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Characterization of solid lipid nanoparticles produced with carnauba wax for rosmarinic acid oral delivery

Ana Raquel Madureira[1*], Débora A. Campos[1], Pedro Fonte[2], Sara Nunes[3], Flávio Reis[3], Ana Maria Gomes[1], Bruno Sarmento[2, 4,5], Maria Manuela Pintado[1]

[1] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal*

[2] *CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, 4585-116 Gandra PRD, Portugal*

[3] *IBILI - Laboratory of Pharmacology and Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences, Faculty of Medicine, Sub-Unit 1 (Pólo III), University of Coimbra, 3000-548 Coimbra, Portugal.*

[4] *I3S, Instituto de Investigação e Inovação em Saúde, 4150-180 Porto, Portugal*

[5] *INEB- Institute of Biomedical Engineering, University of Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal*

*rmadureira@porto.ucp.pt

Telf: 00351225580044, Fax: 00351225580000

Abstract

In the last decade, research studies have increased, on the development of delivery systems for polyphenols, for protection, improvement of stability and increase of their bioavailability. Rosmarinic acid is a polyphenol with described bioactivities, such as antioxidant, anti-mutagenic, anti-bacterial and anti-viral capabilities. Thus, the aim of this research work was to produce stable solid lipid nanoparticles (SLN) using carnauba wax as lipidic matrix, for delivery of rosmarinic acid, to be further incorporated into food matrices. Hence, different concentrations of wax (0.5, 1 and 1.5%, w/v) and percentages of surfactant (1, 2 and 3%, v/v) were tested. Physical properties, surface morphology and association efficiencies were studied at time of production and after 28 d at refrigerated storage. Thermal properties and the nature of the chemical interactions between the lipids waxes and rosmarinic acid were also evaluated. The particles showed range size between 35-927 nm and zeta potentials of ca. - 38-40, showing high stability, with no risk of aggregation due to electric repulsion of SLN. High association efficiencies % (ca. 99%) were also obtained. FTIR analyses proved the association of rosmarinic acid and lipidic matrix. The low lipid and high surfactant concentrations leads to small SLN. The surfactant, Polysorbate 80 decreases the interfacial tension in the SLN surfaces, preventing aggregation, leading to the development of small particles. These properties were maintained throughout the 28 d of refrigerated storage, and no rosmarinic acid was released by the particles during refrigeration, indicating good compatibility between rosmarinic acid and the waxy core of SLN. The optimum range values to obtain the desirable features for incorporation in a functional food suggest formulations containing 1.0 and 1.5% (w/v) of lipid and 2% (v/v) of surfactant.

Keywords: SLN, carnauba wax, polyphenols, rosmarinic acid, oral route

1. Introduction

The food and beverage sector is a global and high financed industry and the major food companies have been investing in research to improve production efficiency, food safety and nutritional properties. Studies have been made to develop functionalized foods with ingredients bearing biological activity for the human body. These foods could be named functional foods, which differ from the common foods because they bring an additional and improved nutritional content, by the incorporation of bioactive compounds or ingredients. Nanotechnology has been used as tool to develop and in particular stabilize these ingredients [1]. However, oral delivery of nanosized ingredients included in foods for regular consumption still brings several doubts owing to toxicity issues. Solid lipid nanoparticles (SLN) were introduced by the pharmaceutical industry for the first time in the 90's, as an alternative and stable carrier system for controlled drug delivery [2]. Since then, a wide variety of SLN have been developed, and it has been determined that in their formulation, lipid, emulsifier and water are needed as essential components. The lipids used range from triglycerides, mono, di and triglycerides mixtures to waxes, hard fats and other types of lipids, having a special feature of melting point above room and body temperature [3]. The structural features of the dynamics of the topological transitions in these amphiphilic materials, preventing the impact in the development of nanosystems with these lipids was deeply studied in several research works[4,5]. The favorable physical and chemical properties stability, good tolerability and biodegradability and the possibility of large-scale production of SLN, have demonstrated that they can be a good vehicle to functional ingredients in the food industry. Additionally, these SLN-based systems include compounds that hold a Generally Recognized as Safe (GRAS) status for oral and topical administration [6]; [3].

Carnauba wax or Brazilian wax is naturally extracted from the leaves of a particular palm tree known as *Copernicia cerifera*, a plant native of the northeast of Brazil. Carnauba wax is already used by several industries for different purposes, as gelling, releasing and glazing agent. It possesses a high melting point (between 82.0-85.5 °C) making it a good candidate to be used in food systems, such as in the production of SLN [7]; [8]; [9]; [10].

Rosmarinic acid is a natural polyphenol carboxylic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. This acid commonly appears in higher amounts in families such as *Boraginaceae* and *Lamiaceae*, but, in the latter, it is restricted to a subfamily, the *Nepetoideae*. Rosmarinic acid present a number of potential biological properties associated therewith, such as antioxidant, anti-mutagenic, anti-bacterial and anti-viral capabilities [11]; [12]; [13]. Additionally, rosmarinic acid exhibits various pharmacological properties, including prevention of oxidation of low density lipoprotein, inhibition of murine cell proliferative activity and of cyclooxygenase, anti-inflammatory and anti-allergic actions, however protection against cancer and the high antioxidant activity have been the most important activities [14].

