Design, characterization and optimization of a novel liposomal based food supplement against chronic fatigue syndrome

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A mi familia,
por estar siempre.

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ABSTRACT

In today's society, people's interest against health care is growing every day. On a health-concerned population, food supplements and nutraceuticals are products with an increasing interest among the global community. This thesis arises as an industrial doctorate project, with the purpose of strengthen the bioavailability and effectivity of the active substances in Reconnect®, a current product commercialized by Vitae Health Innovation, S.L.

Coencapsulation of different actives has been widely studied and reported on the last decades, and concretely liposomes have already been used for the encapsulation of the isolated antioxidant actives phosphatidylserine (PS), coenzyme Q10 (CoQ10), [redacted] nicotinamide adenine dinucleotide (NAD[redacted]) and vitamin C, but not as a combinatory oral therapy. Coadministration of these actives on a combinatory therapy within the liposomal encapsulation technology may provide a wide and complete supplementation with promising results on chronic fatigue syndrome (CFS) patients or people suffering from general fatigue, among others. Therefore, the design and development of a novel liposomal-based food supplement against chronic fatigue syndrome is of great industrial interest.

This thesis is structured on different parts. On first place, the development and validation of a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of CoQ10, PS, and vitamin C was essential for the analysis and characterization of the selected drugs.

The second part of the thesis, separated in two chapters, is focused on the design, characterization and optimization of two methods of liposomal production, one based on the thin-film method, being the main axis of the

thesis, while the other method based on microfluidic technology was developed on a short research stay within Prof. Jing Fan's research group of Complex Fluids and Soft Materials at the City College of New York – City University of New York (CCNY – CUNY).

Finally, the third part is focused on the scaling up optimization of the most promising results of the liposomal formulation.

TABLE OF CONTENTS

ABBREVIATIONS

AA: Ascorbic Acid

Acetyl-CoA: Acetyl Coenzyme A

ADP: Adenosine Diphosphate

ANOVA: Analysis of Variance

AOAC: American Association of Official Analytical Chemists

ATP: Adenosine Triphosphate

Caco-2 cells: Human Epithelial Colorectal Adenocarcinoma cells

cADPR: Cyclic Adenosine Diphosphate Ribose

Cavin 1: Caveolae Associate Protein 1

CCC: Canadian Clinical Criteria

CDC: Centers for Disease Control

CDP-diacylglycerol: Cytidine Diphosphate Diacylglycerol

CFS: Chronic Fatigue Syndrome

Chol: Cholesterol

Chol-PEG: Cholesterol-(polyethylene glycol-600)

CoQ10: Coenzyme Q10

Cryo-TEM: Cryogenic Electron Microscopy

DAD: Diode-Array Detection

DC-Cholesterol·HCl: 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride

DHPE: 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine

DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate

DLS: Dynamic Light Scattering

DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DNA: Deoxyribonucleic Acid

DOPS: 1,2-dioleoyl-sn-glycero-3-phospho-L-serine Sodium Salt

DQ: Design Qualification

DSC: Differential Scanning Calorimetry

EE: Encapsulation Efficiency

EFSA: European Food Safety Authority

EQ: Equipment Qualification

FAD: Flavin Adenine Dinucleotide

$FADH_2$: Reduced Flavin Adenine Dinucleotide

FAT: Factory Acceptance Test

FDA: U.S. Food and Drug Administration

FR: Response Factor

GTP: Guanosine-5'-triphosphate

GUVs: Giant Unilamellar Vesicles

HDL: High-density Lipoprotein

HL: Hemolysin

HPLC: High-Performance Liquid Chromatography

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

ID: Inner Diameter

IQ: Installation Qualification

IR: Infrared Radiation

J: Flux

Kp: Permeability Coefficient

LDL: Low-density Lipoprotein

LOD: Limit of Detection

log P: Partition Coefficient

LOQ: Limit of Quantification

LRT: Lipid Replacement Therapy

LUVs: Large Unilamellar Vesicles

ME: Myalgic Encephalomyelitis

MLVs: Multilamellar Vesicles

MWCO: Molecular Weight Cut-Off

N: Theoretical plate numbers

NaA: Sodium Ascorbate

NAD: Nicotinamide Adenine Dinucleotide

NAD+: Oxidized Nicotinamide Adenine Dinucleotide

NADH: Reduced Nicotinamide Adenine Dinucleotide

NO: Nitric Oxide

NOAEL: No-observed-adverse-effect Level

O/W/O: Oil in Water in Oil

O/W: Oil in Water

OD: Outer Diameter

OQ: Operational Qualification

PA: Palmitoyl Ascorbate; 6-O-Palmitoyl-L-ascorbic Acid

PARPs: Poly ADP-ribose polymerases

PAS: Periodic Acid Schiff

PC: Phosphatidylcholine

PdI: Polydispersity Index

PE: Phosphatidylethanolamine

PEG: Polyethylene Glycol

pH: Potential for Hydrogen

pHBA: p-hydroxybenzoic acid

pKa: Acid Dissociation Constant

PQ: Performance Qualification

PS: Phosphatidylserine

PSS: Phosphatidylserine Synthase

PVA: Polyvinyl Alcohol

PVDF: Polyvinylidene Fluoride

Qt: Cumulated amount of active permeated

RES: Reticuloendothelial system

ROS: Reactive Oxygen Species

RSD: Relative Standard Deviation

RT: Retention Time

SAT: Site Acceptance Test

SDS: Sodium Dodecyl Sulfate

SFG: Simulated Gastric Fluid

SLNs: Solid Lipid Nanoparticles

SOD: Super Oxide Dismutase

Src: Sarcoma

SRPE: Supercritical Reverse Phase Evaporation

SUVs: Small Unilamellar Vesicles

Tg: Transition Temperature

TRP: Transient receptor potential

URS: Users Requirement Specifications

USP: United States Pharmacopeia

UV light: UltraViolet light

Vis-light: Vissible Light

W/O/W: Water in Oil in Water

W/O: Water in Oil

WHO: World Health Organization

ZP: Zeta Potential or ζ Potential

CHAPTER 1. Introduction

1.1 Food supplements and nutraceuticals

In today's society, people's interest against health care is growing every day. Life expectance is rising up due to the improvements on medicine and its growing ability on curing many diseases, and people is more mindful everyday about maintaining a wholesome condition. In this line, food supplements and nutraceuticals are products of increasing interest on a health-concerned population.

