Chapter

Why Produce Food-Bioactive Compounds to Generate Functional Grade Foods?

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Abstract

Functional foods are those with health benefits but cannot incorporate and protect from oxidation or deterioration, maintaining the bioactive compounds (BC) activity. The liposomes have several advantages for BC encapsulation: ease of obtention, characterization, scaling-up, lipid protection for hydrophilic and lipophilic BC, and best, they are made with natural lipids of alimentary grade. In our studies, liposomes were made of soy phosphatidylcholine (SPC) with Stearic Acid or Calcium Stearate as membrane stabilizer. They encapsulated BC as vitamin E, vitamin C and folic acid (B9). The liposome's design strategy is that SPC lipid's components are BC like choline and essential fatty acids. These liposomes preserved and maintain the activity of the thermolabile vitamins C and B9. Like milk and fruit juice, in various food types can incorporate liposomes protecting BC. A series of laboratory studies will be performed to select the most stable liposomal formulations, like characterization, encapsulation efficiency, physicochemical, microbiological, thermal and sensory stability. Liposomes- BC design and development are discussed in the chapter. The food heat treatment and the conditions/storage time are also crucial and must be considered in these studies. Finally, incorporating the BC into a food production line is feasible with an excellent economic prospect until supermarket shelves are reached, like our food product proposal.

Keywords: bioactive compounds, liposomes, nutrition, stability, industry

1. Introduction

Functional foods are those with health benefits that must demonstrate that: a) they have a beneficial effect on one or more specific functions of the organism, beyond their usual nutritional effect, b) they improve the state of health and well-being, and c) they reduce disease risk [1]. Functional food must contain functional ingredients or bioactive compounds (BC), which are natural constituents that are generally found in small amounts in food. These compounds provide health benefits beyond the essential nutritional value of the product and for that reason, they are intensively studied to evaluate their effects on human health [2, 3].

BC include flavonoids, phytoestrogens, isoflavones, resveratrol, lycopene, organosulfur compounds, soluble dietary fibers, and isothiocyanates monoterpenes, plant sterols, olive oil. Among the BC of hydrophobic nature are carotenoids, tocopherols, flavonoids, polyphenols, and phytosterols stand out [3].

Regarding the importance of particular BC is the folic acid (FA) or vitamin B9, which belongs to the folates family and act as cofactors in carbon transfer reactions (formyl, hydroxymethyl, and methyl) in nucleotide biosynthesis (purine and pyrimidine bases), amino acid metabolism (methionine, histidine) and metabolism neurotransmitters (serine, choline) [4]. Animals and humans cannot synthesize folates. Thus, it is necessary to incorporate them into the diet from plant sources or food FA fortified [5].

The consumption of FA is critical since the deficiency of this vitamin is related to neural tube defects, coronary heart disease, and megaloblastic anemia, similar to that generated with the deficiency of vitamin B12, which occurs more frequently in pregnancy [5–7].

Scientific studies link low folate intake with neurocognitive dysfunctions. Folates play an essential role in developing the central nervous system and in the metabolism of some neurotransmitters; low folate concentrations may also be related to dementia and decreased cognitive function [6].

Various studies explain that FA is being used as a potential agent for preventing cancer and reducing the risk of heart disease [8, 9]. Epidemiological studies have shown that folate supplements can significantly reduce pancreatic cancer and breast cancer [10]. Besides, some studies link the intake of FA with a decrease in colon cancer and neurological diseases such as Alzheimer's [11]. The protective effect of FA on cardiovascular diseases, hematological and neurological diseases, and cancer has been associated with the antioxidant activity of this vitamin [12].

Another important BC is vitamin E (VE), also called α-tocopherol [13]. There are also foods such as eggs, seeds, nuts, and whole grains, which also contribute to VE's daily intake [5].

VE is the main fat-soluble antioxidant in the body. Its action is based on capturing peroxide radicals produced in cells by oxidative metabolism [7, 14]. Hence, it prevents the formation of hydroperoxides, delaying the initial phase of the oxidative process [13, 15]. This action protects cell membranes and other lipids from severe alterations produced by peroxidation [7, 14]. Various clinical studies also describe the beneficial effects of VE, alone or in combination with other vitamins, in some types of tumors such as prostate, gastric and lung. This fact is based on experimental studies that show the role of free radicals as a critical factor associated with the development of cancer. It is precisely the effectiveness of antioxidants from the diet such as tocopherols that have an essential role in the prevention of the development and progression of this disease [16].

Another group of BC are polyunsaturated fatty acids (PUFAs). There are two families of PUFAs: the n-6 family and the n-3 family. The PUFAs n-6 family is derived from linoleic acid, with two double bonds, and is characterized by having its first double bond at the carbon chain number 6, counting from the methyl at the end of the chain [17]. The n-3 PUFAs family derives from α-linolenic acid (with three double bonds), whose fatty acids have a first double bond at carbon number 3 in the chain. Besides being a source of energy, the PUFAs n-6 and n-3 families incorporate into cell membranes. They are precursors of eicosanoids like prostaglandins, prostacyclins, thromboxanes, and leukotrienes, that intervene in several physiological processes, like blood clotting or inflammatory and immune responses [17].

