THE INFLUENCE OF VEGETABLE OIL AND SELF-ORGANIZING AGENTS' COMPOSITION ON OBTAINING STABLE NANOSTRUCTURED LIPID CARRIERS

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The scope of this research was to identify optimal compositions for both lipid and aqueous phases, suitable for obtaining nanostructured lipid carriers (NLCs) based on vegetable oils – primrose oil (Po) and soybean oil (So) – with average dimensions of about 200 nm and to evaluate their physical stability by determining the mean particle size (Z_{ave}) and zeta potential (ξ) at different time intervals (3 days, 1÷4 weeks and 60 days). Results showed that the composition of 2.5% surfactant mixture, in a weight ratio Tween20: Poloxamer 188: Phosphatidylcholine = 4.66:1:1, is the most suitable for obtaining NLCs as efficient drug distribution systems.

Keywords: stable nanostructured lipid carriers, primrose oil, soybean oil.

1. Introduction

Nanostructured Lipid Carriers (NLC) represent a widely explored generation of lipid nanoparticles developed in the early 2000s by *Muller et al*. for lipophilic drug delivery [1]. NLC are composed by a lipid core made of biocompatible solid and liquid lipids, stabilized by an outer shell of surfactants [2]. The lipid core itself determines the drugs' pharmaceutical properties as it is the structure that preserves, delivers and releases the drug [3]. In addition to their excellent physical stability, biocompatibility and biodegradability, NLCs provide emphasized features in terms of multiple pharmaceutical and food applications [4, 5, 6]. Nanostructured lipid carriers have been employed for improving drug therapy, due to their ability to augment drug stability, decrease the toxicity of drugs [7], solubilize poorly soluble drugs [8], and achieve controlled and sustained delivery in addition to drug targeting at site of action [9]. NLC exhibit superior controlled drug release capacity over other lipid carriers in the gastrointestinal tract and different release mechanisms by adjusting the solid form, by means of appropriate liquid-solid lipids content [10]. For the promotion of low water-soluble drug absorption, NLC can maintain sufficient solubility in the intestinal

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absorption site on account of the small particle size and lipid solubilization [11]. By interacting with bile salts to form micelles, the lipids from NLC degrade to mono- and diacylglycerols triggered by digestive enzymes to improve drug solubility by the intestinal enterocytes [12].

NLC are fabricated by mixing solid lipids with spatially incompatible lipids leading to nanoparticulate structures with improved drug loading and controllable release properties [13]. To obtain NLC, there are several methods of preparation, microemulsions [14], high-shear homogenization/HSH [15, 16], high-pressure homogenization/HPH [17, 18], sonication [19], solvent diffusion [20] etc. Out of the numerous methods reported to prepare NLC, melting emulsification coupled with HPH is the most simple and preferred method [18, 21]. This last preparation method of NLC implies the existence of high-water content. Any product containing water must be preserved; for example, inhibition of bacterial growth and preservation of the initial properties (e.g. size and zeta potential of suspended nanoparticles) must be ensured. There are mainly two possibilities available for NLC preservation [22]: (i) removal of water content and transformation of the aqueous suspension of nanoparticles into a solid, by lyophilization; (ii) addition of specific preservatives.

Among the obstacles encountered in the development of NLC, their long-term physical instability is undoubtedly an important factor of concern. Certain NLC stabilized with surfactants and co-surfactants, even in aqueous dispersions, show good long-term physical stability, which can sometimes reach several months. The stability of these NLC systems depends on the composition in lipids and surfactants, as well as on storage conditions: type and ratio between lipids that form the lipophilic network [22], choice of surfactants that stabilize the lipid core [23], temperature, light exposure, type of containers storage etc. Phenomena such as particle aggregation, increasing particle size or gelling are the most common phenomena that can occur during the storage of aqueous dispersions of NLC [24]. As a result, the objectives of this research were as follows: *i.* firstly, to prepare consecutive series of NLC in order to identify optimal compositions for solid lipids and vegetable oils as well as a weight ratio between surfactants, suitable for the preparation of lipid nanocarriers systems with average dimensions less than 200 nm. *ii.* secondly, tracking the time evolution of physical stability of NLC prepared with primrose oil and soybean oil (stored at a temperature of 4°C), by determining at pre-established time intervals (3 days, 1÷4 weeks, and 2 months) of the average diameters and the electrokinetic potential of the lipid nanocarriers.