The motivation on consumers for trusting and using food supplements and nutraceuticals are broad, such as a general well-being, aesthetic pursuits, longevity, sport performance or prevention or treatment of many diseases, among others[1,2].

Approximately one third part of U.S. Food and Drug Administration (FDA)-approved drugs over the past 20 years are based on natural products or their derivatives[3], that being said to highlight how powerful is nature and the potential a correct food supplementation may have if the right molecules, compounds and nutrients are selected.

Generally, any active substance can be used as a therapeutic drug depending on the given dose, and any food contains potential active compounds that may be used beyond its value as a nutrient. So that's where food supplements / nutraceutical's potential emerge.

The nutraceutical revolution started in the early 80s with the publication of some clinical trials in distinguished journals regarding the benefits of calcium, fish oil and fiber. The terminology nutraceutical was first coined

by De Felice in 1989, who defined it as "*any substance that is a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease*"[4]. He pointed out that this applied to all categories of foods and parts of foods, ranging from dietary supplements such as folic acid, used for the prevention of spina bifida, or bioengineered designer vegetable food, rich in antioxidant ingredients, to chicken soup, taken to lessen the discomfort of the common cold. Nevertheless, this definition has been evolving during years and differences have been pointed out separating nutraceuticals from functional foods and food supplements, although there is not a stablished official definition for none of them.

Nutraceuticals is the terminology stablished in the frontier between a food and a drug. Active substances either extracted from plants or animals, concentrated and administered in a pharmaceutical form (as instance pills, tablets, powders or capsules), creating a promising tool of proven efficacy useful to prevent or treat diverse pathological conditions[5].

Food supplements have sometimes been differentiated from nutraceuticals saying both can be administered on the same pharmaceutical form, but food supplements do not necessarily have a specific proven action on a health condition beyond their nutritional value. Currently, European Food Safety Authority (EFSA) consider nutraceuticals equal as a food supplement. EFSA uses the term food supplement in relation to all concentrated sources of nutrients or other substances with a nutritional or physiological effect whose purpose is to supplement the normal diet. The definition includes non-nutrient compounds that usually are marketed as nutraceuticals[5].

The European Nutraceutical Association defines the term "nutraceutical" as "*nutritional products which have effects that are relevant to health. However, in contrast to pharmaceuticals these are not synthetic substances or chemical compounds formulated for specific indications*", while the American Nutraceutical Association uses the De Felice definition[6].

In contrast, functional foods are similar to ordinary foods which are consumed as part of a regular diet, but exert a health-beneficial effect when frequently consumed that goes beyond their nutritional value[7].

All this nutraceutical revolution is leading the market into a new era of health and medicine, where the food industry is everyday more research oriented towards the same way than the pharmaceutical industry. Nutraceuticals progressively provides more means to improve society's health on treatment of conditions and its prevention, and future perspectives will keep on the aim of improving together with the increasing demand and the interest of the public.

1.2 Chronic fatigue syndrome

Chronic fatigue syndrome (CFS), also named myalgic encephalomyelitis (ME), is a cluster of clinical symptoms which is defined as the presence of unexplainable fatigue lasting more than 6 months accompanied by further conditions like musculoskeletal pain, multiple joint pain without swelling or redness, headaches or unrefreshing sleep, among others. In consequence, CFS additionally affects productivity, since CFS patients had discontinued employment because of fatigue-related symptoms[8-11].

In the last version of the World Health Organization (WHO) International Classification of Diseases[12], both CFS and ME are included under the same code and they are considered synonym terminologies of the disease, despite of the different origin of the two terminologies.

Although fatigue has been a condition that has always concerned humanity, it was firstly described as an illness by Acheson E.D. in 1956 with the proposed name "benign myalgic encephalomyelitis"[13]. In parallel, on the early 80's a chronic debilitating affection was associated to a series of viral and immunological alterations, naming the clinical picture as "chronic Epstein-Barr virus syndrome"[14]. In 1988, Holmes G.P. *et al.* proposed a different term for this syndrome, since any concluding result demonstrated the direct correlation of the syndrome with the Epstein-Barr virus. "Chronic fatigue syndrome" was the suggested alternative, being with this event redefined and standardized as an idiopathic condition providing the bases for further studies in the future[15]. The Centers for Disease Control (CDC) recommended lately the use of CFS as the best and more adequate terminology among the existing proposes, and removed the "benign" from the ME[16]. Additionally, with Holmes G.P. *et al.* it was conceptually established the first definition of a case to be diagnosed as a CFS case.

The subject must first of all be discarded from other medical or psychiatric causes for the illness. Afterwards, the patient must manifest fatigue lasting for 6 months or more that does not disappear with bed resting, and severe enough to reduce daily activity below 50 %. Apart of this, the subject must exhibit eight of the following symptoms: moderate fever, sore throat, general muscle debilitation or myalgia, arthralgia, recurrent headache,

sleeping alterations and neurophysiologic complaints like irritability; or at least 6 of the previously described symptoms plus 2 of the following ones reported by a physician: low fever, non-exudative pharyngitis or palpable lymph nodes.

