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β-sitosterol Lipid Nano Carrier Based on Propolis Wax and Pomegranate Seed Oil: Effect of Thermal Processing, pH, and Ionic Strength on Stability and Structure

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The β-sitosterol-loaded nanostructured lipid carrier (NLC) is formulated using propolis wax (PW) alone or in the mixture (1:1 w/w) with glyceryl behenate (GB), and pomegranate seed oil stabilized with Tween 80 and lecithin. The influence of thermal treatment (60-90 °C, pH 7, 20 min), pH (2, 4, 6, and 8) and ionic strength (0–200 mM NaCl, pH 7) on physical stability, structure, and β-sitosterol degradation is evaluated. The NLCs are physically and chemically stable during heating at low temperatures (≤70 °C) while thermal treatment at 80–90 °C leads to an increase in size and a slight decrease in β-sitosterol content. The particle size of PW and PW + GB NLCs increases from 96 and 105 nm at pH 7 to 108 and 114 nm at pH 2, respectively, which may limit NLC long-term stability in highly acidic products. On the other hand, there is no significant difference in particle size and PDI with respect to electrolyte concentration. The NLC does not undergo polymorphic transitions by changes in pH and ionic strength. However, crystallinity decreases with increasing electrolyte concentration which is further confirmed by differential scanning calorimetry results. The β-sitosterol NLC relatively showed a suitable potential for industrial applications.

Practical Applications: **β-sitosterol has many valuable physiological functions in humans which has made it an attractive nutraceutical ingredient for food fortifications. Nevertheless, incorporation of β-sitosterol in food and beverages is rather challenging since it is almost insoluble in water and only slightly soluble in oils. NLC based on PW and PSO offer a promising means for delivery of β-sitosterol to aqueous-based foods and developing functional health promoting products by virtue of supplementary health benefits in addition to those of β-sitosterol. However, functionality and dispersibility of formulations could be affected by environmental stresses typically experienced by foods during production and storage. Thus, behavior of emulsions at different conditions should be examined. NLC with relatively good physical and chemical stability to thermal processing, pH, and salt can be created using Tween 80 and lecithin as interfacial coating and PW as lipid matrix which ensures the successful delivery of β sitosterol to various food formats.**

1. Introduction

In recent decades, there has been an emerging interest from health and nutrition scientists toward nutraceuticals such as phytosterol compounds mainly β sitosterol. β sitosterol is a cholesterol-like molecule found in all plant foods which when taken at $2\,g\,day^{-1}$, can cause a significant reduction in intestinal cholesterol absorption. Moreover, β sitosterol has attracted attention due to the several other beneficial effects such as anti-tumor, antioxidant, antidiabetic, anti-inflammatory, and gallstone reducing activities.[1]

Given the low natural dietary intake of phytosterols ($167–437\,mg\,day^{-1}$), food fortification can be a good approach to meet the recommended daily dose. However, the high melting point and low solubility of β sitosterol in both water and fat limit its use as a functional ingredient in many food products.[1] Esterification with long-chain polyunsaturated fatty acids increases fat solubility by tenfold and allows delivery of several grams of phytosterols daily in high-fat foods such as spreads and margarines. However, for further applications as nutraceuticals to supplement aqueous products like juices, yogurts or milk, they need to be either suspended or emulsified.[2] Therefore, it is necessary to formulate/design an effective carrier able to solubilize crystalline phytosterols and maintain the stability of the formulation in different environmental conditions.

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In the past few decades, a wide variety of lipid-based colloidal dispersions including liposomes, microemulsions, nanoemulsions, multilayer emulsions, solid lipid nanoparticles, and nano structured lipid carriers (NLC) have been developed to improve solubility and bioaccessibility of lipid-soluble bioactive substances. NLC are oil-in-water (O/W) emulsions in which a major portion (70–99.9%) of the lipid matrix is constituted by solid lipids.[3] NLC offer several advantages as a delivery system due to the possibility of cost-effective large-scale production, non-toxicity, and good stability against sedimentation and creaming as well as oxidation.[3,4] However, the major advantage of this type of delivery system is its ability to incorporate large quantities of drugs as a result of the formation of a less ordered lipid phase with many imperfections.[5] NLC are also interesting for the food industry because they allow the introduction of lipophilic substances with a negligible impact on product appearance and stability.[1]

Currently, different lipophilic bioactive compounds such as carotenoids, flavonoids, vitamins, essential fatty acids, and plant sterols are successfully encapsulated by NLC and introduced for food applications.[1,6–13] However, the effectiveness of developed dispersions could become highly dependent on the food system involved, and each encapsulation system must be carefully designed for a particular food product.[14] Any aggregation or degradation of nanocarriers can impair the purpose of delivery system and reduces the encapsulant efficiency.[15]

A number of studies have examined the functionality of bioactive loaded nanoparticles against different environmental stresses and reported their good or poor stability, depending on stress condition, interfacial coating, lipid phase composition, and type of the encapsulated compound.[3,7,16–19]

In previous study, we developed and characterized novel water dispersible suspensions of β sitosterol using natural propolis wax (PW) and pomegranate seed oil (PSO). The small size (approximately 100 nm), high encapsulation efficiency of β sitosterol (≥90%) and lower crystallinity of optimized formulations as well as numerous potential health benefits manifested by PSO and PW confirmed the NLC applicability for food fortification.[1] PSO supplies a relatively high amount of conjugated linolenic acid (punicic acid). Therefore, small supplement of PSO can significantly increase the dietary intake of conjugated fatty acids, which normally does not exceed 0.5–1 g in a normal diet, while the recommended intake for delivering health-promoting effects ranges from 1.5 to 3.5 g day^{-1} for an adult person.[20] This unique conjugated fatty acid presents outstanding functions related to human health, such as anti-carcinogenic, anti-diabetes, anti-hyperlipidemia, anti-obesity, and anti-atherosclerotic properties.[21] On the other hand, PW contains complex composition and fatty acids of different chain length which favors the formation of highly disordered crystal lattice that can better accommodate large amounts of bioactive components with long-term physical stability.[22]

In the present study, an attempt has been made to investigate the influence of environmental stresses, typically encountered by food products (heat, pH, and ionic strength) on the stability and properties of the PW NLC containing β sitosterol. Dynamic light scattering was used for the determination of physical stability of the fabricated NLC. Lipid structure and polymorphism was studied using X-ray diffraction and differential scanning calorimetry (DSC). Precise quantification of β sitosterol was done by high-performance liquid chromatography (HPLC) method. The outcome of this investigation could assist in identifying which food processing, formulation, and condition might be important to consider when developing functional foods containing the β sitosterol NLC formulations.

