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Influence of vegetable oil on the synthesis of bioactive nanocarriers with broad spectrum photoprotection+

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Abstract: Due to their unique features, most nanostructured lipid carriers (NLCs) in association with vegetable oils that exhibit UV filtering properties and bioactivity could be used in many cosmetic formulations. Therefore, in this work, a new application of pomegranate seed oil (PSO) in the cosmetic sector was developed, based on the synthesis of bioactive lipid nanocarriers loaded with various UV filters by the hot high pressure homogenization technique. To get broad spectrum photoprotection, different UVA and UVB filters have been used (Avobenzone - AVO, Octocrylen -OCT, Bemotrizinol - BEMT). The influence of the solid lipids combined with PSO on the particle size, physical stability and entrapment efficiency was investigated using 8 nanocarrier systems. An improved physical stability and an appropriate size were obtained for NLCs prepared with Emulgade, carnauba wax and PSO (*e.g.* -30.9÷-36.9 mV and 160÷185 nm). NLCs showed an entrapment efficiency above 90% and assured slow release rates of UV filters, especially for BEMT (5%). The developed nanocarriers have been formulated into safe and effective sunscreens containing low amounts of synthetic UV filters coupled with a high percent of natural ingredients. The highest SPF of 34.3 was obtained for a cream comprising of 11% PSO and 3.7% BEMT

Keywords: *Lipid nanocarriers • Pomegranate seed oil • Effective sunscreens • Critical wavelength • Release kinetics*

1. Introduction

Nanostructured lipid carriers (NLCs) are among the most promising colloidal systems that can be readily formulated in water-based systems and have been intensively studied as drug delivery systems for various lipophilic drugs due to their multiple advantages when compared to other lipid and polymeric nanosystems [1-3]. These nanocarriers are submicron particles, usually with spherical shape and mean diameters ranging between 50 and 500 nm, composed of a mixture of solid and liquid lipids dispersed in an aqueous medium and stabilized by the presence of surfactants [4]. These lipid systems are safe and biodegradable carriers because they are produced using non-toxic and non-irritating ingredients [5]. Due to the solid nature of the lipid matrix at body and room temperature NLCs present several advantages which are manifested in the improved efficacy, enhanced permeation, chemical stability, the ability to reduce the mobility of incorporated drugs and so on [6]. Therefore, the expulsion of encapsulated drug molecules during storage is minimized and a controlled rate of the active ingredients at the site of action can be achieved [7]. In addition, due to their solid lipid matrix they can act as physical sunscreen by scattering the UV rays and an improved photoprotection can be obtained by a synergistic effect between the UV filters loaded into NLCs and its lipid matrix [8-10]. All these aspects are of great interest to sunscreen formulation technology because the conventional sunscreen formulations based on high amount of synthetic UV filters proved to have some drawbacks. For instance, the UV filters are known to bioaccumulate in humans [11] and aquatic media [12,13], where they present toxicity [14,15] and

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estrogenic activity [16]. Applied to skin, they could present adverse side effects such as allergic and irritant contact dermatitis [17], they can penetrate the skin and arrive in the systemic circulation [18,19]. Furthermore, some UV filters are photo-unstable and after sun exposure they lose the UV filtering properties [20-22], thereby the efficacy of the sunscreen is impaired. Despite these drawbacks, the use of synthetic UV filters is still needed to ensure the level of photoprotection required by European regulations [23], as natural compounds cannot fulfill this requirement. Therefore, lowering the amounts of UV filters and encapsulating them into lipid nanocarriers could lead to safe sunscreens while maintaining the same protection level as a conventional formulation.

Different studies on NLCs loaded with UV filters have proven their enhanced photoprotective properties when compared to a reference system [24-26] and also a very low skin irritation potential has been observed [27]. The photoprotective properties of the NLCs proved to be significantly influenced by the type and concentration of solid and liquid lipids used for the nanocarriers synthesis. Thus, Nikolic *et al.* synthesized NLCs with different solid lipids and concluded that carnauba wax – NLCs offer the best photoprotection [28]. Carnauba wax, which is isolated from leaves of palm tree, presents UV absorption properties by having in its composition substituents of cinammic acid [29]. The synthesis of several nanocarriers prepared with a synthetic lipid matrix, able to co-encapsulate two UV filters (*e.g.* avobenzone and octocrylene) by using the high shear homogenization method, has been reported in previous studies of the authors [30,31]. The creams developed based on NLCs presented strong photoprotective properties, with a sun protection factor (SPF) of 17 and a protection in the UVA of 50 [30]. Starting from these results, safe and effective sunscreens based on low amounts of synthetic UV filters coupled with natural compounds which possess UV filtering properties and bioactivity, co-loaded into lipid nanocarriers could be obtained. In this respect, the aim of the paper is to obtain bioactive nanocarriers with high a percent of natural ingredients for the development of sunscreens with increased SPF and broad spectrum photoprotection.

The bioactive ingredient used for NLCs production is pomegranate seed oil (PSO). PSO is an attractive nutraceutical ingredient possessing an enriched phytochemical composition with an ideal highly unsaturated fatty acid content of punicic acid. PSO has a potential biological effect by improving immune function in vivo, exhibiting antivirus, antioxidant, anticancer [32], anti-inflammatory [33] and UV absorptive properties [34,35]. Furthermore, PSO is available at low cost, as the seed is a byproduct of the pomegranate juice industry.

To get a broad spectrum photoprotection, two systems of UV filters were used; the first is composed of an UVA filter (avobenzone) coupled with an UVB filter (octocrylene), and the second of an UV filter which absorbs both UVA and UVB rays (bemotrizinol). The developed NLCs were characterized in terms of mean particle size, physical stability, entrapment efficiency, thermal analysis, antioxidant activity, *in vitro* UV absorptive properties and *in vitro* release. The evaluation of the NLCs cream formulation in developing effective sunscreen has been achieved after subjecting them to UV radiation and determining the *in vitro* SPF and the parameter of broad spectrum photoprotection - critical wavelength (λ*c*).

