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Soy PC liposomes as CLA carriers for food applications: Preparation and physicochemical characterization

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ABSTRACT

Soy phosphatidylcholine (PC) liposomes added with conjugated linoleic acid isomers, (CLA, 9c, 11t, and 10t, 12c) at two PC/CLA ratios, 2:0.4 and 2:1, were prepared by ethanolic injection, and followed during 30 days of storage. These systems were investigated in encapsulation efficiency and fatty acid composition by gas chromatography, size by dynamic light scattering, morphology by TEM images, and membrane fluidity by electron paramagnetic resonance (EPR). Both type of formulations showed highly significant stability and protective effect on CLA isomers with encapsulation efficiencies over 80% during storage. Liposome sizes increased at increasing CLA content. TEM showed evidence of oligolamellar vesicles formation. Regarding membrane fluidity, two behaviors were distinguished in the bilayer: CLA at both formulations disordered the outer membrane zone increasing its fluidity, but the formulation with higher CLA content caused a decreased fluidity near the center of the membrane. The method is safe and easily scalable, and formulations are suitable for future applications in food industry.

1. Introduction

Functional foods are those that generate a health benefit to the consumer beyond their basic nutritional contribution (Korhonen and Pihlanto, 2007; Korhonen, 2009; Park and Oh, 2010).

Many of them contain components known as bioactive: chemical compounds that may be naturally present, formed or added during food processing, and exert specific biochemical/physiological functions when consumed by humans. Conjugated linoleic acid (CLA) is a bioactive compound that describes a group of positional and geometric isomers of Linoleic Acid (LA, C18:2 9c12c). Mainly, their major isomers 9c,11t and 10t,12c have beneficial effects, which include the reduction of body fat content and mass muscle increment, stimulation of the immune system, reduction of plasma cholesterol, inhibition of carcinogenesis and possibly antioxidant activity (Belury, 2002; Hur et al., 2007; Kim et al., 2016).

CLA usage in food is limited because it is susceptible to spoilage, especially oxidation, leading to the loss of bioactivity and the appearance of undesired compounds. One approach to achieve foods enriched in this bioactive compound without the defects associated with its deterioration would be the addition of CLA protected by encapsulation. In this sense, liposomes have appeared as an innovative encapsulation technology. These structures are phospholipid bilayers enclosing water that favor the solubility and bioavailability of several hydrophobic or hydrophilic compounds. Biocompatibility and biodegradability make liposomes a suitable delivery system to be utilized not only in food industry but also in a variety of areas such as drug delivery, cosmetic formulations and diagnostic agents (Andhale et al., 2016; Laouini et al., 2012; Mozafari et al., 2008).

In the literature, several methods were reported for liposome preparation (Maherani et al., 2011). In particular, ethanol injection technique offers many advantages as it is easily scalable: it is simple, it does not include the use of hazardous solvents, and small liposomes are obtained without excessive technical requirements (Gharib et al., 2016; Jahn et al., 2004; Wagner et al., 2002).

The adequacy of a liposome formulation to be used as a carrier system depends on the physicochemical properties of their membranes, on the nature of their components, on their size, surface charge, and lipid organization (Bozzuto and Molinari, 2015). Indeed, liposomes are very susceptible to the type and concentration of phospholipids, nature of the encapsulated compound, method of preparation, presence of cholesterol, ionic lipids, etc.(Sekhon, 2010). Moreover, their physical stability is an important parameter for applications, as is related with the capability of maintaining the size distribution and the encapsulation efficiency of the compound of interest (Torchilin and Weissig, 2003).

The effect of the large number of variables above mentioned on the characteristics of the vesicles and the possibility of designing structures according to the desired application is an emerging research field that has not yet been fully explored. Therefore, the aim of the present work was to study two liposomal formulations in which the proportion of phospholipid: CLA is varied, with a view to potential applications in functional foods. For this, liposomes were pre-

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pared by ethanol injection technique and characterized in size, efficiency of incorporation of CLA, and membrane fluidity, at 3 and 30 days of cold storage.

