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Evaluation of additives containing omega-3, omega-6 and vitamins E and C to generate a functional food in chocolate milk

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Running title: Characterization and application of additives with lipids

Keywords: Lipid-SPC, functional-food, characterization, rheology, sensory-analysis.

Abbreviations: CaS: calcium stearate; **EE%:** percentage of encapsulation efficiency; **LTLT**: low temperature long time; **MC540:** merocyanine 540; **n:** flow behavior index; **ORAC:** oxygen radical absorbance capacity; **PC:** partition coefficient; **SA:** stearic acid; **SPC:** soy phosphatidylcholine; **TBA:** thiobarbituric acid; **TBARS:** thiobarbituric acid reactive species; **VC:** vitamin C; **VE:** vitamin E; **ZP:** zeta potential.

Abstract

Additives were made by soy phosphatidylcholine (SPC)-based liposomes to encapsulate vitamin E (VE) and vitamin C (VC), and incorporated in pasteurized chocolate milk to generate a functional food. SPC is a natural lipid which contains essential fatty acids like omega-3 and omega-6. Stearic acid (SA) or calcium stearate (CaS) was incorporated as bilayer stabilizer.

Size and surface charge were studied by light scattering and zeta potential, respectively. Morphology and structure were analyzed by optical and transmission electron microscopy. Membrane packing was studied with probe merocyanine 540 and oxidative stability was analyzed by the TBA and ORAC methods. The studies were made before and after pasteurization to obtain information about the thermal stability. All formulations showed significant stability of analyzed parameters even after pasteurization. Also, they presented an important protective effect over thermolabile VC which demonstrated an antioxidant action after pasteurization.

Additionally, release of vitamins was studied by 72 hours of dialysis. All additives were able to retain a high percentage of VE (all over 85%) and VC (c.a. 30%).

For food application, a sensory evaluation was performed in chocolate milk with liposomes and the rheological behavior of additives was studied. Data supports the applicability of the additives especially for SPC:SA.

1. Introduction

It is important to consume essential fatty acids because they are necessary for the normal functioning of the body and must be ingested through the diet [1]. Example of essential fatty acids like linolenic acid (ω-3) and linoleic acid (ω-6) are contained in soy phosphatidylcholine (SPC) which is a natural lipid. Also, the vitamins have important functions in certain metabolic processes in the human body. For example the vitamin E (VE) or α-tocopherol is the major liposoluble antioxidant. It protects cellular membranes against oxidative damage and is related to the decrease of atherosclerosis. Another antioxidant hydrosoluble vitamin is the ascorbic acid or vitamin C (VC) that participates in various oxidation reactions such as the proline and lysine which are essential components of collagen. It also has the property of capturing peroxyl radicals of lipids in cells favoring their stability [2].

However, it is not easy to add these vitamins to aqueous foods while retaining their activity. For instance, VE is liposoluble and destroyed by UV light [2], while VC is thermolabile and it is dramatically reduced by different heat treatment process [3]. The application of liposomes as carriers for vitamins solves the problem of adding liposoluble vitamins in foods of high water activity. 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 [4]. Besides, for VE, it was demonstrated that this vitamin mixes perfectly with the phosphatidylcholine of the bilayer [5]. Also, absorption and bioavailability is increased when the VE is encapsulated by liposomes [6]. If liposomes are made with SPC allow the incorporation of essential fatty acids, VE and VC in food generating a functional food. Typically, a food marketed as functional contains added, technologically developed ingredients with a specific health benefit [7]. The liposome will incorporate a nutritious substance which chemically modifies the food, thus they are considered as additives (Argentine Food Code). It is very important that additives also showed additional functional effects [8].

In the food industry, for a given industrial application, membrane stability and structure are important factors when designing liposomes [4], where several studies are pursued including characterization of liposomes. Besides, for an industrial application and production line of a new food, it is very important to assess its acceptability. In case consumers have a negative

perception of additives [9] it is very important to know the product acceptance where additives are incorporated.

The aim of this work was the design, characterization and application of additives made of different liposomal formulations based on SPC. The liposomes allow the incorporation of VE, VC, omega-3 and omega-6 to fortify pasteurized chocolate milk. Stearic acid (SA) and calcium stearate (CaS) were incorporated to stabilize the lipid bilayer by increasing rigidity. Besides, CaS incorporates a mineral (calcium ion) increasing the food nutritional value. Size and surface charge were analyzed by light scattering and zeta potential, respectively. Morphology and structure were studied by optical and transmission electron microscopy. Membrane packing was studied with probe merocyanine 540 and oxidative stability of liposomal formulations was determined using two independent methods: thiobarbituric acid (TBA) and oxygen radical absorbance capacity (ORAC). All studies were performed before and after pasteurization in order to have information about the effect of heat treatment. The experiments mentioned were made in a food model system, to avoid fluctuations in data due to the presence of other components of the food product. In addition the percentage encapsulation efficiency (EE%) of each vitamin was determined after dialysis for 72 hours after pasteurization. Finally, for food application, the rheological behavior and the organoleptic properties were study in the liposomes.

2. Materials and methods

2.1. Materials

SPC was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). SPC had purity higher than 99% and respect to the fatty acid composition contains palmitic (14.9%), stearic (3.7%), oleic (11.4%), linoleic (63%), and linolenic (5.7%) acids, according to the company's specifications. SA and CaS were purchased from Vitalquim (Buenos Aires, Argentina). All these components comply with local food regulations and are classified as additives according to Good Manufacturing Practices (Mercosur Resolution No. 31 of 1992) which may be used without restrictions. VE was obtained from Parafarm (Buenos Aires, Argentina) and VC was obtained from Baker (New Jersey, USA). Merocyanine 540 was obtained from Sigma-Aldrich (Missouri, USA).

2.2. Preparation of liposomes

Multilamellar liposomes were prepared by the dehydration-rehydration method [10]. 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 ºC. 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 of 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 taken and 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 VC, fresh solutions of this vitamin were prepared at the moment of rehydration. VC was weighted and diluted with distilled water to reach a 90 mM concentration.

2.3 Application of food model system

Assays were performed in distilled water which that simulates aqueous food having a pH higher than 5 (Mercosur Resolution No. 30 of 1992), such as chocolate milk.

2.4. Structure and morphology determination

2.4.1. Optical microscopy

Micrographs of liposomes with vitamins were obtained with an optical microscope operating at 400x magnification and using an adapted digital camera (Canon A570 IS; Malaysia) at 4x optical zoom.

2.4.2 Transmission electron microscopy

Negative stain micrographs were prepared on copper grids covered with a formvar/carbon film, 300 mesh (TED PELLA, INC, USA). A 1 μl drop of the liposomal dispersion was set onto the copper grid and, after one minute, liquid was adsorbed with filter paper down to a thin film. Negative staining was performed with a drop of a 1% uranyl acetate solution. After 1 minute,

this drop was removed with filter paper and the resulting stained film was viewed and photographed with a Zeiss EM 109 Turbo transmission electron microscope (TEM), at an accelerating voltage of 80 kV [11].

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 defined as:

$$D_{3,2} = \frac{\sum_{i=1}^{n} S_i d_i}{\sum_{i=1}^{n} S_i} = \frac{\sum_{i=1}^{n} n_i d_i^3}{\sum_{i=1}^{n} n_i d_i^2} \quad (1)$$

$$D_{4,3} = \frac{\sum_{i=1}^{n} V_i d_i}{\sum_{i=1}^{n} V_i} = \frac{\sum_{i=1}^{n} n_i d_i^4}{\sum_{i=1}^{n} n_i d_i^3} \quad (2)$$

where *ni* is the number, *Si* the surface, and *Vi* the volume corresponding to all particles with diameter *di* [11].

