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Bioactive compounds as functional food ingredients: characterization in model system and sensory evaluation in chocolate milk

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ABSTRACT

The objective was the design and the structural and functional characterization of bioactive compounds (BC) as functional food ingredients for a future industrial application in chocolate milk. Liposomes were made of soy phosphatidylcholine, containing BC (omega-3, omega-6, vitamin E) and encapsulating folic acid (FA). Stearic acid and calcium stearate were added as liposome stabilizers.

The oxidative stability, size and shape were analyzed by thiobarbituric acid method, light scattering, and light microscopy, respectively. Membrane packing was also studied. Rheological behavior of liposomes and encapsulation efficiency of FA were analyzed after pasteurization. Studies were performed in food-model. Liposomes showed significant stability of all parameters and a protective effect over thermolabile FA remaining half of this vitamin encapsulated.

Sensory evaluations were studied in chocolate milk with BC, demonstrating positive effects on acceptability. For all the above, BC formulations are suitable for a future application and/or scaling up in dairy industry.

1. Introduction

Bioactive substances present as natural constituents in food provide health benefits beyond the basic nutritional value of the product (Biesalski et al., 2009). They are extranutritional constituents that typically occur in small quantities in foods and they are being intensively studied to evaluate their effects on health (Kris-Etherton et al., 2002). For example, it is important to consume essential fatty acids because human body cannot produce those (López and Suárez, 2003). However it is not the only quality because they have proven benefits in preventing cardiovascular disease (Lee and Lip, 2003), schizophrenia (Sivrioglu et al., 2007) and cancer (Jenski et al., 1995), among others. Also, they have properties of vasodilators, antihypertensive, anti-inflammatory, and anti-atherothrombotic (Baguma-Nibasheka et al., 1999). Essential fatty acids like linolenic acid (ω-3) and linoleic acid (ω-6) are contained in soy phosphatidylcholine (SPC) which is a natural lipid.

Besides, vitamins have important functions in certain metabolic processes in the human body. Vitamin E (VE) or α-tocopherol is the major fat soluble antioxidant in the body. It protects the lipids against oxidative damage (Atkinson et al., 2008), reduces the formation of hydroperoxides and delays the initial phase of the oxidative process (Ordóñez et al., 1998). Also, it is related to the decrease in blood cholesterol, having a positive effect on the incidence of atherosclerosis and cardio-circulatory system (Primo Yúfera, 1998). Another antioxidant vitamin, which is hydrosoluble, is the folic acid (FA) or vitamin B9. This vitamin acts as a cofactor in carbon transfer reactions (formyl, hydroxymethyl, and methyl) nucleotide biosynthesis (purine bases and pyrimidine), amino acid metabolism (methionine, histidine) and metabolism

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neurotransmitters (serine, choline) (Bekaert et al., 2008). Deficiency of FA is related to neural tube defects, heart disease and megaloblastic anemia (López and Suárez, 2003). And an important discovery in recent years is that there are studies linking FA intake with a decreased risk of heart disease and cancer (Bailey et al., 2003; Malinow et al., 1998).

However, most of the bioactive compounds: e.g. fatty acids, carotenoids, tocopherols, flavonoids, polyphenols, phytosterols, oil soluble vitamins have hydrophobic nature (Kris-Etherton et al., 2002). Besides it is not easy to add vitamins to aqueous foods while retaining their activity. For instance, VE is easily oxidized in the air (Ordóñez et al., 1998), while FA is thermolabile and is degraded after thermal treatments like pasteurization and light exposure (Fennema, 2000). The addition of these vitamins to aqueous foods using liposomes appears to be a promising solution. Liposomes are microscopic spherical vesicles composed of polar lipids like phospholipids, which enclose liquid compartments within their structure (consisting of lipid bilayers) and enable the encapsulation of both hydrophilic and lipophilic materials (Keller, 2001). With respect to VE, it was demonstrated that this vitamin mixes perfectly with the phosphatidylcholine of the bilayer (Wang and Quinn, 2000). With respect to hydrosoluble vitamins, liposomes promote the protection and activity of vitamins like vitamin C (Marsanasco et al., 2011). Liposomes could be done with SPC allowing the incorporation of bioactive compounds (essential fatty acids, VE) and FA in food like milk, generating a functional food. In recent years, functional foods are defined as the substances which provide benefits to the health of the consumers. Typically, a food marketed as functional contains added, technologically developed ingredients with a specific health benefit (Niva, 2007).

In the food industry, for a given industrial application, membrane stability and structure are important factors when designing liposomes (Keller, 2001), considering that phospholipids can be oxidized, limiting their lifetime (Grit and Crommelin, 1993). That is why several studies include characterization of liposomes by several methodologies. Besides, for preservation of liposomes is necessary to apply a heat treatment used in food industry like pasteurization. And it is very important that liposomes remain stable after a heating process because the higher the stability, the higher the protection of vitamins (Marsanasco et al., 2011) and bioactive compounds.

For an industrial application and production line of a new food, it is very important to assess its acceptability and ease of production. No one would be willing to invest in the development and production on a larger scale of a food that is not acceptable for potential consumers.

The aim of this work was the design and the structural and functional characterization of different liposomal formulations based on SPC with bioactive compounds (omega-3, omega-6, VE) and VC for developing a functional food in pasteurized chocolate milk. Essential fatty acids like linolenic acid (ω-3) and linoleic acid (ω-6) are contained in SPC and in the formulations, the stearic acid (SA) and the calcium stearate (CaS) were incorporated for stabilize the lipid bilayer by increasing rigidity. Besides, addition of CaS incorporated a mineral (calcium) that increases the nutritional value. Oxidative stability was determined with the thiobarbituric acid method. The size and the shape were analyzed by light scattering and light microscopy, respectively. And the packing of membrane was studied with two probes (merocyanine 540 and Laurdan). All studies were performed with or without vitamins and before and after pasteurization in order to have information about the effect of the heat treatment. Pasteurization was applied for preserve the liposomes. Also, encapsulation efficiency of FA was determined after 72 h of dialysis in pasteurized formulations. The experiments mentioned were made in a food model system in order to avoid fluctuations in data due to other components of the food product. Finally, applicability in the product was studied by the rheological behaviors of liposomes and by the sensorial tests. The sensory evaluation in commercial chocolate milk, with and without liposomes, was done by the overall acceptability with hedonic scale and triangular tests with 40 and 78 potential consumers, respectively.