The main reasons for the vast range of the industrial applications of rosmarinic acid are associated with the low production cost (extraction from natural sources is a relatively cheap process), low toxicity and its recognition as an important antioxidant [15]. The technological handling of such type of compounds is sometimes limitative since they are very reactive, and their incorporation in a food formulation may bring some difficulties if these compounds are not protected. The creation of a nanocarrier to protect the rosmarinic acid from these events, will permit the inclusion of such polyphenol in food matrices bearing reactive components e.g. proteins, allowing the appearance of more products with polyphenols, and increasing the nutritional value of

such products. Additionally, conditions prevailing in digestion can be negative for the stability of these compounds as well their bioavailability can be compromised. Hence, the polyphenol entrapped in a nanoparticle will be protected from the harsh events of the gastrointestinal tract, reaching intact to the gut and absorbed by the intestinal epithelium.

The objective of this research work was to optimize the formulation of SLN loaded with rosmarinic acid, using carnauba wax as matrix material. The obtained SLN will be characterized in terms of their physical, morphological and thermal properties.

2. Materials and methods

Carnauba wax yellow no. 1, Polysorbate (Tween 80) and rosmarinic acid (96% pure, 10 mg/mL solubility in water) were purchased from Sigma-Aldrich Chemistry (St. Louis, Missouri, USA). According several manufactures, carnauba wax ($C_7H_5HgNO_3$) has a mean composition of aliphatic and aromatic (cinnamic acid based) mono- and di-esters (75-85 %), free wax acids (3-6 %), free wax alcohols (10-15 %), lactides (2-3 %), hydrocarbons (1-2 %) and resins (4-6 %). Methanol (Panreac, Barcelona, Spain) and formic acid (Merck, Darmstadt, Germany) were used for HPLC analyses.

2.1. Production of Carnauba wax SLN loaded with rosmarinic acid

Three batches of SLN were produced according the scheme of Figure 1, in duplicated using a hot melt ultrasonication method, by loading rosmarinic acid at a final concentration of 0.15 mg/mL. Three different concentrations of carnauba wax (0.5, 1 and 1.5%, w/v) and three different percentages of surfactant, Polysorbate 80 (1, 2 and 3%, v/v) were used in the production. The ultrasonicator used was a VCX 130 (Sonics

& Materials, Newtown, USA). For further analyses, where dried SLN are need, a lyophilization of final emulsions at the time of production was made using a Vacuum Freeze Drier (Model FT33, Armefield, UK), under a vacuum pressure of 100 militorr; the temperature in the freezing chamber was -46 ˚C and the temperature in the sample chamber was 15 ˚C. The resulting emulsions were left to cool at room temperature (20 ˚C), and then stored at 5 ˚C throughout 28 d until further use.

2.2.Physical and morphological characterization

2.2.1. Particle size and zeta potential analyses

Before being dried, the liquid samples were subject to the analysis of the physical properties. Dynamic light scattering (DLS) (ZetaPALS, Zeta Potential Analyzer, Holtsville, New York, USA) was used for evaluation of the mean particle size (PS) and polydispersity index (PI). Zeta potential was measured using DLS in combination with an applied electric field (electrophoresis). For evaluation of PS and PI, SLN were diluted 1:10 with MilliQ-water, while in the latter samples were used directly. All analyses were carried out with an angle of 90˚ (angle at which the detector is located with respect to the sample cell) at room temperature of 25 ˚C. These properties were evaluated in triplicate at 0 and 28 d of storage.

2.2.2. Association efficiencies

A separation process of free rosmarinic acid (that was not loaded) from the emulsions of SLN was performed using Centrifugal Filter Units with a cut-off of 10 K (Amicon® Ultra-4, Millipore; Billerica, Massachusetts, USA), by centrifugation at 3000 rpm, during 20 min and at 4 ˚C. The resulting supernatant was removed and used to evaluate the percentage of encapsulated rosmarinic acid.

The association efficiency (EE%) values were calculated by the difference between the total amount of incorporated rosmarinic acid (RA) and the amount of the polyphenol present in the supernatant of SLN formulations. The calculations were performed according to the following formula:

$$AE\% = \frac{Total\ amount\ of\ RA - Amount\ of\ RA\ in\ Supernatant}{Total\ amount\ of\ RA} \times 100$$

(*Equation 1*)

The quantification of rosmarinic acid was performed by HPLC using a diode-array detector (Waters Series 600, Mildford, Massachusetts, USA), in triplicate. Separation was done in a C18 reverse-phase column at room temperature, coupled with a guard column containing the same stationary phase (Symmetry® C18, Waters, Mildford, Massachusetts, USA). Chromatographic separation was carried out with mobile phase A - water, methanol and formic acid (92.5:5:2.5) - and mobile phase B – methanol, water and formic acid (92.5:5:2.5) - under the following conditions: linear gradient elution starting at 0 to 60% mobile phase B in 60 min at 0.65 mL/min, 60 to 10% in 5 min at 0.5 mL/min and from 5 to 0% in 5 min. Injection volume was 20 µl. Detection was achieved using a diode array detector (Waters, Mildford, Massachusetts, USA) at wavelengths ranging from 200 to 600 nm measured in 2 nm intervals, while the detection of rosmarinic acid was performed at 280 nm [10, 16]. All samples were measured in triplicate and filtered with a 0.22 µm pore membrane filter (Millipore) before being injected.