2.6. Determination of surface charge

Liposomal suspensions were used at 1/100 dilution of the formulations with distilled water at 5 mM concentration for the determinations of zeta potential (ZP). Measurements were made on a Nanosizer (Malvern Instruments) with a 1 ml cuvette. Each measurement was performed in quintuplicate, and each event was recorded by accumulation of 10 consecutive scans, which were processed to obtain an average value. The analysis software provided by the manufacturer was used.

2.7. Membrane packing

The probe, merocyanine 540 (MC540) is located in the membrane phospholipids with its polar sulphonated group towards 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 [12].

Liposomes formulations (with or without pasteurization) were diluted with distilled water until a concentration of 0.868 mM. MC540 stock concentration was 4.344×10^{-3} mM was incorporated into the vesicles; probe/lipid ratio was 1/200 [13].

A scan of each sample between 400 and 600 nm was obtained with a UV-VIS spectrophotometer (Shimadzu), at room temperature as previously described [14].

The partition coefficient (PC) of the probe was calculated from the equation: PC= A570/A530, relating absorbance of the monomer in the non-polar phase with the absorbance of the monomer in the aqueous phase [14].

2.8. Oxidative stability of liposomes

2.8.1. TBA method

Lipid peroxidation was followed by the TBA method, as described before [11].

2.8.2. ORAC method

For each determination, an aliquot of a sample dilution (1/250) with distilled water was prepared at room temperature. Each aliquot was incubated for 2 min at 37ºC with 75 mM Buffer Phosphate (pH=7) and 10 µM fluorescein. After the addition of 275 mM 2,20-azo-bis(2-amidinopropane)-dihydrochloride (AAPH), fluorescence intensity was determined at 37ºC every 60 seconds for 15 minutes. The consumption of fluorescein was assessed from the decrease in the sample fluorescence intensity (excitation at 493 nm and emission at 515 nm). This consumption was associated to its incubation in presence of AAPH and estimated from fluorescence (f), employing an F-3010 Fluorescence Spectrophotometer (HITACHI). Calculated values of f/f_0 were plotted related to time. To obtain f_0, distilled water was used instead of a sample. The area under the curve (AUC) was calculated up to 5 % of the initial value and was used to obtain the ORAC values according to the following equation [15]:

$$ORAC = \frac{(AUC - AUC_0)\ f.\ [Trolox®]}{(AUC_{Trolox®} - AUC_0)}$$

AUC= Sample area under the curve between time cero and final time corresponding to 5 % of the initial value.

AUC_0= Control area under the curve.

$AUC_{Trolox®}$= Trolox® area under the curve.

F= dilution factor, ratio between each sample concentration volume and final volume.

[Trolox]= Trolox® concentration (μM).

Trolox® equivalents were corrected by the dilution factor and by the sample volume used, expressed as ORAC units (U ORAC = loss of fluorescence from 1 μM Trolox® solution). The calibration Trolox® curve was y=0.5533 x-0.2512 considering 9 data points in μM (0, 0.5, 1, 2, 3, 6, 10, 20, 30, 40) with an R^2=0.9849.

2.9. Percentage of encapsulation efficiency

Leakage of VC and VE (separately) was analyzed after 72 hours of dialysis. 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 hours at room temperature to remove the non-encapsulated vitamin remaining in the dispersion medium.

For determination of VC, at different times 0.2 mL of sample was withdrawn. For maintenance dialysis volume was replaced with 0.2 ml of distilled water in it.

For determination of VE, 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 ethanol was added to aliquot withdrawn for the measurement and 0.2 ml of distilled water was added to dialysis medium to maintain final volume (5 mL).

VC or VE were also measured by absorbance at 247 and 286 nm, respectively in an UV-Vis spectrophotometer (Nanodrop 1000, USA).

After 72 hours of dialysis, the remaining sample containing liposomes and retained VC or VE were disrupted by the addition of two volumes of absolute ethanol. The VC or VE in liposomes after the disruption with ethanol was also measured.

The percentage of encapsulation efficiency (EE%) was calculated according to the following equation [16]:

$$\% \text{ EE} = \frac{\text{vitamin encapsulated in liposomal formulation x 100}}{\text{Total vitamin incorporated in liposomal formulation}}$$

2.10. Rheology

The behavior of liposomal dispersions in food model system was 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) at 21 °C.

Besides, the flow behavior index (n) value was obtained using the model of power law. Ostwald's equation or power law is calculated as follows: $\tau = k.D^n$, where τ is the shear stress (MPa) and D is the shear rate (s^{-1}). If n=1, the fluid is Newtonian; if n<1, the fluid is pseudoplastic; and if n>1, the fluid is dilatant [17].

2.11 Effects of heat treatment

In order to analyze the effects of increasing temperature on liposomal systems, they were suspended in distilled water, and incubated at 65° C for 30 minutes to simulate the pasteurization process of low temperature long time (LTLT). 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.

2.12. Organoleptic properties

2.12.1. Processing of liposomes incorporated in chocolate milk

Chocolate milk of an Argentine trademark was used (Cindor® of Danone S.A.). The day before the sensory evaluation test, liposomes were prepared and pasteurized fulfilling the Good Manufacturing Practices. It is important to mention that in the preparation of liposomes, the rehydration was made with safe drinking water. Pasteurized liposomal formulations with 5 mM of VE and 90 mM of VC were added to chocolate milk in a 1/100 ratio and they were kept at 4 °C until the sensory evaluation.

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

2.12.2. Overall acceptability test

The purpose of this study was to evaluate the acceptability of chocolate milk with liposomes. The evaluators knew beforehand which commercial chocolate milk had the additives. Thus, the essay will provided information about the acceptability of the product and the effect of such knowledge in sensory perception of potential consumers.

For affective tests, like overall acceptability, it is necessary to have a minimum of 30 untrained judges and they must be habitual or potential consumers and buyers of the type of food in question [18]. For that, the panel was conformed of 40 potential consumers; men and women over 18 years old that usually consumers the commercial chocolate milk. The evaluators were instructed to perform the tests after they had signed a consent form. They were called and it was presented two samples: commercial chocolate milk with liposomes as additives (with vitamins) and the commercial chocolate milk without liposomes.

Samples at room temperature were given to each evaluator at the same time in 200 mL disposable cups; each cup had 30 mL of chocolate milk with or without liposomes. The

randomness of the samples was ensured throughout the test. Between samples, evaluators were instructed to drink mineralized water to avoid sensory fatigue. Hedonic rating scales associated with score were 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 [19].

2.13 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. Characterization of liposomes in food model system

3.1.1. Structural and morphological characteristics

TEM is one of the adequate methodologies to obtain information about the lamellar structure of liposomes [20]. The three liposomal formulations with vitamins (Figures 1a, 1b, 1c, 1d, 1e and 1f) presented a lamellar structure with a central core with spherical and non-spherical shapes. Moreover, the liposomes proposed in this work showed thermal stability maintaining their structure even after the pasteurization process (Figures 1d, 1e, and 1f).

Micrographs of liposomes with vitamins, before and after pasteurization, in distilled water are shown in Figures 1g, 1h, 1i, 1j, 1k and 1l. Size heterogeneity was observed in all cases with or without pasteurization, which is related to the preparation method and the composition of the formulation [11]. All formulations showed isolated liposomes as well as aggregation. Besides, the three liposomal formulations showed aggregation which remained after pasteurization. Other authors [21] reported liposome aggregation (mainly phosphatidylcholine and phosphatidylethanolamine) even at different pH. Liposome aggregation is a physicochemical mechanism that depends on pH, heat treatment, external load and the presence of cations among others [11, 21].