2. Materials and methods

2.1. Raw materials

SPC was purchased from Avanti Polar Lipids (Alabaster, USA). SA and CaS were purchased from Vitalquim (Buenos Aires, Argentina). VE was obtained from Parafarm (Buenos Aires, Argentina) and FA was obtained from Anedra (Buenos Aires, Argentina).

2.2. Liposome preparation

Multilamellar liposomes were prepared by the dehydration–rehydration method (Bangham, 1972). Briefly, 40 μmol of lipids were dissolved in 500 μL ethanol in a round bottom flask, solvent was dried in a rotary evaporator at 37 °C. Dry lipid film composed by SPC, SPC:SA (1:0.25, mol ratio), and SPC:CaS (1:0.25, mol ratio) was rehydrated with 2 mL distilled water to a final 50 mM lipid concentration.

In order to prepare liposomes with VE, a stock solution of this vitamin diluted in ethanol was prepared. Stock concentration was 22.4 mM. Then, 0.445 mL of this stock was mixed with a proper amount of lipids. Solvent was evaporated and lipid film was obtained. When the film was rehydrated in 2 mL of distilled water, a final concentration of 5 mM was reached.

In the case of FA, fresh solutions of this vitamin were prepared at the moment of rehydration. FA was weighted and diluted with distilled water to reach a 0.136 mM concentration.

Samples were prepared with the main goal of fortify food with mentioned vitamins. According to Argentina regulations (Article 1363 of the Argentine Food Code), the percentage of recommended daily intake (RDI) in a portion of fortified food must be between 20% and 50% for fat soluble vitamins and between 20% and 100% for hydro-soluble vitamins. The RDI of VE is 10 mg and for FA is 400 μg. In this work, chocolate milk was used for the sensory evaluation assay. In order to fortify this beverage, 2 mL of liposome suspension (50 mM) with vitamins was added in each serving of chocolate milk (200 mL), which implies that it was fortified with 4.3 mg of VE (5 mM) equivalent to 43% of the RDI and 120 μg of FA equivalent to 30% of the RDI. Thus, 1 L of chocolate milk contained 21.5 mg of VE and 600 μg of FA.

2.3. Preparation of food model systems

Assays were performed in distilled water, classified as food simulant according to local food regulations (Mercosur Resolution No. 30 of 1992). This solution simulates aqueous food having a pH higher than 5, such as chocolate milk.

2.4. Lipid peroxidation stability

Lipid peroxidation was followed by the thiobarbituric acid (TBA) method, as described before (Marsanasco et al., 2011).

2.5. Particle size distribution

Particle size distributions were determined in the range 0.1–1000 μm by laser scattering using a Particle Analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd, UK).

Liposomal suspensions were diluted in 500 mL of distilled water. The dispersion was carried out at 2000 rpm and the degree of obscuration was between 10% and 15%. Sauter mean diameter ($D_{3,2}$) and De Brouker mean diameter ($D_{4,3}$) are the mean diameters from the surface and volume distributions, respectively, and they were defined before (Marsanasco et al., 2011).

2.6. Light microscopy

In order to take the images, 50 mM lipid concentration was used with both vitamins. Micrographs were obtained with a light microscope operating at 400× magnification and using an adapted digital camera (Canon A570 IS; Malaysia) at 4× optical zoom.

2.7. Membrane packing

Two types of probe, merocyanine 540 (MC540) and Laurdan were used. MC540 is located in the membrane phospholipids with its polar sulphonated group toward the more polar outer surface of the head group region and the rest of the rodlike dye ranging through the ester bonds, anchored with the two butyl groups in the hydrocarbon chain region (Lelkes and Miller, 1980). The other probe used is the Laurdan (6-Dodecanoyl-2-Dimethylaminonapht halene), located at the interface of the bilayer with the hydrocarbon portion aligned parallel to the acyl chains of the phospholipids and naphthalene fluorescent residue located at glycerol level (Parasassi et al., 1998). With these two packing probes, the entire membrane can be analyzed.

2.7.1. Membrane packing followed by MC540

Liposomes formulations (before and after pasteurization) with a 50 mM concentration were diluted with distilled water until they reach a 0.868 mM concentration. Probe concentration was 4.344×10^{-3} mM, incorporated into the vesicles with a probe/lipid ratio of 1/200 (Bernik and Disalvo, 1993).

A scan of each sample between 400 and 600 nm was obtained with a UV–VIS spectrophotometer (Shimadzu). Measurements were performed at room temperature and baseline correction was done as previously described (Bernik and Disalvo, 1996).

Partition coefficient (PC) was calculated as: PC = A570/A530, this parameter relates concentration of monomer in non-polar environment with respect to monomer in an aqueous phase (Disalvo et al., 2003).

2.7.2. Membrane packing followed by Laurdan

For these assays, lipid concentration was 1 mM and Laurdan concentration was 0.33 mol% with respect to the total lipids (Hollmann et al., 2010). The probe was added to the lipids with the organic solvent prior to evaporation and the samples were measured in a Fluorospectrometer Nanodrop 3300 (Thermo Scientific, Wilmington USA).

When lipids are in the gel phase, Laurdan maximum is located at 440 nm; and when lipids are in the liquid crystalline phase, the maximum emission of this probe is at 490 mm. Generalized polarization (GP) can be calculated as follows:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

where I_{440} is the fluorescence intensity at 440 nm and I_{490} is the fluorescence intensity at 490 nm.

GP value varies between −1 and +1; a high GP value indicates less dipolar relaxation, which corresponds to a rigid environment (Parasassi et al., 1990, 1994).