2.2.3. Thermal properties determination

The thermal properties of the SLN were evaluated by Differential Scanning Calorimetry (DSC-60, Shimadzu, Columbia, USA). Briefly, 3 mg of freeze-dried SLN

183 were placed on an aluminum pan and the thermal behavior was determined in the range
184 20-100 ˚C at a heating rate of 10 ˚C/min. Enthalpy values and melting temperatures
185 were calculated by the equipment software (ta60 version 2.10, DSC software,
186 Shimadzu, Columbia, USA). The crystallinity indexes percentages (CI%) of SLN was
187 calculated according to Kheradmandnia *et al.*, (2010), using the following equation:

188 $$CI\% = \frac{Melting\ enthalpy\ (SLN\ dispersion)\ (J/g)}{Melting\ enthalpy\ (bulk\ material\ without\ RA)\ (J/g) \cdot Concentration\ of\ lipid\ phase\ (\%)} \times 100$$

189 (*Equation 2*)

190 All SLN formulations, either loaded with rosmarinic acid or unloaded, as well as
191 all the raw materials used in the formulations, individually and in combination, were
192 tested.

193

194 **2.2.4. Morphological properties of nanoparticles**

195 Morphology of nanoparticles was evaluated by Scanning Electron Microscopy
196 (SEM) and Transmission Electron Microscopy (TEM) techniques. For SEM, a JEOL-
197 5600 Lv microscope (Tokyo, Japan) was used. For both techniques, the freeze dried
198 SLN at the time of production were used. Briefly, a small amount of freeze-dried SLN
199 was placed in metallic stubs with carbon tape and coated with gold/palladium using a
200 Sputter Coater (Polaron, Bad Schwalbach, Germany). SEM was operated at the high
201 vacuum mode, using a spot size of 36-37 and a potential of 20-22 kV. All analyses were
202 performed at room temperature (20 ˚C). For TEM, a TECNAI G^2 12 microscope was
203 used. The SLN were examined after suspension in water and subsequent deposition onto
204 copper grids (Formvar/Carbon Support Film, 100 mesh, *3.05mm* diameter, TAAB).
205 After 4 times washed with sterile water, the grids were negatively stained, i.e. contrast
206 of sample with an optically opaque fluid where the background is stained, leaving the

actual specimen untouched, and thus visible. This optically opaque fluid was sterile-filtered 1% (w/v) uranyl acetate solution. Digital images were acquired using Analysis version 3.2 software.

2.2.5. Fourier transform infrared (FT-IR) spectroscopy

The freeze-dried formulations of SLN with and without rosmarinic acid, pure rosmarinic acid and pure carnauba wax, as well as their physical mixtures, i.e. mixtures of these compounds in their pure form and state (solid) were evaluated using an ABB MB3000 FT-IR spectrometer (ABB, Zürich, Switzerland) equipped with a horizontal attenuated total reflectance (ATR) sampling accessory (PIKE Technologies, Madison, Wisconsin, USA) with a diamond/ZnSe crystal, obtaining different spectra. All samples were run in triplicate. A background run was performed to remove the background noise of the instrument.

The mid-infrared absorbance region was settled between 4000-700 cm^{-1} and the spectra were measured at a spectral resolution of 4 cm^{-1} with 200 scans co-added, to minimize differences between spectra due to baseline shifts. In order to perform the spectra comparison, spectra were truncated at from 1800 to 700 cm^{-1}, since this region displays typical absorption bands for the used compounds. In addition, baseline 4-5 point adjustment and spectra normalization was performed. Treatment of all spectra was carried out with the Horizon MBTM FTIR software (ABB, Zürich, Switzerland) [17].

2.3.Statistical analyses

The statistical significance at a 5% level of differences between the means values of the tested parameters, viz. PS, PI, ZP, EE%, and crystallization index values obtained. Test between subjects were useful to determine the weight of the effect of

232 both factors - lipid and surfactant % - in the variation of tested parameters. Multiple
233 comparisons were determined by Tukey's test. Also, the statistical difference between
234 the values obtained for time 0 and 28 days was also determined. All tests were
235 performed running a Multivariate ANalysis Of VAriance (MANOVA) carried out with
236 the aid of SPSS (v. 20, Chicago IL, USA).
237

238 **3. Results and discussion**

239 **3.1. Physical properties**

240 The lipid nanoparticles are complex systems, and the optimization of the
241 conditions used during their formulation is critical. When these nanoparticles are
242 incorporated on a food product, it is essential that they do not aggregate and that they do
243 not interact with other compounds of the matrices. Also that after ingestion, and passage
244 by the gastrointestinal tract, they reach the gut and release the loaded compound and
245 that this compound is intact and bioavailable to be adsorbed intact by the intestinal
246 epithelium. Analyses concerning the physical properties of the SLN developed (particle
247 size, polydispersity index and zeta potential) at the time of production (0 d) and stored
248 during (28 d), were performed. These analyses were done to assess the stability of each
249 formulation throughout the storage time. The surfactant, lipid type, concentration and
250 compound loaded, as well as production methods and conditions such as sonication
251 time, melting temperature, equipment used are production conditions that can affect the
252 physical properties of the SLN.