3.1.2 Liposomal size

The volume size distributions were bimodal for all the systems (both pasteurized and unpasteurized). The exception was for pasteurized SPC:SA and SPC:CaS (before and after pasteurization), in presence of vitamins which showed multimodal size distributions (Figure 2). The range of particle sizes in liposomes without vitamins which was between 0.5-200 μm and the values of $D_{3,2}$ and $D_{4,3}$ (Table 1) were in agreement with those reported for multilamellar liposomes [4] and the size of the morphology study (Figure 1).

In systems without vitamins, the addition of SA or CaS in liposomes did not produce changes in particle size distributions in comparison to the SPC system (Figure 2) while this effect was not detected by the $D_{3,2}$ and $D_{4,3}$ values, as they did not show significant differences between SPC:SA or SPC:CaS with respect to SPC (Table 1, Dunnett test, statistic not shown). This result shows that with CaS or SA addition, the basic SPC system is stable.

Addition of vitamins to SPC system did not modify size, even after pasteurization (Table 1, Figure 2a), revealing a high stability of this formulation.

The size distribution of the SPC:SA formulation with both vitamins, either with or without pasteurization (Table 1 and Figure 2b) shifted to higher values. These results are consistent with the $D_{4,3}$ values showed in Table 1 which presented a significant increment respect to the control, and they indicate increment of aggregation of particles (with or without pasteurization). These results are in accordance with the morphology in Figures 1b and 1e, where the system presented a higher aggregation. It is worth mentioning that the volume size distribution is more sensitive to larger particles, so this is a very useful parameter to study liposome aggregation [11]. Also, the addition of vitamins showed a significant increase in the $D_{3,2}$ value before and after pasteurization, related to an increment of liposomal size.

In SPC:CaS, the addition of both vitamins showed a significant shift in the size distribution to higher values(Table 1 and Figure 2c) and a significant increase in the $D_{4,3}$ values were show with respect to the control (before and after pasteurization). The same happened in the $D_{3,2}$ values. This system showed a higher aggregation corroborated in the particle size distribution, $D_{4,3}$ value and morphology (Figures 1c and 1f). This results are related to the charge effect of VC (pKa_1 = 4.04) in distilled water lowering the pH from 5.7 to 3.26 (measured by pH meter)

and the acidic environment promotes the dissociation of CaS. According to other studies [22] they demonstrated that lower the pH, greater the dissociation of the CaS and the release of calcium ion to the medium. In this situation, Ca^{2+} is free to bind with two adjacent phosphatidylcholines, inducing more compact areas in the bilayer packing, accompanied by a structural reorganization of the molecules of phosphatidylcholines. The calcium ion is located at the bilayer level-interface, where negative charges of phosphate groups attract the Ca^{2+}, while the positive charges of the trimethylammonium group repels it. This additional repulsion supports the position of calcium along the area of the phosphate group [23]. Besides, Also, Ca^{2+} ions favor the dehydration at the interfacial region facilitating vesicle aggregation by lowering the repulsive hydration forces [24]. These results indicate that the concentration of VC could be exerting a significant influence on the system with CaS, favoring the liposome aggregation and increased of size.

Comparing each system before and after pasteurization, only SPC:SA with vitamins showed a significant increase in the $D_{4,3}$ values, which means that size and aggregation of liposomes have increased as well. In SPC:CaS systems, the heat treatment decrease the $D_{4,3}$ value related to a reduced aggregation tendency. The aggregation is an example of physical instability [25], for that it can be inferred that a decrease in aggregation favors the stability of SPC:CaS system. And for the other systems, no significant differences between samples with or without heat treatment were observed, demonstrating good thermal stability of them in terms of particle size distribution and tendency to aggregate.

3.1.3. Surface Charge

In the results of ZP (Figure 3), the SPC system provided values of -36.62 ± 0.77. Such a value agrees with those reported by other authors. According to other studies [16], in Egg phosphatidylcholine liposomes at pH of 6.8 showed a -45 mV value of ZP. Phosphatidylcholine in water at a neutral pH presented values of ZP of about -40 mV /-50 mV [26]. When analyzing the effect of the composition of the liposomal formulations without vitamins, SPC:SA increased significantly its ZP value regarding SPC. The SA (pKa=4.5) in the pH of distilled water provide an extra negative charge to the system. This explains the increase in the negative value of ZP. Instead, SPC:CaS had a significantly lower value compared to SPC. This result may be related

to two factors: the first is that the pH of distilled water in the system without VC did not favor the dissociation of CaS, so that there is no contribution charge; the second is that SPC:CaS has a 1:0.25 ratio, there is a 25% lower rate of SPC that is responsible for providing the load favoring the negative value.

The addition of VE showed the same trend in the results without vitamins but with a decrease in the negative values of ZP. This result coincides with the studies reported by other authors [27], where it was shown that zeta potential has decreased by increasing the α-tocopherol content in nanoparticles.

The sign of ZP changed in systems with VC, becoming positive. The same effect systems presented with both vitamins E and C. VC (PKa_1 = 4.04) in distilled water is charged negatively by reducing the pH of distilled water from 5.7 to 3.26, as mentioned above. In this case, the addition of protons from VC could exceed the number of negative charges on the phosphatidylcholine, compensating for these loads and generating a positive sign in the ZP value.

Considering the results of the particle size distribution, the SPC:SA and SPC:CaS systems with vitamins are those with higher aggregation. This physicochemical mechanism is related to the surface charge. The neutralization of the charges favors aggregation, so a lower surface charge induces the presence of aggregates [28]. In our results, SPC:SA and SPC:CaS with vitamins presented the lower ZP value and the highest amount of aggregates.

3.1.4. Membrane molecular packing

In the results of the packing of the membrane with the probe MC540 (Table 1) SPC:SA and SPC:CaS showed a PC value significantly higher related to SPC. The same result was obtained after heat treatment (*** $p < 0.01$ in both cases with the Dunnett test, statistic not shown in Table 1). This result indicates that a greater amount of probe enters in the polar area of the membrane associated with a greater fluidity [14]. Considering that MC540 is located slightly above the domain of the glycerol backbone of phospholipids [12], the addition of SA or CaS favors the entry of this probe. This result could be related to the polar heads of the membrane and the increase of 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 [29]. The formation of the hydrogen bonds in the polar heads favors a greater penetration of water which promotes the formation of more fluid domains [30]. Regarding the SPC:CaS system, the effects of acid pH and packaging generated by phosphatidylcholines and free calcium as mentioned before, were effects which could generate areas in the polar heads of the membrane that favors the entry of MC540. Also, SPC:CaS was the formulation that favored the highest probe entry and the least ZP (Figure 3). It could be related to the effect of ZP value with the fact that the SPC:CaS system presented the greatest value of PC. According to other studies [31] if liposomes have lower surface charge, the incorporation of the anionic MC540 will be favored. But this is not the only factor to be considered in the discussion, taking the case with SPC:SA, that even though it has a more negative value of ZP (Figure 3), it also shows a higher PC value compared with SPC.

In all three systems, the addition of vitamins showed a significant increase in the PC values with respect to the system without vitamins; except the pasteurized SPC:CaS system that did not show any significant difference. This result indicates that the addition of vitamins favors the entry of the probe. With respect to VE, this result is related to the effect generated by this vitamin in the lipid bilayer. The presence of VE in the bilayer produces a general increase in the mobility of the polar head group while molecules of vitamin are inserting between lipids reducing mobility of the bilayer center [32]. This also explains the results obtained for ZP values of systems with VE (Figure 3), wherein a decrease in surface charge related to formulations without vitamins was observed. Having a lower surface charge entering MC540 is favored [31], as mentioned before.