2.8. Encapsulation efficiency

The percentage of encapsulation efficiency (%EE) was analyzed by dialysis subjected to a 1/100 dilution; this last condition is the one that is going to happen in aqueous food. FA concentration was analyzed considering that this vitamin is hydrosoluble and it is easily released from liposomes. This is not the case of VE, which being lipid soluble, it is entirely incorporated in the bilayer (Atkinson et al., 2008; Marsanasco et al., 2011).

Briefly, 50 μL of the pasteurized liposome samples were placed in a cellulose membrane bag (Mol. Wt. of 12,000 Daltons, Sigma, USA) and dialyzed against 5 mL of distilled water for 72 h at room temperature to remove the non-encapsulated vitamin remaining in the dispersion medium. At different times 0.2 mL of sample was withdrawn and concentrated by evaporating the aqueous solvent on a Savant Speed Vac system AES 1010 (GMI, Inc. Ramsey, MN, USA) equipped with a RH 40-11 rotor, in full vacuum (oil-free diaphragm vacuum pump, 10 Torr maximum vacuum). Then, 10 μL of distilled water was added for the measurement. For maintenance dialysis volume was replaced with 0.2 mL of distilled water in it.

Then, after 72 h the remaining sample containing liposomes and the retained FA were disrupted by the addition of two volumes of absolute ethanol to release the encapsulated vitamin. The concentration of FA was determined with a UV–Vis spectrophotometer (Nanodrop 1000, USA) by recording absorbance at 286 nm.

The percentage of encapsulation efficiency was calculated according to the following equation (Xia and Xu, 2005).

$$\%EE \text{ of FA} = \frac{\text{vitamin encapsulated in a liposomal formulation}}{\text{Total vitamin incorporated in a liposomal formulation}} \times 100$$

2.9. Rheology

Liposomal dispersions behaviors were studied using an AR-G2 rheometer (TA Instruments; New Castle, DE, USA) with a cone-and-plate geometry (gap, 55 lm; cone diameter, 40 mm; cone angle, 2). Temperature (21 °C) was controlled with a water bath (Julabo ACW100, Julabo Labortechnik; Seelbach, Germany) associated with the rheometer. Flow behavior was analyzed by increasing the shear rate from 0.1 to 100 s^{-1} over 312 s, then keeping it constant at 100 s^{-1} for 60 s, and finally decreasing it from 100 to 0.1 s^{-1} over 312 s.

Apparent viscosity was determined at 100 s^{-1} in a Haake viscometer (Rotovisco RV2, NV rotor, Karlsruhe, Germany), using 3 mL of formulation at 21 °C.

Besides, n (flow behavior index) and apparent viscosity at 100 s^{-1} values were obtained using the model of power law. Ostwald's equation or power law is calculated as follows: $\tau = k \cdot D^n$ (Sharma et al., 2003).

2.10. Preservation of liposomal formulations: pasteurization

In order to analyze the effects of increasing temperature on liposomal formulations, they were resuspended in distilled water, and incubated at 65 °C for 30 min to simulate the pasteurization process of low temperature long time (LTLT). The same batch before and after the heat treatment was used. This LTLT process is employed as a type of pasteurization of milk apply in the food industry. Besides, this pasteurization favors the preservation of the liposomal formulations and the maintenance of low microbiology flora.

2.11. Sensory evaluation

A chocolate milk of an Argentinean trademark was used (Cindor® of Danone S.A.). The day before the sensory evaluation test, liposomes were prepared and then pasteurized, fulfilling the Good Manufacturing Practices. They were added to chocolate milk in a 1/100 ratio and kept at 4 °C until the sensory evaluation.

Mineralized water and unsalted crackers were used as flavor neutralizers (Meilgaard et al., 1999). Evaluators were instructed to perform the tests after they signed a consent form.

In this work, commercial milk was used with the purpose of analyzing standardized food that always has the same physicochemical, microbiological and sensory characteristics. So, the only variation in the flavor, if it exists, would be induced by the addition of liposomes.

2.11.1. Discriminative test

This test was performed to compare the difference between commercial chocolate milk alone and the same sample with liposomes. As the main goal of this assay is to analyze similarities (if any) between samples, 78 evaluators were used, being the accepted number of evaluators between 50 and 100. The following considerations were taken into account:

H_0: Null hypothesis, there is no significant differences between samples.
H_a: Alternative hypothesis: samples have significant differences between each other.

H_0 was analyzed with 0.10 value of the significance level or α (which is the probability of rejecting H_0 when it is true). This value was high because it is the highest risk to say that the products are different when, in fact, they are similar (Meilgaard et al., 1999).

For the triangular test, evaluators should be familiar with the test and evaluate the product. That is why 78 consumers of commercial chocolate milk (men and women over 18 years old) were selected and instructed in the test (Santa Cruz et al., 2005). In each test two samples were the same and the third one was different (product with and without liposomes). The samples were given to each evaluator in disposable cups of 200 mL; each cup had 30 mL of product. The milk was at room temperature and the randomness of the samples was ensured throughout the test. Thus, each evaluator performed the triangular test for the three formulations, testing a total of 9 samples each. Between samples from the same triangle, evaluators were requested to drink water for neutralization and, prior to move from one triangle to the next, they were requested to eat a cracker and drink water to avoid sensory fatigue. Finally, each evaluator filled a card with their evaluation (Meilgaard et al., 1999).

2.11.2. Overall acceptability

For affective tests, like overall acceptability, it is necessary to have a minimum of 30 untrained judges. So, the panel was composed of 40 potential consumers, men and women over 18 years old, which were habitual or potential consumers of commercial milk (Anzaldúa-Morales, 1994). The evaluators were called and were presented two pairs of samples. In the first pair, they evaluated the acceptability without knowing what they were judging. In contrast, in the second pair of samples, they evaluated knowing what the functional commercial milk was and what the commercial milk was. The samples were given to each evaluator in disposable cups of 200 mL; each cup had 30 mL of chocolate milk with or without liposomes. The milk was at room temperature and the randomness of the samples was ensured throughout the test. Hedonic rating scales associated with score was used and detailed as follows: (1) I really dislike it; (3) I dislike it; (5) I neither dislike nor like it; (7) I like it; (9) I really like it. The evaluators could use these values or intermediate ones (Meilgaard et al., 1999). Between samples, evaluators were instructed to drink water and, between pairs, they were asked to eat unsalted crackers and drink water in order to avoid sensory fatigue, allowing a span of 5 min.