253 In Table 1 are reported the results obtained for the physical properties of SLN.
254 When using the lower carnauba wax concentrations (A-C), the PS increases with the
255 increment of the surfactant concentrations. Siekmann and Westesen [18] described that
256 low lipid concentration leads to SLN with reduced PS. In contrast, when using higher

percentage of lipid (1 and 1.5% (w/v)) and with the increase of the surfactant percentage, the PS decreases (P>0.05). The use of high concentrations of surfactant reduces the surface tension and facilitates the particle division during the process of homogenization, creating SLN with smaller PS [19]. Being the Polysorbate 80 a non-ionic surfactant, creates a steric stabilization of the particles, by penetration of long polyethylene chains, limiting the freedom of the particles and preventing the association with one another [20]. After 28 d of storage period, the PS increased in formulations A and C and decreased in D, G and H (P<0.05). Nevertheless, The PI values at the time of production (0 d) were ca. 0.17-0.40, and in general aligned with the standard value of PI – 0.30 – indicating homogeneity in terms of SLN size. (P<0.05). Statistical significant differences were found between the values of PI of SLN produced with the higher surfactant concentrations (3%) and the intermediated one (2%) (P<0.05). After 28 d of storage the PI values were in general maintained (0.24-0.37), which indicates that the SLN remained with same sizes after this storage period (P>0.05). Zeta potential (ZP) values indicate the repulsion between charged particles in dispersion [19]. The SLN showed ZP values with values range between -37.5 and -40.7 mV (P>0.05). A high absolute value of ZP indicates that there is no risk of aggregation of the particles due to electric repulsion of SLN; for low values of ZP, attraction between the particles would occur enhancing the risk of aggregation. Similar results were obtained for NLC produced with the same wax and surfactant but loaded with benzophenone-3 [21]. Hence, the high values of zeta potential obtained for these particles were expected since this composition is negatively charged and the polysorbate 80, which is neutral is not able to increase the values. The high negative values of zeta are good for avoiding the agglomeration and interaction with other compounds, when these nanoparticles are incorporated in an food formulation, increasing their technological stability.

The AE will give us the information about the percentage of rosmarinic acid that was entrapped in the SLN. The percentages of efficiency were high for all formulations (ca. 99%), even after 28 d of storage, which means that the polyphenol association was efficient and did not change for the different formulations tested and throughout storage time ($P>0.05$). Carnauba wax is a highly lipophilic matrix and also contains 5% of resins which allows almost no water to penetrate into the pores of the lipid structure, and the release of rosmarinic acid is low. In addition, carnauba wax contains low percentages of free fatty acids and hydroxyl groups (acid value: 2-7), which makes a slower degradation rate preventing the penetration of water into the pore of the matrix [22].

3.2.Morphological features

Scanning Electron Microscopy (SEM) uses electron transmission from the sample surface. Using this technique it is possible to confirm the size of the particles, as well as their shape and arrangement. Micrograph (Figure 2) revealed spherical SLN. Furthermore, the sizes measured by SEM are not in agreement with those obtained by DLS. As can be seen in Table 1 the selected SLN formulation (1% carnauba wax: 2% Polysorbate 80) presented average sizes of 438 nm, while microscopic results presented in micrograph shows SLN with sizes ≥1000 nm. Nonetheless, it has been reported that solvent removal may cause modifications, which will influence the particle shape and size. The effect of the highly energetic electron beam in the very labile lipid nanoparticles may also cause artifacts in the images obtained, as observed for the rosmarinic acid-SLN. The results from DLS are more accurate than those from SEM, since fewer artefacts are formed. Nevertheless, the TEM micrographs showed smaller sizes of the SLN obtained which can confirm the maintenance of nanometric sizes (e.g.

500-1000 nm) of the particles produced even after lyophilization, even that some dispersion of sizes is confirmed. The particle shows a crystallized core which may correspond to the wax. This technique showed to be better to visualize this type of nanoparticles than SEM.

3.3.Thermal properties determination

The melting and crystallization behavior (breakdown or fusion of the crystal lattice) are two thermal properties that can be obtained by heating the sample in a DSC, and give us information about polymorphism and crystal ordering [23].

Figure 3 describes the thermal behavior of the raw materials used in the production of the SLN, as well a control without rosmarinic acid. Rosmarinic acid and the surfactant did not present any peaks for the tested temperatures, as expected. Carnauba wax and standard control presented endothermic peaks (lines C and D), which means that for occurrence of polymorphism (melting of the carnauba wax) in the lipid structure, was necessary the absorption of energy [19]. Carnauba wax presented a slightly higher melting point (ca. 92 ºC) than the theoretical one (82-86 ºC), but can be a consequence of the large heating rate (10 ºC/min) used during the measurement. The control SLN presented a lower melting point and an enthalpy (-88.11 ºC, 32.19 J/g) than the wax. This slight reduction in the value of melting temperature is mainly related to the nanocrystalline size of lipids in SLN systems [24]; [25]. It is possible to access the type of polymorphism form of lipids (α-form, β'-form and β-form) through the crystals formed by the melting and cooling of the lipid, the transitions in between and how these transitions affect the encapsulated compound in SLN. A high value for the melting enthalpy suggests a high level of organization in the crystal lattice, because the fusion of a highly organized crystal (perfect crystal) requires more energy to overcome the forces

of cohesion in the crystal lattice. Lipid crystallinity is strongly correlated with compound incorporation and release rate, where thermal behavior is different for pure lipid and the SLN [26]; [27]. The polymorphism of the SLN crystal formed is less organized than the crystal of the pure wax.