The addition of VC in distilled water releases a proton, and influences the entry of MC540 inside the membrane. Being exposed to negative charges of the phosphatidylcholines might interact with protons released by vitamin, corroborated with the change in value of the ZP from negative to positive (Figure 3). This neutralization of charges and lower surface charge favor the entry of the probe.

Finally, by comparing each system before and after the heat treatment only SPC:SA system without vitamins showed a significant increase in the PC value. And in the systems with vitamins and the most important because will be used to fortify the chocolate milk did not show significant differences and for only SPC:CaS presented a significant decrease in the PC value.

The same result was obtained for SPC without vitamins. For the rest of the systems did not show significant differences. This finding is noteworthy because it shows the thermal stability of the membrane regarding the polar area of the molecular membrane packing.

3.2. Oxidative stability of liposomes in food model system

In systems without vitamins, the results showed 0.10 ± 0.01 μM, 0.13 ± 0.01 μM and 0.10 ± 0.02 μM values in TBARS (Figure 4a) for SPC, SPC:SA and SPC:CaS, respectively. Also, SPC:CaS and SPC:SA did not show significant differences in the production of TBARS regarding SPC (Dunnett test). In this work, SPC-based liposomes without vitamins revealed low TBARS values. This result agrees with those published [33], which showed that SPC presented a low tendency in peroxidation with the TBA method. The same result was obtained with the ORAC method in which SPC:SA and SPC:CaS did not show significance difference respect to SPC (Figure 4b). The oxidative stability could be enhanced by lipids, such as SA and CaS that may increase the stability thereof against peroxidation. According to other studies [34], the incorporation of saturated fatty acids in the bilayer increases rigidity causing a decrease in the mobility of alkyl chains of the bilayer and in the water flow. Considering that the reactions are favored by the presence of water in the bilayer, this reduced flow causes the decrease in velocity of oxidative reactions. In our work, the possible stability provided by SA or CaS might not be displayed due to low oxidation in the SPC-based system.

The three liposomal systems with VE did not show significant differences in the results of ORAC (Figure 4b) with respect to their systems without vitamins (controls), probably because of the low peroxidative trend formulations, as mentioned before. The same results were obtained for SPC and SPC:SA with the TBA method (Figure 4a). Only the SPC:CaS system presented a significantly higher peroxidation. According to bibliography, certain vitamins such as VE and VC may also have a pro-oxidant or antioxidant action. This action depends on several factors, such as the induction of lipid peroxidation, the composition of the liposomes and the way of introducing these antioxidants to the liposomes. For example, in the case of the VE, the addition of ethanol to form dipalmitoylphosphatidylcholine liposomes generated a pro-oxidant effect in the presence of Cu^{2+}. VE in aqueous media has been attributed the pro-oxidant effect of tocopheryl radicals formed and their interaction in the aqueous zone to reduce Cu^{2+} to Cu^{+} [35].

In this work, the presence of VE could generate a pro-oxidant effect reducing Fe^{+3} to Fe^{+2} only in the SPC:CaS system.

The three formulations with VC or along with VE showed a significantly higher TBARS in relation to their respective controls (Figure 4a). This result can be related to the pro-oxidant effect of VC, which depends on several factors, as mentioned above [35]. Even also in the case of the VC, other studies [36] demonstrated that SPC liposomes suspended in neutral pH and VC added in the presence of Fe^{2+} are able to generate a pro-oxidant effect. VC can promote lipid oxidation as it can reduce catalytically metals such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, respectively [37]. This result was corroborated comparing systems with both vitamins with respect to the same formulations with VC and SPC:SA and SPC:CaS did not show significant differences (statistic not shown in Figure 4a, Tukey test), demonstrating that these results are due to the action of VC. Only SPC with vitamins presented a significantly decrease in TBARS with respect to their system with VC. This result could be related to the thermal degradation of VC due to the pasteurization or as an antioxidant effect of VE, both counteracting the pro-oxidant effect of VC.

The ORAC results showed that systems with VC alone or with VE showed a significantly higher value compared to controls (Figure 4b). Also, systems with both vitamins did not show significant differences compared with VC systems, putting forward the idea that the effect is only related to the action of VC (statistic not shown in Figure 4b, Tukey test). In these results, the antioxidant activity of VC was demonstrated effectively concluding that the study of TBARS in the presence of Fe^{3+} favored the pro-oxidant effect of the vitamin. What is striking about these results is the observed pro-oxidant and antioxidant activity of the VC after pasteurization. The effect of heat treatment, including the LTLT, dramatically reduced this vitamin stability [3]. Then, on the basis of our results, it is possible to infer a protective effect of liposomal systems on this thermolabile vitamin, which is encapsulated in the aqueous interior (see point 3.4).

It is important to mention that the incorporation of vitamins favor the incorporation of MC540 (Table 1), related with associated with greater fluidity in the polar area of the membrane [14]. The peroxyl radical's phospholipids become more polar and locate near by the polar area of the membrane [38]. Thus the polar region of the membrane is more fluid favoring the interaction of the vitamin with peroxyl radicals, which would help to the antioxidant activity of VC.

3.4 Encapsulation efficiency

Dialysis data obtained for liposomal formulations with VC are shown in Table 2. The liposomes retained a percentage of VC and the system with SA showed the highest initial %EE for this vitamin (***p<0.001 respect to SPC and SPC:CaS with the Tukey test, statistic not shown in table 2). According to the discussion mentioned above, in relation to peroxidation stability and activity of this vitamin; it is possible to infer that encapsulation efficiency allowed vitamins to maintain their activity. VC being active after pasteurization means that all liposomal formulations effectively protected the vitamin throughout the process as mentioned before. Moreover, the three systems showed a similar release of the vitamin in the dialysis without significant differences in the measures respect to the first hour. The only exception was the SPC:SA system which presented significant reduce in the value after 2 hours.

It is important to mention that the release of VE during the dialysis essay was not detectable. These results were as expected since VE is a liposoluble vitamin and it is incorporated almost entirely in the bilayer [5].

Table of supplementary material (Table S1) shows data of %EE after 72 hours of dialysis liposomes and the disruption with ethanol. The three liposomal formulations showed a high and similar EE% of VE, without significant differences between them. With respect to the VC, comparing the % EE alter 72 hours respect to the first hour of dialysis, only the SPC:SA system showed a significant decrease in the value (***p <0.001, Dunnett's test, statistic not shown in Table S1) possibly related to the highest baseline vitamin encapsulation presented by this formulation. On top of that, after 72 hours of dialysis the three systems had a EE% similar without significant differences between them.

Besides, although dialysis was made in the food model, it should be mentioned that liposomes are made to be incorporated to the milk in the ratio 1/100. As the trial of dialysis was performed with the same liposome/volume ratio, the obtained results offer information about the encapsulation efficiency and the effect of dilution with the milk.

3.5. Rheology

Although the apparent viscosity values did not present significantly different between the systems, before and after pasteurization (Table 3); a tendency of higher viscosity in the SPC system followed by the SPC:SA formulation was observed.

Further, while the values of n did not show significantly different between the systems, also before and after the heat treatment (Table 3), SPC (with or without pasteurization) and SPC:SA formulations presented a trend similar to Newtonian fluid behavior. This result was corroborated by the values of n (with or without pasteurization) which were lower than 1, but close to this value. While in the pasteurized SPC:SA system and SPC:CaS (with or without pasteurization), present a trend towards more pseudoplastic behavior with a value lower than 1.

