UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão do Curso de Farmácia

Development of cosmetic formulation containing sunscreen nanoencapsulated and evaluation of cutaneous penetration *in vivo* and *in vitro* safety

Cláudia de Melo Oliveira

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Cláudia

Este artigo foi elaborado segundo as normas da Revista “Skin Pharmacology and Physiology” apresentadas em anexo na qualidade de “Artigo Original”.

O artigo apresentará uma formulação fotoprotetora semissólida inédita utilizando nanocarreadores poliméricos submetidos a um processo de secagem. A suspensão desenvolvida, o pó obtido bem como a formulação final foram adequadamente caracterizados. O produto semissólido foi avaliado quanto sua segurança por meio de potencial de irritação, utilizando um teste *in vitro*. Além disso, realizou-se a avaliação da penetração cutânea da formulação por meio de *tape stripping in vivo.*

Development of cosmetic formulation containing sunscreen nanoencapsulated and evaluation of cutaneous penetration *in vivo* and *in vitro* safety

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Running head: Sunscreen nanoencapsulated penetration *in vivo* and *in vitro* safety

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Development of cosmetic formulation containing sunscreen nanoencapsulated and evaluation of cutaneous penetration *in vivo* and *in vitro* safety

Aim: The aim of this study was develop semisolid formulation containing spray-dried nanostructured system with the association of three organic UV filters - Octyl Methoxycinnamate (OMC), Octyltriazone (OCT) and Bemotrizinol (BMT) - using Titanium Dioxide (TiO_2) as drying excipient. The formulation was tested by means of cutaneous penetration *in vivo* and *in vitro* irritation.

Method: Polymeric nanocapsules were prepared by interfacial deposition of polymer and characterized before the spray drying process. The powder obtained was incorporated in an emulsion formulation. A formulation containing free organic UV filters were also prepared by means of comparison. *In vitro* irritation was evaluated on chorioallantoic membrane (HET-CAM). *In vivo* tape stripping study was conducted with 10 volunteers.

Results: The powder was efficiently developed maintaining their nanotechnologies properties. Presence of nanocapsules were observed by scanning electron microscopy. Formulations produced were non-irritant to the chorioallantoic membrane. There were no statiscal differences between the formulation containing the nanostructured system and free organic filters. However, results with the nanostructured system were more homogeneous.

Conclusion: An innovative non-irritant cosmetic input was developed. The formulation produced remains in the stratum corneum layer, where they are designed to act.

Key words: Nanoencapsulated sunscreen, HET-CAM, Tape Stripping

1. Introduction

Excessive sun exposure is associate to many damages to skin such as sunburn, photoaging, and DNA damages [1,2]. The skin is the organ most affected by the harmful effects of ultraviolet radiation, and there are studies showing the association between sun exposure and skin cancer [3]. Solar damages caused by UV exposure can be minimized by using sunscreen [3,4]. Sunscreens are generally divided in two groups. Organic UV filters are formed by organic molecules which have the ability to absorb

one or more specific wavelengths, transforming it into another type of energy. While inorganic UV filters work through physical process by reflecting and scattering UV radiation [4,5]. Once that sunscreens are widely used in world population, it is always useful to search new approaches to improve their efficacy and security. Colloidal carriers, such as polymeric nanocapsules, are being study as promising to load UV organic filters [6–9]. Polymeric nanocapsules are a class of nanostructures containing an oil core surrounded by a polymeric wall and the particle–water interface is stabilized with polysorbate 80 [10]. These structures have been extensively used for cosmetics, since they show many advantages such as adhesiveness, skin hydration by occlusion, skin penetration enhancement [11–13], and offering protection against photodegradation [6,14,15].

Nanostructured systems are designed to deliver drugs in a sustained, controlled and targeted manner to avoid adverse systemic side effects [14–16]. This liberation system allows enhanced delivery of active substances to the out layers of the skin, and also allows prolonged contact at the site of action, which could be interesting to sunscreen products [13,17,18]. Therefore, nanostructured systems containing sunscreens may remain longer on the surface of the skin where they are designed to act [7,9,17–21].

Although all features of nanocapsules above cited, aqueous formulations might show instability issues, such as microbiological growth [22]. Spray-drying technique is being applied as an alternative to overcome this drawback by turning suspensions into powders. Common excipients used to this technique are lactose and mannitol [23,24]. Whereas, once that this study works with a sunscreen formulation the use of Titanium dioxide (TiO_2), as drying excipient, could be interesting. Using an inorganic UV filter, it might improves the UV-block activity and sunscreen efficacy [25].