In rosmarinic acid loaded SLN formulations, endothermic peaks were visualized (Figure 4), and the enthalpy and melting values were in general lower than the ones obtained for the pure wax and the control SLN (Figure 3). This indicates a less ordered structure, being required lower energy to breakdown the internal connections of the wax [28]. There is no trend in what concerns the lipid or the surfactant concentrations used to produce the SLN. Nevertheless, the existence of a shoulder in the curves is more pronounced in the SLN produced with higher lipid concentration. This can be a consequence of different polymorphisms, the lipid modifications are not always solved with the forms α, β, and β' and the differences could be from several subspecies already detected from the interaction between the lipid and the emulsifier. When compared with other carnauba wax DSC thermograms, such as those showed by [2], it was possible to observe the same behavior, i.e. the appearance of a more pronunciated shoulder when higher concentrations of lipid are used. Also there is a direct correlation of PS with the thermal behavior of SLN [29].

Crystallinity index (CI%) allows the understanding of the thermal behavior of materials. As in *equation 2*, the percentage is calculated using the enthalpy value of pure lipid (normally high) and the value of the SLN (value normally lower than the pure compound). Lipid crystallinity is also strongly correlated with drug incorporation and release rates. Thermodynamic stability and lipid packing density increase, whereas drug incorporation rates decrease in the following order: supercooled melt, α-modification, β′-modification, and β-modification. Hence, high values of CI% leads to faster

compound release, but it also means that more energy is required to melt the crystal lipids. Furthermore, when the SLN are formed with ordered crystals, the more difficult is the release of the bioactive compound into the medium. Hence, the ideal value of CI% should be sufficiently high to make sure that most of the SLN are not unstable in the formation of new particles, but it also has to be sufficiently low to ensure the release of rosmarinic acid. The most CI appropriate values are those close to 50% [19]. In Table 1, it is possible to observe that the increase of percentage of lipid leads to a decrease of the CI% ($P>0.05$).

In order to select the formulations, the desired features can be analyzed taking into account the final application. These SLN will be further incorporated in an oral formulation/food product. The PS should be ≥ 300 nm to decrease the possibility of adsorption of the SLN by the intestinal epithelium, and only permit the compound to be absorbed instead of the entire nanoparticle [3]. Hence, all percentages of carnauba wax can be used and the percentage of surfactant should be below 2-3% (v/v). The use of 1.0 and 1.5% of wax and 2% of surfactant showed good CI% values.

3.4.Fourier transform infrared (FT-IR) spectroscopy

The main bands for rosmarinic acid identification are located between wavenumber 1800 and 700 cm^{-1} [30], as shown in Figure 5.

The three bands at 1605, 1520 and 1445 cm^{-1} are due to the presence of aromatic ring stretching, as can be seen in the graphs for FTIR spectra of rosmarinic acid (A) and physical mixtures between SLN and rosmarinic acid (C and E) (Coates, 2000). The spectra from SLN (B and D) did not present these peaks, probably due to the interaction of the lipids and the reactive connections O-H of aromatic rings of rosmarinic acid. Other evidences for presence of phenolic groups were delivered through the bands at

382 1360 and 1180 cm^{-1} resulting from O-H and C-O stretches; these peaks were presented
383 in all spectra of the wax, evidencing the presence of phenolic groups and the
384 incorporated polyphenol. Therefore, an overlap of two bands in the region is very likely
385 to have occurred. Carboxylic acid groups show a characteristic band in the range 1725-
386 1700 cm^{-1}, as can be seen in Figure 5 for rosmarinic acid and for both physical mixtures
387 between the SLN and the rosmarinic acid (C and E); these results confirm the
388 maintenance of structure of the polyphenol. When the rosmarinic acid was
389 encapsulated, these characteristic peaks generally disappeared, mostly due to chemical
390 interactions between the reactive groups of polyphenol and the matrices; hence these
391 typical bands probably were masked by the matrices.
392
393 **4. Conclusions**
394 The purpose of this research work was to optimize the production of SLN with
395 rosmarinic acid and to characterize these ones in terms of its physical, association
396 efficiencies, thermal and morphological properties. In the production of rosmarinic acid-
397 SLN, the percentage of carnauba wax and surfactant used were important factors
398 influencing their physical properties. The concentration of lipid, i.e. of carnauba wax
399 showed to have effect in the size of the generated particles. Increasing the
400 concentrations of carnauba wax leads to small sized particles, but the surfactant
401 concentrations must be high (> 2% (v/v)). The final particles are negative highly
402 charged, which allows concluding that they are stable during the storage of 28 d in
403 refrigerated conditions. With FTIR analysis, it was possible to confirm the physical
404 encapsulation of rosmarinic acid in the SLN. The optimum range values to obtain the
405 desirable features for incorporation in a functional food suggest formulations containing
406 1.0 and 1.5% (w/v) of lipid and 2% (v/v) of surfactant. These formulations will

guarantee SLN with sizes >300 nm to avoid adsorption by the intestinal epithelium and will allow high association efficiency (>99%) combined with high fusion point (>86 ºC). These SLN characteristics will be maintained throughout at least 28 d of storage.