2. Materials and methods

2.1. Materials. Polyoxyethylenesorbitan monolaurate (Tween 20) was purchased from Merck (Germany); Phosphatidylcholine (PC) and Synperonic PE/P84 (P188) from Sigma Aldrich Chemie GmbH (Munich, Germany). Glycerol monostearate (GMS) and cetyl palmitate (CP) were purchased from Cognis GmbH (Germany) and

Acros Organics (USA), respectively. The vegetable oils – primrose oil (Po) and soybean oil (So) were from Textron Plimon S.L.U. (Barcelona, Spain).

GC-MS analysis: SRM®2377 (National Institute of Standards &Technology, SUA), F.A.M.E. Mix., C4–C24 (18919-1AMP, Sigma Aldrich), concentrated methanolic solution of sodium methoxide 5.4 M from Acros Organics, New Jersey, methanolic solution of boron trifluoride, 14%, and sodium chloride (Sigma Aldrich) and methanol and isooctan obtained from LGC Standards GmbH (Wesel, Germany).

2.2. Preparation of NLC. The NLC were prepared by employing a melting-emulsifying procedure accompanied by a high-pressure homogenization (HPH), consecutively applied on the emulsion obtained from an aqueous and a lipid phase.

Table 1

NLC composition prepared with different weight ratio of surfactants and different type of vegetable oil, primrose oil (Po) and soybean oil (So)

	Sample name	Solid and liquid lipids, g				Surfactants and co-surfactants, g		
		GMS	CP	*Po*	*So*	Tween 20	P188	PC
1	**NLC-*Po*-2%-1**	3.5	3.5	3.0	-	1.40	0.30	0.30
2	**NLC-*Po*-2%-2**	3.5	3.5	3.0	-	1.60	0.20	0.20
3	**NLC-*Po*-2%-3**	3.5	3.5	3.0	-	1.70	0.15	0.15
4	**NLC-*Po*-2.5%-1**	3.5	3.5	3.0	-	1.75	0.375	0.375
5	**NLC-*Po*-2.5%-2**	3.5	3.5	3.0	-	2.00	0.250	0.250
6	**NLC-*Po*-2.5%-3**	3.5	3.5	3.0	-	2.125	0.187	0.187
7	**NLC-*So*-2%-1**	3.5	3.5	-	3.0	1.40	0.30	0.30
8	**NLC-*So*-2%-2**	3.5	3.5	-	3.0	1.60	0.20	0.20
9	**NLC-*So*-2%-3**	3.5	3.5	-	3.0	1.70	0.15	0.15
10	**NLC-*So*-2.5%-1**	3.5	3.5	-	3.0	1.75	0.375	0.375
11	**NLC-*So*-2.5%-2**	3.5	3.5	-	3.0	2.00	0.250	0.250
12	**NLC-*So*-2.5%-3**	3.5	3.5	-	3.0	2.125	0.187	0.187

Note: NLC systems prepared with *Po/So* and 2%, respectively 2.5% mixture of surfactants, coded with 1, 2 and 3 indicate the different ratios between the selected surfactants and co-surfactants

Table 2

	Tween 20 (%, w/w)	PC (%, w/w)	P188 (%, w/w)
NLC-*Po/So*-1	**70**	**15**	**15**
NLC-*Po/So*-2	**80**	**10**	**10**
NLC-*Po/So*-3	**85**	**7.5**	**7.5**

The method was detailed by the authors in a previous study [25, 26]. Briefly, the aqueous phase and the lipid phase were separately supposed to a magnetic stirring at 73°C for 2 minutes. The two phases were mixed at 73°C for 20 minutes and the obtained pre-emulsion was subjected to a high homogenization by applying 12.000 rpm for 1 min (High-shear homogenizer PRO250, Germany) and further subjected to

six homogenization cycles at 500 bar (HPH, APV 2000 Lab Homogenizer, Germany). After cooling the nanoemulsions these were kept at 4°C for 60 days and analysed regarding the size of the lipid population and their physical stability over time. The synthesis process has been presented in Fig. 1.