2. Materials and methods

2.1. Liposome preparation

Liposomes were prepared according to the ethanolic injection technique (Wagner et al., 2006). For this purpose, PC (Phospholipon 90 G, Lipoid, Switzerland, 97.2% phosphatidycholine) and CLA (conjugated linoleic acid isomers 9*c*, 11*t* and 10*t*, 12*c*, Tonalin BASF, 80% CLA, Germany) were used. Two formulations (4 mM total PC + CLA concentration) with different proportions PC: CLA were assayed: formulation A (2:0.4) and formulation B (2:1). PC and CLA were dissolved in ethanol in the desired proportions, and the ethanolic solution was injected into distilled water (0.1 mL/min at 37 °C), up to a final ethanol/water volume ratio of 0.1. In addition, controls without CLA (empty liposomes) were included for each formulation (Controls A and B: with 3.3 and 2.7 mM PC concentration, respectively). Three replicates were performed for each formulation. Liposomal suspensions were stored at 4 °C and analyzed at 3 and 30 days of storage.

2.2. Size distribution

Average particle size and size distribution (polydispersion index, PDI) of liposome preparations were measured by dynamic light scattering (DLS, Zeta Sizer Nano ZS90, Malvern Instruments) at 25 °C and 90° scattering angle. The analysis was performed in triplicate. Results were given as Z-average size, which is the mean value of the hydrodynamic diameter, and polydispersity index (PDI), which is a measure of the width of the particle size distribution.

2.3. TEM analysis

A drop of each liposome sample was placed on a formvar coated copper TEM grid. The samples were allowed to settle to the grid surface for 60 s, and excess liquid wicked away with a filter paper. The grids were then stained with a drop of aqueous 2% uranyl acetate for 30 s after which the excess stain was wicked away. The samples were observed with a JEOL-JEM 1200 EX II TEM (Laboratorio Integral de Microscopia, CICVyA, INTA) at 80 KV.

2.4. Encapsulation efficiency and fatty acid composition

The percentage of CLA incorporated into the liposomes was measured after free CLA had been separated from the liposomes by centrifugation. Approximately 2 mL of CLA liposomes were centrifuged (using Amicon Ultra filter units, 10 KDa molecular weight cutoff) at 4000×*g* for 45 min. CLA content was determined by gas chromatography (GC) analysis, both in the suspension (3 days, C_{total}), and in the retained fraction (3 and 30 days, $C_{retained}$). The procedure was as follows. Fatty acids (FA) were in situ methylated according to the P. Park and Goins (1994) method with some modifications. For that, 100 µl of the retained fraction, or 500 µl of the formulations were transferred into a test tube. For quantification, C17:0 was used as internal standard at a dose of 0.3 mg (Sigma-Aldrich, St. Louis, MO), and calibration curves were made for each fatty acid. Then, 200 µl of methylene chloride were added and agitated. Two mL of 14% boron trifluoride in methanol were added, and tubes were heated at 50 °C for 30 min in a water bath, with agitation. Then, tubes were cooled to 25 °C, and 1 mL of saturated NaCl solution and 1 mL of hexane were added. After that, tubes were shaken in vortex for about 1 min and left at 4 °C in refrigerator for 15 min. The aliquots were dried with anhydrous sodium sulfate; the top layer was transferred into a vial, and analyzed by GC. For that, a Perkin Elmer model GC-9000 series gas chromatograph (Perkin Elmer Corp., Waltham, MA) equipped with a flame ionization detector (FID) and with a split/splitless injector was used. FFA methyl esters were separated on a fused silica capillary column (60 m × 0.25 mm; HP-INNOWax, Agilent J&W, USA) coated with a bonded polyethylene glycol stationary phase (0.25 µm layer thickness); carrier gas H2 flow at 2 mL/min; 1 µL injection (splitless); injector and detector temperatures at 250 and 300 °C, respectively; oven temperatures running from 75 °C (1.5 min) up to 150 °C (10 min) at 8 °C/min, then increased to a final temperature of 245 °C (15 min) at 10 °C/min. Analyses were performed by duplicate.

Percent encapsulation efficiency (EE%) was determined as follows:

$$EE\% = \frac{C\ retained\ (mg/ml)}{C\ total\ (mg/ml)} \times 100 \quad (1)$$

Fatty acid composition of CLA liposomes suspensions (expressed as percentages) was also determined at 3 and 30 days of storage as described above in order to check their stability.

