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Preparation of betulinic acid nanoemulsions stabilized by ω-3 enriched phosphatidylcholine

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ABSTRACT

Bioactive compounds such as ω-3 fatty acids and terpenes, have been associated with beneficial health effects; however, their solubility in the gastrointestinal tract and its bioavailability in the body are low. Nanoemulsions offer a viable alternative to disperse lipophilic compounds and improve their dissolution, permeation, absorption and bioavailability. Enzyme modified phosphatidylcholine (PC) with ω-3 fatty acids was used as emulsifier to stabilize oil-in-water nanoemulsions generated using ultrasound device. These systems were used as carriers of betulinic acid, which has reported anti-carcinogenic activity. Phospholipase-catalyzed modification of PC allowed the incorporation of 50 mol% of ω-3 fatty acids. Formation variables such as oil type and ultrasound amplitude had effects on nanoemulsion characteristics. Incorporation of betulinic acid affected globule size; however, betulinic acid nanoemulsions below 200 nm could be prepared. The conditions under which betulinic acid nanoemulsions were obtained using the modified phosphatidylcholine with the smaller globule size (91 nm) were 10% PC, 25% glycerol, medium chain oil and 30% amplitude for 12 min in the sonicator. Storage temperature had an effect on the stability of the nanoemulsions, at 5 °C we observed the smallest growth in globule size. The use of olive oil decreased the globule size growth during storage of the nanoemulsion stabilized with modified phosphatidylcholine, although globule size obtained was greater than 200 nm. Medium pH had a significant effect on the nanoemulsions; alkaline pH values improved storage stability. These results provide useful information for using this type of carrier system on the formulation of products in the pharmaceutical or food industry.

1. Introduction

Consumption of polyunsaturated omega-3 (ω-3) fatty acids, such as eicosapentaenoic acid (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) has been reported to have beneficial physiological effects, among which are reduction in the incidence of cardiovascular disease, cancer, diabetes, arthritis, central nervous system (schizophrenia, depression, Alzheimer's disease), as well as in brain and retina development in the fetus and infants [1–5], so the development of products containing them are of interest for the development of nutraceuticals. Many other bioactive molecules present in foods and other natural products can exert a beneficial effect on health; some of these are considered functional compounds and have been commercially branded as nutraceuticals. This group of compounds includes vitamins, carotenes, phytosterols, polyphenols, phospholipids, probiotics and prebiotics, among others. Some of these compounds are lipophilic; hence have poor water solubility, so that their absorption and bioavailability is limited [6].

Different approaches and formulations have been developed to improve the absorption of lipophilic compounds, in which the use Nanotechnology for the development of carrier systems, such as nanoparticles, nanospheres, nanocapsules, solid lipid nanoparticles (SLN), self-emulsifying drug delivery systems (SEDDS) and nanoemulsions are becoming relevant [7]. The latter nanoscale system, nanoemulsions, has been employed as a delivery system, with improved solubility, stability, and enhancement of their bioavailability and pharmacological activities [8,9], Additionally, it has been reported a correlation between globule sizes below 0.2 μm with improved absorption of the compound of interest and the desired pharmacological activity [10]. Nanoemulsions are colloidal

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dispersions with globule sizes in the range of 20 to 200 nm, in which oil droplets that contain the bioactive compounds are dispersed in a hydrophilic phase [11]. Different processes have been used for nanoemulsion formation, although, the most widely used for the preparation of oil-in-water (O/W) nanoemulsions comprise high-energy emulsification methods such as high-pressure homogenization, microfluidization, and ultrasonication, all of which can reduce the globule size to nano-scale, obtaining traslucent nanoemulsions [12–14].

In ultrasonic devices the shear is provided predominantly by cavitation, defined as the process of bubble formation, growth and implosive collapse of the bubbles in a liquid medium [15]. Compared with the traditional mechanical methods, ultrasound is a superior tool to obtain nanoemulsions with smaller and homogeneous globule size and physical stability; the energy consumption was considerably lower than other classical devices. Ultrasound is more versatile in terms of operation and cleaning and is considered a cost-effective method to generate the pharmaceutical grade nanoemulsions [16]. Many studies have reported the formation of pharmaceutical nanoemulsions developed by this technique at laboratory scale [17–22], however, studies on a large scale have also been performed by continuous flow emulsification equipment such as the liquid whistle hydrodynamic cavitation reactor (LWHCR) with the preparation of highly stable simple as well as multiple emulsions in an industrial size production [23,24].

Nanoemulsions have served as delivery systems in a variety of formulations such as foams, creams, liquids and sprays for many industrial products, and the pharmaceutical field is perhaps the area of greatest applications that illustrate their use [25]. In the pharmaceutical field the main usage is in the treatment of cancer; nanoemulsions have been developed for many applications such as tissue targeting, imaging analysis, and cancer therapy. Nanoemulsions have improved stability, solubility and exposure of lipophilic anticancer compounds; have reduced the required dosage and the side effects. Nanoemulsions have achieved a greater inhibition in cancer cell proliferation and increased cell apoptosis [26–28].

A natural compound with reported anticancer activity is betulinic acid (BA); it is a pentacyclic triterpene (Fig. 1) widely distributed in the plant kingdom. BA showed to suppress the growth and induce apoptosis in various tumor cell lines, among which are melanomas, brain, colon, breast, neuroblastoma, ovarian carcinoma, lung, cervical and leukemias. There are also reports in which BA concentrations of 1 to 4 μg/mL have acted as a selective inhibitor of *in vitro* as well as in live cancer cell proliferation [29–32]. Betulinic acid was used at concentrations up to 500 mg/kg body weight, showing low toxicity [33]. BA is a liphophilic compound with a water solubility of 0.02 μg/mL, so its incorporation in O/W nanoemulsions would increase its solubilization [34].

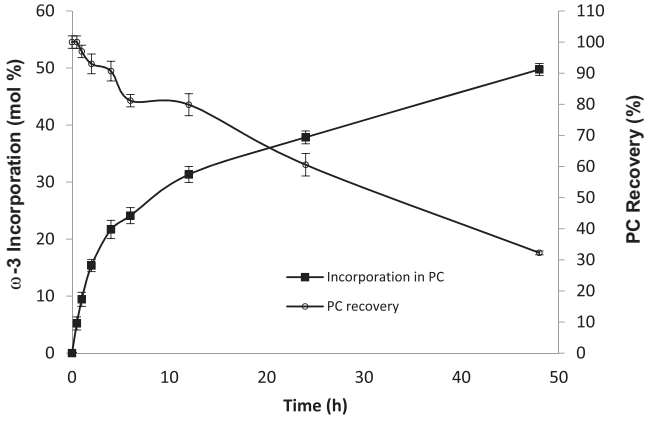

Fig. 1. Incorporation of ω-3 fatty acids in PC and PC recovery during acidolysis reaction. Reaction conditions: 50 °C; 10% enzyme, molar ratio PC:fatty acids 1:8. Results for a mixture of phospholipid products.

The use of ultrasonic equipment for preparing nanoemulsions has allowed to obtain nanoemulsions with globules sizes of 40 nm, translucent appearance and can be used in pharmaceutical and food products; therefore, the purpose of this study was to prepare betulinic acid nanoemulsions stabilized by modified phosphatidylcholine using ultrasound emulsification that serve as delivery systems for betulinic acid. The first part of the study was the modification with ω-3 fatty acids and purification of PC. It is known that the fatty acids in PC have an effect on the emulsifying capacity; however, the formulation of an emulsion may also influence its characteristics [35]. Hence, the second part of this work was dedicated to evaluate the emulsifying properties of modified PC on the characteristics and stability of betulinic acid nanoemulsions varying the formulation and preparation procedure. It is expected that results of this study may be useful for the possible application of this release system in either food or pharmaceutical matrices.

2. Materials and methods

2.1. Materials

Soybean lecithin (95% phosphatidylcholine, PC) was purchased from Avanti Polar Lipids (Alabaster, AL). The ω-3 fatty acids were obtained from a fish oil concentrate kindly provided by Ocean Nutrition (Nova Scotia, Canada). Immobilized lipases from *Thermomyces lanuginosus* (TL IM), *Rhizomucor miehei* (RM IM) and *Candida antarctica* (Novozyme 435) were a gift from Novo Nordisk (Franklinton, NC), and phospholipase A1 (PLA_1) (Lecitase Ultra) was kindly provided by Novozymes (Salem, VA). Duolite A568 was a gift from Rohm and Haas (Barcelona, Spain) and Macroporous polypropylene (EP 100) was from Akzo (Obernburg, DE). Betulinic acid (purity $\geqslant$ 95%) was purchased from Indofine Chemicals. Medium chain oil of pharmaceutical grade was obtained from Now Foods (Bloomindale, IL) and olive oil was obtained from the local market. Both oils mixed with betulinic acid were used as dispersed phase. For all the experiments, Milli-Q water was employed. All reagents used were analytical grade or better and solvents were HPLC grade from Baker (Mexico city).