2. Experimental Section

2.1. Materials

β sitosterol was supplied by Sigma–Aldrich. Propolis sample obtained from Espadana Mokamel Co. (Isfahan, Iran). PW (melting point of 62–64 °C) was extracted using soxhlet apparatus and petroleum ether as solvent. PSO (with fatty acid composition of 78.12 ± 0.16% punicic acid, 7.6 ± 0.04% linoleic acid, 7.12 ± 0.04% oleic acid, 3 ± 0.04% palmitic acid, and 2.52 ± 0.02% stearic acid) was provided by the local supplier. Compritol 888 ATO US/NF (glyceryl behenate (GB), a mixture of ~15% monoglycerides, 50% diglycerides and 35% triglycerides of behenic acid, melting point of 71–74 °C) was kindly provided by Gattefossè (Saint-Priest, France). Lecithin (L-α-phosphatidylcholine) was purchased from Daejung Co. (Korea). All other chemicals were analytical grade and supplied by Merck Co. (Darmstadt, Germany).

2.2. Methods

2.2.1. Evaluation of Complete β sitosterol Solubility in the Lipid Phase

The solubility and miscibility of β sitosterol in lipid phase were confirmed by observing the transparency of the obtained lipid phase hot solutions first visually and then by microscopy method. This was done by putting lipid droplet on a glass slide, observing the presence/absence of crystals under the microscope after the mixtures had cooled down to room temperature (BME Monocular, LEICA, Solms, Germany).[10]

2.2.2. β sitosterol NLC Preparation

In this study, two NLC formulations different in composition of solid lipid phase with optimum contents of drug and PSO were developed, based on our previous work.[1] In brief, an aqueous phase (a solution of Tween 80 in phosphate buffer solution (10 mM; pH = 7) and disperse phase (β sitosterol, PSO, lecithin, and solid lipid: PW alone or its binary mixture (1:1) with GB and) were separately prepared and heated under stirring at 85 °C for 5 min. The aqueous phase was gradually added to the oil phase with intense stirring. The resulted emulsions were subjected to a hot high shear homogenization stage (Ultra-Turrax T25 basic, IKA Staufen, Germany), by applying 14 000 rpm for 10 min. After shear homogenization, the samples were processed by probe-type sonicator (Bandelin, Berlin, Germany; amplitude:

50%; power: 100W; probe: MS 72) for 8 min (on for 2 s at intervals of 2 s, 250 W) while maintaining the temperature around the melting point of the lipids. The obtained emulsion was cooled down in an ice bath for 30 min to recrystallize lipid and form NLC. The total concentration of lipid phase (the mixture of solid lipids, PSO, and β sitosterol) and surfactant mixture (Tween 80: lecithin with the ratio of 1:0.25 w/w) was 10 and 6% of total formulation weight, respectively. β sitosterol and PSO concentration was 10 and 50% of total lipid phase, respectively. The freshly prepared NLC formulations were diluted (1:1) with respective buffer solution, frozen at −80 °C for 24 h, and then lyophilized (ALPHA 2–4, Martin Christ Inc., Osterode, Germany) at −70 °C and 0.001 bars for 24 h for evaluation of crystalline structure and thermal behavior.

2.2.3. Influence of Heat Treatment

The thermal stability was determined by immersing 10 mL of each emulsion in a water bath heated to fixed temperatures ranging from 60 to 90 °C for 20 min and then cooling to ambient temperature. Samples were analyzed for mean particle size, polydispersity index (PDI), zeta potential and β sitosterol content on the following day. The percentage of remaining β sitosterol was calculated by comparing the content of β sitosterol in NLC after and before heat treatment.

2.2.4. Influence of pH and Ionic Strength

The pH stability was determined by adjusting the aqueous phase of the NLC to desired final values (2–8) by adding either NaOH or HCl solution. The salt stability was determined by dissolving different amounts of NaCl crystals in NLC (pH: 7) to obtain concentrations of 0, 50, 100, 150, or 200 mM NaCl. After every treatment, the samples were stored for 24 h at ambient temperature before being analyzed.

2.2.5. Dynamic Light Scattering

The analysis of particle size (Z-average) and PDI of NLC formulations was performed by photon correlation spectroscopy (PCS) using a Zetasizer (NanoSizer 3000, Malvern Instruments, Malvern, UK) at an angle of 90° in 0.01 m width cells at 25.0 ± 0.1 °C. The electrical charge (zeta potential) of lipid nanoparticles was determined in a capillary cell using the same instrument. Prior to analysis, NLC were diluted 1:100 with proper buffer solutions to avoid multiple scattering effects. The buffers used for dilution had the same pH and salt concentration as the samples being analyzed. The analysis of particle size, PDI and zeta potential was performed after production and environmental stresses.

2.2.6. β sitosterol Extraction and Quantification

Sample preparation: Firstly, the saponification was performed to extract unsaponifiable matter (USM) by adding 50 mL of ethanolic KOH (2M) to 1 g of the sample. The sample was heated for 60 min in a silicone oil bath set at 90 °C, under continuous agitation and refluxing. After the addition of 50 mL of hexane to the saponified sample, the bottle was shaken and the contents carefully transferred to a 250 mL separatory funnel for liquid–liquid extraction. The flask was washed with 15 mL of deionized H_2O and the washes were transferred to the same funnel. The organic phase was again washed three times with deionized H_2O and water removed. The hexane was dried with anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 50 °C. The dried USM was then dissolved (solubilized) in 2 mL of chloroform and the volume was made up to 10 mL with methanol.[23]

Standard solutions preparation and validation of the HPLC assay: The separate stock solution of β sitosterol standard was prepared by weighing out appropriate amount into volumetric flasks and filling to volume with methanol. Serial dilutions were made to prepare six concentrations over the concentration range of 250–1500 μg mL^{-1} in methanol. These solutions were used for the linearity experiments. The solutions with low, medium and high concentrations (210, 980, and 1750 μg mL^{-1}) were analyzed three times on the same day and repeated on three consecutive days for determination of intra-day and inter-day accuracy and precision, respectively. Accuracy was calculated by comparing the averaged measured concentration to the nominal concentration and was expressed in percentage. Precision was evaluated by calculating the relative standard deviation (RSD%) of measured concentrations in each sample. The extraction recoveries of the drug from NLC at levels of 210 and 980 μg mL^{-1} were determined by comparing mean peak area of samples to the respective standards in methanol and expressed in percentage.[24]

Chromatographic conditions: The β sitosterol content of NLC after production and thermal treatment was analyzed using reverse-phase HPLC methods. The HPLC system (Merck Hitachi, Darmstadt, Germany) was composed of a L-2130 isocratic pump and UV detector. The analytical column was Hibar 150–4,6 Purospher STAR C18 (5 μm). The injection volume was 20 μL; the mobile phase was methanol-water (98/2, v/v) at a flow rate of 1.0 mL min^{-1}; the wavelength was 210 nm; the column temperature was 40 °C.[25] For these conditions, β sitosterol was eluted at a retention time of 11.5 min.

