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Article

Development and characterization of photoprotective formulations containing keratin particles

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This work deals with development and evaluation of MFQ protective formulation, which contains two organic filters, namely: octyl-p-methoxycinnamate (OMC) and benzophenone-3 (BP-3); a photostabilizing agent called ethylhexylmethoxycrylene (EHMCR) and keratin particles. The MFQ formulation was evaluated in order to measure its pH, spin-spin lattice relaxation time (T_2H), occlusivity factor, formulation efficacy, photostability and skin permeation, as well as keratin particle properties. Keratin particle size increased when incorporated to formulation, however, it did not affect pH. The MFQ formulation was found to be photostable and photoprotective, as evidenced by sunlight photostability test, sun protection factor (SPF), UVA/UVB ratio and critical wavelength. Interaction between keratin particles and active substances (OMC, BP-3 and EHMCR) was evidenced by T_2H measurements. Evidences suggest that keratin reduces the permeation of both UV filters employed along this study, therefore, it can be stated that keratin has a promising potential for use in sunscreen formulations.

Keywords: Sunscreen. Keratin. Octylmethoxycinnamate. Benzophenone-3. Ethylhexylmethoxycrylene.

INTRODUCTION

Ultraviolet (UV) radiation is responsible for a variety of acute and chronic effects on the skin, which could be either positive acute effects as D vitamin synthesis or negative, such as erythema; on the other hand, chronic UV radiation effects include photoaging and photocarcinogenesis, caused by immunosuppression induction and mutations. An enhancement of public awareness concerning the damaging effects of UV radiation has resulted in an increased interest in sunscreens. Photoprotective agents can be classified into organic and inorganic; inorganic sunscreens (e.g. zinc oxide and titanium oxide) act by absorbing or reflecting UV radiation whereas organic sunscreens are classified into UVA, UVB or wide-spectrum absorbers; the absorption at specific UV radiation wavelengths depends on the chemical structure of the organic sunscreen active molecules (Cerqueira-Coutinho *et al.*, 2015; Cerqueira-Coutinho, Santos, Mansur, 2015).

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Organic sunscreen can include *p*-methyl benzoates, salicylates, cinnamates, dibenzoylmethanes, benzophenones, anthranilates, camphor derivatives and various other types of molecules, which could be used at different maximum permitted concentrations. Sunscreens must be photostable when irradiated with sunlight, since the photoprotective agent must ensure constant efficacy during the entire exposure period. Moreover, the sunscreen product safety is related to the evaluation of the effects of photoprotective active agents in the skin, as well as the possibility of cutaneous permeation, which could imply product leaking into the bloodstream, generating systemic toxicity (Cerqueira-Coutinho, *et al.*, 2015; Cerqueira-Coutinho, Santos, Mansur, 2015). Thus, sunscreens should be retained on the upper layer of the skin, known as the stratum corneum, where chromophores can absorb or reflect the UV radiation, preventing skin damage.

The UV filter benzophenone-3 (BP-3), which is commonly used in personal care products, is an aromatic ketone with the ability to absorb UVB and UVA, whose absorption is influenced by hydrogen bonding; however, previous studies have shown that due to its small size, BP-3 penetrates into deeper skin

layers compared to other filters, showing also cutaneous and systemic absorption, which could lead to contact dermatitis and skin sensitization when applied topically (González *et al.*, 2017). Octyl-*p*-methoxycinnamate (OMC), an organic UVB filter developed in the 1950s, has been one of the most widely used sunscreens for decades, and its use in pharmaceutical formulations is allowed by several agencies. Recent studies have detected OMC in deep skin layers, urine and milk after application on the skin; different approaches have been studied to ensure proper efficacy for this sunscreen agent (Monteiro *et al.,* 2012). An alternative to improve sunscreen photostability and consequently its efficacy, is the ethylhexyl methoxycrylene (EHMCR) incorporation in photoprotective products. The EHMCR is a photostabilizer agent for broad spectrum (UVA/UVB) sunscreens (e.g. octylmethoxycinnamate and avobenzone), preventing some photochemical reactions induced by sunlight exposure that can prejudice its efficacy. Between EHMCR properties, its oil solubility, its ability to dissolve organic filters such as BP-3 and its absorption of UVA and UVB radiation are worth to highlight. EHMCR is formed by *cis* and *trans* isomers in the same proportion, as shown in Figure 1 (Hallstar, 2015; Bonda, Zhang, 2011; Kikuchi *et al.*, 2013).

Keratin is an insoluble protein found in the skin, hair, feathers, nails, horn, wool and other dermis structures. The stability of its structure is due to disulfide cross-links, hydrogen bonds and hydrophobic interactions. A chicken feather is composed by approximately 90% of keratin in the β-pleated-sheet (β-form), which is considered a natural polymer. Due to its composition, keratin fibers from chicken feathers are a valuable biomaterial that can be used as structural reinforcement for other polymers, as well as several other applications in the biomedical field; they have been used as a protein source (Moritz, Latshaw, 2001), in hair cosmetics after enzymatic hydrolysis (Villa *et al.*, 2013), as composites of keratin and cellulose (Wrześniewska-Tosik*et al.*, 2007) and as composite for dental application (Salehuddin *et al.*, 2014).

In addition, keratin is the most abundant protein in the stratum corneum and has chemical groups capable of absorbing UV radiation (e.g. amino acids aromatic, tryptophan, tyrosine and phenylalanine). Sionkowska *et al.* (2011) observed that the hydrolyzed keratin presents absorption in the UV region, with a maximum absorption peak between 250 to 280 nm. Consequently, the use of keratin in photoprotective formulations seems to be very promising, since keratin is capable of making chemical bonds with other substances, reducing the permeation of chemical filters in human skin and also presents the ability to absorb the UV radiation. Moreover, keratin is a particle and may act as physical filter.

The aim of this work was to develop and evaluate a photoprotective formulation, containing two organic filters, OMC and BP-3, a photostabilizer agent, EHMCR, and keratin particles extracted from chicken feather.