2.12. Statistical analysis

ANOVA was carried out with GraphPad Software (version Prism 5.0, Statistical Analysis System, La Jolla, USA). Dunnett, Tukey and paired samples tests were performed for a mean comparison test at a significance level of 5%.

3. Results and discussion

3.1. Lipid peroxidation

In liposomes without vitamins (Fig. 1), results showed that in SPC:CaS and SPC:SA, with or without pasteurization, there were no significant differences in the production of thiobarbituric acid reactive species (TBARS) compared to SPC (Dunnett test). The SPC based liposomes revealed a low peroxidative trend. According to our results and Monroig et al. (2007) SPC liposomes have also a good oxidative stability in comparison to other lipids. The addition of SA or CaS to SPC favors such stability. Studies carried out by Soto-Arriaza et al. (2008) revealed that 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylcholine, a synthetic phospholipid with two chains derived from palmitic acid, causes increased rigidity in the hydrophilic–hydrophobic region of the vesicle when it is incorporated to an egg phosphatidylcholine-based liposome. According to these authors, the increased rigidity affects propagation of the radical initiation and produces a reduction in the water flow rate, decreasing lipid oxidation. A lower oxidative value was expected in SPC:SA and SPC:CaS in comparison to SPC due to an increase in rigidity, but this difference was not detected probably because of the low production of TBARS given by SPC.

The three formulations with FA showed significantly higher values of TBARS in comparison with controls (before and after pasteurization). These results could be related to a prooxidant effect of FA. Several studies have revealed that this vitamin could have an antioxidant action that lies on the elimination of free radicals (Joshi et al., 2001) or a prooxidant action (Gupta et al., 2012).

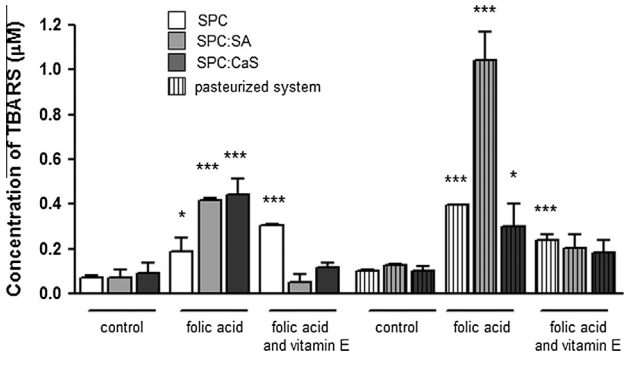

Fig. 1. Peroxidation assay in liposomal formulations. Data correspond to SPC (white), SPC:SA (gray), SPC:CaS (dark gray) without vitamins (control), with 0.136 mM of FA and with 5 mM VE and 0.136 mM of FA. Each column represents the mean ± SD of three independent assays before and after pasteurization in distilled water. Statistical comparison was made: (1) Between each system with vitamin/s with respect to the same system without vitamins (control) through the Dunnett test, before and after pasteurization. Significant differences from control are shown as $^{*}p < 0.05$, $^{***}p < 0.001$. (2) Respect to SPC in systems without vitamins before and after pasteurization with the Dunnett test. No significant differences were observed.

This action depends on several factors, such as the induction of lipid peroxidation and its concentration, the composition of the liposomes and the way of introducing these antioxidants to the liposomes and its concentration (Schnitzer et al., 2007). The FA can promote lipid oxidation as it can reduce catalytic metals such as Fe^{3+} (using in the TBA method), regenerating Fe^{2+} that are involved in oxidation. These results are in concordance with the study made by Gupta et al. (2012) that showed a prooxidant action of FA with the TBA method.

Regarding the systems with FA and VE (with or without pasteurization), only the SPC system presented a significantly higher TBARS value in comparison with formulation without vitamins. In the other two systems, FA prooxidant effect would have been counteracted by the antioxidant action of the VE. Also, in the presence of VE, the system with FA had a good oxidative stability, demonstrating the feasibility of application in commercial chocolate milk.

3.2. Size distribution and morphology

3.2.1. Morphology

Light micrographs of the liposomal systems with both vitamins before and after pasteurization are presented in Fig. 2. The three systems showed a variety of sizes, both before and after pasteurization, which could be related to the preparation method and composition of the liposomes (Marsanasco et al., 2011). Besides, the three liposomal formulations showed aggregation that remained after pasteurization. These results agree with those obtained by other authors. De la Maza et al. (1998) reported liposomal aggregation of vesicles composed by egg phosphatidylcholine at pH of 7.2.

3.2.2. Size distribution

The three formulations with vitamins (before and after pasteurization) had expected values for multilamellar vesicles according to Keller (2001) (Table 1 and Fig. 3A–C). Also, all formulations showed a bimodal distribution, except SPC:SA with vitamins that was multimodal (Fig. 3C).

The SPC system with vitamins presented a significant decrease of the $D_{4,3}$ value in comparison to the same system with FA (Table 1), revealing a lower tendency to liposomal aggregation. This trend remained after pasteurization (Fig. 3A). Also, this system did not present significant differences in the $D_{3,2}$ value (with or without pasteurization), demonstrating a similar liposomal size (Marsanasco et al., 2011).

The SPC:SA system with vitamins showed a significant increase in the $D_{4,3}$ value before pasteurization with a consequent shift to larger values in the particle size distribution (Table 1 and Fig. 3B), with respect to the same system with FA. After the heat treatment, the system with vitamins did not present significant difference in the $D_{4,3}$ value respect to the same formulation with FA. Addition of VE favored the liposomal aggregation increment whereas the pasteurization generated a decrease of the above-mentioned parameter (Table 1, Figs. 2 and 3B). Also, in the $D_{3,2}$ value the system with vitamins did not present significant differences respect to the same system with FA, demonstrating that the addition of VE not changed the size in liposomal formulation.