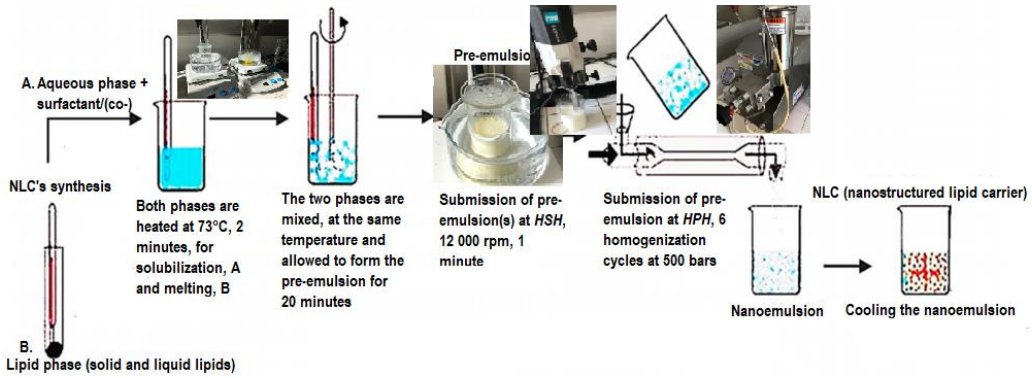

Fig.1. Scheme of NLC preparation

2.3. Primrose oil and soybean oil characterization. Po and So were characterised regarding the composition in fatty acids by determining the methyl esters of these fatty acids using the SR EN ISO 12966-2:2017, adjusted. For GC-MS determination, two reference standards SRM®2377 containing 26, respectively F.A.M.E. Mix., C4–C24 with 37 methyl esters of fatty acids (FAME), with specified/known values were used. These served for the quantification phase too.

Preparation of fatty acids methyl esters involved the saponification of the fat using methanolic solution of sodium hidroxide, followed by the acid transesterification with a methanolic solution of boronfluoride and separation of FAME using isooctan, as solvent [27].

The analysis and identification of fatty acids methyl esters was carried out using the Trace GC Ultra gas chromatograph coupled with a mass spectrometer TSQ Quantum XLS, TriPlus Autosampler, from Thermo Fisher Scientific, SUA. A TR-FAME (Thermo Fisher Scientific, SUA), high polar capillary column, 60 m length, 0.25 mm internal diameter and 0.25 µm film thickness was used for the GC system. The temperature program was set up to 100°C for 0.2 minutes, then raised to 240°C with a growth of 2°C/min and isothermally held for 15 minutes (total run time: 85.20 minutes). Both the injector and detector temperatures were set at 250°C and He, 99.9995% purity, at a constant flow of 1 mL/min, was used as carrier gas. The injection volume was 0.5 µL with a split ratio of 1:50. The mass spectrometer used for the analysis and detection of compounds worked in EI+ ionization mode of 70 eV. The spectra acquisition and data processing were made using Xcalibur program (Thermo Fisher Scientific Inc).

2.4. Particle size analysis. Particle size measurements were statistically determined by photon correlation spectroscopy, using a Zetasizer ZS 90 (Malvern

Instruments Inc., UK), equipped with a solid-state laser (670 nm) at a scattering angle of 90°. The mean particle size (Z_{ave}) and the polydispersity index (PdI) of the NLC aqueous dispersions were measured at a scattering angle of 90° and a temperature of 25 ± 0.1°C. Before conducting the measurements, the concentrate NLC dispersions were diluted with deionized water to an adequate scattering intensity. The particle size data were evaluated using intensity distribution. The average diameters and the polydispersity index were given as average of three individual measurements.

2.5. Determination of zeta potential. Zeta potential corresponds to the electric charge on the particle surface. Zeta potential or electrokinetic potential is an important constraint to predict the physical stability of colloidal systems. The electrical charge of aqueous dispersions of NLC was determined in a capillary cell, using the appropriate accessory of Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom) which utilizes the Helmholtz–Smoluchowski equation to convert the measured electrophoretic mobility (of dispersed lipid particles) into zeta potential. Prior to analysis, the concentrated NLC aqueous dispersions were diluted 1:100 with deionized water and were adjusted with 0.9% NaCl solution (to avoid multiple scattering effects and to reach a conductivity of 50 μS/cm). All measurements were performed at 25°C, in triplicate and the mean value was reported.