2.2.7. XRD Analysis

The XRD patterns of freeze-dried formulations were obtained by collecting intensity data measured by Bruker D8 GmbH diffractometer (Karlsruhe, Germany) using Cu Kα radiation ($\lambda = 1.540598$ Å; voltage 40 kV; current 40 mA) with a step width of 0.05°/s over the range of 5–40°2θ.

2.2.8. DSC Analysis

The thermal behavior of β sitosterol loaded nanocarriers was studied by differential scanning calorimetry using a Mettler TA 4000 Star^e system apparatus equipped with a 25 DSC cell (Mettler Toledo, Milano, Italy). The samples (5–10 mg) were weighed (Mettler M5X Balance) into standard aluminum pans and heated from 25 to 160 °C with nitrogen gas flow at

Table 1. Accuracy and precision (RSD%)[a] of the HPLC assay in β sitosterol quantification.

	Intra-day ($n=3$)			Inter-day ($n=3$)		
Added concentration [μg mL^{-1}]	Measured concentration [mean ± S.D, μg mL^{-1}]	Precision [%]	Accuracy [%]	Measured concentration [mean ± S.D, μg mL^{-1}]	Precision [%]	Accuracy [%]
210	213.75 ± 1.23	0.54	101.79	220.16 ± 13.04	4.1	104.84
980	1012.39 ± 13.87	1.35	103.3	1007.12 ± 5.66	0.45	102.77
1750	1776.59 ± 6.31	0.35	101.52	1741.28 ± 45.41	0.30	99.5

[a] RSD% reflects method repeatability.

20 mL min^{-1} and the scan rate of 10 °C min^{-1}. An empty aluminum pan was used as a reference.

2.3. Statistical Analysis

All measurements were carried out in triplicate and reported as means ± SDs. Data collected in this study were analyzed using a SAS statistical software package (version 9.4) by one-way analysis of variance (ANOVA), followed by comparisons with a least significant difference (LSD) procedure and the results of the statistical analysis were considered significant if their corresponding p-values were ≤ 0.05.

3. Results and Discussion

3.1. Evaluation of β sitosterol Solubility in the Lipid Phase

For evaluating the ability of the applied lipid phase to solubilize β sitosterol, lipid screening was carried out via microscopic assessment. The mixture was observed by the likely presence of crystals. Microscopic assessment and/or visual observation with the naked eyes was used by several researchers to determine drug solubility in a solid lipid matrix and considered it suitable when the mixture had an amorphous image without crystal under optical microscope.[10,26–29] Through observing transparency of the hot mixtures visually and then microscopically, no crystals were detected and so good solubility and miscibility of β sitosterol with the solid lipids and other components was considered. Moreover, the absence of β sitosterol endothermic peak in DSC thermograms (Section 2.2.8) confirmed its high solubility (figures are not shown). This could be explained by mixed lipid matrix (solid fat and liquid oil) that confer more imperfection crystal structure and improve drug incorporation.[30] Furthermore, GB and PW (with very complex composition) have many imperfections in the crystalline lattice which ensures high encapsulation efficiency and uniformity of lipid nanoparticles.[22]

3.2. Validation of HPLC Assay

The method was found to be precise, accurate, and sensitive. A good linearity was achieved in the concentration range. The linearity experiment was performed three times and the mean was used for the calculations. The regression equation and correlation coefficient were $y = 23903x + 376645$ and $R^2 = 0.999$, where x was the peak area, and y was the drug concentration. The variation of the method was validated performing repeated analyses of decreasing analyte amounts. The limit of quantification (LOQ) and limit of detection (LOD) were 18.9 and 5.67 mg L^{-1}, respectively. The summaries of other evaluating parameters are listed in Tables 1 and 2. Single-laboratory validation method should include an intra and inter-day component determined by the evaluation of a minimum of three replicates at three different concentrations, representing the entire range of the calibration curve. The intra- and inter-day accuracies ranged from 99.5 to 104.84%. Accuracy refers to the trueness of the experimental procedure by evaluating the closeness of the true value of the analyte concentration and the mean result obtained, while the precision characterizes the closeness of agreement between the measured values. The intra-day precision (being referred as repeatability) and inter-day precision (being referred as intermediate precision) describe how much variation occurs within a day and between separate days, respectively, and could be expressed by RSD%. As a general rule, within-day RSD values should be lower or in the same order of magnitude of between-day values. The within-day and between-day RSD ranged between 0.35–1.35% and 0.3–4.1%, respectively, which satisfies the validation criteria and indicates the method repeatability, showing both within and between day values lower than 5%.[23] The high extraction recoveries (95.41–102.62%) showed that β sitosterol could

Table 2. The extraction recovery (analyzed/certificate value*100) of β sitosterol from nanocarriers ($n=3$).

	PW NLC[a]		PW + GB NLC[b]	
Sample concentration [μg mL^{-1}]	Analyzed concentration [mean ± SD, μg mL^{-1}]	Recovery [%]	Analyzed concentration [mean ± SD, μg mL^{-1}]	Recovery [%]
210	215.5 ± 6.36	102.62	203.5 ± 7.78	96.9
980	935 ± 7.07	95.41	973.2 ± 9.55	99.31

[a] Nanostructured lipid carriers made with propolis wax as solid lipid.
[b] Nanostructured lipid carriers made with mixture of propolis wax and glyceryl behenate (1:1) as solid lipid.

accurately and efficiently be extracted and quantified using the applied method.

3.3. Stability Under Different Stress Conditions

Among the major challenges for the successful utilization of edible delivery systems for food fortification, is to remain stable throughout the anticipated shelf life of the final product. During their ideal shelf life, nanocarriers should be able to conserve the therapeutic activity of the drug and to maintain the physical integrity of the lipid matrix and dispersion stability.[15] Flocculation, coalescence, Ostwald ripening, and gravitational separation are the main instability mechanisms of lipid nanoparticle suspensions which are influenced by storage conditions such as pH, salt concentration, and temperature and lead to particle size enhancement and dispersion gelation. Also, drug loading capacity can decrease during process or storage by lipid conversion into a more stable and organized β form. This process can lead to drug expulsion from the lipid matrix and, consequently, resulting in the loss of unprotected drug.[15,31] Moreover, the occurrence of chemical reactions, like hydrolysis or oxidation of lipid components can affect product quality.[15]

Studies show that the physical properties of the lipid phase have a significant impact on lipid nano particle stabilization. For example, large proportion of long chain triglycerides provides a kinetic barrier to Ostwald ripening.[19] Nature of the colloidal interactions operating between oil droplets also considerably affects the stability. Generally, emulsions primarily stabilized through electrostatic repulsion mechanism tend to become unstable when pH, salt content, and temperature changes; while lipid droplets are much less sensitive to these changes when the primary stabilization mechanism is steric repulsion.[32] The stability of emulsion systems can be improved considerably by using different combinations of emulsifiers in stabilizer mixture.[18]

We therefore examined the effect of different environmental factors on stability and structure of β sitosterol NLC stabilized by combination of steric and electrostatic repulsion, using Tween 80 and lecithin.