It is essential to highlight the contribution benefits of essential fatty acids. Man needs these fatty acids for normal functioning but must ingest them since body cannot produce them. That is why they are called essential [5]. Both linoleic and α-linolenic are essential fatty acids that cannot be synthesized by the body. Therefore, both fatty acids must be provided in the diet [17]. Research published has established that these fatty acids are very beneficial in the prevention of

cardiovascular diseases [18–20], schizophrenia [21], and cancer [22], among others. Also, they have vasodilator, antihypertensive, anti-inflammatory, and anti-atherothrombotic properties [23].

The problem is that many of these BC are not consumed in the necessary amounts for the body's normal functioning. According to the recent 2015–2020 Dietary Guidelines for Americans, VE and choline consumption is below the daily requirements, so it must be compensated with the intake of supplements in 50% of adults [24].

2. Developing formulations with bioactive compounds

The BC amount that must be added to food and its nutritional values detected are useful in preventing certain diseases. Then there is a need to study and bring to the industry the incorporation of matrices/carriers that permit BC in food. Various types of matrices in the food industry, like liposomes, nanoemulsions, microemulsions, solid lipid nanoparticles, and polymeric micro and nanoparticles have been studied for BC encapsulation [25]. A whole new market has even been generated related to nano-foods associated to nanotechnological techniques or tools, or to which manufactured nanomaterials should be added, either during their starting point or production processing or packaging [26].

Liposomes are considered within an emerging trend in the market called nano-feeding. They offer a series of competitive advantages compared to other matrices. For example, their production on a larger scale is of good feasibility. They are easy to obtain and scale-up, which allows the incorporation of BC compounds in this type of matrices in food production lines. Besides, their characterization and physicochemical, microbiological, and sensorial stability can be studied with different techniques. Also, liposomes components are low cost, and natural food can be easily obtained [25, 27].

Liposomes are microscopic spherical vesicles, formed by lipids that enclose liquid compartments in their structure [28], allowing the encapsulation of molecules, whether they are liposoluble or water-soluble BC [29, 30].

The liposomes can be formulated with phospholipids, which are polar lipids characterized by having hydrophilic and lipophilic groups on the same molecule [30]. These spherical vesicles are formed under certain conditions. After flash evaporating solvent, phospholipids are hydrated and organized into lipid bilayers. These lipid bilayers called lamellae, unite to form the phospholipid sphere that encloses the water [31]. Liposomes can have one or multiple concentric lamellae called a vesicle or multilamellar liposome [30].

These types of matrices have broad applications in the industry to transport BC or other types of compounds. For example, in accelerating cheese ripening, the vesicles offer a uniform distribution of hydrophilic enzymes [30]. In the encapsulation of flavorings, acidulants (citric acid, ascorbic acid, buffer, and alkalis), antioxidants, colorants, essential oils, vitamins, and minerals. Furthermore, these systems are used to encapsulate lactoferrin, a bacteriostatic glycoprotein, and nisin z, an antimicrobial polypeptide, to increase dairy products' shelf life. Liposomal systems are also used to trap Phosvitin (antioxidant), which inhibits lipid oxidation in various dairy products and ground pork. Besides, they are used to capture antioxidants like vitamin C, maintaining 50% of activity after 50 days in refrigerated storage and non-encapsulated vitamin C, which loses its activity after 19 days [32].

In this way, liposomes can encapsulate all kinds of BC. Besides, specific BC can be part of the carrier itself if a strategy is applied in these matrices' design.

To implement liposomes with BC in the food industry, the research and development of these matrices should be deepened to ensure the compounds' stability to be encapsulated and incorporated into food. It is essential to mention that for an industrial application, membrane stability and structure are important factors when designing liposomes [30], and must always ensure that they are food grade [33].

In our research line, we sought to incorporate saturated fatty acids that can act as membrane stabilizers and intervene against lipid oxidation processes [33]. Based on the research carried out by Hsieh and collaborators [28], stearic acid (SA) is an excellent alternative to prepare stable liposomes, structural benefits, and increase the efficiency of liposomal encapsulation, as well as oxidative stability. SA is an 18-carbon saturated fatty acid, insoluble in water, so it is located between the hydrophobic chains of the fatty acids in the bilayer. The authors carried out studies on liposomes formed with egg phosphatidylcholine (EPC) and SA in the molar ratio of 1: 0.25. The problem was that EPC has a much higher cost than soy phosphatidylcholine (SPC) in Argentina since EPC is a specific raw material obtained in the bench lab at a laboratory scale.