The SPC:CaS system with vitamins showed a similar size distribution and did not present significant differences in $D_{4,3}$ and $D_{3,2}$ values compared to the same system with FA, before and after pasteurization (Table 1 and Fig. 3C). The only exception was after pasteurization in the $D_{3,2}$ value, where this formulation with vitamins showed a significantly decrease, which is a consequence of a decrement of the liposomal size (Marsanasco et al., 2011).

When comparing each system before and after pasteurization, in SPC:SA formulation with vitamins, the heat treatment induced a significant decrease of $D_{4,3}$ value, compared to the same formulation without pasteurization, thus contributing to the formulation stability. And SPC:SA system with FA presented an increment in the liposomal size after pasteurization, with a significant increase in the $D_{4,3}$ value. The rest of the formulations before and after pasteurization showed no significant differences, demonstrating a good thermal stability of the systems in terms of particle size distribution and tendency to aggregate.

Size and tendency of aggregation were stable throughout 28 days of storage at 4 °C for all formulations, with the exception of the system with CaS, where a mild size increment and aggregation was observed (data not shown).

3.3. Membrane packing

Regarding the addition of FA, SPC:SA and SPC:CaS presented a significantly higher PC value with respect to the SPC system. Same result was maintained after pasteurization ($^{***}p < 0.01$ in both cases, Dunnett test; data not shown in Table 1). The addition of SA or CaS could be favoring the entry of MC540 as it is located slightly above the domain of the glycerol backbone of phospholipids (Lelkes and Miller, 1980). One possible explanation would be related to the effects on the polar heads of the membrane and the increased hydration. The polar heads of phosphatidylcholines bind water molecules by hydrogen bonding and the polar heads of the fatty acids, as the stearic acid, interact with water as well (Müller-Sterl, 2008). The formation of the hydrogen bonds in the polar heads favors a greater penetration of water which promotes the formation of more fluids domains (Peng et al., 2012). Regarding the SPC:CaS system, FA ($pKa_1 = 2.3$) also provides protons to the medium, decreasing pH of distilled water from 6.9 to 3.88 (measured by pHmeter). An acidic environment promotes dissociation of CaS. Sukhija and Palmquist (1990) demonstrated that the lower the pH, the greater the dissociation of the CaS and the release of calcium ion to the medium. Free Ca^{2+} ions are located at the bilayer level-interface, interacting with the phosphate groups of two adjacent phosphatidylcholines, inducing more compact areas in the bilayer packing, and accompanied by structural reorganization of phosphatidylcholine molecules. Negative charges of phosphate groups attract Ca^{2+}, while the positive charges of the trimethylammonium group repel it (Marsanasco et al., 2011; Yeap et al., 2008). The previously mentioned factors would generate areas near by the polar heads of the membrane that favor the entry of MC540.

In the three liposomal formulations, the addition of the vitamins showed a significant increase in the PC values with respect to the system with FA (before and after pasteurization, Table 1). This result indicates that a greater amount of probe is entering the membrane (Disalvo et al., 2003) and this is related to the effect that this VE generates in the lipid bilayer. According to studies reported by Hincha (2008), the presence of VE increases mobility near the area of the polar heads while decreases mobility in the center of the bilayer.

With regard to the GP values, comparing SPC:SA and SPC:CaS to SPC (Dunnett test, data not shown in Table 1), only SPC:SA showed a positive value with a significant difference with respect to SPC, demonstrating that rigidity is provided by the SA at the hydrocarbon chains level of the lipid membrane. This result was maintained after pasteurization, so packing and structure were maintained after the heat treatment. It is also noteworthy that although the SA favors the polar area hydration, the non-polar part of the saturated fatty acid increases the rigidity in the hydrocarbon zone, measured with Laurdan. This result agrees with those obtained by Soto-Arriaza et al. (2008) which showed that in liposomes of egg phosphatidylcholine, GP value increased as percentage of 1,2 -dipalmitoyl-sn-glycerol-3-phosphatidylcholine increased, going from a negative value (GP = −0.2) to a positive one (GP = 0.4). In

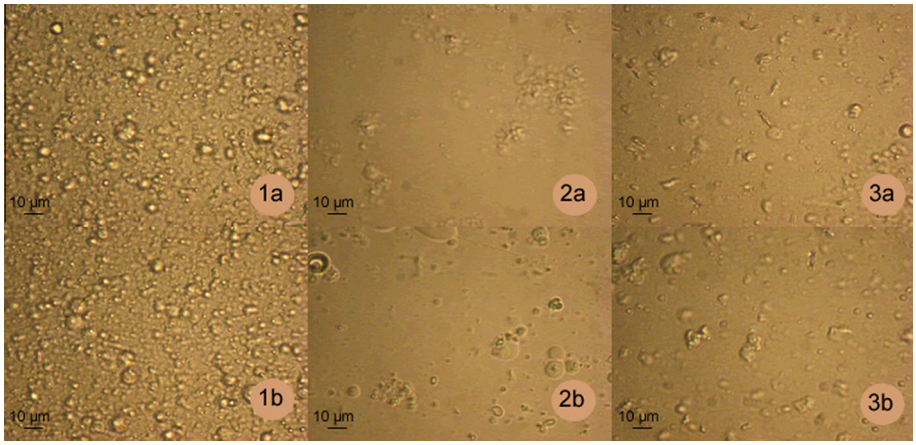

Fig. 2. Morphology of liposomal formulations. Light micrographs of the liposomal system with 5 mM of VE and 0.136 mM of FA in distilled water before and after pasteurization (PAST). 1a: SPC; 1b: SPC PAST, 2a: SPC:SA, 2b: SPC:SA PAST, 3a: SPC:CaS, 3b: SPC:CaS PAST.