2.2. Phospholipid modification

Preparation of free, ω-3-rich fish oil fatty acids by saponification, immobilization of PLA_1 and phosphatidylcholine enrichment with ω-3 fatty acids were performed according to Garcia et al. [36], with slight modifications.

2.2.1. Preparation of ω-3-rich free fatty acids by fish oil saponification

Fish oil was saponified to obtain the ω-3 free fatty acid concentrate. Fish oil (100 g) was mixed with 300 mL ethanol and 100 mL of 1 M NaOH in methanol; the mixture was heated under reflux for 30 min, then cooled and transferred to a separatory funnel. 200 mL of water and 400 mL of hexane were added to the mixture, stirred and the phases allowed to separate. The lower layer was transferred to another funnel and the pH was adjusted to 1-2 using concentrated HCl. The lower layer was discarded and the free fatty acids obtained were washed twice using 400 mL hexane and 200 mL of water. The mixture was shaken manually, allowed to separate and the lower phase was discarded. The upper layer containing the ω-3 free fatty acids was filtered through a bed of Na_2SO_4 and cleaned by passing through a glass column packed with silica gel (60–200 mesh). Hexane was evaporated under Nitrogen and the concentrated ω-3 free fatty acids were stored in a freezer under Nitrogen. Gas chromatography (GC) analysis of FAME was made to estimate the molecular weight of the concentrate and thus

estimate the mol ratio of PC to ω-3 fatty acids for the acidolysis reactions.

2.2.2. Phospolipase A1 immobilization

The PLA_1 is distributed in a liquid form containing the enzyme. The PLA_1 (5 g) was mixed with a volume of Tris–HCl adjusted to pH 7 or 8. The immobilization carrier (1 g Duolite or EP 100) and enzyme solution were stirred in an orbital shaker at 300 rpm and 50 °C for 12 h, according to Garcia et al. [36]. After the incubation time the mixture was filtered to recover the remaining soluble enzyme. The support was washed with 15 mL of buffer and dried at 37 °C in a vacuum oven. Immobilized PLA_1 was stored at 5 °C prior to use. The quantification of the adsorbed enzyme on the support was calculated starting from the difference of protein content remaining in the supernatant after incubation compared to the initial concentration in the initial solution. Protein concentration was determined by the Bradford method [37], and bovine serum albumin was used as standard.

2.2.3. Acidolysis reaction for phospholipid modification

The acidolysis reactions between soybean phosphatidylcholine and the free fatty acids were carried out using an orbital shaker set at 300 rpm and 50 °C, for 48 h. Different commercial immobilized lipase preparations (TL IM, RM IM or N 435) and the immobilized PLA_1 were tested at a rate of 10% of the total substrates weight. The reaction mixture consisted of phosphathidilcholine and free fatty acids in a 1:8 mole ratio. The reaction mixtures were placed into glass vials and incubated under nitrogen atmosphere. Analysis of the incorporated fatty acids was made throughout the reaction by selective methylation and GC.

2.2.4. Gas chromatographic fatty acid analysis

The fatty acids contained in the fish oil concentrates after saponification, in PC and the modified PC were analyzed on a HP6890 gas chromatograph (GC) equipped with a HP-INNOWax capillary column (60 m x 0.25 mm x 0.25 mm film thickness) and a flame ionization detector (FID). Methylation of esterified fatty acids was carried out by placing 100 μL of the sample and 1 mL of sodium methoxide (Supelco, Bellefonte, PA) in test tubes and allowed to rest for 5 min at room temperature. For methylation of total fatty acids, 100 μL of sample were mixed with 1 mL of 1 N methanolic HCl (Supelco) and heated for 2 h at 60 °C in a dry block. Both methylation reactions were stopped by addition of 100 μL water, and the methyl esters were extracted with 2 mL hexane. The organic phase was centrifuged; separated and anhydrous sodium sulfate was added to remove water. Subsequently, 1 μL of the sample was injected into the GC. For the separation of the methyl esters, the temperature program started at 190 °C, increased by 4 °C/min to 210 °C, then increased by 2 °C/min to 230 °C and it was subsequently maintained for 16 min. The temperature of the injection port and the FID detector were 240 and 250 °C, respectively. UHP nitrogen was the carrier gas at a constant flow rate of 1 mL/min.

2.2.5. Phosphatidylcholine analysis and recovery after modification

PC was analyzed according to Hossen and Hernandez (2005) using a Waters HPLC System equipped with a UV-Visible detector (Waters 2487). A normal phase 5 μm, 250 × 4.6 mm silica column (Econosil Sílica, Alltech Associates, Inc.) was employed. The mobile phase consisted of acetonitrile/methanol/phosphoric acid (130:5:1.5 v/v/v) running isocratically at a flow rate of 1 mL/min and the detector was set at 205 nm.

The PC recovery after modification was calculated as follows:

$$\text{PC recovery } (\%) = [\text{recovered PC(g)/used PC (g) on the acidolysis reaction}] \times 100$$

2.2.6. Separation of modified PC

The modified PC was isolated from the free fatty acids (FFA), lysophosphatidylcholine (LPC) and glycerophosphatidylcholine (GPC), present in the reaction mixture after the acidolysis reaction. Column chromatography was used to purify the modified PC from the reaction mixture after acidolysis, using the method reported by Vikbjerg et al. [38] with some modifications. The column (l = 46 cm, i.d. = 57 mm) was packed with silica and the solvents used were chloroform and methanol. FFA's were eluted with chloroform, then PC was eluted with chloroform/methanol (65:35 v/v) and then LPC and GPC were eluted with methanol. The fatty acids in the modified PC were analyzed by CG and the peroxide value (PV) was quantified according to the AOCS method Ja 8-87 [38] to monitor PC oxidation.

2.3. Betulinic acid nanoemulsion formation stabilized by modified phosphatidylcholine

BA nanoemulsion were prepared using ultrasonication. Oil in water nanoemulsions were formulated using BA dispersed in oil as the dispersed phase (Medium chain oil or olive oil); the continuous phase consisted of water with 25% glicerol and PC was used as emulsifier. BA concentration was fixed for all the formulations to 50 mg/g nanoemulsion and oil was fixed at 5%. Betulinic acid and oils were pre-mixed in a sonication bath for 1 min. First coarse emulsions were prepared by high speed stirring using an UltraTurrax T25 (IKA Works, Inc., Wilmington, NC) at 20,000 rpm for 3 min; then the coarse emulsion was subjected to ultrasonic emulsification using a 20 kHz S-450D Branson sonicator (Emerson Electric Co., St. Louis, MO). The probe diameter used was 13 mm and the sonication process was carried out under different emulsification conditions described in the experimental design below.

A set of nanoemulsion formulations was conducted to evaluate the effect of three variables using a 2^3 factorial design. The variables studied were: type of oil, amplitude during ultrasonication and the use of modified PC in the nanoemulsions. Formulations are depicted in Table 1. Treatments were characterized and the storage stability at 5 °C was examined. Ostwald ripening was evaluated in nanoemulsions plotting the cube of the mean droplet radius.

2.4. Nanoemulsion characterization

Characterization of the nanoemulsions was described by the mean globule size diameter and globule size distribution (reported as polydispersity index, or PDI), together with the globule electrical charge (ζ-potential). These properties were all determined using a Zetasizer Nano S90 (Malvern Instruments Inc., Worcestershire, UK). All the measurements were made in triplicate at 25 °C.

2.5. Storage stability study

The effect of storage time on the characteristics of the nanoemulsions was analyzed. Samples were maintained in incubation

Table 1
Treatments of factorial design for the preparation of nanoemulsions with betulinic acid using modified PC.

Treatment	Oil	Amplitude	PC
1	Olive oil	30	Unmodified PC
2	Olive oil	30	Modified PC
3	Olive oil	40	Unmodified PC
4	Olive oil	40	Modified PC
5	Medium chain oil	30	Unmodified PC
6	Medium chain oil	30	Modified PC
7	Medium chain oil	40	Unmodified PC
8	Medium chain oil	40	Modified PC

chambers at 5 °C for 12 weeks. Nanoemulsion stability was determined by measuring the change in the globule size, distribution and zeta potential. Nanoemulsions considered as stable did not show phase separation and had globule sizes within the range stated to be considered nanoemulsions (<200 nm).