In comparison, SPC is a lipid product of the country's intense soy farming activity. It is a raw material that is easily obtained at a large scale and has a low cost. Therefore, SPC with SA was used in the molar ratio 1:0.25. A second strategy is developing liposomal formulations using the SPC base system and incorporating calcium stearate (CaS), with the double benefit that CaS can act as a possible stabilizer of the liposomal bilayer. CaS also incorporates a mineral such as calcium that increases the nutritional value [27, 29, 33, 34]. CaS is a salt composed of two 18-carbon saturated fatty acids linked to a calcium cation. The concentration used was the same as in SPC and SA. In this way, the possible effects of the stability provided to the liposomal bilayer incorporating saturated fatty acids are preserved, and at the same time, extra calcium is added to the formulation.

The formulations proposed to obtain liposomes that encapsulate and protect BC were the following [27, 29, 33, 34]:

- SPC.
- SPC:SA in a mol/mol ratio of 1: 0.25.
- SPC:CaS in a mol/mol ratio of 1: 0.25.

Besides, SPC is a natural lipid that generate the liposome's transporter, has essential fatty acids such as linoleic acid (omega-3) and linolenic acid (omega-6).

Table 1 shows the percentage composition of fatty acids in SPC and EPC considered essential fatty acids. Furthermore, SPC is also the source of choline, an essential nutrient needed to synthesize neurotransmitters (acetylcholine). It plays an essential role in the fetus's brain and memory development, and some researchers have indicated that choline and methionine intake may be necessary for reducing the risk of neural tube defects [35].

Multilamellar liposomes were prepared by the dehydration–rehydration method [36]. Briefly, 40 μmol of lipids were dissolved in 500 μL ethanol in a round bottom flask, and the solvent was dried in a rotary evaporator at 37 °C. Dry lipid film composed of SPC, SPC:SA (1:0.25, mol ratio), or SPC:CaS (1:0.25, mol ratio) was rehydrated with 2 mL distilled water to a final 50 mM lipid concentration.

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. The solvent was evaporated until the lipid film was obtained. Any liposoluble BC to be incorporated into the liposome

Fatty acids	% Fatty acids in SPC	% Fatty acids in EPC
Myristic acid 14:0	—	0.2
Palmitic acid 16:0	14.9	32.7
Sapienic acid 16:1	—	1.1
Stearic acid 18:0	3.7	12.3
Oleic acid 18:1	11.4	32.0
Linoleic acid 18:2*	63	17.1
Linolenic acid 18:3*	5.7	—
Eicosatetraenoic acid 20:4*	—	2.7
Docosahexaenoic acid 22:6*	—	0.4

Table 1.
*Composition of fatty acids in SPC and EPC (*essential fatty acids).*

must always be done in this step. Moreover, it is essential to use only ethanol to dissolve lipids since it is a solvent that is approved as an additive at a national and international level of the Food Committee with concentrations (possible trace) that do not exceed the maximum permitted [37–39]. When the film was rehydrated in 2 mL of distilled water, a final concentration of 5 mM was reached, and this is the step to incorporate the hydrosoluble BC's. In the case of FA, recently prepared solutions of this vitamin needed when the experiment is on the rehydration step. FA was weighed and diluted with distilled water to reach a 0.136 mM concentration.

Samples were prepared with the primary goal of fortifying food with the mentioned vitamins. According to Argentina regulations [40], 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 order to fortify aqueous food like chocolate milk, regular milk, or juice, 2 mL of liposome suspension (50 mM) with vitamins was added to each serving of food (200 mL), which implies that it was fortified with 4.3 mg of VE (5 mM) equal to 43% of the RDI and 120 μg of FA (0.136 mM) equivalent to 30% of the RDI. Thus, 1 L of aqueous food will contain, for example, liposomes of SPC:CaS and BC in the proportion seen in **Table 2**.

Liposomal formulation SPC: CaS (mol / mol ratio of 1: 0.25) with 5 mM VE and 0.136 mM of FA	Mass of component
SPC	303.20 mg
Choline from SPC	41.66 mg
Linoleic acid from SPC	191.05 mg
Linolenic acid from SPC	17.30 mg
CaS	60.70 mg
Calcium from CaS	4.00 mg
VE	21.50 mg
FA	600 μg

Table 2.
Composition of SP:CaS liposomal formulation as carries of BC: VE, FA, choline and essential fatty acids in 1 L of product for example chocolate milk [27, 29, 33, 34].

The design, research, and development of carriers for BC, including all aspects, like the final quality of the food product and its feasibility, have to be considered until reaching the consumer's market. Thus, the liposomal formulation must be stable from a physicochemical, microbiological, and sensory perspective. The interaction of these matrices with BC must ensure their stability and protection until consumption. This data is not minor given that in the food industry, a series of treatments, usually thermal, must be applied to ensure the useful shelf life and safety of the product.