Fukuda K. *et al.*[10] revised the case definition of the syndrome in 1994 and developed a guideline for the better clinical evaluation and diagnosis of the illness, which is nowadays the most used guideline among physicians worldwide. They also suggested diagnosing with "idiopathic chronic fatigue" the clinical cases that do not fit with all the established criteria for a CFS case. In 2003, a group of CFS experts created the Canadian Clinical Criteria (CCC) after a Consensus Workshop was held with selected researchers and physicians[17], and it was lately refined in 2011 with the International Consensus Criteria[18]. The International Association for Chronic Fatigue Syndrome/Myalgic Encephalomyelitis developed a clinical guideline in 2012[19], and another one was released in 2015 by the American Institute of Medicines with the aim of unifying criteria and definitions stablished until the moment[20]. Even with so many case definitions and guidelines, it is still challenging to properly identify the illness because of the lack of any diagnostic tool/biomarker, which is urgently needed for an accurate diagnosis.

It is estimated that CFS will have a prevalence of 857/100,000 habitants (meaning 2.8 M and 6.4 M people in USA and Europe, respectively) by July 2020[21-22]. In particular, gender demographics of the diagnosed population show a higher prevalence on women than in men with a reported average of 60-80 % female compared to males[22-23]. Thus, identifying the potential CFS population is crucial for early intervention.

Three decades after it was firstly described, CFS etiology remains unclarified. The cause of CFS remains unknown, although some theories have been suggested. Some investigators relate it to abnormal function of the immune system[24], or with viral infections with effects on the immune system, as there are many cases reported after an episode of the Epstein Barr virus or after hepatitis B vaccination, for example[25,26]. Other recent studies have reported higher levels of cytokines or hsCR as biomarker, suspecting of a pathogenesis related to inflammation[27,28]. The potential role of transient receptor potential (TRP) ion channel in CFS pathogenesis has also been described, since a reduced expression of the ion channels together with their dysfunction has been identified[29-31]. There is also evidence of relation with genetic predisposition, with reported dysfunctional or abnormally expression of genes[32-34], premature telomere attrition associated with accelerated aging[35], or polymorphisms in the mitochondrial Deoxyribonucleic Acid (DNA)[36]. Some other results suggest as well that the central nervous system plays a key role on CFS pathogenesis, with significant results on a reduction of the grey matter volume directly linked with physical activity[37]. Lastly, dysfunctional mitochondria has been lately popularized as a key factor for the development of CFS, since mitochondria are responsible of producing up to 90 % of the human body energy in form of adenosine triphosphate (ATP)[38-42]. Excellent reviews have been published resuming the exposed hypothesis until the moment[43-44], although this thesis will focus on the mitochondrial damage and its treatment.

1.2.1 Energy and mitochondria

From Greek, *mitos* meaning "threat", plus *khondrion* as a diminutive of *khondros*, meaning "little granule", mitochondria are dynamic intracellular organelles with essential roles on numerous biochemical processes[45]. Their structure and morphology variates among the different kind of cells, with a size ranging from 0.75 to 3 μm^2 approximately, although it is always composed by the same elements (Figure 1)[46-49]. It consists on an outer and inner membrane, where the inner is extremely invaginated with deep grooves –cristae– that provide a large surface area, thus permitting chemical reactions to occur. The space within the inner membrane, the matrix, is a gelled solution that contains the mitochondrial DNA, ribosomes, substrates and enzymes enabling the ATP production[46].

Mitochondria are responsible of producing up to 90 % of human body energy in form of ATP, thanks to the oxidative phosphorylation of reduced cofactors by the ATP synthase on the respiratory chain. Additionally they are involved on cell signaling cascades, metabolism, antiviral responses, cell-cycle control and death and also on generating Reduced Nicotinamide Adenine Dinucleotide (NADH) and Reduced Flavin Adenine Dinucleotide ($FADH_2$) through the oxidation of substrates derived from glucose, fatty acids and aminoacids[46,50-52].

Most of the human energy production in form of ATP starts in the mitochondrial matrix with the citric acid cycle, also known as Krebs cycle or tricarboxylic acid cycle (TCA). The Krebs cycle is a circular catalytic complex of reactions that oxidizes acetyl coenzyme A (Acetyl-CoA) molecules derived from the breaking down of aminoacids, fatty acids, or the glycolysis of glucose, producing reduced NADH, $FADH_2$ and ATP

(Figure 2). The net balance of products after one complete citric acid cycle is three NADH molecules, one $FADH_2$, and one Guanosine-5'-triphosphate (GTP) (equivalent to an ATP) molecule. These NADH and $FADH_2$ molecules will lately contribute to the formation of more ATP energy on the electron-transport chain.

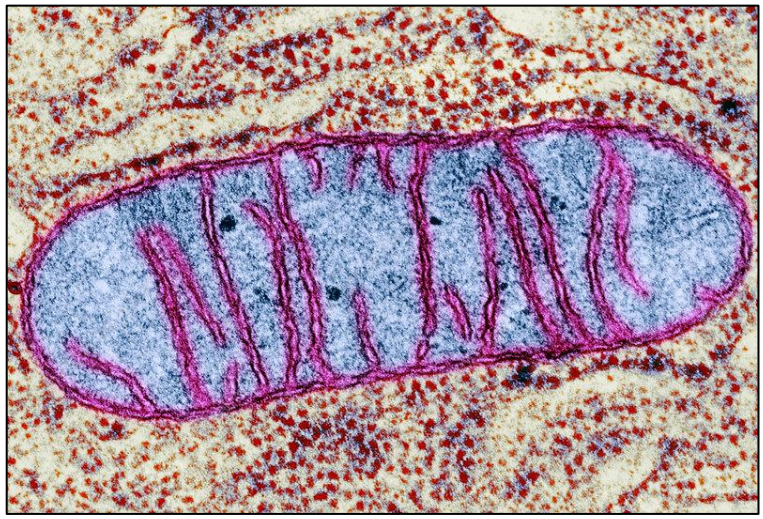

Figure 1. Coloured TEM image of a mitochondrion. Membranes and cristae are coloured in dark magenta and intermembrane space appears in light magenta, while mitochondrial matrix is coloured in blue[49].