2.6. Lipid oxidation in nanoemulsions

To monitor lipid oxidation in nanoemulsions, the secondary oxidation products were measured by the Thiobarbituric acid reactive substances (TBARS) method as described by Alamed et al. [39]. The solution of TBARS was prepared with trichloroacetic acid (15% w/v), thiobarbituric acid (0.375% w/v) dissolved in HCl (0.25 M); and then 3 mL of butylated hydroxytoluene (2%) in ethanol was mixed with the first solution. The TBA reagent in an amber vial was mixed in a vortex with the nanoemulsion and water. The mixture was placed in a boiling water bath for 15 min. The mixture was cooled to room temperature for 10 min and then centrifuged at 3500 rpm for 15 min. Absorbance was measured in a diode-array spectrophotometer (Agilent 8453) at 532 nm. Concentration of TBARS in nanoemulsions was determined using a 1,1,3,3-tetraethoxypropane standard curve.

2.7. Influence of pH on storage stability

Nanoemulsions prepared using both unmodified and modified PC as emulsifier and medium chain oil were prepared and adjusted to different pH levels (pH 2–12) using 1 M HCl or NaOH. A pH meter (Thermo Orion 5 star, Thermo Fisher Scientific Inc.) was used to measure pH values and the nanoemulsions were stored in glass vials at 5 °C. During the storage time (12 weeks) globule size and zeta potential were determined.

2.8. Statistical analysis

Analysis of variance of the experimental designs and comparison of means by Tukey's test ($p < 0.05$) were carried out using the Minitab v.16 statistical software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. ω-3 fatty acid obtention by fish oil saponification

Fatty acids profile from the free fatty acid concentrate used as ω-3 donor is shown in Table 2. The content of ω-3 fatty acids (mol%) reported in this study was composed by 74% of EPA, DPA and DHA. These results were used to calculate the molecular weight of the concentrate and to prepare the mixture at the specified mole ratio PC:ω-3 for the acidolysis reactions.

3.2. Immobilization of phospolipase A1

Phospholipase A_1 is currently available only in the free form as a solution. Hence, its immobilization onto an inert support provides several advantages in its use as a catalyst. Several studies focused on the immobilization of phospholipases A_1 and A_2 have been performed using immobilization by adsorption due to its simplicity, but also because it brings about only minor changes in the structure of the enzymes [36,40,41]. The supports we employed are different in nature, and diverge in the way in which the enzyme is adsorbed. Duolite A568 is an anion exchange resin that exposes charges capable of adsorbing enzymes. The EP 100 is composed of macroporous polypropylene so that the enzyme is mainly trapped in the pores of the support.

Table 2
Fatty acid composition (mol%) in ω-3 fatty acid concentrate, medium chain oil and olive oil.

Fatty acid	ω-3 fatty acid concentrate	Medium chain oil	Olive oil
C8:0		55.81 ± 0.13-	–
C10:0		44.19 ± 0.13	–
C 14:0	0.26 ± 0.00	–	1.10 ± 0.07
C16:0	0.69 ± 0.00	–	13.06 ± 1.04
C16:1	0.63 ± 0.00	–	1.03 ± 0.07
C18:0	2.25 ± 0.04	–	2.68 ± 0.19
C18:1	5.56 ± 0.03	–	75.76 ± 2.49
C 18:2	0.48 ± 0.02	–	5.60 ± 0.02
C 18:3	0.42 ± 0.10	–	0.77 ± 0.03
C 18:4	0.58 ± 0.00	–	–
C 20:0	0.37 ± 0.01	–	–
C 20:1	3.47 ± 0.01	–	–
C 20:2	0.37 ± 0.16	–	–
C 20:4	4.11 ± 0.19	–	–
C 22:1	6.59 ± 0.19	–	–
EPA	39.97 ± 0.09	–	–
DPA	4.56 ± 0.07	–	–
DHA	29.77 ± 0.44	–	–

The enzyme solution used at the beginning of the immobilization contained 75.8 mg protein/g support. After the equilibrium between enzyme and support was reached it was estimated that the EP 100 adsorbed 85.6%, while Duolite adsorbed 95% of the available protein (represented by PLA_1) (72.2 mg protein/g of support). The factor most likely responsible for the percentage of adsorption in Duolite may be the ionic interactions between the carboxyl groups of the amino acids from the surface of the PLA_1 and the positive charges of the surface in the support [42]. The concentration of immobilized enzyme reported in previous studies by Vikbjerg et al. [41] and Garcia et al. [36] were near 70%. In these studies the percentage of adsorption by varying the pH of the enzymatic solution was evaluated using buffers with different pH values. The authors found the maximum adsorption at pH values of 7 and 9, similar to those at which the enzyme exhibits its highest hydrolytic activity [41]. However, the authors did not take into consideration the pH variation of the medium when the concentrate containing the enzyme (pH 4–5) was added. In our study we observed the same effect and because of this, the pH of the enzyme solution was adjusted with buffer to reach pH values of 7 and 8. Table 3 shows the protein adsorption at pH values of 7 and 8, and the highest adsorption was at pH 8 (ca. 98%). Duolite has been widely used in the immobilization of commercial lipases such as Lipozyme RM IM and other immobilization studies authors observed good results [40,41].

3.3. Phospholipid modification by acidolysis

Lipases can effect the hydrolysis in the PC as PLA_1 does. In this work, in addition to the immobilized PLA_1 on Duolite and EP 100, lipases TL IM, RM IM and N-435 were also used for the incorporation of ω-3 fatty acids into PC. Lipases can perform the incorporation of ω-3 fatty acid residues into the PC; however, this was achieved in a much smaller extent compared with the PLA1

Table 3
Enzyme adsorption in different carriers and pH values.

Carrier	Enzyme adsorbed (mg protein/g support)
EP 100 (pH 8)	64.82 ± 0.06[a]
Duolite (pH 7)	72.15 ± 0.03[b]
Duolite (pH 8)	73.91 ± 0.01[c]

Different letters in the same column indicate statistically significant differences ($p < 0.05$).

immobilized in the two supports, as it can be noted in Table 4. In the reactions catalyzed by lipases, differences in incorporation of ω-3 fatty acids for each lipase were observed. These results may be caused by the stereospecificity of each lipase, TL IM and RM IM are *sn-1,3* specific, so the catalytic site in both enzymes will act only on the primary positions of the PC, while for lipase N-425 which had the lowest incorporation, it was probably attributed to its random specificity, without a particular preference for *sn-1* and *sn-2* positions in the PC. The use of PLA_1 allowed greater incorporation of ω-3 fatty acids into PC, and the differences observed between the immobilization supports could be related to the amount of adsorbed enzyme; the greatest incorporation (ca. 50%) was obtained with Duolite which adsorbed the greatest amount of PLA_1. The results obtained with PLA_1 in Duolite in this work were higher than those obtained by Hosokawa et al. [43] and García et al. [36], who at the same reaction time (48 h) incorporated 38 and 33 mol%, using phospholipase A_2 and A_1, respectively. Fig. 1 depicts the acidolysis reaction between the PC and the ω-3 fatty acids using the immobilized PLA_1 in Duolite. During the first 4 h of reaction there was a fast incorporation of ω-3 fatty acids (22 mol%); the rate of incorporation of these fatty acids was higher likely because of a higher concentration of substrate available to react with PLA_1; as reaction time proceeded, the active site of the enzyme may have become saturated, so that in the last 24 h, the incorporation rate was slow, so that at the end of the acidolysis reaction (48 h) almost 50% of ω-3 fatty acids were esterified in PC. During the acidolysis reactions, production of intermediates are involved due to hydrolysis of PC and further migration of the acyl group, causing the formation of 1- and 2-Lysophosphatidylcholine (1-LPC and 2-LPC), and glycerophosphatidylcholine (GPC); therefore, a net reduction in PC content occured [43–45]. The results of PC analysis during acidolysis catalyzed by PLA_1 immobilized on Duolite are shown in Fig. 1. The data show tendencies similar to those reported by other researchers [43–45]. As acidolysis reactions proceed and fatty acids were incorporated, an increase in the hydrolysis products from PC occurred, which had an impact on the PC recovery. After 48 h of acidolysis, very low yields were attained, and only 32% of the PC was measured. After 24 h of reaction, an incorporation of nearly 40 mol% of ω-3 fatty acids in the PC was reached, and at this time the PC yield was above 60%. This yield was greater than yields obtained by other researchers employing PLA_1, Kim et al. [44] after 6 h of acidolysis reaction incorporated 28 mol% of ω-3 fatty acids into PC with yields of less than 20%; Garcia et al. [36] reported the incorporation of 35% of these fatty acids but the PC content was 34%. By carrying out the acidolysis reaction for less than 24 h, higher yields of PC could be obtained; however, the incorporation of ω-3 fatty acids was lower than those obtained by other authors.

