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RESEARCH ARTICLE

Melatonin-based pickering emulsion for skin's photoprotection

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Abstract

Context: Based on its antioxidant activity, melatonin was recently found to have a protection effect against photocarcinogenesis.

Objective: This work aimed to develop an innovative sunscreen formulation based on the Pickering emulsions concept, stabilized by physical UV filters, modified starch and natural oils associated to melatonin as a key strategy for prevention against UV-induced skin damage.

Materials and methods: For this purpose, melatonin was incorporated in Pickering emulsions that were characterized using physicochemical, *in vitro* and *in vivo* testing. Physicochemical studies included physical and chemical stability by a thorough pharmaceutical control. The possible protective effects of melatonin against UV-induced cell damage in HaCaT cell lines were investigated *in vitro*. The safety assessment and the *in vivo* biological properties of the final formulations, including Human Repeat Insult Patch Test and sunscreen water resistance tests were also evaluated.

Results and discussion: These studies demonstrated that melatonin sunscreen Pickering emulsion was beneficial and presented a powerful protection against UVB-induced damage in HaCaT cells, including inhibition of apoptosis. The inclusion of zinc oxide, titanium dioxide, green coffee oil and starch ensured a high SPF (50+) against UVA and UVB.

Conclusion: The combination of melatonin, multifunctional solid particles and green coffee oil, contributed to achieve a stable, effective and innovative sunscreen with a meaningful synergistic protection against oxidative stress.

Keywords

Antioxidant, melatonin, photodamage, pickering emulsion, topical delivery

History

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Introduction

Skin cancer is one of the most common types of cancer worldwide with an increasing incidence, mainly the non-melanoma skin cancer (NMSC). In addition, there is an arising group of high-risk patients submitted to surgeries, chemotherapy and radiotherapy, which have an increased risk of developing NMSC, either as basal cell carcinoma or squamous cell carcinoma (Hofbauer et al., 2010; Rangwala & Tsai, 2011). Ultraviolet radiation (UVR) from sun exposure is the main etiological agent for skin cancer (Hadshiew et al., 2000; Yaar & Gilchrest, 2001) and the UV-induced cell damage occurs by two mechanisms: (a) development of cyclobutane–pyrimidine dimers (Ahmed et al., 1999); (b) generation of reactive oxygen species (ROS), which are also involved in mutagenesis and carcinogenesis events. Therefore, avoiding sun exposure, covering the skin and applying sunscreens with a high degree of protection are the leading strategies recommended to prevent UV-induced cell damage.

Melatonin (N-acetyl-5-methoxytryptamine, AMK) is a well-known neuroendocrine mediator discovered by Lerner (1960), and mostly produced by the pineal gland that follows a circadian light-dependent rhythm of secretion (Lerner et al., 1960; Marshall et al., 1996; Papagiannidou et al., 2014). This hormone can be also produced by retina, bone marrow, gastrointestinal tract, gonads, immune system and skin (Rezzani et al., 2014). In skin, melatonin can be synthesized by normal and malignant keratinocytes (*stratum corneum*), melanocytes (epidermis) and fibroblasts (dermis). This synthesis may be induced or modified by UVB irradiation (Kleszczynski & Fischer, 2012; Rezzani et al., 2014; Kim et al., 2015a). Melatonin receptors are G protein-coupled receptors, and there are two subtypes in humans (MT₁, MT₂). MT₁ receptors expression has been detected by immunocytochemistry in skin (*stratum granulosum*, *stratum spinosum*, upper and inner epithelial root sheath, eccrine sweat gland and blood vessel endothelium). The MT₂ receptors expression has been detected in the inner epithelial root sheath, eccrine sweat gland and blood vessel endothelium (Słominski et al., 2002, 2012b; Kim et al., 2015b).

The melatonin-biosynthesis pathway in skin, similar to what happens in other organs, is divided into four stages, initiated by the uptake of the essential amino acid

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L-tryptophan by pineal parenchymal cells (Slominski et al., 2008, 2014a). After this, L-tryptophan is converted to another amino acid, 5-hydroxytryptophan due the action of tryptophan hydroxylase enzyme (TPH), which is dependent on (6R) 5,6,7,8-tetrahydrobiopterin (6-BH4). There are two isoforms of tryptophan hydroxylase identified as TPH1 and TPH2. The first one is expressed in many peripheral tissues including the skin, whereas TPH2 is expressed predominantly in the central nervous system (Zhang et al., 2004). Thus, TPH1 is the responsible enzyme for the production of melatonin at skin level (Slominski et al., 2005a).

Melatonin is metabolized through enzymatic as well as chemical reactions in a total of three major metabolic pathways: classic, indolic and kynuric (Slominski et al., 2014a,b). All these pathways are operative in skin (Kim et al., 2015b). The main metabolites are 5-methoxytryptamine (5-MTT), Cyclic 3-hydroxymelatonin (3-OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), AMK, 6-hydroxymelatonin sulfate (epidermis major metabolite), 2-hydroxymelatonin, 1-nitromelatonin and nitrosomelatonin. Due to its biological significance, three of these metabolites are more widely studied, namely, 3-OHM, AFMK and AMK (Fischer et al., 2006; Galano et al., 2015). Moreover, the metabolism of melatonin is cell-type dependent and expressed in all three main cell populations of human skin – keratinocytes, melanocytes and fibroblasts. The skin cell type-specific metabolism, which determines the biological effects of melatonin, has not been established up to the Kim et al. work. The main conclusions of this multidimensional experimental study were as follows: (a) identification of 6(OH)M and AFMK as the main metabolites of the melatonin degradation; (b) identification of the indolic pathway as the predominant route of melatonin metabolism; (c) importance of the kynuric pathway in organs exposed to environmental stress such as skin, in contrast to the systemic metabolism, in which AFMK may be below or at the limit of detection; (d) differences in the rate of melatonin metabolism dependent on cell type (Kim et al., 2013).

Probably, this metabolization exhibits species-, site- and tissue compartment-dependent differences which are modulated by several factors, such as environmental UVR or local adrenergic stimulation levels and heme proteins (Slominski et al., 2012a).

As it is involved in several physiological processes related to circadian rhythms, seasonal reproduction, retinal function, etc melatonin also seems to participate in seasonal and non-seasonal skin changes, hair growth and pigmentation (Papagiannidou et al., 2014; Rezzani et al., 2014). It acts as an immunomodulator, anti-inflammatory and potent antioxidant agent by down-regulating several pro-inflammatory cytokines and up-regulating antioxidant enzymes, besides being an extra- and intracellular free radical scavenger of ROS and RNS, able to quench the hydroxyl radical, among others (Fischer et al., 1999; Rodriguez et al., 2004; Fischer et al., 2013). The ability of melatonin to act as an antioxidant, by itself and through its active metabolites, makes it particularly efficient either *in vitro* or *in vivo*, even at a low concentration (Kostoglou-Athanassiou, 2013; Janjetovic et al., 2014; Slominski et al., 2014a). Several reports in the literature emphasize the superior antioxidant activity of this

molecule, when compared to other antioxidants (e.g. ascorbic acid or vitamin C; vitamin E, etc.) (Martín et al., 2000; Yalcinkaya et al., 2009). Moreover, it also inhibits the cAMP signal transduction pathway when it binds to the MT_{1A} receptor and activates phospholipase C, potentiating the release of arachidonate (Slominski et al., 2012b; Singh & Jadhav, 2014). By activating cytoprotective pathways, counteracting oxidative stress and suppressing the damaged cells proliferation, melatonin has been shown to provide cellular protection against UVR damage. In fact, numerous studies have recognized the photoprotective effects of melatonin after topical application (Reiter et al., 1997; Slominski et al., 2005b; Kleszczynski & Fischer, 2012), including reduction of UV-induced erythema, protection against oxidative damage caused by UVA rays and prevention of both photoageing and photocarcinogenesis (Papagiannidou et al., 2014).