This fact presents a challenge in the development and research of foods with BC because many of these degrade or lose activity before reaching the industry's scale up regular treatments. VE is heat stable but oxidizes quickly in the air, with consequent loss of vitamin activity, especially in the presence of ferric ions and other metals [13]. Furthermore, VE is destroyed by exposure to UV light and to a great extent, during oil refining process [7]. Also, concerning food storage, during the storage of plant foods, VE has a weak antioxidant character, being much more active against animal fats, especially in the presence of synergistic substances [13]. On the other hand, FA is stable to alkalis under anaerobic conditions. However, under aerobic alkaline conditions, its hydrolysis occurs, separating the side chain and yielding glutamic acid, and pterin-6-carboxylic acid. Acid hydrolysis under aerobic conditions yields 6-methylpterin [41]. For this reason, to favor the stability of the FA, it must remain at a pH close to neutrality [42]. None of the degradation compounds mentioned shows biological activity; therefore, during the formulation of pharmaceuticals, nutraceuticals, or foods enriched with FA, it is necessary to protect this vitamin against environmental factors such as extreme light and pH [7, 43]. Specifically, in acidic media, it was shown that FA is unstable [44]. Furthermore, FA solutions decompose when exposed to light, forming glutamic acid, and pterin-6-carboxylic acid [41].

The milk pasteurization by the high temperature and short time method (2–3 seconds, 92 ºC) causes a loss of around 12% of total folates, and the loss caused by boiling the milk for 2–3 minutes is in the order of 17%. Sterilization of milk in bottles (13–15 minutes at 119–120 ºC) is the treatment that causes the most significant losses, about 39% [41].

Based on those aspects mentioned above of unfavorable conditions, if the objective is to add BC such as FA and VE, the liposomes must have oxidative stability not to affect the vitamin antioxidant activity, and they must protect the FA from the applied food heat treatments. Also, the liposomes can be food-incorporated at a neutral pH due to FA's stability.

The application of advanced liposomal formulation has shown that they can be applied in pH foods such as chocolate milk and orange juice. They demonstrate that liposomes can protect thermolabile vitamins such as vitamin C and FA [29, 33]). Other authors demonstrated that the capture of antioxidants like vitamin C in liposomes maintains 50% of activity after 50 days in refrigerated storage, and non-encapsulated vitamin C loses its activity after 19 days [32].

It should also be mentioned that the liposomal formulations of our research line presented values of oxidative stability under quality food parameters [45]. According to what is established by the authors [45], for a food to have a good quality, it must have an oxidative value below 0.2 mg of malondialdehyde (MDA) per Kg of food. In SPC, SPC:SA and SPC:CaS with VE-FA after pasteurization thiobarbituric acid reactive species (TBARS) value were 0.2380 ± 0.0248 μM, 0.2017 ± 0.0645 μM, 0.1816 ± 0.0581 μM, respectively. The results are shown as the mean ± SD of three independent assays; as published in Marsanasco and collaborators [29]. Taking as a reference the average value of SPC of 0.2380 μM that was the highest of the three formulations, if the transition from μM of TBAR to mg of

MDA/Kg of food is performed, it gives a value of 0.0166 mg per 1 Kg of chocolate milk (density was 1,033 Kg/L). Thus, is below 0.2 mg of MDA/Kg established by the authors, complying with the excellent quality parameter.

3. Incorporating bioactive compounds into different types of food

Liposomes have the particularity that they require an aqueous medium for their stability [30]. Based on the above, it is essential to consider that the choice of food to incorporate the liposomes with the BC must have a high water-activity (a_w) to maintain the liposomal formulations' stability. After considering this factor, the second thing is how the functional food to be produced will be positioned in the market. Alternatively, may be the product production has a social focus related to the country's nutritional deficiencies.

Our line research, based on the social requirements of our country and the market niche that we want to cover, the incorporation of liposomal formulations with BC in the following types of food was proposed:

- Milk/flavored milk.
- Fruit juices.

Thus, chocolate milk fortified with VE and FA was implemented with essential omega-3 and omega-6 FAs and choline (from the SPC-based liposome). Besides, when using the liposomal formulation SPC:CaS, calcium is added to the product. This type of food will provide energy and nutritional contribution to the consumer.

This research was complemented by studying the same liposomal formulations but with 5 mM of VE and 50 mM of vitamin C (VC).

In all cases, the focus of incorporating vitamins was towards the food fortification with the incorporation of these vitamins that are considered as BC.

The objective will be to offer a massive product such as milk, with an essential nutritional addition, in response to a growing demand for products with high nutritional value and nutritional deficiencies in the country's specific sectors.

Another type of food prepared with the addition of liposomal formulations-BC was orange juice. In this case, FA could not be incorporated; as explained in the previous section, considering it is not stable at extreme pHs. In this case, liposomal formulations were used with VE and VC in the concentrations already mentioned.

The liposomes in the food were incorporated in the volumetric ratio 1/100. Furthermore, they did not modify the base food's density, pH, or visual characteristics [27, 29, 33].