The electron-transport chain (Figure 3) not only allows the ATP synthesis but also promotes the transport of Adenosine Diphosphate (ADP) and phosphate from the cytosol to the matrix. Protein complex I passes electrons from NADH to coenzyme Q10 (CoQ10) through its oxidation, pumping to the intermembrane space four protons. These electrons are transported along the electron-transport chain through the different complexes by the CoQ10 and the cytochrome c, with a final balance of 10 protons introduced to the intermembrane space for a complete electron-transport chain that will lately be processed on the proton-pumping ATP

synthase (or ATPase) to generate ATP energetic molecules. The energy provided by a single NADH molecule enables the synthesis of 2.5 ATP molecules, approximately.

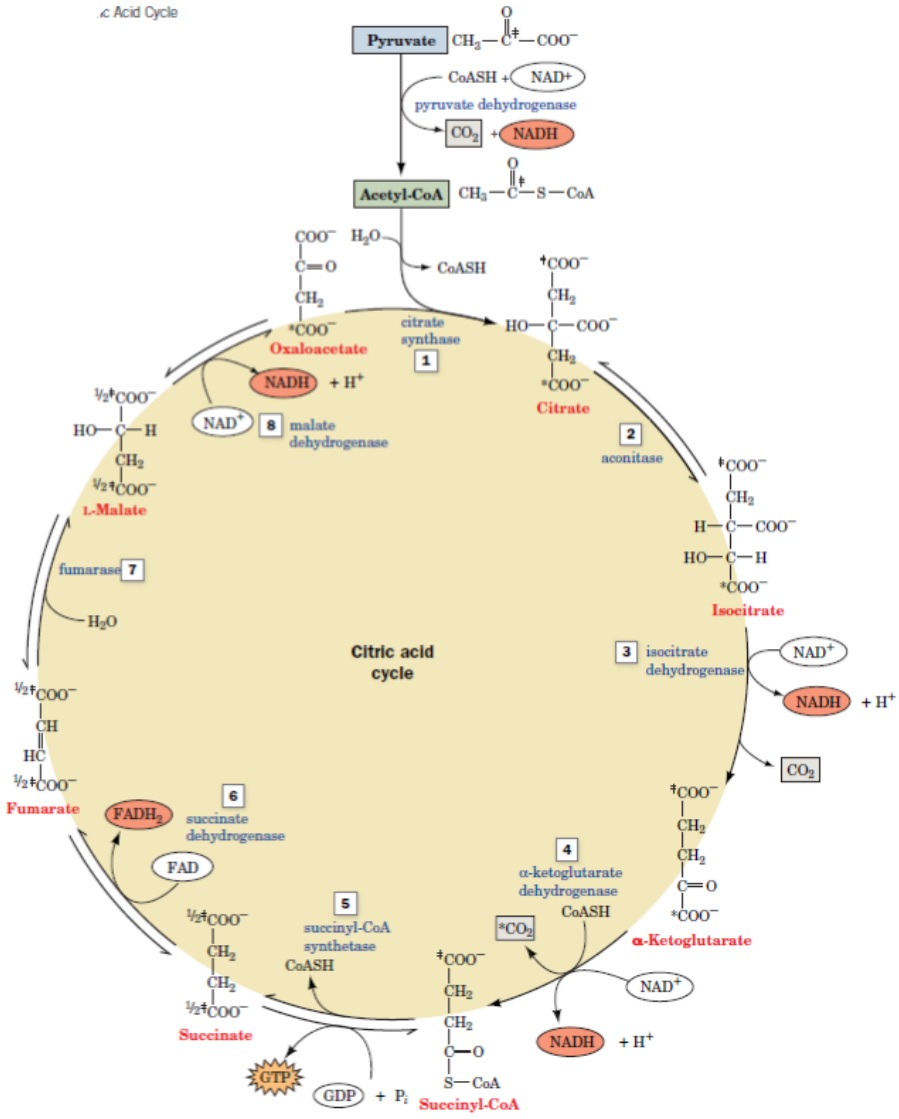

Figure 2. Schematic representation of the 8 reactions forming the citric acid cycle. All catalytic enzymes appear in blue, while substrates appear in red[46].

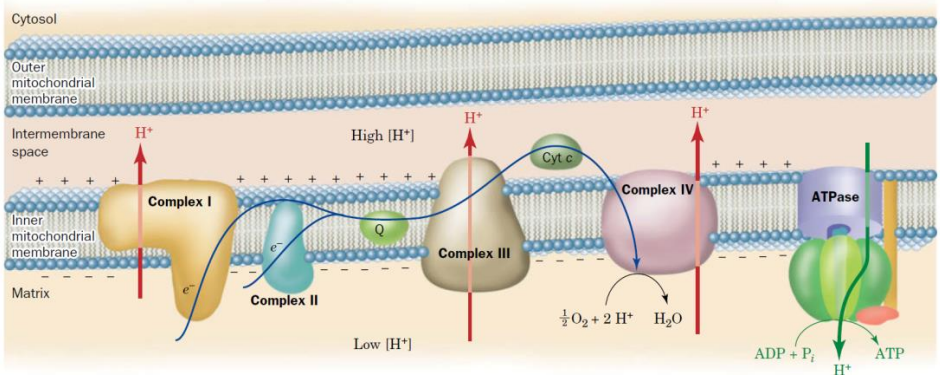

Figure 3. Diagram representing the mitochondrial electron-transport chain[46].