3. Results and discussion

Starting from the impact of the hydrocarbon chains existent in the composition of So and Po on the formation of disordered lipid networks that will be later suitable for entrapping lipophilic active principles, the two vegetable oils were characterized by GC-MS method. The results obtained indicated that the two vegetable oils possess high amount of polyunsaturated fatty acids, such as linoleic acid (e.g. So contains 53.45% and Po 61.01%), quite small amounts of α and γ-linolenic acids, saturated fatty acids, like palmitic and stearic acids (e.g. So contains 16.81% and Po 8.06%) and variable rates of ω-3 and ω-6, Fig.2.

3.1. The influence of primrose oil and soybean oil in obtaining aqueous dispersions of lipid nanocarriers. To observe the influence of the percentage of vegetable oil on the formation of a lipid core that can accommodate adequate amounts of active principles, NLC with variable composition of vegetable oil - solid lipids were prepared, respectively, GMS:CP:So/Po = 3.5:3.5:3; 4:4:2; 4.5:4.5:1. The influence of the oil type on obtaining lipid nanocarriers with dimensions of 100 nm was evaluated based on the dynamic light scattering method (DLS). The synthesized lipid nanocarriers showed average diameters between 140 and 250 nm, without observing significant differences associated with the type of vegetable oil (Fig. 3).
As expected, while the vegetable oil concentration increased, a significant decrease in the mean diameters of the lipid nanocarriers was determined.

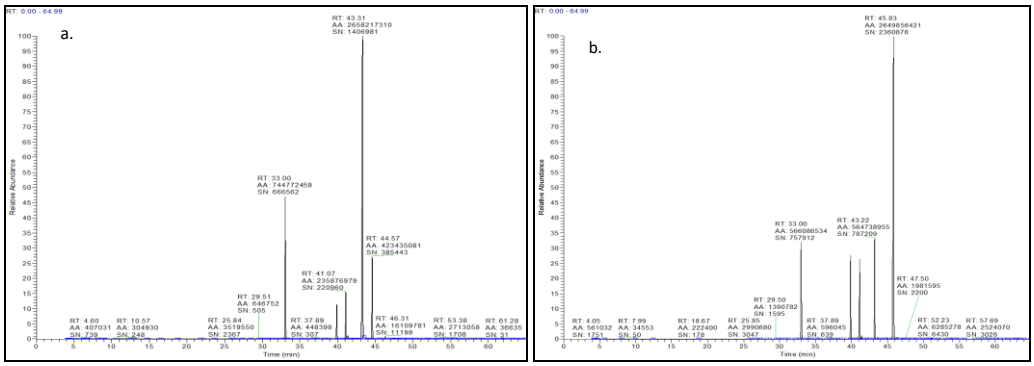

Fig. 2. Chromatograms of primrose oil (*Po*) (a) and soybean oil (*So*) (b)

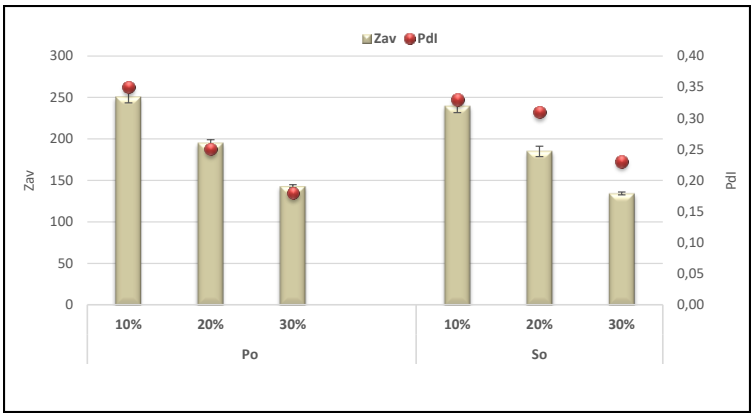

Fig. 3. The influence of the type and amount of vegetable oil on the average diameters of lipid nanocarriers

For instance, 143 ±1.74 nm (for NLC system prepared with Po) and 134.3 ± 1.65 nm (for NLC system prepared with So). These results indicate an optimal lipid composition of 70% solid lipids (GMS and CP): 30% vegetable oil, out of a total of 10% of lipid mass used to prepare 100g of aqueous NLC dispersion.

Regarding the physical stability, an amount of 30% Po or So (from the total lipid mixture used in the preparation of NLC) ensures values of zeta potential more electronegative than those determined for concentrations of 10 and 20% Po / So (e.g. ξ = - 46.50 ± 0.35 mV for the **NLC-30% So** system and respectively ξ = - 48.8 ± 0.95 mV for the **NLC-30% Po**), through the efficient environment of the 3 categories of non-ionic and ionic surfactants (Fig. 4).

