness analysis (CEA) is an economic evaluation technique to describe an intervention impact in terms of decrees/increase of cost-effectiveness ratio (incremental costs divided by its incremental health benefits). CEA was applied previously in oncology (Shih Y.C., 2008; Neumann P.J., 2015).

Louisa G. Gordon et al. (Gordon L.G., 2009) estimate, over 5 years, a total saving of $ 88 203 for the health care in randomized daily sunscreen treatment groups versus discretionary sunscreen treatment groups, at the same, preventing 11 BCCs, 24 SCCs, and 838 actinic keratoses among 812 residents. On this line other studies showed similar result (Vallejo J.J., 2011; Hirst N.G., 2012). The focus, in the future, should be in substantial investments in prevention affords, eliminating wasteful spending, including well-designed campagnas from foundations focused on skin cancer prevention to affect the incidence of skin cancer with an equivalent advantage of costs saving.

**11 A new environmental risk: marine pollution**

Sunscreen products have been used for nearly 80 years, and in the past decades an increased use of sunscreen cosmetic products leading the introduction of new chemical compounds. There are around 45 UV chemical filters subjected to regulation in different countries, in addition to UV filters, sunscreen contains other ingredients such as preservatives, film forming agents, surfactants, viscosity controllers etc. During bathing and showering etc., the new chemicals went down household drains, made their way into rivers, lake and oceans. It has begun to raise concerns regarding marine pollution and its consequences on flora and fauna. Some effects of marine pollution are visible but other contaminants are less-apparent. The sunscreen chemicals have a preferential association with particulate organic matter in the environment (Rodil R., 2008) making them very persistent overtime and making them very targeted when the topic is the marine pollution. Once there, UV filters can bioaccumulate in biota as fish, aquatic mammals etc. Bachelot in 2012 (Bachelot M., 2012), studied the organic UV filter concentration in marine mussels from French coastal regions finding detectable accumulation of 2-ethyl-hexyl-4-trimethoxycinnamate, octocrylene and octyl-

dimethyl-PABA in two spices of marine mussels. These findings were conformed in other parts of the word as Asia (Huang W., 2016; Sang Z., 2016), Iberian rivers (Gago-Ferrero P., 2015), Brazil (Molins-Delgado D., 2018), Atlantic coast (Pintado-Herrera M.G., 2017), Norway (Langford K.H., 2015) etc. Since 1970s, coral reefs have been devastated on global scale form climate events (Carpenter K.E., 2008) but the toxicological effects of pollution compared as predominant cause for ecological resilience of coral reefs in the latest years. Downs in 2016, studied the toxic pathological effects and environmental contamination of Oxybenzone (Benzophenone-3) on coral planulae showing an increasing rate of coral bleaching in response to increasing concentrations of oxybenzone and exhibiting a close relationship between DNAAP lesions and increasing oxybenzone concentrations. An accumulation of DNA damage has implication for the success of coral recruitment, juvenile survival and reproductive effort (Downs C.A., 2016). Laboratory experiments were made to evaluate the impact of inorganic filters, such as zinc and titanium dioxide uncoated and coated, on corals Acropora spp. Severe and fast coral bleaching were observed due to the alteration of the symbiosis between coral and zooxanthellae (Corinaldesi C., 2018). Direct release of UV filters chemicals into the aquatic environments from bathing and swimming was reported as the main environmental source of those chemicals (Giokas D.L., 2007). Marine water from six coastal South Carolina, USA sites was analyzed in order to evaluate the relationship between beach use of sunscreen and the distribution of organic compounds showing a close link between the organic compounds measured and the site. Sites with the highest percentage of tourism showed the highest concentration of UV filters (Bratkovics S., 2015).

The development of sensitive and selective analytical methods used for UV filters determination in environmental matrices is of high interest. The methods used to determinate UV filters in cosmetics sometimes are not applicable for trace analysis in environmental matrices. The most popular analytical techniques employed to the detection of UV filters form cosmetics are the chromatographic techniques (thin layer chromatography, gas chromatography, liquid chromatography), spectroscopic techniques, electrochemical techniques (Salvador A., 2005) etc. But the detection form environmental matrices required a suitable LODs, the ability to determinate simultaneously organic chemicals and a sorbent-based or solvent-based extraction methods that it doesn't required a complex sample preparation manipulation with

strong solvents. Extraction techniques are playing key role in sample preparation in this analysis when it comes to separation of the of analytes from potentially interfering compounds, concentrating the analytes improving the limit of detection and conditioning the sample to the analytical instrument. To this regard, even if the most popular analytical technique are in continuous development in order to improve their speed and versatility, microextraction technique have acquired popularity in the last decades. Chisvest in 2018, collected a total of 70 articles for the determination of organic UV filters in environmental water samples based on microextraction techniques from 2002 and 2017 (Chisvert A., 2018), showing the powerful expansion that this technique is getting over the years. The main advantages are the possibility to: reduce the consumption of organic solvents from milliliters to just a few microliters, remove additional cleaning steps and improve selectivity and the enrichment factors.

In the last decades, cosmetic and hygiene companies had a huge expansion using plastic microbeads in cleanser, exfoliant, shower gel, toothpaste etc. Ingestion of microplastics has been reported for several marine organisms as mussels (Browne M.A., 2008), marine mammals (Denuncio P., 2011) and seabirds (Avery-Gomm S., 2012). The microbeads are made in polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polymethyl methacrylate (PMMA) or nylon (Gouin T., 2015) with a diameter up to 500 μm and due to their small size, a consisted part of them will pass through filtration systems and enter aquatic environments. By 2015, significant amount of microbeads was found in North America, Japan, China (Eriksen M., 2013; Isobe A., 2016; Cheung P.K., 2017).

## 12 Conclusion

The major strength of the current study is to underline the discrepancy between sun-protective behaviors and sunscreen application patterns in-real life versus the “ideal” sunscreen employment. In order to achieve the labelled SPF, and with it the claimed sunscreen efficacy, several factors should be evaluated. The recommended sunscreen application thickness of 2 mg/cm$^2$, most of the time, does not correspond to the real amount applied by consumers, even the type of sunscreen application method may influence the amount used. The frequency of application and re-application become critical factors to reach for a full covered body. In vivo studies showed how the type of the sunscreen application method can affect the quantity of product used, uncomfortable application methods or products with an unsatisfying tactile profile can

modify the amount of sunscreen used bringing issues as the “missing area”. Sun exposure behaviors such as the intentional exposure to UV radiation, with the desire to acquire a tan, came up as essential factors in cases of prolonged exposure when protected with high SPF sunscreen. In the next future, the efforts should be focused to delete the gap between in-real life use and the “theoretical” SPF values, considering the ideal sunscreen employment versus consumer habits in real-life conditioners. Studies showed the total saving of a prevention action instead of the skin cancer cost, educating the population to a correct use of skin cancer prevention tools can save life and reduce health care expenses. What should be the next direction? Public educational initiatives, investing in the research to obtain product safer and with a low impact on the environment.

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# Chapter IV

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**Topical sunscreen application preventing skin cancer: systematic review**

(Under submission to Cancer Management and Research)

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**Topical sunscreen application preventing skin cancer: systematic review**

**Abstract**

Background: Avoiding extended exposure to direct sunlight and topical application of sunscreen when exposed, are the main techniques to protect the skin form sunburn, photoaging and skin cancer risk (melanoma and non-melanoma skin cancer). Preventive strategies could lead to a significant reduction of the excessive health system cost for the treatment of these conditions. Despite, decades of humane use with health benefits closely related, sunscreen employment and efficacy stay controversial. At the present, few studies found still a connection between the use of sunscreen and not significant long-term benefits from UV induced damages. Objectives: To assess the effects of sunscreens for preventing melanoma, non-melanoma skin cancer (basal or squamous carcinoma and melanoma) and precancerous skin lesions. Method: Published literature (1993-2017) was reviewed and eligible studies reporting the impact of sunscreen use in the prevention of melanoma, non-melanoma skin cancer or precancerous skin lesion were selected. Result: Starting from 532 sources, a total of 8 articles met the inclusion criteria and have been subjected to a systematic review. All the included studies suggest that sunscreen use is associated with a reduction in melanoma, squamous cell carcinoma and precancerous skin lesions, however, the difficulties in evaluating the efficiency of sunscreen was point out. Conclusion: The review of the experimental evidence supports topical application of sunscreen as an efficacies effort in preventing skin cancer and precancerous skin lesions.

**Keywords:** precancerous skin lesions, skin cancer prevention, sunscreen, UV-induced damages.

## 1 Introduction

Skin cancer represent the most common type of malignant neoplasms in Caucasian population, over a million cases diagnosed each year [1]. Nearly 15,000 deaths and 76,380 new cases were estimated in the US in 2016 [2]. The skin cancer development is regulated by intrinsic and extrinsic factors. ~10-30% of lifetime risk to cancer development is represented by DNA replication random errors followed a genetic mutation, but extrinsic factor as prolonged and unprotected UV exposure is accepted as the biggest cause of melanoma (MM) and non-melanoma skin cancer (NMSC) such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [3]. The origin of the melanoma cells is not fully agreed, it has been suggested the MM cells may originated from either from dedifferentiated melanocytes or from melanocyte progenitors. BCC and SCC originates from malignant transformation of keratinocytes and suppression of the cutaneous inflammatory response [4]. BCC and SCC show precursor lesions such as Actinic Keratoses (AK) which are considerate premalignant lesions with 1-20% of rate progression in invasive carcinoma and the risk is appreciably higher in subjects with five or more AK, it is a reliable marker identifying those most predisposed to development of NMSC [5].

Studies shows that timing, pattern and amount of UV exposure seems to be relevant in their development, MM is related to intermittent, infrequent, intense UV exposure, BCC to intermittent, infrequent and intense UV exposure and SCC is been connected to frequent moderate exposure episodes and, usually, presents keratinizing lesions such as actinic keratoses (AKs) [6]. Even if the origin of MM and NMSC seems to be different, studies showed that MM and NMSC incidence is higher for: i) specific phenotypic category: fair-skinned phenotype presents low levels of melanin (skin pigment able to absorb UV radiation) resulting in less protection against UV radiation, usually they are very sensitive to the solar radiation, tending to burn, ii) history of sunburn (in particular during the childhood), iii) personal behavior (e.g. indoor tanning, intentional sun exposure), iv) sun protective attitude (e.g. sunscreen, sun avoiding), v) latitude during UV exposure.

The prevention of MM and NMSC is an essential factor, the measures are divide in sunscreen and physical barriers (special clothes). Sunscreen agents are able to absorb or reflect the UV radiation preventing the skin damages. They are made in a wide range of SPFs, which informs on the time needed to produce sunburn when the sunscreen is applied to the skin compared to the unprotected skin. The efficacy of a sunscreen depends on such

specific characteristics such as: ingredients, general formulation (e.g. water-resistance), broad-spectrum, application patterns, sunscreen amount applied, exposure time etc. [7].

## 2 Materials and methods

### 2.1 Criteria for considering studies

- Types of studies

Any randomized controlled trial, case control, population-based cohort study that assessed incidence rate of MM, NMSC and precancerous skin lesion (such as actinic keratosis, AK) were included.

- Types of participants

General population, including children and special population, was included in the following systematic review.

- Types of interventions

Experimental studied measuring UVR induced damages in humane skin using sunscreen with sun protection factor (SPF) 15+ or more versus placebo or other interventions.

### 2.2 Type of outcomes measures

Primary outcomes: melanoma confirmed clinically or histopathologically at any follow-up, basal-cell carcinomas (BCC) confirmed clinically or histopathologically at any follow-up, squamous-cell carcinomas (SCC) confirmed clinically or histopathologically at any follow-up. Secondary outcomes: actinic keratoses (AK) confirmed clinically or histopathologically at any follow-up. Studies conducted on animals, animal models and cell lines were excluded.

### 2.3 Search strategy for identification of studies

A comprehensive search strategy was developed, on-line searches, electronic searches and searches in clinical trial registers were made. Relevant papers were searched using following key words, or a combination of them, to identify relevant papers: skin cancer, cutaneous tumor, melanoma, non-melanoma skin cancer (NMSC), basal cell carcinoma (BCC), squamous cell carcinoma (SCC), actinic keratoses (AK), sun-protection, sunscreen, UV filters, clinical trials, incidence, epidemiology, skin group. No restrictions on language were imposed during the search strategy. An investigator (C.A.C.)

independently reviewed titles, abstract, text and abstracted data from identified studies. On-line searchers: Google Scholar and Madeline; electronic searches: the Cochrane database browse and the Cochrane Central Register of Controlled Trial (CENTRAL); clinical trial register: Australian New Zealand Clinical Trials Registry, WHO International Clinical Trials Registry Platform and the EU Clinical Trial Register.

**2.4 Extraction and unification data**

An extraction form was developed to collect the relevant information from included papers:

- General data: author and year of publication, study design and characteristic of selected population.
- Treatment strategy.
- Sunscreen information: SPF, brand, UV filters and their percentage, spectrum and type of formulation.
- Additional information: phototype, intentional exposure, sunscreen amount, latitude, reapplication.
- Statistical information: statistical method used, adjustment for factors.

**3 Result**

With the search strategy defined, 532 publications were identified, 97 were potentially eligible for inclusion based on title, after abstract reviewing 23 papers were excluded because they did not focus exclusively on MM/NMSC or application of sunscreen SPF 15 or more as prevention tool. 9 papers were duplicates. We included 8 studies: 2 for MM, 3 for NMSC and 3 for AK (**Figure 1**). Papers included in this systematic review provide data on 4 different countries over the period 1993-2017. 5 included studies were based on Australian population, 1 Norwegian population, 1 German population, 1 Canadian population. In all the papers, population ages were evaluated in a range of 20-77. Data acquired form the experimental studies considerate, were heterogeneous in terms of: i) sunscreen SPF, ii) UV filters actives, iii) sunscreen brand, iv) sunscreen application directive, v) additional info. Outcomes for melanoma, BCC/SCC and precancerous skin lesions were analyzed separately.

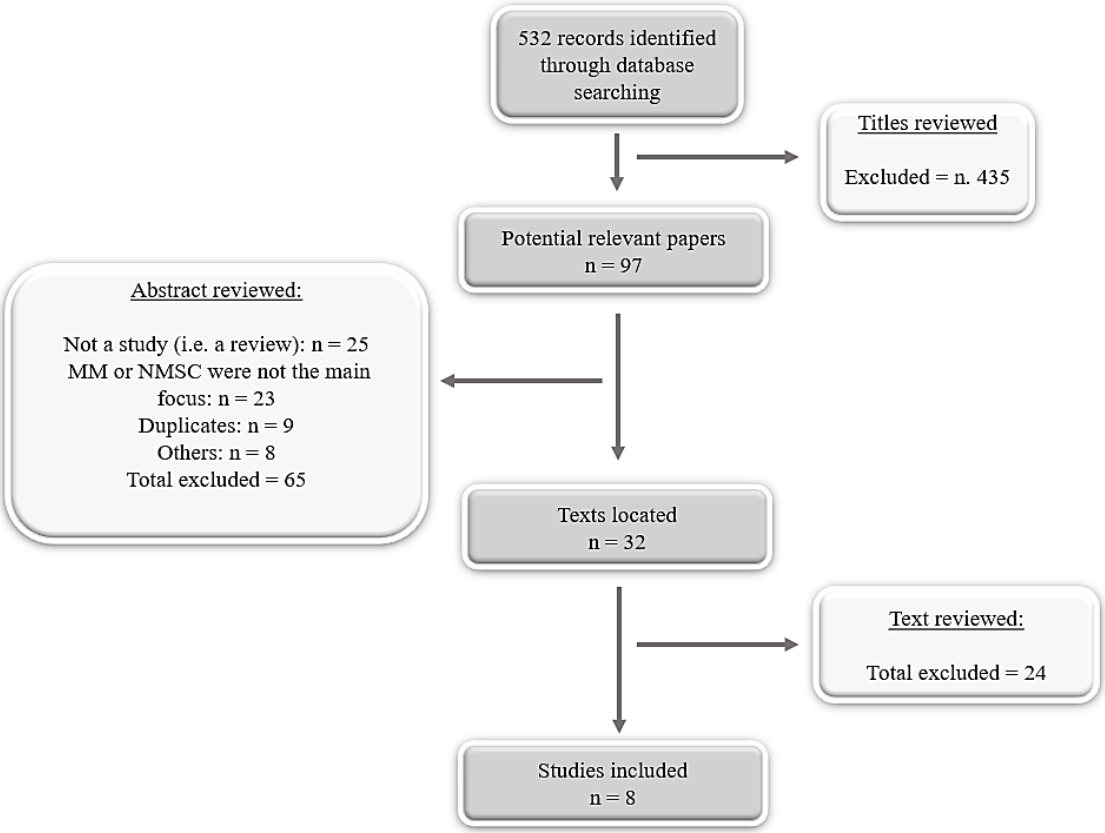


**Figure 1.** Study flow diagram.

### 3.1 Included studies description

#### 3.1.1 Melanoma (MM)

Only 2 papers claimed a potential reduction of melanoma incidence after using of sunscreen SPF > 15+. One study used randomized controlled trial (RCT) and the other one the population-based cohort study (P-BCS). Both enrolled general population from Australia or Norway.