Table 1
Size distribution and membrane packing values of liposomal formulations. The results are shown as the mean ± SD of three independent assays in systems with 0.136 mM of FA or with 0.136 mM of FA and 5 mM of VE, before and after pasteurization (PAST) in distilled water. A statistical comparison was made: (1) Between each system with vitamins respect to the same system with FA (control) through the Tukey test before and after pasteurization. Significant differences from the control are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (2) In each system before and after pasteurization with the Tukey test. Significant differences from the control are shown as $^{\blacktriangle}p < 0.05$, $^{\blacktriangle\blacktriangle\blacktriangle}p < 0.001$.

Liposomal formulation	$D_{4,3}$	$D_{3,2}$	PC	GP
SPC with FA	11.46 ± 0.58	5.43 ± 0.43	1.15 ± 0.03	−0.10 ± 0.06
SPC:SA with FA	13.96 ± 0.30	5.73 ± 0.43	1.88 ± 0.05	0.02 ± 0.01
SPC:CaS with FA	11.92 ± 0.50	6.61 ± 0.20	2.15 ± 0.02	−0.03 ± 0.01
SPC with VE and FA	9.20 ± 0.04***	5.16 ± 0.01	1.48 ± 0.03***	0.13 ± 0.06**
SPC:SA with VE and FA	54.05 ± 2.49***	6.55 ± 1.50	2.18 ± 0.05**	0.35 ± 0.04***
SPC:CaS with VE and FA	11.19 ± 0.40	6.26 ± 0.06	2.27 ± 0.02*	0.28 ± 0.05***
SPC with FA PAST	12.58 ± 0.24	5.90 ± 0.09	1.19 ± 0.01	−0.09 ± 0.04
SPC:SA with FA PAST	14.54 ± 1.44▲	6.47 ± 0.23	2.05 ± 0.09▲	0.02 ± 0.01
SPC:CaS with FA PAST	12.40 ± 0.13	6.77 ± 0.09	2.17 ± 0.05	−0.03 ± 0.01
SPC with VE and FA PAST	8.66 ± 0.11***	5.38 ± 0.11	1.47 ± 0.02***	0.19 ± 0.01**
SPC:SA with VE and FA PAST	11.19 ± 1.04▲▲▲	4.71 ± 0.08	2.29 ± 0.04**	0.28 ± 0.04***
SPC:CaS with VE and FA PAST	10.92 ± 0.55*	6.05 ± 0.23**	2.30 ± 0.03**	0.21 ± 0.03***

this work, the same effect was observed: GP in the SPC system was negative and the addition of SA induced the increment to a positive value of this parameter. Regarding the SPC:CaS system, it did not show significant differences in the value of GP in comparison to SPC; this result would be related to the effect of FA in the decrease of pH and the association of calcium with phosphatidylcholines mentioned above.

The three formulations with vitamins showed a significant increase of the GP values with respect to the systems with FA (before and after pasteurization). This result agrees with those obtained by other authors, who have shown that VE increases membrane rigidity (Fukuzawa et al., 1980; Urano et al., 1988.) especially in level of fatty acids chains (Hincha, 2008).

Finally, by comparing each system before and after pasteurization, in both PC and GP parameters, there were no significant differences except for the SPC:SA system with FA, which presented a significant increase in the value of PC. This finding is noteworthy because it shows the thermal stability of the membrane with regard to packaging.

3.4. Encapsulation efficiency

As mentioned before, dialysis emulates the dilution that liposomes would be subjected in chocolate milk (1/100). So EE% in this assay is expected to be similar to the same parameter in chocolate milk. Table 2 shows the results of the %EE of FA. The three formulations presented a high initial %EE that favored the protection of FA, maintaining the prooxidant effect of this vitamin after pasteurization. Other authors showed reductions of 12% in the folate content of pasteurized milk and a loss of 51% and 56% in the folate content in raw foods when boiling them for typical time periods (Fennema, 2000; Lešková et al., 2006). We previously reported that a protective effect over thermolabile vitamin C can be exerted by SPC, SPC:SA and SPC:CaS (Marsanasco et al., 2011). However, the presence of another antioxidant vitamin such as VE, counteracts this prooxidant effect giving as a result a good oxidative stability. It can be inferred that these liposomal systems protected the FA and that the oxidative stability remains unchanged in the presence of VE after pasteurization.

The SPC and SPC:SA systems showed a significant decrease in the %EE from 8 h on of dialysis. Instead, the SPC:CaS formulation presented a value significantly lower of %EE from 3 h on of dialysis. Besides, of the three systems, SPC:SA was the formulation that gave the higher encapsulation efficiency in the dialysis. This result could be related to the greater membrane rigidity in this formulation with FA (Table 1, GP value).

It should be noted that at the end of the dialysis, when the liposomes were disruptured after 72 h, the values of EE% showed that the three systems retained a high percentage of FA without significant difference between them (Tukey test, statistic not shown in Table 2).

3.5. Rheological behavior

In SPC system with vitamins behavior was similar to a Newtonian fluid (Fig. 4) because the shear stress was virtually proportional to the shear rate (McClements, 1999). This result was manifested by the values of n (before and after pasteurization),