Once the essential foods have been established, the first phase of the study in the research and development of these functional foods is the characterization and physicochemical stability on a laboratory scale of the liposomal formulations with BC. At this stage, the following parameters should be studied: size, shape, electrical charge, encapsulation efficiency, oxidative stability, packing and lipid membrane stability, and rheological behavior of liposomal formulations with and without the addition of vitamins. Also, the heat treatment of the selected food must be considered, in our research line LTLT process was selected. LTLT is low temperature and longtime pasteurization process, that is 65 °C for 30 minutes applied to milk and juices.

There is a question of whether all liposomal studies with BC can successfully fortify the base food. Food has components that can interfere with analytical

determinations. It is for this reason that in this study stage, food simulants were used. Milk and flavored milk are found within foods with a pH > 5. Therefore, the food simulant distilled water was used instead of milk, while fruit juices are within pH <5, so the food simulant of 3% m/v acetic acid was employed [46].

Among the studies to be carried out at this stage, transmission electron microscopy is one of the most suitable methodologies to obtain information on the morphology of liposomes [47]. This technique must be complemented with optical microscopies that allow analyzing the shape and distribution of the liposomes, respectively. In the formulations developed, explicitly mentioning those with VE-FA, the liposomes presented a structure in which an outermost zone and a central nucleus were differentiated (**Figure 1**). They also presented a variety of sizes with spherical shapes and non-spherical related to the method of preparation and composition of liposomes. Moreover, isolated and aggregated liposomes were also observed [29, 33]. Data discussed coincide with those obtained by other authors [48], where the unilamellar liposomes of egg Lecithin at pH 7.2 showed liposomal aggregation. Nacka and collaborators [49] also demonstrated liposome aggregation of mainly phosphatidylcholine and phosphatidylethanolamine at various pH. In liposomal systems, aggregation is a physicochemical mechanism that can occur under certain conditions influenced by pH, heat treatment, external load, and cations' presence, among others [29, 33, 49].

It is important to mention the surface and viscosity behavior study of the matrices that will encapsulate the BC. The matrix that encapsulates the BC has a behavior like that of the product. It represents a great advantage to apply to the food industry, if visualizing an industrial-scale production. The size and shape of the liposomes with the BC are related to the final product's stability. But there is also another aspect that is related to the composition, shape, morphology distribution, tendency of aggregation, and membrane packing of the liposomal formulation with BC, and that is the rheological behavior and viscosity that they will contribute to the food product [29, 34]. Newtonian behavior occurs in almost all common liquids such as water, milk, orange juice, apple juice, and corn syrup. Furthermore, the pseudoplastic behavior is also present in common foods such as sauces and orange juice concentrate [50, 51].

For example, liposomal formulations SPC, SPC:SA, all with VE-FA and pasteurized, presented a behavior similar to that of a Newtonian fluid. While the SPC:CaS formulation with VE-FA pasteurized presented a behavior with a tendency towards a pseudoplastic fluid. This result is related to various factors as the membrane packing with the viscosity of formulation and rheology. According to the results obtained by other authors [52], a greater rigidity of the membrane of the phosphatidylcholine L-α-dipalmitoyl liposomes increased in viscosity. In the system with CaS, FA's addition would favor the association of two adjacent phosphatidylcholines with the calcium cation. It must be considered that FA (pka1 = 2.3) was shown to decrease the pH of distilled water from 6.0 to 3.88 by releasing the protons to the medium. Moreover, the

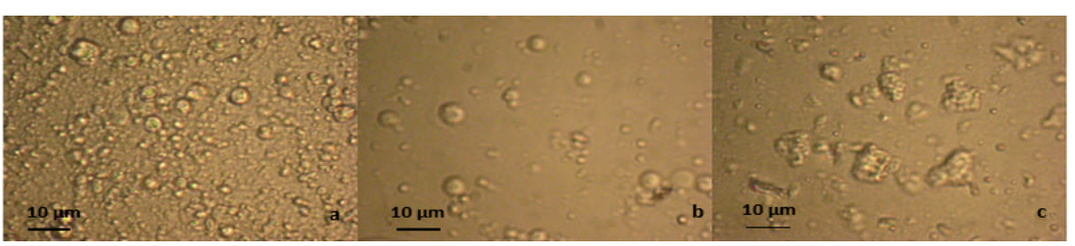

Figure 1.
Light micrographs of the liposomal system with 5 mM of VE and 0.136 mM of FA in distilled after pasteurization: a) SPC; b) SPC:SA, c) SPC:CaS.

low pH favors the dissociation of calcium from CaS, which generates bonds between calcium cations and two adjacent phosphatidylcholines [53, 54]. This greater stiffness of the membrane would also explain the pseudoplastic behavior of the SPC: CaS system [29]. Newtonian or pseudoplastic behavior in liposomal formulations with BC represents an advantage if, in the future, it is desired to apply liposomes on an industrial scale, especially the foods to be fortified, such as milk and orange juice.