It is important to mention that the heat treatment did not change the viscosity and the behavior of the three systems, corroborated in the statistic of the table 3. Besides, a relationship between aggregation and rheological behavior of the systems was established. SPC:SA formulation with vitamins showed an increment in aggregation after pasteurization; this was corroborated by the $D_{4,3}$ value and multimodal particle size distribution. This result could be directly related to the pseudoplastic behavior in this formulation (Figure S1 of supplementary material). In the case of SPC:CaS formulation with vitamins also showed an increase in aggregation, reflected in a higher $D_{4,3}$ value and multimodal size distribution related to a pseudoplastic behavior (Figure S1).

As reported by other authors [25] when the flocs decrease their effective volume fraction it contributes to a decrease in the viscosity. SPC:SA system and especially SPC:CaS formulation showed the lowest tendency in the viscosity in comparison to SPC (Table 3). This is a result linked to the liposomal aggregation: when viscosity did not increase the effective volume fraction of the aggregates was less than the total volume. Morphology results obtained (see Figures 1h, 1i, 1k and 1l) with SPC:SA and SPC:CaS showed isolated aggregates which reduce the effective volume fraction respect to the total volume.

The Newtonian behavior occurs in almost all the ordinary liquids like water, milk, apple juice, corn syrup, etc. The pseudoplastic behavior is present also in ordinary industrial foods like sauces and concentrates orange juice [17]. Thus, the behavior of this type of liposomes which resembles a Newtonian or pseudoplastic fluid is a great advantage to implement in the food industry, considering a production thereof at larger scales.

3.6 Acceptability

In Figure 5, the acceptability test results are shown. Commercial chocolates milk with SPC and SPC:CaS showed lower acceptability. In contrast, the chocolate milk with the SPC:SA system did not change the acceptability with respect to the commercial milk and did not change the sensory perception considering that the evaluators knew what they are judging . These results allow inferring that the additive made of SPC:SA is the most suitable to fortify milk with vitamins E and C. It is noteworthy that the increasing aggregation after pasteurization presented by SPC:SA with vitamins (see Table 1 and Figure 2b) did not affect the sensory characteristics of the final product.

4 Conclusions

In this work, all the additives showed high VE and VC encapsulation, being maximum in SPC:SA for the initial %EE. And in the case of VC, the pro-oxidant or antioxidant activity was persistent even after treatment. This dependence was reflected by the protection of the liposomes even for the thermolabile VC. These results allowed us to infer that all are excellent candidates to be used in aqueous pasteurized food like milk; they enable vitamins food activity and at the same time the addition of liposoluble VE.

Furthermore, additives maintained oxidative, membrane packing, structure, morphology and size stability even after the heat treatment.

Also, additives presented rheological behavior similar to Newtonian or pseudoplastic fluid. The last behavior was favored by liposome aggregation. In SPC:SA with vitamins increased in aggregation after pasteurization but did not affect the sensory characteristics of the final product.

For all the above mentioned, the SPC:SA system showed the best applicability for an industrial level application in chocolate milk and presented great stability after pasteurization.

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6 Conflict of interest

All the authors declare that have no financial and commercial conflicts of interest.

7 References

[1] L. B. López, M. M. Suárez: Fundamentos de Nutrición normal. El Ateneo (Argentina) 2003.

[2] E. Primo Yúfera: Química de los Alimentos. Síntesis (España) 1998.

[3] Abioye, A. O., Abioye, V. F., Ade-Omowaye, B. I.O., Adedeji, A. A., Kinetic modeling of ascorbic acid loss in baobab drink at pasteurization and storage temperatures. *J. Environ. Sci. Toxicol. Food. Tech*. 2013, *7(2)*, 17-23.

[4] Keller, B. C., Liposomes in nutrition. *Trends in Food Sci. & Technol.* 2001, *12*, 25–31.

[5] Wang, X., Quinn, P. J., The distribution of α-tocopherol in mixed aqueous dispersions of phosphatidylcholine and phosphatidylehanolamine. *Biochim Biophys Acta.* 2000, *1509*, 361-372.

[6] Gonnet, M., Lethuaut, L., Boury, F. New trends in encapsulation of liposoluble vitamins. *J. Controlled Release*. 2010, *146*, 276–290.

[7] Niva, M., 'All foods affect health': Understandings of functional foods and healthy eating among health-oriented Finns. *Appetite*. 2007, *48*, 384–393.

[8] Hancsóka, J., Bubálika, M., Töro, M., Baladincz, J. Synthesis of fuel additives on vegetable oil basis at laboratory scale. *Eur. J. Lipid Sci. Technol*. 2006, *108*, 644–651.

[9] Varela, P., Fiszman, S. M. Exploring consumers' knowledge and perceptions of hydrocolloids used as food additives and ingredients. *Food Hydrocolloid.* 2013, *30(1),* 477–484.

[10] Bangham, A. D., Model membranes. *Chem. Phys. of Lipids*. 1972, *4*, 386–392.

[11] Marsanasco, M., Márquez, A. L., Wagner, J. R., Alonso S del V., Chiaramoni, N. S., Liposomes as vehicles for vitamins E and C: An alternative to fortify orange juice and offer vitamin C protection after heat treatment. *Food Res. Int.* 2011, *44*, 3039–3046.

[12] Lelkes, P. I., Miller, I. R., Perturbations of membrane structure by optical probes: I. Location and structural sensitivity of merocyanine 540 bound to phospholipid membranes. *The J. Membr. Biol.* 1980, *52*, 1-15.

[13] Bernik, D. L., Disalvo, E. A., Gel state surface properties of phosphatidylcholine liposomes as measured with merocyanine 540. *Biochim. Biophys. Acta*. 1993, *1146*, 169-177.

[14] E. A. Disalvo, J. Arroyo, D. L. Bernik: Surface properties of liposomes as measured with fluorescent methods. In Methods in Enzymology: Vol-367, Part-A: Liposomes. Ed. N. Duzgunes, Academic Press (United States) 2003, pp. 213 – 233.

[15] Atala, E., Vásquez, L., Speisky, H., Lissi, E., López-Alarcón, C., Ascorbic acid contribution to ORAC values in berry extracts: An evaluation by the ORAC-pyrogallol red methodology. *Food Chem*. 2009, *113*, 331-335.

[16] Xia, S. Q., Xu, S. Y., Ferrous sulfate liposomes: preparation, stability and application in fluid milk. *Food Res. Int.* 2005, *38(3)*, 289-296.

[17] S. K. Sharma, S. J. Mulvaney, S. S. H. Rizvi: Ingeniería de los alimentos. Operaciones prácticas y de laboratorio. Limusa S. A. (México) 2003.

[18] A. Anzaldúa-Morales: La evaluación sensorial de los alimentos en la teoría y la práctica. Acribia (España) 1994.

[19] M. Meilgaard, G. V. Civille, T. B. Carr: Sensory Evaluation Techniques. CRC Press (United States) 1999.

[20] Chen, C., Zhu, S., Huang, T., Wang, S., Yan, X., Analytical techniques for single-liposome characterization. *Anal. Methods.* 2013, *5(9)*, 2150-2157.

[21] Nacka, F., Cansell, M., Gouygou, J. P., Gerbeaud, C., Méléard, P., Entressangles, B. Physical and chemical stability of marine lipid-based liposomes under acid conditions. *Colloids Surf. B: Biointerfaces.* 2001, *20*, 257-266.

[22] Sukhija, P. S., Palmquist, D. L., Dissociation of calcium soaps of long-chain fatty acids in fumen fluid. *J. Dairy. Sci.* 1990, *73*, 1784-1787.

[23] Yeap, P. K., Lim, K. O., Chong, C. S., Teng, T. T., Effect of calcium ions on the density of lecithin and its effective molecular volume in lecithin–water dispersions. *Chem. Phys. of Lipids.* 2008*, 151*, 1-9.