2. Experimental procedure

2.1. Materials

The solid lipids, Emulgade SE/PF – Em (Glyceryl Stearate, Ceteareth-20, Ceteareth-12, Cetearyl Alcohol, Cetyl Palmitate from Cognis GmbH, Dusseldorf, Germany) and carnauba wax – Wax, were provided by Elmiplant S.A. (Romania). The pomerganate seed oil – PSO was purchased from Elemental Company (Romania). The oil has a fatty acid content determined by: 71.1% punicic acid, 8.4% linoleic acid, 7.9% oleic acid, 4.8% palmitic acid, 3.6% stearic acid, 1.3% eicosenoic acid, 0.8% arachidic acid, 0.3% acid behenic, 0.1% lignoceric acid. Tween 80 (2-[2-[3,5-bis(2-hydroxyethoxy)oxolan-2-yl]-2-(2-hydroxyethoxy)ethoxy]ethyl(E)-octadec-9-enoate) was purchased from Merck, Germany. The UV filters, Avobenzone (1-(4-Methoxyphenyl)-3-(4-tert-butylphenyl)propane-1,3-dione) – AVO (Merck, Germany), octocrylene (2-ethylhexyl 2-cyano-3,3-diphenyl-2-propenoate) – OCT and bemotrizinol (5-[(2-ethylhexyl)oxy]-2-(4-{4-[(2-ethylhexyl)oxy]-2-hydroxyphenyl}-6-(4-methoxyphenyl)-1,3,5-triazin-2-yl)phenol) – BEMT have been purchased from Sigma Aldrich. The cream base (which contains stearats, glycerine, fatty alcohols, emulsifier, emollients and an antioxidant – butylhydroxyanisole) was provided by Elmiplant S.A (Romanai). Tris[Hydroxymethyl] aminomethane, 5-Amino-2,3-dihydro-1,4-phthalazinedione (Luminol) were purchased from Sigma Aldrich Chemie GmbH and hydrogen peroxide was obtained from Merck (Germany). All other chemicals used were of analytical grade.

2.2. NLCs production

Nanostructured lipid carriers (NLCs) were prepared using the high pressure homogenization method (HPH).

Table 1. Composition of NLCs loaded with UV filters.

Composition	Tween 80	PSO%	Em%	Wax%	AVO%	OCT%	BEMT%
NLC 1	2.5	3	7	-	-	-	-
NLC 2	2.5	3	7	-	0.3	0.7	-
NLC 3	2.5	3	7	-	-	-	1
NLC 4	2.5	3	3.5	3.5	-	-	-
NLC 5	2.5	3	3.5	3.5	0.3	0.7	-
NLC 6	2.5	2	3.5	3.5	0.3	0.7	-
NLC 7	2.5	1	3.5	3.5	0.3	0.7	-
NLC 8	2.5	3	3.5	3.5	-	-	1
NE 9	2.5	10			0.3	0.7	
NE 10	2.5	10			-	-	1

The lipid phase consisting of carnauba wax, emulgade and pomegranate seed oil was heated under stirring at 85°C. The UV filters, OCT and AVO or only BEMT, were added into the lipid phase, which was stirred until a clear molten solution was formed. The aqueous phase consisting of 2.5% Tween 80 was also heated under stirring at the same temperature (85°C). The lipid phase was gradually added into the aqueous phase and stirred for 0.5 h at 85°C. The formed pre-emulsion was mixed for 1 min at 12000 rpm using a high-shear homogenizer (PRO250 type; 0~28.000 rpm; power of 300 W, Germany) and was further subjected to a high-pressure homogenization (APV 2000 Lab Homogenizer, Germany) at 600 bar for 196 s. The nanoemulsions were cooled at room temperature to obtain solid lipid nanocarriers. In order to remove the excess of water, the dispersions were frozen at - 25°C overnight and they were lyophilized for 72 h at - 55°C, using an Alpha 1-2 LD Freeze Drying System (Germany). The reference nanoemulsions were produced in the same manner as NLCs, the lipid phase being entirely composed of liquid lipids, with only pomegranate seed oil. Table 1 gives an overview of the formulations produced.

2.3. Formulation characterization

2.3.1. Particle size and zeta potential

Particle size measurements were performed by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom). The mean diameter (Zave) and the polydispersity index (PdI) of the lipid nanoparticles in dispersion were determined at an angle of 90°. Before measurements, all samples were diluted with double distilled water to produce an adequate scattering intensity. The zeta potential (ZP), which reflects the electric charge on the particle surface and indicates the physical stability of colloidal systems, was determined by measuring the electrophoretic mobility of the nanoparticles in an electric field with the appropriate accessory of the Zetasizer Nano ZS. Prior to the measurements, the lipid nanoparticle dispersions were diluted with double distilled water and the conductivity was adjusted to 50 µS cm^{-1} with a sodium chloride solution (0.9%, w/v). All measurements were performed at 25°C and data was given as average of three individual measurements.

2.3.2. Entrapment efficiency and drug loading

The entrapment efficiency of AVO, OCT and BEMT into NLCs was determined by measuring the concentration of free UV filters in the aqueous phase of the nanoparticle dispersion. The loaded NLC dispersion was uniformly mixed by gentle shaking with ethanol and then centrifuged for 20 min at 15000 rpm. The supernatant was filtered using a Millipore membrane. The filtrate was collected, diluted with ethanol and the spectrum measured at λ_{max} = 356.5 nm for AVO, λ_{max} = 303 nm for OCT and λ_{max} = 344 nm using a UV–Vis–NIR spectrophotometer type V670 (Jasco, Japan). The entrapment efficiency (EE) and drug loading (DL) were calculated using the following equations [36]:

$$\%EE = \frac{W_a - W_s}{W_a} \times 100 \qquad (1)$$

$$\%DL = \frac{W_a - W_s}{W_L} \times 100 \qquad (2)$$

where, W_a is the weight of UV filters added into NLCs, W_s is the analyzed weight of UV filters in supernatant and W_L is the weight of the lipid phase.

2.3.3. Thermal analysis

Thermal curves were recorded by a differential scanning calorimeter (DSC Jupiter, STA449C, Netzsch, Germany). The samples (lyophilized lipid nanoparticles unloaded or loaded with UV filters and their corresponding lipid bulk materials) were scanned at a heating rate of 10°C min^{-1} over the range of 20 – 110°C. The samples (10 mg) were weighed in a standard alumina pan using an empty pan as reference.