MATERIAL AND METHODS

Material

The chemicals UV filters OMC and BP-3 were provided by Merck (Germany) and Guangzhou Shiny CO. Ltd. (China), respectively. The EHMCR was obtained from Hallstar (EUA). The keratin powder was obtained from chicken feathers according to the method described by Wawrzkiewicz, Lobarzewski and Wolski (1987) and modified by Mazotto *et al.* (2011), with dimethyl sulfoxide (DMSO) as solvent, in partnership with Laboratory of Proteases of Microorganisms at Paulo de Goés Microbiology Institute from Rio de Janeiro Federal University. The ammonium acryloyldimethyltaurate/VP copolymer (Aristoflex™AVC) was purchased from Clariant S/A (Germany); methylisothiazolinone/phenoxyethanol solution (Conserve NovaMit MF™) from IPEL (Brazil); sorbitanoleate (Span™80), polysorbate 80(Tween™80) and disodium phosphate PA (Na_2HPO_4) from Farmos (Brazil); propylene glycol from Dow Chemicals Company (Brazil); potassium phosphate PA

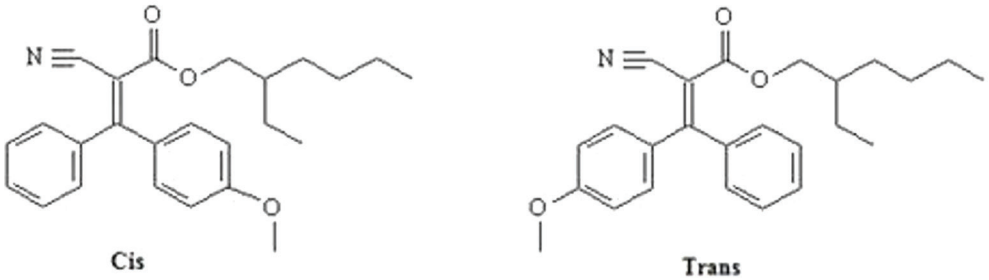

FIGURE 1 - Chemical structure of *cis* and *trans* isomers of ethylhexylmethoxycrylene (EHMCR) (Source: adapted of Bonda, Zhang, 2011).

(KH_2PO_4) from Sigma-Aldrich (Brazil), sodium chloride (NaCl) from Natural Pharma (Brazil), acetonitrile PA and ethanol PA was obtained from Tedia Company INC (USA).

Methods

Development of the Formulations

The materials used for formulations are listed in Table I. In all formulations, the aqueous gel was composed by 3 wt% of ammonium acryloyldimethyltaurate/VP copolymer and others excipients, such as methylisothiazolinone/phenoxyethanol solution and propylene glycol.

The MFQ and MFL formulations were employed to evaluate the effect of keratin incorporation. The BM and BMQ were developed for the photostability test, to verify the activity of keratin and EHMCR against natural sunlight. The Q formulation containing only keratin was used to evaluate its intrinsic photoprotective efficacy.

Evaluation of keratin particles

The mean diameter of keratin particles and dispersity (Đ) were determined in triplicate by photon correlation spectroscopy Zetasizer™ nano ZS (Malvern instruments, UK). Measurements were performed at 25 °C at an angle of 90°. All samples were obtained by emulsions diluted in distilled water. Scattering intensity data were analyzed by digital correlation. The MFQ formulation was also evaluated.

Evaluation of photoprotective formulations

The pH, spin-spin lattice relaxation time (T_2H), occlusivity factor, sun protection factor (SPF), photostability and the skin permeation assay of formulations were evaluated.

- *pH assessment*

The pH values of MFQ, MFL and aqueous gel were determined in triplicate using a HANNA potentiometer. Analyses were performed submerging the probe directly into the samples. The mean ± standard deviation (SD) was assessed.

- *NMR relaxation measurements*

The interaction between sunscreens, OMC and BP-3, photostabilizing agent, EHMCR, and keratin in MFQ, MFL and the aqueous gel, was evaluated by spin-spin relaxation time (T_2H), in a MARA Ultra low field NMR spectrometer (Oxford Instruments, Oxford, UK), at 28 °C, using an 18 mm NMR tube, operating at 23 MHz for the hydrogen nucleus. The pulse sequence used to measure the spin-spin relaxation time was Carr-Purcell–Meiboom-Gil (CPMG) and the 90° pulse of 7.5 μs was calibrated automatically by the instrument´s software; τ was 1000 μs. The relaxation values and relative intensities were obtained by fitting the exponential data with the aid of the WINFIT program. Distribution exponential fittings were performed using the WINDXP software. The precision of equipment is ± 2%. The relaxation values were calculated employing the following equation (1) (Silva, Menezes, Tavares, 2016; Bakhmutov, 2004).

$$M_y = M_0 \exp^{-\tau/T2H} \quad (1)$$

where, M_y is a longitudinal component of the magnetization vectors, M_0 is an equilibrium value, τ is time period delay and T_2H is a spin-spin relaxation time.

- *Occlusivity test*

In order to evaluate the effects of the occlusive

TABLE I - Formulations developed: MFQ, MFL, BM, BMQ and Q

Materials	Percentage in the formulations (w/w)				
	MFQ	MFL	BM	BMQ	Q
Ammonium acryloyldimethyltaurate/VP copolymer	3.0	3.0	3.0	3.0	3.0
Methylisothiazolinone/phenoxyethanol solution	0.3	0.3	0.3	0.3	0.3
Propylene glycol	3.0	3.0	3.0	3.0	3.0
Keratin	10.0	-	-	10.0	10.0
OMC	10.0	10.0	10.0	10.0	-
BP-3	6.0	6.0	6.0	6.0	-
EHMCR	5.0	5.0	-	-	-
Distillate water	100.0 q.s.				

OMC, octyl-p-methoxycinnamate; BP-3, benzophenone-3; EHMCR, ethylhexyl methoxycrylene.

properties, an *in vitro* occlusion test was performed. A glass recipient (40 mL) with a diameter of 4.6 cm was filled with 30 g of water and covered with filter paper (cellulose filter, 90 mm, Whatman number 6, cutoff size: 3 µm, USA) (Müller *et al.*, 2007; Wissing, Lippacher, Müller, 2001), then 220 mg of samples was spread homogenously with a spatula on the filter surface (13.3 mg.cm^{-2}) and the samples were subsequently stored at 38 ºC for up to 48 h. The weight of water remaining in the glass recipient was weighted at 6, 24 and 48 h. The glass recipient without sample on the filter paper was used as a reference. All experiments were done in triplicate (n = 3). The occlusion factor (F) was computed by means of the following equation (2):

$$F = [(A - B)/A] \times 100 \quad (2)$$

where, A stands for the water loss without sample (reference) and B is the water loss with sample. An F value of 0 means no occlusive effects compared to the reference, while an F value of 100 means maximum occlusiveness.

- *In vitro SPF evaluation assessment*

In vitro SPF evaluation is usually performed to estimate the *in vivo* SPF. To the in vitro SPF assessment formulations MFQ, MFL and Q were evaluated.

Samples were diluted in ethanol at a final concentration of 0.2 mg.mL^{-1} and analyzed by UV spectrophotometry (Jasco V-630) from 290 to 320 nm, with 5 nm.s^{-1} step, according to Mansur´s method (1986), which is simple and easily reproducible; SPF determination, which is the correlation between the erythematogenic effect (EE) and the radiation intensity (I) at each wavelengths between 290 and 320 nm, are adjusted according to (3).

$$SPF = CF \sum_{290\,nm}^{320\,nm} EE(\lambda)\, I(\lambda)\, Abs(\lambda) \quad (3)$$

where CF corresponds to a correction factor (= 10), EE(λ) stands for the erythematogenic effect of radiation with wavelength λ, I(λ) is the intensity of sunlight at each wavelength and Abs(λ) is the absorbance obtained by spectrophotometric measurements of the solutions at each wavelength (Mota et al., 2013; Velasco et al., 2011; Mansur et al., 1986).