Aiming to improve UV protection effects, polymeric nanocapsules containing the combination of three organic UV filters - Octyl Methoxycinnamate (OMC), Octyltriazone (OCT) and Bemotrizinol (BMT) were developed and dried by spray-drying using TiO_2 as support. Powders were incorporated in semisolid formulations and evaluated as the cutaneous penetration *in vivo* and *in vitro* safety.

2. Materials and Methods

2.1 Materials

Octyl methoxycinnamate, octyltriazone and bemotrizinol were purchased from Deg (São Paulo, Brazil), Daltomare (São Paulo, Brazil) and Cosmetrade (Porto Alegre, Brazil), respectively. Titanium dioxide (Eusolex® T-AVO) was obtained from Merck (São Paulo, Brazil). Poly(methacrylic acid-co-methyl methacrylate) (Eudragit S100®) and sorbitan monostearate (Span 60®) were purchased from Degussa (Paulínia, Brazil) and Sigma-Aldrich (São Paulo, Brazil), respectively. Caprylic/capric triglycerides (CCT) and polysorbate 80 were acquired from Delaware (Porto Alegre, Brazil). All reagents and solvents used in this study were of pharmaceutical or HPLC grade and used as received.

2.2 Sunscreens-loaded Nanocapsules Preparation

Polymeric nanocapsules were prepared by interfacial deposition (n=3) of the preformed polymer according to the method described by Fessi and co-workers [26]. Theorical sunscreens content were respectively: 1mg mL^{-1} OMC, 2mg mL^{-1} OCT and 2mg mL^{-1} BMT. The organic phase was consisted by above mentioned organic UV filters (0.100 g of OMC, 0.200 g of OCT and 0.200 g of BMT), 1.0 g of Eudragit S100®, 1.6 mL of CCT, 0.383 g of sorbitan monostearate dissolved in a solution of 240 mL of acetone and 30 mL of ethanol. The lipophilic phase was injected into aqueous phase containing 0.770 g of polisorbate 80 in 500 mL of water, under moderate stirring and 40°C of controlled temperature. The suspension was submitted to reduced pressure to eliminate acetone and excess water and the final volume adjusted to 100 mL.

2.3 Sunscreens-loaded Nanocapsules Characterization

Nanocapsules suspensions were characterized before drying. Samples were analyzed according to their pH values, mean average size, polydispersity index, zeta potential and sunscreens content. PH values were measured using a calibrated pH meter (VB-10, Denver Instrument, USA). Granulometric profiles were determined by laser diffraction (Mastersizer 2000®, Malvern Instruments, Malvern, UK). The particle *z*-average, polydispersity index (PDI) and zeta potential were determined using a Zetasizer Nano ZS® (Malvern Instruments, Malvern, UK). Particle size was evaluated by photon correlation spectroscopy after diluting 20 microliters of samples in 10 mL of ultrapure water. For measurements of zeta potential, suspensions were diluted (500 X) in 10 mM

NaCl aqueous solution and analyzed by electrophoretic mobility.

A previously validated high-performance liquid chromatography (HPLC) method was used to quantify sunscreens content. The method used to determine OMC, OCT and BMT were described by Santos in 2014 [27]. The mobile phase in this method was acetonitrile:metanol solution (50:50 v/v). The column used was a Phenomenex Gemini C18 (150 mm x 4.6 mm x 5 µm particle size) thermostatically at 35°C and with a guard column. Mobile phase flow was 1.0 mL min^{-1}, volume of sample injected was 100 µL and all three sunscreens were detected with wavelength of 310 nm. Total sunscreen content was obtained by dissolution of the suspension (50µL) in 10 mL of acetonitrile and filtration through a 0.45 µm pore size membrane before HPLC analysis.

2.4 Preparation Spray-drying Nanocapsules

Spray-dried polymeric nanocapsules (SP-NC powder) were prepared in triplicate batches using a Mini Spray-Dryer Büchi B-290 (Flawil, Switzerland). The drying adjuvant used was TiO_2 at 3% (w/v) and it was added to the nanocapsule suspension under magnetic stirring. The stirring was maintained during all the formulation fed into the spray-dryer. A two-fluid nozzle with a cap orifice diameter of 0.8 mm was used. Inlet temperature in the drying chamber was maintained at 150 ± 1 °C, feeding rate was set at 0.5 L h^{-1} and airflow rate was 600 N L h^{-1}(18 ,27).