[24] Hincha, D. K., Effects of calcium-induced aggregation on the physical stability of liposomes containing plant glycolipids. *Biochim Biophys Acta*. 2003, *1611*, 180– 186.

[25] D. J. McClements: Food emultions. Principles, practice and techniques. CRC Press (United States) 1999.

[26] Mosca, M., Ceglie, A., Ambrosone, L., Effect of membrane composition on lipid oxidation in liposomes. *Chem. Phys. of Lipids.* 2011, *164*, 158-165.

[27] Naghibzadeh, M., Amani, A., Amini, M., Esmaeilzadeh, E., Mottaghi-Dastjerdi, N., Mohammad, A. F., An Insight into the Interactions between α-Tocopherol and Chitosan in Ultrasound-Prepared Nanoparticles. *J. Nanomater.* 2010, *2010*, 1-7.

[28] Henriksen, I., Våagen, S. R., Sande, S. A., Smistad, G., Karlsen, J., Interactions between liposomes and chitosan II: Effect of selected parameters on aggregation and leakage. *Int. J. Pharm.* 1997, *146(2)*, 193–203.

[29] W. Müller-Sterl: Bioquímica. Fundamentos para Medicina y Ciencias de la Vida. Reverté, (España) 2008.

[30] Peng, A., Pisal, D. S., Doty, A., Balu-Iyer, S. V. Phosphatidylinositol induces fluid phase formation and packing defects in phosphatidylcholine model membranes. *Chem. Phys. Lipids*. 2012, *165(1),* 15–22.

[31] Mateašik, A., Šikurová, L., Chorvát Jr., D., Interaction of merocyanine 540 with charged membranes. *Bioelectrochem.* 2002, *55*, 173–175.

[32] Hincha, D. K., Effects of alpha-tocopherol (vitamin E) on the stability and lipid dynamics of model membranes mimicking the lipid composition of plant chloroplast membranes. *FEBS Letters.* 2008, *582*, 3687-3692.

[33] Monroig, O., Navarro, J. C., Amat, F., González, P., & Hontoria, F., Oxidative stability and changes in the particle size of liposomes used in the Artemia enrichment. *Aquaculture.* 2007, *266*, 200–210.

[34] Soto-Arriaza, M. A., Sotomayor, C. P., Lissi, E. A., Relationship between lipid peroxidation and rigidity in L-α-phosphatidylcholine-DPPC vesicles. *J. Colloid Interface Sci.* 2008*, 323*, 70–74.

[35] Schnitzer, E., Pinchuk, I., Lichtenberg, D., Peroxidation of liposomal lipids. *Eur. Biophys. J. Biophy.* 2007*, 36*, 499–515.

[36] Liebler, D. C., Kling, D. S., Reed, D. J., Antioxidant Protection of Phospholipid Bilayers by α -Tocopherol, control of α-tocopherol status and lipid peroxidation by ascorbic acid and glutathione. *The j. Biol. Chem.* 1986*, 261,* 12114-12119.

[37] Decker, E. A., Hultin. H. O., Factors Influencing Catalysis of Lipid Oxidation by the Soluble Fraction of Mackerel Muscle. *J. Food Sci.* 1990*, 55(4),* 947-950.

[38] Fukuzawa, K., Dynamics of Lipid Peroxidation and Antioxidion of α-Tocopherol in Membranes. *J. Nutr. Sci. Vitaminol.* 2008, *54(4)*, 273-285.

Liposomal formulation	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	PC
SPC	11.10 ± 3.38	5.55 ± 0.62	1.40 ± 0.04
SPC:SA	10.62 ± 0.36	6.19 ± 0.54	1.57 ± 0.01
SPC:CaS	12.23 ± 0.72	6.40 ± 0.56	2.15 ± 0.02
SPC with VE and VC	11.40 ± 0.30	6.06 ± 0.16	2.12 ± 0.02***
SPC:SA with VE and VC	82.28 ± 9.77*	15.56 ± 0.69***	2.39 ± 0.01***
SPC:CaS with VE and VC	300.50 ± 31.27***	13.91 ± 5.54*	2.57 ± 0.08***
SPC PAST	12.69 ± 1.98	5.98 ± 0.59	1.31± 0.02▲
SPC:SA PAST	11.18 ± 0.64	5.63 ± 0.44	1.79 ± 0.01▲▲▲
SPC:CaS PAST	11.20 ± 1.91	6.16 ± 1.02	2.15 ± 0.03
SPC with VE and VC PAST	10.17± 0.09	5.73 ± 0.09	2.11± 0.02***
SPC:SA with VE and VC PAST	159.50 ± 47.65***▲	14.59 ± 1.81***	2.41± 0.01***
SPC:CaS with VE and VC PAST	245.5 ± 8.51***▲	14.18 ± 0.67*	2.22± 0.04▲▲▲

Table 1: Size distribution (first and second column) and partition coefficient (PC) (third column) values of the liposomal formulations without vitamins or with 90 mM of VC and 5mM of VE, before and after pasteurization (PAST) in distilled water. The results are shown as the mean ± SD of three independent assays. A statistical comparison was made:

-Between each system with vitamins respect to the same system without vitamins (control) through the Tukey test before and after pasteurization. Significant differences from the control are shown as *p<0.5, **p <0.01, ***p <0.001.

-In each system before and after pasteurization with the Tukey test. Significant differences from the control are shown as ▲p<0.05, ▲▲p<0.01, ▲▲▲p<0.001.

	%EE of VC by dialysis					
Time (hours)	[a]1	2	8	10	24	48
SPC	34.5 ± 3.5	23.3 ± 4.6	41.9 ± 17.8	45.5 ±14.8	36.9 ± 4.5	41.5 ± 3.5
SPC:SA	52.3 ± 2.0	37.2 ± 2.6**	45.2 ± 5.2	46.0 ± 6.6	43.2 ± 7.2	41.7 ± 6.1
SPC:CaS	34.3 ± 2.5	21.7 ± 2.4	28.3 ± 3.7	30.34 ± 4.2	28.6 ± 3.1	27.8 ± 5.9

Table 2: %EE of VC after dialysis at room temperature at 1, 2, 3, 8, 10, 24, 48 hours in distilled water. Values are expressed as mean ± SD of three independent measurements. Statistical comparison was done in the liposomal formulation at each time of measurement respect of the measure of the first hour, through the Dunnett Test. Significant difference respect to the control are shown as **p <0.001.

Liposomal formulation	n	Apparent viscosity (Pa.s) (10^{-3})
SPC-VE-VC	0.92 ± 0.01a	3.13 ± 0.06a
SPC-VE-VC PAST	0.93 ± 0.01a	3.51 ± 0.04a
SPC:AE -VE-VC	0.92 ± 0.06a	2.49 ± 0.93a
SPC:AE -VE-VC PAST	0.86 ± 0.01a	2.89 ± 0.92a
SPC:ECa -VE-VC	0.81 ± 0.11a	2.07 ± 0.24a
SPC:ECa -VE-VC PAST	0.80 ± 0.15a	2.25 ± 0.15a

Table 3: It depicts n (flow behavior index) and apparent viscosity at 100 s^{-1} values in liposomal formulations with 5 mM of VE and 90 mM of VC 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 the three systems together, with or without heat treatments. Different letters show significantly different results at least with *p <0.05, the samples with the same letters show no significant differences.