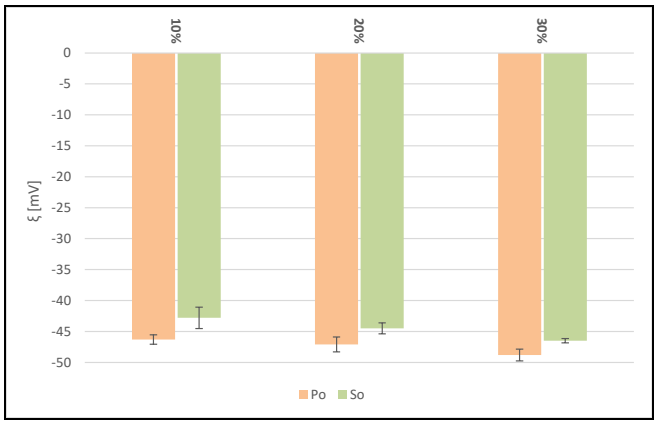

Fig. 4. The influence of the type and amount of vegetable oil on zeta potential values of NLC

3.2. Effect of the variable surfactant composition on obtaining NLC. The optimal lipid compositions resulted from the previous activity, corresponding to a lipid percentage of 70% (GMS and CP) and 30% vegetable oil (in a weight ratio of 1.16:1.16:1) were associated with two different concentrations of the surfactants and co-surfactants mixture (2% and 2.5%) in different weight ratios (according to Table 1) and the average diameters determined for the NLC samples are shown in Fig. 5.

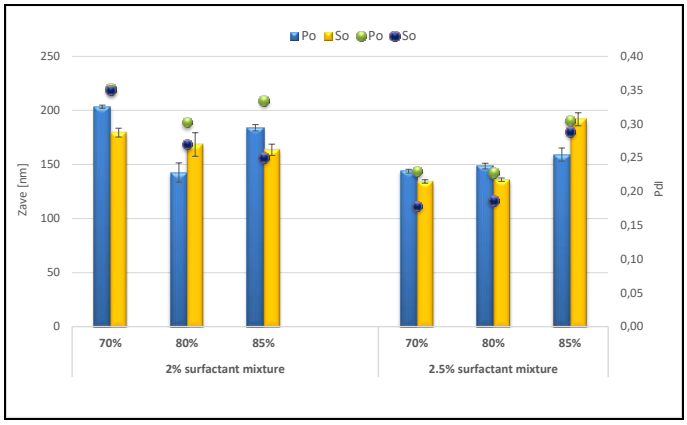

Fig.5. Z_{ave} and PdI variation depending on the concentration of Tween 20 and vegetable oil type

Both types of lipid matrices (formed with Po and So) led to the obtaining of NLC with average diameters between 140 and 200 nm and variable polydispersities. The best results were obtained by using 2.5% mixture of surfactants in ratios of Tween 20: PC: P188 = 70:15:15 (ratio 1) and 80:10:10 (ratio 2):

NLC_Po_2.5%: Z_{ave} = 143.8 ± 1.74 nm/PdI = 0.23 ± 0.01 (for ratio 1), respective 148.5 ± 2.58 nm/PdI = 0.228 ± 0.01 (for ratio 2).

NLC_So_2.5%: Z_{ave} = 134.3 ± 1.65 nm/PdI = 0.18 ± 0.01 (for ratio 1), respective 135.9 ± 1.63 nm/PdI = 0.19 ± 0.01 (for ratio 2).

For appropriate physical stability over time, NLC should have a very narrow size distribution to avoid the growth of crystals and the occurrence of Ostwald maturation phenomena [28]. An adequate physical stability of the colloidal systems is ensured by the existence of steric and electrostatic repulsions, the latter being responsible for the presence of surface charges that ensures more electronegative or electropositive zeta potential values of -40mV. The determinations of the zeta potential performed on the 12 categories of NLC developed (according to Table 1) with the selected vegetable oils (Po and So), prepared with two different concentrations of mixture of surfactants and co-surfactant, showed that these lipid nanocarriers has adequate values of this parameter, zeta potential being in the range -39 ÷ -62 mV.