The *in vitro* SPF measurements were also performed using UV transmittance analyzer (Labsphere® UV-2000S) and quartz plates with an area of 25 cm^2 covered by Transpore™ tape on one surface. It was applied 0.75 mg.cm^{-2} of each formulation with a micropipette in the support, and manually spread with circular movements in order to obtain a uniform film. Glycerin was spread in the support as reference for 100% of transmission. After drying for 15 min in dark chamber, the samples were analyzed. SPF, UVA/UVB ratio and critical wavelength (λ_c) of the formulations were determined in triplicate (Ruvolo Junior, Kollias, Cole, 2014; Hojerová, Medovcíková, Mikula, 2011; Springsteen *et al.*, 1999).

- *Photostability assay*

MFQ, MFL, BM and BMQ formulations were evaluated after exposure to natural sunlight employing three parameters: UVA/UVB ratio, critical wavelength (λ_c) and SPF. The 1.2 mg.cm^{-2} amount of each formulation was applied on PMMA Helioplates HD6 with 25 cm^2 area and homogeneously distributed. Then, the plates were protected from light for 15 minutes; then, they were exposed to natural sunlight between 09 AM and 03 PM (6 h), and analyzed every two hours. The measurement performed before exposure to sunlight corresponded to time zero (Hojerová, Medovcíková, Mikula, 2011, Ferrero, Pissavini, Doucet, 2010; Garoli *et al.*, 2009; Couteau *et al.*, 2007).

The UVA/UVB ratio and the λ_c were determined by Integration Sphere Transmittance Spectrophotometry, Labsphere UV-2000S, analyzing nine points on the PMMA plates (Velasco et al., 2011; Ferrero, Pissavini, Doucet, 2010).

Then, the PMMA plates were treated for SPF evaluation, using UV-Visible Spectrophotometer (Jasco, V-630) and quartz cubete with 1 cm light path. The plates were immersed in 15.0 mL of ethanol and the dry film was dissolved by ultrasound (Thornton–T14); then, 1.0 mL of this solution was diluted to 10.0 mL in ethanol and the SPF of final solution was evaluated by means of Mansur method (Gaspar, Campos, 2006); the assay was performed in triplicate. Results were expressed as mean ± standard deviation, with n = 3.0.

The test was performed on a sunny day, at sea level, between 09 AM and 03 PM, during the winter season (June 2015), in Rio de Janeiro, Brazil. The maximum temperature was 31ºC, according to the National Institute of Meteorology (INMETRO). The UV radiation index was measured at each hour, using the Digital UV Light Meter with Photosensor Probe (µW.cm^{-2}), MRU-201-Instrutherm. Observed average UV index during 6 h of experiment was 2280 µW.cm^{-2}, while maximum UV index of 3290 µW.cm^{-2} was obtained at noon.

- *Franz cells permeation assay*

Franz cells are vertical diffusion glass devices

used to evaluate the release, retention and permeation of drugs through skin. The system consists of donor and a receptor compartments, which are separated by a synthetic membrane or skin. The system simulates what occurs when a formulation is applied on the skin (Freitas *et al.*, 2015; Santis *et al.*, 2013; Monteiro *et al.*, 2012).

The pig ears were obtained from a slaughterhouse in Juiz de Fora, Brazil. The ears were removed from four-month-old female pigs and transported to the laboratory under refrigeration. The ears were washed with running water and distilled water. The skin of the dorsal region was excised with the aid of a scalpel. The adipose tissue, blood vessels and hairs were removed with scissors; then, skin discs of 11 cm^2 were cut, rolled in PVC film and aluminum foil, then stored at -20 °C for up to four weeks prior to use. Before the test, the skin discs were completely thawed at room temperature (25 °C).

Receptor fluid was prepared with phosphate buffer (PB) at pH 7.4 containing 2.0 wt% polysorbate 80 and 30 wt% ethanol. The solubility of OMC, BP-3 and EHMCR in the receptor fluid was confirmed before the beginning of assay. Franz cells were placed in a water bath with magnetic stirring (900 rpm) at 32 °C (Freitas *et al.*, 2015; Santis *et al.*, 2013; Monteiro *et al.*, 2012).

Skin discs were set on the top of the Franz cells, which enabled the epidermis to be exposed to the formulation (nonocclusive conditions) and dermis to be exposed to the receptor fluid, with a volume of 7.5 mL. Using an automatic pipette, 400 µg of each formulation, MFQ and MFL, was distributed homogeneously on the skin surface (1.54 cm^2) (Freitas *et al.*, 2015; Santis *et al.*, 2013; Monteiro *et al.*, 2012).

Aliquotes (1 mL) were collected with 1 h frequency for 6 h of experiment. Every volume collected was replaced with receptor fluid. After being collected, samples were analyzed by high-performance liquid chromatography (HPLC). By the end of the experiment, the skin samples were removed from the respective cell and the excess of formulation on the skin surface was removed with surgical cotton; then, epidermis and dermis were separated using a scalpel. Each layer was placed into an eppendorf with 1 mL of mobile phase (acetonitrile:water, 90:10 v/v) for OMC, BP-3 and EHMCR extraction. Then the eppendorfs were stirred in a vortex mixer three times for 30 s each and the tubes were then centrifuged at 6400 rpm for 10 minutes. The supernatant from each tube was filtered in 0.45 µm Millipore membranes and analyzed by HPLC after 24h.

The in vitro permeation assay was performed in four Franz cells for each sample; mean ± SD were assessed.

- *HPLC analysis*

A Gilson HPLC equipment was used, equipped with a 321 model pump, a 152 model ultraviolet-visible detector, a 7725i model injector (Shimadzu, Canby, OR, USA) with 50 µl loop, a 506C model system interface module, and Gilson Unipoint 3.0 software system controller (Gilson, Bedfordshire, UK).

OMC, BP-3 and EHMCR were quantified at 40 °C using an HPLC Kromasil reverse-phase column (C18), with dimensions of 250 x 4.6 mm. The mobile phase was acetonitrile:water (90:10) at a 1.0 $mL.min^{-1}$ flow rate. The detector wavelength was set at 320 nm to detect OMC, BP-3 and EHMCR.

A calibration curve of each compound (OMC, BP-3 e EHMCR), ranging from 5 to 50 $\mu g.mL^{-1}$, diluted in mobile phase was plotted.

Statistical analysis

Experimental data are presented as the mean ± SD or standard error of the mean using Origin Pro 8 (OriginLab, USA) software and $p< 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Keratin particle size

The average diameter and dispersity (Đ) of isolated keratin particles and keratin particles included in MFQ formulation were evaluated. The obtained average diameter ± SD (n=3) of keratin particles was 487.1 ± 61.4 nm and Đ ± SD (n=3) was 0.687 ± 0.094. Keratin particles included in MFQ formulation showed an average diameter ± SD (n=3) of 3065.3 ± 682.9 nm and Đ ± SD (n=3) value of 0.227 ± 0.169. Keratin particles included in the MFQ formulation showed a higher diameter and lower Đ value than the keratin powder. The aqueous gel, could have hydrated the particles, leading to increased diameter and homogeneity, which is reflected in its lower Đ value. In the keratin particles the high value of Đ indicates a heterogeneous particle size distribution.