2.5. Lipid bilayer fluidity

The study of fluidity at different depths of the liposome membranes was carried out by electron paramagnetic resonance spectroscopy (EPR), incorporating 5-doxyl stearic acid or 16-doxyl stearic spin labels (5-SASL and 16-SASL, Sigma, USA). These labels incorporate readily to the liposomes. 5-SASL senses the outer part of the membrane, near the lipid polar heads, and 16-SASL is located in the middle part of the lipid bilayers. Liposome suspensions were labeled at a label/lipid molar ratio of 1%. The procedure was as follows: 1.7 µl of 5- or 16-SASL stock solution in ethanol was added to a plastic tube, and the solvent was evaporated under nitrogen flux. Then, 60 µl of acetate buffer, pH 5.4 were added, followed by 60 µl of a concentrated liposome suspension (25 mM lipids in distilled water). The samples were incubated 30 min at room temperature, and subsequently concentrated by centrifugation (4000×*g*, 40 min, 20 °C) in Amicon tubes (10 kDa). Concentrated samples were loaded into flame sealed capillary tubes. EPR spectra were recorded at 25 °C and X band frequency in a Bruker EMX-Plus spectrometer with temperature control by nitrogen circulation. From the spectra of both labels the order parameter S was calculated, and for 16-SASL the correlation time Tc was also calculated (Pincelli et al., 2000).

2.6. Statistical analysis

Variance analysis (ANOVA) was performed to detect significant differences among the formulations and controls, and to determine the influence of storage time. The means were compared by the Tukey test. We used the SPSS program (10v SPSS Inc., USA).

3. Results and discussion

3.1. Size distribution

Fig. 1 shows the DLS size distributions for all formulations, at the beginning and at the end of storage. Although distributions are unimodal, all samples show a slight positive asymmetry, which could be due to a second population of higher size that is not detectable by the technique employed. However, in all cases the polydispersity index (PDI) was lower than 0.25 (Table 1), which indicates largely homogeneous distributions (Wagner et al., 2006). No significant changes were seen in mean size and PDI during storage in all preparations. Accordingly, all liposome suspensions were visually stable, as no sedimentation was observed during the time of study.

Table 1 lists the mean size and PDI of the liposomes at the two storage times. Liposome average sizes vary between 148 nm and 270 nm. For all formulations, no significant size changes are observed at 30 days storage.

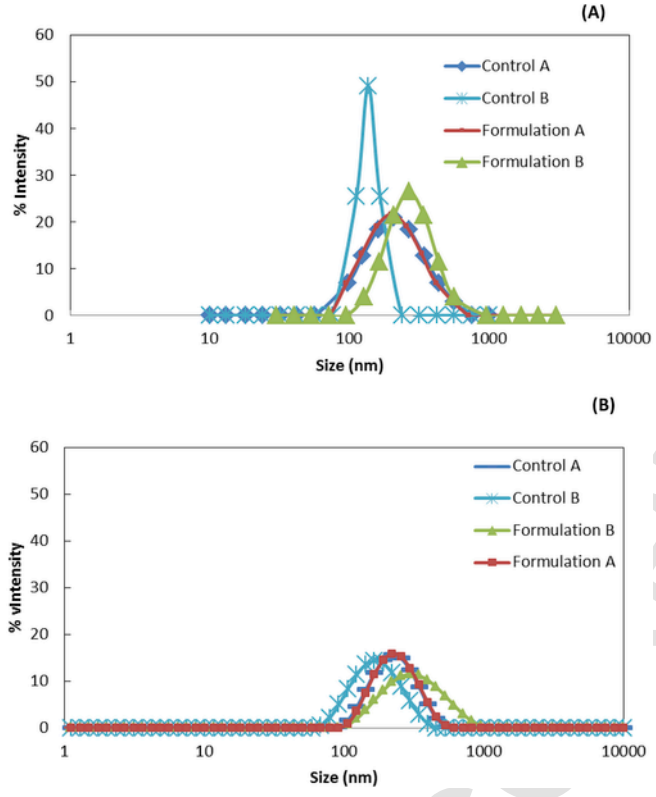

Fig. 1. Size distribution of liposome suspensions at 3 days (A) and 30 days (B).