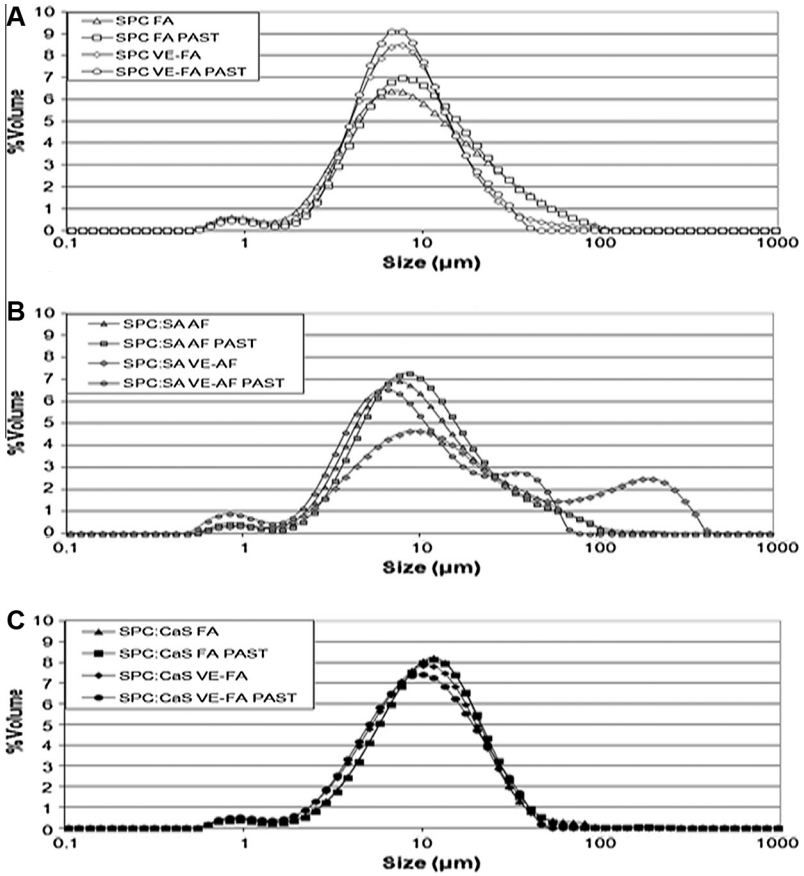

Fig. 3. Particle size distribution expressed as volume percentage for liposomal formulations. Data correspond to A: SPC, B: SPC:SA and C: SPC:CaS with 0.136 mM of FA or 0.136 mM of FA and 5 mM of VE before after and after pasteurization (PAST) in distilled water.

Table 2
Percentage of encapsulation efficiency of FA. Values are expressed as mean ± SD of three independent measurements after dialysis at room temperature at 2, 3, 6, 8 and 10 h in distilled water (the first five columns). Percentage of encapsulation efficiency after disruption with ethanol after 72 h of dialysis was showed in the last column. Statistical comparison was done in the liposomal formulation at each time of measurement respect of the measure of the second hour, through the Dunnett test. Different letters show significant difference results at least with $^{*}p < 0.05$, the samples with the same letters show no significant differences.

%EE by dialysis						%EE at 72 h
Time (hours)	[a]2	3	6	8	10	72
SPC	90.9 ± 3.5a	84.0 ± 3.4a	72.7 ± 4.5a	68.4 ± 16.2b	62.2 ± 9.6 b	37.6 ± 7.4b
SPC:SA	97.1 ± 2.1a	91.4 ± 4.2a	89.0 ± 4.4a	80.3 ± 0.7a	61.8 ± 13.9b	50.4 ± 13.8b
SPC:CaS	78.9 ± 4.0a	64.9 ± 2.1b	49.0 ± 0.4b	47.1 ± 1.0b	33.5 ± 4.1b	48.5 ± 0.9b

[a] In the measurement of the initial time (2 min) and of the first hour of dialysis the release of vitamin was not detectable. The only exception was in the SPC:CaS system in the first hour which presented a %EE of 91.80 ± 2.80.

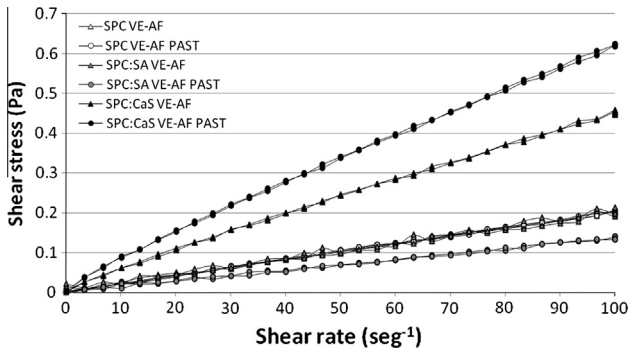

Fig. 4. Rheology of liposomal formulations. Results showed the systems with 5 mM of VE and 0.136 mM of FA before and after pasteurization (PAST) in distilled water.

Table 3
Rheological behavior of liposomal formulations. Depicts *n* (flow behavior index) and apparent viscosity at 100 s^{-1} values in liposomal formulations with 5 mM of VE and 0.136 mM of FA before and after pasteurization (PAST) in distilled water. Values were shown as mean ± SD of three independent samples. A statistical comparison with the Tukey test was performed comparing all the systems. Different letters show significant difference results at least with $^{*}p < 0.05$, the samples with the same letters show no significant differences.

Liposomal formulation	Apparent viscosity (Pa s) (10^{-3})	*n*
SPC VE FA	1.98 ± 0.04a	0.96 ± 0.01a
SPC VE FA PAST	2.04 ± 0.05a	0.98 ± 0.01a
SPC:SA VE FA	2.38 ± 0.35a	0.86 ± 0.04b
SPC:SA VE FA PAST	1.41 ± 0.07b	0.95 ± 0.03a
SPC:CaS VE FA	4.55 ± 0.09c	0.89 ± 0.01b
SPC:CaS VE FA PAST	5.97 ± 0.28d	0.87 ± 0.01b

even though they were lower than 1, they were very close to this value (Table 3). The Newtonian behavior remained after pasteurization, which confirms that heat treatment did not modify the viscosity of the system. This was corroborated with the apparent viscosity measurements which did not show a significant difference before and after pasteurization. These results are also related to the morphology and size distribution: when a low aggregation tendency was observed, favors a Newtonian behavior.

Before pasteurization the SPC:SA formulation with vitamins showed an increment of aggregation corroborated by the high $D_{4,3}$ value, morphology and multimodal particle size distribution. This result is related to a pseudoplastic behavior; as the n value was lower than 1. However, after pasteurization this system showed less aggregation, corroborated by a decrease of the $D_{4,3}$ value and reduction of aggregation observed by morphology and multimodal particle size distribution. This effect is related with the decrease of viscosity and a Newtonian behavior since the n value was close to 1 respect to the same unpasteurized system (Table 3).