3.3.1. Influence of Thermal Processing on Physical Stability and β sitosterol Content

Foods fortified with nanodispersions may experience thermal treatment during sterilization, pasteurization, or cooking. Therefore, it is essential to assess the influence of heat processing on emulsion stability. NLC were held at temperatures ranging from 60 to 90 °C for 20 min (pH = 7) and then stored for 1 day at 25 °C prior to analysis. The change in mean particle diameter (Z-average), PDI and zeta potential of NLC stabilized

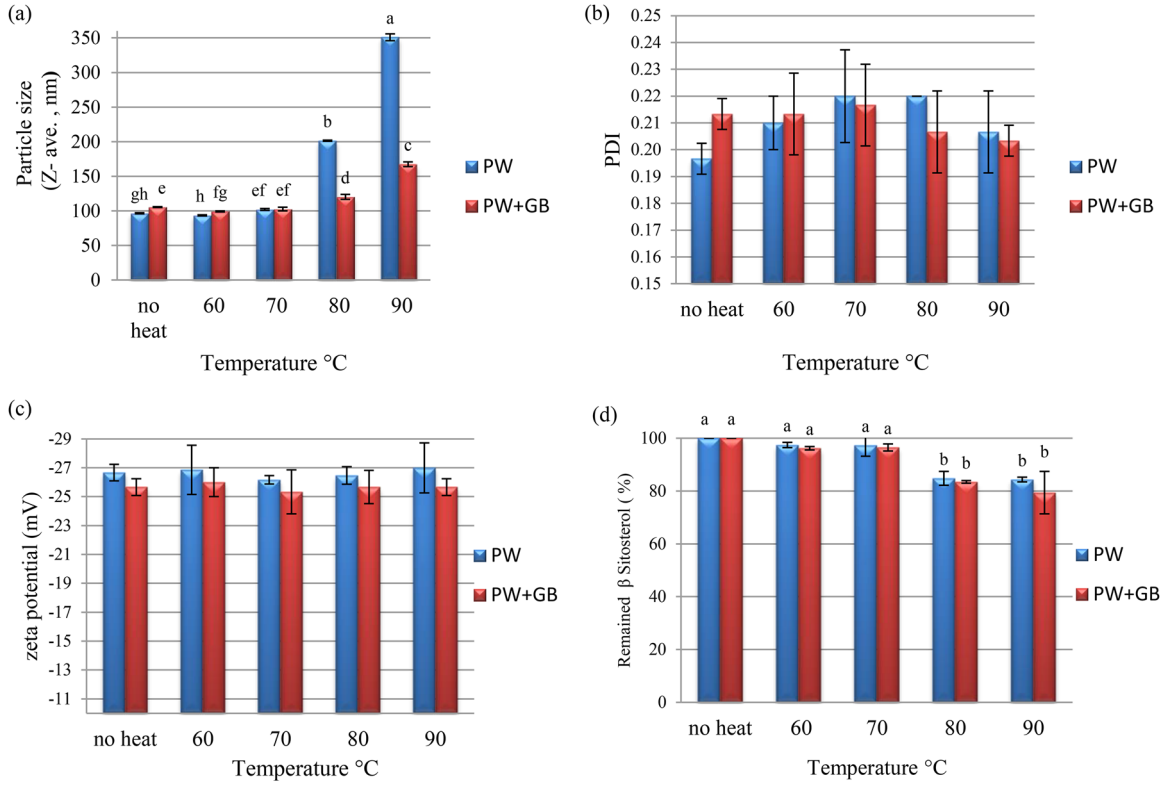

Figure 1. Particle size (a), polydispersity index (PDI) (b), zeta potential (c), and β sitosterol content (d) of heat treated (60–90 °C, 20 min, pH = 7) β-sitosterol nano structured lipid carriers made with either propolis wax (PW) or mixture of (1:1) propolis wax and glyceryl behenate (PW + GB). In each parameter, the means that have no superscript in common are significantly different from each other ($P \leq 0.05$). The means without superscript are not significantly different ($P > 0.05$).

with Tween 80 and lecithin are illustrated in **Figure 1**(a–c). The particle size and PDI are the most important characteristics of nano dispersions which govern the physical stability, solubility, biological performance, release rate, and turbidity.[33] The small size of dispersed particles can ensure a long-term kinetic stability.[19] Heat treatment and lipid type had a significant effect on the particle size (Figure 1a). Our results suggested that PW and PW + GB NLC were stable to aggregation during relatively short term heating at low temperatures (≤70 °C); however, growth in particle size was observed at higher temperatures.[3] The particle size of the original PW + GB sample was 105 ± 0.75 nm which increased to 120 and 167 nm after heating at 80 and 90 °C, respectively. Larger values of the average particle diameter were observed for NLC droplets made with PW alone (from 96 nm to 200 and 350 nm after heating at 80 and 90 °C, respectively). The superior stability of PW + GB NLC could be mainly dependent on the chemical composition of GB. GB contains approximately 15% monoglycerides and 50% diglycerides of behenic acid. It has been proposed that partial glycerides possess surfactant properties (HLB 2–5) and can improve the surfactant film around the nanoparticles and thus prevent particle aggregation.[34] These results confirmed that the stabilization effect differs between the applied lipid compositions and reflect the importance of lipid selection and interfacial composition when developing delivery systems.

Visual observation of NLC samples indicated that they remained fairly transparent and homogeneous at 60–80 °C. However, the transparency of PW and PW + GB NLC deteriorated at 90 °C and flocculation occurred (**Figure 2**).