The SLN developed in the present work, could be incorporated as oral bioactive/nutraceutical ingredient in oral formulations/foods, that can be taken to provide antioxidant, anti-inflammatory or even antimicrobial bioactivities. Since, rosmarinic acid has proved antioxidant and anti-inflammatory activities, the developed formulations could be used to prevention of anti-inflammatory states such as cancer, or other inflammations. Moreover, it is proved that rosmarinic acid has antimicrobial activity, so these formulations could be used as prevention of bacterial infections. The bioactivities of these systems would be in the future search, in order to predict the effects that these systems will have when they are orally administrated. For this purpose, the stability of these SLN will be also studied, at a simulated gastrointestinal conditions and when at intestinal epithelium, where they are adsorbed to the lymphatic blood stream and distributed to body organism.

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Table 1: Mean values ± SD of the physical properties (PP), particles size (PS), polidispersity index (PI), zeta potential (ZP) and percentage of entrapment efficiency (EE%) of the produced nanoparticles (in emulsion) throughout storage time, and thermal properties (TP) such as entalphy (ΔH), melting temperature (MT) and percentage of cristallinity (CI%) at the time of production.

Properties	Storage time (d)	A	B	C	D	E	F	G	H	I
PS (nm)	0	43±3^{a}	722±41^{a}	887±426^{a}	907±438^{a}	582±392^{a}	587±392^{a}	945±211^{a}	897±189^{a}	35±2^{a}
	28	124±72^{a}	892±60^{a}	542±380^{a}	585±307^{a}	438±338^{a}	527±285^{a}	605±248^{a}	491±210^{a}	49±6^{a}
PI	0	0.33±0.06^{ab}	0.23±0.02^{ab}	0.17±0.12^{a}	0.33±0.04^{ab}	0.31±0.08^{ab}	0.34±0.12^{ab}	0.36±0.02^{ab}	0.40±0.05^{a}	0.27±0.03^{ab}
	28	0.32±0.01^{a}	0.24±0.06^{a}	0.29±0.13^{a}	0.31±0.07^{a}	0.36±0.02^{a}	0.29±0.19^{a}	0.34±0.02^{a}	0.37±0.09^{a}	0.30±0.04^{a}
ZP (mV)	0	-38.1±0.7^{a}	-38.7±0.4^{a}	-37.6±0.6^{a}	-40.7±1.6^{a}	-38.7±0.8^{a}	-37.6±0.4^{a}	-37.9±0.1^{a}	-38.2±0.0^{a}	-38.7±0.6^{a}
	28	-39.1±1.0^{a}	-38.1±0.4^{ab}	-38.4±2.1^{ab}	-38.4±2.5^{ab}	-37.6±0.9^{ab}	-38.2±0.8^{ab}	-38.1±3.9^{ab}	-38.3±3.3^{ab}	-37.8±0.4^{b}
EE (%)	0	99.93	99.96	99.92	99.94	99.88	99.84	99.89	99.95	99.89
	28	99.93	99.94	99.94	99.87	99.97	99.94	99.98	99.99	99.98
ΔH (J/g)	0	-24±5	-21±4	-15±5	-39±3	-30±12	-10±3	-14±4	-51±16	-24±3
MT (˚C)	0	86.14	86.14	85.91	85.26	88.25	86.95	87.56	85.41	87.04
CI%	0	66.46	58.59	42.07	54.88	41.72	14.65	12.72	47.73	22.44

Designations of the letters are as follows (CW %, w/v: Polysorbate 80 %, v/v): **(A)** 0.5: 1, **(B)** 0.5: 2, **(C)** 0.5: 3, **(D)** 1.0: 1, **(E)** 1.0: 2, **(F)** 1.0: 3, **(G)** 1.5: 1, **(H)** 1.5: 2, **(I)** 1.5: 3. ^{a,b,c,d} The differences between the means in the same row labelled with the same superscript are not statistically significant (P>0.05) (n=9).