During glycolysis, where glucose is converted to two pyruvate molecules, also two molecules of ATP and two NADH are formed. Subsequently, pyruvate molecules are converted to acetyl-coA with the production of 2 more NADH molecules. These cytosolic NADH molecules will eventually produce more ATP energy as well on the mitochondrial electron-transport chain, although they will need an additional step since there is no NADH transport protein on its membrane. Only the reducing protons and electrons of the cytosolic coenzyme will cross the membrane through the glycerophosphate shuttle[46]: NADH is oxidized by dihydroxyacetone phosphate catalyzed by 3-phosphoglycerol dehydrogenase, which is afterwards oxidized by flavoprotein dehydrogenase by reduction of FAD to $FADH_2$. Then, $FADH_2$ is oxidized passing in this way the electrons to the complex III on the electron-transport chain on the inner mitochondrial membrane. This process will yield in a final balance of six pumped protons to the intermembrane space, four less than on the NADH originated at the citric acid cycle, and consequently leading to 1.5 ATP equivalents per cytosolic NADH molecule.

Oxidative metabolism and energy production collaterally has an occasional production of Reactive Oxygen Species (ROS) that may damage cells[53]. Cytochrome C oxidase and protein complexes I, II and III sometimes generate non-completely reduced metabolites of oxygen like the superoxide radical, a very reactive intermediate that may put in danger cell integrity. Nevertheless, mitochondria is equipped with an antioxidant defense system in charge of the detoxification of the species created by the ROS, like the superoxide dismutase (SOD) or the formation of other antioxidant species, like vitamin C or vitamin E.

1.2.2 Mitochondrial oxidative damage

The human body contains usually less than 100 g of ATP at any instant, but can consume up to 100 kg per day. For this reason, the ATP recycling process in mitochondria is extremely important for cellular energy production. If the mitochondrial source of energy is dysfunctional, many symptoms may appear, including CFS symptoms[54].

Dysfunction of mitochondria is usually defined as the inability of the organelle to appropriately carry out its predominant physiological activity to produce ATP in response to energy demands. Depending on the context though, it may also be related with the incapability of abnormally detoxifying the ROS or their excessive production and accumulation, the incorrect synthesis and catabolism of substrates and metabolites, or the calcium regulation of homeostasis and transport on the matrix[55]. Given the essential role that mitochondria have on many biological mechanisms, any change or alteration may lead to various dysfunctions or even cell death. In general, damaged mitochondria are fragmented, round, and swollen,

but they may also apparently look normal[48]. Many tests can be undergone to evaluate mitochondrial functionality: ATP turnover, proton leak, lactate concentrations, coupling efficiency, maximum respiration rate, apparent respiratory control ratio spare respiratory capacity and non-mitochondrial respiration, among others[55,56].

1.2.3 Mitochondria as drug targets in CFS

Considerable evidence has been reported regarding mitochondrial dysfunction in CFS cases[54]. Studies of serum analyses on CFS patients have demonstrated a significant reduction of oxidative metabolism and of the concentration of essential compounds related with ATP synthesis when compared with healthy subjects, like CoQ10, carnitine and acylcarnitine, together with an increase in the concentrations of xenobiotics, lactate, pyruvate, pro-inflammatory cytokines, elastase and intracellular lactic acid and oxygen/nitrogen species[27,54,56-60]. This may inhibit mitochondrial activities and cause their shutdown. Other studies of muscle biopsies have shown proof of abnormal mitochondrial degeneration in CFS, with atrophy on fibers and fusion/branching on mitochondrial cristae, and it has also been found evidence of gene deletion in mitochondrial DNA with genome involving energy production[61,62]. Membrane potentials on mitochondrial membranes has been observed in ME/CFS patients[59,63]. Mitochondrial dysfunction on mononuclear blood cells[54,64] and on the neutrophils of CFS patients[54] was described, with also an increased apoptosis on neutrophils in another study. Additionally, it has been reported a decrease of intracellular pH and a lower synthesis rate of ATP after moderate exercise on CFS patients, suggesting problems on the recycle of ADP or on mitochondrial functionality[65]. Lastly, significant

correlation on the severity level of the illness and the degree of the mitochondrial dysfunction was observed[54,66].

Mitochondrial dysfunction is an attractive hypothesis for the pathology of Chronic Fatigue Syndrome, since it would explain a substantial fraction of its symptomatology. The similarities between mitochondrial dysfunction and CFS are numerous between the multisystem symptoms of both disorders. Both patients appear to have muscle weakness, cramps or pain. Both patients usually suffer from migraine, neurocognitive symptoms with impairments in attention or memory, neuro-psychiatric disorders, neuropathic pain or dysautonomia. And both patients show also alterations on general exercise tolerance, fatigue and diminished cardiac response to exercise, with a progressive course on the disease[58].

The complex synergistic impairments of mitochondrial oxidative phosphorylation, ROS system and lowered CoQ10 suggest that a multicomponent drug cocktail treatment may be a promising approach against CFS. To date, few therapies have been developed for the treatment of CFS. Among them, some interesting results have been obtained in CFS patients treated with mitochondrial drug therapy based on oral supplementation with CoQ10, NAD and Phosphatidylserine (PS)[67-76].