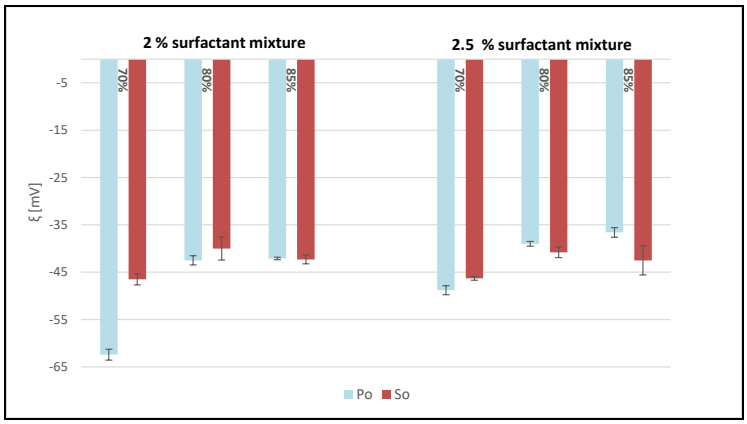

Fig. 6. Influence of Tween 20 and of the surfactant concentration on physical stability of NLC

3.3. Tracking the evolution of physical stability over time of NLC prepared with Po and So. The stability of NLC depends mainly on the physico-chemical stability of the solid lipid core because lipids are prone to coalescence and sometimes degradation. The physical stability of NLC can be assessed by monitoring changes in particle size, zeta potential, active ingredient content, appearance, and viscosity over time [29]. In general, it is considered that for the physical stability of colloidal systems, more electronegative or more electropositive potential values of +/-40 mV are required. However, some research recommends for NLC systems that zeta potential values should generally remain above -60 mV for the aqueous dispersion of NLC to remain perfectly physically stable [30]. Therefore, to evaluate the physical stability of the colloidal systems by NLC type, it is necessary to observe in time the changes related both to the average particle size and to the values of the zeta potential, over a determined period of time. In this study we followed the evolution of the two parameters of NLC prepared (average diameter and potential zeta) with different compositions of surfactant and co-surfactants (kept in the form of aqueous dispersions at a temperature of 4°C), over a period of 3 days, 1 ÷ 4 weeks, and 2 months.

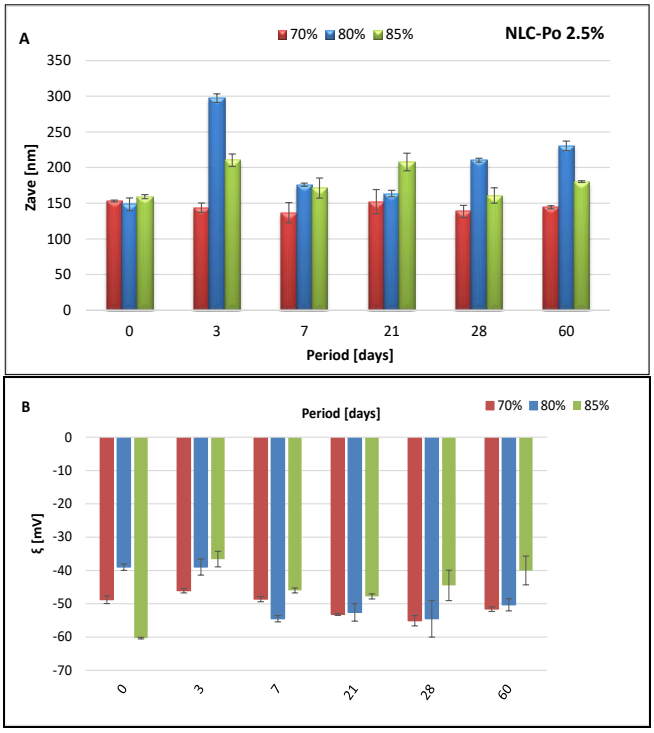

Fig. 7. Changes in average diameters (A) and zeta potential values (B) over time of NLC prepared with Po and 2.5% surfactant mixture (at 3 different weight ratios between Tween 20: PC: P188)

As it can be seen in Figs. 7A and 8A, the Z_{ave} values have undergone some changes, sometimes quite significant, as in the case of NLC prepared with So (Fig. 8A), where significant increases in average diameters values have been determined, compared to the initial ones. Not the same behaviour was followed by NLC prepared with Po; in this case it was observed the almost constant value for Z_{ave} (with non-significant shiftings) for a ratio of Tween20: P188: PC = 70%:15%:15% (Fig. 7, 8A).