In addition, melatonin may function as an antimutagenic and anticarcinogenic agent, capable of suppressing all three stages of carcinogenesis (initiation, promotion and progression) (Pugazhenthil et al., 2008; Papagiannidou et al., 2014; Rezzani et al., 2014). Therefore, these properties suggest that melatonin may be a promising candidate as a new and effective sun protective agent especially for immunosuppressed patients (Scheuer et al., 2014).

However, the short half-life (1 h) and chemical instability of melatonin may limit its therapeutic use. Consequently, melatonin is a challenging drug for stabilization in a suitable prolonged-release sunscreen formulation. In this context, Pickering emulsions are excellent candidates to stabilize this radical scavenger on a safe and effective manner. Pickering emulsions, i.e. surfactant-free liquid or semi-solid systems stabilized by solid particles (SP) present important advantages over the classic surfactant-based emulsions, such as a higher tolerability, and easy production (Laredj-Bourezg et al., 2012).

Therefore, the major aim of this research study was to develop and characterize a melatonin-based innovative sunscreen formulation based on semi-solid Pickering emulsions stabilized by physical UV filters and natural oils associated to melatonin as a key strategy to prevent UV-induced skin damage. Physicochemical characterizations as well as *in vitro* and *in vivo* testing were performed. Studies included physical testing and chemical stability of melatonin by a thorough pharmaceutical control. The Sun Protection Factor (SPF) was determined and the skin permeation and retention was evaluated *in vitro* using newborn pig skin. The ability of melatonin-containing sunscreens to decrease ROS production was assessed *in vitro*, using cell cultures. *In vivo* biological properties of the final formulations, including the Human Repeat Insult Patch Test (HRIPT) and sunscreen water resistance were also evaluated.

Material and methods

Materials

Melatonin was purchased from Alfa Aesar (Ward Hill, MA). Green coffee oil (GCO) was supplied by Cooxupé – Cooperativa de Cafeicultores de Gauxupé (Minas Gerais, Brazil). Triethoxycaprylylsilane titanium dioxide (mTiO₂) (Unipure White LC 987) was a gift from Sensient

(Milwaukee, WI). Aluminum starch octenylsuccinate (ASt) (DryFlo® Plus) was obtained from AkzoNobel (Amsterdam, Netherlands). Zinc oxide (ZnO) (Tego® Sun Z 500) was obtained from Evonik Industries AG (Essen, Germany). Ethanol was obtained from Merck® (Kenilworth, NJ). All other reagents were HPLC grade. Purified water was obtained by reverse osmosis (Millipore, Elix 3, Millipore Corporation, Billerica, MA).

Methods

Sunscreen formulations – PhotoMel 1 (PM1) and PhotoMel 2 (PM2)

Preparation of the PM1 and PM2. According to the pre-formulation studies (results not shown), two final melatonin-containing formulations were selected (Table 1) based on macroscopic appearance, stability and SPF value. The continuous oil phase (Phase A) consisted of green coffee oil, and the aqueous phase (Phase B) was composed by purified water, ethanol and melatonin. Solid particles – mTiO₂, ZnO and ASt were firstly dispersed in the oil phase. The oil and aqueous phases were then mixed using a high-speed homogenizer (UltraTurrax®, IKA-Werke GmbH & Co. KG, Germany) at room temperature (cold process). Two batches of PM1 and PM 2 emulsions were stored during 3 months at room temperature (25 ± 2 °C/60 ± 5% RH) and under accelerated conditions (40 ± 2 °C/75 ± 5% RH).

Efficacy of the sunscreen formulations

In vitro SPF determination. The SPF was assessed using the Optometrics SPF-290S Analyzer (Optometrics Corporation, Essex, UK). Samples were prepared by spreading 110 mg of each formulation over a Transpore® tape (70.7 × 70.7 mm) to obtain a film of 2 mg/cm², as specified by the European legislation (EC, 2009). Each sample was exposed to a xenon arc solar simulator, and the analyzer performed scans in 6 different spots on the Transpore® tape substrate. Each scan takes a transmittance (T) measurement every 2 nm from a wavelength ranging from 290 to 400 nm. The Monochromatic Protection Factor (MPF) was determined for the selected wavelengths using Equation (1). The SPF value was calculated using Equation (2).

$$\text{MPF} = \frac{1}{T} \quad (1)$$

$$\text{SPF} = \frac{\sum_{290}^{400} E_{\lambda} \cdot I_{B\lambda}}{\sum_{290}^{400} \frac{E_{\lambda} \cdot I_{B\lambda}}{\text{MPF}_{\lambda}}} \quad (2)$$

where E is the spectral irradiance of terrestrial sunlight under controlled conditions and B is the erythema effectiveness (Kale et al., 2010).

Stability of sunscreen formulations

Two batches of PM1 and PM 2 emulsions were stored during 3 months at room temperature (25 ± 2 °C) and under accelerated conditions (40 ± 2 °C). Samples were analyzed for chemical, physical (melatonin assay, macroscopic

Table 1. Qualitative and quantitative composition of the final melatonin-containing formulations.

Ingredients	Quantitative composition (%, w/w)	
	PM 1	PM 2
Phase A (external)		
Triethoxycaprylylsilane titanium dioxide	20	20
Zinc oxide	15	15
Aluminum starch octenylsuccinate	5	5
Green coffee oil	35	35
Phase B (internal)		
Melatonin	–	1
Ethanol	6	6
Purified water	19	18

appearance, pH by potentiometry, droplet size distribution) and microbiological stability before the storage period and after 14 days, 1 and 3 months.

Melatonin chemical stability. Melatonin assay was performed by liquid chromatography using a HPLC system (Shimadzu®, Japan) with a SPD-10A UV-VIS detector at 300 nm according to a previously validated method reported in the literature (Zhang et al., 2012). Briefly, chromatographic separation was obtained using a reverse-phase chromatography column (Lichrospher 100 RP-18, 250 × 4 mm, Merck®, Germany) with a flow rate of 1.0 mL/min and an injection volume of 10 µL. The run time was approximately 10 min. The mobile phase for melatonin assay consisted of methanol:acetonitrile:0.5% acetic acid solution (40:10:50, v/v/v).

Droplet size distribution. The emulsions were observed using an optical microscope (Olympus CX40, Japan) equipped with a video camera. One drop of each emulsion was added to a glass slide without covering glass, and diluted with two drops of green coffee oil. The droplet size was determined using the image analysis software Olympus Stream Essentials®. The size data was expressed in terms of relative size distribution of particles, and given as diameter values corresponding to percentiles of 50% (BS, 1993).

Microbiological stability. The microbiological stability was performed according to the ISO 16212:2008, ISO 21149:2006 and ISO 21148:2005 (ISO Standard, 2005, 2006, 2008).