2.3.4. Antioxidant activity

The in vitro antioxidant activity of lipid nanocarriers was determined by the chemiluminescence method using a Chemiluminometer Turner Design TD 20/20, USA. Luminol and H_2O_2 in Tris-HCl buffer solution (pH 8.6) have been used as a generator system for free radicals. Luminol has been used as a light amplifying substance that emits light when it is oxidized and it is converted into an excited amino-phthalate ion in the presence of reactive oxygen species such as H2O2. Starting from lyophilized NLC with a concentration of 2.2% AVO and 5.2% OCT (NLC 2, 5, 6, 7) or 7.4% BEMT (NLC 4, 8), and with an oil concentration of 22% (NLC 1 – 5, 8) or 14.8% (NLC 6) or 7.4% (NLC 7) ethanol solutions were prepared having a concentration of 3.3 mg L^{-1} AVO and 7.8 mg L^{-1} OCT, 11.1 mg L^{-1} respectively and 33 mg L^{-1} AO and 22.2 mg L^{-1} PO 11.1 mg L^{-1}, respectively. Prior to the measurements, the samples were ultrasonicated for 5 min. The antioxidat activity (the percent of free radicals scavenged) was calculated using the following formula:

$$\%AA = (I_0 - I_s)/I_0 \times 100 \tag{3}$$

where I_0 and I_s are the chemiluminescence maximum for the standard, and for the sample at t = 5 s.

2.3.5. In vitro UV absorptive properties

The photoprotective properties of NLCs were preliminary assessed by evaluating their UV absorptive capacity. Thus, the lipid nanocarriers in dispersion loaded with AVO and OCT or only with BEMT (2 mg cm^{-2}) were evenly applied onto a quartz plate covered with Transpore™ tape (3M Health Care, U.S.A.) and the absorption spectrum was registered from 290 to 400 nm with a UV – Vis V670 Spectrophotometer equipped with an integrated sphere. A quartz plate covered with Transpore™ tape and without a sample was used as reference support [37]. The absorption spectrum of each sample was obtained from six measurements in different points.

2.3.6. In vitro release studies and kinetic modeling

The UV filters in vitro release was studied in phosphate-buffered saline, pH 5.5/ethanol (70:30, v/v), by applying the vertical Franz glass diffusion cells (Hanson Reasearch Corporation, USA) method at 37°C and 400 rpm. The Franz diffusion cell consists of a donor and a receptor chamber between which a cellulose nitrate membrane filter (0.1 µm; Whatman, Germany) is placed. The release medium from the receptor chamber consisting of ethanol buffer solution had a volume of 6 mL. Prior to the analysis, the membrane was previously hydrated in ethanol buffer solution for 1 h, and then 200 µL of nanoparticles in dispersion were placed onto it, in the donor chamber. During a period of 8 hours, after each hour, 1 mL of sample was withdrawn from the receptor chamber and diluted with ethanol for examination. The volume of release medium was maintained constant throughout the study. The amount of released AVO, OCT and BEMT from the samples was determined spectrophotometrically at λ_{max} 356.5 nm, 303 nm and 344 nm, respectively. The release data of UV filters from NLCs were fitted into various kinetics models by using the following mathematical models equations [38,39]: zero order:

$$\%R = k_0 t \tag{4}$$

first order:

$$\log(100 - \%R) = k_1 t / 2.303 \tag{5}$$

Higuchi:

$$\%R = k_2 \sqrt{t} \tag{6}$$

Korsmeyer–Peppas:

$$\%R = k_3 \sqrt[n]{t} \tag{7}$$

where,%R is the percent of UV filter released at time t; k_0, k_1, k_2, k_3 and k_4 are the rate constants for zero order, first order, Higuchi, Korsmeyer–Peppas and Hixson–Crowell respectively; and n is the release exponent. The mathematical model equation with the highest correlation coefficient (R^2) was selected as the best release kinetic model.

2.4. Characterization of cream based lipid nanocarriers

2.4.1. In vitro SPF and critical wavelength (λ_c) determinations

Cream formulations based on pomegranate seed oil – NLCs were developed by incorporating the lyophilized lipid nanocarriers into a cream base. The SPF (sun protection factor) is a parameter used to estimate the UVB protection efficacy of sunscreens. The critical wavelength is used to evaluated the broad spectrum photoprotection efficacy and it represents the wavelength at which 90% of the area under the absorbance curve (AUC), which is set at 100%, is reached in the range

290 – 400 nm. It is considered as a broad spectrum sunscreen if the formulation achieved λ_c higher than 370 nm [40]. The in vitro determination of SPF and λ_c was performed according to Diffey and Robson methodology [41]. The method is based on the measurement of the radiation intensity transmitted through the substrate of the applied sample, by recording the photocurrent in 5 nm steps from 290 to 400 nm. Each sample was spectrophotometrically registered in six different points and the average value was used to calculate the SPF and the *λc* by using the following equations:

$$SPF = \sum_{290}^{400} E_\lambda B_\lambda \Big/ \sum_{290}^{400} \frac{E_\lambda B_\lambda}{MPF_\lambda} \tag{8}$$

$$\int_{290\,nm}^{\lambda_c} A(\lambda) \times \delta\lambda = 0.9 \times \int_{290\,nm}^{400\,nm} A(\lambda) \times \delta\lambda \tag{9}$$

where, E_λ is the spectral irradiance of terrestrial sun light under defined conditions; B_λ is the erythemal effectiveness; MPF_λ is the monochromatic protection factor at each wavelength increment (the ratio of the detector signal intensity without formulation applied to the substrate to that with formulation applied to the substrate) and A is the absorbance of the sample.

2.4.2. Photostability studies

The photostability of the developed creams was evaluated by exposing them to radiation at two wavelengths, 365 nm (UVA) and 312 nm (UVB), using the BioSun system (Vilver Lourmat, France). The creams contained 11% PSO and 3.7% UV filters or their equivalent amounts encapsulated into NLCs (1% AVO and 2.6% OCT – NLC 2, 5, 6, 7 or 3.7% BEMT – NLC 3, 8). The samples were irradiated over two stages: irradiation I - with energy of 19.5 J cm^{-2} and irradiation II with energy of 39 J cm^{-2}. The SPF and λc of the samples were evaluated after irradiation.