Green A.C. in 2011 made a randomized controlled trial (RCT) follow-up suggest that melanoma may be preventable by regular sunscreen use in adults. A general population of 1,339 (~50% female and ~50% male, age between 20-69) from Nambour (Australia, latitude 26 °S) residents was selected and independently randomly assigned to 4 groups: 1) daily application of sunscreen broad-spectrum SPF 16 plus 30 mg betacarotene, 2) daily application of sunscreen broad-spectrum SPF 16 plus placebo tables, 3) betacarotente only, 4) placebo only. Placebo sunscreen was considerate unethical and was avoided. In the first group, free and unlimited supply of broad-spectrum

sunscreen containing 8% of Octinoxate and 2% of Avobenzone was given. Participants not assigned to daily application of sunscreen were asked to continue application of sunscreen at their usual discretionary rate, which for most was recreational use. Information about risk factors for skin cancers, such as skin color (fair, medium, olive/brown), outdoor behavior (mainly outdoors, indoors and outdoors, mainly outdoors) and sunburn history (none, once, 2-5, > 5) was obtained at baseline. Self-application of a layer to all exposed sites every morning was requested, and reapplication was suggested for heavy sweating or after long exposure. The amount of sunscreen applied during exposure was estimated by weighting sunscreen returned battels. None information about relative latitude during the sun exposure was recorded [8].

In the second paper, Ghiasvand R. in 2016 with a P-BCS enrolled Norwegian general population with age between 30 and 75 years. 171,725 subjects were enrolled. Specific information about time spent under the sun and the relative latitude were asked. The sunscreen brand and SPF were selected by the single participant, but precise information about the occasion of use of sunscreen were asked in order to know which sunscreen was used in high or low latitude condition. Base on the fact that sunscreen SPF 15 is considered sufficient to prevent sunburn if applied properly, the population was divide in 3 groups: 1) sunscreen non-users, when they did not indicate sunscreen use or they indicate SPF 0; 2) sunscreen users SPF < 15; 3) sunscreen users SPF ≥ 15. The participates phenotype were recorded by a color scale graded from 1 (very fair) to 10 (very dark brown) in relation to their skin color. Participants getting a score from 8 to 10 were excluded from the study. Other phenotype characteristics were recorded as: hair color, freckles and nevi. History of indoor tanning were reported. Skin reaction before, during and after sun exposure were recorded. The study excluded participants who had been given a diagnosis of melanoma. The study evaluated the patterns and intensity of the sun exposure for European people who receive intense UV exposure mainly during summer vacation [9].

### 3.1.2 Non-melanoma skin cancer (NMSC)

Three studies claimed a potential reduction of BCC and SCC incidence after using of sunscreen. A RCT and its follow up 7 years late with randomly selected population form Australia and a case-control (C-C) with 120 immunocompromised organ transplant form

Berlin. Green A.C. et al. [10] and in Van Der Pols J.C. et al. [11], conducted a RCT in 1999 and its follow-up in 2006 with the specific outcomes which were reported previously in the study made form Green A.C. et al. in 2011 [8].

Ulrich C. et al. in 2009 randomly selected 120 patients immunocompromised, with age between 40 to 77, form Charite´ University Hospital in Berlin, Germany. The population was divided in relation to the Fitzpatrick's skin type and was considerate only patients with type II and III avoiding population with very fair or very dark skin. In order to make 2 groups comparable, the type of immunosuppression was evaluated and equally distributed. Specific information about sunscreen formulation used: water-resistant cream Daylong actinica, Spirig Pharma Ltd (Switzerland) with several UV filters (bemotrizinol, octyl Triazone, isoamyl p-methoxycinnamate, ethylhexyl methoxycinnamate, methylene bis-benzotriazolyl tetramethylbutylphenol, avobenzone) rated as sunscreen SPF > 60 for UV-B, according to the EU commission recommendation (26/9/2006), and according to the Australian Standards (AS/NZS 2604–1997) the product delivers a good UV-A protection. The population was divided in sunscreen and control groups. In both groups, information about sun intense unprotected UV exposure risks and sun protection behaviors, in specific the use of sunscreen, were given. In specific all the patients were awarded to apply at least 2 mg/cm$^2$ on the exposed areas 20-30 minutes before UV exposure [12].

### 3.1.3 Precancerous skin lesions (PSL)

Were identified 3 studies focusing on the prevention PSL using sunscreen. Two RTC and one prospective, double-blind, controlled trial (PCT).

Jolley D. in 1993 enrolled 588 subjects living in Maryborough (Australia) and randomly assigned to the sunscreen or base-cream groups. The sunscreen composition was specified as broad-spectrum sunscreen cream containing 8% of Octinoxate and 2% of Avobenzone with SPF 17 (according to Australian Standard 2604 1986). The instructions were to apply 1.5 ml of sunscreen on exposed spots of the skin every day and it was suggest reapplying it if necessary. The number of new lesions appeared, and the remission of existing ones were evaluated [13].

Naylor M.F., in 1995, set up a PCT from 1987 to 1990. 90 participants with clinical evidence of AKS or NMSC were divided in treatment group or placebo group. The

treatment received sunscreen SPF 29 contained methoxycinnamate, benzophenone-3 and octyl salicylate (UVB protection). Information about the negative side of sun overexposure were given but none information about sunscreen amount or reapplication was allowed, the participates were encouraged to use their usual routines. The amount of sunscreen used was approximal estimated counting the bottle request during the trial (max 2 battle 120 mL per month) [14].

Darlington S., in 2003 [15]**,** conducted a RCT in conjunction with a trial all the specific outcomes were reposted previously in the study conducted by Green A.C. in 1999 [10].

### 3.2. General data results and statistical evaluation

**Figure 2** shows the 8 datasets included with a total of 177,104 subjects. In 6 cases the population was randomly enrolled collecting general population, 1 consist of high risk population and the last one focused on immunocompromised population. Most of the selected articles concerned randomized controlled trial, population-based cohort and prospective double-blind controlled trial. Studies were performed in Australia, Norway, Germany and Canada. The ages of the subjects were in the range of 20 to 77 years old, sunlight susceptibility was determinate following Fitzpatrick classification or authors suitable protocol. Data such as hair color, freckling, nevi etc. were examined and recorded by the authors. The findings were reported as hazard ration 95% confidence intervals [8,9] in 2 studies, relative risk 95% confidence in 3 studies [10, 11, 15] and in mean differences 95% confidence intervals in one study [13].

In the study conducted by Green A.C. et al. in 2011, 11 new primary melanomas were identified in the daily application sunscreen versus 22 new primary melanomas detected in control group and a substantial reduction of invasive melanoma (3 versus 11 in control group) was observed. Invasive melanoma was reduced by 73% in the daily sunscreen group, the diagnosticated melanoma average thickness was 1.2 mm in the control group and 0.5 mm in the intervention group. In the control group 38% of the subjects didn't use sunscreen or applied it maximum twice a week (35%), in the intervention group 75% of the subjects claimed to use sunscreen. Time spent under the sun was close for both groups during the trial, 79% for intervention group and 77% for control group, spent less than 50% of weekend time outdoors. Around 60% of both groups used sunscreen-alternative sun protection actions (shade avoiding, hat etc.) [8].

Ghiasvand R. in 2016 conclude that the use of sunscreen with SPF 15+ or more could potentially reduce the melanoma incidence by 18%. Significative differences were observed between the sunscreen group and the control group, sunscreen users were mainly the youngest part of the selected population, living in the areas with higher ambient UV radiation, higher education and closer to phenotype I/II. Skin reaction such as sunburn were correlated to a higher risk of melanoma even in sunscreen group, sunscreen users with no history of sunburn tended to have lower incidence of melanoma. In 10.7 years follow-up 722 new cases of melanoma were diagnosticated, the most common areas were: limb, trunk, head and neck with 56% of spreading melanoma and 15% of nodular melanoma [9].

For Green A.C. et al. in 1999 detected a prevention action for daily application of sunscreen for SCC but not for BCC because lower incidence for SCC (1508 versus 1146 per 100 000) was showed in the daily sunscreen group compared to the control group but none significative difference was detected in BCC incidence. Until 1996, new skin cancer cases were 1343 in 441 subjects, 67% were histologically confirmed or reviewed on medical records (33%). SCC incidence was significative lower in intervention groups compared with control group, instead, BCC incidence didn't show difference between intervention group and control group [10]. Some years later, during the follow-up in 2006, previous findings were confirmed. No significant decrease (25%) in BCC incidence was observed in the sunscreen users group compared with the control group, but SCC incidence rates presented a significant value (38%) in sunscreen users group compared to the control one [11].

Ulrich C. also found that daily application of sunscreen could prevent the development of AK and invasive SCC, but the same positive result was confirmed for BCC. In 24 months study, in the sunscreen group significative less lesions were detected compared to the control group (89 vs. 273; $P < 0.01$) with a good tolerance for the sunscreen formulation. Also, vitamin D levels were monitored showing a lower level of vitamin in the sunscreen group compared to the control group (53 ng/mL1 vs. 60 ng mL). During the 24 months trial, 19 new invasive NMSC were histologically confirmed in 22 subjects, 8 new SCC and 2 new BCC cases were identified in control group versus, respectively, 0 and 3 new cases in intervention group [12].

Three studies reported positive result about the action of sunscreen on the progression of AK. In the RCT conducted by Jolley D. a mean increase of 1.0 ± 0.3 in AK count for control group compared to sunscreen group (0.6 ± 0.3). The control group had 508 new lesions and 18% of lesion remission compared to the 333 new lesions and 25% remission detected in sunscreen group, so, the sunscreen use prevents the development if AK and promoted the regression of the existing ones. The amount of cream during the study used was similar in both the intervention and control group, only a difference between the two sexes was observed: the man used more cream than woman. The incidence of new lesions was correlated to the amount of sunscreen used, 23% of the new lesions were found in subject using less than 500g of sunscreen, the percentage is reduced at 12% in subject using more than 1000g [13]. In its study, Naylor M.F reported a less appearance rate of AK in the sunscreen group compared to the control group. The control group shown an average of 27.9 AK per year compared to the intervention group where the average was 13.6 AK per year [14]. Darlington S. conclusions were that there is a decrease in the ratio of AK counts for sunscreen group compared with the control group (24%) [15].

| **Study, Year** | **Population** | **Findings** |
|---|---|---|
| **MELANOMA** | | |
| [8] Green A.C., 2011, RTC | Australia (Nambour, Queensland), n. 1339, general population, age 20-69 | Risk of melanoma reduced in daily sunscreen application compared with discretionary use, HR 0.50; 95% CI, 0.24 to 1.02. Invasive melanoma was reduced by 73% in the daily sunscreen group HR 0.27; 95% CI, 0.08 to 0.97 |
| [9] Ghiasvand R., 2016, P-BCS | Norway, n. 171,725, general population, age 30-75 | Risk of melanoma reduced in sunscreen SPF > 15 group compared with sunscreen SPF < 15, HR 0.67; 95% CI, 0.53 to 0.83 |
| **NMSC** | | |
| [10] Green A.C., 1999, RTC | Australia (Nambour, Queensland), n. 1621, general population, age 20-69 | The incidence of SCC reduces in sunscreen group compared with control group, RR 0·61, 95% IC, 0·46–0·81. |
| [11] Van Der Pols J.C., RTC follow-up 2006 | Australia (Nambour, Queensland), n. 1621, | The incidence of SCC reduces in sunscreen group compared with no-sunscreen-group, RR 0.65; 95% CI, 0.43-0.98 |

| | | |
|---|---|---|
| | general population, age 20-69 | |
| [12] Ulrich C., 2009, C-C | Berlin, n. 120, immunocompromised organ transplant, age 40-77 | 8 new cases of SCC were developed in control group compared 0 diagnosticated in the intervention group ($P < 0.01$) and 2 new BCC cases in intervention groups compared to 3 cases in control group (n.s.). 11 BCC (2 vs. 9; ns). |
| **AK** | | |
| [13] Jolley D., 1993, RCT | Australia, n. 588, general population, age over 40 | The incidence of AK reduces in sunscreen group compared with placebo group, MD 1.53, 95% CI, 0.8 to 2.25 |
| [14] Naylor M.F., 1995, PCT | USA, n. 90, high-risk population, age 39-70 | Reduction of 51% in appearance rate of AK in intervention group compared to the control group |
| [15] Darlington S., 2003, RTC | Australia (Nambour, Queensland), n. 1621, general population, age 20-69 | The AK incidence decreases in sunscreen group compared to no-sunscreen-group, 1992-1994: RR 0.78, 95% CI, 0.64-0.96; 1994-1996: RR 0.94, 95% CI, 0.75-1.19 |

**Figure 2.** Study's findings. RTC: randomized controlled trial, P-BCS: population-based cohort study, C-C: case-control, CT: controlled trial, CI: confidence interval, HR: hazard ratio, RR: relative risk, MD: mean difference, PCT: prospective controlled trial.

## 4 Discussion

### 4.1. Comments on included studies

Few observations about the exanimated studies can be made (**Figure 3**). This systematic review wants to evaluate the effectiveness of sunscreen to prevent melanoma, non-melanoma skin cancer (basal or squamous carcinoma and melanoma) and precancerous skin lesions. The effectiveness of a sunscreen is regulated by multifactorial model. It depends not only on its SPF, UV spectral absorption or sunscreen actives but also amount applied, type of sunscreen formulation, coverage of sun-exposed parts, reapplication, latitude etc.

A sunscreen is defined "broad-spectrum" when it is able to protect against both UVA and UVB offering a full covered protection. In 5 studies [8,10,11,13,15] were given specific information about sunscreen such as SPF rating, sunscreen brand, type of sunscreen formulation and UV filters percentage. For those studies the formulation was the same and it was identified as a water-resistant sunscreen SPF 16 broad-spectrum

(Auscreen Ultrablock Lotion SPF 15-plus, Ross Cosmetics, Melbourne, Australia) with 8% octinoxate and 2% avobenzone. In one study [12] was specified the sunscreen UV filters but the percentages weren't available and was used a sunscreen protection with SPF 60+ for UVB and only a "good" UVA protection according to the Australian Standards (AS /NZS 2604–1997). In the study n. 14 it has been used a sunscreen SPF 29 absorbing only in the UVB range and the sunscreen brand, the type of formulation and the percentage of UV filters were not specified and in the study n. 9 the sunscreen choice was made following the usual participants sunscreen routine.