Liposomal formulation	VC	VE
SPC	28.4 ± 1.7	91.18 ± 7.79
SPC:SA	24.1 ± 1.4	76.96 ± 17.66
SPC:CaS	27.6 ± 15.3	85.72 ± 10.80

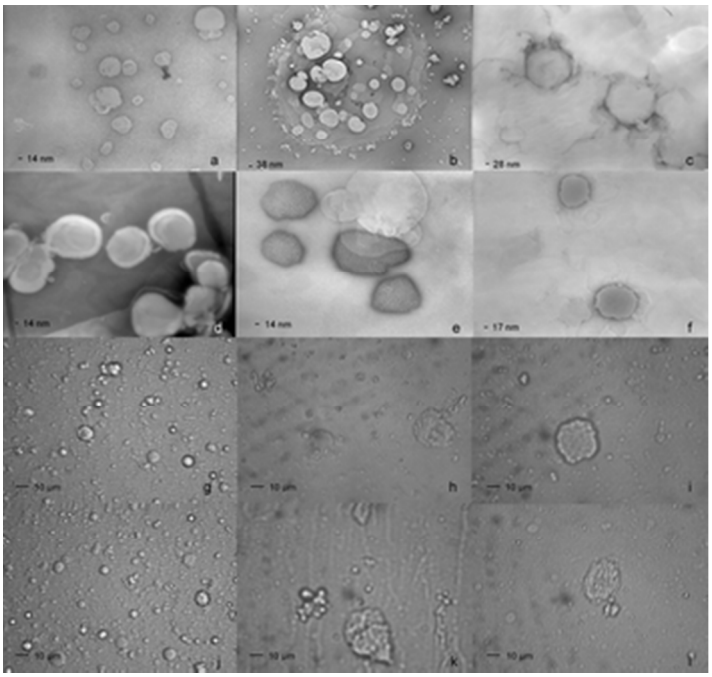
Table S1: %EE of VC or VE after disruption with ethanol after 72 hours of dialysis in distilled water. Values are expressed as mean ± SD of three independent measurements. A statistical comparison between the three formulations together by the Tukey test was performed. The systems did not present significant differences.

Liposomal formulation	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	PC
SPC	11.10 ± 3.38	5.55 ± 0.62	1.40 ± 0.04
SPC:SA	10.62 ± 0.36	6.19 ± 0.54	1.57 ± 0.01
SPC:CaS	12.23 ± 0.72	6.40 ± 0.56	2.15 ± 0.02
SPC with VE and VC	11.40 ± 0.30	6.06 ± 0.16	2.12 ± 0.02***
SPC:SA with VE and VC	82.28 ± 9.77*	15.56 ± 0.69***	2.39 ± 0.01***
SPC:CaS with VE and VC	300.50 ± 31.27***	13.91 ± 5.54*	2.57 ± 0.08***
SPC PAST	12.69 ± 1.98	5.98 ± 0.59	1.31± 0.02▲
SPC:SA PAST	11.18 ± 0.64	5.63 ± 0.44	1.79 ± 0.01▲▲▲
SPC:CaS PAST	11.20 ± 1.91	6.16 ± 1.02	2.15 ± 0.03
SPC with VE and VC PAST	10.17± 0.09	5.73 ± 0.09	2.11± 0.02***
SPC:SA with VE and VC PAST	159.50 ± 47.65***▲	14.59 ± 1.81***	2.41± 0.01***
SPC:CaS with VE and VC PAST	245.5 ± 8.51***▲	14.18 ± 0.67*	2.22± 0.04▲▲▲

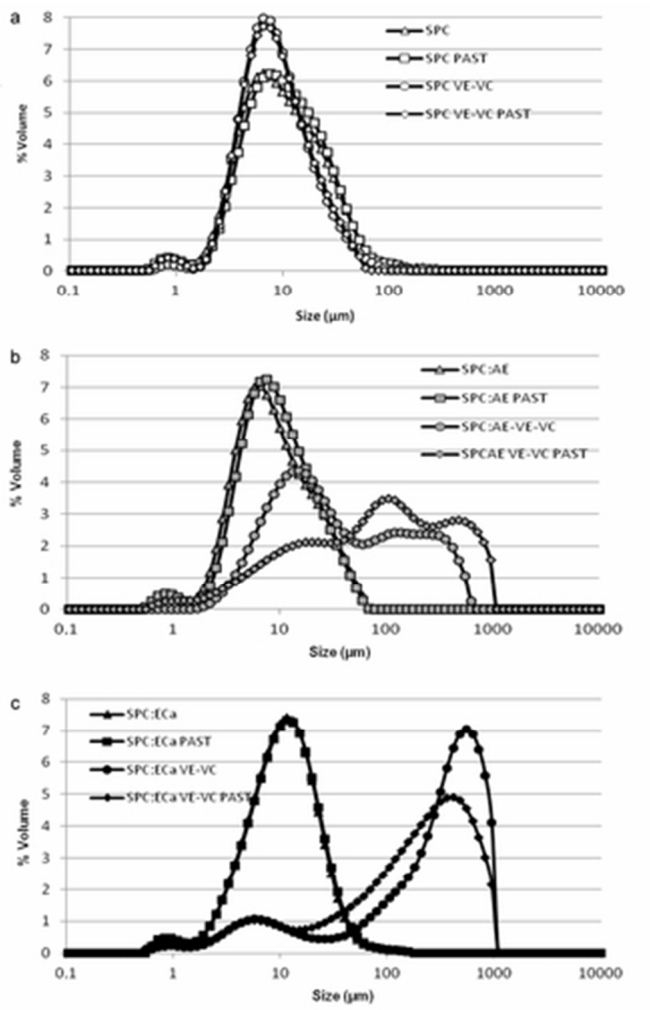
	%EE of VC by dialysis					
Time (hours)	[a]1	2	8	10	24	48
SPC	34.5 ± 3.5	23.3 ± 4.6	41.9 ± 17.8	45.5 ±14.8	36.9 ± 4.5	41.5 ± 3.5
SPC:SA	52.3 ± 2.0	37.2 ± 2.6**	45.2 ± 5.2	46.0 ± 6.6	43.2 ± 7.2	41.7 ± 6.1
SPC:CaS	34.3 ± 2.5	21.7 ± 2.4	28.3 ± 3.7	30.34 ± 4.2	28.6 ± 3.1	27.8 ± 5.9

[a]In the measurement of the initial time (2 minutes) of dialysis the release of vitamin was not detectable.

Liposomal Formulation	n	Apparent viscosity (Pa.s) (10^{-3})
SPC-VE-VC	0.92 ± 0.01a	3.13 ± 0.06a
SPC-VE-VC PAST	0.93 ± 0.01a	3.51 ± 0.04a
SPC:AE -VE-VC	0.92 ± 0.06a	2.49 ± 0.93a
SPC:AE -VE-VC PAST	0.86 ± 0.01a	2.89 ± 0.92a
SPC:ECa -VE-VC	0.81 ± 0.11a	2.07 ± 0.24a
SPC:ECa -VE-VC PAST	0.80 ± 0.15a	2.25 ± 0.15a



Optical micrographs and negative stained TEM of the liposomal formulations with 5 mM of VE and 90 mM of VC in distilled water before and after pasteurization (PAST). Data corresponded to optical micrographs: a:SPC, b:SPC:SA, c:SPC:CaS, d:SPC PAST, e:SPC:SA PAST, f:SPC:CaS PAST; and negative stained TEM: g:SPC; h:SPC:SA, i:SPC:CaS, j:SPC PAST, k:SPC:SA PAST, l:SPC:CaS PAST.
14x14mm (600 x 600 DPI)



Particle size distribution expressed as volume percentage for liposomal formulations without vitamins and with both vitamins (5 mM of VE and 90 mM of VC) in distilled water before and after pasteurization (PAST). Data correspond to a:SPC, b:SPC:SA and c:SPC:CaS.