2.5 Characterization Spray-dried Nanocapsules

The process yield was calculated from the relation of total weight of powder recovered in the sample collector and the amount obtained by the initial concentration of solids in the nanocapsule. Results were expressed in percentage. Moreover, the residual moisture content of powders was analyzed using Ohaus® MB25 Halogen kept at 105 °C until constant weight.

The size of spray-dried powder particles was measured by laser diffractometry using the refraction index of TiO_2 (Mastersizer 2000, Malvern, UK). Analysis were performed by dry powder method. Width of the distribution of particle size (SPAN) values were used, and they were calculated by $(d0.9 - d0.1)/d0.5$ where $d_{0.1}$; $d_{0.5}$ and $d_{0.9}$ represent the mean volume diameter and lower sizes of 10, 50 and 90 % of particles,

respectively. Same analysis conditions were used for commercial TiO_2 in order to compare the results.

Sunscreens content were measured after dispersion of SP-NC powders in acetonitrile. Those were stirred 15 minutes, sonificated in ultrassom at 40°C for 45 minutes and centrifuged for 10 min at 1890 *x* g. Samples were filtrated (Millipore®, 0.45 µm) and the same HPLC method previously described was used for dosages.

Morphological analyses of polymeric microparticles were performed using a scanning electron microscope (SEM) (Zeiss EVO HD15, Germany). Samples of spray-dried nanoparticles were added in aluminum stubs with carbon conductive double-sided tape and sputter-coated with layer of gold. Analysis were conducted at the Electron Microscopy Center of the Federal University of Rio Grande do Sul (Centro de Microscopia Eletrônica - UFRGS).

2.6 Emulsion preparation

A semisolid formulation was prepared using 9.5 % (w/w) glyceryl monostearate, 4 % (w/w) cetyl alcohol, 1.5 % (w/w) ethoxylated cetostearyl alcohol, 0.2 % (w/w) methylparaben, 0.1 % (w/w) propilparaben, 2 % (w/w) polysorbate 80 and distilled water. Two different formulations were prepared in order to compare the analysis: a) an emulsion base containing spray-dried sunscreens-loaded nanocapsules (SS-SP-NC), and b) Commercial TiO_2 and free OMC, OCT and BMT organic UV filters (OF) (SS-TiO_2-OF). Active igredients were incorporated in semisolid base by levigation using 10 % (w/w) propyleneglycol. One gram of spray-dried powders was added in 20 g of base emulsion to produce SS-SP-NC, corresponding approximately to 0.0143 g of OMC, 0.0266 g of OCT, 0.0276 g of BMT, 0.138 g of Eudragit S100®, 0.0528 g of sorbitane monostearate, 0.221 g of CCT, and 0.106 g of polysorbate 80. SS-TiO_2-OF was prepared by adding same quantity of sunscreens content in SP-NC powder determined by previously described HPLC method. Moreover, 0.413 g of TiO_2 was added corresponding, theoretically, the same amount present in SP-NC powder, considering a homogenous process and a proportional constituents loss during spray-drying.

2.7 Emulsion characterization

SS-SP-NC and SS-TiO_2-OF were characterized by means of pH values, sunscreens content and *in vitro* irritation potential by HET-CAM technique. A calibrated potentiometer was used to determine pH measurements (VB-10, Denver Instrument, USA) after dilution of emulsions in ultrapure water (10 %, w/v).

In order to determinate sunscreens content in emulsions, 100 mg of samples were diluted in 10 mL of acetonitrile and the same extraction method for spray-dried polymeric nanocapsules was utilized.