Guidelines from the FDA [16] and the international Organization for Standardization [17] agreed the amount of sunscreen applied for testing SPF should be 2 mg/cm$^2$. This is the amount of sunscreen necessary to achieve the labeled SPF rating. Also, applying the recommended quantity of sunscreen is not a guarantee for proper protection against UV radiation. The sunscreen application and the relative body coverage after application are premises for a full activated protection. The sunscreen should stay stable during the UV exposure on the superficial part of the skin in order to create a protective film. In included studies, only in one case [12], in the sunscreen group, the participates were trained to apply 2 mg/cm$^2$ on the exposed areas, 20-30 minutes before leaving the shade. In all the other studies followed a self-application of a layer to all exposed sites every morning or following the participants usual routine, and in almost all the studies the sunscreen application was only a suggestion. The reapplication of the sunscreen every two hours or after working, swimming, playing or exercising outdoors is mandatory to guarantee a complete protection over all the exposure time.

In literature, studies showed that there is a close relation between the incidence rate of MM and NMSC and ambient solar latitude gradient. The skin cancer incidence increases with decreasing of the latitude, where there is the greater UV energy to which they are exposed [18]. Also, ambient factor as ozone layer depletion play a role. Ozone layer is a region of the Earth's stratosphere that it is able to absorb some of sun UVR radiation, its depletion lead to region overexposed to UVR [19]. Ozone depletion is most evident in polar regions, studies have related close correlation between an increase of the skin cancer incidence in Caucasians living near those regions [20]. Only in one study [9] there was specific information about where the participants used sunscreen within Norway or other location, during vacation, in low or high latitude and which sunscreen they used in that occasion.

We should consider that trial conduced in subtropical areas consist of mainly unintentional sun exposure because the population is well aware about the hazard risk about exposure. Instead, Europeans and North Americans occasionally expose themselves to UV light, mainly during the summer. So, we considered that the intentional exposure to the sunlight is not related to the time spent outside, but it has to be connected with the intention of the single person. Two different type of sun exposure patterns came up: non-intentional sun exposure (NISE) and intentional sun exposure (ISE). The NISE type doesn't have an interest to acquire a tan, the exposure is related to the daily life activities or occasional exposure enjoying the time spend outside in the sun but avoiding uncovered long and intense exposure. The ISE type stays under the sun a big number of hours per day with uncover skin with the porpoise to acquire [21].

Also, skin pigmentation has a strong impact on skin cancer development [22]. Light-skinned population presents less melanin resulting in low protection versus UV light hazard risk, rather than dark-skinned population that presents bigger level of melanin production resulting in more protection against UV-induced damages. So, following the Fitzpatrick scale, subject phenotype I has a higher intrinsic risk factor for skin cancer compared to phenotype VI subjects. In all the studies information about population phenotype were express, but only in [12] were included population with lower intrinsic risk of skin cancer: phenotype II and III. In another study [12] was excluded population with very dark skin (phenotype VI).

The sun-protective behaviors and sunscreen application patterns are fundamental and should be not left outside of a study. All the factors above pointed should be considerate in order to have a result that express the real effect of sunscreen action and not just the expression of intrinsic risks.

| Study, Year | Approaches | Sunscreen | Application | Additional info | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Phototype | Intentional Exposure | Sunscreen amount | Latitude | Re-application |
| **MM** | | | | | | | | |
| [8]Green A.C., 2011 | 1) sunscreen SPF 16 plus 30mg betacarotene<br>2) sunscreen SPF 16 plus placebo tables<br>3) betacarotente only<br>4) placebo only | SPF: 16, Sunscreen brand: Auscreen Ultrablock Lotion SPF 15-plus, Ross Cosmetics, Melbourne, Australia, Type of formulation: lotion water-resistant, UV filters percentage: 8% Octinoxate and 2% Avobenzone, Spectrum: broad-spectrum rated according to Australian Standard 2604.1. | Daily group: Self-application of a layer to all exposed sites every morning (suggestion: reapplication for heavy sweating or long sun exposure), Discretional group: continue application of sunscreen at their usual discretionary rate | | | Measured weights of returned bottles | | Suggestion |
| [9] Ghiasvand R., 2016 | 1) sunscreen SPF<15<br>2) sunscreen SPF>15 | Participants usual routine | Participants usual routine | Excluded: Very dark skin | | | High or low latitude | |
| **NMSC** | | | | | | | | |
| [10]Green A.C., 1999<br>[11]Van Der Pols J.C., 2006 | 1) sunscreen SPF 16 plus 30mg betacarotene<br>2) sunscreen SPF 16 plus placebo tables<br>3) betacarotente only<br>4) placebo only | SPF: 16, Sunscreen brand: Auscreen Ultrablock Lotion SPF 15-plus, Ross Cosmetics, Melbourne, Australia, Type of formulation: lotion water-resistant, UV filters percentage: 8% Octinoxate and 2% Avobenzone, Spectrum: broad-spectrum rated according to Australian Standard 2604.1. | Daily group: Self-application of a layer to all exposed sites every morning (suggestion: reapplication for heavy sweating or long sun exposure), discretional group: continue application of sunscreen at their usual discretionary rate | | | Measured weights of returned bottles | | Suggestion |
| [12]Ulrich C., 2009 | 1) sunscreen SPF 50 plus education | SPF: 60+, Sunscreen brand: Daylong actinica; Spirig Pharma Ltd. Switzerland, Type of formulation: | Both groups: Written and oral information on sun protection, Sunscreen group: trained 2 mg | Included: only | | 2mg/cm2 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2) sunscreen SPF 50 self-responsible application | water-resistant cream lotion, UV filters: Bis-ethylhexyloxyphenol methoxyphenyl triazine, ethylhexyl triazone, isoamyl p-tetramethylbutylphenol, butyl methoxydibenzoylmethanemethoxycinnamate,ethylhexyl methoxycinnamate and methylene bis-benzotriazolyl , Spectrum: SPF over 60 for UVB, good UVA protection according to the Australian Standards. | cm)2 to the head, neck, forearms, and hands. | phenotype II and III | | | | |
| **AK** | | | | | | | | |
| [13]Jolley D., 1993 | 1) sunscreen SPF 17, 2) placebo. | SPF: 16, Sunscreen brand: Auscreen Ultrablock Lotion SPF 15-plus, Ross Cosmetics, Melbourne, Australia, Type of formulation: lotion water-resistant, UV filters percentage: 8% Octinoxate and 2% Avobenzone, Spectrum: broad-spectrum rated according to Australian Standard 2604.1. | apply 1.5 ml of sunscreen on exposed spots of the skin every day and it was suggest reapplying it if necessary. | | | 1.5 ml per day | | Suggestion |
| [14]Naylor M.F., 1995 | 1)sunscreen SPF 29, 2) placebo. | SPF: 29, Sunscreen brand: n.d., Type of formulation: n.d., UV filters presented: methoxycinnamate, benzophenone-3 and octyl salicylate, Spectrum absorption 280-320 nm (UVB). | participants usual routine. | | | Estimation n. battles ordered per month | | Participants usual routine |
| [15]Darlington S., 2003 | 1) sunscreen broad-spectrum SPF 16 plus 30mg betacarotene, | SPF: 16, Sunscreen brand: Auscreen Ultrablock Lotion SPF 15-plus, Ross Cosmetics, Melbourne, Australia, | Daily group: Self-application of a layer to all exposed sites every morning (suggestion: | | | Measured weights of | | Suggestion |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2) sunscreen broad-spectrum SPF 16 plus placebo tables, 3) betacarotente only, 4) placebo only. | Type of formulation: lotion water-resistant, UV filters percentage: 8% Octinoxate and 2% Avobenzone, Spectrum: broad-spectrum rated according to Australian Standard 2604.1. | reapplication for heavy sweating or long sun exposure), discretional group: continue application of sunscreen at their usual discretionary rate. | | | returned bottles | | |

**Figure 3.** Studies characteristic.

## 5 Conclusion

In the current socio-economic scenario, there is a significant increase in skin cancer cases per year followed by a constant pressure to reduce costs related to medical treatments (direct costs), extra-medical expenses and humane intangible costs. In the health system, the assessment of these costs is essential to concentrate the resources. Pharmacoeconomic evaluation models are able to identify the value (convenience) and the economic impact of a specific intervention providing the better facility at minimum cost. This systemic review intended to be a scientific tool that uses a reproducible and transparent approach to evaluate the results of individual studies making them available to the health care decision makers. Sunscreen use as strategy for sun protection has been criticized for its long-term activity such as protection against MM and NMSC development. The studies included in this systematic review support the beneficial effects of the sunscreen as tool to prevent the harmful effect of UV radiation, however, several comments about sunscreen selection and application were made. Sunscreen spectrum, its modality of application and the amount applied should considerate essential factors for a sunscreen activity. It is needed studies conducted with a standardized protocol to evaluate the real efficacy of a sunscreen and not just the variation of sunscreen intrinsic factors.

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# Chapter V

cosmetics

MDPI

*Article*

## Packaging Evaluation Approach to Improve Cosmetic Product Safety

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**Packaging evaluation approach to improve cosmetic product safety**

**Abstract**

In the Regulation 1223/2009, evaluation of packaging has become mandatory to assure cosmetic product safety. In fact, the safety assessment of a cosmetic product can be successfully carried out only if the hazard deriving from the use of the designed packaging for the specific product is correctly evaluated. Despite the law requirement, there is too little information about the chemical-physical characteristics of finished packaging and the possible interactions between formulation and packaging; furthermore, different from food packaging, the cosmetic packaging is not regulated and, to date, appropriate guidelines are still missing. The aim of this work was to propose a practical approach to investigate commercial polymeric containers used in cosmetic field, especially through mechanical properties' evaluation, from a safety point of view. First of all, it is essential to obtain complete information about raw materials. Subsequently, using an appropriate full factorial experimental design, it is possible to investigate the variables, like polymeric density, treatment, or type of formulation involved in changes to packaging properties or in formulation-packaging interaction. The variation of these properties can greatly affect cosmetic safety. In particular, mechanical properties can be used as an indicator of pack performances and safety. As an example, containers made of two types of polyethylene with different density, low-density polyethylene (LDPE) and high-density polyethylene (HDPE), are investigated. Regarding the substances potentially extractable from the packaging, in this work the headspace solid-phase microextraction method (HSSPME) was used because this technique was reported in the literature as suitable to detect extractables from the polymeric material here employed.

**Keywords:** safety evaluation; polyethylene; packaging; mechanical properties

## 1 Introduction

Packaging can be defined as an economical means of providing presentation, protection, identification, information, containment, convenience, and compliance for a product during storage, carriage, and appearance until the product is consumed. Packaging must provide protection against climatic conditions and biological, physical, and chemical hazards and must be economical. The package must ensure adequate stability of the product throughout the shelf life [1].

In recent decades, the interest of research and industry towards plastic packaging, both environmentally friendly and safe for the consumer, has exponentially grown. In the cosmetic and pharmaceutical packaging field, one of the most used plastic materials is polyethylene (PE), a thermoplastic resin obtained by polymerization of ethylene. As a numerical example, the worldwide production capacity of PE is estimated to be 79,106 metric tons per year. Of this amount about 21,106 tons are low-density PE (LDPE), 22,106 tons linear LDPE (LLDPE), and the remaining 36,106 tons is high-density PE (HDPE).

All types of polyethylene are semi-crystalline polymers. Their densities and melting temperatures decrease with the increase of ramification. Many hundreds of grades of PE, differing in their properties, are actually available [2]. PE possesses good chemical stability [3,4,5]. The mechanical properties are dependent on the molecular weight and on the degree of chain branching. With increasing density, the barrier properties increase as well as the stiffness, hardness, and strength, as a result of the higher crystallinity. At the same time, there is a decrease in the impact resistance, toughness, resistance to stress cracking, cold resistance, and transparency [2]. Furthermore, polyethylene can be produced from renewable resources and it is readily recyclable if it has not been coated with other materials [6].

Blown containers from LDPE are used as packaging in the pharmaceutical and cosmetic industries as well as for food, toys, and cleaning agents. The most important application area of HDPE is the production of containers and injection-molded articles [2].

Despite the excellent characteristics of this polymer as packaging material, both plastic and its additives used in the production process can migrate from the packaging to the content over time as a result of an increase in temperature, mechanical stress, or aging. Like in the food field, the presence of plastic components or additives in

cosmetics, if not properly controlled, can affect the organoleptic properties of the product, or its safety, if the levels exceed the legislated or toxicological values [7].

Furthermore, in contrast to glass or metal packaging materials, polymeric packaging are permeable at different degrees to small molecules like gas, water vapor, and to other low-molecular weight compounds like aromas, flavors, and additives present in the formulation; this is an important point, as contamination from external environment could cause reactions within the contained product (oxidation of lipids, degradation of actives, etc.) or the absorption of ambient vapor or liquid could cause an increase of polymer plasticization, resulting in a decrease in mechanical properties [8].

In particular, PE it is able to retain large amounts of nonpolar compounds, such as most of the volatile molecules, because of its polyolefin nature: this phenomenon, known as aroma scalping, causes a loss of aroma content and or/aroma imbalance. On the other hand, other plastic materials (e.g., ethylene-vinyl alcohol copolymer, EVOH) are medium to poor water barrier plastics and their hydrophilic nature promotes the sorption of large amounts of water, which results in plasticization of the polymers and the subsequent loss of mechanical and barrier properties [9].

Evidence in literature show that changes in mechanical behavior causes changes on the barrier properties [10]. These kinds of modifications in packaging can greatly affect the safety of consumers. In fact, it is well known that some substances can migrate from packaging to the formulation, but it is not well disseminated; yet, the knowledge about the influence of packaging mechanical changes on product safety would be improved. For example, the presence of microcracks can modify oxygen permeability and thus lead to a degradation of substances in the formulation, like preservative, reducing their activity.

For this reason, in the development of a cosmetic product safety assessment, besides the packaging raw materials information issue, other aspects related to packaging functionality should be evaluated, like possible interactions between material and product in relation to primary packaging [11].

In fact, packaging made from the same starting polymeric material but with different additives or produced by different manufacturing processes, although apparently similar, can interfere differently with the content, causing unwanted reactions on the consumer [12]. Recently, a new preservative ingredient was placed on the market to be used as an additive in the preparation of "active" packaging composed

of glass beads in which silver ions are dispersed. This material received a positive opinion from the Scientific Committee on the Consumer Safety (SCCS) [13]. It is clear that any change, also mechanical, of this kind of packaging, will affect in a decisive way the release of the preservative in a cosmetic product and consequently influence the safety of the finished product.

Compatibility tests should be performed on the product, once transferred to the final container. The container-content relationship should be explored for all the packaging materials, as the final quality of the goods is always the result of a delicate balance between these two components.

Despite the importance of these aspects, there is too little information about the possible chemical-physical interactions between formulation and packaging, because, differing from food packaging, the cosmetic one is not regulated and, to date, appropriate guidelines are still missing. However, with Regulation 1223/2009 coming into full entry force, among the voices of the Cosmetic Product Safety Report of the Product Information File (PIF), a section pointing out "Impurities, traces, information about the packaging material" has become obligatory.

This work aims to propose a protocol to characterize final packaging for underlining possible critical issues in order to assure a completely safe product to consumers.In particular, next to analysis of the extractables, of which a lot of methods and protocols are present in literature [14,15,16], this work focuses on the mechanical analysis step since, as said before, changes in mechanical properties could provoke alterations of packaging performance, like barrier properties, with a consequent risk for the product's integrity.

As an example of application, a study conducted on two types of polyethylene with different densities is reported.

A simple experimental design, in order to minimize the number of trials, was employed [17,18]. Polyethylene containers were filled with standard formulations and submitted to different degradation tests (photostability test and accelerated stability test) to mimic stress conditions that products can meet during their shelf life, according to European guidelines for stability tests on cosmetic products.

Standard monophasic formulations (pH 2 and pH 10) were used, in order to carry out the test in extremes conditions.

After this treatment, the samples were analyzed by tensile test, to verify possible changes of mechanical properties. "Bone-shape" specimens, obtained from empty and

filled bottles [19], were analyzed with a tensile machine until their break, obtaining stress-strain curve. The comparison between treated and untreated materials permitted the underlining of any mechanical change.

Afterwards, an extraction method was used in order to detect all the potentially extractable substances.