PC was separated from LPC and free fatty acids by column chromatography and the ω-3 fatty acids incorporated in the modified PC fraction after 24 h of acidolysis were quantified. The results are shown in Table 5. The modified PC fraction was composed of 35 mol% of ω-3 fatty acids and the fatty acids that were primarily exchanged during interesterification were linoleic acid and palmitic acid.

The modified PC fraction obtained was the emulsifier during the formulation of nanoemulsions and involved several steps that may expose the PC to oxidation reactions. This process may cause oxidation of unsaturated fatty acids in the phospholipid. Because of this possibility, the peroxide value was determined. Quantification of peroxide values in PC is an important concern in the chemical stability of a nanoemulsion, given that lipid oxidation is a chain reaction, a PC with a high peroxide values can lead to oxidation of fatty acids present in the oil of the dispersed phase in nanoemulsions, and probably the bioactive compound. Peroxide values obtained for the unmodified and modified PC were 0.30 ± 0.14 and 0.59 ± 0.14 meq/kg sample, respectively. These data show that the oxidative stability of the modified PC is not too far from that of the unmodified PC, and thus the process of modification and fractionation of PC was not deleterious to the polyunsaturated fatty acids present.

3.4. Betulinic acid nanoemulsions preparation and stability

Ultrasound is a convenient method for producing nanoemulsions where the dispersed phase is composed of triacylglycerols that can be used in the food and pharmaceutical sector [46]. Nanoemulsions employing modified PC as emulsifier were prepared and storage stability was examined at 5 °C (Fig. 2). By employing the modified PC was observed an increased in globule size during storage, compared with the unmodified PC. After four weeks, globule size had increased 20% of the initial value and at 16 weeks the globule size had exceeded the 200 nm. Stability of the nanoemulsions formed can be confirmed by determining the rate of Ostwald ripening from the plot of the cube of globule radius of nanoemulsions (r^3) as a function time (t). Fig. 3 shows the variation of r^3 against time (weeks). It is observed that for unmodified PC there is a linear relationship; which according to the Liftshitz–Slezov–Wagner (LSW) theory for Ostwald ripening, a linear variation indicates that oil droplets grow through molecular diffusion and Ostwald ripening is the destabilization mechanism. Ostwald ripening affect globule sizes of less than 100–500 nm, additionally, the dispersed phase was composed of medium-chain oil can diffuse into the continuous phase favoring this mechanism [47]. An alternative to minimize this mechanism is the use of less soluble oils composed of long chain fatty acids as olive oil. For this reason, an experimental design was devised to evaluate the effect of the type of oil on the characteristics and stability of the nanoemulsions. Regarding the plot for nanoemulsions stabilized with modified PC, there is no linear relationship, Urbina-Villalba et al. [48] reported a similar behavior where state that when the plot is divided into intervals a linear relationship is observed in the initial interval, the increase in globule radius is not due to molecular diffusion, this is a result of the combined process of flocculation and

Table 4
ω-3 incorporation (mol%) in PC using different enzymes.

Enzyme	Incorporation of ω-3 (mol%)
N -435	4.235 ± 0.03[a]
RM IM	11.80 ± 0.34[b]
TL IM	22.776 ± 0.94[c]
EP 100	34.012 ± 0.10[d]
Duolite	49.749 ± 0.04[e]

Reaction conditions were 50 °C; 10% enzyme, molar ratio PC:fatty acids 1:8 during 48 h. Results for a mixture of phospholipid products. Different letters in the same column indicate statistically significant differences ($p < 0.05$).

Table 5
Fatty acid composition (mol%) in unmodified and modified PC.

Fatty acid	Unmodified PC	Modified PC
C16:0	14.50 ± 0.04	3.09 ± 0.06
C18:0	3.51 ± 0.02	1.66 ± 0.01
C18:1	12.00 ± 0.02	9.25 ± 0.10
C 18:2	63.83 ± 0.06	40.22 ± 0.61
C 18:3	6.16 ± 0.01	3.70 ± 0.02
Other	–	7.12 ± 0.78
EPA	–	19.07 ± 0.12
DHA	–	1.53 ± 0.01
DPA	–	14.36 ± 0.09
Total ω-3	–	34.96 ± 0.21[b]

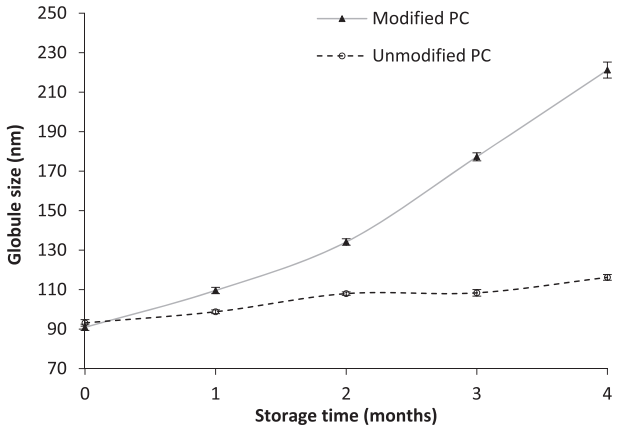

Fig. 2. Globule size comparison of betulinic acid nanoemulsions stabilized with modified and unmodified PC. The values presented are mean ± standard deviation (n = 6). Nanoemulsions prepared with 10% PC, 25% glycerol, 5% medium chain oil at 30% amplitude for 12 min. Storage at 5 °C.

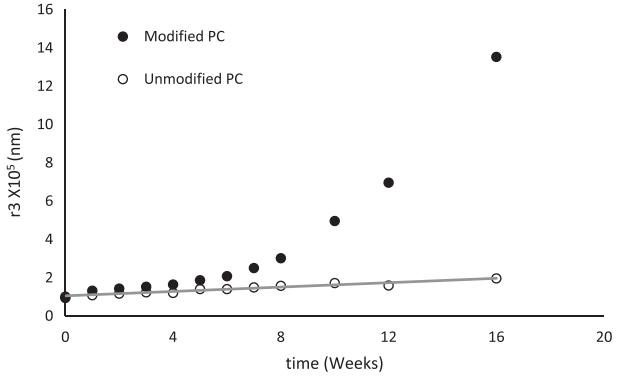

Fig. 3. Change of the cube of radious as a function of time exhibited by betulinic acid nanoemulsions. Nanoemulsions prepared with 10% PC, 25% glycerol, 5% medium chain oil at 30% amplitude for 12 min. Storage at 5 °C.

coalescence [48,49]. In the second interval is observed a linear variation, a greater increase in r^3 and a high slope, is then due to the contribution of flocculation and coalescence. Thus, it is possible to obtain a linear relation of r^3 versus time as a consequence of flocculation and coalescence, and not necessarily by Ostwald ripening [48–50]. Coalescence is determined by the ability of the emulsifier to form a rigid film covering the globule, then this mechanism in nanoemulsion stabilized by modified PC was probably caused by the long chain unsaturated fatty acids contained in the modified PC. It has been reported that the type of fatty acid influences the phospholipid packing geometry, then the presence of long chain unsaturated fatty acids in the phospholipids may provide high fluidity in the membrane globule causing diffusion of the dispersed phase [51].

During ultrasonic nanoemulsificacion factors as the composition of the emulsion, equipment configurations and the energy delivered by the equipment has effect on the final globule size. In this study using experimental design, the effect of the oil (O), amplitude in the ultrasound device (A) and PC used as emulsifier (PC) on the globule size and distribution of nanoemulsions were evaluated (data not shown). An analysis of the estimated values of p (α error = 0.05) indicated that all the variables and their interactions had significant effects on globule size and distribution. The experimental results of the factorial design are contained in Fig. 4, where it can be seen that nanoemulsions with unmodified PC were within the nanometer range and there were no significant differences in the treatments at the same amplitude where olive oil or medium chain were used.