3. Results and discussion

Mean particle size, polydispersity index and zeta potential of the lipid nanocarriers developed with pomegranate seed oil are illustrated in Figs. 1 and 2, respectively. Lipid nanocarriers based on different lipid matrices with particle sizes ranging from 130 to 280 nm and with relatively uniform particle size distribution (PdI of 0.13 ÷ 0.25) were obtained (Fig. 1). Free - NLC 1 prepared with only one solid lipid, Emulgade, presents the smallest particle size of 133 nm, while for the free - NLC 4 prepared with two solid lipids, Emulgade and carnauba wax, an increase in the particle size has been observed (*e.g.*190 nm). The larger particle size of free - NLC 4 is attributed to the higher melting point of carnauba wax (82-86°C) than Emulgade (49-52°C), a fact which determines the formation of a more viscous dispersed phase. Because of the more viscous dispersed phase, the surface tension increases and leads to the formation of larger particles [27,42].

By encapsulating the UV filters into NLCs, the particle size of nanocarriers prepared with Emulgade is significantly increased, by 150 nm, (NLC 2 and 3), while for nanocarriers prepared with Emulgade and carnauba wax, the size is slightly decreased (NLC 5 and 8), as compared to the free NLCs. The increase in particle size of loaded NLCs prepared only with Emulgade and pomegranate oil could be attributed to the higher melting point of AVO (81–86°C) and BEMT (83-85°C) when compared to that of Emulgade or could be due to an inadequate spatial arrangement of the UV filters inside the lipid core. The slight decrease of particle size in case of NLCs based on Emulgade/carnauba wax and pomegranate oil could be a result of many imperfections present in the lipid core which allow a better fitting of the UV filters inside the lipid core, between the lipid layers of the crystalline lattice, without disturbing the lipid arangament.

For the NLCs with the optimum particle size (*e.g.* lipid matrix composed of Em, Wax and PSO), the vegetable oil concentration was varied in order to study their influence on the NLCs properties. One can note that by reducing the oil concentration, larger particles are formed since the oil contributes to a decrease of the dispersed phase viscosity.

Regarding the type of UV filters encapsulated into NLCs, BEMT determines the formation of particles with only a few nanometers smaller than those of NLCs co-loaded with OCT and AVO.

The particle charge is one of the factors that determines the physical stability of colloidal systems and it is quantified as a function of zeta potential value. The magnitude of the zeta potential determined on the synthesized NLCs ranged between −21.7 and − 36.9 mV (Fig. 2). Typically, particles with zeta potential values more negative than –30 mV are considered physically stable, while –20 mV provide only a short-term stability. Interesting results were obtained when using the two types of lipid matrices. For instance, the NLCs prepared with the lipid matrix consisting in Em, Wax and PSO are more stable than NLCs prepared with a mixture of Em and PSO. The zeta potentials of NLC 1÷3 ranged between −21.7 and −24.3 mV and of NLC 4÷8 ranged between −30.9 and − 36.9 mV. This significant difference could be associated to the carnauba wax presence. The main compounds found in carnauba wax are aliphatic esters, α-hydroxyl esters, cinnamic

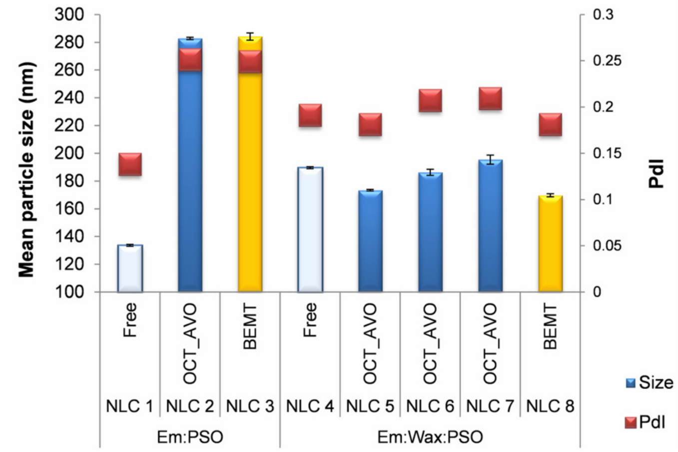

Figure 1. Mean particle size and polydispersity index (PdI) of the lipid nanocarriers synthesized with different lipid matrices.

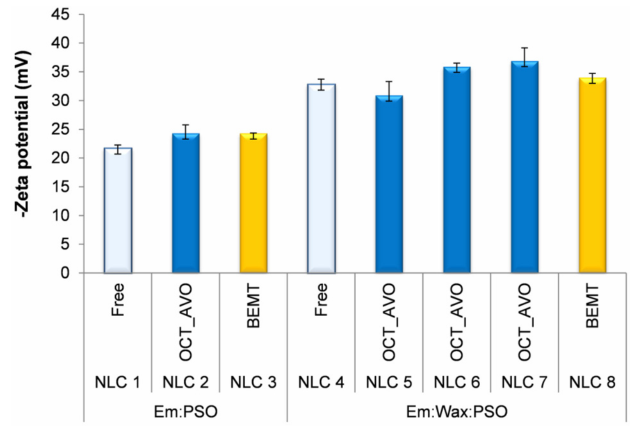

Figure 2. Zeta potentials values of lipid nanocarriers synthesized with different lipid matrices.

aliphatic diesters and free fatty acids. This composition may lead to a disruption of the surfactant shell which occurs with a partial rearrangement of surface charge and results in a change of zeta potential values.

Regarding the influence of the oil, by decreasing the oil concentration the physical stability of the nanocarriers was slightly increased by about 5 mV in absolute value.

3.1. Entrapment efficiency and drug loading

The developed NLCs based on pomegranate seed oil showed a high ability to entrapp both kinds of UV filters, with high entrapment efficiency values ranging between 89 and 99% (Fig. 3). Good drug loading values (Fig. 4) were also obtained for the synthesized nanocarriers (Fig. 4). For instance, NLC 5 showed a DL of 9.6% (3% AVO and 6.6% OCT) and NLC 8 of 9.5% (BEMT) from the maximum 10% DL which could be reached.

These values demonstrate an efficient capacity of the lipid matrices of NLCs to load different types of UV filters, presenting similar loading capacity for both systems, loaded with one UV filter (BEMT) or with two UV filters (AVO and OCT).

Out of the two types of lipid matrices used, the NLCs with Em:Wax:PSO have presented better entrapment efficiency values (94–99%) than the NLCs with Em:PSO (89–92%). This could be explained by the fact that in using more different lipid structures for NLCs synthesis, the resulted lipid core presents a more disordered lipid lattice, with space between the lipid layers that can be used for the active compund to fit in.