UV degradation studies

Three UVB lamps (Sankyo Denki G8T5E, Kanagawa, Japan) with a peak emission at 312 nm were used as the UVB source, and measured with a VLX 312 radiometer equipped with a UVB sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). The formulations were exposed to a UV-B radiation dose (26 mJ/cm²) for 2 h to simulate the solar radiation exposure (Gonzalez et al., 2007). Non-irradiated PM2 and melatonin solution (water:ethanol, 50:50) (Mel Sol) formulations were used as negative controls of the irradiated group. Following UVB irradiation, the samples were analyzed at pre-determined time points (15, 30, 60 and 120 min) using HPLC.

Topical delivery studies of sunscreen formulations

In vitro skin permeation. The skin permeation of sunscreen formulations was measured using Franz diffusion cells and newborn pig skin obtained from a local slaughterhouse. The entire skin was cut into sections (1 cm² permeation area). Ethanol/water (1:1) was used as the receptor phase that assured perfect sink conditions during all experiment period. The cells were immersed in a bath system at 37 ± 2 °C under stirring (200 rpm). The formulations samples were applied (0.2–0.4 g, an infinite dose) on the skin surface in the donor compartment further sealed by Parafilm® in order to prevent the water evaporation (occlusive conditions). Samples were collected from the receptor fluid at pre-determined time points – 2, 4, 8, 12 and 24 h and replaced with an equivalent amount (200 µL) of fresh receptor medium. The melatonin content in the withdrawn samples was analyzed by HPLC. The Mel Sol was used as a control. Six replicates for each sample were used (*n* = 6). The cumulative amount of melatonin permeated (*Q_t*) through excised newborn pig skin was plotted as function of time and determined based on the following equation:

$$Q_t = \frac{V_r \times C_t + \sum_{i=0}^{t-1} V_s \times C_i}{S} \quad (3)$$

where *C_t* is the drug concentration of the receiver solution at each sampling time, *C_i* is the melatonin concentration of the sample applied on the donor compartment, *V_r* and *V_s* are the volumes of the receiver solution and the sample, respectively and *S* is the skin surface area (1 cm²).

In vitro skin retention. *In vitro* skin retention or penetration study was performed by tape stripping according to the method recommended by OECD Guideline 428 (OECD, 2004). Both formulations (0.2–0.4 g) were spread over the newborn pig skin (1 cm²) in contact with 4 mL of receptor phase as described before. After 24 h, the skin samples were rinsed to remove the excess formulation and dried with filter paper. After the skin samples had been attached and fixed on a smooth surface, the *stratum corneum* (SC) was removed using 20 adhesive tapes (Scotch® 3M, UK). In order to improve the reproducibility of the tape stripping technique, a cylinder (2 kg) on foam and an acrylic disk were used and the pressure was applied for 10 s for each tape. All the tapes (excluding the first one) with the SC removed and the remaining skin (viable epidermis and dermis, ED) were cut into small pieces used for the extraction process previously validated. In this extraction process, 3 mL of mobile phase for melatonin assay was added to the SC tapes and ED pieces. Both samples were vigorously stirred for 2 min in a vertical mixer (Kinematica AG), and sonicated for 20 min to obtain the cell lysis. The final solution was centrifuged (30 000 rpm, 10 min) and the supernatant was filtered (0.2 µm) and injected in HPLC to quantify the amount (% w/w) of melatonin retained in these skin layers (SC + ED).

In vitro studies of sunscreen formulations

The cytotoxicity was assessed in HaCaT cells (CLS, Germany), a spontaneously immortalized human keratinocyte

cell line, using a general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay (Cadete et al., 2012; Lopes et al., 2012). Inhibitory concentrations (IC₅₀) were calculated using GraphPad Prism software v5.0 (GraphPad Software, Inc., La Jolla, CA) by the sigmoidal curve fitting method.

The HaCaT cells were seeded (2 × 10⁵ cells/mL) in sterile flat bottom 96 well tissue culture plates (Greiner, Germany) with RPMI 1640 culture medium (Life Technologies, UK) supplemented with 10% Fetal serum bovine, 100 units of penicillin G (sodium salt) and 100 µg of streptomycin sulfate and 2 mM L-glutamine (Life Technologies, UK). Cells were incubated at 37 °C and 5% CO₂. The medium was replaced (after 24 h) by fresh medium containing PM1, PM2 and Mel solution at various concentrations. Cells were incubated for another 48 h period. After cells exposure, the medium was replaced by medium containing 0.5 mg/mL MTT, and the cells were further incubated for 3 h. After that time, the medium was removed and the intracellular formazan crystals were solubilized with dimethylsulfoxide (DMSO). After 15 min at room temperature, the absorbance was measured at 570 nm in Microplate Reader (FLUOstar Omega, BMGLabtech, Germany). The relative cell viability (%) was compared to control cells (culture medium and sodium dodecyl sulfate (SDS) at 1 mg/mL as negative and positive controls, respectively).

Measurement of ROS production. The HaCaT sub-confluent cells were incubated for 30 min with 20 µM 2,7-dichlorodihydrofluorescein diacetate (H₂-DCFDA, Life Technologies, UK) in the dark at 37 °C, with 5% CO₂. Fresh medium was added to the cells before exposure to PM1, PM2 and melatonin solutions (1% w/v) and ascorbic acid (1% w/v) for 1 h. After hydrogen peroxide (H₂O₂, 500 µM) exposure, ROS levels were determined at 485 nm (excitation) and 520 nm (emission) wavelengths using a fluorescence microplate reader (FLUOstar BMGLabtech, Germany) (Seida et al., 1984). Data from six replicates were reported as relative fluorescence units (RFU) percentage and expressed as mean fluorescence ratio (fluorescence of exposed cells/fluorescence of unexposed control from the same experiment).

UV-irradiation dose and measurement of caspase-3 activity. Three UV-B lamps (Sankyo Denki G8T5E, Kanagawa, Japan) with a peak emission at 312 nm were used as the UV-B source, and measured with a VLX 312 radiometer equipped with a UV-B sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). Twenty-four hours after seeding cell culture (~300 000 cells/2 mL well), the cells were exposed to formulations and controls for 2 h and then irradiated with a UV-B single dose of 26 mJ/cm². The caspase-3 fluorimetric assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. As caspase-7 shares the same substrate sequence as caspase-3, this assay also detects caspase-7 activity.

After the incubation period, cells were washed with 10 mM PBS at pH 7.4, and 100 µl of lysing buffer (50 mM HEPES, pH 7.4, with 5 mM CHAPS and 5 mM DTT) were added to

each well and cells incubated for 15 min on ice. After lysis, the samples were removed and added to white 96-well plate and 100 μ l of assay buffer containing Ac-DEVD-AMC substrate (20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 20 mM Ac-DEVD-AMC) was added to each well. The reaction was followed by reading the fluorescence in a kinetic mode every minute for 60 min at 37 °C in a fluorescence microplate reader (FLUOstar Omega, BMGLabtech GmbH, Germany). Results are presented as the rate of enzyme activity measured in relative fluorescence unites (RFU) by minute and normalized to the protein content. Protein concentration was assayed by the BCA kit (Micro BCA Kit, Applichem, Germany) using bovine serum albumin (BSA) as the standard.

Safety assessment of PM emulsions

The safety assessment of the PM emulsions was accomplished according to the Scientific Committee on Consumer Safety's (SCCS) Notes of Guidance for Testing of Cosmetic Ingredients and their Safety Evaluation (SCCS/1416/11, 2011). The information for each ingredient was obtained from the respective supplier.

Hazard identification. The results of the *in vitro* and *in vivo* tests, clinical studies, physical, chemical and toxicological properties of each ingredient were considered to recognize if the ingredient has the potential to damage human health.