The intense shear force applied during the formation of an emulsion depends on the energy input, which is the product of power delivered and the residence time [46]. The variation in amplitude from 30% to 40% represented an increase in the energy delivered to the emulsion. It was expected that the increase in the applied energy would result in a decrease of globule size, however 40% amplitude supplied more energy that caused intense turbulence promoting a higher rate of collision between globules, their coalescence and larger globule sizes, phenomenon reported as over-processing. This effect has been described by Desrumaux and co-workers [52], wherein a clear growth of globule size is caused by an increase in the emulsification energy (increase in shear intensity), and similar trends have been reported [46,53,54]. The globule size in treatments where modified PC and olive oil as dispersed phase were used exceeded 200 nm. The unsaturated long chain fatty acids in PC and olive oil have a broad molecular distribution, so the packing of the molecules were not as compact as with saturated fatty acids, causing the formation of larger globules. In addition, during the process of globule formation in nanoemulsions involves the rupture of large globules into smaller, which is governed among other factors by the relative viscosities of the dispersed (η_D) and continuous (η_C) phases [55]. Braginsky et al. [56] reported an optimal range of η_D/η_C between 0.1 and 5, where droplet disruption is most efficient. The viscosity of long chain oils such as olive oil is higher than the medium chain oils, so the olive oils nanoemulsions η_D/η_C is 90, which is located outside the Braginsky's range, then droplets are more resistant to disruption and globule size in olive oil nanoemulsions were greater than those prepared with medium chain nanoemulsions. Similar results were shown by Wooster et al. [55] where nanoemulsions using long chain triacylglycerols with η_D/η_C of 56 had globule sizes larger than hexadecane nanoemulsion with η_D/η_C of 3.4.

The stability study of this design is depicted in Fig. 4. Betulinic acid nanoemulsions where the dispersed phase consisted of olive oil, showed greater stability, the use of oils with long-chain fatty acids in the dispersed phase allows for less diffusion into the continuous phase and nanoemulsion instability is reduced; so in nanoemulsions with olive oil the greater increase of globule size obtained was 20% of the initial size, compared to nanoemulsions prepared with medium chain oil, where a 95% increase of the initial

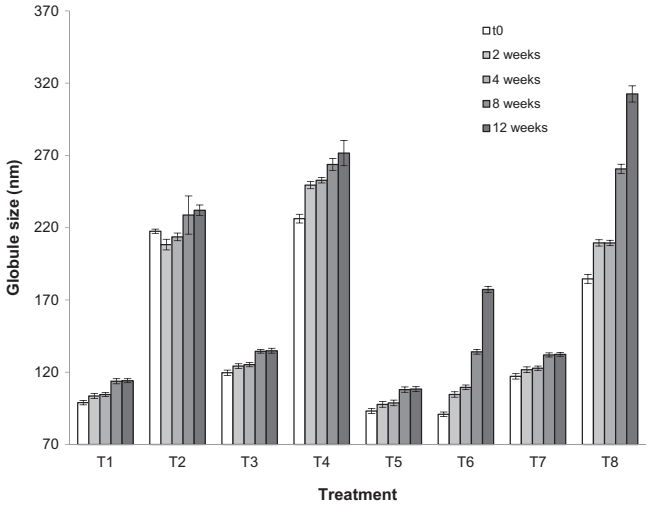

Fig. 4. Globule size of nanoemulsions at the different conditions of the experimental design during storage.

size was reached. Similarly, Wooster et al, [55] used long chain triglyceride oil as dispersed phase, which is insoluble in water, thus preventing the Ostwald ripening and increasing the physical stability.

The amplitude effect was influenced by the type of PC and oil used. In the treatments with olive oil stabilized with modified PC at 40% amplitude the increase in emulsification energy caused a further growth of globule size during storage, but when unmodified PC was used at this amplitude the stability during storage was better, probably caused by a more suitable geometry packing of the fatty acids in the PC, which in the case of the modified PC composed by long-chain unsaturated fatty acids is more flexible and fluid. When a greater force of disruption was applied, instability was caused in the binding forces, which maintained the globule covered by the emulsifier membrane. Hence, in the case of the nanoemulsions prepared with modified PC and olive oil, with the application of less ultrasonic energy (30% amplitude) the stability was better. In betulinic acid nanoemulsions with medium chain oil as dispersed phase stabilized using PC (both modified and unmodified) the use of 40% amplitude reduced the globule size growth during storage. Saturated fatty acids have been reported to have a cylindrical shape and together with the fatty acids in PC form a globule with a rigid membrane, which is more estable to drainage during storage [57].

Betulinic acid nanoemulsions prepared with olive oil and stabilized by modified PC using 30% amplitude showed the smallest growth in globule size (ca. 7%); the presence of long chain fatty acids in the emulsifier and the dispersed phase made the globule insoluble into the continuous phase, and avoided draining during storage. However, the initial globule size in these nanoemulsions was greater than 200 nm. Betulinic acid nanoemulsions containing medium chain oil and stabilized with modified PC were within the globule size considered for this study as nanoemulsions and displayed a translucent visual appearance, which is characteristic of nanoemulsions. Further studies of the effects of the type of oil on the betulinic acid digestive absorption should be pursued. These studies could include the usage of long chain oils [58] or the possible effect of globule size on bioavailability [59].

3.5. TBARS in betulinic acid nanoemulsions

It is very important to monitor the oxidative stability of a nanoemulsion for its use in foods or drugs, especially when ω-3 polyunsaturated fatty acid (PUFA) susceptible to oxidation are used. Lipid oxidation occurs mediated by reactions that proceed on the surface of the globules. Given this, characteristics of a globule O/W emulsion such as globule size (nanoemulsions vs emulsions), ζ-potential, dispersed phase used (saturated or polyunsaturated fatty acids) and the properties of the interfacial membrane have a direct impact on the oxidative stability of the emulsions [39,60]. The ability of phospholipids for scavenging free radicals acting as metal chelator, and retarding lipid oxidation has been reported [61]. When phospholipids are used to stabilize oil globules in a nanoemulsion, this behavior could be affected by the polyunsaturated fatty acids on the PC. Fig. 5 shows the results for the TBARS formation in betulinic acid nanoemulsions. An increased content of TBARS in nanoemulsions with PC modified at time zero was observed; the presence of oxidation products is possibly attributed to the nanoemulsion preparation process, provided that a high energy method was applied, causing an increase in temperature and a possible exposure to metals that lead to oxidation reactions of polyunsaturated fatty acids in modified PC. For the original PC, values of 0.0029 mM at zero time were obtained, compared with 0.024 mM for modified PC. Despite the higher TBARS content in modified PC, this value increased slowly until the 12th week (0.055 mM), without differences in the values

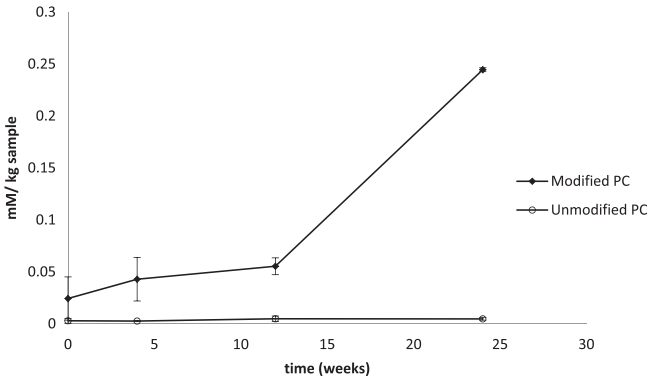

Fig. 5. TBARS formation during storage time of betulinic acid nanoemulsions stabilized with modified and unmodified PC. Nanoemulsions prepared with medium chain oil as dispersed phase at 30% amplitude for 12 min.

obtained from the first month until this time. TBARS values obtained at week 24 in betulinic acid nanoemulsions with modified PC, was probably caused by globules coalescence. During this process, rupture of the emulsifier membrane that covers the globule occurs and consequently exposure and diffusion of fatty acids through the aqueous phase allows them to be exposed to components that may lead to their oxidation. Lower TBARS values for unmodified PC may be caused by its metal chelating ability and its content of polyunsaturated fatty acids, which is lower than the modified PC. In addition to the difference in oxidation stability due to the content of polyunsaturated fatty acids, the surface charge of the globules in the nanoemulsions can also be responsible. The modified PC nanoemulsions are more negative, and may attract more positively charged metals [62,63], so the ζ-potential can also influence the oxidative stability.

Initial TBARS values obtained in this work were smaller than those reported by Wang and Wang [64], who prepared emulsions using PC from egg and soy, and obtained values of 0.2 mM/kg oil after 6 days. Similar values were found in this study after 6 months; these differences were most likely caused by the storage temperature of 60 °C as was reported by Wang and Wang [68] and 5 °C employed in this work. Lee et al. [65], found initial values of 0.24 mM/kg oil to 1 mM/kg oil after 20 days; these values should be attributed to the dispersed phase used that contained polyunsaturated fatty acids, the storage temperature (37 °C) and the emulsifier, which forms part of the globule membrane (protein) that has no antioxidant ability.