It is observed that formulation B has the highest diameter values, followed by formulation A, which is similar to control A. These results may be related to the increased CLA amount fraction in formulation B (33%) compared to formulation A (17%) and to controls (0%). They can be rationalized in the frame of the theory of Israelachvili (1992). Having one double bond in *cis* conformation and a small polar moiety, the molecules of CLA isomers can be described by a conical shape. When incorporated to bilayer membranes at an adequate concentration, this kind of molecules would promote a decrease in membrane curvature, leading to an increased particle radius. Our results lead us to conclude that 17% CLA is not enough to promote this effect, as formulation A has similar liposome size to controls, but 33% CLA promotes decreased curvature and increased size in PC/CLA liposomes (formulation B).Varona et al. (2011) also found that liposome size was strongly dependent on lavandin oil/lipids ratio, increasing size with increased ratio.

Our findings demonstrate good stability of liposome suspensions for at least one month. Other studies found an increase of mean size or PDI during storage at 4 °C in different formulations (Rasti et al., 2012; Sahari et al., 2017; Sebaaly et al., 2015). According to our knowledge, there are no previous reports of CLA encapsulation in liposomes. Sizes obtained in the present work were slightly higher than the ones obtained by Gharib et al. (2017, 2016) who reported the encapsulation of essential oils at laboratory scale using a syringe injection and at pilot scale using membrane contactor, respectively (sizes varied between 153 and 164 nm for the first and 151–168 nm for the second work). These authors employed soybean phosphatidylcholine and cholesterol at a volume ratio between ethanol and the aqueous phase of 0.5, higher than that employed in the present work (0.1). In this sense, it is known that hydration water also plays an important role, since liposome diameter increases as the amount of water increases (Varona et al., 2011).

On the other hand, the mean values obtained by us were lower than those reported by Rasti et al. (2012), who prepared liposomes using soy phospholipids and polyunsaturated fatty acids (docosahexaenoic acid DHA, and eicosapentaenoic acid EPA) by the conventional method of film hydration (mean particle size obtained: 362.5 nm) and by Mozafari method (mean particle size, 316.5 nm). It is known that liposomes larger than 200 nm in diameter tend to have a multilamellar structure, which is usually obtained by the conventional method. Besides, mean values obtained in the current study are higher than those obtained when sonication is applied on liposome suspensions: Rasti et al. (2012) produced nanoliposomes of 73.2 nm and Sahari et al. (2016) obtained suspensions with diameters lower than 110 nm. Mohammadi et al. (2014) used homogenization followed by sonication cycles and obtained vesicles in the rage 86–89 nm. Bochicchio et al. (2016) produced small or gigant unil-

Table 1
Size, polydispersity index (PDI), and CLA encapsulation efficiency of liposomal suspensions during storage. (Mean ± SD).

	Time (days)	Control A 3.3 mM (PC)	Control B 2.7 mM (PC)	Formulation A 2:0.4 (PC:CLA)	Formulation B 2:1 (PC:CLA)
Size (nm)	3	$192^{bA} \pm 30$	$150^{cA} \pm 17$	$199^{bA} \pm 10$	$270^{aA} \pm 17$
	30	$174^{bA} \pm 21$	$148^{cA} \pm 17$	$200^{bA} \pm 10$	$253^{aA} \pm 11$
PDI	3	$0.21^{aA} \pm 0.06$	$0.24^{aA} \pm 0.03$	$0.16^{aA} \pm 0.03$	$0.18^{aB} \pm 0.03$
	30	$0.16^{aA} \pm 0.03$	$0.24^{aA} \pm 0.04$	$0.149^{aA} \pm 0.008$	$0.20^{aA} \pm 0.01$
Encapsulation Efficiency (%)	3	–	–	$81.5^{aA} \pm 0.4$	$83^{aA} \pm 6$
	30	–	–	$93^{aA} \pm 23$	$71^{aA} \pm 11$

[a,b] Different lowercase letters show statistically significant differences between columns ($p < 0.05$).
[A,B] Different capital letters show statistically significant differences between rows ($p < 0.05$), for each column.