Dose–response assessment. The dose–response assessment describes the change in effect on an organism caused by different levels of exposure to a chemical after a certain exposure time. In the case of an effect with a threshold, the dosage at which No Observed Adverse Effect Level (NOAEL), is determined (SCCS/1416/11, 2011).

Exposure assessment. The amount and the frequency of human exposure to the PM emulsions were determined, using the systemic exposure dose (SED) that was calculated for each ingredient, according to the Equation (4).

$$\text{SED} = E(\text{mg/kg/bw/day}) \times \frac{C(\%)}{100} \times \frac{DA(\%)}{100} \quad (4)$$

where E is the amount expected to enter the blood stream per kg body weight and per day, C is the concentration of the ingredient in the PM emulsions, DA is the dermal absorption reported as a percentage of the test dose to be applied under conditions simulating those of real-life.

Risk characterization. The probability that the substances under study cause damage to human health was considered. In the case of a threshold effect, the margin of safety (MoS) was calculated according to the Equation (5).

$$\text{Mos} = \frac{\text{NOAEL}}{\text{SED}} \quad (5)$$

In vivo studies of sunscreen formulations

Human Repeat Insult Patch Test. A safety evaluation study was performed on the melatonin-containing sunscreen

formulations, using the Marzully & Maibach (1976) Human Repeat Insult Patch Test (HRIPT protocol as described in detail elsewhere (Marto et al., 2015). This protocol was approved by the local Ethical Committee and respected the Helsinki Declaration and the AFSSAPS regulations on performed HRIPT studies on cosmetic products. The study was conducted under the supervision of a dermatologist.

***In vivo* sunscreen water resistance.** The water resistance of sunscreens was tested on three subjects (Fitzpatrick skin type II). Panelists cleanse their forearms using a mild cleanser and leave them to air dry for 30 min before starting the test. Initial cross polarized images are taken after the sunscreens application (2 mg/cm²) on the inner forearm (4 cm²).

The amount of each sunscreen formulation left before and after water bath immersion was quantified via cross-polarized imaging by means of the Visia[®] CA (Canfield Scientific, Fairfield, NJ). Panelists immerse their forearms into a water bath system (29 ± 2 °C) and washed away by the flow of water (150 rpm) during 40 min (Ahn et al., 2008). Their forearms were allowed to air dry for 15 min and the amount of sunscreen was measured again. This procedure was repeated and the amount of sunscreen was measured again to calculate the water resistance of the sunscreen formulations.

This method yields a series of three cross-polarized images for each panelist: clean skin (without sunscreen), immediately after sunscreen application and post-water bath. Water resistance information was obtained from the cross-polarized Visia[®] CA imaging mode in visible light. The RGB color space of the raw bitmap images was converted to relative luminance using ImageJ[®]. From these images, average L changes for each sunscreen area were obtained from histograms. Skin whiteness was defined as the change in L value before and after water immersion, and the percentage of water resistance retention (%WRR) of the sunscreens was determined according to Equation (6).

$$\%WRR = \frac{L_{\text{washed protector}} - L_{\text{skin}}}{L_{\text{protector}} - L_{\text{skin}}} \quad (6)$$

Statistical analysis

The data were expressed as mean and standard deviation (mean ± SD) of experiments. Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Tukey–Kramer multiple comparison test (GraphPad PRISM 5 software) was used to compare the significance of the difference between the groups ($p < 0.05$).

Results and discussion

Strategy to prevent skin photodamage using melatonin

UV radiation from sun exposure is the main etiological agent for skin cancer, causing DNA damage by increasing the levels of ROS. Hence, increased free radical action can contribute to the development of cutaneous diseases. Thus, new strategies for skin protection comprise the synergistically use of sunscreen and antioxidants to counteract oxidative stress.

Table 2. *In vitro* and *in vivo* efficacy tests of the PM emulsions.

Formulations	<i>In vitro</i> Sun Protection Factor (SPF) – Optometrics SPF-290S Analyzer			<i>In vivo</i> sun product water resistance %WRR
	SPF	UVA/UVB	UVA	
PM1	82.3 ± 10.3	0.9 ± 0.1	71.1 ± 10.1	71.2 ± 0.5
PM2	75.4 ± 11.7	0.9 ± 0.1	66.4 ± 11.4	70.1 ± 2.2

WRR – water resistance retention.

Sunscreens are usually composed of synthetic chemical filters with a high capacity to absorb sun light at the region of UVB (320–290 nm) and UVA (400–320 nm) spectrum. The reduction of filter concentration in sunscreen formulations is a strategy to improve quality without affecting their properties as well as to reduce the adverse effects (e.g. estrogenic effects, disruption of human endocrine activity, etc.). In addition, the inclusion of natural products with antioxidant activity (e.g. GCO and melatonin) may also improve the photoprotective activity of sunscreen formulations. In fact, these natural products present several advantages, such as the bioactivity, relative safety and obtainment from renewable sources, low cost, besides the feasibility for application in a wide range of health care products. In this context, melatonin was associated to GCO, a rich source of antioxidants and polyphenols, which has arisen as a potential candidate to replace the chemical filters in sunscreen PM formulations (Ribeiro et al., 2013). As reviewed by Scheuer et al. (2014), topical application of melatonin conducted to a dose-dependent decrease of erythema degree and a formulation containing 1% (w/w) of melatonin proved to be effective in preventing UV-induced erythema. However, it is important to note that the ideal concentration of melatonin is still to be defined, so further studies will be needed to assess the required dose to achieve an effective photoprotection. Thus, attending to pre-formulation studies (data not shown), two formulations (PM1 and PM2) were characterized and selected for further assessment. Concerning the composition, the only difference between these two emulsions was the presence (PM2) or absence (PM1) of 1% (w/w) of melatonin. Based on the results, in Table 2, both emulsions showed high values of SPF with a suitable UVA/UVB ratio, assuring the water resistance claim ($p > 0.05$).

Improvement of long-term stability of melatonin

In previous studies, the degradation profile of melatonin has already been evaluated (data not shown). These results confirmed the high degradation tendency of melatonin, particularly in the presence of an oxidizing agent. Therefore, Pickering emulsions were formulated to deliver this radical scavenger on a safe and effective manner. The stability of these emulsions in terms of melatonin content, pH and macroscopic characteristics was assessed for 3 months at room temperature (25 ± 2 °C) and under accelerated conditions (40 ± 2 °C; relative humidity $75 \pm 5\%$). After this period, both PM1 and PM2 emulsions remained white with a creamy and homogeneous aspect for both storage conditions. In fact, no instability-related processes, such as creaming (or sedimentation), flocculation, coalescence or

phase inversion were observed. The pH values ($\text{pH} \sim 5$) did not significantly vary over time as well as the droplet size (Table 3). Melatonin assay remained within the pre-established limits (90–110%) throughout the study.

Microbiological testing revealed that PM formulations were stable for 3 months as the total aerobic microbial, yeast and mold count presented accepted values following the established criteria (<10 cfu/g). Thus, Pickering emulsions showed to be an effective vehicle to stabilize melatonin.

Enhancement of melatonin photostability

The photo-instability of melatonin is well documented. As a result of its absorption at 254 nm, this compound is quite vulnerable to UV light, originating N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) as its main photodegradation product (Fischer et al., 2006; Bromme et al., 2008). Melatonin can also be attacked by the $\cdot\text{OH}$ radical, which interacts similarly with AFMK. The concentrations of these photodegradation products are directly proportional to UVR-dose and melatonin content, and their accumulation are time-dependent (Andrisano et al., 2000).