The amount of UV filters encapsulated into NLCs has influenced the entrapment efficiency, by having small quantities of active compund, a better entrapment in the lipid core of the NLCs was obtained. Thus, the best entrapment efficieny value of 99% is obtained for AVO – NLC 5 which is introduced in a concentration of 0.3% (w/w of the NLCs in dispersion).

By evaluating the influence of the oil concentration used for the NLCs synthesis (NLC 5÷7), it can be observed that the EE values were not affectted by the amount of oil found in the lipid matrix.

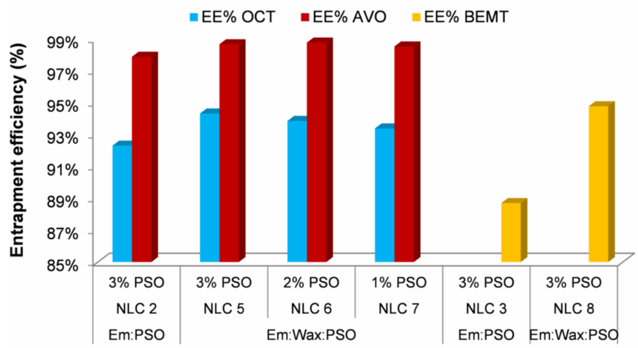

Figure 3. Encapsulation efficiency of UV filters into the developed lipid nanocarriers synthesized with different lipid matrices.

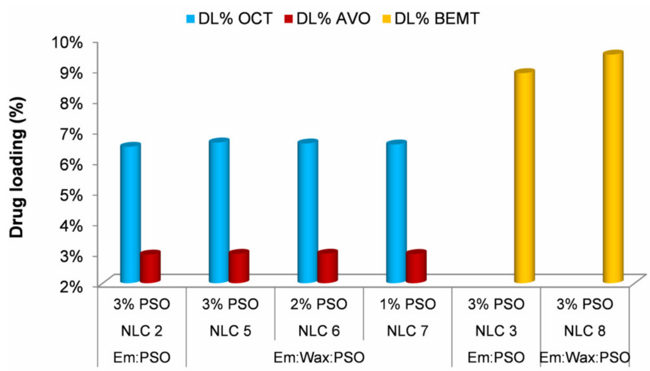

Figure 4. Drug loading capacity of lipid matrix of the developed lipid nanocarriers synthesized with different lipid matrices.

3.2. Differential scanning calorimetry analysis

In order to evaluate the changes in the crystalline state of the developed lipid nanocarriers differential scanning calorimetry analysis was performed. The DSC parameters, melting enthalpy and melting peaks, of the free and loaded – NLCs prepared with the two types of lipid matrix and of their coresponding physical lipid mixtures are presented in Table 2.

The DSC curves of the physical mixtures, free NLCs and OCT_AVO – NLCs were compared in Fig. 5. The endothermic peaks of the lipids from the physical mixtures shifted to lower temeratures in the free and loaded - lipid nanocarriers. This shift could be due to the lipid matrix formation at nanometer size, previously reported by Westesen and Bunjes (1995), as well as to the presence of surfactants distributed into the lipid phase (free – NLCs) and UV filters (loaded – NLCs), according to Das *et al*. (2012) [43,44].

Also, a reduction in crystallinity of the lipid matrix of free NLCs was observed when compared to the physical mixture, indicated by the reduction of the enthalpies [45]. This reduction is attributed to the partial formation of lower energy lipid modifications.

This crystallinity is further reduced by encapsulating the UV filters into NLCs synthesised with the lipid matrix Em:PSO. On thecontrary, the loaded NLCs synthesized with the Em:Wax:PSO present a more crystalline lattice, having larger enthalpies than those corresponding to free – NLCs, but still manifest a shift to lower melting temperatures.

The melting peaks of AVO (81–86°C) and BEMT (83–85°C), indicated by literature data, were absent in both loaded NLCs, a fact which is more evident for the NLCs with Em:PSO. This indicates a complete solubilization or an amorphous state of the UV filters inside the lipid matrix of NLCs, which determines a decrease of the lipid molecules cooperativity [46] as a result of a less crystalline structure for the NLCs with Em:PSO.

For the NLCs with Em:Wax:PSO this behaviour could indicate that the UV filters formed clusters entrapped inside the NLCs, between the lamellar lattice structure of the lipids, considering that carnauba wax is a more crystalline lipid than Emulgade. The last aspect was also presented by Lukowsky *et al.* (2000), which studied by crystallographic analysis the solid lipid nanoparticles prepared using cetyl palmitate as solid lipid and observed that cetyl palmitate was arranged in a lamellar lattice structure in nanoparticles [47]. These results are in agreement with the previous particle size results.

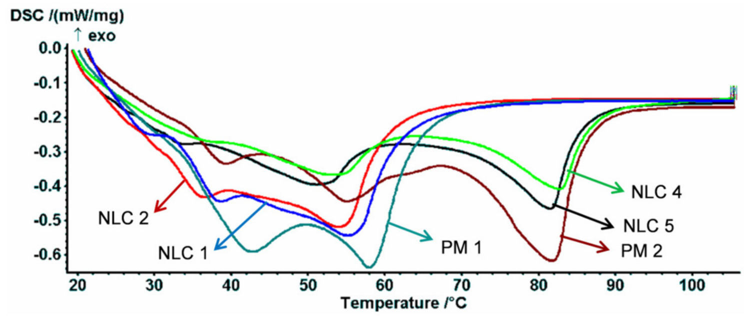

Figure 5. DSC curves of free (NLC 1 and 4) and loaded NLCs (NLC 2 and 5) in comparison to their physical lipid mixtures (PM 1 and 2).

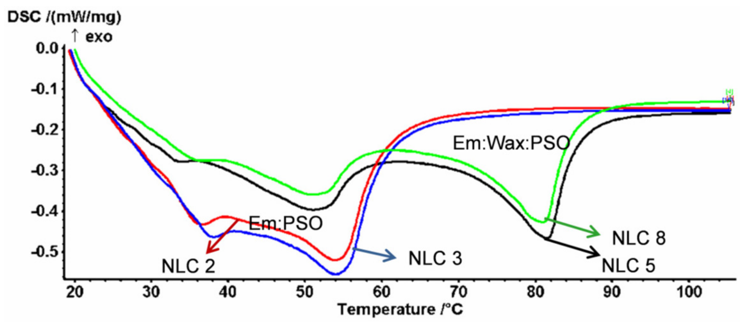

Figure 6. DSC curves of the NLCs loaded with AVO and OCT (NLC 2 and 5) in comparison to BEMT – NLCs (NLC 3 and 8).