Other studies carried out are the membrane packaging and its behavior, for this type of study; techniques are implemented that allow detecting the hydrophobicity factor (FH). This factor determines the degree of hydrophobic sites exposed at interface level and the possibility that the probe used, which is merocyanine MC540, is inserted into the bilayer. For this reason, the higher the value of this parameter, the higher the number of membrane surface defects [29].

In liposomes obtained, specifically those with VE and FA, considering that MC540 is slightly located above the domain of the main glycerol chain of phospholipids [55]. It can be inferred that the addition of vitamins favored membrane fluidity (specifically in the phospholipid polar zone) with the consequent greater probe entry and a greater number of defects in a said membrane appearance (**Table 3**).

Regarding VE, the result would be related to the effect of this vitamin that produces a general increase in the mobility of the headgroup in the lipid bilayer [56], which would favor the entry of the probe MC540 [57]. In the case of the SPC:SA (1: 0.25 molar ratio) or SPC: CaS (1: 0.25 molar ratio) system, the polar part of the fatty acids alternates with 21% SA or CaS, respectively. As the amount of saturated fatty acids increases, it is expected a membrane stiffness increment and fewer probes entering. But considering the membrane, the MC540 location; the addition of the SA or CaS could be favoring their income. In the case of SPC:SA, a possible explanation would be related to the fact that the polar heads of phospholipids such as phosphatidylcholine bind to water molecules through hydrogen bonds, and the polar heads of fatty acids such as SA also interacting with water molecules [58]. Although SA is an anion (as occurs in the pH of distilled water), it can also affect the hydrogen bonds with water [59]. The greater the hydration and formation of hydrogen bonds in the polar heads, the greater the water's penetration, which promotes the formation of more fluid domains [60]. In the case of the SPC:CaS system, the presence of FA (pka1 = 2.3) and the pH descent in distilled water with the consequence of the release of calcium ions from CaS and the association with close next phosphatidylcholines, increase the surface fluidity of the membrane and favors the entry of the probe [29].

Liposomal formulation	**HF**
SPC	2.02 ± 0.05
SPC VE-FA	2.61 ± 0.06***
SPC:SA	3.19 ± 0.02
SPC:SA VE-FA	5.310 ± 0.13***
SPC:CaS	5.12 ± 0.13
SPC:CaS VE-FA	6.16 ± 0.14***

*Values of the membrane packing of SPC, SPC:SA and SPC;CaS with 5 mM of VE and 0.136 mM of FA after pasteurization in distilled water. Results are shown as the mean ± SD of three independent tests for MC540 hydrophobicity factor (HF). Statistical comparison was made in each system with vitamin / s compared to the same system without vitamins (control), by means of the Dunnett's Test. The significant differences with respect to the control are shown as * p <0.5; ** p <0.01; *** p <0.001 [29].*

Table 3.
Values of the membrane packing of SPC, SPC:SA and SPC;CaS with 5 mM of VE and 0.136 mM of FA after pasteurization in distilled water.

In this case, the incorporation of SA or CaS increased the lipid membrane's fluidity and defects, but this does not imply ruling out the developed carrier. Because it can generate this effect but have important oxidative stability, protection, and encapsulation of BC, that is why all the characterization and stability studies must be carried out to have a precise knowledge of how the carrier interacts with the BC and, based on that, define which ones will continue in the second study stage.

Once the studies of physicochemical stability, encapsulation efficiency, and characterization of the BC liposomal formulations have been carried out, start the second study stage, the final product application. For that type of study, two points of great importance must be evaluated: the final functional food's microbiological and sensory stability. The purpose of microbiological tests is to analyze whether the composition, preparation, and incorporation of matrices with BC in food contributes to microbial load. It is crucial to ensure that certain factors such as manufacturing techniques, acceptable manufacturing practices and especially heat treatment contribute to the fact that liposomal formulation with BC does not provide bacterial load and is safe.

The other aspect of studying is the sensory evaluation of the product with the carrier and BC. Sensory evaluation is needed because changes in food can be physical or chemical and can affect the product's appearance, texture, taste, smell, aroma, taste, and safety.

In these studies, the product's heat treatment and shelf life must be considered, and in these cases, they must be carried out on the base food and not on a food simulant as applied in the previous stage of characterization.