Thus, photodegradation studies were performed using an UVB lamp (wavelength that mostly affects the melatonin stability). An acute exposure was simulated for 2 h correspondent to the reapplication time of sunscreen, as recommended (FDA, 2012). The melatonin solution (Mel Sol) presented a significant decrease ($p < 0.05$) of the melatonin content to values below the inferior limit of the 90–110% specification after 15 min of UVB exposure (Figure 1). In contrast, PM2 remained within the established range until the end of exposure time (120 min).

Comparing the photodegradation profiles of PM2 and Mel Sol, it is possible to conclude that this novel sunscreen formulation is suitable for the protection of melatonin.

Improvement of melatonin's efficacy during sun exposure

Skin permeation and retention

In vitro studies with Franz diffusion cells are a useful tool to determine the drug flow before performing *in vivo* assays (Gujjar & Banga, 2014). Skin permeation occurs via two main routes: the transappendageal (through the sweat glands and across the hair follicles associated with the sebaceous glands) and the transepidermal (intercellular route through intercellular lipid domains and transcellular pathway through the keratinocytes). The relative importance of which route will be followed depends on the molecules physicochemical characteristics.

Figure 2 shows the permeation profiles of melatonin from PM2 and solution (Mel Sol) through newborn pig skin, considering the cumulative amount of melatonin permeated to the acceptor compartment as a function of time for 24 h. There was a significant increased permeation of melatonin with time for PM2 and Mel Sol ($p > 0.05$). However, permeation profiles showed that, only at 12 h PM2 is statistically different from Mel Sol ($p < 0.05$). These profiles are typical of infinite dose experiments where the applied dose is so high that the depletion of the permeant in the donor

Table 3. Droplet size distribution of the PM1 and PM2 emulsions ($n = 625$; mean \pm SD) and percentage of melatonin recovered in batches 1 and 2 (mean \pm SD; $n = 3$) stored at 25 and 40 °C during 90 days.

	Time (days)	Temperature(°C)	PM 1		PM 2	
			Batch 1	Batch 2	Batch 1	Batch 2
Droplet size distribution $d(0.5)$ (μm)	1	25	6.2 \pm 4.1	6.7 \pm 4.8	5.7 \pm 4.3	6.2 \pm 3.6
	14	25	6.4 \pm 5.8	6.4 \pm 4.7	5.6 \pm 6.3	6.5 \pm 4.1
		40	7.1 \pm 8.5	6.5 \pm 4.2	5.3 \pm 3.8	5.9 \pm 5.7
	30	25	5.9 \pm 7.2	6.8 \pm 3.9	6.5 \pm 5.7	6.1 \pm 3.5
		40	6.7 \pm 3.4	6.2 \pm 3.8	6.2 \pm 5.9	5.4 \pm 4.1
	90	25	6.4 \pm 3.9	5.9 \pm 3.8	6.1 \pm 6.2	5.6 \pm 5.5
Melatonin recovery (%)		40	5.7 \pm 4.1	5.8 \pm 4.5	5.8 \pm 3.9	6.3 \pm 6.7
	1	25	–	–	92.8 \pm 0.6	99.3 \pm 1.5
	14	25	–	–	93.0 \pm 2.2	94.7 \pm 3.1
		40	–	–	96.6 \pm 0.7	95.0 \pm 2.8
	30	25	–	–	100.4 \pm 3.3	100.1 \pm 11.2
		40	–	–	101.2 \pm 1.1	99.6 \pm 0.6
	90	25	–	–	94.2 \pm 1.6	101.3 \pm 0.3
		40	–	–	94.7 \pm 0.4	104.2 \pm 1.8

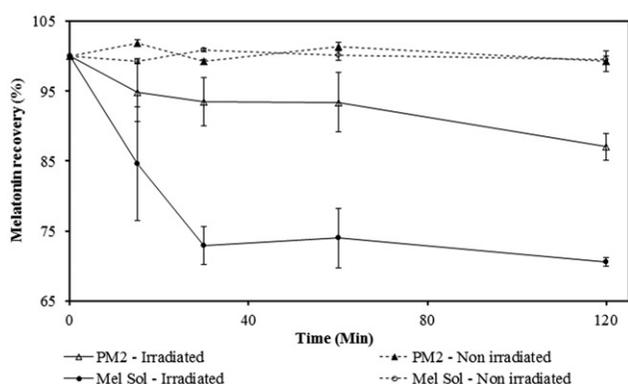
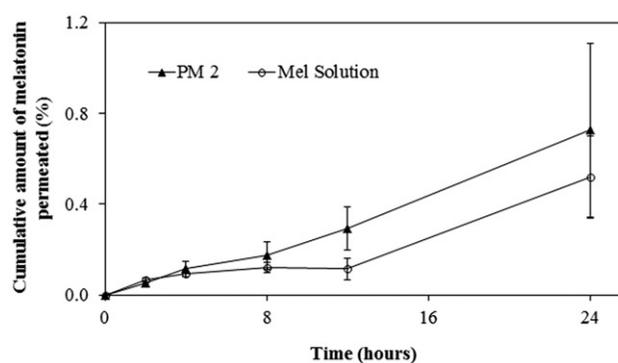


Figure 1. UV degradation studies of PM2 and melatonin solution (Mel Sol).

chamber caused by evaporation or diffusion into and through the barrier is negligibly low (Selzer et al., 2013). The high permeation through the skin was also due to the small molecular size (232.3 Da) and to the octanol-water partition coefficient ($\log P$) of melatonin. Although melatonin is usually considered an amphiphilic molecule, its $\log P$ (≈ 1.4) is relatively close to that of amphoteric molecules ($\log P \geq 1.7$). Finally, the presence of ethanol in the tested formulations also justifies the permeation enhancement. In fact, low molecular weight alkanols, like ethanol, act by enhancing the solubility of the drug in the SC lipid matrix by extraction of hydrophobic alcohols, disrupting its integrity (Kikwai et al., 2002; Marto et al., 2014). Besides lowering the skin barrier function, ethanol also increased the melatonin solubility in the vehicle.

However, it should be noted that this data obtained using newborn pig skin cannot be translated to *in vivo* delivery in humans, as other factors significantly alter its permeation profile, such as cutaneous microvasculature, which prevents the accumulation of melatonin in the skin, and the cutaneous metabolism of fatty alcohols (Kikwai et al., 2002).

The tape stripping method is well-suited to determine the dermatopharmacokinetics of topically applied substances especially if all procedures are standardized and validated, as it was previously performed in this work.

Figure 2. Permeation profile of melatonin from PM2 and Mel Solution (Sol) through newborn pig skin (mean \pm SD, $n = 6$).

Melatonin extraction from the skin by tape stripping was performed for PM2 and Mel Sol (Figure 3).

The amount of melatonin extracted from the SC was significantly higher than from viable skin layers (epidermis and dermis) for all tested formulations, as expected attending that SC is the first main skin barrier. This is a required outcome, as the target activity of sunscreens is in the outermost layers of the skin, reflecting UVR in order to reduce penetration to deeper viable skin layers where damage may occur.

The statistical analysis indicated that the amounts of melatonin extracted from the SC and viable skin layers are significantly lower when using Mel Sol compared to PM2 ($p < 0.05$). However, PM2 probably due its composition, i.e. w/o emulsions, could be more retained due to the lipophilicity of the upper skin layers, suggesting that w/o emulsions may provide advantages with respect to low penetration rates and high substantivity to the SC (Benson et al., 2005).