Evaluation of the formulations

pH assessment

The obtained pH±SD for MFQ and MFL were 5.9 ± 0.06 and 5.8 ± 0.06, respectively while aqueous gel showed a pH ± SD of 5.9 ± 0.06, therefore, it can be inferred that the addition of OMC, BP-3, EHMCR and keratin did not modify the pH of formulations. There

were no statistical differences in the pH value between formulations and aqueous gel ($p>0.05$). Taking into consideration that skin surface is covered by a hydrolipidic film which is slightly acidic, the values obtained in this assay were in agreement with the pH recommendations for topical formulations, which should present a pH value in the range of 5.0-6.0, similar to the pH of *stratum corneum* to prevent skin irritation.

NMR relaxation measurements

In spin-spin lattice relaxation, the decay to equilibrium is based on the spins of nuclei in neighboring molecules. Because all nuclei in the sample have varying spin, the spin of the nuclei in neighboring molecules produce magnetic fields that affect the spin of the nuclei in other molecules. So, the relaxation time T_2H is related to the molecular dynamics of the system, that is to say, any change in the molecular mobility in the system may be reflected in this parameter. The rigid and restricted systems present a smaller and less intense signal of T_2H (Kock, 2013; Bakhmutov, 2004).

As a consequence of the aforementioned, when molecules have high viscosity, they have slow movements and their magnetic fields oscillate slowly, presenting lower values of T_2H. Although not directly related, viscosity is the simplest intrinsic property of a liquid to be correlated to T_2H. Therefore, NMR relaxation correlates well with viscoelastic characteristics of formulation, consequently, as viscosity increases the value of T_2H decreases (Kock, 2013; Bakhmutov, 2004).

The peaks observed in Figure 2 represent different proton populations present in each sample. The T_2H value of aqueous gel was used as reference in this analysis and it was compared to the T_2H values of MFL and MFQ formulations.

Pristine aqueous gel, MFL and MFQ formulations presented T_2H values of 1810 ms, 1487 ms and 671.1 ms, respectively. As mentioned before, the reduction of T_2H indicates a lower freedom of movement; thus, the addition of keratin powder was able to restrict the hydrogen chemical environment present in the aqueous gel. Furthermore, the reduction of T_2H value can indicate an increase in viscosity.

In MFQ formulation, three values of T_2H (20.85 ms, 100.0 ms and 671.1 ms) were observed and it can be correlated with three different proton populations with different molecular mobility. In MFL formulation, two values of T_2H (61.57 ms e 1487 ms) were observed, which can be correlated with two hydrogen populations with different molecular mobility. The most intense T_2H peak (1810 ms, 1487 ms and 671.1 ms) corresponds to the protons in the aqueous gel phase. The peak at 61.57 ms corresponds to the hydrogen population of OMC, BP-3 and EHMCR, since it presents a higher restriction in the hydrogen mobility compared to the hydrogen nuclei in the aqueous gel. The keratin is a solid; consequently, it has a small T_2H value, which cannot be observed. However, it was observed a broadening in the peaks base with the appearance of new T_2H values (20.85 ms and 100 ms). This behavior indicates there was chemical interaction between keratin and OMC, BP-3 e EHMCR.

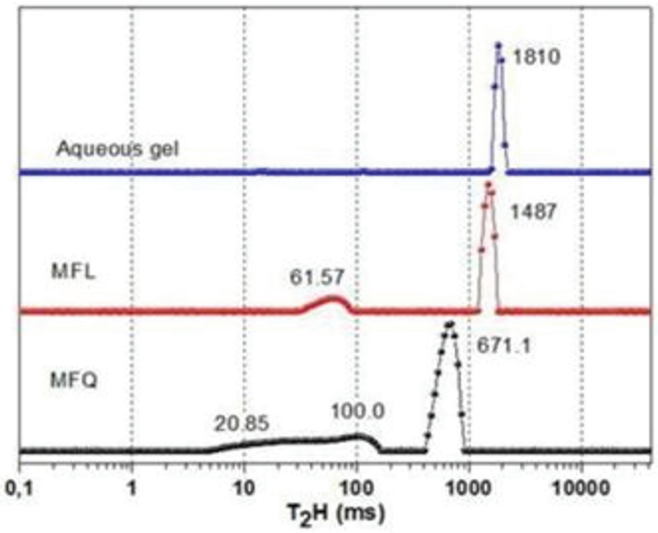

FIGURE 2 - T_2H D domain distribution curves. Abbreviation: MFQ - formulation containing octyl-*p*-methoxycinnamate (OMC), benzophenone-3 (BP-3), ethylhexylmethoxycrylene (EHMCR) and keratin; MFL - formulation containing OMC, BP-3 and EHMCR.

Occlusivity test

The occlusion factor (F) is a parameter that evaluates the ability of the formulation to form a barrier in the skin, promoting a reduction of water evaporation and the maintenance of skin hydration. Occlusion factor is dependent upon the sample volume, particle size, crystallinity, lipid concentration and type of colloidal systems. This factor varies from zero, non-occlusive effect, to 100, which is the maximum occlusivity (Müller *et al.*, 2007; Wissing, Lippacher, Müller, 2001).

The MFL formulation presented a mean occlusion factor ($n = 3$) of 26.30% ± 0.05, 28.33% ± 0.08 and 29.66% ± 0.26 at 6, 24 and 48 h, respectively. The MFQ formulation showed a mean occlusion factor of 24.22% ± 0.02, 24.82% ± 0.11 and 27.16% ± 0.18 at 6, 24 and 48 h, respectively. Consequently, the occlusion factor of both formulations was similar at 6, 24 and 48 h and there were no statistical differences between them ($p> 0.05$).

Both formulations presented occlusion factor around 25%, indicating a low occlusive action; this result is expected since both formulations are aqueous gel based, with low percentage of lipophilic content, which is usually responsible for the occlusive effect. Furthermore, the presence of keratin particles did not alter the occlusion factor of MFQ formulation. Wissing *et al.* (2001) observed that particles with around 3 μm size can diminish occlusion factor below 15, since large particles tend to increase the pores size in the formed film and facilitate water evaporation. So, the mean diameter of keratin particles, 3065 nm, may have hindered the occlusivity factor of MFQ formulation.

The low occlusion factor in photoprotective formulations helps to reduce the sunscreen permeation throughout skin. Several chemical filters, especially the OMC and BP-3, are capable of causing toxic effects to the human body, mainly by endocrine disruption. Therefore, a formulation with low occlusive effect is desirable (Jisuk *et al.*, 2016; Klimová, Hojerová, Beránková, 2015; Rodrígues-Gómez *et al.*, 2015; Zhang *et al.*, 2013).