The sensory analysis allows knowing the organoleptic properties of food because it is done through the senses. Sensory evaluation is innate in man since from the moment a product is tried, a judgment is made about it, whether it likes or dislikes, and describes and recognizes its characteristics of taste, smell, and texture. Discriminatory tests should be used when it is necessary to determine if two samples are significantly different. Within this type of test is the triangular one that consists of presenting to the evaluator three suitably coded samples, of which two are the same, and the third is different [61]. This test was used in our research line, considering that there were three liposomal formulations used with the vitamins: SPC, SPC:SA, and SPC:CaS. For that reason three triangles were used for each combination of vitamins, for example VE and FA. Each rater was presented with three coded samples in each triangular trial, two of which were identical, and one was different, all numbered with random three-digit numbers for identification. The same samples could be the product (e.g., chocolate milk) with or without liposomes-BC, and the difference could be the product (chocolate milk) without or with liposomes-BC, respectively [29, 34]). The results obtained concluded that the addition of the liposomal formulations with the BC produced significant sensory changes in some products, such as chocolate milk. For this reason, it was proposed to carry out global acceptability tests to analyze whether the differences found were positive or negative in the base food.

The global acceptability test is within the affective tests, which are those in which the evaluator expresses his subjective reaction to the product, indicating whether he likes or dislikes it, whether he accepts or rejects it, or whether he prefers it to another. The primary purpose of effective methods is to evaluate the response (reaction, preference, or acceptance) of a product's actual or potential consumers. Hedonic scales are used to carry out these tests. The word hedonic comes from Greek and means pleasure. In this sense, hedonic scales are instruments for measuring the pleasant or unpleasant sensations produced by a portion of food to those who taste it. Hedonic scales can be verbal and graphic, and the choice of the type of scale depends on the age of the evaluators and the number of samples to be

evaluated. These scales are those that present a verbal description of the sensation that the sample produces. They must always contain an odd number of points, and the central point, "I neither like nor dislike," must always be included [61, 62].

Objectivity is achieved in the evaluators' responses to the sensations caused by a food product using the hedonic scales [61]. The evaluators were summoned, and two samples were presented. One was the chocolate milk without liposomes-BC, and the other was the chocolate milk with the liposomes and BC. In this case, they did not know what they were evaluating. **Table 4** shows the global acceptability test results where the SPC and SPC:CaS liposomes in the chocolate milk do not modify its acceptability concerning the raw food. Only the SPC:SA formulation in chocolate milk generates a decrease in its acceptability, coinciding with the fact that it had shown significant differences in the triangular test.

This global acceptability test can be performed using the pairs of product samples with or without BC-liposomes but informing the evaluator who the functional food is and the base food—obtaining very positive results that demonstrate the excellent acceptability of potential consumers regarding wanting to consume a food with different nutritional properties [29].

Thus, the implementation of sensory tests that are already effective to evaluate matrices with BC in food allow inferring the impression that a consumer would have of this functional food, and the achievement is that it is positive, which implies a significant advantage at the moment for producing BC-fortified food on an industrial scale. Because if we consider the importance and above all the cost that would imply launching a product for sale, this task cannot be carried out without being sure that the incorporation of BC matrices does not modify the base foods' sensory aspect.

4. Elaborating carriers/bioactive compounds to a larger scale, at the level of a food production line

Once the research stage study, development and application of matrices with BC at laboratory scale is finished, the final stage begins. The valuable part of this procedure is that if its production feasibility is analyzed when determining liposomes' production or any other matrix on a laboratory scale, it is easy to bring it up to a higher volume. Once the laboratory stage has succeeded, it is possible to upgrade to a larger production scale.

Sample	Total assay acceptability
Chocolate milk with SPC-VE-FA	7.30 ± 1.24
Chocolate milk	7.10 ± 2.81
Chocolate milk with SPC:SA-VE-FA	6.18 ± 3.17
Chocolate milk	7.2 ± 1.76**
Chocolate milk with SPC:SA-VE-FA	6.83 ± 1.99
Chocolate milk	7.13 ± 1.70

*Total assay acceptability of liposomal formulations. Qualifications of 40 panelists in commercial chocolate milk with or without liposomes SPC, SPC:Sa or SPC:CaS or with 5 mM of VE and 0.136 mM of FA pasteurized. Statistics were performed using the test for paired samples between each chocolate milk, with and without liposomes-BC. The results with significant differences are shown as * p <0.5; ** p <0.01; *** p <0.001 [29].*

Table 4.
Total assay acceptability of liposomal formulations.

The larger-scale production phase comprises a series of studied, analyzed, and calculated stages in what is called project evaluation. The purpose of which is to verify whether the functional product can be produced and developed at an industrial level, which can also be positioned in a specific market niche according to consumers' needs and that it is economically profitable to produce and commercially market it. In this way, in our line of research, the evaluation of the project has been carried out, which analyzed the feasibility of installing a pilot plant that produces functional chocolate milk being fortified with VE and FA, and added with essential fatty acids omega-3 and omega-6, choline and calcium (from the liposomal formulation). This type of study should consider the project idea as a starting point, and that it is related to the product to be marketed, its definition and characteristics, the type of BC, the nutritional benefits, and the deficiencies and needs of the country population. Subsequently, a market study of the functional product must be carried out, analysis of supply and demand, and analyzing the future projection in the market.