Although PM2 is a sunscreen formulation usually considered as cosmetic products, in this case it was also incorporated an active molecule (melatonin) to offer DNA protection against UVR damage. Attending to the fact that melatonin receptors are located in epidermis layer, inner epithelial root sheath, sweat glands and blood vessel endothelium (Slominski et al., 2012b), the skin permeation and penetration of these formulations were desirable. Fischer et al. (2004) also related that topical melatonin had a depot effect in

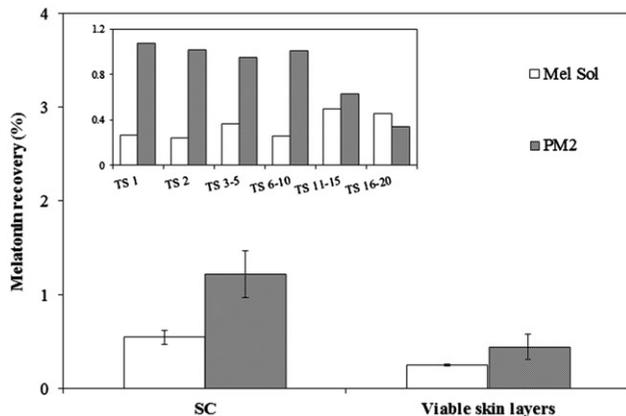


Figure 3. Penetration of PM2 and melatonin solution (Mel Sol) in the SC (from tape stripping, TS) and viable skin layers (epidermis and dermis) after 24 h. Results are expressed as mean \pm SD ($n=6$). Statistical analysis was performed using one-way ANOVA with Tukey's *post-hoc* test ($p < 0.05$).

the SC being continuously released to the rest of the skin and dermal vasculature.

Melatonin improves sunscreen photoprotection against UV-induced reactive oxygen species

Keratinocytes represent the major population in the skin and UVR causes damage to these cells. Thus, the possible protective effect of melatonin against oxidative damage in HaCaT cell lines was investigated *in vitro*.

To investigate the potential cytotoxicity of the melatonin, the cell viability was evaluated using HaCaT cell lines in a MTT assay. Melatonin is not cytotoxic in HaCaT cells on the range of concentrations tested, 1–0.015% w/v.

Effect of melatonin on generation of ROS

The intracellular production of reactive oxygen species (ROS) within cells was assessed with a fluorimetric technique using 2,7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA, Life Technologies, UK). H₂-DCFDA is non-fluorescent molecule that is hydrolyzed by intracellular esterases to non-fluorescent 2,7'-dichlorodihydrofluorescein (H₂-DCF), which is oxidized in the presence of H₂O₂, hydroxyl radicals and diverse peroxides (Soh, 2006) to a highly fluorescent compound (DCF) (Wardman, 2007). Ascorbic acid was used as a positive control due to its potent antioxidant properties.

Figure 4 refers to the difference between ROS generation in ethanol/water sample (vehicle) and in the tested formulations. It is clear that all formulations decreased the formation of ROS, particularly the melatonin loaded emulsions due to its antioxidant activity.

All formulations and melatonin solution led to an increase of ROS formation when compared to ascorbic acid and the statistical analysis indicated that the Mel Sol provides a significant better protection than PM2. This phenomenon can be explained probably due to the presence of ethanol, once ethanol may induce ROS production *per se* and due to the incomplete release of melatonin from PM2 (Helkin et al., 2010; Ma et al., 2014). Furthermore, some studies refer the cytotoxicity of ZnO nanoparticles, which may be associated not only with its action as an antibacterial, disinfecting and

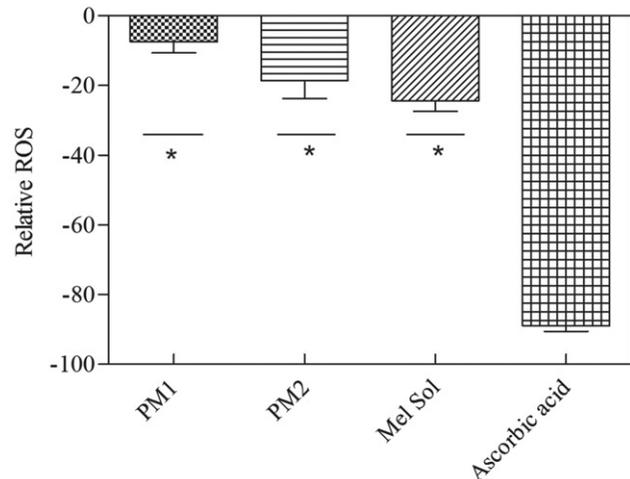


Figure 4. Relative ROS determination of HaCat cell line measured by the H₂-DCFDA assay. Melatonin concentration is 1% w/w in all cases. Results are expressed as mean \pm SD ($n=6$). Statistical analysis was performed using one-way ANOVA with Tukey's *post-hoc* test ($*p < 0.05$).

drying agent but also with its capacity to enhance ROS production (Ma et al., 2014). This suggests that the excipients of these formulations could have an oxidant activity, and that by themselves may induce ROS formation. On the other hand, ascorbic acid (vitamin C) was used as a positive control due to its potent antioxidant properties and because it possesses a variety of other topical advantages including photoprotection from UVR, inhibition of melanogenesis and improvement of a variety of inflammatory skin disorders. However, despite of its potential as a topical antioxidant, the instability of this water-soluble vitamin, together with difficulties associated with its topical delivery, proved ascorbic acid unattractive for topical application (Murray et al., 2008).

Effect of melatonin on apoptosis

This study investigates the effects of a broad-spectrum melatonin-containing sunscreen formulation on the ability of sunscreen filters and melatonin to protect HaCaT from UVR.

To confirm the inhibitory effect of melatonin on ROS synthesis and apoptotic cell death, the activity of caspase-3/7 using Ac-DEVD-pNa was performed. The levels of the activity of the two enzymes caspase3/7 decreased in melatonin-treated HaCaT cells after UVB irradiation compared to those of UVB-treated HaCaT cells without melatonin treatment (Figure 5). The decrease of the activity of these enzymes indicates that melatonin reduces the generation of ROS, thereby reducing oxidative stress in UVB-irradiated HaCaT cells and, finally, decreasing apoptotic cell death. Also, caspase 3/7 activity was very low for PM2. Thus, topical application of an efficiently broad-spectrum melatonin-containing sunscreen formulation almost completely protect against photodamage.

Safety assessment of PM formulations

Hazard identification

Many variables affect the skin penetration and permeation, including the physicochemical parameters of each ingredient

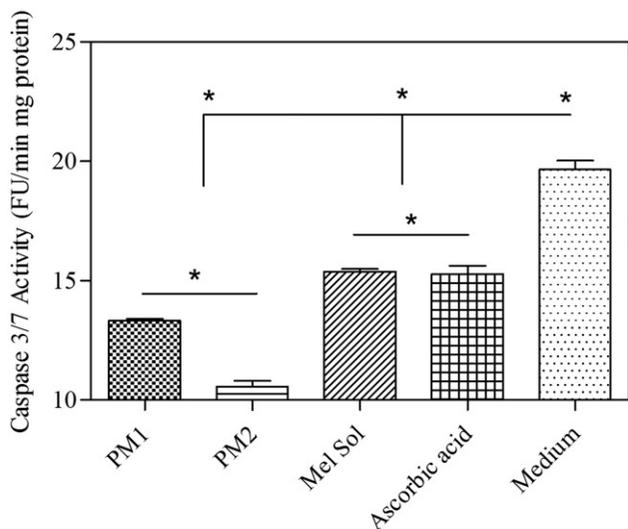


Figure 5. Levels of expression of caspase 3/7 activity in HaCat cell. Results are expressed as mean \pm SD ($n=6$). Statistical analysis was performed using one-way ANOVA with Tukey's *post-hoc* test ($*p<0.05$).