## 2 Materials

Packaging materials, the object of this study, were commercial containers of 250 mL capability: HDPE bottles and LDPE tubes obtained from different suppliers. The thicknesses of containers are around 500 µm and 1 mm for LDPE and HDPE, respectively.

The filling solutions were set up with the following substances: potassium chloride, 37% hydrochloric acid, borax, and potassium hydroxide drops, all provided by CARLO ERBA reagents (Cornaredo, MI, Italy).

## 3 Experimental

The proposed approach foresees different steps.

### 3.1. Provision of data

The first step is the collection of all data regarding the considered packaging. Companies operating in the cosmetic industry provide information about packaging for the CPSR (Cosmetic Product Safety Report), for example, the food grade certificate and test reports according to the Regulation (EC) No. 1935/2004 on Food Contact Materials [20]; the declaration/certificate of compliance according to Annex IV of Regulation (EU) No. 10/2011 (plastic materials and articles) [21]; the composition, with the specification/technical data for each raw material, based on knowledge of the process for manufacturing the raw material (origin of substance, production process, synthesis Cosmetics 2016, 3, 32 4 of 12 route, extraction process, solvent used, etc.) and with a physical and chemical analysis of possible impurities in raw materials and, if necessary, in the final product (e.g., nitrosamines); and the SVHC (substances of very high concern) declaration/certificate and test report to comply with REACH regulations (packaging being considered an article under REACH).

The comparison with the requirements of food packaging could be useful because the food grade of packaging is mentioned in several EU cosmetic guidelines;

there are migration tests and limits and a positive list of allowed monomers and additives. However, some substances are not included in the Union list, but they may be present in the plastic layers of plastic materials or articles, like non-intentionally added substances and additives for polymerization; furthermore, in food packaging, different from cosmetic field, colorants are not of concern and there are some substances that are allowed in Food, but regulated in EU Cosmetic Regulation (e.g., hydroquinone, phenoxyethanol, etc.).

### 3.2 Experimental design

In order to maximize the information while reducing the number of analyses, an appropriate experiment design (screening design) has to be developed.

In this study, a simple full factorial design was chosen to investigate the effect of three experimental factors on two response parameters. The results of mechanical tests, such as the variation of stress and the percentage of elongation at break point of containers, compared to non-treated empty ones, were chosen as response parameters. In fact, we have already demonstrated that these parameters can be good indicators of any change occurring in the mechanical behavior of polymeric materials [22]. The three factors of interest were varied on two levels according to the experimental plan showed in the **Table 1**. The density of polyethylene (low or high density), the pH of contained solutions (2 and 10), and the kind of treatment (accelerated aging and solar simulated irradiation) were chosen as factors, to determine the influence of these parameters on mechanical properties of polyethylene used as packaging material in the pharmaceutical and cosmetic field.

**Table 1.** Investigated experimental factors and levels experimental design.

| **Experimental Factors** | **Level** | |
|---|---|---|
| | −1 | 1 |
| Density of polyethylene | LDPE | HDPE |
| Buffer pH | 10 | 2 |
| Treatment | 30 days climatic chamber | 24 h solar box |

The order of the experiments was randomized to avoid any bias. Statistical calculations were carried out using the software StatGraphics (Statpoint Technologies,Warrenton, VA, USA).

### 3.3 Degradation testing procedures

The HDPE and LDPE containers (object of this work) were numbered, weighed, and washed according to a standard washing procedure [19]. Afterwards, 10 bottles for each polymer filled with standard solutions were used for each degradation test:

- Photostability test by simulating UV-visible ray irradiation using SUNTEST XLS +II (Atlas®, URAI, Assago, MI, Italy) for 24 h.
- Accelerated stability test by incubation in climatic room (ClimaCell 111 MMM) at 40 ºC with 75% Relative Humidity (R.H.) for 30 days.

SUNTEST instrument was set up in according to standard European procedures, with the following parameters:

- Time: 4 h corresponding to 192 h solar light.
- Irradiation control: 300–800 nm.
- Irradiation (W/m2): 750.
- Room temperature: 35 ºC.
- Black standard temperature (BST): 45 ºC.

Photostability test was performed in according to Colipa guidelines about cosmetic products [23]. At least three specimens were obtained from each bottle to carry out mechanical and morphological analyses in triplicate.

### 3.4 Mechanical test

The investigation of the mechanical properties of the bottles was performed using a tensile machine, AGS 500ND (Shimadzu Corporation, Kyoto, Japan) equipped with a 500 N load cell; the test was performed using a strain rate, specific for each material, evaluated by preliminary trials:

- LDPE: 5 mm/min
- HDPE: 10 mm/min

Five “bone-shape” specimens were obtained from each container; the feature of the specimens followed the principles of the European Standard EN ISO 527 [24], suitably modified for bottle containers [19]. Briefly, an optimized dog bone shape obtained by

punchcutting was used. This design was developed in order to obtain a localized stress region 3 mm width and thick. Wall thickness distributions for each sample were measured at 3 different points using a digital microscope Duratool model BW1008-500x (Farnell element14 Trade Counter, Leeds, UK). The section of each sample was calculated from thickness and width using a suitable software program (micromeasure vers. 1.2). Samples were kept under constant temperature (23 ºC) and humidity (52% R.H.) for a week until tension tests started and during the entire test time.

This procedure permitted the obtainment of a stress versus strain curve. From each set of results, it was possible to estimate the tendency of materials to oppose to deformation, and to evaluate the curve profile in elasticity regime, the elongation percentage in elasticity regime, and the absolute elongation elasticity.

A critical analysis and comparison of parameters derived from diagrams allowed a qualitative but also a quantitative assessment of any significant change that occurred in the packaging due to interactions between the material they are made of and the conditions or substances with which they are in contact.

### 3.5 Extractables' analysis

The next step aims to obtain and interpret data from a controlled extraction's study starting from the several methods proposed in the literature.

In this work the headspace solid-phase microextraction (HSSPME, fiber: PDMS 100 micron, Supelco, Sigma-Aldrich, Gallarate, MI, Italy) was the extraction method considered for obtaining information about extractable substances from packaging. Briefly, 500 mg of polymer was put into a vial and the HSSPME conditions used were the following: fiber: PDMS 100 micron (Supelco); adsorption temperature: 90 ºC; extraction time: 60 min; desorption temperature: 250 ºC; desorption time: 4 min, 30 s. After extraction, for the identification of compounds a gas chromatography-mass spectrometry (GC-MS, Termo Scientific Trace DSQ II, Fisher Scientific Italia, Rodano, MI, Italy) was used. The GC conditions were the following: column: Restek Rtx-5MS, 30 m x 0.25 mm ID x 0.25 µm; gradient: 60 ºC for 4.5 min, 20 ºC/min until 280 ºC, 280 ºC for 5 min; injector: PTV 250 ºC, split time 4.5 min, split flux 10 mL/min; gas: He, constant flux 1 mL/min; transfer line: 270 ºC.

The MS conditions were: source: 250 ºC; ionizing mode: EI 70 eV; scansion mode: full Scan; scansion range: 50–650 amu; scansion rate: 870 amu/s.

After analyses, a search in the spectra library, using databases like NIST/EPA/NIH Mass Spectral Library (Wiley Registry of Mass Spectral Data 8th Edition) with Search Program (MSSP) (Data Version: NIST 2008, Software Version 2.0) was performed in order to identify all substances recovered in the sample.

**4 Results and discussion**

The safety assessment of a cosmetic product can be successfully carried out only if the safety assessor can obtain all information concerning the product, including the specific area of application (face, mucosa, periocular area, etc.), the people for whom the product is intended (baby, elderly people, etc.), and the conditions of use, but it is extremely important also to evaluate the hazard deriving from the use of the designed packaging.

Furthermore, commercial packaging is varying widely and it is very difficult to have complete information about it. For this reason, it is very important to define a general protocol that every manufacturer can apply, modifying it in a suitable way to its own formulation-packaging system for the development of an “in house” stability test. Following the protocol developed in this study, it is possible to evaluate both the behavior of container itself and the possible interactions between content and container in order to ensure the quality of product and the safety for consumers. This study case, in particular, focuses on the evaluation of one of the most used plastic packaging materials, polyethylene, to understand which are the most influential factors that could cause variations in their properties, as a starting point to extend the knowledge in this field. After finding all the information about these packaging materials, the second step aims to evaluate the mechanical properties, designed as behavior to tensile testing, of final containers. In particular, adapted “bone-shape” specimens [19] were obtained from LDPE and HDPE bottles and then analyzed with a tensile machine.

Here parameters obtained from the tensile test are shown and discussed in order to make a comparison between the different materials.

During the tensile test, the specimen presents five basic stages, resulting in the five areas of a typical stress-strain curve:

- Elastic behavior: this corresponds to the first phase of material deformation; deformations that occur during this phase are reversible, so if at this stage the applied stress is stopped there are no residual deformations of the specimen, which restores its initial length. In this phase the elongation is directly

proportional to the load (in the stress-strain diagram it is represented by a straight portion).

- Continuing the tensile test, it adopts a more linear behavior; this step is called the yield point and it corresponds to a fall of the strength of the material due to the formation of "microcracks" within the material. The yield corresponds to the initial part of the plastic behavior.
- Plastic behavior: in this phase there are both elastic (reversible) and plastic (permanent) deformation; this means that if resetting the load during this phase, there will be residual deformations associated with the contribution of plastic deformation, for which the specimen will have a greater length than at the start of the test.
- During the test, there is a localized deformation of the specimen, for which a small part of the specimen quickly decreases the area of its cross-section; this is called necking phase and it characterizes the descending part of the stress-strain curve.
- After necking there is the specimen break, which occurs in correspondence with the so-called breaking load, which corresponds to the maximum stress that the specimen can withstand.
- The reported graphs in Figures 1 and 2 show, as an example, a different mechanical behavior depending on the considered material, according to the UNI EN ISO 527 [24].

As it can be seen, the mechanical behavior of these two polymers is greatly different, in terms of elongation percentage and stress (MPa); so, it is not numerically possible to compare one material with the other. For this reason, every change in mechanical properties has been evaluated, comparing each material untreated with itself after treatment. Furthermore, it is important to underline that the approach described in this work can be successfully employed to evaluate modification of packaging during aging or during contact with the packed formulation in order to define the shelf life of the product or any interactions between formulation and the packaging.

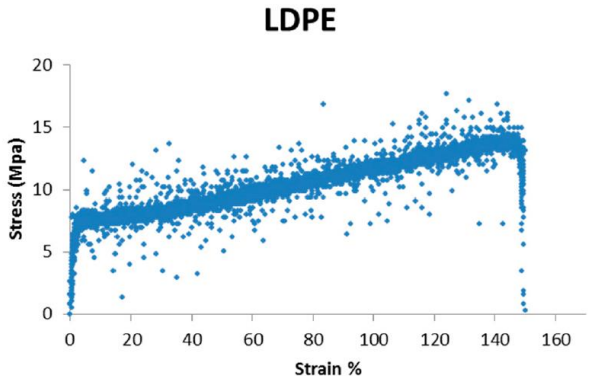


**Figure 1**. Mechanical behavior of low-density polyethylene (LDPE).

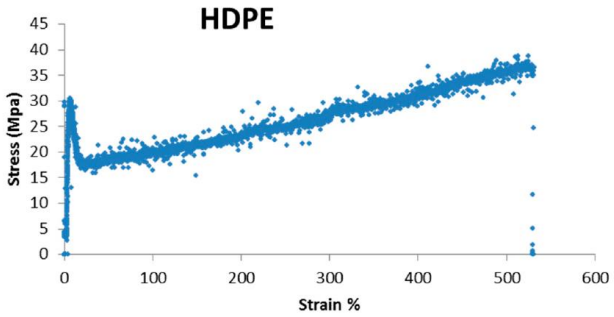


**Figure 2.** Mechanical behavior of high-density polyethylene (HDPE).

For this purpose, mechanical properties of empty and filled bottles, before and after stress testing procedures, were investigated. The stress-strain curve profile is useful to compare specimens subjected to environmental and chemical stress. HDPE presents major strength, maybe due its linear structure, that makes the polymer more resistant, while LDPE presents a greater ability to stretch, with a lower stress value. Results of tensile tests for different materials are reported in **Tables 2** and **3**, in terms of tensile stress and strain at break.

**Table 2.** Results obtained by mechanical analyses for low-density polyethylene (LDPE) containers.

| LDPE | Tensile Stress at Break (σB) (MPa)* | Tensile Strain at Break (εtB) (%)* | Δ Tensile Stress at Break (%)* | Δ Tensile Strain at Break (%)* |
|---|---|---|---|---|
| Empty | 21.30 | 150.97 | - | - |
| pH 2 sun 24 h | 17.44 | 122.62 | −18.09 | −18.78 |
| pH 2 chamber 30 days | 22.45 | 189.07 | 5.41 | 25.24 |
| pH 10 sun 24 h | 18.42 | 148.95 | −13.52 | −1.34 |
| pH 10 chamber 30 days | 21.80 | 189.20 | 2.35 | 25.33 |

* S.D. ≤ 10%.

**Table 3.** Results obtained by mechanical analyses for HDPE containers.

| LDPE | Tensile Stress at Break (σB) (MPa)* | Tensile Strain at Break (εtB) (%)* | Δ Tensile Stress at Break (%)* | Δ Tensile Strain at Break (%)* |
|---|---|---|---|---|
| Empty | 29.65 | 391.70 | - | - |
| pH 2 sun 24 h | 26.47 | 399.06 | −10.74 | 1.88 |
| pH 2 chamber 30 days | 24.88 | 331.21 | −16.10 | −15.44 |
| pH 10 sun 24 h | 25.31 | 325.23 | −14.65 | −16.97 |
| pH 10 chamber 30 days | 23.62 | 289.35 | −20.33 | −26.13 |

* S.D. ≤ 10%

Observing the values, it can be said that for LDPE there is a general reduction of the yield stress at break point. The major reduction is observable for samples treated with irradiation, regardless of the type of solution contained. So, the light has the bigger influence on material changes; this influence is exacerbated by extreme pH.

Also, regarding HDPE, we can observe that there are some changes in stress and elongation at break. The bigger variation can be observed for the samples treated in climatic chamber. It can be underlined that the container filled with the pH 10 solution has undergone the bigger changes.

Results are very interesting and they agree with literature data. In fact, it is well known that PE polymers are quite stable to degradation depending of their molecular weight, but it is also known that UV irradiation and thermal exposure can increase surface hydrophilicity of these polymer [25]. Furthermore, in all final PE packaging available in the market, antioxidants and stabilizers, in smaller or bigger amount, are present. The presence of these substances products containing PE become susceptible to degradation and subsequent oxo-biodegradation. They cause initiation and propagation of free radical chain reactions taking place in the presence of atmospheric oxygen, which leads a polymer to gradually reduce its molecular weight [26,27]. These processes cause a change in the hydrophilicity of a polymer surface, that can be more susceptible to extreme pH.

Here the Pareto Charts and the Factor Means Plots of statistical analysis of the mechanical test's results, obtained by the simple screening experimental design descripted above, are reported in **Figures 3** and **4**.