Formulation efficacy: SPF assessement

Only the MFL formulation was evaluated by the Mansur´s method and by transmittance analyzer (Labsphere® UV-2000S). The presence of keratin, which is insoluble in solvent, became impossible the analysis of MFQ and Q formulations, using Mansur´s method.

It has been observed that the MFL formulation presented different SPF values for the two techniques employed (Table II). Both techniques could be used to determine SPF of formulations, however, the results obtained can be quite different due to the nature of analyses. The evaluation by Mansur´s method requires complete solubilization of the sunscreen in the solvent for SPF determination. On the other hand, the evaluation by Labsphere® transmittance analyzer presents variables related to the analyst that directly influence the final result (Ferrero, Pissavini, Doucet, 2010; Garoli *et al.*, 2009).

The formulations are spread in Labsphere® plates to evaluate SPF, while Mansur´s method only takes into consideration the sunscreen concentration, since the method only requires the solubilization of the formulation and dismisses the fact that the product could form a film on the plate (e.g. gel). Because of that, analyses with Labsphere® are more representative of real use conditions.

Formulation Q had a SPF value of 1.0 ± 0.0. This result was expected since this formulation did not have photoprotective/photostabilizing agents in its composition. SPF is related to the protection against UVB radiation, consequently, keratin did not influence in UVB photoprotection since the SPF value was 1.0.

Boots Star Rating System classifies a product according to its UVA/UVB ratio. The higher this ratio the better the protection against UVA radiation. According, to Boots Star Rating System the formulations MFQ, MFL and Q would be considered as products with good UVA protection. It was observed that the absence of organic filters (OMC and BP-3) and photostabilizing agent (EHMCR) did not cause a reduction in the UVA/UVB ratio. Thus, keratin may have been responsible for maintaining the UVA/UVB ratio, indicating a protection against UVA radiation.

According to COLIPA/JCIA/CTFA-SA (2006) and to FDA (1999) a photoprotective product with a λ_c of 370 nm or higher is considered a broad-spectrum product. The Q formulation showed a broad-spectrum behavior. On the other hand, MFL and MFQ showed intermediate broad-spectrum protection. As a consequence of the last mentioned results, it can be inferred that keratin particles have a potential protective effect against UVA radiation and could be incorporated into a photoprotective formulation.

Photostability

The stability of organic filters against solar radiation is an important factor that should be considered during the development of a sunscreen, since it directly influences the efficacy of sunscreen and prevents the formation of

TABLE II - *In vitro* assessment of Solar Protection Factor (SPF^M) (according to Mansur´s method) and SPF^T (UV transmittance analyzer), UVA/UVB ratio and critical wavelength (λ_c)

Formulations	$SPF^M \pm SD$	$SPF^T \pm SD$	UVA/UVB ratio	λ_c (nm)
MFQ	-	-	0.454	361
MFL	21.7 ± 0.9	31.5 ± 3.5	0.493	366
Q	-	1.0 ± 0.0	0.568	383

MFQ: formulation containing octyl-p-methoxycinnamate (OMC), benzophenone-3 (BP-3), ethylhexyl methoxycrylene (EHMCR) and keratin; MFL: formulation containing OMC, BP-3 and EHMCR; Q: formulation containing only keratin.

toxic products in the skin. Some substances employed as UVA and UVB filters are not photostable. Sunlight is able to modify the efficiency of the formulation and also cause the production of free radicals and reactive oxygen species (Kockler *et al.*, 2012; Hojerová, Medovcíková, Mikula, 2011; Hallstar, 2015).

The formulations were irradiated for 2 h, which is the period recommended by the FDA for the reapplication of a photoprotective product. The formulations were also irradiated for 4 h and 6 h in order to evaluate if they would be photostable for an extended period, since users can forget to reapply the photoprotective product.

The SPF value, UVA/UVB ratio and λ_c were evaluated before (zero time) and after six hours of sun exposure. It was evaluated the influence of the keratin and EHMCR in photostability.

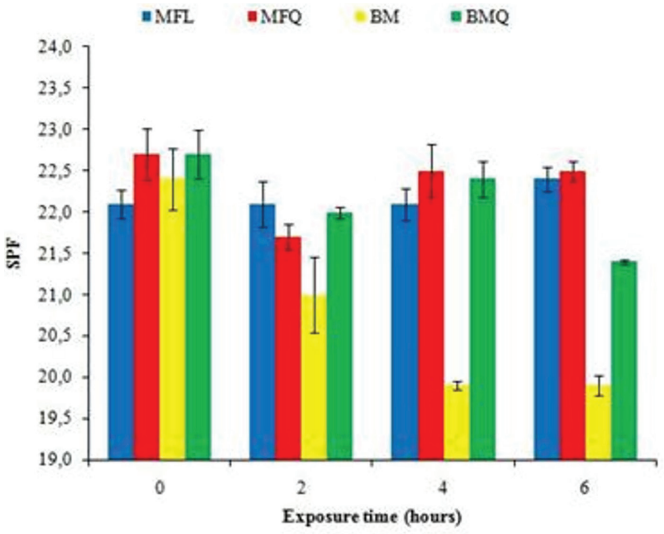

FIGURE 3 - *In vitro* Sun Protection Factor (SPF) of MFQ, MFL, BM and BMQ formulations, after six hours of sun exposure. The results were expressed as mean ± DP, with n = 3.0. Abbreviation: MFQ - formulation containing octyl-p-methoxycinnamate (OMC), benzophenone-3 (BP-3), ethylhexylmethoxycrylene (EHMCR) and keratin; MFL - formulation containing OMC, BP-3 and EHMCR; BM - formulation containing OMC and BP-3; BMQ - formulation containing OMC, BP-3 and keratin.

The MFL and MFQ remained photostable after 6 hours of sun exposure (Figure 3), no statistical differences were observed for comparison between MFL and MFQ formulation ($p > 0.05$).

After 2 h of sun exposure the BM formulation presented lower SPF value than the BMQ formulation. After 6 hours of sun exposure, the SPF values decreased around 11.1% and 5.7%, respectively. Thus, it can be observed that keratin contributed to the maintenance of photostability, since the BQM formulation presented a higher SPF than the BM formulation. There was a statistically significant difference ($p<0.05$) between the SPF values of both formulations along testing time.

A comparison between BM and MFL formulations was also made, aiming to verify the EHMCR photostabilizing action, and it was observed that the MFL formulation did not present a reduction of SPF value after 6 hours of sun exposure. BM formulation presented a SPF reduction of 11.1% and showed a statistically significant difference ($p<0.05$) between SPF values of both formulations.