Table 2. DSC parameters, enthalpy (ΔH, J g^{-1}) and melting peak (Mp, °C) of free and loaded – NLCs, and of their corresponding physical mixtures (PM).

Sample	ΔH (J/g)	Mp (°C)	Mp (°C)	Mp (°C)	CI
PM 1 Em:PSO	114.4	42.8	58	-	100
NLC 1	88.34	38.8	55.2	-	77.2
NLC 2	76.42	36.7	53.9	-	66.8
NLC 3	84.9	38.1	54	-	74.2
PM 2 Em:Wax:PSO	144.1	39.4	55.2	81.8	100
NLC 4	93.42	36.8	53.3	82.7	64.8
NLC 5	98.21	34.1	51.2	81.6	68.1
NLC 6	109.7	35.6	53.8	79.8	76.1
NLC 7	132.3	35.1	53.9	80.9	91.8
NLC 8	98.11	36.9	51.1	81	68.0

Regarding the thermal behaviour of the NLCs loaded with different types of UV filters, there are not significant differences in their calorimetric curves, as shown in Fig. 6. This suggests that all the lipid matrices used for NLCs present enough space to entrap different types and numbers of active compounds, a fact which is in accordance with the entrapment efficiency results.

On observing the behavior of the NLCs prepared with different amounts of oil (NLC 5–7), it was found that a higher concentration of oil produced modifications in the lipid network with a more disordered lattice, suggested by the decrease of the enthalpies and of the melting points (Table 2). Thus, the use of a high concentration of pomegranate seed oil in the NLCs synthesis is desired due to the fact that a disordered lipid matrix could provide more space and a better entrapment of the active compounds. In the present study, according to the entrapment efficiency results, by increasing the oil concentration an insignificant modification of the UV filters loading inside the NLCs was obtained.

3.3. Antioxidant activity

The pomegranate seed oil is a natural ingredient known for its antioxidant compounds, anthocyanidins (such as delphinidin, cyanidin, and pelargonidin) and hydrolyzable tannins [48]. In order to evaluate its in vitro antioxidant activity and that of the developed NLCs the chemiluminescence method was used. The results in Fig. 7 revealed good antioxidant properties of the developed NLCs with values ranging between 67÷74.6%. The free NLC 1 and 4 developed with different lipid matrices presented an enhanced AA of 67% and 71.3%, respectively, when compared to the pure pomegranate oil (AA = 63.9%). Therefore, by bringing the oil at nanometer scale into NLCs, the

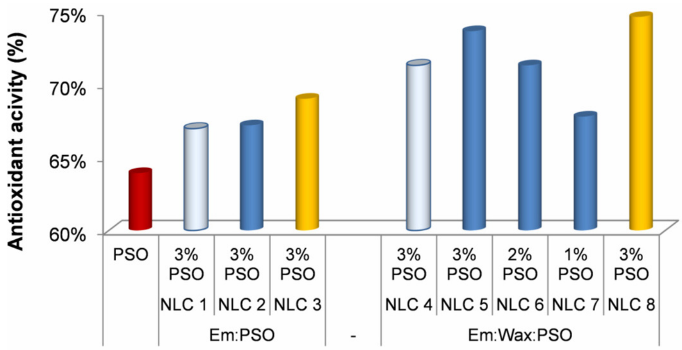

Figure 7. The antioxidant properties of the lipid nanocarriers synthesized with various solid lipids and oil concentrations.

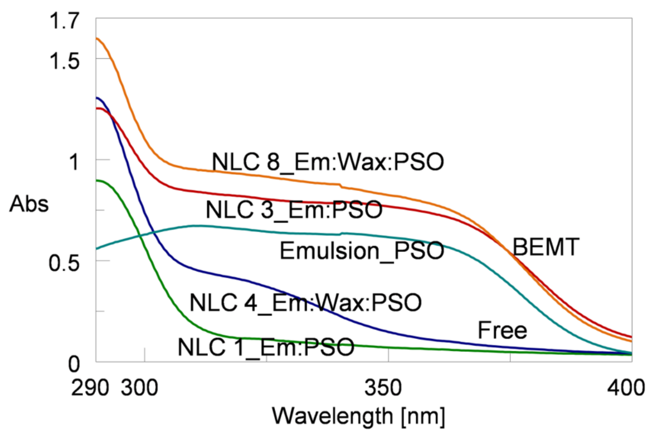

Figure 8. UV absorbance of free and BEMT – NLCs in dispersion in comparison with reference nanoemulsion.

antioxidant properties of the oil were enhanced. In addition, the presence of an antioxidant, the cinnamic acid derivative, in the composition of carnauba wax has determined a greater antioxidant activity of the NLCs with the lipid matrix Em:Wax:PSO.

The UV filters have contributed to an improved AA of loaded NLCs compared to those of free-NLCs, between which the BEMT – NLCs exhibited better values than OCT_AVO – NLCs.

Also, it was noticed that by increasing the oil concentration in NLCs synthesis an enhancement of the antioxidant activity was obtained, thus the NLC 5 (3% oil) presented an AA of 73.6% and NLC 7 (1% oil) presented an AA of 67.8%.

3.4. *In vitro* UV absorptive properties

The ultraviolet spectral analysis of free and loaded NLCs in dispersion and of reference nanoemulsions o/w, with the same concentration of UV filters, was performed in the UVB and UVA ranges, from 290 to 400 nm.

In Fig. 8, the UV absorbance of free and BEMT – NLCs in dispersion in comparison with a reference nanoemulsion is shown. It is observed that the NLCs with the lipid matrix Em:Wax:PSO present the highest absorbance as compared to the NLCs with Em:PSO matrix, followed by the reference nanoemulsion. The NLCs synthesized with both types of lipid matrix have better absorptive properties than the reference nanoemulsion as the solid lipid core of the NLCs assures an additional photoprotective effect by scattering the UV filters. Furthermore, the more crystalline structure and the presence of cinnamic acid derivatives in the composition of carnauba wax have determined an improvement of the UV scattering and filtering properties as compared to NLCs prepared only with Emulgade as solid lipid.