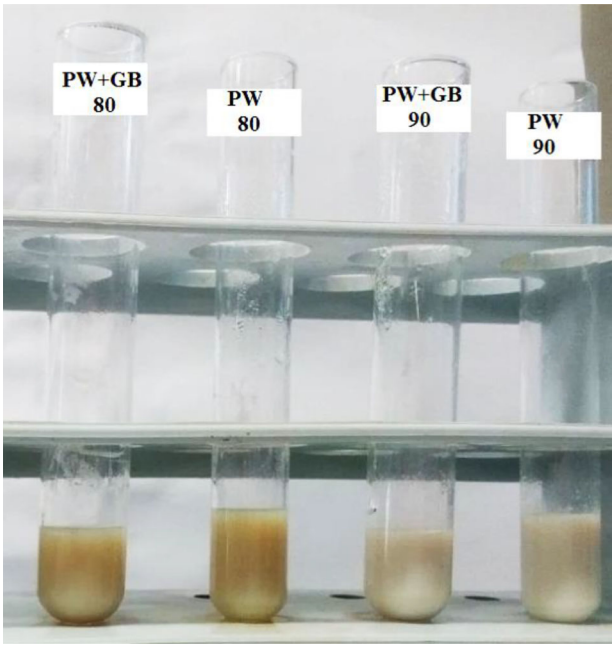

Figure 2. Influence of thermal processing on the appearance of β-sitosterol nano structured lipid carriers made with either propolis wax (PW) or mixture of (1:1) propolis wax and glyceryl behenate (PW + GB) after 24 h storage. Droplet flocculation occurred in emulsions treated at 90 °C (20 min, pH = 7) and the color changed.

The surfactant nature plays a prominent role in emulsion stability. Emulsions prepared using non-ionic surfactants such as Tween 80 are stable to thermal processing when held at temperatures below critical temperature which is called phase inversion temperature (PIT). However, elevated temperatures (above PIT) could have a negative effect on the stability by dehydration of the hydrophilic surfactant head groups and alteration of optimum curvature of the surfactant monolayer at the oil-water interface.[35] Dehydration changes the hydrophilic–lipophilic balance (HLB) of surfactants which favors coalescence.[3] Coalescence is the process where two or more liquid droplets merge together to form a single larger droplet.[31] Furthermore, the increased kinetic energy and the reduced viscosity of continuous phase at evaluated temperatures could accelerate the number of droplet collisions per unit time and area, and the rate of coalescence. At low temperatures the surfactant molecules can form a rigid layer that is more resistant to coalescence.[3]

The obtained results agreed with observations of Tamjidi et al.[3] who reported that astaxanthin NLC stabilized with Tween 80 and lecithin were stable to aggregation at low holding temperatures but size growth was observed at high temperatures due to the changes in interfacial characteristics, kinetic energy, and melting of the lipid phase.

The values of zeta potential and PDI were −25.5 mv and 0.21 in PW + GB NLC and −26.5 mv and 0.2 in PW NLC, respectively. Zeta potential and PDI are parameters related to surface charge and particle size distribution, respectively. PDI values close to 0.2 are usually associated with more homogeneous particle size distribution.[36] The PDI of the heat treated NLC was in range of 0.2–0.22 and had no significant difference with unheated sample. Moreover, no significant difference ($P > 0.05$) was observed among the zeta potential values of original and heat-treated NLC formulations (Figure 1b and c). This was in agreement with findings of Tamjidi et al.[3] and Charoen et al.[32] who reported unchanged electrical characteristics of all developed emulsions by heating. The high zeta potential value suggests that heat-treated emulsions may have a good stability against aggregation during storage.

The application of heat treatment can affect the stability of sterols. Figure 1(d) shows the percentage of remaining β sitosterol after the thermal process. There was little influence of solid fat composition on thermal stability, and β sitosterol content of both NLC composed of PW and/or PW + GB remained relatively stable during heating at low temperatures. The total β sitosterol content decreased by increasing temperature ($P \leq 0.05$) and the highest degree of deterioration was found after 20 min of heating at 90 °C (19% for PW + GB NLC and 15% for PW NLC). A possible explanation for this reduction could be the degradation of sterols into other compounds. Oxidation is the main mechanism for phytosterols degradation. Sterols are well-known to be prone to oxidation by heat, reactive oxygen species, light, UV light, ionizing radiation, chemical catalysts, lipid hydroperoxides, and enzymatic reactions. Oxidative susceptibility is higher in oil/water emulsions than in bulk fat systems. Investigation of stability of sterols in phytosterol-enriched milk showed that drastic heating of enriched milk (15 min at 90 °C) caused a 60% reduction of total phytosterol. However, phytosterol content maintained during usual heating

condition.[37] Although it was not statistically significant, the lower destruction of β sitosterol in PW NLC could be related to antioxidant properties of PW due to the presence of pentacyclic triterpenoids which are considered among the components with biological and antioxidant activity.[22]

3.3.2. Influence of pH and Electrolyte Concentration on Stability

The aqueous stability of NLC is strongly affected by two distinctly different processes: the ability of the suspension to remain homogeneous (suspension stability) and the ability of the crystal matrix to resist recrystallization (matrix stability).[31] Changing in the pH or ionic strength of the system can influence the charge of interface and recrystallization rate and behavior.[4,31] Therefore, the effect of varying pH and electrolyte concentration on physical stability, lipid structure, and polymorphism was examined.

Mean particle diameters and PDI (**Figure 3**a) changed significantly with pH variation ($P \leq 0.05$). The results of particle size were in agreement with the observation found on the zeta potential values obtained at different pH (Figure 3a). The absolute zeta potential of the PW NLC prepared at pH 8 was about −26.5 mV which reduced to −3 at pH 2. The same pattern was observed for PW + GB NLC. However, despite very low surface charge density, there was little change in the mean particle diameter of the droplets under acidic conditions and size of the PW and PW + GB NLC increased from 96 nm and 105 nm at pH 7 to 108 and 114 nm at pH 2, respectively (Figure 3a). This indicates the importance of steric repulsion due to a Tween 80 layer which decreases the nanoparticles aggregation induced by weak electrostatic repulsion.

In general, protein-stabilized emulsions are sensitive to pH changes since the degree of electrostatic repulsion depends on the magnitude of surface charges.[38] The surface charge reduction in the case of our study (Tween and lecithin stabilized emulsions) could be attributed to several factors. Lecithin is an amphoteric lipophilic surfactant which is negatively charged above pH of 3.5. As pH decreases, the anionic groups of lecithin ($-PO_4^{-3}$) protonate and the net charge of the interface and therefore, electrostatic repulsion decreases.[3] Furthermore, decreasing pH leads to increasing protonation and decreasing deprotonation of the functional groups (e.g., $NH_3/NH_4^+$and $COOH/COO^-$) on the surface of lecithin stabilized emulsions. On the other hand, although Tween 80 is a non-ionic surfactant and would therefore not be expected to have any impact on surface charge, the ether linkage in the polyoxyethylene group of Tween 80 can be protonated at a very low pH and neutralizes the negatively charged interface.[4] Therefore, the increase in particle size under an acidic condition could be attributed to decrease in the surface active properties of Tween 80 and thus insufficient interfacial adsorption between the lipid and water phase as well as reduction of the charge density due to protonation of lecithin and polar groups of Tween 80.