amellar vesicles (49 nm - 1.416 μm) by controlling different duty cycle sonication rounds. Therefore, several techniques, such as extrusion, ultrasonication, homogenization and freeze-thawing, have been extensively employed to further treat the liposome suspensions obtained from the thin film dispersion method in order to diminish size (Liu et al., 2013). Sizes reported for unilamellar vesicles are in the range 20–200 nm (Rasti et al., 2014). In the present work, it seems that oligo lamellar vesicles were formed, as intermediate diameters were obtained, which is in concordance with TEM images (see the following section). The fact that no energy was applied in order to reduce size, is advantageous for industrial applications. Besides, compared to the conventional preparation method, the size reduction could enhance bioactivity of encapsulated compounds due to larger interfacial contact area with biological tissues (Wang et al., 2014).

3.2. TEM

TEM images (Figs. 2–3) provide evidence of liposome formation; they show oligo lamellar vesicles. No differences are noticed between formulations and through time of storage. Liposome sizes are near 150–200 nm, which is compatible with DLS determinations. In most cases, agglomerations are noticed, which could be due to sample treatment (Torchilin and Weissig, 2003; Zhang et al., 2011).

3.3. Entrapment efficiency and FA composition

Table 1 shows the percent encapsulation efficiency values obtained for the studied formulations. At the beginning of storage, %EE for both formulations were similar and above 80%, and no significant differences were observed at 30 days. These results suggest that CLA isomers have a much higher affinity for liposomes structures than for the surrounding media.

As mentioned above, there are no published results of CLA encapsulation in liposomes. However, similar results were found in previous studies which reported high yields of liposomes encapsulating fish oil, having %EE ranges between 73.5 and 92% (Ghorbanzade et al., 2017; Rasti et al., 2012; Sahari et al., 2016). As for the time effect, Rasti et al. (2012) found an insignificant effect of 30 days storage (4 °C) on DHA and EPA encapsulation efficiency of liposomes. On the other side (Sahari et al., 2016), found a decrease in encapsulated DHA and EPA in different liposome formulations during storage at different temperatures, after 30 or 90 days.