The SPC:CaS system with vitamins showed the highest viscosity in comparison to the other two formulations, which increased after pasteurization (Table 3). The viscosity increment cannot be related to the values of $D_{4,3}$ and morphology, due to the low aggregation tendency obtained for this system. However, this result can be attributed to the effect of calcium associated to phosphatidylcholines causing membrane compaction (Marsanasco et al., 2011; Yeap et al., 2008) and the increment of membrane rigidity (Table 1, GP value). These latter factors are probably the responsible of increase in viscosity. The changes in the membrane packing modify viscosity of liposomes, because a higher membrane rigidity of L-α-Dipalmitoyl phosphatidylcholine liposomes increased viscosity (Mady, 2013). This higher membrane rigidity would also explain the pseudoplastic behavior of the SPC:CaS system, as it showed lower values of n respect to SPC (before and after pasteurization) and respect to SPC:AE before heat treatment.

The Newtonian behavior occurs in almost all the ordinary liquids like water, milk, apple juice, and corn syrup. And the pseudoplastic behavior is present also in ordinary foods like sauces and orange juice concentrate (Sharma et al., 2003). Thus, the behavior of this type of liposomes which resembles of Newtonian or pseudoplastic fluid is a great advantage to implement these systems to be applied in the food industry, considering a production thereof at larger scales.

3.6. Sensory evaluation

3.6.1. Triangular test

The numbers of the correct answers in triangular test for liposomes with vitamins were 28 for SPC, 42 for SPC:SA and 35 for SPC:CaS. The correct answer means that the evaluator could find the difference between commercial chocolate milk samples with or without liposomes.

Applying the statistical table for the triangular test with a significance level of 0.10 and 78 reviewers, the minimum number of correct answers from which the samples show significant differences is 32 (Meilgaard et al., 1999). From the above, it is concluded that SPC did not change sensory perception in chocolate milk. Instead, SPC:SA and SPC:CaS in commercial chocolate milk presented significant differences with respect to the same product without liposomes. So it was necessary to analyze if this difference was negative or positive with the test of acceptability.

3.6.2. Total assay acceptability

Results of the acceptability test are shown in Fig. 5. In the case of commercial chocolate milk with SPC and vitamins, it did not show changes in the acceptability with respect to the commercial product without liposomes. This result was maintained with or without knowledge of what was being evaluated. This result agrees with the triangular test which showed that commercial chocolate milk with this liposomal formulation did not provide sensory changes with respect to the commercial milk without liposomes.

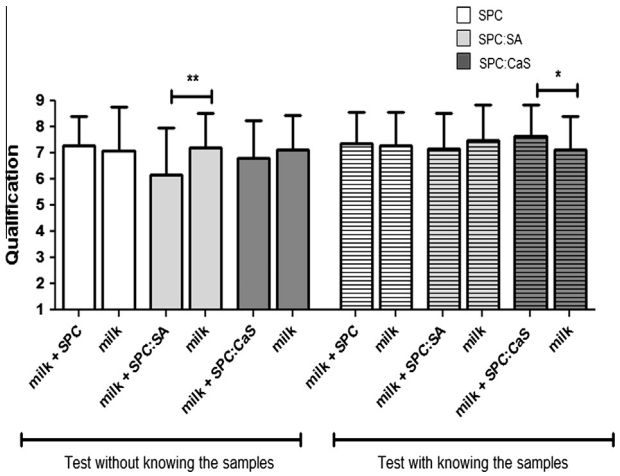

Fig. 5. Total assay acceptability of liposomal formulations. Qualifications of 40 panelists in commercial chocolate milk without liposomes or with 5 mM of VE and 0.136 mM of FA for each type of liposomal formulation. Statistics were performed using the test for paired samples between each milk sample with and without liposomes. The results with significant differences are shown as $^{*}p < 0.05$, $^{**}p < 0.01$.

In chocolate milk with SPC:SA and vitamins, although there were significant differences presented in the triangular test, in this evaluation it was found that this difference could modify acceptability. Thus, commercial milk with this liposomal formulation had significantly lower acceptability with respect to the commercial milk without liposomes, when the panelists did not know what was evaluating. Although it should be noted that milk with a nutritional promise seems to generate a positive effect based on the fact that when the evaluators knew what they were judging, there were no significant differences in milk samples with or without liposomes.

Finally, in milk with SPC:CaS and vitamins, although the triangular test showed differences, this liposomal formulation would not be affecting the acceptability because both milk samples with or without liposomes were not significantly different in trials with panelists that did not know what they were tasting. Again, as in the case of milk with SPC:SA, knowledge of the nutritional promise produced a positive effect with a score significantly higher respect to the milk without liposomes.

From the obtained results, it can be concluded that the addition of liposomes in milk not only adds nutritional value but also, in the case of SPC, does not affect its acceptability or differs from commercial milk. Moreover, in the case of SPC:CaS, it does not only affect the acceptability but it is increased when the nutritional promise is known. Finally, the SPC:SA system, although it has differences and showed lower acceptability, knowledge of the nutritional promise generated a positive effect on the acceptability of milk. Our results demonstrate an excellent acceptability of potential consumers when knowing that they are drinking the functional milk, which means an advantage in the sale and consumption of the product.

4. Conclusions

The three systems showed a high encapsulation of FA and a protective effect over this thermolabile vitamin that remained active after pasteurization.

The liposomes with vitamins remained stable after pasteurization, as demonstrated by oxidative stability, membrane packing, morphology and size. Besides, SPC system had a rheological behavior similar to a Newtonian fluid whereas that SPC:SA and SPC:CaS presented a pseudoplastic one. Adding vitamins to SPC:SA and SPC:CaS systems favored liposomal aggregation and membrane rigidity, respectively. A relationship was observed between these properties: if membrane rigidity and/or aggregation increase, favor the pseudoplastic behavior and the increment of apparent viscosity.

The incorporation of SPC or SPC:CaS did not change the acceptability of commercial milk. Also, when the potential consumer beforehand knew the addition of nutritional bioactive compounds, it generated a positive effect on acceptability for all the formulations.

From all the above discussed, it can be concluded that these liposomal formulations are suitable for food industry application, incorporating bioactive compounds and generating functional chocolate milk.

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