NLC samples with higher pH values (6–8) were stable to size increment. At high pH values, the zeta potential and thereby the electrostatic repulsion is sufficiently strong to overcome attraction forces and prevents nanoparticle aggregation. Similar changes in size and zeta potential of particles (stabilized by Tween 80 or mixture of Tween 80 and lecithin) by varying pH were also reported by other researchers.[3,4,39]

The above results indicate that developed NLC suspensions may have no enough long-term stability in products with high acidity as low pH can trigger the destabilization by reducing the electrical repulsive force between particles and influencing the surfactant efficiency.

Ionic strength is one of the most important factors which influence the stability of emulsified foods and beverages.[19] Figure 3(b) shows the effect of different NaCl concentrations (0–200 mM, pH = 7). NLC were stable at all concentrations, exhibiting no significant change in mean particle diameter (PW NLC–95 nm; PW + GB NLC–105 nm) and PDI value. The surface charge remained negative at all NaCl concentrations. However, the PW NLC charge magnitude (−27) fell to −5 mV by increasing NaCl concentration from 0 to 200 mM ($P < 0.05$). A similar pattern of behavior was also observed in PW + GB NLC.

The surface electrical potential depends on the ionic composition of the surrounding medium and usually decreases as the ionic strength of the aqueous phase increases (electrostatic screening effects).[2] Therefore, a reduction in charge of PW NLC can be attributed to the binding tendency of

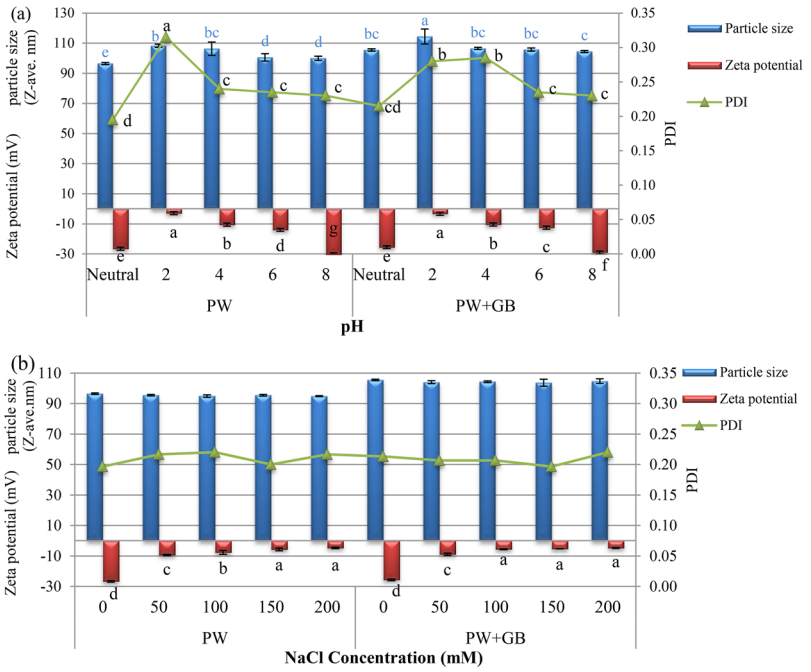

Figure 3. Influence of pH (2–8) (a) and ionic strength (0–200 mM NaCl) (b) on particle size, polydispersity index (PDI), and zeta potential of β sitosterol nano structured lipid carriers made with either propolis wax (PW) or mixture of (1:1) propolis wax and glyceryl behenate (PW + GB). In each parameter, the means that have no superscript in common are significantly different from each other ($P \leq 0.05$). The means without superscript are not significantly different ($P > 0.05$).

Na^+ to the negatively charged groups of lecithin ($-PO4^{-3}$). However, NLC formulations were physically stable and no particle growth was detected at all investigated NaCl concentrations. It shows that additional steric stabilization through non-ionic surfactant (Tween 80) was sufficient to avoid aggregation and can compensate for the decrease of electrostatic repulsion.

Our results are in good agreement with Yang et al.[35] who reported that emulsions containing Tween 80 were stable to electrolyte concentrations (0–500 mM), exhibiting no change in mean particle diameter. Zimmermann and Muller[40] also investigated pH and electrolyte stability of different SLN dispersions consisting of various lipids and surfactants and reported that in general the influence of low pH was stronger than electrolyte concentration and some formulations remained stable with respect to electrolytes but were pH sensitive.

However, the decrease of zeta potential and the increase of the particle size of Tween 80-stabilized SLNs and NLC by adding NaCl to the aqueous phase have been described in other literatures.[3,4] It should be noted that in general the stabilizing properties depend on factors such as molecular characteristics of surfactant, specific interactions of the lipid matrix with the emulsifier, and anchoring and density of the stabilizer on the lipid surface.[40]

3.3.3. Influence of pH and Electrolyte Concentration on Structure and Polymorphism

To determine if stress condition have any impact on lipid crystal type and crystallinity, the stability of the nanoparticles in low and high pH (2 and 8) and low and high salt concentrations (50 and 200 mM) was further investigated by XRD and DSC.

The X-ray diffraction profiles of NLC samples prepared at different pH and electrolyte concentrations are shown in **Figure 4**. Lipid crystals (α, β′, and β crystals) differ in the structural arrangement of fatty acid chains of triglycerides and therefore, in their lattice spacing. The lipid composition will determine the type of crystal that is generated upon cooling, thereby influencing the stability and the release characteristics of the encapsulated bioactive.[31] Generally, pure homogeneous lipids tend to form thermodynamically most stable and highest melting β modification (triclinic unit structures) while β′ (orthorhombic subcell) is the dominant crystal form of waxes.[34]

PW NLC samples revealed a high intensity reflection at 21.25° and a medium intensity reflection at 23.4° indicating short spacings of fatty acid chains at 4.2 and 3.8 A°, respectively, which are typical for the orthorhombic metastable polymorph (β′). Another reflection at 31.73° was also found in diffractograms of samples containing electrolyte which corresponds to XRD spectrum of NaCl crystal.[41]

Changing the aqueous pH did not yield polymorphic behavior change and peak location and intensity of both PW and PW + GB samples resembled original NLC prepared at neutral pH (Figure 4a and b). Peak intensity decreased greatly with increasing ionic strength. Reduction in intensity of the diffraction peaks of samples in the presence of salt compared to original NLC (0 mM NaCl) might indicate the higher proportion of β′ polymorph and lower degree of crystallinity.[22] Jenning and Gohla[34] reported that in contrast to glyceride nanoparticles, polymorphic transition does not occur in waxes even under stress conditions such as exposure to electrolytes. Recrystallization, due to polymorphic transitions, can lead to network formation of crystallized particles resulting in a gel-like behavior.[31]