The irritation potential of both semisolid formulations (SS-SP-NC and SS-TiO_2-OF) was examined by the hen's egg test (HET) an *in vitro* technique, which uses the chorioallantoic membrane (CAM), a fetal vascularized respiratory tissue, to mimic the conjunctive ocular cavity. Fertilized chicken eggs on the tenth day of incubation (37.5 °C, 60 % relative humidity) were used. The assay is based on the observation of phenomena vasoconstriction, hemorrhage and coagulation, which results in a scale that considers the irritation potential of formulations [28,29]. Egg shells were opened at the air chamber of the egg, the white membrane was removed and 300 µL of the formulation was applied to the CAM (n=5). Considering the opacity of formulation, the CAM was rinsed with saline solution 20 seconds after the application and then the time of first occurrence of irritant phenomena was monitored until 300 s. Positive controls, NaOH (0.1 N) and sodium lauryl sulfate (1 %), and a non-irritant control, NaCl (0.9 %) were also tested (n=3). The irritation score (IS) was determined according to the follow equation:

$$IS = \frac{5\times(301-hemorrhage\ time)}{300} + \frac{7\times(301-vasoconstriction\ time)}{300} + \frac{9\times(301-coagulation\ time)}{300}:$$

Lesions were classified as non-irritant (0-0.9); slightly (1-4.9); moderate (5-8.9) and extremely irritant (9-21)[28].

2.8 Clinical trial

Tape stripping is a simple and efficient technique for demonstrate the penetration profile of topical formulations, by using adhesives to remove cell layers of the stratum corneum [30]. Ten healthy volunteers participate in a phase I randomized controlled crossover experiment. Inclusion criteria included: age 20-40 years, both sexes.

Exclusion criteria include allergies to sunscreens or any other formulation ingredients, allergy to adhesive tape, skin sensitization medications (retinoic acid and its derivatives, peeling products) and presence of dermatoses. All volunteers signed an informed consent before the study was conducted. This protocol was approved by Federal University of Rio Grande do Sul Ethics Commitee, number 787334.

Application areas of formulations were delimited with adhesive plasters, totalizing an area of 16 cm^2. Volunteers were instructed to wash their arms before the experiment. SS-SP-NC and SS-TiO_2-OF (0.4 g) were applied alternately on the right or left arm of volunteers, and they stood for 30 minutes. Thereon, the excess sample was removed with cotton and five scotch tapes were used, successively, for each arm, to remove stratum corneum cells. For the determination of sunscreens content, tapes were placed in test tubes and treated with 8 mL of acetonitrile. Then, tubes were vortexed for 2 min and sonificated in ultrassom at 40 °C for one hour. Samples were filtrated (Millipore®, 0.45 μm) and the same HPLC method previously showed was used to quantify the amount of sunscreens present in each volunteer.

2.9 Statistic Analysis

Analysis of the data was performed by t-test with GraphPad Prism® software.

3. Results and Discussion

This study showed the feasibility of a process to obtain polymeric nanocapsules containing three organic UV filters, combined with a spray-drying process with an inorganic UV filter - the TiO_2.

3.1. Physicochemical characteristics

Characterization of nanocapsule suspensions is an essential step in the development of strategies to guaranteeing their nanotechnological properties and physicochemical stability [31]. Laser diffraction was used to ensure that only submicrometric particles were present in formulations prepared in this study, since this technique can detect the presence of nano and microparticles (20 nm to 2000 μm). According to granulometric profiles, formulations produced showed monomodal size distribution

down to the nanoscale, without micrometric particles. Three batches of nanocapsule suspensions produced were overlapped, approving the nanotechnology quality of the system (1). The absence of micrometric particles in these formulations ensure that organic UV filters used - OMC, OCT and BMT are not over concentrate or agglomerate. Measurements of samples indicated that D4,3 was 174 ± 4 nm, with low span (1.56 ± 0.03), confirming a narrow size distribution. By the Zetasizer Nano ZS® analysis, the *z*-average was 172.30 ± 5.15 nm and showed a low polydispersity index (0.133 ± 0.014). All formulations presented negative zeta potentials (-3.81 ± 2.11 mV). Although the value of zeta potential was low, the presence of Polisorbate 80 in the interface oil-water indicates that the stabilization mechanism of nanocapsules is by steric hindrance [31]. The pH mean value was 4.61 ± 0.20. Sunscreens were quantified by high performance liquid chromatography, showing values close to expected for each sunscreen loaded: 0.93 ± 0.08 mg mL^{-1} for OMC, 1.93 ± 0.04 mg mL^{-1} for OCT, and 2.05 ± 0.07 mg mL^{-1} for BMT. As can be seen, particle size was not affected by co-encapsulation of sunscreens with Eudragit S100, keeping an average diameter similar to previous described by Hoffmeister and co-workers [24].