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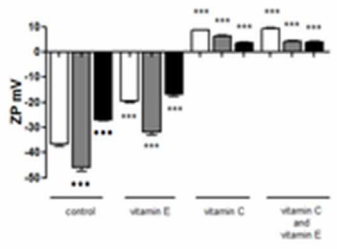
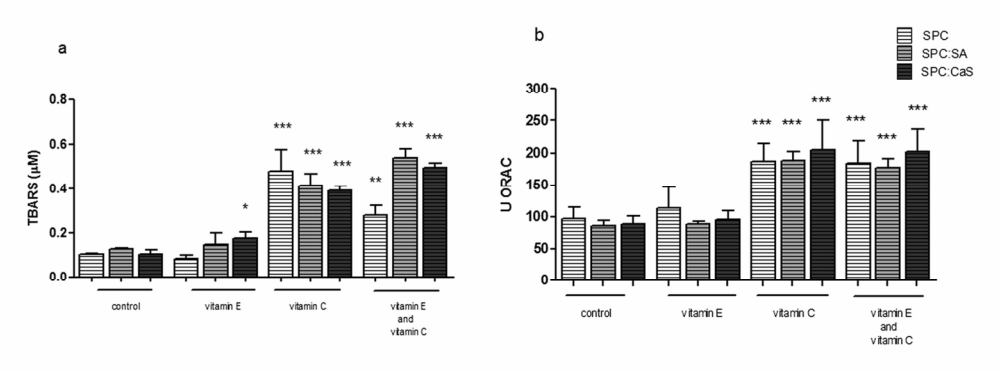


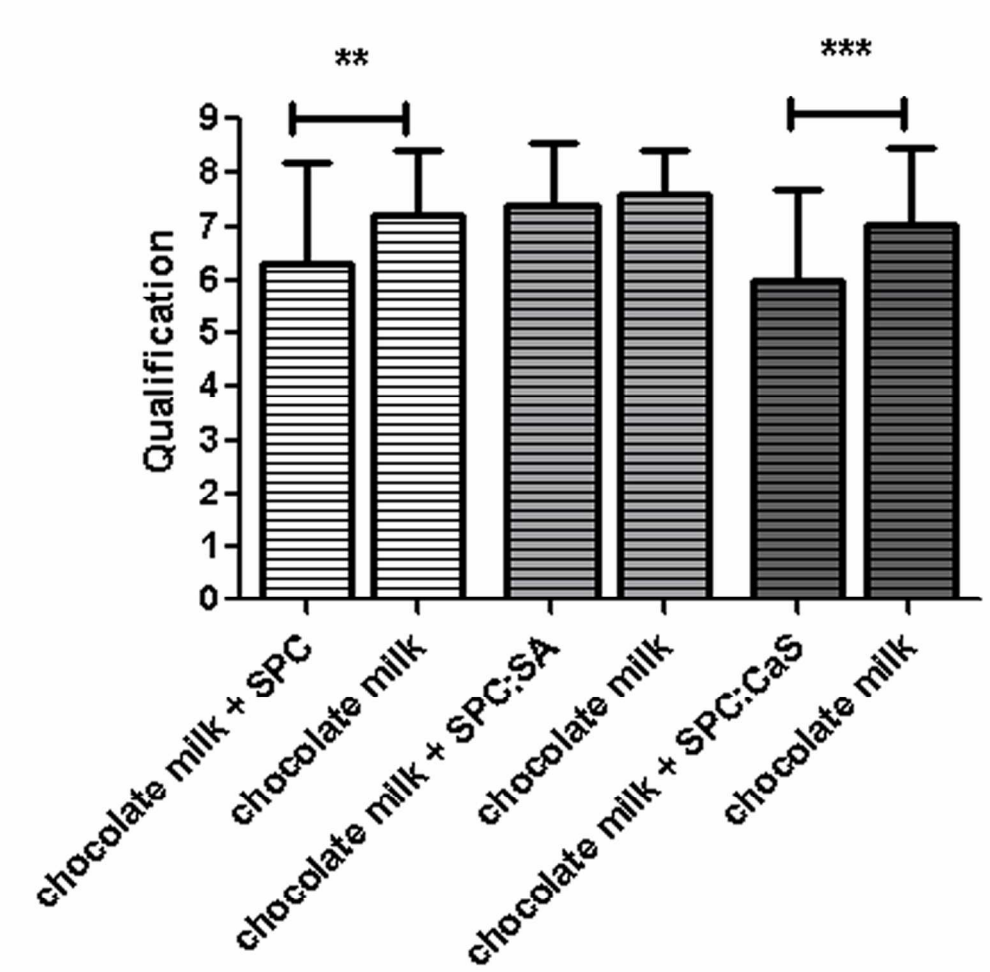
Zeta potential (ZP) values of the liposomal formulations without vitamins, with 5 mM of VE, with 90 mM of VC and with 90 mM of VC and 5 mM of VE in distilled water. The results are shown as the mean ± SD of five measures. The following statistical comparison was made:
-Among the formulations without vitamins with respect to SPC (control) with the Dunnett test. Significant differences from the control are shown as ●●●p <0.001.
-Between each system with vitamin/s with respect to the same system without vitamins (control) with the Dunnett test. Significant differences from control are shown as ***p <0.001.

7x5mm (600 x 600 DPI)

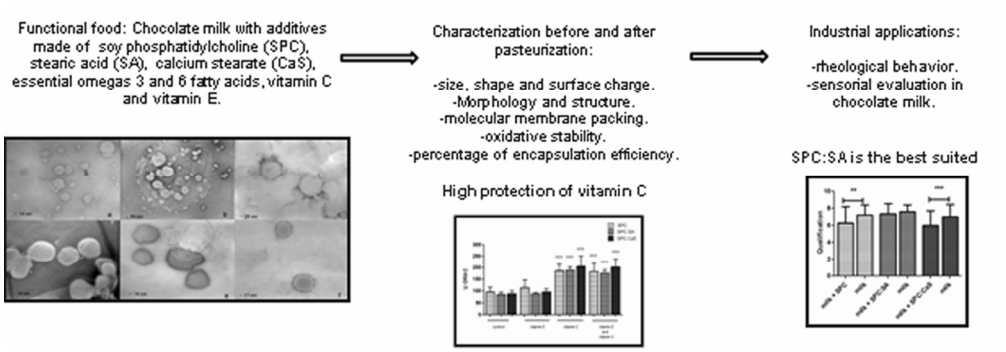


Peroxidation assay in liposomal formulations after pasteurization in distilled water with TBA (a) and ORAC (b) methods. Data correspond to SPC (white), SPC:SA (grey), SPC:CaS (dark grey) without vitamins (control), with 5 mM of VE, 90 mM of VC and both vitamins (5 mM for VE and 90 mM for VC). Each column represents the mean ± SD of three and four independent assays for TBA and ORAC methods, respectively. A statistical comparison was made:
-Among the formulations without vitamins with respect to SPC (control) with the Dunnett test. The systems did not present significant differences.
-Between each system with vitamin/s with respect to the same system without vitamins (control) through the Dunnett test. Significant differences from the control are shown as *p<0.5, **p <0.01, ***p <0.001.

148x55mm (300 x 300 DPI)



Qualifications of 40 panelists for commercial chocolate milk with or without liposomes with 5 mM of VE and 90 mM of VC, for each type of formulation. Statistics were performed using the test for paired samples between each commercial chocolate milk sample with and without the additive. The results with significant differences are shown as **p <0.01, ***p <0.001.
74x74mm (600 x 600 DPI)



56x19mm (600 x 600 DPI)