CoQ10 is a strong antioxidant and an essential nutrient of the mitochondrial respiratory chain that also has been described as an anti-inflammatory agent[67]. Supplementation with CoQ10 has been tested in double-blinded clinical studies in CFS patients, and several CFS symptoms like depression, tiredness, weakness and sleep quality seemed to be significantly improved[67,68].

On other hand, NAD is a coenzyme known for triggering ATP production in mitochondria that has also been reported to reduce fatigue in clinical assays with CFS patients[69-70]. Recent studies have tested the coadministration of CoQ10 and NAD as mitochondrial antioxidant therapy in double-blind trials, obtaining an impressive recovery on biochemical parameters in CFS and a significant improvement in fatigue impact scale total score[71-72].

Additionally, it has been proven that PS is able to reduce muscle soreness and induce well-being and exercise capacity[73], and some interesting works describe the supplementation with cell membrane forming lipids like PS (Lipid Replacement Therapy, LRT) in order to avoid the damage of mitochondrial membranes, and therefore, the loss of cellular functionality[74-75]. In fact, in another clinical trial, 58 patients were administer with a cocktail of CoQ10, NAD and PS, resulting in a 30.7 % statistically significant reduction of the fatigue symptomatology after 60 days of study[76].

Lastly, strong and widely known antioxidant activity has been used and reported on vitamin C, which has been very used on both pharmaceutical, cosmetic and food supplement industry[77,78].

1.3 Coenzyme Q10

The vitamin-like CoQ10 or Ubiquinone is a highly hydrophobic molecule that plays an essential roll on multiple processes for the proper function of the human body.

Chemically, coenzyme Qn molecules consist on a benzoquinone nucleus prenylated to an isoprenoid chain of various lengths, and the subscript

refers to how many isoprenoid units does the molecule has. With a molecular weight of 863.34 g/mol, CoQ10 is formed by a side chain of 10 isoprenoid units (Figure 4). Interestingly, CoQ10 is also known as ubiquinone due to its ubiquitous presence among nature, and the quinone structure[79-82].

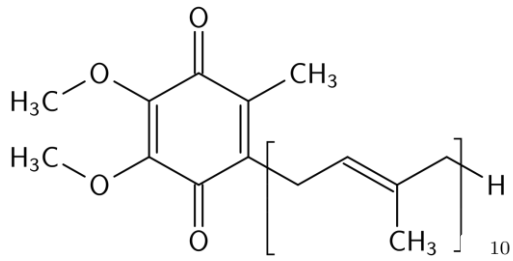

Figure 4. Detail of the chemical structure of CoQ10.

CoQ10 molecules are located along the hydrophobic cellular membranes intertwined with the phospholipids forming the bilayer. It is a non-essential nutrient that is endogenously synthesized in the human body: Briefly, the 4-hydroxybenzoate nucleus is formed from the essential aminoacid tyrosine, while the isoprenoid chain is build up from isoprenoid units proceeding from the metabolism of three molecules of acetyl-CoA in the Mevalonate pathway. Prenylation takes place thanks to the enzyme p-hydroxybenzoic acid-decaprenyltransferase (pHBA-decaprenyltransferase), and some other modifications are afterwards made to the ring by other enzymes[80].

Due to its extremely hydrophobic behavior, absorption on the small intestine is very low and thus leads to a low bioavailability when orally administered. Because of its lipid characteristics, absorption mechanism may be very complex and dependent upon both active and passive

transports. CoQ10 has a demonstrated "formulation-dependent" bioavailability: multidosage seems to be several fold more bioavailable than single dosages, and it has also been suggested that solubilization might be a key parameter on absorption[83].

The administration of CoQ10 as a supplement was reported as safe with very low toxicity and no genotoxic potential, having a non-observed adverse effect level (NOAEL) of 1.2 mg/kg/day. As a reference, the average daily intake is 5.4 and 3.8 mg on men and women, respectively. Additionally, no signals of plasma/tissues accumulation were found after the suspension of the administration, nor was found any evidence of influencing its endogenous synthesis[84,85].

Strongly attached to the inner mitochondrial membrane, the main function of CoQ10 is the contribution to the energy formation on the electron transport chain in form of ATP[46]. For this reason, the coenzyme can be mainly found located on tissues with high energy demand like heart, muscles, kidney or liver[86]. CoQ10 also plays an essential role on preventing oxidative damage of DNA, lipid and proteins, due to the remarkable antioxidant properties that neutralize ROS[87].

Many factors can contribute to lower its concentrations on the body system, such as aging, genetic alterations, insufficient nutrients intake or pharmacological treatments like statins. Statins inhibit the formation of mevalonate, that is not only a precursor of cholesterol (Chol) but also a CoQ10 precursor[88].

CoQ10 deficiency phenotypes could be an encephalomyopathy, cerebellar ataxia, severe infantile multisystem disease, nephrotic syndrome or

isolated myopathy[89]. Additionally, deficiency on CoQ10 bioavailability may lead to many disease states, and its supplementation has been proved to show clinical improvements on various studies[67,90,91].

It has been considered a great candidate for the treatment of mitochondrial dysfunction on CFS with abnormal energy metabolism or increased oxidative stress. Double-blinded clinical studies have been carried out in CFS patients, and several CFS symptoms like depression, tiredness, weakness and sleep quality improved significantly[67,68].

Its supplementation has also demonstrated positive results on the treatment of cardiovascular diseases: statins-induced myopathy; heart failure, since it is an energy depletion status with high oxidative stress that produces a reduced contractile function; hypertension, since oxidative stress reduces nitric oxide availability and therefore produces vasoconstriction that elevates blood pressure; and atherosclerosis, due to the antioxidant properties of CoQ10 that may reduce Low-density Lipoprotein (LDL) peroxidation and endothelial dysfunction, together with a reduction on total Chol and an increase on High-density Lipoprotein (HDL)[91-94].