In the second part of the project evaluation, technical analysis of the plant must be carried out, which includes determining the factory's location, the quantity, and characteristics of the industrial equipment necessary according to the process—also considering the capacity of the machines according to the volume of production, process, and space. The shifts and number of working days must be analyzed according to the production volume and established product demand. We are also carrying out an administrative study considering the various aspects such as organization chart, functions, and responsibilities of the plant's employees.

The last part of the project consists of carrying out an economic analysis considering the initial investment, total operating costs, financial and economic budgets, income statement, and breakeven point and culminating with the economic evaluation of investment and determination of the economic profitability of the project [63].

Regarding the type of plant to produce the functional food, in the beginning, a small-scale pilot plant can be established, which will grow as the functional foods demands increase. It is important to highlight that the capacity of the machinery and production volume are related to the supply and demand of functional foods, in which the area to be commercialized the functional product maters, as well as with the total and variable fixed costs of production and the price of sale that will have the functional food. This last aspect is not minor, given that a consistent and competitive sale price is needed, which allows the functional food to enter and position itself in the market. All these points mentioned will be considered in the equilibrium point calculation, which allows identifying the number of functional food units that must be marketed per month to cover the sum of the total fixed and variable costs of the enterprise and to be found in balance. The equilibrium point is a reference point that indicates the minimum level of sales that must be produced not to suffer losses [64]. If the functional product's sales exceed the breakeven point, the plant will make a profit. Nevertheless, to analyze the project's profits and profitability in-depth, a series of indices such as the net present value and the internal rate of return must be calculated [65].

Of all the detailed points of the evaluation of a project, the technical analysis of the plant has a close relationship with the production and incorporation of the matrices that the BC will carry in a food production line. A technical study analysis, studies and calculates how the production line will be assembled, its lay-out, machinery and capacity, and the production volume, among other aspects.

And it is at this stage that you should consider how to incorporate BC into food production line. In the technical study of the project evaluation in our line of chocolate milk with the incorporation of liposomal formulations with BC, and

considering that it is a pasteurized and homogenized chocolate milk, it was proposed that the liposomal formulations would be incorporated after the milk homogenization stage. This decision based preventing liposomes from damaging their structure and prior to pasteurization, to ensure food safety of the entire product.

In other words, the liposomes will be incorporated into the pasteurization tank with the chocolate milk so that the heat treatment is carried out on the functional food. The low temperature heat treatment will be applied for a long time, at a temperature of 65 ºC for 30 minutes, in order to eliminate pathogenic microorganisms and decrease the bacterial flora that cause the deterioration of the product. Subsequently, a cooling will be carried out in the same pasteurization tank in order to avoid intermediate temperatures that favors the growth of microorganisms and/or unwanted effects produced in the nutrients present in the milk resulting in a greater unnecessary exposure to heat. The milk will be cooled by circulating water in the same jacketed tank where the pasteurization was carried out.

A high-pressure homogenizer will be used for the formation and incorporation of the liposomes. It is the high-pressure homogenizer. The turbine's high rotation speed and the deflection of the materials through the plate create a continuous flow through the stator. The result is a smooth surface without lumps, a mixture of both emulsion and dispersion components. A microfluidizer will also be used to obtain a uniform distribution of particles with a built-in heat exchanger for product discharge at a constant temperature.

The necessary procedure will consist of adding 500 g SPC: CaS with VE to 9.5 liters of drinking water with the AF and dispersing the sample using the homogenizer at a temperature of 35 °C at 8000 rpm for 30 minutes. Next, the liposomal suspension obtained will be processed with the microfluidizer at a pressure of 100 MPa for 5 minutes [66]. The incorporation of the liposomes will be carried out from the microfluidizer, where they have been formed towards the batch pasteurization tank. The liposomal suspension will be added to the milk with the correct dilution since the concentration of the preparation is notably higher than that added to the milk.

It is essential to consider the implementation of equipment designed to work on a pilot scale, but with the possibility of expansion to production volumes. In this way, it will be possible to start with small production volumes but increase them as functional food is increasingly positioned in the food market.

5. Conclusion

It is evident that the knowledge and importance that BC are having in nutrition and especially in people's health is increasing. And as a result of the life system and / or socio-economic situations, it is of industrial relevance that a greater quantity of functional foods with specific BC needs to be incorporated into the market, due to the deficiencies and/or nutritional needs of the population. In this emerging production line, the need arises to investigate and develop matrices that allow the incorporation of BC in all types of products, but that also protect them and preserve their functional activity. And in addition, the laboratory scale that produces those matrices must be scalable to a greater volume like that of a production line.

This chapter has exposed all the various stages of research, development, production and incorporation into foods of liposomal formulations that allow encapsulation and contain BC. This allows us to conclude that when investigating and developing a functional food, not only the results obtained at the laboratory scale in relation to its stability and characterization are of utmost importance, but also whether it is feasible, profitable and marketable should be evaluated in industrial scale production.

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Conflict of interest

None of the authors have no competing interests to declare.

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