3.2 Spray dried polymeric nanocapsules

Nanocapsule suspensions were spray-dried using micronized TiO_2 and then characterized. Process yields was 81.85 ± 1.60 % and dried formulations showed a low moisture content (0.97 ± 0.30 %). The process yield was very high being much over than 50% [24].

Sunscreen-loaded content in powders were analyzed after spray-drying process, and recovery values were calculated (in percentage) considering their contents in nanocapsule suspensions. These values corresponds to a content of 16.01 mg g^{-1} for OMC, 28.55 mg g^{-1} for OCT and 29.79 mg g^{-1} for BMT indicating 80.97 ± 8.03 %, 81.14 ± 4.42 % and 78.39 ± 3.50 % of recuperation, respectively.

Spray-dried polymeric nanocapsules were analyzed by laser diffraction (n=3) and had shown a diameter $D_{4,3}$ of 2.60 ± 0.09 µm with a span value of 1.20 ± 0.15 and monomodal granulometric profile distribution. While the commercial TiO_2 had a diameter $D_{4,3}$ of 3.24 ± 0.46 µm with a span value of 1.84 ± 0.1 and bimodal

population (2). Comparing these values, we can assume that drying technique decreased the particle size and, it also homogenizes size population. Previous reports have described that segregation of materials can occur during spray-drying process, depending on the raw material or the process'parameters [32,33]. Therefore, operational conditions used were approved once that powders obtained had a high yield, satisfactory sunscreen-loaded content and a low percentage of humidity.

Morphological analysis of the excipient used (TiO_2) and spray-dried powders were performed using scanning electron microscopy (SEM) to verify their morphology (3). Commercial TiO_2 morphological analysis showed irregular shaped microparticles with high variability (3a) and at a higher magnification we can observe some cavities and rugged surface (3b). Meanwhile the SP-NP showed spherical shaped microparticles (3c). At a higher magnification we can observe nanostructures adsorbed in a microscale network structure (3d). Morphological analysis support the data obtained of the laser diffraction technique, where the polidispersity index of commercial TiO_2 decreases after the spray drying process. Furthermore, the observed change in TiO_2 particle morphology might benefit skin application due to their major homogeneity when incorporated into emulsions.

3.3 Semisolid formulations

Characterization of emulsion formulations were made by mean of pH values, sunscreen-loaded content and HET-CAM technique. Emulsions had a pH value being close to neutral: 6.92 ± 0.20 for SS-TiO_2-OF and 6.21 ± 0.40 for SS-SP-NC. The slight acidity of the SP-NC emulsion is similar to the pH value of the stratum corneum, being considered suitable formulation for topical application [34].

Sunscreens content were close to the theoretical value being, respectively, for SS-TiO_2-OF 0.94 ± 0.9 mg g^{-1} for OMC, 1.36 ± 0.2 mg g^{-1} for OCT and 1.22 ± 0.1 mg g^{-1} for BMT. While for SS-SP-NC values were 0.71 ± 0.04 mg g^{-1} for OMC, 1.33 ± 0.07 mg g^{-1} for OCT and 1.38 ± 0.07 mg g^{-1} for BMT.

The *in vitro* assay HET-CAM was utilized for evaluate irritation potential. The choroallantoic membrane of each egg was applied directly with 0.3 mL of positive controls: NaOH (0.1 N) and SLS (1%) being classified as extremely irritant with values of

IS being, respectively, 10.24 ± 0.80 and 12.50 ± 1.20. On the other hand, the negative control as well both formulations tested did not show any of phenomena observed. Thus, according to the data from CAM assay, formulations were classified as non-irritant. Therefore, formulations produced are promising to be utilized for topical application.

3.4 Clinical Trial

Tape stripping technique was performed to establish the amount of sunscreen retained in human stratum corneum in both formulations developed. The efficacy of sunscreens formulation depends on the interaction with the stratum corneum [16] and studies have demonstrated that the use of carriers increase the amount retained and permanence' time of substances topically applied, including OMC [7,11,13,15]. Values of sunscreens retained in the stratum corneum, for SS-TiO_2-OF, were 0.287 ± 0.10 μg cm^{-2} for OMC, 0.580 ± 0.19 μg cm^{-2} for OCT and 0.471 ± 0.11 μg cm^{-2} for BMT. While for SS-SP-NC, values obtained were 0.257 ± 0.09 μg cm^{-2} for OMC, 0.561 ± 0.11 μg cm^{-2} for OCT and 0.516 ± 0.07 μg cm^{-2} for BMT. In this clinical trial there were no statiscal difference between SS-TiO_2-OF and SS-SP-NC (4). Analyses showed a lower standard deviation for SS-SP-NC, it indicates that the formulation is more homogeneous. The use of polymeric nanocapsules leads to a depot effect of actives in the more superficial layers of the skin due to their larger and rigid structure [21]. Nonetheless, the high molecular weight of sunscreens used - OMC 290 Da; OCT 823 Da and BMT 629 [35,36] - might also decrease their penetration retaining these filters in the out layers of the skin.