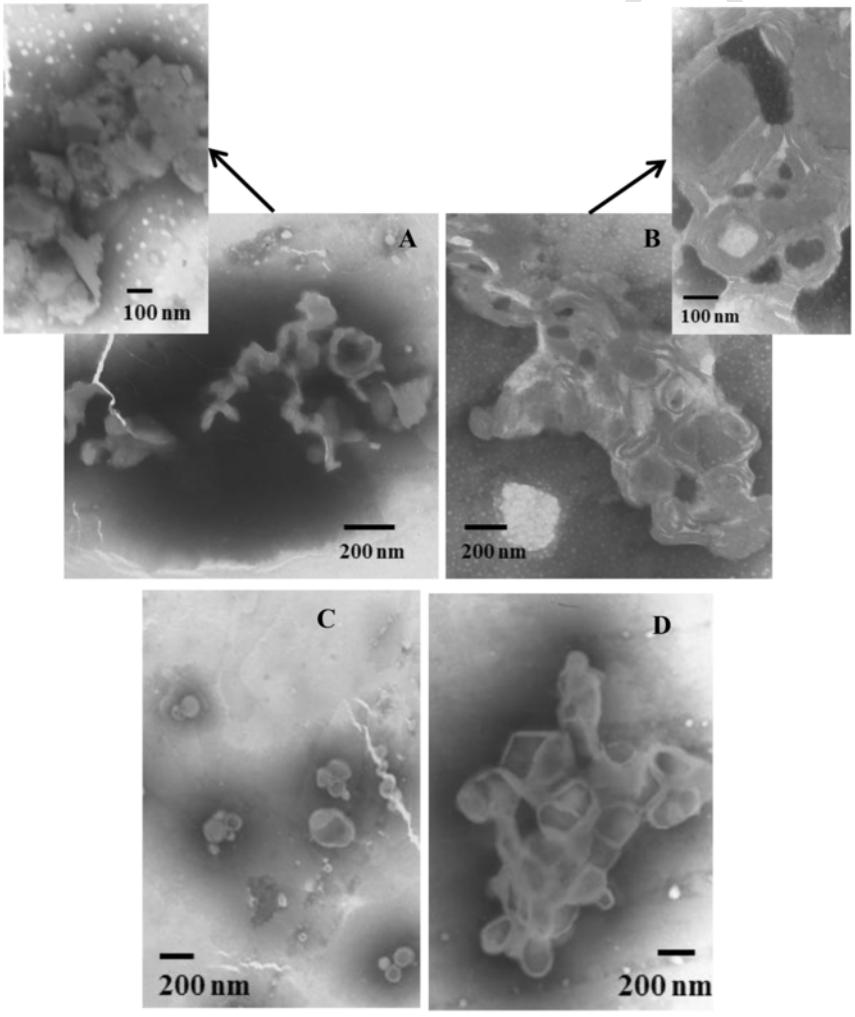

Fig. 2. TEM micrographs of liposomes at 3 days of storage: controls of formulation A (A) and controls of formulation B (B); CLA loaded liposomes from formulation A (C) and CLA loaded liposomes from formulation B (D).

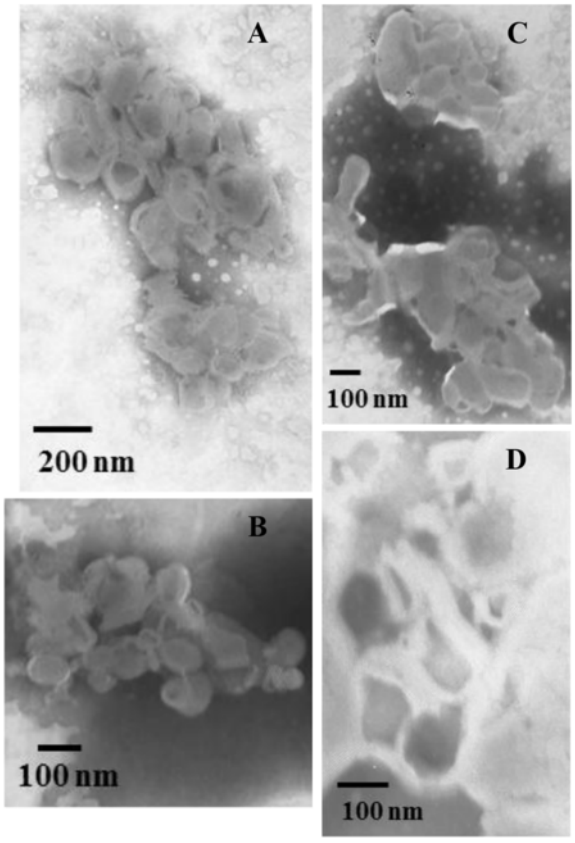

Fig. 3. TEM micrographs of liposomes at 30 days of storage: controls of formulation A (A) and controls of formulation B (B); CLA loaded liposomes from formulation A (C) and CLA loaded liposomes from formulation B (D).

Fig. 4 shows the percent composition of total fatty acids at the two storage times. In this figure, 100% corresponds to the total amount of FA, including those of the PC. It is evidenced a good stability of the formulation CLA-liposomes, as the composition remained without changes through storage ($p > 0.05$). CLA proportions were in concordance with theoretical values (17 and 33% total CLA isomers in formulation A and B respectively). Linoleic acid ($C_{18:2}$) was the main fatty acid in the formulations, in consonance to fatty acids distribution of soybean phospholipids. As it can be seen in the FA profiles from both liposome suspensions, not only CLA isomers would be incorporated in the manufacture of functional foods using these formulations, but also a high level of linoleic acid (essential FA) and minor quantities of palmitic, stearic and oleic acids will be increased in the food matrix. In this sense, scaling studies for dairy food applications are being carried out in our laboratory.

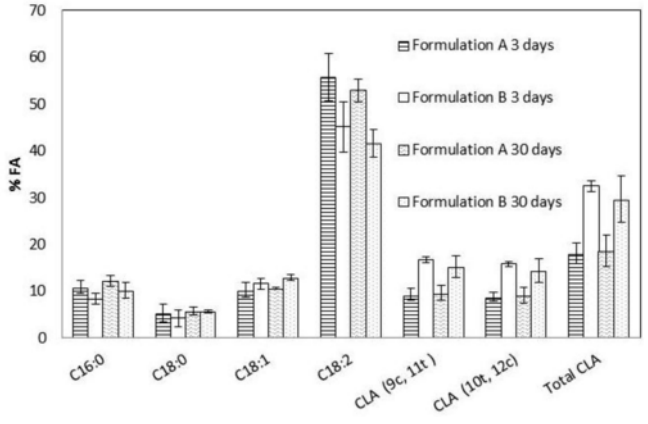

Fig. 4. Fatty Acids composition of CLA-liposomes.