A neuroprotective activity of the molecule has also been evidenced in literature on various studies: when neuronal cells suffer from oxidative stress that could cause cell dysfunction or death, CoQ10 has a vital importance on stabilizing mitochondrial membranes[94]. Additionally, lowered serum levels of CoQ10 were found on many neurodegenerative diseases. Promising results have been reported against glaucoma[96], together with reported slowing in the progression of Parkinson's, Alzheimer's and Huntington's disease, Friedrich's ataxia or amyotrophic

lateral sclerosis[97,98]. There are studies suggesting migraine could be a result of mitochondrial impairment, and therefore CoQ10 may be a successful tool to reduce the severity or frequency of the symptoms[99].

CoQ10 activity on prevention of lipid peroxidation and DNA and protein oxidative damage may be a powerful tool to reduce the susceptibility to develop cancer, together with a reduction of metastases risk. Immunomodulators used to reduce metastases after surgery require large amounts of ATP: it has been reported 10 times reduction of metastases with the cotreatment of CoQ10 and immunomodulators, in comparison with the treatment with just the immunomodulators[100,101]. ROS could be responsible on infertility due to DNA damage and lipid peroxidation on the membrane of sperm[102]. In this sense, antioxidant properties of CoQ10 have been reported to improved semen parameters after its supplementation[103].

Hyperglycaemia on diabetes cause an increase on the production of oxygen free radicals, which are the responsible on the pathogenesis of many diabetic complications due to oxidative stress. Therefore, CoQ10 can also be a promising supplement to mitigate part of its symptomatology[104].

Lastly, due to the strong antioxidant activity of the molecule, CoQ10 has recently gained popularity among its use on the cosmetic industry.

1.4 [redacted] NAD[redacted]

Nicotinamide adenine dinucleotide (NAD) is a coenzyme with a fundamental function in the metabolism of energy in form of ATP and other essential biological processes[46].

Structurally consists in a ribosylnicotinamide 5'-diphosphate conjugated to adenosine 5'-phosphate by pyrophosphate linkage[105]. The coenzyme, as shown in Figure 5, can be expressed in two different interconvertible states, the oxidized and the reduced one, being named as NAD+ and NADH, respectively. The transformation between both species by hydride transference makes it a recycling process where one specie can be converted to the other one without being destroyed[106].

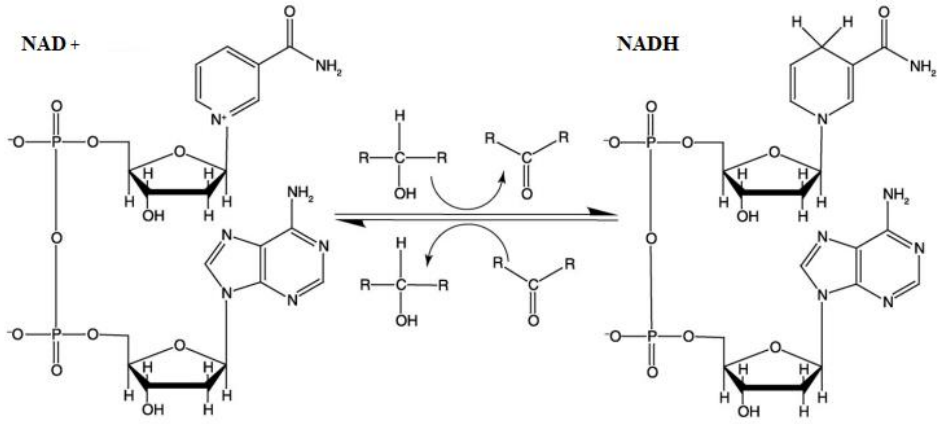

Figure 5. Chemical structure of NAD+ and NADH, with detail on the interconvertible hydride transfer reaction: the moment when NAD+ is converted to NADH, the molecule gains two electrons and a charged hydrogen molecule (H+)[106].

When no recycled, NAD can be *de novo* synthesized from the essential aminoacid tryptophan or can be recovered from breakdown products like niacin (salvage biosynthetic pathway). NAD is mostly located in the mitochondria, thus being found in higher concentrations on tissues with energy demand like muscle or heart.

A challenge to undergo when thinking on using the coenzyme NAD[redacted] for the production of medicinal products or food supplements, is the sensibility of the molecule when exposed to heat, light, pH and oxygen.

This water-soluble molecule may easily degradate and particularly when in solutions, although it may be stable if properly stored protected from light in dry and cold conditions[107].

There are no toxicity reports available, although the safety of NADH was evaluated on beagle dogs on doses up to 500 mg/kg/day without any significant adverse effect observed. However, attention should be paid off at ≥ 10 mg/day doses of NADH, which may produce anxiety, jitteriness or insomnia[105,108].

The redox cycling of NAD is an essential reaction on metabolic pathways such as mitochondrial oxidative phosphorylation, glycolysis and citric acid cycle, therefore constituting a basic for the synthesis of energy in form of ATP. Additionally, it is indispensable for the homeostatic functioning of around 500 enzymes, such as sirtuins, poly ADP-ribose polymerases (PARPs) and cyclic ADP-ribose (cADPR) synthases. This makes the molecule indispensable for cellular viability, signaling, DNA repairing, transcription, muscle function, neuroprotection and oxidative stress resistance, among others[46,109-111].

Supplementation with NAD is therefore an interesting option in several conditions and pathologies. Defects on mitochondrial function, due to age-related factors or any other cause, may lead to a NAD concentration decline. The restorement of NAD levels might increase ATP synthesis rate, increase oxidative stress defenses, delay senescence, and treat many neurodegenerative diseases such as Huntington's disease, Alzheimer's, Parkinson's and CFS. NAD has been reported to reduce fatigue in clinical assays with CFS patients, with the obtaining of an impressive recovery on biochemical parameters[69,72].