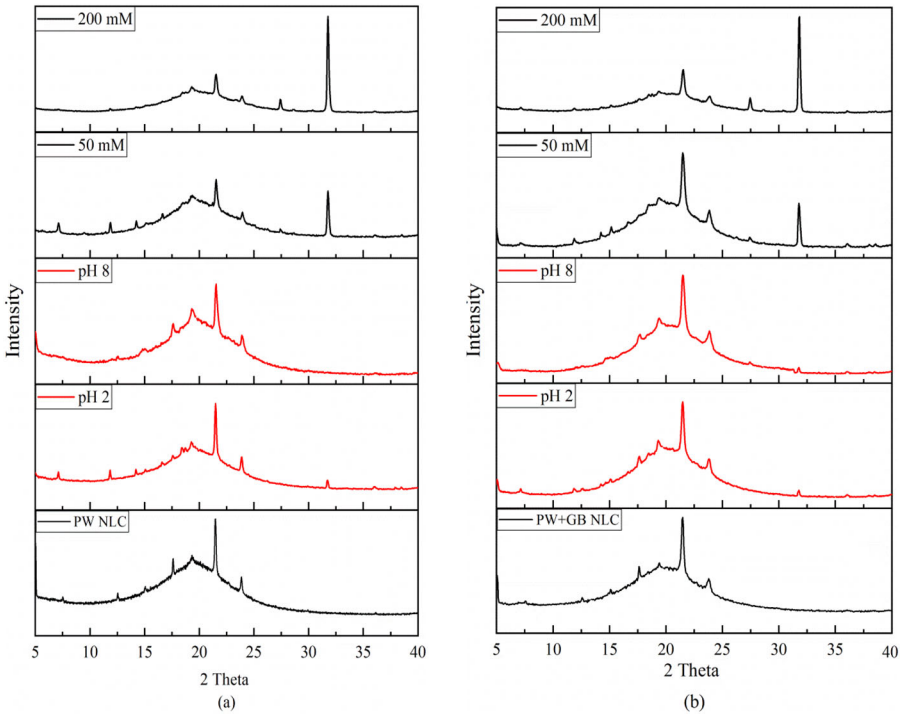

Figure 4. X-ray diffraction pattern of β-sitosterol nano structured lipid carriers made with either propolis wax (PW) (a) or mixture of (1:1) propolis wax and glyceryl behenate (PW + GB) (b) subjected to different pH (2 and 8) and electrolyte content (50 and 200 mM) and original sample (pH = 7, 0 mM).

Table 3. Effect of pH and electrolyte concentration on the melting temperature and enthalpy of nano carriers.

	PW NLC[a]				PW + GB NLC[b]			
	OT [°C]	PT [°C]	ET [°C]	Enthalpy [J g^{-1}]	OT [°C]	PT [°C]	ET [°C]	Enthalpy [J g^{-1}]
pH								
Neutral	50.54	58.12	60.92	15.12	46.01	54.99	58.14	30.01
2	49.65	57.37	60.01	16.57	46.22	54.21	58.10	29.23
4	49.7	57.77	60.47	16.68	47.10	54.56	58.32	30.20
6	50.23	58.12	60.96	16.73	45.98	54.99	58.15	30.42
8	50.03	58.96	61.53	16.40	45.24	55.53	59.06	30.78
NaCl concentration [mM]								
0	50.54	58.12	60.92	15.12	46.01	54.99	58.14	30.01
50	50.05	58.02	60.12	10.57	44.26	54.82	57.49	25.84
100	50.56	57.77	59.76	10.37	44.28	54.54	57.45	25.74
150	49.12	57.79	59.76	10.79	44.05	54.51	57.16	25.83
200	49.13	57.7	59.85	10.68	44.67	54.62	57.26	24.4

OT, onset melting temperature; PT, peak melting temperature; ET, endset melting temperature.
[a] Nanostructured lipid carriers made with propolis wax as solid lipid.
[b] Nanostructured lipid carriers made with mixture of propolis wax and glyceryl behenate (1:1) as solid lipid.

DSC was also employed to check any variation of crystalline properties of lipid carriers after adjusting the aqueous phase to different pH values and adding different electrolyte concentrations. The melting behavior and melting enthalpy of samples prepared at different pH and electrolyte concentrations are presented in **Table 3**.

The melting peaks reflect the crystal structure in NLC systems. For the PW and PW + GB NLC, the melting process takes place at 58.18 °C and 54.99 (peak maximum), respectively. A decreased melting point and onset temperature of PW + GB NLC indicates a more imperfection crystal structure and less ordered arrangement of oil within the carrier compared to PW NLC.[42]

Regarding electrolyte concentration, DSC data confirmed XRD results by showing a reduction in melting enthalpy and therefore crystallinity of both NLC formulations in the presence of salt compared to original NLC. However, the onset temperatures and melting points were not influenced appreciably. It seems that electrolytes suppress crystallization and most lipid droplets remain in a liquid state. pH did not affect the crystalline state of NLC. This could be attributed to the insensitive nature of saturated fatty acids and the non-ionic surfactant to the pH of the aqueous medium.[4]

4. Conclusion

This study characterized the influence of thermal processing, pH, and salt on the stability and structure of PW and PW + GB (1:1) NLC containing β sitosterol. At relatively low temperatures (≤70 °C), the particle size of NLC remained unchanged while some aggregation occurred at high temperatures (80 and 90 °C). Heat stability strongly depended on solid lipid type, with a mixture of PW and GB being more effective than PW alone at preventing flocculation, which was attributed to the stabilizing effect of GB. β sitosterol content maintained during heating at low temperatures while decreased (≈15–20%) by increasing temperature. These results suggested that the freshly produced β sitosterol NLC formulations can be thermally pasteurized. Lipid particle size increased with decreasing pH. The results were supported by the decrease of zeta potential in lower pH values which may limit long-term stability of developed NLC in low pH products such as acidic soft drinks. PW and PW + GB NLC proved to be stable to aggregation at different salt concentrations (0–200 mM NaCl), indicating that steric stabilization can compensate for the decrease of electrostatic repulsion. Submitting the suspensions to different stress conditions did not affect the lipid structure (β′crystal form); however, peak intensity decreased greatly with increasing ionic strength. Overall, despite being essential several further studies to ensure the efficacy of obtained lipid nanoparticles for food fortification, these initial results showed that PW NLC stabilized with mixture of lecithin and Tween 80 were stable to a wide range of environmental stresses which makes them promising carrier system for delivery of β sitosterol into a variety of food and beverage products.

Abbreviations

DSC, differential scanning calorimetry; GB, glyceryl behenate; NLC, nanostructured lipid carriers; PDI, polydispersity index; PSO, pomegranate seed oil; PW, propolis wax.

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Conflicts of Interest

The authors declare no conflicts of interest.