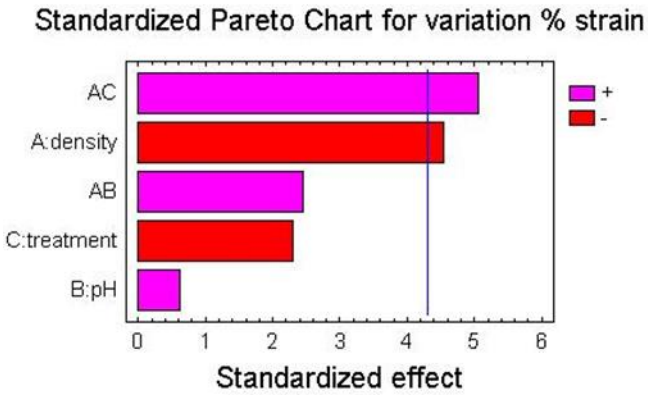


**Figure 3.** Standardized Pareto Chart for percentage variation of strain.

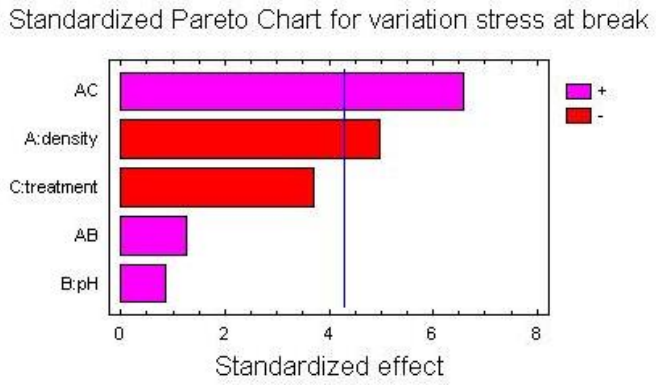


**Figure 4.** Standardized Pareto Chart for percentage variation of stress at break.

As it is possible to see from the graphs, the only factor that has a significant influence on the mechanical variations after treatment is the density of polyethylene, both regarding the variation of percentage elongation and the variation of the stress at break point.

For both for the variation of percentage elongation and for the variation of the stress at break point, the interactions between two factors—the density of the polymer and the kind of treatment (UV-vis irradiation and climatic chamber)—are significantly influential.

The main effect represents the average result of varying one factor at a time from low to high and keeping the other one constant. The interaction term shows changes in the response when both factors are varied concurrently, as this is possible to observe in the **Figures 5** and **6** below reported.

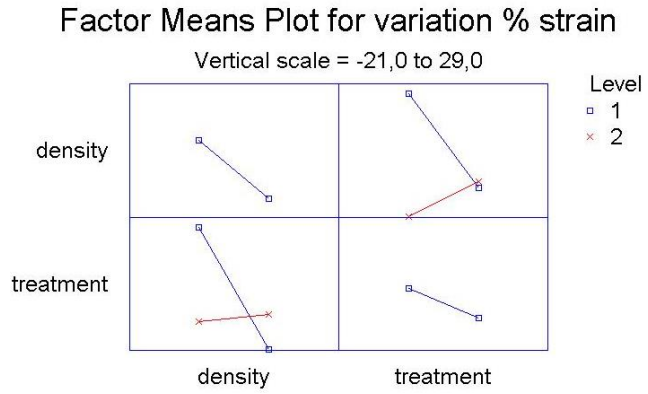


**Figure 5.** Factors Means Plot for percentage variation of strain.

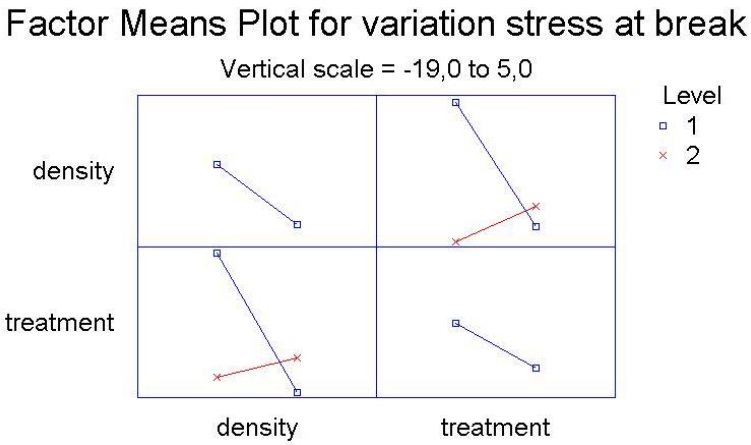


**Figure 6.** Factors Means Plot for variation of stress at break.

The considered extraction method was headspace solid-phase microextraction (HSSPME). After extraction, for the identification of compounds a gas chromatography–mass spectrometry was used. **Figure 7** shows an example of the chromatogram obtained by GC/MS.

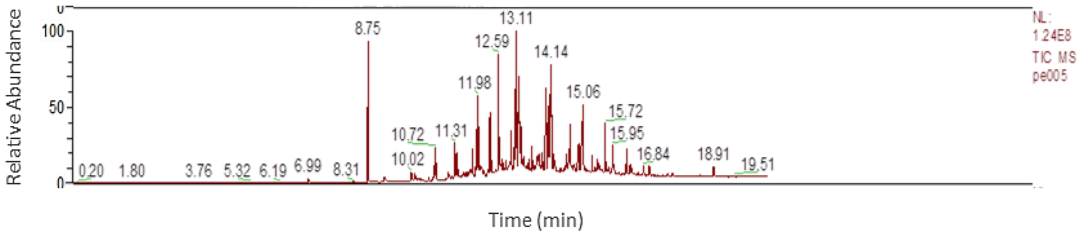


**Figure 7.** Chromatograms obtained with headspace solid-phase microextraction (HSSPME) on untreated LDPE.

The deconvolution of the chromatographic peaks leads to the identification of more than 100 substances. Many of these substances are linked to the bleeding of stationary phase of the chromatographic column to the SPME fiber coating, and to characteristic analytes also present in blanks used as references. By eliminating the interfering peaks, a list of compounds that can be identified as extractable that were released from the analyzed polymer can be obtained. In this way it is possible to split the substances into several categories, as reported in the Table 4. From the analysis of chromatographic profiles of extraction process and of relative percentage of the different substances present in the packaging it is possible:

- To choose the better packaging for the specified cosmetic product.
- To define which substance has to be quantitatively evaluated in the final cosmetic product as leachable after stability and interaction studies.

**Table 4.** Categories of extractable type released from PE polymer.

| **Extractable Type** | **Example** |
|---|---|
| Initial ingredients | Antioxidants (e.g., Terbutylphenol, Irganox), additives (phtalaths), amides (exadecanammide) |
| Impurities related to processing | Oligomers, residual solvents, esters (miristyl miristate), siloxane |
| Degradation products of the polymer | Fragments of saturated and unsaturated hydrocarbons, ketones, acids |

During compatibility testing it is also possible to detect products adsorbed by the formulation contained in the packaging material.

The data show that the sample obtained from head space microextraction (HSSPME) is representative, and it also identifies numerous nonpolar organic compounds, even the most significant polar substances.

## 5 Conclusions

This work aims to provide necessary tools and a practical approach to evaluate commercial polymeric containers used in cosmetic packaging in order to assure the safety of the finished product.

In fact, it is well known that packaging can greatly affect the safety of the product by both losing its barrier property and containing substances potentially

harmful for the consumer, especially for products for children or containing sunscreens.

Despite the importance of this aspect, there is too little information about the possible chemical-physical modifications of the packaging itself during aging or about the interactions between formulation and packaging.

The correct approach involves the provision of all possible information about the packaging material from suppliers' data sheet and from literature; then, an appropriate design of experiment has to be successfully used in order to obtain relevant indications minimizing the number of trials that must be carried out in order to perform an effective safety evaluation of the finished packaging used and of the interaction between each couple packaging-formulation.

Actually, the main problem is related to the actual composition of the packaging at the end of the production process. For this reason, it is essential to collect information about the container and not only the polymer raw materials used in the packaging production.

In this work the results of mechanical tests are chosen as predictive system's parameters, but this kind of approach can be used also for describing other system's parameters, for example the viscosity or other characteristics of the contained product. After mechanical analysis, it is important to perform also an extractables' analysis; in this case the used technique was the headspace solid microextraction (HSSPME), since, compared to other techniques used in preliminary studies, this one allows the definition of almost the total extraction profile of the analyzed material.

The reported study case regards two types of polyethylene containers with different densities, HDPE and LDPE; the commercial containers made of these materials were treated in extreme conditions of pH and accelerated aging, in order to evaluate which factors have the most influence on the mechanical properties of these materials.

This work has shown that the most influential factor is the density of polyethylene, but also that the interaction between the kind of polyethylene and the kind of treatment has significant influence on the mechanical answer of the material in comparison with the same untreated material.

So, these polymers cannot be considered as completely inert and stable. Some particular conditions (for example heat, UV radiation, and humidity) may alter the chemical, physical and mechanical properties of these polymeric materials.

In conclusion, it would be very important to apply this kind of experimental approach in the development phase of a new cosmetic product before its introduction into the market.

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# Chapter VI

*pharmaceutics*

MDPI

*Article*

## Evaluation of Mechanical Properties and Volatile Organic Extractable to Investigate LLDPE and LDPE Polymers on Final Packaging for Semisolid Formulation

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**Evaluation of mechanical properties and volatile organic extractable to investigate LLDPE and LDPE polymers on final packaging for semisolid formulation**

**Abstract**

Plastic material is used for a wide variety of commercial packaging due to being inexpensive, lightweight, and due to its resistance. In pharmaceutics, container-content compatibility studies are required for product authorization. Many guidelines and publications are available; however, the information is often only related to the raw materials used to produce packaging. During the manufacturing process, substances can be added to improve the product characteristics and performance, resulting in a processed material that is considerably different from the unprocessed material. In this study, the mechanical properties of low-density polyethylene (LDPE) and linear low-density polyethylene (LLDPE) specimens fabricated according to standard ISO 527 and specimens fabricated with the same materials, but obtained from final packaging, were evaluated. Furthermore, we examined the interaction between a semisolid formulation and LLDPE and LDPE as a final packaging, by subjecting two samples to accelerated degradation testing. Then, mechanical properties and volatile organic extractable were evaluated. Simulated solar radiation did not induce changes in the packaging mechanical properties and no extracts were detectable. The thermal shock strongly influenced the mechanical behavior, and interactions between packaging contents were identified. The present work underlines the difference between analyzing the standard ISO specimens versus samples obtained from final packaging in order to evaluate the packaging under real use conditions. An evaluation on the final packaging, instead on standard specimens, can provide information about the plastic material after the manufacturing process and the interaction between packaging and content.

**Keywords:** packaging evaluation; mechanical properties; extractable testing; content-container interaction; LDPE; LLDPE

## 1 Introduction

Packaging plays a key role in preventing spoilage, extending shelf life, and facilitating storage and transport, but the packaging has to fulfil more than these primary containment, preservation, and protection functions. Packaging also influences the sale of products with a promotional function, delivering information about the convenience and outlining claims about the final products [1–4]. Even if the packaging should preserve and protect the content from external physical, chemical, and microbiological hazard in order to maintain the safety, quality, and effectiveness of the product, studies about the interactions between the packaging material and the product contained have highlighted possible migration of chemical substances across the packaging sourced from the contained or the sorption of product ingredients by the packaging. Moreover, the loss of volatile compounds or the diffusion of volatiles from the environment (O2, CO2) toward the contents could result in degradation (e.g., oxidation) or a microbiological contamination [5–11].

Pharmaceutics packaging involves two critical aspects: the product's quality and the safety. Quality is evaluated using a stability study performed on the product; safety is partially evaluated with a stability study and toxicity testing. The toxicity is the key focus of the container-content compatibility study and for patient safety. For cosmetic products, Regulation 1223/2009 commits to reporting information about "impurities, traces, and information about the packaging material" [12]. However, no guidelines are currently available for testing cosmetic packaging. Testing is only performed on packaging materials used for food. The food sector is regulated by European Regulation No. 1935/2004 for materials and articles intended to contact food, and Commission Regulation (EU) No. 10/2011 is specific to food-contact plastic materials. This regulation establishes a list of compounds authorized for use in plastic formulation and migration tests performed on food simulants because the packaging and the contained product are not two separated entities, but they may interact, especially in presence of varying environmental conditions. Penetration of content components into the packaging or migration of packaging substances into the product could produce significant variation in the packaging properties or affect product safety and efficacy.

One of the main and often measured properties of plastic materials used in structure applications is the ability to resist breaking under tensile stress. Tensile testing provides information about yield strength, ultimate tensile strength, modulus

of elasticity, and elongation. It is a reliable method used to obtain data about how different conditions (exposure to various temperature and humidity conditions, ultraviolet (UV) radiation, etc.) may affect the performance of the final product. Universal testers are available: International Organization for Standardization (ISO) system (ISO 527) and the ASTM (American Society for Testing and Materials) system (D638) [13,14]. Usually, this testing is performed on standardized specimens. During the manufacturing process, substances such as plasticizers, thermal stabilizers, lubricant, light stabilizers, and pigments are added to improve product characteristics and performance. These additives have been shown to alter the material characteristics [15–17]. As such, the unprocessed material could be considerably different from the final packaging, underling the importance of a valuation on the plastic material obtained from the final plastic packaging and not from the unprocessed material or a standardized specimen.

In this paper, low-density polyethylene (LDPE) and linear low-density polyethylene (LLDPE) were selected as plastic materials. They are common plastic materials used for flexible bags, battels, single-dose, caps, blister packs, etc. [18–20], widely applied for their suitable production characteristics.

## 2 Materials

Xanthan gum transparent 8 mesh (ACEF SpA, Fiorenzuola d'Arda, Italy), sodium bicarbonate, borax, 37% hydrochloric acid, sodium hydroxide pellet (CARLO ERBA reagents, Cornaredo, Italy). Dimethicone KF995 (Prodotti Gianni srl, Milan, Italy). Tegosoft diethylhexyl carbonate (DEC) and Abil CARE XL 80 (Bis-PEG/PPG-20/5 PEG/PPG-20/5 Dimethicone; Methoxy PEG/PPG-25/4 Dimethicone; Caprylic/Capric Triglyceride) (Evonik Industries, Essen, Germany). Type 2 purified water obtained from the Milli-Q® purification system (Merck, KGaA, Darmstadt, Germany) was used.

### 2.1 Polymeric ISO specimens

ISO (ISO 527-1:1996) specimens of low density polyethylene (LDPE-ISO) were provided by Lameplast S.p.A. (Rovereto s/S, Novi di Modena, Italy) from polymer pellets (Purell 1840) provided by Lyondell Basell (Lyondell Basell, Rotterdam, The Netherlands). ISO (ISO 527-1:1996) specimens of linear low density polyethylene (LLDPE-ISO) were provided by Lameplast S.p.A. (Rovereto s/S, Novi di Modena,

Italy) from polymer pellets (Stamylex 2258) provided by DEXplastomers (DEXplastomers, Borealis, The Netherlands).

### 2.2 Final Packaging

Single dose containers (5 mL) of low-density polyethylene (LDPE) were provided by Lameplast (Lameplast, Rovereto s/S, Novi di Modena, Italy), and 5 mL single dose containers of linear low-density polyethylene (LLDPE) were provided by Lameplast (Lameplast, Rovereto s/S, Novi di Modena, Italy).

## 3 Methods

### 3.1 Simulant production and characterization

In order to detect some criticities in semisolid formulation, such as high pH and silicone presence, an appropriate emulsion was prepared. Silicone chemistry plays a key role in personal care and cosmetic formulations due to a multifunctional set of properties [21,22]. **Table 1** shows the qualitative and quantitative composition of the formulation intended to fill the final packaging and used as the simulant. Phases A and B were stirred separately. Phase B was added to phase A using a Silverson SL2T High Shear Laboratory Stirrer Mixer (Silverson Machines Ltd., Chesam, UK) for 10 min, 6700 rpm, and at 50 ºC.

**Table 1.** Qualitative and quantitative simulant composition used to fill the low-density polyethylene (LDPE) and linear low-density polyethylene (LLDPE) single dose containers.

| Phase | Ingredient | INCI | % |
|---|---|---|---|
| A | Xanthan gum | Xanthan gum | 0.8 |
| | pH 10 buffer solution (FUI XII Ed.) | 37% hydrochloric acid, borax, sodium hydroxide | 59.2 |
| B | Tegosoft DEC | Diethylhexyl carbonate | 15 |
| | Abil Care XL 80 | Bis-PEG/PPG-20/5 PEG/PPG-20/5 Dimethicone; Methoxy PEG/PPG-25/4 Dimethicone; Caprylic/Capric Triglyceride | 5 |
| | Dimethicone KF995 | Dimethicone | 20 |

* pH 10 buffer solution (F.U.I XII Ed.) composition: 19.07 g borax, 1.4 mL hydrochloric acid 37%, 4 g sodium hydroxide pellet in 1000 mL purified water, and adjusting pH, if needed.