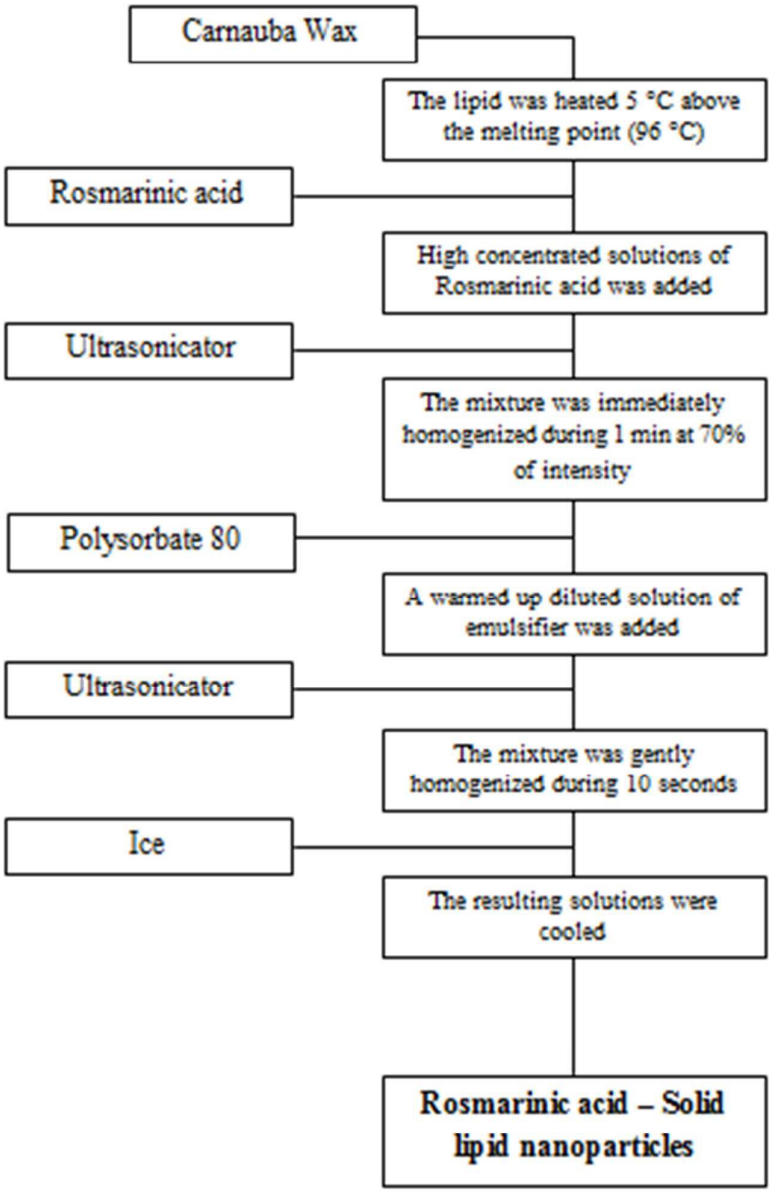

Figure 1

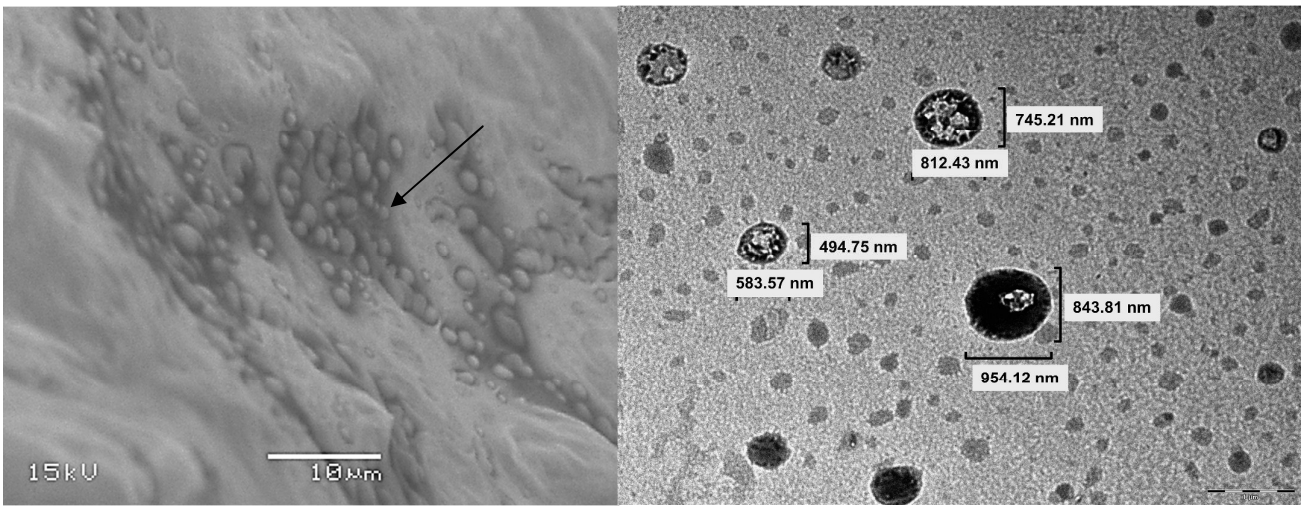

Figure 2

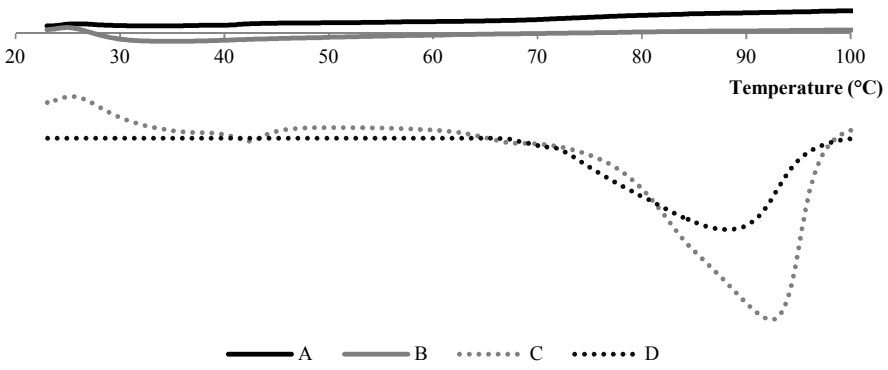

Figure 3

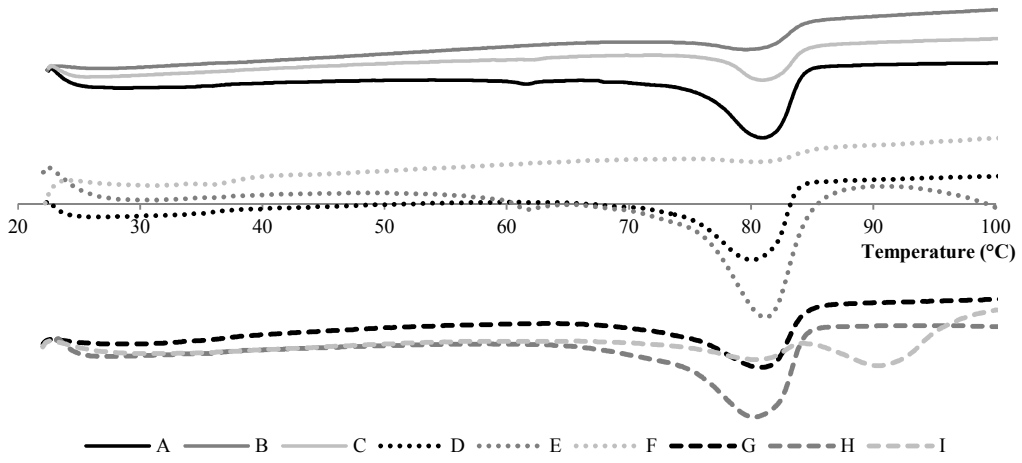

Figure 4

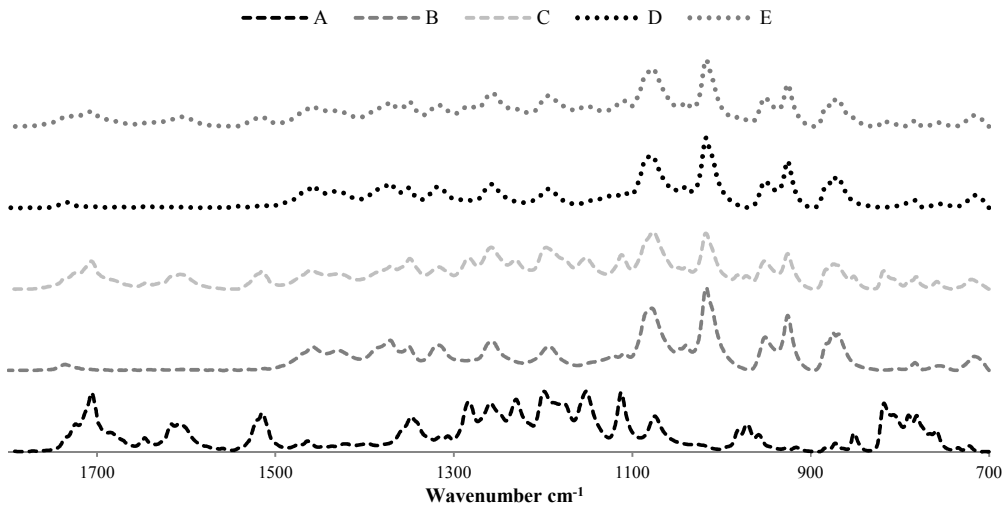

Figure 5

Figure 1: Schematic representation of the production of RA-SLNs using carnauba wax as lipidic matrix.

Figure 2: Micrographs of carnauba wax rosmarinic acid-SLN, 1.0% of carnauba wax (w/v) and 2% (v/v) of polysorbate 80. At the left figures lyophilized SLN, visualized by scanning electron microscope (SEM). Row indicate a group of SLN. At the right figures SLN lyophilized, visualized by transmission electron microscopy (TEM) technique.

Figure 3: Thermograms of the bulk materials (A) rosmarinic acid, (B) polysorbate 80 and (C) carnauba wax. Standard formulation without rosmarinic acid (D) 1.0% (w/v) of carnauba wax and 2% (v/v) polysorbate 80. Thermograms information about melting points and enthalpies.

Figure 4: Thermograms of carnauba wax rosmarinic acid-SLN (carnauba wax %, w/v: polysorbate 80 %, v/v): **(A)** 0.5: 1, **(B)** 0.5: 2, **(C)** 0.5: 3, **(D)** 1.0: 1, **(E)** 1.0: 2, **(F)** 1.0: 3, **(G)** 1.5: 1, **(H)** 1.5: 2, **(I)** 1.5: 3.

Figure 5: FTIR spectra of **(A)** rosmarinic acid **(B)** SLN formulation (0.5% carnauba wax: 1% polysorbate 80), the corresponding **(C)** physical mixture of SLN (0.5: 1) and rosmarinic acid, **(D)** SLN formulation (0.5% carnauba wax: 2% polysorbate 80) and the corresponding **(E)** physical mixture of SLN (0.5 : 2%) and rosmarinic acid.