It is interesting to note that the free - NLCs with the lipid matrix composed of carnauba wax present significant filtering of UV rays. Fig. 9 shows that by increasing the oil concentration used in NLCs synthesis, the UV absorbance shifts to higher values leading to improved UV filtering properties.

By comparing the UV properties of the different types of UV filters encapsulated into particles, the NLCs and the reference nanoemulsion loaded with BEMT have a higher absorbance than those loaded with OCT coupled with AVO (Fig. 10). These results are in good agreement with the previous entrapment efficiency values.

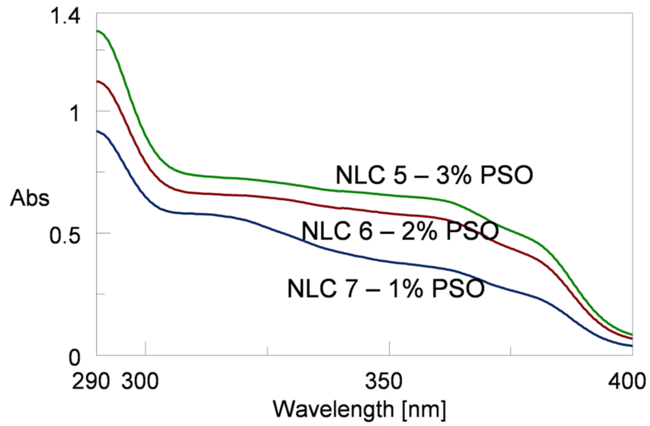

Figure 9. UV absorbance of OCT_AVO – NLCs based on Em:Wax:PSO in dispersion synthesised with diferent concentrations of pomegranate seed oil.

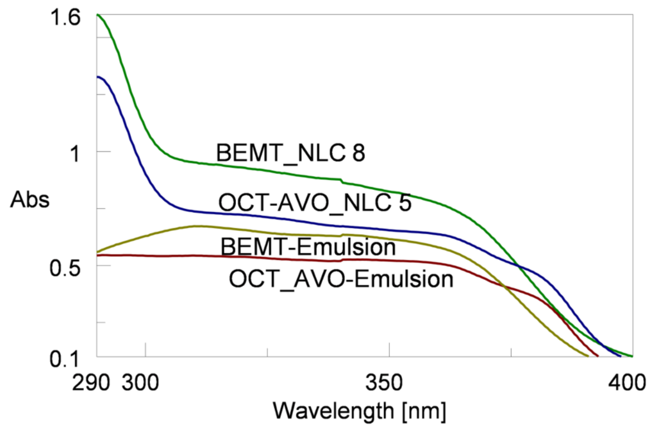

Figure 10. UV absorbance of BEMT – NLCs dispersions and BEMT – NE 10 in comparison with OCT_AVO – NLCs dispersions and BEMT – NE 10.

3.5. *In vitro* release and kinetic modeling

The lipid nanocarriers with the best photoprotective properties were selected for the in vitro release study. The release profiles of AVO and OCT from NLC 5 and of BEMT from NLC 8 are shown in Fig. 11. After 8 hours of study, the slowest release rate of 5% was obtained for BEMT. The NLC 5 presented a release of 6.6% AVO and 23.7% OCT.

The release profiles of UV filters showed a biphasic pattern with a relatively rapid release in the first hour, followed by a sustained release for the next hours. This release pattern is associated with the homogenization method used for the NLC synthesis, as it was previously stated by other studies [49,50]. The release rate of OCT is faster than the release rate of AVO and BEMT, a fact which could be attributed to a higher solubility of OCT in the release medium than the other UV filters. Thus, the percentage of OCT released from NLC 5 does not represent a risk to the human skin because the release percent is still in acceptable limits.

The release data have been incorporated into various release kinetics models including zero order, first order, Higuchi, and Korsmeyer-Peppas. The correlation coefficient (R^2), the rate constant (k) and the release exponent (n) of the UV filters are presented in Table 4. For all UV filters, the data best fits the Peppas-Korsmeyer release kinetic model with $n < 0.5$. This release model describes a Fick diffusion process from a homogenous matrix system.

This study demonstrates that the developed NLCs present appropriate nanoconfinements able to incorporate and to assure a slow release rate of toxic UV filters resulting in minimal side effects.

3.6. Characterization of cream based lipid nanocarriers

The broad spectrum photoprotective properties and the photostability of the developed NLCs were assessed by means of SPF and critical wavelength, after the NLCs were formulated into creams. These results are

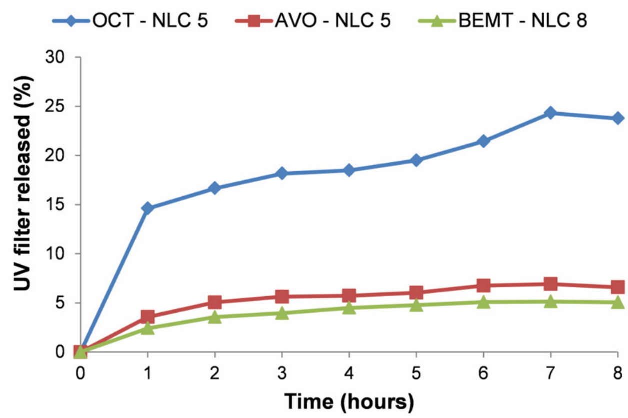

Figure 11. The release profiles of OCT, AVO and BEMT from the developed lipid nanocarriers.

in accordance with the UV absorbance measurements. Thus, the NLCs with the solid lipids carnauba wax and Emulgade have higher SPF values than the NLCs with Emulgade and reference creams, values which are shown in Fig. 12.

The highest SPF of 34.3 was obtained for the cream with a content of 11% PSO and 3.7% BEMT based on NLC 8 with the lipid matrix of Em:Wax:PSO. The SPF of NLC 8 is twice as high as the reference cream containing the same amount of BEMT and oil (SPF 15).

The influence of the pomegranate seed oil on the SPF values of the creams based on OCT_AVO – NLCs is shown in Fig. 13. By increasing the oil concentration, the SPF values are not only significantly improved, but they are also slightly higher after the UV irradiation.