The loss of photoprotective efficacy in BM formulation was confirmed by the SPF reduction after 6 hours of sun exposure. This result can be related to the OMC, which is an organic filter that absorbs UVB radiation and exhibits maximum wavelength of 310 nm. It has been then confirmed that BM formulation is unstable under UV radiation, where it becomes susceptible to photochemical reaction and its isomers are converted from *trans* to *cis*. The *trans* isomer has a high absorption coefficient (Kikuchi *et al.*, 2013; Kockler *et al.*, 2012; Pattanaargson and Limphong, 2001). The OMC photoinstability was also described by Pattanaargson and Limphong (2001), where they exclusively detected the presence of *cis* isomer after the exposure to sunlight.

The BP-3 absorbs UV radiation in the range of 270 to 350 nm and it can be classified as a UVA and UVB filter. This molecule does not exhibit the same OMC photoinstability. Couteau *et al.* (2007) carried out tests with several chemical filters, including BP-3, and they incorporated the filters in oil-water emulsions and exposed them to the solar simulator ($\lambda > 290$ nm) for $t_{90\%}$ evaluation. It was observed that BP-3 presented $t_{90\%}$ equal to 320 minutes, an indicative of its photostability (Kockler *et al.*, 2012; Balogh *et al.*, 2011).

Tarras-Wahlber *et al.* (1999) (apud Kockler *et al.*, 2012) evaluated the photostability of BP-3 and OMC, separately, with vaseline. It was used UVA and UVB radiation generated from solar simulator and fluorescent lamp, respectively. No significant change in the absorption spectrum of BP-3 was observed, therefore, it was considered photostable. On the other hand, OMC showed approximately 30% diminution of its absorption, so, it was considered unstable.

The MFL and MFQ formulations presented UVA/UVB ratio higher than BM and BMQ formulations (Figure 4). Consequently, the MFL and MFQ formulations have a greater anti-UVA protection. The presence of the photostabilizing agent (EHMCR) in MFL and MFQ formulations, was determinant for the maintenance of

UVA protection. According, to Boots Star Rating System, MFL and MFQ formulations would be considered as products with good UVA protection, while BM and BMQ formulations would be considered as products with moderate UVA protection (Velasco *et al.*, 2011; Hojerová, Medovcíková, Mikula, 2011).

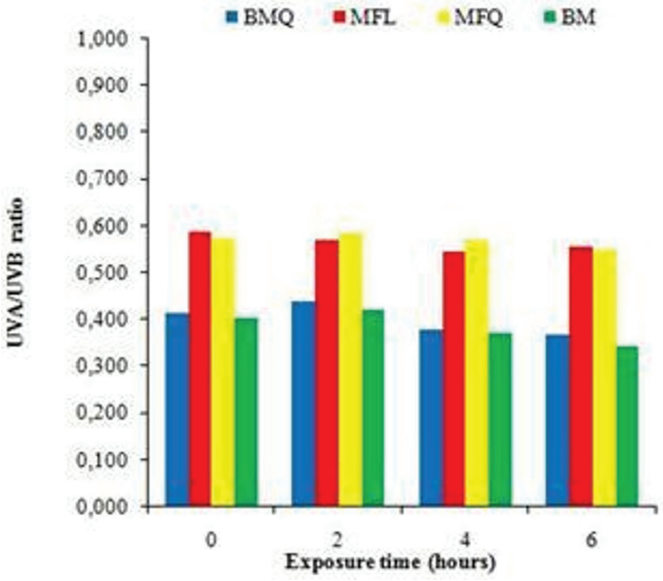

FIGURE 4 - UVA/UVB ratio of MFQ, MFL, BM and BMQ formulations after six hours of sun exposure. The results were expressed as mean ± DP, with n = 3.0. Abbreviation: MFQ - formulation containing octyl-p-methoxycinnamate (OMC), benzophenone-3 (BP-3), ethylhexylmethoxycrylene (EHMCR) and keratin; MFL - formulation containing OMC, BP-3 and EHMCR; BM - formulation containing OMC and BP-3; BMQ - formulation containing OMC, BP-3 and keratin.

The formulations with λ_c higher than 370 nm can be classified as broad-spectrum and presented action against UVA and UVB radiations. When λ_c has a value between 340 nm and 370 nm, the sunscreens show intermediate protection against UVA and UVB radiation. It was observed that the MFL and MFQ formulations maintained the λ_c higher than 370 nm while the BM and BMQ formulations presented λ_c lower than 370 nm after six hours of sun exposure. Based on the aforementioned results, the MFL and MFQ formulations provide a broad-spectrum protection and BM and BMQ formulations provide a intermediate protection (Velasco *et al.*, 2011; Springsteen *et al.*, 1999).

Skin permeation assay

Two days of analysis were necessary to evaluate the MFL and MFQ formulations. A calibration curve was also prepared for each substance, with five concentrations (5, 10, 15, 20 and 30 μg. mL^{-1}) for each day of analysis, as shown in Table III.

Table IV shows the detection and quantification limits for each active substance.

The presence of interfering substances in pig ear skin was evaluated. A small peak was observed in 6.85 minutes, with 1336.67 area units (AU). This was not considered an interfering agent.

Then, the pig ear skin was contaminated with a mixture of active substances, each substance at 20 μg.mL^{-1}. The extracted solution was evaluated and Figure 5 shows its chromatogram with the presence of five peaks.

The peak 1 shows a very small area and retention time different from the active substances OMC, BP-3 and EHMRC, as observed in Figure 6. Thus, the pig skin did not show any substance that can interfere in the analysis. The retention time of BP-3 in 4.43 min. (A), *trans* isomer of EHMCR in 7.22 min. and *cis* isomer of EHMCR in 7.83 min. (B) and OMC in 9.04 min. (C). According to Bonda and Zhang (2011), the *trans* isomer of EHMCR present a shorter retention time compare to the *cis* isomer. Both are well defined in the chromatogram, but it was observed that *trans* isomer has peak area 39% lower than *cis* isomer.

TABLE III - Equations of the analytical curves, y = ax + b, and determination coefficients (r^2)

Active substance	Equation of the analytical curve[1]	Determination coefficient (r^2)[1]	Equation of the analytical curve[2]	Determination coefficient (r^2)[2]
BP-3	y = 12856x + 2188	0.999	y = 13193x - 1269	0.999
OMC	y = 19050x + 2920	0.999	y = 19871x + 1524	0.999
EHMCR (*trans*)	y = 3399x + 1640	0.999	y = 3352x - 182	0.999
EHMCR (*cis*)	y = 5669x + 2234	0.999	y = 5664x - 544	0.999

[1]*In vitro* permeation assay of MFL formulation; [2]*In vitro* permeation assay of MFQ formulation. Data obtained in the *in vitro* permeation assays of MFL and MFQ formulations for the actives substances octyl-*p*-methoxycinnamate (OMC), benzophenone-3 (BP-3) and ethylhexyl methoxycrylene (EHMCR *cis* and *trans* isomers). MFQ: formulation containing OMC, BP-3, EHMCR and keratin; MFL: formulation containing OMC, BP-3 and EHMCR

TABLE IV - Limit of detection and limit of quantification of octyl-*p*-methoxycinnamate (OMC); benzophenone-3 (BP-3) and ethylhexylmethoxycrylene (EHMCR)

Active substance	Limits of detection ($\mu g.mL^{-1}$)	Limits of quantification ($\mu g.mL^{-1}$)
BP-3	0.44	1.33
OMC	0.77	2.33
EHMCR (*trans*)	1.04	3.16
EHMCR (*cis*)	0.82	2.50

The result for each active substance (OMC, BP-3 and EHMCR *cis* and *trans* isomers) was based on the parameters of the analytical curve.