The effect of the amplitude during ultrasonication and the oil used as the dispersed phase on the oxidative stability of betulinic acid nanoemulsions with modified PC was evaluated. The results can be described in Fig. 6. After 12 weeks, no significant difference was observed in treatments using the same oil; when ultrasonication amplitude was increased, treatments in which olive oil was used as disperse phase showed significant higher TBARS content than treatments with medium-chain oil at 30% amplitude, due to the presence of unsaturated fatty acids in olive oil which are susceptible to oxidation during storage.

It may be understood then, that it is necessary to control diverse factors involved with TBARS formation in nanoemulsion globules, such as preparation conditions, the oil selected as the dispersed phase (saturated or unsaturated fatty acids mainly present in oils), use of emulsifiers with antioxidant capacity, surface charge and storage at low temperatures. Control of these factors is an alternative to improve the oxidative stability of the nanoemulsions and facilitate their application in different food and pharmaceutical systems.

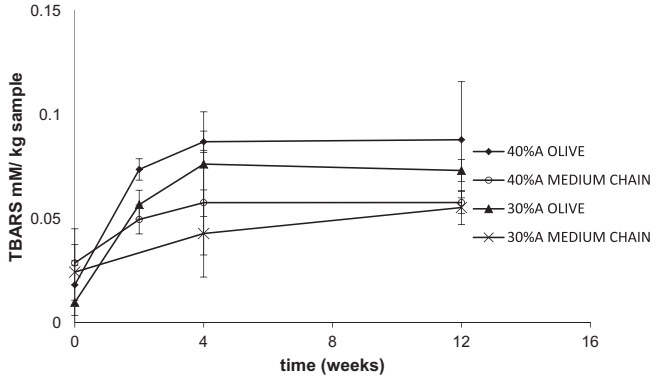

Fig. 6. TBARS content in betulinic acid nanoemulsions stabilized with modified PC varying the disperse phase and amplitude of ultrasonication. Nanoemulsions prepared with modified PC.

3.6. Influence of pH on betulinic acid nanoemulsion stability

The pH represents the amount of ions in the medium and exerts an important effect on the ζ-potential of the globule and other changes that influence the stability of the nanoemulsion. Some studies that have evaluated the effect of pH on emulsion stability under different pH conditions that resemble those found in food products, nutritional supplements and pharmaceuticals; these products may have pH values ranging from acidic beverages and dairy products to neutral and mildly alkaline pH levels as in infant formulae and tea [66]. It is then important to understand the behavior of the nanoemulsions at different conditions of pH and the implications of its characteristics and stability during storage.

Betulinic acid nanoemulsions with modified and original PC freshly made had a pH around 4. In Fig. 7 it can be observed that all nanoemulsions had a negative ζ-potential at pH 2, and as the medium approached alkalinity, the ζ-potential decreased concomitantly to reach values of −44 and −45 mV for nanoemulsions with modified and original PC, respectively. Reduction in ζ-potential have been reported as a preferential adsorption by the OH^- groups in ion layers around the globule, causing the surface charge of the globule to turn more negative as the ionic force increased [54,65,67]. Nanoemulsions prepared with modified PC with pH values from 6 to 10 had significantly lower surface charge than those prepared with unmodified PC. The behavior of ζ-potential during storage at different pH values in the nanoemulsions with modified PC is depicted in Fig. 8, where a significant reduction in the ζ-potential at pH 2 is noted. The ζ-potential of betulinic acid nanoemulsions at pH 8–12 remained constant, without significant differences until 12 weeks of storage. The surface charge of globules can be an important influence in stability; the electrical charge of globule causes repulsion with other globules, keeping them away from each other and they will not be able to form aggregates. It has been reported that ζ-potential values near ±30 mV confer stability to the nanoemulsion [68]. Then, based on the results obtained, to have the ζ-potential value that provides stability are required conditions around pH values of 7 and 10 in the nanoemulsions stabilized with modified PC and unmodified PC respectively.

The globule size in nanoemulsions provides additional evidence to explain the effect of pH on the ζ-potential, globule aggregation and stability of nanoemulsions. An increase in globule size was observed during the storage at pH 2 (Fig. 9), this could be explained by the fact that surface charge of the globule is not sufficient to cause repulsion with other globules, at this pH value the fraction of choline in PC is positively charged and most of the phosphate groups are neutral, bringing the globule to a net charge near zero. With the reduction in surface charge of the globule, the electrostatic repulsion that would keep globules away from each other is reduced, It is reported that the point at which the nanoemulsion exhibits no net charge is termed as the iso-electric point (IEP) and the zero net charge poses a detrimental effect on the interfacial stabilization forces promoting droplet aggregation and the increase in globule size [54]. Similar trends have been reported by other researchers, where bimodal distributions were observed in nanoemulsions with charge near zero, and it was considered that the increase in globule size was caused by flocculation [65]. Flocculation in this study was confirmed by adjusting the pH to 14 in betulinic acid nanoemulsions were at pH 2 after eight weeks of storage, this with the purpose of increase the number of OH^- groups surrounding the globule and to provide a higher negative surface charge that favors the interglobular repulsion. Nanoemulsions at pH 2 after storage changed the globule size from 96 to 250 nm and PDI value from 0.07 to 0.5; when pH was adjusted to 14, there was a reduction in the floc size, which was demonstrated by a reduction in globule size (from 250 nm to 146 nm) and the PDI value decreased from 0.5 to 0.33. During storage of nanoemulsions at pH values above 2 the globule size did not exceed 200 nm and nanoemulsión at pH values from 10 to 12, where the superficial charge of the globule in the nanoemulsions employing modified PC was greater than −30 mV, no significant increases in globule size were found and greater stability to the nanoemulsions was measured. According to the above data, an

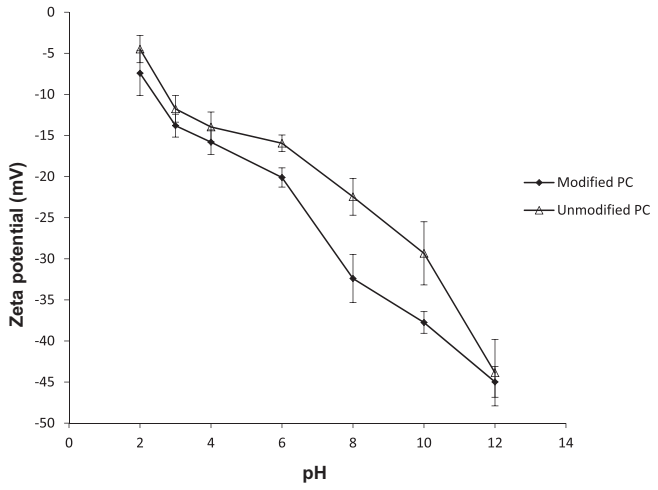

Fig. 7. Effect of pH on the ζ-potential of betulinic acid nanoemulsions. Nanoemulsions prepared with 10% PC, 25% glycerol, 5% medium chain oil at 30% amplitude for 12 min. Storage at 5 °C.

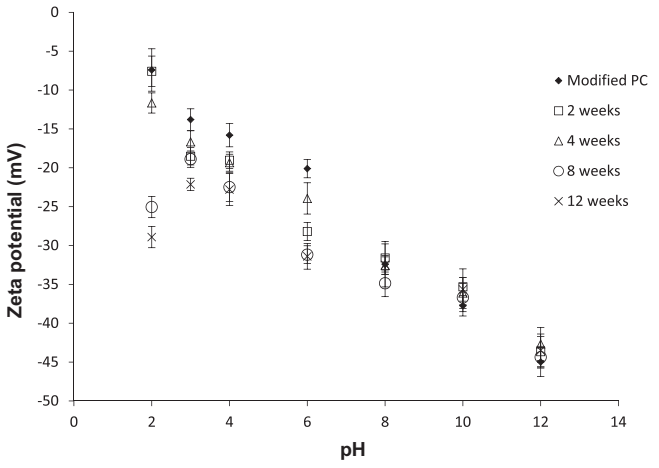

Fig. 8. ζ-Potential at different pH values.