The critical wavelength of the developed creams is indicated in Table 3. It is observed that all the creams based on NLCs, with both types of lipid matrices and UV filters, and on reference creams present initial λ_c with values greater than 370 nm. Thus, all the developed creams are broad spectrum photoprotective formulations.

Exposed at different stages of UV radiation, after each stage the SPF of NLCs increased while the SPF of the reference creams decreased. The λc was also affected; the creams which still present broad spectrum photoprotection after the irradiation are based on loaded NLC 4÷8 with the lipid matrix Em:Wax:PSO and on NLC 2 – Em:PSO loaded with BEMT.

It is important to highlight that by using only natural ingredients, like pomegranate seed oil and carnauba wax, it was possible to obtain a sunscreen formulation based on free NLC 4 – Em:Wax:PSO with minimum SPF (5.6) and with protection on both UVA and UVB ranges. This was possible because of a synergistic effect of the solid lipid matrix formed at the nanoscale and of the UV filtering properties of the compounds found in the pomegranate oil and in carnauba wax.

Table 3. The critical wavelength of creams based on NLCs exposed to UV radiation.

Sample	λ_c initialy	λ_c irrad I	λ_c irrad II
NLC 1	370	368	369
NLC 2	376	371	368
NLC 3	374	371	371
NLC 4	371	355	355
NLC 5	377	372	370
NLC 6	376	373	370
NLC 7	378	373	370
NLC 8	374	371	370
Reference OCT_AVO	376	367	368
Reference BEMT	374	369	369

Table 4. The release kinetics parameters of AVO, OCT from NLC 5 and of BEMT from NLC 8.

UV filter		Order 0	Order 1	Higuchi	Peppas
OCT	R^2	0.7194	0.7529	0.9156	0.9171
	k	2.5296	0.0127	8.0378	14.1318
	n				0.23
AVO	R^2	0.7562	0.7645	0.9484	0.9544
	k	0.8034	0.0036	2.5341	3.7661
	n				0.31
BEMT	R^2	0.8032	0.809	0.9725	0.9749
	k	0.6357	0.0028	1.9701	2.5603
	n				0.38

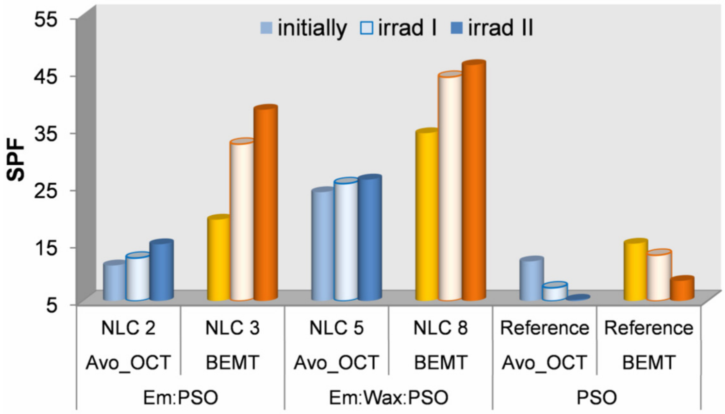

Figure 12. SPF values of the creams based on lipid nanocarriers synthesized with different lipid matrices exposed to UV radiation.

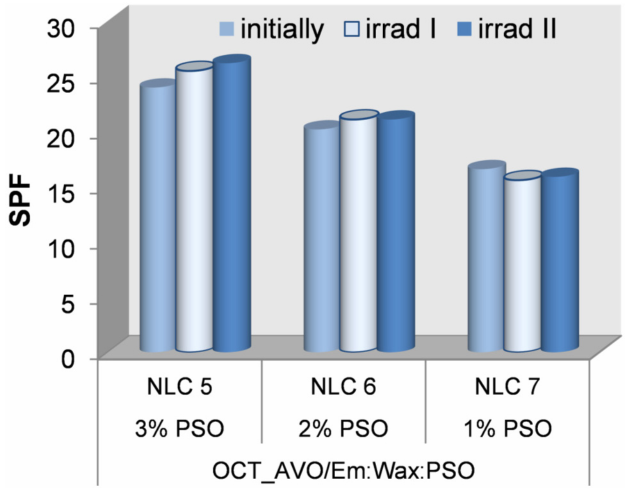

Figure 13. SPF values of the cosmetic creams based lipid nanocarriers co-loaded with UV-filters (2.6% OCT and 1.1% AVO).

4. Conclusions

The use of natural ingredients is highly pursued in human health, particularly in cosmetics, with an ongoing search for developing efficient health products with broad biological relevance. Safe and effective sunscreens based on lipid nanocarriers containing low amounts of synthetic UV filters and high amounts of natural ingredients, which possess UV filtering properties and bioactivity, were developed in the current work.

Lipid nanocarriers based on different lipid matrices formed with pomegranate seed oil, with particle sizes ranging from 130 to 280 nm and with a narrow particle size distribution were obtained by the high pressure homogenization technique. An improved physical stability and a size less than 160 nm were obtained for nanocarriers prepared with Emulgade, carnauba wax and pomegranate seed oil. The developed NLCs based on pomegranate seed oil showed entrapment efficiency values for the UV filters ranging between 89 and 99%. A more crystalline structure and the presence of cinnamic acid derivatives from the NLCs prepared with carnauba wax and Emulgade have determined an improvement of the UV scattering and filtering properties, when compared to the NLCs based only on Emulgade. The in vitro evaluation of the antioxidant activity has revealed the ability of NLCs to scavenge between 67 and 74.6% of free oxygen radicals.

The release study showed, after 8 h of experiments, a faster release rate for OCT (23.7%) and a slower release rate for BEMT (5%) and AVO (6.6%). These results indicate that NLCs present a lower toxicity by retarding the penetration of organic UV filters through the skin.

The developed nanocarriers have been formulated into safe and effective sunscreens containing low amounts of synthetic UV filters coupled with a high percent of natural ingredients. The highest SPF of 34.3

was obtained for the NLCs based-cream containing 11% PSO and 3.7% BEMT. This SPF value is two times higher than the reference cream containing the same amount of UV filter and pomegranate seed oil (SPF 15).

Based on the results of this study and the scientific literature on the use of nanotechnology in cosmetics, this work shows that this emerging approach to replacing synthetic UV filters with natural ingredients originated from vegetable sources becomes a crucial tool, not only for scientific research but also for industrial development of new cosmetic products.

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