3.4. Fluidity of liposomal membranes

EPR is a valuable technique for determining the fluidity and structural changes of the lipid bilayers. The fluidity of a liposomal membrane characterizes the mobility and molecular rotation rate of lipids. The order parameter S, calculated from the EPR spectra as shown in Pincelli et al. (2000), is a joint indicator of order/mobility restrictions. This parameter varies in the range $0<S<1$. Values near 0 indicate high mobility in a completely disordered environment, while values near 1 correspond to highly ordered, low mobility environments (Griffith and Jost, 1976). On the other side, rotational correlation time Tc, which can be calculated from the 16-SASL spectra, is proportional to the viscosity of the label environment, being lower at higher fluidity (Pincelli et al., 2000). Fig. 5 A shows the order parameter for control and formulation samples labeled with 5-SASL, at the two storage times. This label senses order at outer region of the bilayer, near the lipid polar heads. For both times of ripening, significant lower S values are observed for formulations A and B ($p < 0.05$), revealing that the presence of CLA disorders the outer region of the lipid bilayer, increasing its fluidity. As for time effect, no influence was noticed through storage, except for Formulation A, where a small increase of S parameter is observed ($p < 0.05$). By contrast, at the bilayer center, sensed by16-SASL, formulation B have a higher S parameter at both times studied ($p < 0.05$, Fig. 5 B). The effect is consistent with correlation time (Fig. 5 C), which is significantly higher in formulation B than in formulation A and controls, at both times studied ($p < 0.05$). These results show that at the highest CLA content (33% in formulation B), the central portion of the lipid bilayer is rigidized, presenting less fluidity. This property is preserved along time. On the other side, control samples showed the opposite behavior, as correlation time diminished from 3 days to 30 days ($p < 0.05$).

Studies in biological membranes and composite model systems have shown that the insertion of free fatty acids induces the transformation of the lipid structures into new microdomains that differ in size and composition from the initial ones (Ibarguren et al., 2014). Indeed, the addition of oleic acid, arachidonic acid and DHA, all of them unsaturated, has shown to increase cell membrane fluidity (Ibarguren et al., 2013; Onuki et al., 2006). The structure modification of cell membranes by lipids appears to involve regulations on cell signaling, which is of particular importance in medicine (Awad et al., 1993; Mullen et al., 2010). Through modifying the membrane fluidity and ordering state, CLA could generate changes in the permeation of molecules into the membrane. In fact, there are studies which suggest that membrane permeability is related with membrane disorder, as it has been reported that certain unsaturated FA affect membrane areas that prevent ions from crossing the bilayer (Ibarguren et al., 2014). However, the obtained results depend both on the composition of the model membranes, and on the type of FA (chain length and unsaturation degree) (Ehringer et al., 1990; Langner and Hui, 2000; Muranushi et al., 1981).

Another important aspect regarding liposomes usage as carriers systems in food is the possibility to increase bioavailability after oral consumption (Takahashi et al., 2009). This is influenced by the physical stability of liposomes, which in turn is influenced by the state of membrane lipids, as packing and ordering (Frenzel and Steffen-