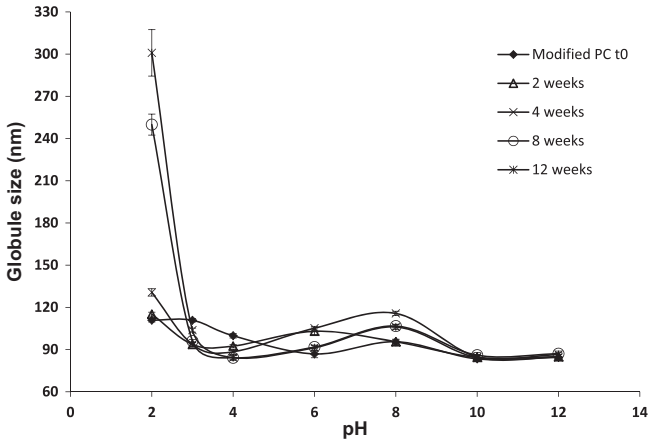

Fig. 9. Globule size during storage at different pH values of the nanoemulsions stabilized with modified PC.

alternative to enhance the stability of the betulinic acid nanoemulsions would be to adjust pH to values that provide charges far from zero.

4. Conclusions

This study provides information concerning the modification of PC, its use as a stabilizer in betulinic acid nanoemulsion and the variables that can influence their characteristics and stability. Incorporation of ω-3 fatty acids in the PC is dependent on the type of enzyme used and its specificity; ω-3 fatty acids were successfully esterified into PC employing PLA_1 immobilized on Duolite and the modification process and purification did not affect the PC's oxidative stability.

Regarding the preparation of nanoemulsions, most of the variables evaluated for the betulinic acid nanoemulsions exerted effects on their characteristics, while storage temperature had effects on nanoemulsions stability; high temperatures accelerated globule size growth, thereby causing phase separation. At 5 °C smaller globule size growth and greater stability was favored. Globule sizes in nanoemulsions stabilized with PC modified with ω-3 fatty acids fell within the nanometer scale; in these nanoemulsions the type of oil employed affected their globule size and storage stability. When olive oil was used as dispersed phase, globule size increased to values greater than 200 nm; however, the stability was favored during storage, while emulsions prepared with medium chain oil as dispersed phase had globule sizes of 91 nm and a traslucent appearance. The pH had a marked effect on the characteristics of the nanoemulsions; alkaline pH values improved storage stability. BA nanoemulsions applications in any food or pharmaceutical matrix may be affected by other factors and components in the system, so their characteristics and stability must be determined for each particular system.

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References

[1] F. Shahidi, Nutraceutical and Specialty Lipids and Their Co-products, Marcel Dekker Inc, New York, 2006. p. 563.
[2] F. Shahidi, H. Miraliakbari, Omega-3 (n-3) fatty acids in health and disease: 1, cardiovascular disease and cancer, J. Med. Food 7 (2004) 387–401.
[3] H. Tapiero, B.G. Nguyen, P. Couvreur, K.D. Tew, Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies, Biomed. Pharmacother. 56 (2002) 215–222.
[4] J. Delarue, C. LeFoll, C. Corporeay, D. Lucas, N-3 long chain polyunsaturated fatty acids: a nutritional tool to prevent insulin resistance associated to type-2 diabetes and obesity, Reprod. Nutr. Dev. 44 (2004) 289–299.
[5] S.C. Larsson, M. Kumlin, S.M. Ingleman, A. Wolk, Dietary long chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms, Am. J. Clin. Nutr. 79 (2004) 915–945.
[6] C.P. Tan, M. Nakajima, β-Carotene nanodispersions: preparation, characterization and stability evaluation, Food Chem. 92 (2005) 661–671.
[7] P.D. Marcato, N. Duran, New aspects of nanopharmaceutical delivery systems, J. Nanosci. Nanotechnol. 8 (2008) 2216–2229.
[8] H. Chen, J. Weiss, F. Shahidi, Nanotechnology in nutraceuticals and functional foods, Food Technol. 60 (2006) 30–36.
[9] N. Sozer, J.L. Kokini, Nanotechnology and its applications in the food sector, Trends Biotechnol. 27 (2009) 82–89.
[10] J.S. Schwarz, M.R. Weisspapir, D.I. Friedman, Enhanced transdermal delivery of diazepam by submicron emulsion (SME) creams, Pharm. Res. 12 (1995) 687–692.
[11] T. Tadros, P. Izquierdo, J. Esquena, C. Solans, Formation and stability of nanoemulsions, Adv. Colloid Interface Sci. 108–109 (2004) 303–318.
[12] S. Pinnamaneni, N.G. Das, S.K. Das, Comparison of oil-in water emulsions manufactured by microfluidization and homogenization, Pharmazie 58 (2003) 554–558.
[13] L. Lee, I.T. Norton, Comparing droplet breakup for a high-pressure valve homogeniser and a microfluidizer for the potential production of food-grade nanoemulsions, J. Food Eng. 114 (2013) 158–163.
[14] S.Y. Tang, P. Shridharan, M. Sivakumar, Impact of process parameters in the generation of novel aspirin nanoemulsions – comparative studies between ultrasound cavitation and microfluidizer, Ultrason. Sonochem. 20 (2013) 485–497.
[15] M. Sivakumar, S.Y. Tang, K.W. Tan, Cavitation technology – a greener processing technique for the generation of pharmaceutical nanoemulsions, Ultrason. Sonochem. 21 (2014) 2069–2083.
[16] B. Abismail, J.P. Canselier, A.M. Wilhelm, H. Delmas, C. Gourdon, Emulsification by ultrasound: droplet size distribution and stability, Ultrason. Sonochem. 6 (1999) 75–83.
[17] H.J. Doh, Y. Jung, P. Balakrishnan, H.J. Cho, D.D. Kim, A novel lipid nanoemulsion system for improved permeation of granisetron, Colloid Surf. B. Biointerfaces 101 (2013) 475–480.
[18] S. Kentish, T.J. Wooster, M. Ashokkumar, S. Balachandra, R. Mawson, L. Simons, The use of ultrasonics for nanoemulsion preparation, Innov. Food Sci. Emerg. Technol. 9 (2008) 170–175.
[19] S. Sugumar, V. Ghosh, M.J. Nirmala, A. Mukherjee, N. Chandrasekaran, Ultrasonic emulsification of eucalyptus oil nanoemulsion: antibacterial activity against Staphylococcus aureus and wound healing activity in Wistar rats, Ultrason. Sonochem. 21 (2014) 1044–1049.
[20] L. Wang, J. Dong, J. Chen, J. Eastoe, X. Li, Design and optimization of a new self nanoemulsifying drug delivery system, J. Colloid Interface Sci. 330 (2009) 443–448.
[21] P. Balakrishnan, B.-J. Lee, D.H. Oh, J.O. Kim, Y.-I. Lee, D.-D. Kim, J.-P. Jee, Y.-B. Lee, J.S. Woo, C.S. Yong, H.-G. Choi, Enhanced oral bioavailability of Coenzyme Q10 by self-emulsifying drug delivery systems, Int. J. Pharm. 374 (2009) 66–72.
[22] T.S.H. Leong, T.J. Wooster, S.E. Kentish, M. Ashokkumar, Minimising oil droplet size using ultrasonic emulsification, Ultrason. Sonochem. 16 (2009) 721–727.
[23] Shridharan Parthasarathy, Tang Siah Ying, Sivakumar Manickam, Generation and optimization of palm oil-based oil-in-water (O/W) submicron emulsions and encapsulation of curcumin using liquid whistle hydrodynamic cavitation reactor (LWHCR), Ind. Eng. Chem. Res. 52 (2013) 11829–11837.
[24] S.Y. Tang, M. Sivakumar, A novel and facile liquid whistle hydrodynamic cavitation reactor to produce submicron multiple emulsions, AIChE J. 59 (2013) 155–167.
[25] A. Maali, M.T. Hamed Mosavian, Preparation and application of nanoemulsions in the last decade (2000–2010), J. Disper. Sci. Technol. 34 (2013) 92–105.
[26] S. Ganta, M. Amiji, Coadministration of paclitaxel and curcumin in nanoemulsion formulations to overcome multidrug resistance in tumor cells, Mol. Pharm. 6 (2009) 928–939.
[27] J.B. Tagne, S. Kakumanu, R.J. Nicolosi, Nanoemulsion preparations of the anticancer drug dacarbazine significantly increase its efficacy in a xenograft mouse melanoma model, Mol. Pharm. 5 (2008) 1055–1063.
[28] A.V. Kabanov, E.V. Batrakova, V.Y. Alakhov, Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery, J. Control. Release 82 (2002) 189–212.
[29] E. Pisha, H. Chai, I.S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, C.W.W. Beecher, H.H.S. Fong, A.D. Kinghorn, D.M. Brown, M.C. Wani, M.E. Wall, T.J. Hieken, T.K. Gupta, J.M. Pezzuto, Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis, Nat. Med. 1 (1995) 1046–1051.
[30] S. Fulda, I. Jeremias, T. Pietsch, K.M. Debatin, Betulinic acid: a new chemotherapeutic agent in the treatment of neuroectodermal tumors, Klin. Padiatr. 211 (1999) 319–322.
[31] V. Zuco, R. Supino, S.C. Righetti, L. Cleris, E. Marchesi, P. Gambacorti, F. Formelli, Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells, Cancer Lett. 175 (2002) 17–25.