The formulation was stored overnight at 25 ºC. Successively, pH organoleptic characteristics and rheological properties were evaluated. The pH measurement was performed using a pH meter model 3510 (Jenway, Staffordshire, UK), and viscosity properties were evaluated using a Kinexus Pro+ rheometer (Malvern, Worcestershire, UK), equipped with Peltier Plate Cartridge, with cone geometry CP40/4.

### 3.2 Accelerated stability testing

The final packaging, both empty and filled with simulant, were subjected to accelerated degradation testing in order to simulate the "in use" stress conditions that the final product could encounter during its life. Simulated solar irradiation and thermal shock cycles were used.

#### 3.2.1 Simulated solar irradiation

Simulated solar irradiation was performed using SUNTEST XLS + II (Atlas®, Chicago, IL, USA) according to standard European procedures [23] with the following parameters: irradiation control 300–800 nm, irradiation 750W/$m^2$, room temperature 35 ºC, and black standard temperature (BST) 45 ºC. The samples were irradiated for 48 h on each side of the final packaging, for a total of 96 h of irradiation.

#### 3.2.2 Thermal shock cycles

Thermal shock cycle testing was performed in the Clima Cell 111MMM Medcenter Einrichtungen GmbH climatic room, Munchen, Germany. The samples were exposed to 4 ºC for 7 days and then to 37 C for other 7 days. This cycle was repeated twice.

### 3.3 Mechanical and migration tests

After accelerated stability testing, the final packaging filled with simulant was emptied and washed with 1% bicarbonate solution and rinsed with distilled water in order to eliminate the simulant excess. The same treatment was performed on the final empty packaging to maintain the same experimental conditions. Those samples were subjected to mechanical tests and evaluation of extractables and then compared with controls.

### 3.3.1 Mechanical test

We investigated the mechanical properties using a tensile machine, AGS 500 ND (Shimadzu Corporation, Kyoto, Japan) equipped with a 500 Newton (N) load cell, with a crosshead stroke of 1100 mm, and tensile stroke of 740 mm. The test was performed using a strain rate of 10 mm/min. The test protocol for the mechanical measurements was reported in a previous work [24]. Estimating the following is possible: elastic portion by a linear regression procedure (Et); stress properties: yield stress (σy), tensile strength (σM), and tensile stress at break (σB); and tensile strain expressed as the increase in length: at yield (εy), at tensile strength (εM), and at break (εtB). From each set of results, we estimated the tendency of materials to oppose deformation, and evaluated the curve profile in elasticity regime, the elongation percentage in the elasticity regime, and the absolute elongation elasticity.

## 3.4 Tensile test specimens

A total of 25 dog bone shaped specimens, for each kind of container, were obtained horizontally from the central part of the 5 mL untreated and treated single dose containers. We made this choice because the small size of the containers did not allow us to obtain vertical specimens. An optimized dog bone shape was obtained by punchcutting as described in a previous work [24]. We created the specimens in accordance with European Standard EN ISO 527 [25].

The only standard reference found for tensile testing on plastic material was ISO 527. ISO 527 standard specimens are created by injecting the melted polymeric pellets in a standard mold. In the considered ISO, no information about the injection region was found. Consequentially, 10 specimens for each material (LDPE and LLDPE) were obtained by molding following the ISO 527 standard: 5 ISO specimens for each material were created with a polymeric injection from the side of the mold (H-ISO), and 5 ISO specimens for each material were created with a polymeric injection from the bottom of the mold (V-ISO). Using this method, horizontal and vertical polymeric chain alignments were considered in the production, and two types of specimens were obtained: horizontal (H-ISO) and vertical (V-ISO) specimens, respectively.

Each specimen was characterized in terms of thickness and width of this region using a digital microscope, model BW1008-500x (Farnell element14 Trade Counter, Leeds, UK). The section of each sample was calculated from thickness and width using

a suitable software program (Micro-Measure version 1.2, Colorado State University, Fort Collins, CO, USA).

### 3.5 Statistical analysis

The data obtained from the mechanical test on specimens were processed via the Mann-Whitney test and comparing specific tests for parametric and nonparametric data. We chose a confidence range of 95%, so the changes were considered statistically significant for $p < 0.05$.

### 3.6 Extractable testing

In order to evaluate the extractable profiles, plastic materials before and after specific treatments were subjected to different extraction conditions and the resulting extractions were analytically characterized by gas chromatography-mass spectrometry (GC/MS) to establish the material profile of each extracted volatile organic compounds. An optimization of the controlled extraction method was studied. This test procedure could provide a good baseline to determine a method for controlled extraction studies specifically relevant for the plastic materials investigated.

### 3.7 Extraction methods

Multiple extraction processes were evaluated to maximize the predominant extractable. The extraction process and extraction solvents were chosen in relation to the plastic materials investigated and according to the literature [26]. Table 2 shows the specific of extraction methods used: sonication, Sealed Vessel, Soxhlet, and Head Space Solid Phase microextraction (HS-SPME). Extraction solvents included: low pH water buffer solution pH = 2, high pH water buffer pH = 10, 1:1 isopropanol/water mixture, and hexane. All extraction processes were conducted in duplicate. **Table 2** provides the extraction methods specifications.

**Table 2.** Extraction methods specification.

| Extraction Methods | Tested Article | Condition | Specification |
|---|---|---|---|
| Sonication | 500 mg | 150mL in buffer solution pH2 and pH10 | - |
| Sealed Vessel | 500 mg | 10mL 1:1 isopropanol/water 55°C for 3 days | - |
| Soxhlet | 500 mg | 150 mL Hexane 140°C for 30 min | Soxtherm/Multistat Rapid Soxhlet Extraction System (Gerhardt) |
| HSSPME | 500 mg | Incubation temperature: 90°C, Extraction time: 60 min | Headspace-mode, Fiber 100 µmPolydimethylsiloxane (PDMS), Supelco |

### 3.8 Characterization by Gas Chromatography-Mass Spectrometry (GC/MS)

The extracts obtained from the extraction method were characterized by gas chromatography-mass spectrometry (GC/MS). Analyses were performed by using a Thermo Fisher Scientific, (Waltham, MA, USA) GC/MS system (TraceDSQII mass spectrometer, TraceGCUltra gas chromatograph, CTC Analytics COMBIPAL autosampler), and Xcalibur MS Software Version 2.2. Operating parameters are reported in **Table 3**. The mass spectra of the detected extractable compounds were compared with the GC/MS NIST Mass Spectral Library (NIST 08) databases and the 8th Edition Wiley Registry of Mass Spectral Data. Although databases were used, some classes of compounds, such as alkanes, yielded similar fingerprint patterns or fragments, and thus we were not always able to cleanly identify every peak (compound) detected.

**Table 3.** Operating parameters of gas chromatography-mass spectrometry (GC/MS) analysis.

| **Operating Parameters** | **Organic extracts** | **Headspace(HS-SPME)** |
|---|---|---|
| Column | Restek capillary column Rtx-5MS 30 m x 0.25 mm ID x 0.25 µm | Restek capillary column Rtx-5MS 30 m x 0.25 mm ID x 0.25 µm |
| Oven Program | Start 50 °C, hold for 1 min; ramp 12 °C/min to 315 °C, hold for 16 min | Start 60°C, hold for 4.5 min; ramp 20 °C/min to 280 °C, hold for 5 min |
| Injector | CT Split/Splitless 300 °C, Split flow 10 mL/min, Split ratio 1:10 | PTV Splitless 250°C, Splitless time 4.5 min |
| Injection | Split, 1µL | - |
| Carrier Gas | He, 1 mL/min constant flow | He, 1 mL/min constant flow |
| MS Transfer line temperature | 290 °C | 270°C |
| MS Detection Details | 70 eV (+EI), Ion source 250 °C, Mass range 35-650 amu, Scan rate 803.7 amu/sec | 70 eV (+EI), Ion source 250 °C. Mass range 50-650 amu, Scan rate 870 amu/sec |

## 4 Results

### 4.1 Tensile test specimens

Thickness of the LLDPE and LDPE specimens created according to ISO 527 specifications [14] and LLDPE and LDPE dog-bone shaped specimens obtained from the final single-dose packaging containers were calculated to determine if the specimens were uniform. **Table 4** shows the thickness means obtained.

**Table 4.** Thickness data mean obtained on ISO 527 specimens and dog-bone shaped specimens made of linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) expressed as a mean ±% standard deviation.

| **LLDPE (H-ISO)** | **LLDPE (V-ISO)** | **LDPE (H-ISO)** | **LDPE (V-ISO)** | **LLDPE** | **LDPE** |
|---|---|---|---|---|---|
| **Thickness (mm)** | | | | **Thickness (µm)** | |
| 4.1 (±1.69) | 4.2 (±1.01) | 4.2 (±0.79) | 4.1 (±0.92) | 610.4 (±1.73) | 565.6 (±1.17) |

The tensile test was performed on the samples. **Table 5** shows tensile strength (σM), tensile strain at yield (εy), angular coefficient linear portion, tensile stress at break (σB), and tensile strain at break (εtB) data obtained from the tensile test on the ISO and dog-bone shaped specimens expressed as a mean ± standard deviation (%).

**Table 5.** Tensile test data obtained from ISO and dog-bone shaped specimens made of linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) expressed as a mean ±%standard deviation.

| Parameter | Sample | | | |
|---|---|---|---|---|
| | **LDPE (H-ISO)** | **LDPE (V-ISO)** | **LLDPE** | **LDPE** |
| Tensile strength (σM) = yield stress (σy) (MPa) | - | - | 7.5 (±7.97) | 6.7 (±4.29) |
| Tensile strain at yield (εy) = (εM) (%) | - | - | 9.4 (±17.02) | n. d. |
| Angolar coefficient linear portion | - | - | 123.6 (±16.87) | 110.7 (±9.51) |
| Tensile stress at break (σB) (MPa) | 10.0 (±3.00) | 83.5 (±2.96) | 10.8 (±8.53) | 7.7 (±9.15) |
| Tensile strain at break (εtB) (%) | 9.8 (±4.41) | 83.1 (±2.68) | 290.0 (±12.81) | 86.4 (±25.44) |

- Data not obtained. n.d. not determined.

Single-dose LLDPE and LDPE containers, either empty or filled with simulant, were subjected to accelerated stability testing. A total of 25 dog-bone shaped specimens of each sample were analyzed with the tensile test. A graph representing the yield point value trend for LLDPE and LDPE are reported in **Figure 1**.

### 4.2 Extract characterization

The volatile organic extractable profile of the plastic materials obtained from untreated final packaging was investigated using multiple extraction processes. Extraction solvents were used in order to detect the technique able to identify the major class of components in the plastic material.

**Figures 2** and **3** show the Total Ion Current (TIC) chromatograms related to the GC/MS analysis of the various extracts obtained from untreated LLDPE and LDPE single dose containers.

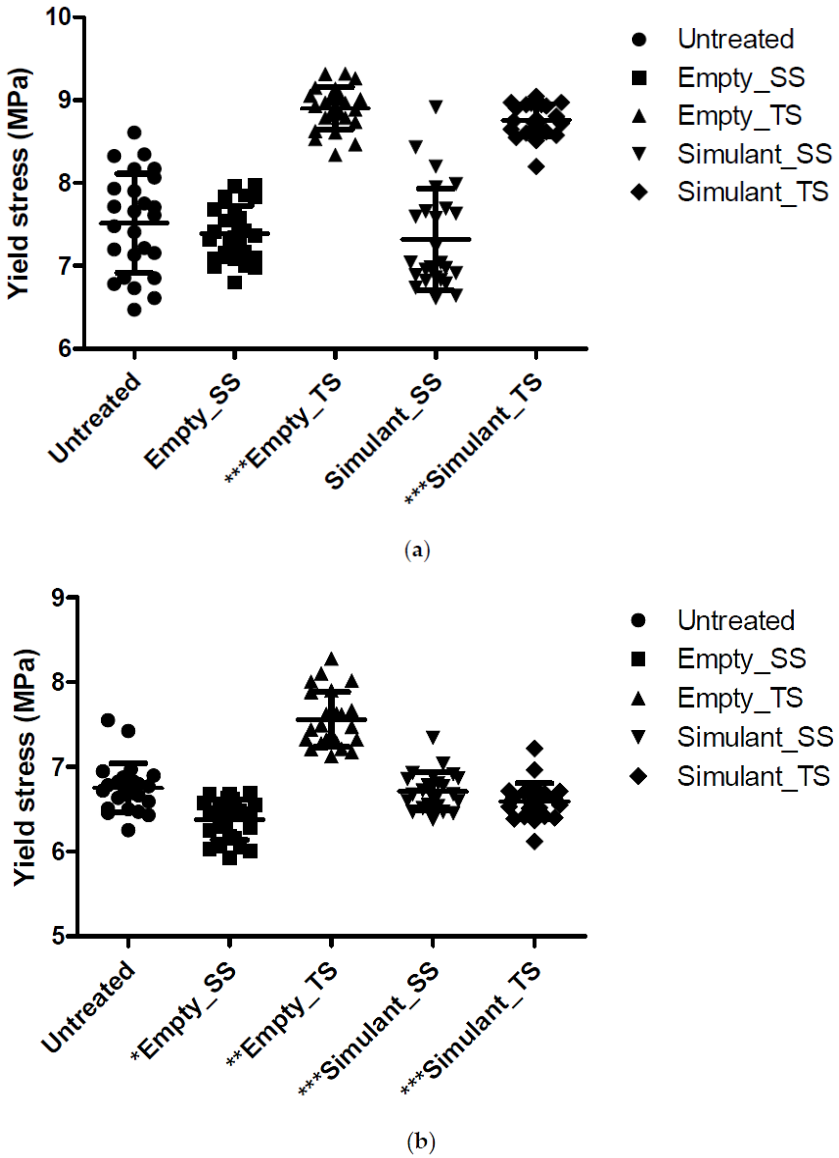


**Figure 1.** Trend of yield stress values for containers filled with simulant after simulated solar irradiation (SS) or thermal shock (TS): (**a**) Linear low-density polyethylene (LLDPE) packaging (statistical significance: *** $p < 0.0001$ compared to Untreated); (**b**) Low-density polyethylene (LDPE) packaging (statistical significance: * $p = 0.2$ compared to Untreated; ** $p = 0.025$ compared to Untreated; *** $p < 0.0001$ compared to Untreated).

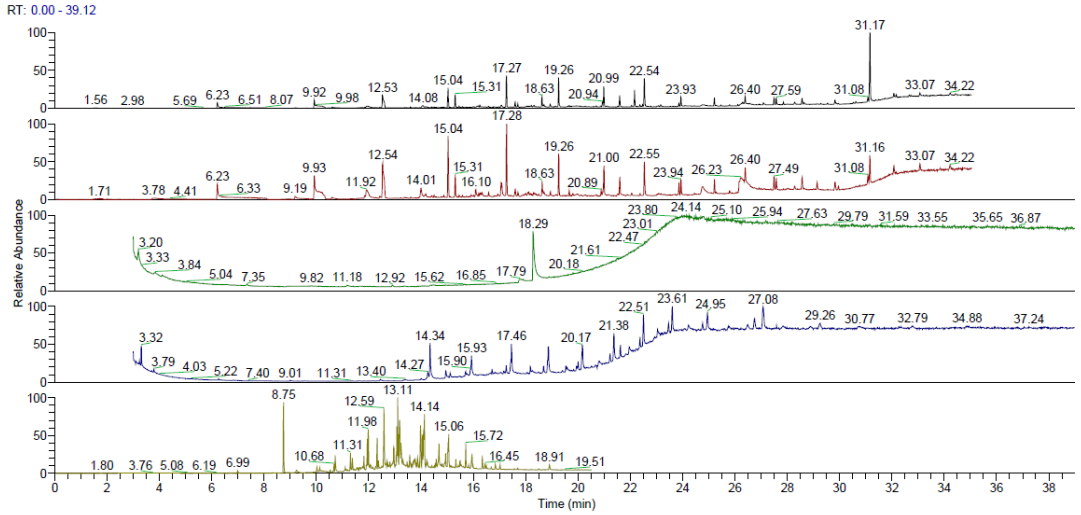


**Figure 2.** Chromatograms obtained by gas chromatography-mass spectrometry (GC/MS) of all the extraction methods on untreated linear low-density polyethylene (LLDPE) single dose containers. From the top: Sonication pH 2, sonication pH 10, Sealed Vessel, Soxhlet, and HS SPME (Head Space Solid Phase microextraction).