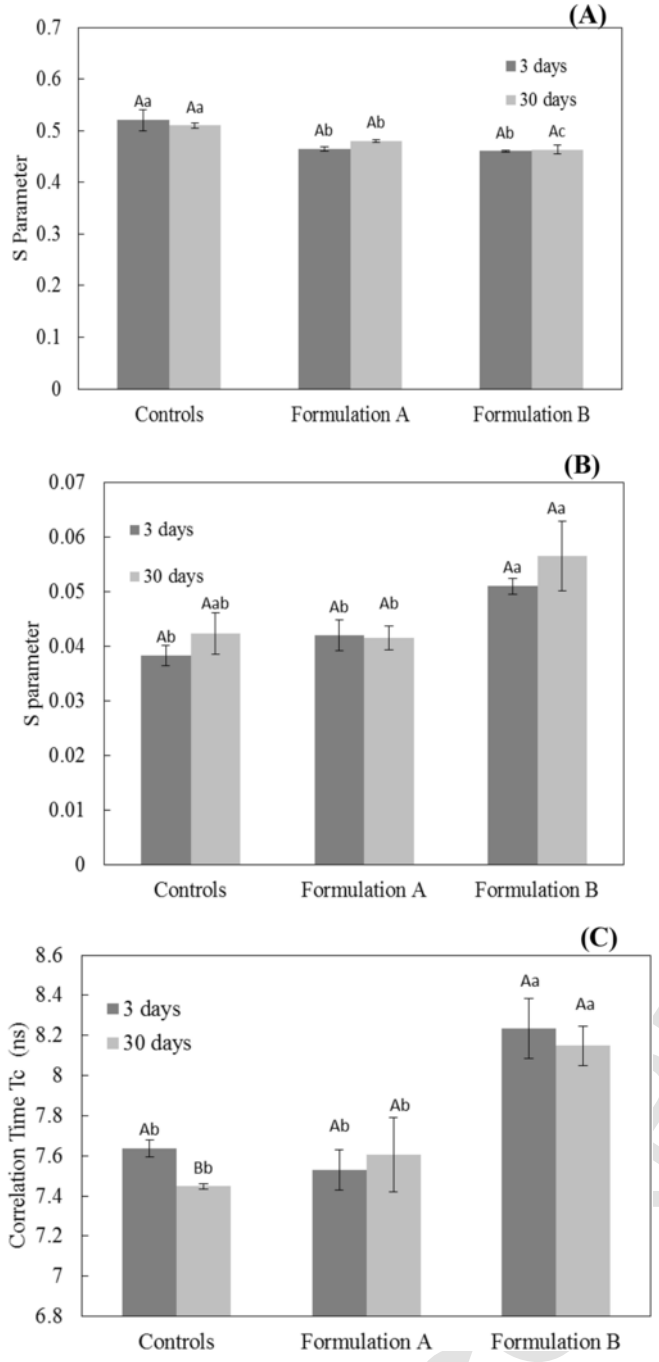

Fig. 5. (A): Order Parameter S corresponding to the spin label 5 – SASL. (B and C): Order parameter S and correlation time Tc corresponding to the spin label 16-SASL. Mean Values ± SD for 3 and 30 days of storage are shown. For each time studied, different lowercase letters (a,b) indicate significant differences ($p < 0.05$). For each liposome formulation, different capital letters (A, B) indicate significant differences through time of storage.

Heins, 2015; Liu et al., 2013). Therefore, differences in fluidity could infer different behaviors during gastro intestinal digestion.

The physicochemical characterization of our liposomes opens the way for selecting the appropriate conditions in order to add these formulations into different food matrices. For example, their stability for 30 days at cold storage could be compatible with the addition of the formulations to certain beverages or dairy food such as cheese or yogurt. However, CLA stability should be checked in the real matrix during manufacture and shelf life.

Moreover, the present work is promising for the development of new encapsulating structures in which other compounds, in addition to CLA isomers, could be included in view of food (vitamins, colorants) or pharma applications.

4. Conclusions

In the present work, two liposomal formulations (with 17 and 33% CLA) were prepared by a safe, easily scalable method, and physicochemically characterized. They showed to be highly stable during 30 days of storage at 4 °C, with no degradation of CLA during this time. Liposome size and membrane fluidity were strongly dependent on CLA content. The inclusion of CLA at the two compositions disordered the external region of the lipid bilayers, locally increasing membrane fluidity. Additionally, the formulation having 33% CLA showed a decreased fluidity in the internal region of the bilayer. These results are promising, since they could have implications in fatty acids digestibility and bioavailabilty. Nowadays, there is an emerging trend in the field of enhancing oral delivery of liposomes by modification of either the liposomal surfaces or the liposomal compositions. This work represents our first step of the study, and more research about digestion of these structures is being done in our laboratory.

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