Therefore, there are studies showing the superior blocking activity of nanocapsules compared to sunscreens non-encapsulated due to synergic effect of absorption by organic UV filters and scattering of radiation by nanosized particles [18,37]. It also improves photostabilization of filters, as previous described in the literature [6,14,15]. Even though that there was no statistical difference between emulsions penetration, advantages of nanostructured systems to overcome some drawbacks in formulations highlight the use of the formulation developed.

Conclusion

Spray-dried nanoencapsuled system containing organic UV filters and TiO_2 as drying excipient was successfully produced and showed consistent properties, sunscreens content close to the theoretical values, pH compatible for topical application, and being classified as non-irritant. Furthermore, the nanostructured system was retained in stratum corneum, due to their size and rigid structure. Therefore, we can highlight that our approach can be an innovative cosmetic input for topical photoprotective formulations. Further studies will be carried out to evaluate the photostabilization and UV blocking activity.

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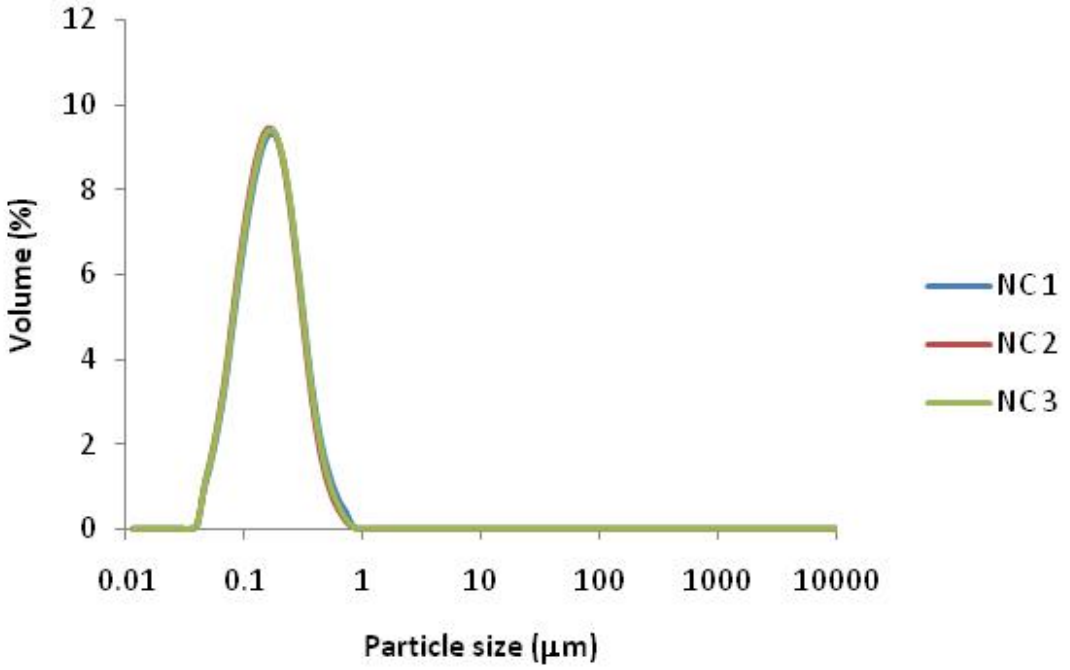

Figure 1: Particle size distribution obtained by laser diffraction (n=3) for sunscreens-loaded polymeric nanocapsules (NC). Results expressed as volume of particle size.