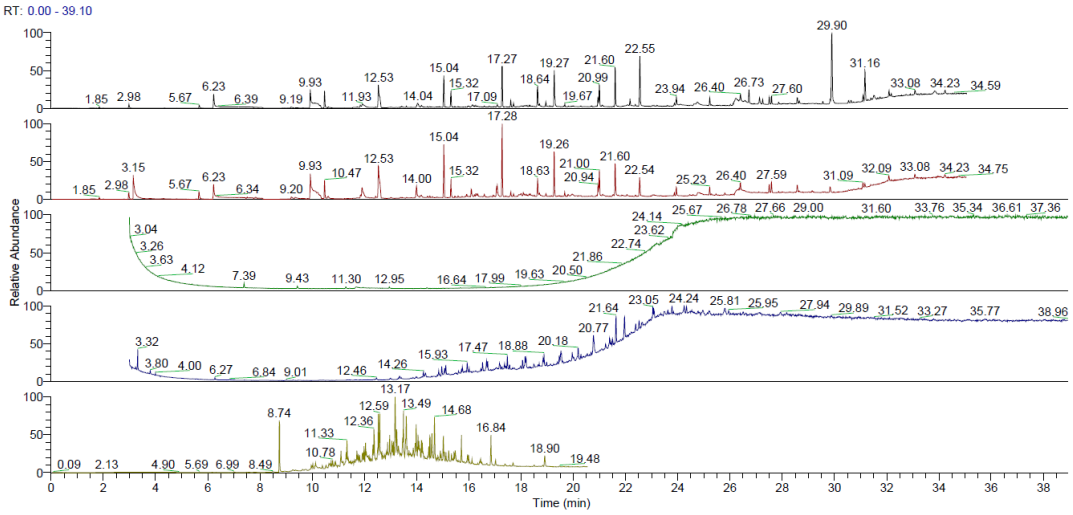


**Figure 3.** GC/MS chromatograms obtained of all the extraction methods for untreated low-density polyethylene (LDPE) single dose containers. From the top: Sonication pH 2, sonication pH 10, Sealed Vessel, Soxhlet, and HS SPME (Head Space Solid Phase microextraction).

After subtraction of the extraction blanks from the samples and removal of the interfering peaks through bleeding the GC capillary column or SPME fiber coating, more than 100 substances were identified. For simplification, the HS-SPME extraction method associated with GC/MS analysis was chosen. The detected substances were divided into three different categories: (1) compounds associated with the initial ingredients (e.g., antioxidants, additives, and amides); (2) impurities related to processing (e.g., oligomers, residual solvents, esters, and siloxane); and (3)

degradation products of polymers (e.g., fragments of saturated and unsaturated hydrocarbons, ketones, and acids), as previously reported [27]. **Table 6** shows some substances detected from untreated LLDPE and LDPE single dose containers.

**Table 6**. Organic extractable profile extracted using the Head Space Solid Phase microextraction (HS-SPME) method and analyzed by gas chromatography-mass spectrometry (GC/MS) expressed as a percentage of linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) from empty untreated final packaging divided into three different categories: (1) compounds associated with the initial ingredients, (2) impurities related to processing, and (3) degradation products of polymers.

| **Compound Categories** | **Identification** | **CAS NR** | **Chemical Formula** | **Molecular Weight** | **LLDPE** (% area) | **LDPE** (% area) |
|---|---|---|---|---|---|---|
| **1** | 2,4-Di-t-butyl phenol | 96-76-4 | $C_{14}H_{22}O$ | 206 | 0.11 | 0.26 |
| | Hexadecanamide | 629-54-9 | $C_{16}H_{33}NO$ | 255 | traces | traces |
| | 9-Octadecenamide, (Z)- | 301-02-0 | $C_{18}H_{35}NO$ | 281 | traces | traces |
| | Hexadecyl 2- | 59130-69- | $C_{24}H_{48}O_2$ | 368 | 12.39 | traces |
| | Diisobutyl phthalate | 84-69-5 | $C_{16}H_{22}O_4$ | 278 | 2.26 | 5.05 |
| | Dibutyl phthalate | 84-74-2 | $C_{16}H_{22}O_4$ | 278 | 2.80 | 3.81 |
| | Irganox 1076 | 2082-79-3 | $C_{35}H_{62}O_3$ | 530 | 11.94 | 3.82 |
| | Diisooctyl phthalate | 131-20-4 | $C_{24}H_{38}O_4$ | 390 | 0.32 | 5.44 |
| **2** | Myristyl myristate | 3234-85-3 | $C_{28}H_{56}O_2$ | 424 | traces | traces |
| | Octinoxate | 5466-77-3 | $C_{18}H_{26}O_3$ | 290 | 3.01 | 1.47 |
| **3** | Aliphatic hydrocarbons* | - | - | - | 57.14 | 61.56 |
| | Olefins* | - | - | - | 7.39 | 10.26 |

CAS NR: Chemical Abstract Service Number, * class of compounds.

LLDPE and LDPE single dose containers, both empty and filled with simulant, were characterized after an accelerated stability test. Extractable profiles of the treated samples were created via HS-SPME extraction and the extracts were analyzed by GC/MS. **Table 7** presents the percent area of each class of extracted substances.

**Table 7.** Percent area of substances extracted with the Head Space Solid Phase microextraction (HS-SPME) method and analyzed by GC/MS from linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) single dose containers, both empty and filled with simulant, after simulated solar irradiation (SS) and thermal shock (TS), compared with untreated samples.

| Compound Categories | LLDPE (%area) | | | | | LDPE (%area) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Empty | Empty | | Filled | | Empty | Empty | | Filled | |
| | Untreated | SS | TS | SS | TS | Untreated | SS | TS | SS | TS |
| **Compounds associated with the initial ingredients** | 17.4 | <0.01 | 2.4 | 0.04 | 0.2 | 18.1 | <0.01 | 2.1 | <0.01 | 0.8 |
| **Compounds related to processing** | 18.0 | 1.3 | 17.5 | 3.4 | 3.3 | 10.1 | 0.8 | 14.7 | 4.0 | 3.1 |
| **Degradation products of polymers** | 64.52 | 98.7 | 80.0 | 1.8 | 1.9 | 71.82 | 99.2 | 83.2 | 1.1 | 1.0 |
| **Compounds absorbed from simulant** | - | - | - | 94.8 | 94.7 | - | - | - | 94.8 | 95.7 |

After simulated solar irradiation or thermal shock of the filled samples, substances closely related to the simulant were detected in the extractable profile of the plastic material. These substances were identified as Decamethylcyclopentasiloxane-Cyclomethicone D5 and Bis(2-ethylhexyl) carbonate, and they represented nearly 95–96% of the total extracted compounds. **Figure 4** shows chromatograms of the LLDPE and LDPE single dose containers filled with simulant after simulated solar radiation.

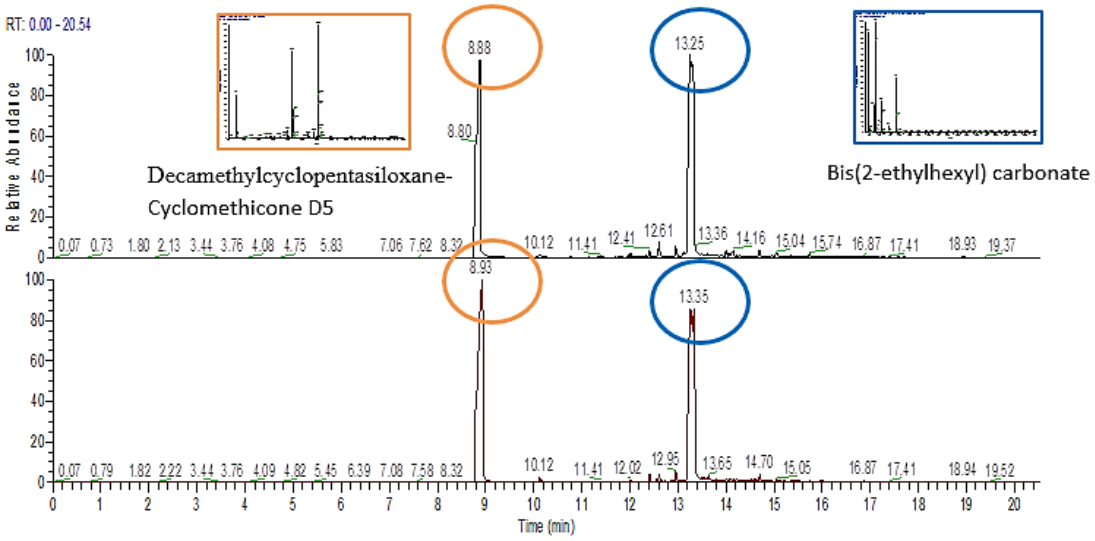


**Figure 4.** Chromatograms for (top) linear low-density polyethylene (LLDPE) and (bottom) low-density polyethylene (LDPE) single dose containers filled with simulant after simulated solar irradiation. (RT: retention time expressed in minutes).

In order to exclude the possibility that the identified substances simply remained on the surface of the polymers in a non-efficient washing system, LLDPE and LDPE samples from the final packaging were placed in the simulant for 30 min. Then, they were cleaned with the washing method used for all the samples. An extractable profile, with the selected test technique (HS-SPME), was created. After GC/MS analysis, no traces of the substances associated with the simulant were found. Successively, a preliminary study was completed to evaluate the substances realized from the final packaging and migrated to the simulant. When the LLDPE and LDPE final packaging were emptied, the simulant was preserved. Samples of simulant (300 mg) treated with simulated solar irradiation or thermic shock in LLDPE and LDPE final packaging containers were analyzed by HS-SPME/GC-MS. No substances related to the polymeric materials were detected within the formulation.

### 4.3 Simulant characterization

pH measurements and organoleptic properties analysis showed no changes between untreated simulant and treated simulant in the LLDPE or LDPE single dose container. The evaluation of the rheological properties of the simulant underlined that the two different types of materials did not change in terms of viscosity or the rheological

behavior of the content. The viscosity of the simulant contained in the two types of plastic material was unaltered after both treatments. **Figures 5** and **6** show the viscosity curve and elastic and viscous modulus curves of the simulant in LLDPE and LDPE vs. untreated simulant.

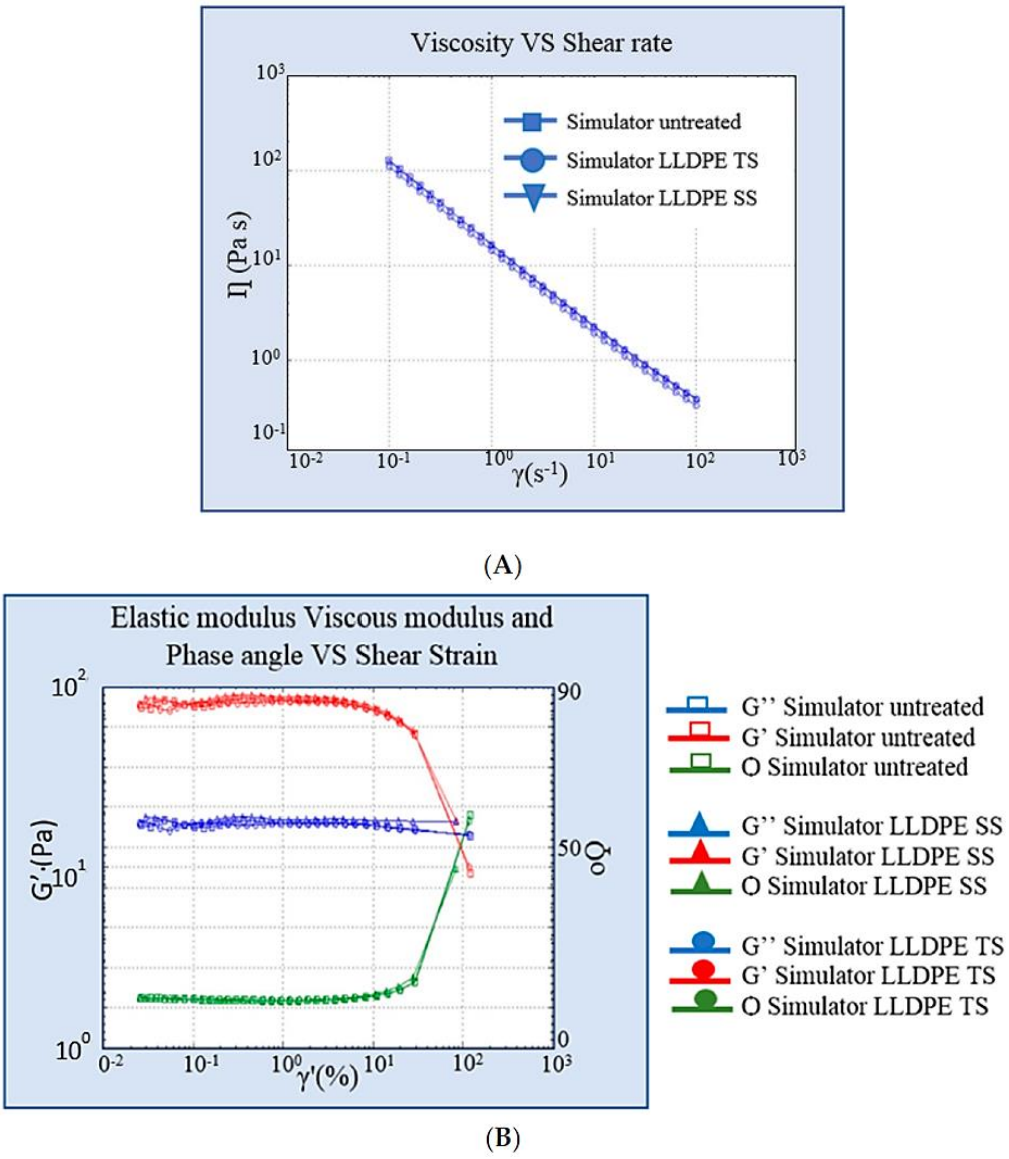


**Figure 5.** (A) Viscosity curve of simulant in linear low-density polyethylene (LLDPE) vs. untreated simulant. (B) Elastic and viscous modulus curves of formulation in LLDPE vs. untreated simulant. SS: simulated solar irradiation, TS: thermal shock.