(Table 4). The chemical structures and physical properties of the ingredients used in the PM emulsions, namely, the emollient and modified solid particles are complex, hindering to predict interactions between them. However, it is recognized that the safety of a final product is determined based on the theoretical safety assessment of its ingredients. Molecules must be in the liquid form, because chemicals in the solid state and with a molecular weight greater than 500 Da do not penetrate the skin. In addition, if a chemical is excessively hydrophilic or too strongly lipophilic, it will not partition or it will partition more readily into the SC, respectively. In general, a Log P of between 1 and 3 is considered to be ideal for skin penetration. Thus, when no permeation data is available, the value considered is 100% according to OCDE, however in case molecular weight >500 Da and log $P<-1$ or >4 , the value of 10% for dermal absorption is considered (Potts & Guy, 1992; Bos & Meinardi, 2000; Brain & Chilcott, 2008).

Assessment of the safety for human health of the finished product shall take into consideration the toxicological profile of each ingredient, its chemical structure and its levels of exposure (Table 5). *In vitro* and *in vivo* studies make it possible to investigate the toxicological profile of a cosmetic ingredient, namely, the cytotoxicity, sensitization and irritation or intracutaneous reactivity, the risk of carcinogenicity, mutagenicity or teratogenicity based on period of exposure of the cosmetic products (SCCS/1416/11, 2011).

The main ingredient present in PM formulations is green coffee oil. Due to the absence of data in the literature for this emollient, it was included information about the fatty acids present in these oils and the safety profile of each ingredient was assessed. Vegetable extracts are widely used is cosmetic products due to their moisturizing, occlusive and emollient properties. Moreover, it is known that after topical application of one product only a very small amount is able to penetrate the skin due to the SC properties. There are several mechanisms of hydration but the majority of the oils act by occlusion, i.e. they avoid the evaporation of endogenous

water, decreasing the transepidermal water loss. The green coffee is considered food oil and thus it is considered safe after topical application (Boekschoten et al., 2004).

Despite the high concentration of mTiO₂ and ZnO, the toxicological profile of these ingredients does not give rise to concern in human use, since the substance is not absorbed through the skin. The ingredients in the solid state and particle size greater than 100 nm are not able to penetrate the SC if not solubilized. mTiO₂ and ZnO are not soluble in water neither in oils. Thus, they are in suspension and they cannot penetrate through the skin. Given the negligible, dermal penetration of mTiO₂ and ZnO when applied on skin, and in consideration of the low toxicity observed, the calculation of a margin of safety (MoS) is not relevant for this assessment (SCCNFP/0649/03, 2003; SCCS/1516/13, 2013).

Concerning the ASt, it was demonstrated that it has unimportant acute toxicity in animals, and no toxicity was reported following chronic administration. This ingredient was not recognized as ocular irritant in rabbits. Oral studies using ASt produced no adverse systemic, reproductive or developmental effects. ASt may produce minor skin irritation, but in general this ingredient is not irritating at concentrations used in cosmetics, and neither sensitizer nor photosensitizer. Based on these data, the author concluded that this ingredient is safe as cosmetic ingredient in the current concentration (Nair & Yamarik, 2002).

In the particular case of ethanol, Pendlington et al. (2001) described the only study in the literature about serum ethanol levels in humans after using a body deodorant spray. Despite the high concentration of ethanol and the high exposure to large surfaces, the concentration of ethanol was 0.4 mg/l. Overall it is concluded that ethanol poses no risk for the consumer in the normal and reasonably foreseeable use of this product (Lachenmeier, 2008).

Considering the molecular weight and the Log P values (when available), the ingredient that most probably penetrates into the SC is melatonin, because PM emulsions contain potent skin enhancers (ethanol and fatty acids).

Exposure assessment

The PM emulsions are proposed for use on intact skin of adults and can be used as a sunscreen. These emulsions are leave-on cosmetic products intended to stay in prolonged contact with the skin and should be applied generously and repeatedly before sun bathing, especially after spending time in water.

According to the SCCS opinion, the exposed skin surface area for a sunscreen is 17 500 cm². The estimated daily amount applied for a sunscreen is 70.0 g/day and the frequency of application is twice a day which is converted in a daily exposure of 1166.7 mg/kg bw/day. Applying the Equation (4), the SED values were calculated for each ingredient (Table 6) (SCCS/1416/11, 2011). Note that both worst scenarios were used: highest skin surface and highest amount applied.

Table 6 shows the estimated SED from the ingredients present in the PM emulsions. In the lack of dermal absorption studies, the worst-case scenario of 100% of dermal penetration should be taken into consideration (SCCS/1416/11,

Table 4. Chemical properties of the ingredients presented in the PM formulations.

Ingredient (INCI name)	CAS number	Molecular weight (g/mol)	Log P
Water (aqua)	7732-18-5	18.02	–
Green coffee oil(Coffea arabica seed oil)	84650-00-0	–	–
<i>Fatty acids*</i>			
Palmitic acid (16:0)	57-10-3	256.42	7.23
Stearic acid (18:0)	57-11-4	284.48	8.02
Oleic acid (18:1)	112-80-1	282.46	7.78
Linoleic acid (18:2)	60-33-3	280.44	7.70
Linolenic acid (18:3)	463-40-1	278.43	6.5
Titanium dioxide* Triethoxycaprylsilane	13463-67-72943-75-1	356.38	–
Zinc oxide*	1314-13-2	81.38	–
Aluminum starch octenylsuccinate	9087-61-0	–	–
Melatonin*	73-31-4	232.3	1.2
Ethanol (alcohol)*	64-17-5	46.06	–0.19

*Data from the literature (EC; SCCNFP/0649/03, 2003; Tetko et al., 2005; SCCS/1416/11, 2011; Ribeiro et al., 2013; SCCS/1516/13, 2013).

2011). In addition, a large number of studies suggest that solid particles do not penetrate healthy or sun burnt human skin deep enough to reach live cells of the epidermis, as a result, the dermal absorption considered for mTiO₂, ZnO and Ast was 0%.

Dose–response assessment

The NOAEL is an important part of the non-clinical risk assessment. The NOAEL values found out for mTiO₂, ZnO and melatonin were 62.5, 53.5 and 200 mg/kg/day, respectively.

Risk characterization

The MoS is used to extrapolate from a group of test animals to an average human being, and subsequently from average humans to sensitive subpopulations. The WHO proposes a value of 100, and it is usually recognized that the MoS should at least be 100 to claim an ingredient safe for human use. The value of 100 consists of a factor 10 for the extrapolation from animal to man and another factor 10 taking into account the inter-individual variations within the human population. The MoS for melatonin was calculated according to Equation (5), and the value obtained was 2500, which is above the threshold value of 100, hence the ingredient may be considered safe.

In vivo safety studies

Safety testing can be used to generate product claims. Thus, HRIPT was conducted to justify the claim “dermatological tested”. During the HRIPT study, no reactions or skin sensitization/irritation were observed in the initial 3 weeks contact and even after the final challenge contact. Thus, very good skin compatibility was obtained for these sunscreen formulations.