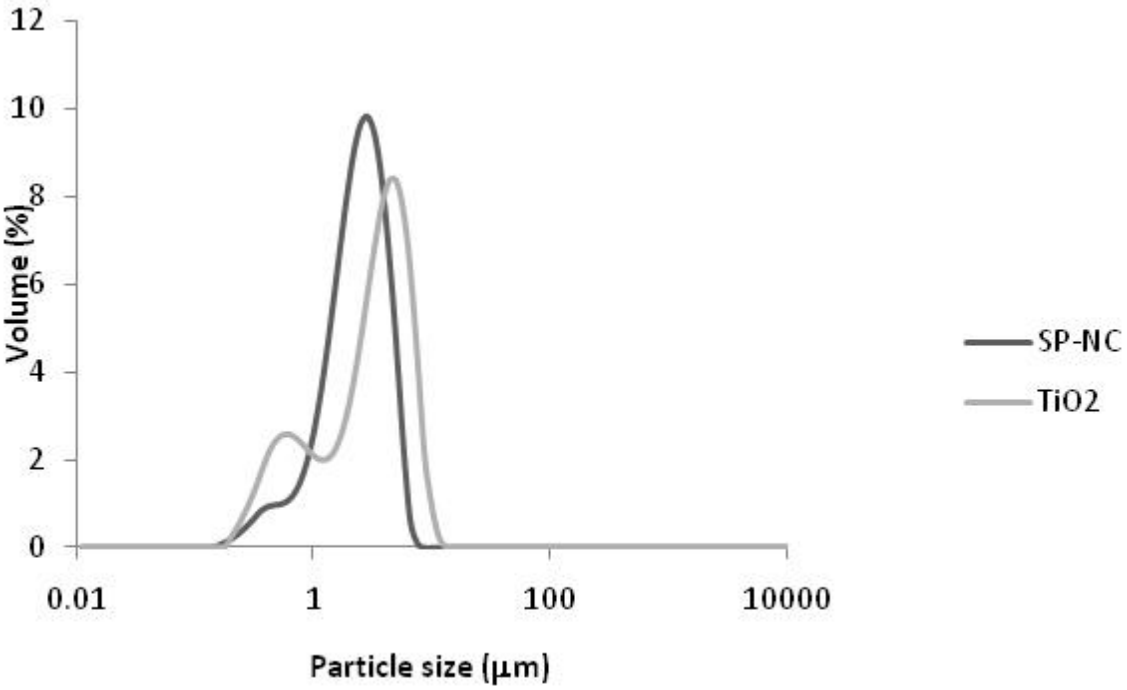

Figure 2: Average particle size distribution obtained by laser diffraction (n=3) for Spray-dried polymeric nanocapsules (SP-NC) and Commercial TiO_2.