The amount of BP-3, OMC and EHMCR, by area ($\mu g.cm^{-2}$), from both epidermis and dermis for formulation MFL and MFQ are presented in Figure 7.

It was not possible to detect the *trans* isomer of EHMCR in the epidermis and dermis for MFL formulation, however, the *cis* isomer of EHMCR was detected and quantified in the epidermis and dermis. In dermis, it was detected and quantified in a single cell (n = 1), which hindered the statistical analysis.

There was a statistical difference ($p<0.05$) between the amounts of BP-3 and OMC present in the epidermis and dermis between MFL and MFQ formulations. It was observed that MFQ formulation, which contains keratin particles, diminished the amounts of BP-3 and OMC amounts in epidermis and dermis. Besides, the receptor solution was evaluated hourly during 5 h and only the BP-3 was detected and quantified from the fourth hour in both formulations. The mean concentration ± SD observed for the MFL and MFQ formulations were 0.90 ± 0.16 $\mu g.mL^{-1}$ and 1.19 ± 0.20 $\mu g.mL^{-1}$ at the fourth hour and 1.48 ± 0.29 $\mu g.mL^{-1}$ and 1.67 ± 0.33 $\mu g.mL^{-1}$ at 5 h, respectively. There was no statistically significant difference ($p>0.05$) between the formulations analyzed at the two times observed.

The epidermis and dermis presented higher amount of BP-3 than OMC, due to their chemical properties such as molar mass and octanol-water partition coefficient ($\log K_{ow}$), which facilitates the BP-3 penetration. The substances with partition coefficient greater than 4 and less than -1 as well as molar mass higher than 500 $g.mol^{-1}$ are difficult to permeate, while substances with low molar mass, less than 500 $g.mol^{-1}$ and lipophilic characteristics have the permeation facilitated by human skin. BP-3 has a molar mass of 228.3 $g.mol^{-1}$ and $\log K_{ow}$ of 3.79. The OMC has a molar mass of 290.4 $g.mol^{-1}$ and $\log K_{ow}$ equal to 5.80. Therefore, BP-3 tends to permeate more easily than OMC because of its lower molar mass and $\log K_{ow}$ (Klimová, Hojerová, Beránková, 2015; Junjie *et al.*, 2015).

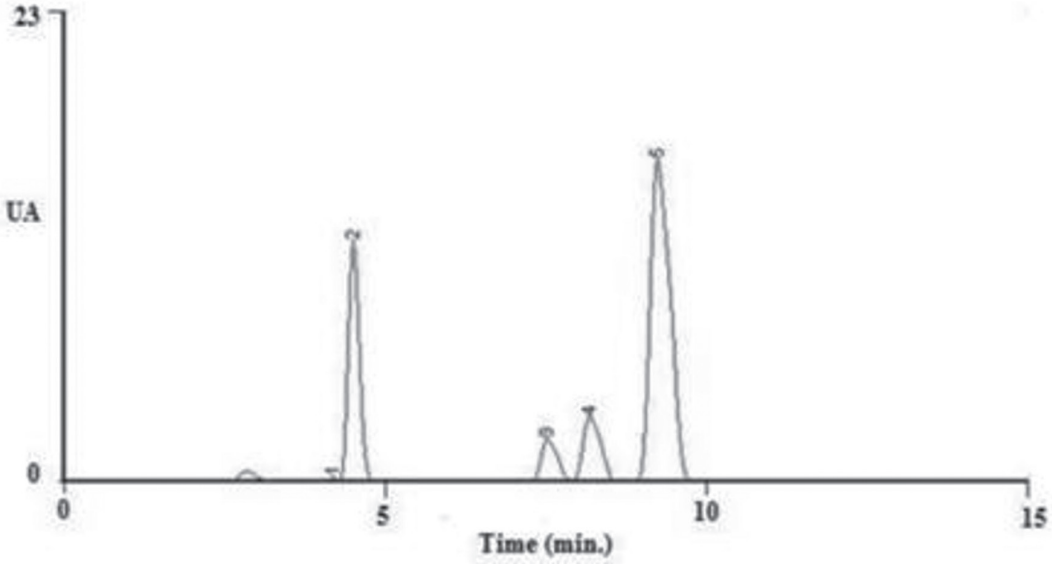

FIGURE 5 - Chromatogram of the solution obtained after the contamination of pig ear skin. The contaminant solution contained a mixture of active substances: octyl-p-methoxycinnamate (OMC), benzophenone-3 (BP-3), ethylhexylmethoxycrylene (EHMCR), each substance at 20 $\mu g.mL^{-1}$. The retention time (min.) and area unit (AU) of each active substance correspond, respectively, to: BP-3, peak 2, 4.50 min. and 236782.08 AU; *trans* isomer of EHMCR, peak 3, 7.53 min. and 63504.15 AU; *cis* isomer of EHMCR, peak 4, 8.18 min. and 104300.85 AU; OMC, peak 5, 9.24 min. and 565367.50 AU. The chromatographic conditions were Gilson equipment (Shimadzu, Canby, OR, USA); Gilson Unipoint 3.0 software system controller (Gilson, Bedfordshire, UK); reverse-phase column (C18); 320 nm; 40 °C; acetonitrile:water (90:10) and 1.0 $mL.min^{-1}$.