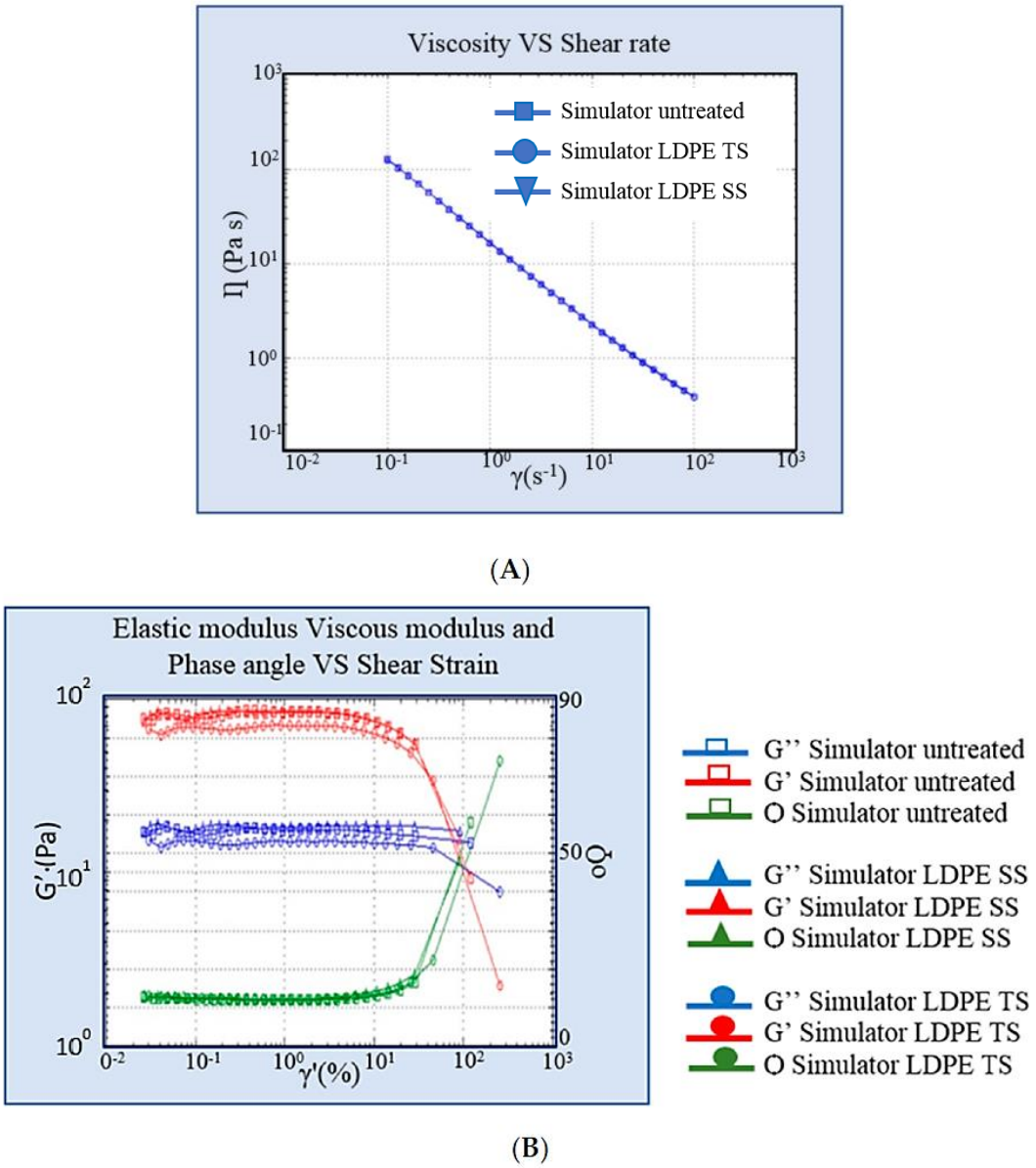


**Figure 6.** (A) Viscosity curve of simulant in low-density polyethylene (LDPE) vs. untreated simulant. (B) Elastic and viscous modulus curves of simulant in LDPE vs. untreated simulant. SS: simulated solar irradiation, TS: thermal shock.

## 5 Discussion

In this study, we investigated the differences between standardized samples and those obtained from final packaging samples. Evaluations of final packaging provided information about the materials after manufacturing processes and the possible interactions between the packaging and the content. Some interactions between packaging and content could occur during in-use conditions. The literature reports a correlation between the changes in the mechanical behavior and changes in the barrier

properties, resulting in changes in quality, efficacy, and safety of the products [28]. Therefore, we evaluated the mechanical characteristics.

In the first place, thickness testing was performed on ISO and dog-bone shaped specimens. Among the different polymer processing techniques used to produce plastic packaging with the desired size, shape, and characteristics, injection molding is the most used in the polymer industry. A critical aspect of the replication is the high precision involved [29,30]. Wall thickness testing allowed us to determine if the plastic material was uniform or, during the blow-molding process, the uniformity was not optimized, compromising the quality of the product [31].

Table 4 shows the thickness data mean obtained on ISO 527and dog-bone shaped specimens expressed as a mean ± % standard deviation. Differences in the thickness values between the two materials are displayed. Those differences are related to the intrinsic characteristics of the polymer and the manufacturing process. ISO specimens are created via the high pressure injection of the melted polymeric pellets in a standard dimension mold. No information about the injection region was specified on ISO 527; thus, ISO specimens were created by injection at the bottom and injection at the side of the mold. Specimens produced by injection at the bottom have vertical polymeric chain orientation (LLDPE and LDPE vertical) and specimens produced by injection at the side have a horizontal polymeric chains orientation (LLDPE and LDPE horizontal). The thickness of the LLDPE and LDPE ISO specimens were homogenous, between 4.1 to 4.2 mm. This homogeneity, however, was hard to attain with specimens obtained from the final packaging, where the thickness is regulated by the packaging production and destination. The mean LLDPE and LDPE single dose container thicknesses were 610.4 and 565.6 µm, respectively.

Table 5 shows the tensile test data obtained from ISO and dog-bone shaped specimens expressed as a mean ± % standard deviation. Obtaining Horizontal and Vertical LLDPE and LDPE stress-strain values at yield was not possible because the stress-strain profile obtained from the tensile test was not clearly detectable. Similarly, it was not possible to obtain Horizontal and Vertical LLDPE stress-strain values at break since the material elongation was greater than the instrument tensile stroke in the set conditions (10 mm/min), showing the lack of versatility of the standardized specimen. **Figure 7** shows the ISO-LLDPE profile as an example.

**Figure 7.** ISO-LLDPE (linear low-density polyethylene) specimen during tensile test.

Data obtained from the LLDPE and LDPE dog-bone shaped specimens tensile testing highlighted the different mechanical behavior in term of elongation percentage and stress (MPa) between the two polymers. The values of stress at yield point and at break had standard deviations within a range of 10%, demonstrating the high

homogeneity. Starting from this low standard deviation, those values can, therefore, be considerate as significant parameters for evaluating possible changes in the mechanical properties of plastic material considered before or after the treatment and/or in contact with a simulant. For the other parameters, like strain and the angular coefficient, a higher standard deviation was found, so these parameters were not further considered as relevant to underline possible changes undergone by the material after stress.

Afterward, we evaluated the plastic material mechanical properties by mimicking the in-use conditions. This step was not possible with the ISO samples. The LLDPE and LDPE single dose container, both empty or filled with the simulant, were exposed to accelerated stability testing and analyzed using the tensile test. The use of silicones in a broad range of products has exponentially increased; thus, the simulant contained a silicon component in order to simulate the performance and the interaction of a formulation commercially available [21,22]. A future work could propose different simulants to reflect a complete range of formulation properties.

Figure 1 shows a graph representing the trend in the yield point values and the statistical analysis completed using the Mann-Whitney test with a 95% confidence interval on LLDPE and LDPE dog-bone shaped specimens before and after the treatments. Simulated solar radiation did not induce significant changes in the LLDPE polymer mechanical characteristics. No significant variations in the mechanical properties were recorded for the polymer in contact with simulant after the treatment. Otherwise, results for the LDPE polymer showed that simulated solar radiation induced statistically significant changes in the mechanical characteristics of this polymer and induced some interactions between the formulation and the container detectable at the level of alterations of the mechanical properties of packaging composed of this polymer.

Conversely, thermal shock treatment significantly influenced the mechanical behavior of both empty polymers. Furthermore, in both polymers, the simulant significantly interacted with the container when subjected to thermal shock.

### 5.1 Extractable characterization

Many protocols used to obtain the extractable profile of plastic material have been studied. All these protocols focus on the raw material (pellets) but no information was found about the extractable profile of the plastic material obtained from the final

packaging. As explained previously, during the manufacturing process, some additives are used, which can create a complex extractable profile. In this part of the study, the volatile organic extractable profile of the plastic materials obtained from untreated final packaging was investigated. Multiple extraction processes and extraction solvents were used to detect the technique able to identify the major class of components. This work group, previously, determined three different categories of detectable components, as reported in Table 6: (1) compounds associated with the initial ingredients, (2) impurities related to processing, and (3) degradation products of polymers [27].

A preliminary study was performed on the untreated LLDPE and LDPE. Multiple extraction processes were evaluated in relation to the plastic materials investigated and according the literature [27]: Sonication, Sealed Vessel, Soxhlet, and HS-SPME. Figures 3 and 4 show the GC/MS chromatograms of all the extraction methods of untreated LLDPE and LDPE single dose containers. Chromatographic analyses showed that HS-SPME and Soxhlet extractions provide complete insight into all the major organic extracts for the analyzed materials. The HS-SPME extraction contained the same extracts as n the Soxhlet extraction, but with higher concentrations, so the methods were optimized. HS-SPME extraction was defined as the best method for performing the controlled extraction of volatile organic extracts in plastic material obtained from the final packaging.

LLDPE and LDPE single dose containers, both empty and filled with simulant, were characterized after accelerated stability testing. The extract profiles of the treated samples were obtained with HSSPME extraction and the extracts were analyzed by GC/MS.

Table 7 shows the percent area of each class of extracted substance. In filled samples after simulated solar irradiation or thermal shock, substances closely related to the simulant were detected in the extractable profile of the plastic material. These substances were identified as Decamethylcyclopentasiloxane-Cyclomethicone D5 and Bis(2-ethylhexyl) carbonate. They represented nearly 95–96% of the total extracted compounds (Figure 4). To exclude the possibility that the identified substances simply remained on the surface of the polymers in a non-efficient washing system, LLDPE and LDPE samples from the final packaging were placed in the simulant for 30 min. Then, they were cleaned with the washing method used for all the samples. An extractable profile, with the selected test technique (HS-SPME), was performed. After

GC/MS analysis, no traces of the substances associated with the simulant were found. This could strongly indicate the efficacy of the washing system used, confirming that some simulant substances could have been adsorbed by the packaging material examined in specific stress conditions.

In addition to assessing the extractable profiles, a first screening was used to evaluate the substances leached from the final packaging that migrated to the simulant. When the LLDPE and LDPE final packaging were emptied, the simulator was preserved. Samples of simulant (300 mg), treated with simulated solar irradiation or thermic shock in LLDPE and LDPE final packaging containers, were analyzed by HS-SPME/GC-MS. No substances related to the polymeric materials were detected within the formulation. The limit of detection is one of the most important topics in extractable and leachable analysis. The Safety Concern Threshold (SCT) below 0.15 µg/day has been defined as the leachable threshold that would present negligible safety concerns from possible carcinogenic to noncarcinogenic toxic effects [32]. Results obtained from this study suggest that the phthalate levels would be below the SCT level of 0.15 µg/day. This work was largely qualitative. Future studies will focus on quantifying the leachable amount according to the safety assessment depending on the product category and exposure levels during use.

### 5.2 Simulant characterization

pH measurements and organoleptic properties analysis showed no changes between the untreated and treated simulants containing LLDPE/LDPE single dose containers. Figures 5 and 6 demonstrate the viscosity curve and the elastic and viscous modulus curves of the simulant after simulated solar testing in LLDPE and LDPE vs. the untreated simulant. The evaluation of the rheological properties of the simulant through the rheometer showed that either the viscosity or the rheological behavior of the content of the two different types of materials did not change. The viscosity of the simulant contained in the two types of plastic material were unaltered after both treatments.

## 6 Conclusions

In this study, we investigated the differences between standardized samples and final packaging samples. We compared ISO and dog-bone shaped specimens. ISO specimens were homogeneous in terms of thickness, but they could not be used to

analyze the final packaging. Instead, the dog-bone shaped specimens used in this work were successfully employed for all packaging shapes, including small packages as single units.

Among the mechanical parameters, the yield stress at and the stress at break point were better indicators for evaluating any changes in the material characteristics before and after treatments.

From results obtained in this study, the thermal shock is the better stress condition for evaluating LLDPE packaging mechanical properties and the possible interaction between content and container. Otherwise, results for the LDPE polymer showed that both simulated solar radiation and thermal shock treatment induced statistically significant changes in the mechanical characteristics of this polymer. Both induced some interactions between the formulation and the container detectable at the level of alterations of the mechanical properties of packaging composed of this polymer.

A preliminary study on the untreated LLDPE and LDPE was performed by multiple extraction processes according to the literature [24]. HS-SPME extracts provided complete insight into all the predominant volatile organic extracts. HS-SPME was selected as the test method to perform the successive controlled extraction studies. Analysis for both untreated and treated empty polymers showed that the largest percentage of compounds extracted are associated with the polymer degradation products. Instead, in both polymers that contacted the simulant after treatments, substances closely related to the simulant were detected at relatively high levels. These substances were identified as Cyclopentasiloxane and Bis(2-ethylhexyl) carbonate. The migration of product components represents an important factor for packaging quality and safety for human health. In the food industry, regulations for materials and packaging expected to contact food are in place to ensure constituents that could affect human health are not transferred. The food approach may also be used in the cosmetic industry.

This work underlined the importance of correctly studying the packaging material in relation to the content, to be able to detect possible interactions.

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## 3 CONCLUSION

Researches conducted during these PhD studies showed that the encapsulation technology represents a high potential system of vehiculation for UV-filters compounds. They allow to stabilize the incorporated substances, reducing the penetration into the skin and modulate its release. More specifically, the incorporation in a sol-gel silica capsule of a synergic combination of UV filters (avobenzone-octocrylene) had improved the sunscreen photostability with a reduction in cutaneous penetration. In this way there is an improvement in the photoprotective efficacy and, also, the toxic effects due to the interaction with the deepest layers of the skin are avoided (Chapter I). Another aspect that has been investigated is that UV exposure deeply disorganize and modify the SC lipid organization and composition, compromising dramatically the skin's nature ability to protect us, thus, its fundamental barrier function. In specific, a modification of the supramolecular order of the lipids was observed over time after the irradiation, showing that the SC lipids are losing their organization/arrangement. Even the content of SC lipids decreased significantly already after short-term exposure, and the decrease proceed with an exposure-dependent behavior. These modifications could alter drastically the skin barrier functions and, often, the abnormal SC lipid profiles is associated with common skin diseases (e.g. actinic dermatosis, psoriasis) presenting faulty permeability barrier function (Chapter II). The knowledges about the effects of UV radiation on skin has been increased, opening the investigation on the impact of different environment stresses and the potential consequences.

This work of thesis, also, has investigated and clarified the sunscreen long-term utility. Results suggest that there is a discrepancy between in real life sun-protective behaviors and sunscreen application patterns versus the "ideal" sunscreen employment. The supposed sunscreen long-term inactivity could be caused by erroneous patterns of use that make inaccurate expectations. An essential passage for an efficient prevention program for skin cancer is an improved public health education to adopt adequate sun exposure attitude (Chapter III). From the results obtained it was possible to demonstrate that sunscreen use is associated with a reduction in melanoma, squamous cell carcinoma and precancerous skin lesions supplying a scientific tool that uses a reproducible and transparent approach to summarize the results of individual clinal trial about the effectiveness of sunscreen as preventive tool, creating a starting point for a future pharmacoeconomic evaluation (Chapter IV).

Research work conducted during this PhD studies evaluated the products loss of efficacy during the conservation period and the possible mass transfer process of organic and lipophilic compounds (such as UV filters) in-real use condition, evaluating the interaction packaging-product contained. In specific, a suitable and practical protocol to evaluate commercial polymeric containers has been defined based on mechanical testing and extractables' analysis. This protocol allowed to perform safety evaluation on the finished product (Chapter V). A successive protocol optimization, consent to evaluate the interaction between a semisolid formulation (simulant) and final packaging, underling the differences between testing standard ISO specimens versus testing finished packaging (Chapter VI). Both works underlined the importance of correctly studying and selecting the packaging material in relation to the content to be able to improve the products efficacy and safety.

In conclusion, innovative technological strategies are able to improve safety and efficacy of prevention products such as sunscreen, the knowledges about the effects of UVR radiation on the skin should be improved in order to fully understand the changes in skin structure and composition after exposure. Primary prevention is essential to reduce the UV induced damaged risks and to instruct the population on the correct sun-protection behaviors. The selection of the packaging in relation to the product contained is an essential step to make in order to guarantee an efficient conservation of the product. For a complete evaluation of product safety, the interaction packaging-contained should be evaluated with a suitable approach.