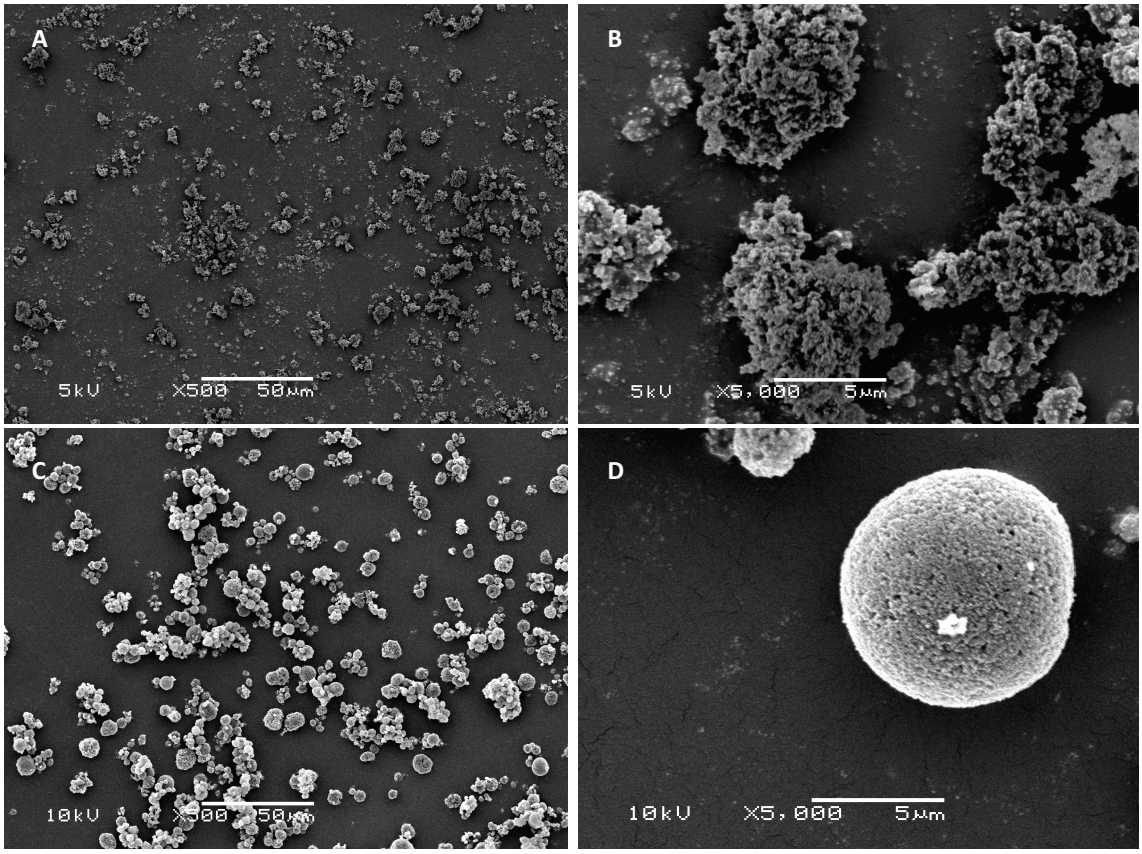

Figure 3: Images obtained by SEM of powders. a) Commercial TiO_2 at x 500; b) Commercial TiO_2 at x 5,000; c) SP-NC at x 500; d) SP-NC at x 5,000.

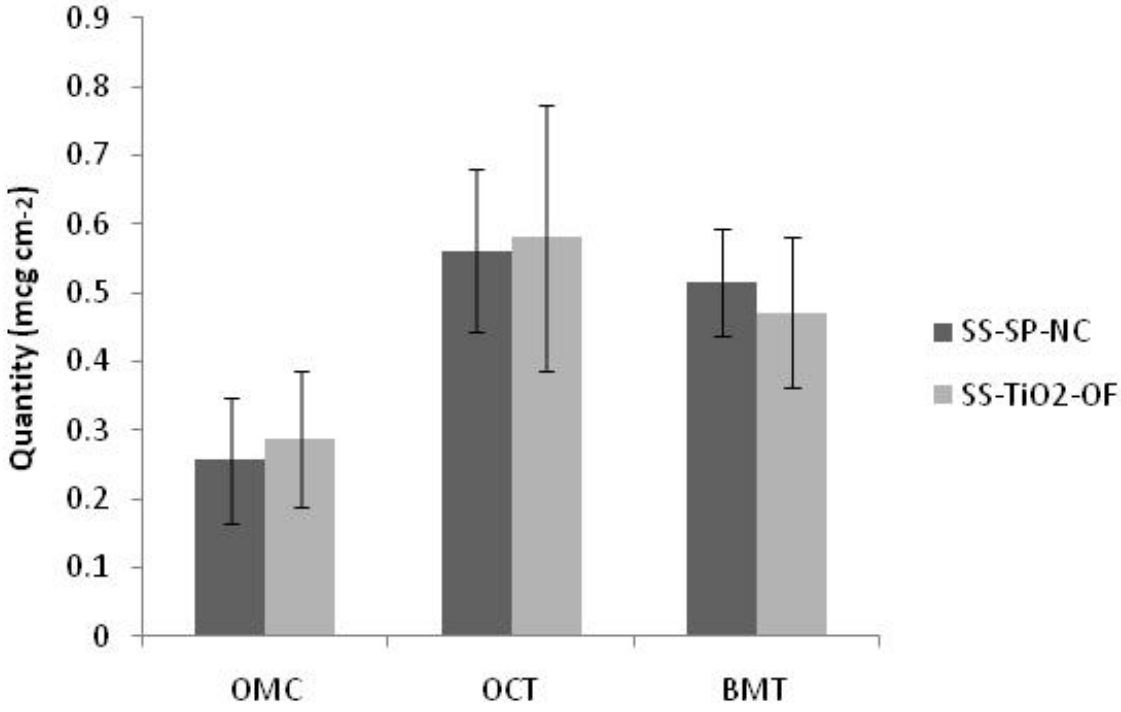

Figure 4: Sunscreen content average quantified in volunteers comparing SS-SP-NC and SS-TiO_2-OF. Results expressed in mcg cm^{-2}.

Anexo:

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