Water resistance performance of sunscreens

After ensuring *in vivo* safety of these formulations, the WRR was evaluated in humans. Human testing is considered to be the most acceptable and definitive method for claiming WRR. Although this method is a new *in vivo* screening approach to measure WRR, it does not allow determining the exact SPF before and after immersion. It rather evaluates the sunscreen

loss due to the action of water immersion. The value of 90% lower unilateral confidence limit must be %WRR \geq 50%. The PM1 and PM2 emulsions possess 84.44 and 80.62% of lightness distribution (L50) without water exposure, respectively. However, when exposed to water for 40 min, both products showed lower values of L50, 76.36% for PM1 and 73.85% for PM2, as a consequence of the water immersion. Nevertheless, both products showed quite similar amounts of L50 on skin with a satisfactory mineral distribution along the irregular skin surface, which means that the presence of melatonin did not significantly influence the water resistance performance (Figure 6). The combination of the multifunctional particles with a suitable cosmetic vehicle usually affords the acceptable distribution of the physical sunscreens onto skin.

Therefore, based on the WRR values (Table 2), it is possible to claim water resistance of this product. Thus, w/o emulsions possess a higher degree of water repellency needed to avoid products to coalesce in contact with water drops, remaining on wet skin.

Conclusions

This study arose from the necessity to fill the gap in the photoprotection whose attention is frequently more focused on cosmetic issues rather than maximum protection against both UVA and B radiation as well as cellular protection. Thus, a novel sunscreen formulation with a high UVB/A protection, biological activity and better tolerability was designed to stabilize and deliver melatonin, based on the Pickering emulsions concept.

As previously outlined, melatonin may be beneficial for sun protection. Based on its free radical scavenger and antioxidant activity, the advantage of including melatonin in PM emulsions became obvious.

Regarding the advantages of melatonin over other antioxidants, these are related to its several mechanisms of action such as immunomodulatory and anti-inflammatory besides potent antioxidant actions, suppressing all three stages of carcinogenesis. Moreover, melatonin can be produced in different skin cells from dermis and epidermis layers which may determine a strong effect on both NMSC and MSC as well as photoaging processes. Pickering emulsions proved to

Table 5. Summary of the biological safety of the ingredients.

Ingredient	Acute toxicity	Dermal Irritation	Ocular irritation	Sensitization	Photostability	Genotoxicity/ Mutagenicity	References
Melatonin	Rat (oral) LD50 > 1600 mg/kg	Human volunteers: no irritant	–	OECD 429: non-sensitizer	–	No evidence of genotoxicity or mutagenicity	Singh & Jadhav (2014)
Green coffee oil	Toxicological information (e.g. acute toxicity, NOAEL, mutagenicity, LD50/LC50-value) is not available for this product. Due to the fact that it is a food grade raw material, there is no reason to determine toxicity data. When applied correctly, the raw material will have no negative adverse effects in humans						Slominski et al. (2008)
Titanium dioxide	Rat (oral) LD50 > 2150 mg/kg	Rabbit (OECD 404): no irritant	Rabbit (OECD 405): no irritant	Guinea pig: non-sensitizer	–	<i>Salmonella typhimurium</i> (OECD 471): no mutagenic	Seida et al. (1984)
Zinc oxide	Rat (oral) LD50 > 2000 mg/kg; Rat (dermal) LD50 > 2000 mg/kg	Rabbit (occlusive patch test): no irritant	Rabbit: no irritant	Guinea pig: non-sensitizer Human: non-sensitizer	UV radiation (30 J/cm ²): photo-stable and non-photoreactive	Bone marrow cytogenetic assay in rodents and host-mediated assay in mice	Selzer et al. (2013)
Aluminum starch octenylsuccinate	–	Human volunteers: no irritant	Ocular irritant in humans	Guinea pig: non-sensitizer	–	No evidence of genotoxicity or mutagenicity	Slominski et al. (2005a)
Ethanol	Mouse (oral) LD50 > 8300 mg/kg	Rabbit (dermal) LD50 > 20000 mg/kg; Rabbit (OECD 404): no irritant	Rabbit (OECD 405): moderately irritating	Guinea pig: non-sensitizer Human: non-sensitizer	–	<i>Salmonella typhimurium</i> (OECD 471): no mutagenic	Slominski et al. (2005b)
Water	n.a.	n.a.	n.a.	n.a.	–	n.a.	–

n.a. – not available.

Table 6. Exposure data of formulation ingredients.

Ingredients	Daily exposure (bw/day)	% in the final product		Dermal absorption (%)	SED (mg/kg bw/day)		NOAEL (mg/kg bw/day)	MoS
		PM1	PM2		PM1	PM2		
Water	1166.7	19	18	100	221.7	210	–	–
Green coffee oil	1166.7	35	35	100	408.3	408.3	n.a.	–
Triethoxycaprylylsilane	1166.7	20	20	0	230.33	230.33	62.5*	n.a.
titanium dioxide								
Zinc oxide	1166.7	15	15	0	170.5	170.5	53.5*	n.a.
Aluminum starch	1166.7	5	5	0	58.3	58.3	n.a.	–
octenylsuccinate								
Melatonin	1166.7	–	1	0.7	–	0.08	200*	2500
Ethanol	1166.7	6	6	0.04[65]	0.03	0.03	2400*	80 000

n.a. – not available.

*Data from the literature (SCCNFP/0649/03, 2003; OECD, 2004; SCCS/1416/11, 2011, SCCS/1516/13, 2013).

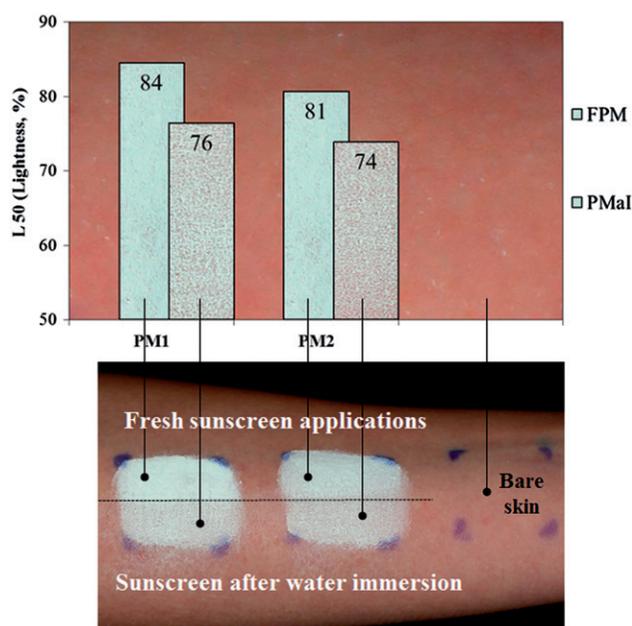


Figure 6. Skin whiteness resulting from sunscreens applied on dry skin and cross-polarized images of two sunscreens (PM1 and PM2) applied to the volar forearm of a subject. Fresh sunscreen applications with 30 min air drying (FPM) and sunscreen after 40 min water immersion (PMaI).

be a promising solution not only for the sunscreen development but also for the photostability of drugs. To the best of our knowledge, this is the first successful formulation able to protect melatonin from photodegradation. All results revealed an excellent compromise between stability, UV protection, topical delivery, efficacy, safety and cosmeticity.

Our findings combined with data reported so far thus enrich existing knowledge about the potent anti-oxidant action of melatonin and highlight that melatonin-containing Pickering emulsion sunscreens could be one of the most promising segments in the personal care industry.

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Declaration of interest

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