Keywords

β-sitosterol, nanodispersions, pomegranate seed oil, propolis wax, stability

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[1] Y. Soleimanian, S. A. H. Goli, J. Varshosaz, F. Maestrelli, *Food Chem*. **2018**, *260*, 97.
[2] D. J. McClements, E. A. Decker, J. Weiss, *J. Food Sci*. **2007**, *72*, 109.
[3] F. Tamjidi, M. Shahedi, J. Varshosaz, A. Nasirpour, *J. Food Sci. Technol*. **2017**, *54*, 3132.
[4] K. Choi, N. P. Aditya, S. Ko, *Food Chem*. **2014**, *147*, 239.
[5] B. Gaba, M. Fazil, A. Ali, S. Baboota, J. K. Sahni, J. Ali, *Drug Deliv*. **2015**, *22*, 691.
[6] A. Akhoond Zardini, M. Mohebbi, R. Farhoosh, S. Bolurian, *J. Food Sci. Technol* **2018**, *55*, 287.
[7] A. Babazadeh, B. Ghanbarzadeh, H. Hamishehkar, *J. Funct. Foods*. **2016**, *26*, 167.
[8] A. Hejri, A. Khosravi, K. Gharanjig, M. Hejazi, *Food Chem*. **2013**, *141*, 117.
[9] F. Tamjidi, M. Shahedi, J. Varshosaz, A. Nasirpour, *Innov. Food Sci. Emerg. Technol*. **2014**, *26*, 366.
[10] S. Ni, R. Sun, G. Zhao, Q. Xia, *J. Food Process Eng*. **2015**, *38*, 93.
[11] A. Pezeshki, B. Ghanbarzadeh, M. Mohammadi, I. Fathollahi, H. Hamishehkar, *Adv Pharm. Bull*. **2014**, *4*, 563.
[12] H. Salminen, C. Gömmel, B. H. Leuenberger, J. Weiss, *Food Chem*. **2016**, *190*, 928.
[13] K. Zheng, A. Zou, X. Yang, F. Liu, Q. Xia, R. Ye, *Food Hydrocoll*. **2013**, *32*, 72.
[14] L. Liang, F. Chen, X. Wang, Q. Jin, E. A. Decker, D. J. Mcclements, *J. Agric. Food Chem*. **2017**, *65*, 4755.
[15] M. Narvekar, H. Y. Xue, J. Y. Eoh, H. L. Wong, *AAPS Pharm. Sci. Tech*. **2014**, *15*, 822.
[16] B. S. Chu, S. Ichikawa, S. Kanafusa, M. Nakajima, *J. Sci. Food. Agric*. **2008**, *88*, 1764.
[17] C. Qian, E. A. Decker, H. Xiao, D. J. Mcclements, *Food Chem*. **2012**, *132*, 1221.
[18] N. Anarjan, H. J. Malmiri, T. C. Ling, C. Ping, N. Anarjan, H. J. Malmiri, *Int. J. Food Prop*. **2014**, *17*, 937.
[19] B. Mehrad, R. Ravanfar, J. Licker, M. J. Regenstein, A. Abbaspourrad, *Food Res. Int*. **2017**, *105*, 962.
[20] F. Siano, F. Addeo, M. G. Volpe, M. Paolucci, G. Picariello, *J. Agric. Food Chem*. **2016**, *64*, 8369.
[21] Y. Cao, L. Wang, M. He, Y. Zhang, H. Wang, *RSC Adv*. **2014**, *4*, 43392.
[22] Y. Soleimanian, S. A. H. Goli, J. Varshosaz, S. M. Sahafi, *Food Chem*. **2018**, *244*, 83.
[23] C. T. Srigley, E. A. Haile, *J. Food Compos. Anal*. **2015**, *40*, 163.
[24] X. Zhang, J. Liu, H Qiao, H Liu, J. Ni, W. Zhang, *Powder Technol*. **2010**, *197*, 120.
[25] I. Lacatusu, N. Badea, R. Stan, a. Meghea, *Nanotechnology* **2012**, *23*, 455702.
[26] L. M. Monteiro, R. Löbenberg, P. C. Cotrim, G. Lima, B. D. Araujo, N. Bou-Chacra, *Biomed Res. Int*. **2017**, *2017*, 1.
[27] S Nikolić, C. M. Keck, C. Anselmi, R. H. Müller, *Int. J. Pharm*. **2011**, *414*, 276.
[28] G. Y. Liu, J. M. Wang, Q. Xia, *Eur. Food Res. Technol*. **2012**, *234*, 391.
[29] N. V. Shah, A. K. Seth, R. Balaraman, C. J. Aundhia, R. A. Maheshwari, G. R. Parmar, *J. Adv. Res*. **2016**, *7*, 423.
[30] M. Zheng, M. Falkeborg, Y. Zheng, T. Yang, X. Xu, *Coll. Surf. A Physicochem. Eng. Asp*. **2013**, *430*, 76.
[31] J. Weiss, E. A. Decker, D. J. McClements, K. Kristbergsson, T. Helgason, T. Awad, *Food Biophys*. **2008**, *3*, 146.
[32] R. Charoen, A. Jangchud, K. Jangchud, T. Harnsilawat, O. Naivikul, D. J. Mcclements, *J. Food Sci*. **2011**, *76*, 165.
[33] F. Tamjidi, M. Shahedi, J. Varshosaz, A. Nasirpour, *Innov. Food Sci. Emerg. Technol*. **2013**, *19*, 29.
[34] V. Jenning, S. Gohla, *Int. J. Pharm*. **2000**, *196*, 219.
[35] Y. Yang, M. E. Leser, A. A. Sher, D. J. McClements, *Food Hydrocoll*. **2013**, *30*, 589.
[36] N. Fachinetti, R. B. Rigon, J. O. Eloy, M. R. Sato, K. C. Santos, M. Chorilli, *AAPS Pharm. Sci. Technol*. **2018**, *19*, 1401.
[37] M. MenEndez-Carreno, D. Ansorena, I. Astiasaran, *J. Agric. Food Chem*. **2008**, *56*, 9997.
[38] J. Surh, E. A. Decker, D. J. Mcclements, *Food Hydrocoll*. **2006**, *20*, 607.
[39] C. W. How, A. Rasedee, S. Manickam, R. Rosli, *Coll. Surf. B Biointerf*. **2013**, *112*, 393.
[40] E. Zimmermann, R. H. Muller, *Eur. J. Pharm. Biopharm*. **2001**, *52*, 203.
[41] S. Addala, L. Bouhdjer, A. Chala, A. Bouhdjar, O. Halimi, B. Boudine, *Chin. Phys. B* **2013**, *22*, 1.
[42] V. Jenning, A. F. Thunemann, S. H. Gohla, *Int. J. Pharm*. **2000**, *199*, 167.