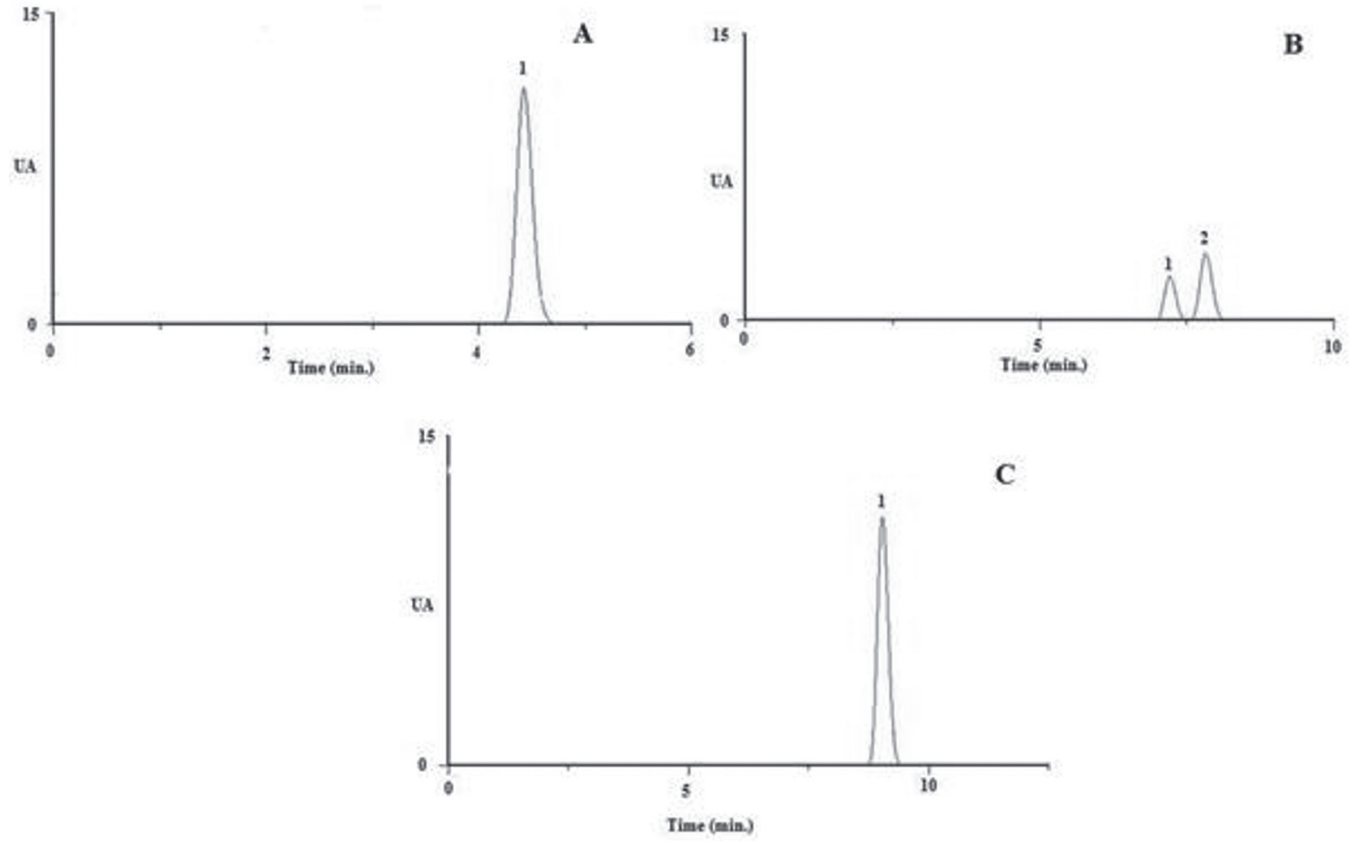

FIGURE 6 - Chromatograms of active substances, benzophenone-3 (BP-3) (A), ethylhexylmethoxycrylene (EHMCR *cis* and *trans* isomers) (B) and octyl-*p*-methoxycinnamate (OMC) (C), at 15 μg.mL^{-1} each. Chromatographic conditions: Gilson equipment (Shimadzu, Canby, OR, USA); Gilson Unipoint 3.0 software system controller (Gilson, Bedfordshire, UK); reverse-phase column (C18); 320 nm; 40°C; acetonitrile:water (90:10) and 1.0 mL.min^{-1}.

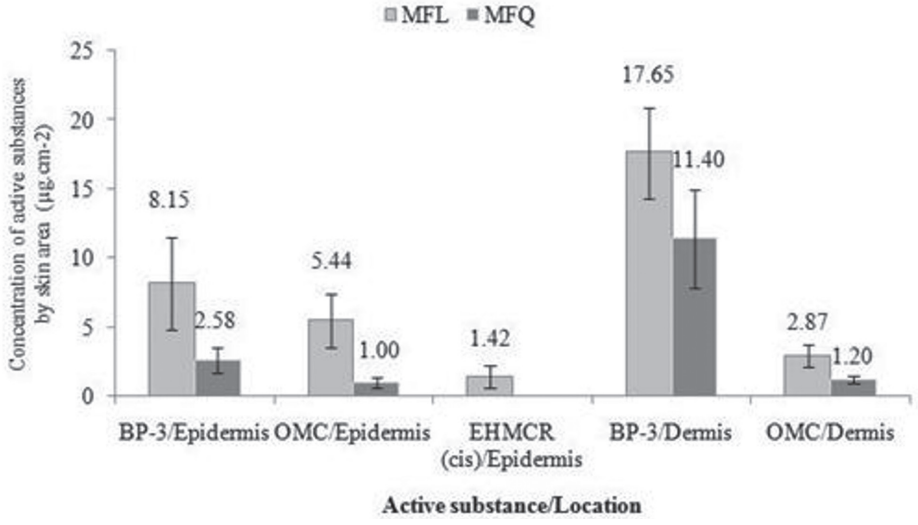

FIGURE 7 - Amount of active substances octyl-p-methoxycinnamate (OMC), benzophenone-3 (BP-3) and ethylhexylmethoxycrylene (EHMCR isomers) per area (μg.cm^{-2}) in epidermis and dermis. The bar represents the standard deviation for n = 4. Abbreviation: MFQ - formulation containing OMC, BP-3, EHMCR and keratin; MFL - formulation containing OMC, BP-3 and EHMCR.

Klimová *et al.* (2015) evaluated the permeation of BP-3 and OMC, observing higher amount of BP-3 in epidermis and dermis, as well as in the receptor solution.

Keratin is the most abundant protein in the stratum corneum and it can be used in topical formulations aiming to keep the formulation retained on the outermost skin

layer, preventing permeation of topical formulation, thus enhancing the product safety. These characteristics are essential for sunscreen formulations, especially when it contains BP-3 and OMC, which can permeate into systemic circulation, causing endocrine disruption and the loss of photoprotection (Jisuk *et al.*, 2016; Klimová, Hojerová, Beránková, 2015; Rodríguez-Gómez *et al.*, 2015; Zhang *et al.*, 2013).

CONCLUSIONS

The MFQ formulation containing the OMC, BP-3, the photostabilizing agent (EHMCR) and keratin particles was effective in photoprotection, as evidenced by SPF value, UVA/UVB ratio and critical wavelength. Keratin was able to absorb UVA radiation and have potential to be incorporated into a photoprotective formulation. Moreover, there was an interaction between keratin particles and UV filters (OMC, BP-3 and EHMCR) as evidenced by T_2H measurements. Thus, the formulation with keratin instead of photostabilizer agent, showed outstanding photostability. Keratin and EHMCR can be considered photostabilizing agents, since the SPF value of the MFQ formulation was maintained during six hours of sun exposure. Keratin also reduced the permeation of both UV filters OMC and BP-3 in epidermis and dermis, making the photoprotective formulation safer. Keratin has a promising potential for use in sunscreen formulations.

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DISCLOSURE STATEMENT

The authors have declared no conflicts of interest.

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