[32] S. Fulda, K.M. Debatin, Sensitization for anticancer drug–induced apoptosis by betulinic acid, Neoplasia 7 (2005) 162–170.
[33] G.O. Udeani, G.M. Zhao, Y.G. Shin, B.P. Cooke, J. Graham, C.W.W. Beecher, A.D. Kinghorn, J.M. Pezzuto, Pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice, Biopharm. Drug Dispos. 20 (1999) 379–383.
[34] S. Jäger, K. Winkler, U. Pfüller, A. Scheffler, Solubility studies of oleanolic acid and betulinic acid in aqueous solutions and plant extracts of *Viscum album* L, Planta Med. 73 (2007) 157–162.
[35] A.F. Vikbjerg, J.Y. Rusig, G. Jonsson, H. Mu, X. Xu, Comparative evaluation of the emulsifying properties of phosphatidylcholine after enzymatic acyl modification, J. Agric. Food Chem. 54 (2006) 3310–3316.
[36] H.S. García, I.W. Kim, A. López-Hernandez, G.C. Hill, Enrichment of lecithin with n-3 fatty acids by acidolysis using immobilized phospholipase A1, Grasas Aceites 59 (2008) 368–374.
[37] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) (1976) 248–254.
[38] AOCS, Official and Tentative Methods of the American Oil Chemists' Society, 3rd ed., AOCS, Champaign, IL, USA, 1993 (vol. 1).
[39] J. Alamed, D.J. McClements, E.A. Decker, Influence of heat processing and calcium ions on the ability of EDTA to inhibit lipid oxidation in oil-in-water emulsions containing omega-3 fatty acids, Food Chem. 95 (2006) 585–590.
[40] A.F. Vikbjerg, H. Mu, X. Xu, Synthesis of structured phospholipids by immobilized phospholipase A2 catalyzed acidolysis, J. Biotechnol. 128 (2007) 545–554.
[41] B. Maroto, C. Camusso, Inmovilización de enzima fosfolipasa A2, Grasas Aceites 51 (2000) 150–156.
[42] I.E. Fuentes, C.A. Viseras, D. Ubiali, M. Terreni, A.R. Alcantara, Different phyllosilicates as supports for lipase immobilization, J. Mol. Catal. B Enzym. 11 (2001) 657–663.
[43] M. Hosokawa, M. Ito, K. Takahashi, Preparation of highly unsaturated fatty acid containing phosphatidylcholine by transesterification with phospholipase A2, Biotechnol. Tech. 12 (1998) 583–586.
[44] I.H. Kim, H.S. Garcia, C.G. Hill Jr, Phospholipase A1-catalyzed synthesis of phospholipids enriched in n-3 polyunsaturated fatty acid residues, Enzyme Microb. Technol. 40 (2007) 1130–1135.
[45] A.F. Vikbjerg, H. Mu, X. Xu, Parameters affecting incorporation and byproduct formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system, J. Mol. Catal. B 36 (2005) 14–21.
[46] T.S. Leong, T.J. Wooster, S.E. Kentish, M. Ashokkumar, Minimising oil droplet size using ultrasonic emulsification, Ultrason. Sonochem. 16 (2009) 721–727.
[47] V. Klang, C. Valenta, Lecithin-based nanoemulsions, J. Drug Del. Sci. Technol. 21 (2011) 55–76.
[48] G. Urbina-Villalba, A. Forgiarini, K. Rahn, A. Lozsán, Influence of flocculation and coalescence on the evolution of the average radius of an o/w emulsion. Is a linear slope of r3 vs. t an unmistakable signature of Ostwald ripening?, Phys Chem. Chem. Phys. 11 (2009) 11184–11195.
[49] G. Urbina-Villalba, K. Rahn-Chique, Short-time evolution of alkane-in-water nano-emulsions, Revista del Centro de Estudios Interdisciplinarios de la Física 1 (2012) 1–14.
[50] T. Sakai, K. Kamogawa, K. Nishiyama, H. Sakai, M. Abe, Molecular diffusion of oil/water emulsions in surfactant-free condition, Langmuir 18 (2002) 1985–1990.
[51] E. Kawaguchi, K.I. Shimokawa, F. Ishii, Physicochemical properties of structured phosphatidylcholine in drug carrier lipid emulsions for drug delivery systems, Colloids Surf. B 62 (2008) 130–135.
[52] A. Desrumaux, J. Marcand, Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): effect of pressure on emulsion characteristics, Intl. J. Food Sci. Technol. 37 (2002) 263–269.
[53] S.M. Jafari, Y. He, B. Bhandari, Nano-emulsion production by sonication and microfluidization – a comparison, Int. J. Food Prop. 9 (2006) 475–485.
[54] S.Y. Tang, M. Sivakumar, T.K. Wei, B. Nashiru, Formulation development and optimization of a novel cremophore EL-based nanoemulsion using ultrasound cavitation, Ultrason. Sonochem. 19 (2012) 330–345.
[55] T.J. Wooster, M. Golding, P. Sanguansri, Impact of oil type on nanoemulsion formation and ostwald ripening stability, Langmuir 24 (2008) 12758–12765.
[56] L.M. Braginsky, M.A. Belevitskaya, Kinetics of droplets breakup in agitated vessels, in: N.N. Kulov (Ed.), Liquid–Liquid Systems, Nova science, Commack, New York, 1996.
[57] T. Nii, F. Ishii, Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method, Int. J. Pharm. 298 (2005) 198–205.
[58] H. Wu, C. Lu, A. Zhou, Z. Min, Y. Zhang, Enhanced oral bioavailability of puerarin using microemulsion vehicle, Drug Dev. Ind. Pharm. 35 (2009) 138–144.
[59] M.P. Desai, V. Labhasetwar, G.L. Amidon, R.J. Levy, Gastrointestinal uptake of biodegradable microparticles: effect of particle size, Pharm. Res. 13 (1996) 1838–1845.
[60] D.J. McClements, E.A. Decker, Lipid oxidation in oil-in water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems, J. Food Sci. 65 (2000) 1270–1282.
[61] Y. Wu, T. Wang, Soybean lecithin fractionation and functionality, J. Am. Oil Chem. Soc. 80 (2003) 319–326.
[62] M.P. Silvestre, W. Chaiyasit, R.G. Brannan, D.J. McClements, E.A. Decker, Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions, J. Agric. Food Chem. 248 (2000) 2057–2061.
[63] J.L. Donnelly, E.A. Decker, D.J. McClements, Ability of emulsifiers to influence iron-catalyzed oxidation of emulsified menhaden oil, J. Food Sci. 63 (1998) 997–1000.
[64] G. Wang, T. Wang, Oxidative stability of egg and soy lecithin as affected by transition metal ions and ph in emulsion, J. Agric. Food Chem. 56 (2008) 11424–11431.
[65] S.J. Lee, S.J. Choi, Y. Li, E.A. Decker, D.J. McClements, Protein-stabilized nanoemulsions and emulsions: comparison of physicochemical stability, lipid oxidation, and lipase digestibility, J. Agric. Food Chem. 59 (2011) 415–427.
[66] C. Qian, E.A. Decker, H. Xiao, D.J. McClements, Physical and chemical stability of b-carotene-enriched nanoemulsions: influence of pH, ionic strength, temperature, and emulsifier type, Food Chem. 132 (2012) 1221–1229.
[67] J.P. Hsu, A. Nacu, Behavior of soybean oil-in-water emulsion stabilized by nonionic surfactant, J. Colloid Interface Sci. 259 (2003) 374–381.
[68] Y. Zhao, C. Wang, A.H. Chow, K. Ren, T. Gong, Z. Zhang, Y. Zheng, Selfnanoemulsifying drug delivery system (SNEDDS) for oral delivery of zedoary essential oil: Formulation and bioavailability studies, Int. J. Pharm. 383 (2010) 170–177.