These results support the choice of a composition consisting of 2.5% surfactants mixture, in a weight ratio Tw20: P188: PC=4.66:1:1, for obtaining nanocarriers systems for efficient drug distribution. As referring to the evolution over time of zeta potentials, two notable aspects are worth to be mentioned: *i.* The values of the zeta potentials are quite uncontrolled, which would suggest a permanent rearrangement of the coating formed by the two categories of surfactants, with variable modifications of the distribution of surface loads. *ii.* A relatively controlled change trend of zeta potential values (Figs. 5 and 7) for NLCs prepared with a surfactant weight ratio of 70:15:15, was reported. These NLC systems kept ξ around values of -50 mV, over the entire analysed time.

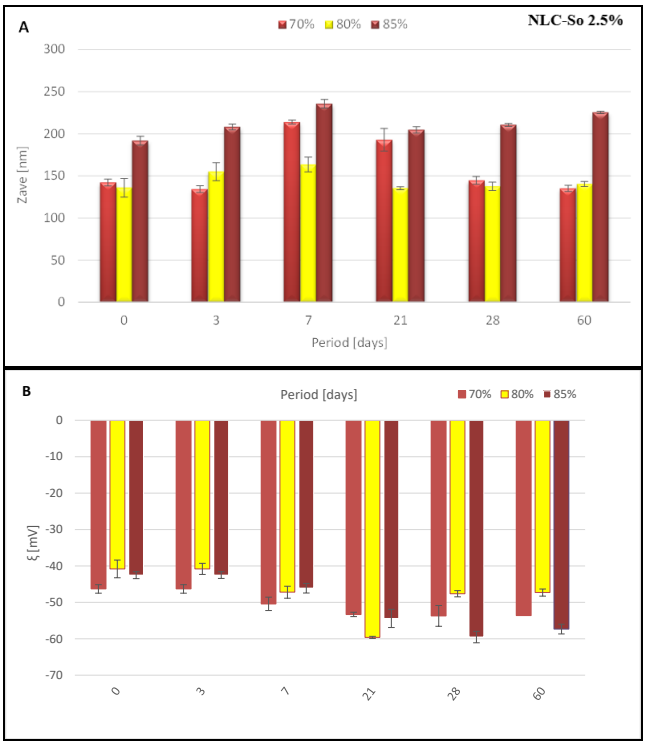

Fig. 8. Changes in average diameters (A) and zeta potential values (B) over time of NLC prepared with So and 2.5% surfactant mixture (at 3 different weight ratios between Tween 20: PC: P188)

The strong electronegative values obtained in the case of NLC prepared with 2.5% mixture of surfactants, in a weight ratio of 4.66: 1:1, suggest an appropriate physical stability, with a low potential for the occurrence of coalescence phenomena over time. The same ratio between lipid components and surfactants was previously reported by authors [4, 26] and proved to be effective in maintaining NLC's stability, promoting high encapsulation efficiency. Also, the mean size of the lipidic nanoparticles [130 ÷ 143] nm, and zeta potential values [–48 ÷ -58] mV showed comparable results with the ones previously published [15, 18].

4. Conclusions

This study aimed at preparing some consecutive lipid nanocarriers, to identify optimal compositions for solid lipids and vegetable oils, as well as a weight ratio between surfactants and co-surfactants. Correlation of the information obtained from the average diameters and the zeta potential determinations, led to the identification of an optimal ratio between the three categories of lipid components: GMS:CP:Po/So: 1.16:1.16:1. Thus, an optimum vegetable oil concentration of 30% of the total amount of lipids mixture used for NLC preparation was identified.

A comparative analysis of the obtained results showed that NLC prepared with 2.5% mixture of surfactants and co-surfactants, corresponding to a weight ratio of 4.66:1:1 (Tween 20: PC: P188) is suitable for obtaining average diameters that fall in the 50 and 500 nm range and maintaining zeta potential values more electronegative than -50 mV. The lipid nanocarriers obtained by using Po and So showed appropriate physical stability during the two months of evaluation, confirmed by nonsignificant changes in average diameters and zeta potential values, detected in several optimal lipids – surfactants compositions.

Funding: This research was funded by the European Union through POC Program 2014-2020, Project SMIS code 120906, contract no. 259/16.06.2020.

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