Metabolic dysfunctions or other conditions like obesity, cancer, diabetes, cardiac ischemia kidney dysfunctions or hyperglycemia may be profitable targets for NAD[redacted] supplementation, since NAD[redacted]-consuming related enzymes are involved in all of these disease's pathogeneses.
Lastly, on healthy individuals, NAD[redacted] may be used to improve simple transitory fatigue, exercise capacity, memory and concentration[106,110-112].

1.5 Phosphatidylserine

PS (Figure 6A) is an essential structural lipid constituent of eukaryotic membranes and organelles, like mitochondria, Golgi complex and endosome, among others. It is the anionic phospholipid with higher content on eukaryotic cells, with a percentage up to 10 % of total lipids, and has a vital role in cell signaling pathways[113].

PS chemical structure consists in a glycerol nucleus, with two acyl chains at sn-1 and sn-2 positions, and a phosphate group attached to position sn-3, which is then linked to a serine (Figure 6B). Acyl chain lengths and saturations variate among the different cells and organelles, although in general, ≥ 16 carbon fatty acids are found in sn-1 positions and unsaturated fatty acids attached to sn-2[114].

The synthesis of PS can be accomplished through two different pathways, schematized in Figure 7. While yeasts use the conjugation of a serine molecule to a Cytidine Diphosphate Diacylglycerol (CDP-diacylglycerol), mammalian cells exchanges the polar group of Phosphatidylcholine (PC) or Phosphatidylethanolamine (PE) with the utilization of the enzymes Phosphatidylserine Synthase (PSS) 1 and 2. PSS1 releases the choline group from PC, while PSS2 releases the

ethanolamine from PE, inserting in exchange a serine group. These PPS are reported to be localized in mitochondria-associated membranes[114,115].

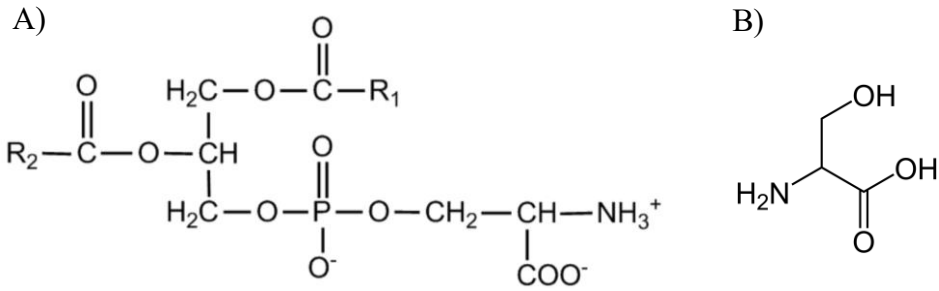

Figure 6. Detail of the chemical structure of PS (A) and Serine (B).

The supplementation with PS has been endorsed with up to 300 mg/day by U.S. Food and Drug Administration, with a demonstrated safety on several studies and clinical trials on human individuals and with no report of adverse reactions upon its oral administration[116-121].

PS has an essential role on eukaryotic cells composition and structure, and in addition some studies suggest that it contributes to the curvature of the membrane and to the formation of endocytic vesicles due to its negative charge[122].

Importantly, PS also plays a key role on the activation and recruitment of various enzymes and proteins at its exposure on membranes. PS has a vital function on signaling in coagulation, since tenase and prothrombinase only activates after binding with PS[123,124]. Erythrocytes clearance by macrophagues is thought to be signaled by the PS in the outer membrane after its translocation by scramblase[125]. It also signals the internalization of viruses by host cells[126], and the recognition and clearance of apoptotic cells: apoptosis enables the renewal of the organism[114].

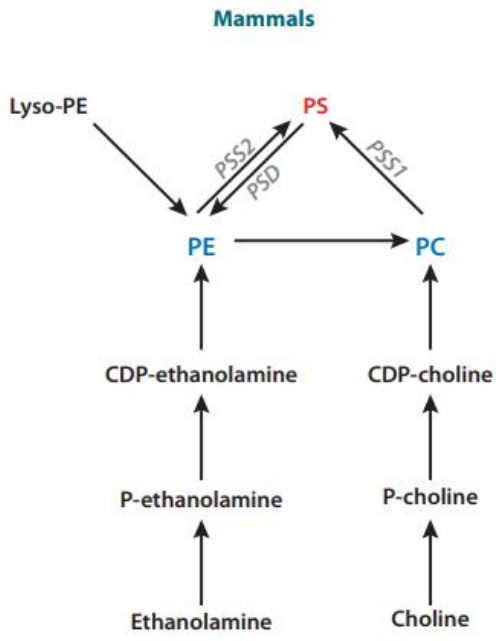

Figure 7. Scheme representing the two biosynthetic pathways for the formation of PS in mammal cells[114].

Some of the other proteins binding or influenced by PS are Rab-GTPases, Na+/K+ ATPases, dynamin-1, neutral sphingomyelinase, K-Ras, Sarcoma (Src), Raf-1 kinase, nitric oxide synthase, Protein Kinase C, TYRO3, AXL, MERTK Akt, Hsp 70, Cavin1 and Evectin-2, among many others: this makes PS a decisive molecule on many different cellular processes, including homeostasis, the control of inflammation processes and other roles in some diseases as cancer[127]. Some interesting reviews have been written deepening on these proteins and their relation with PS[113,114,128].

Regarding the potential of PS as a pharmacological or food supplement candidate, the effects upon its administration are the characteristic effects previously described on the endogenous PS. It may be additionally

effective as an antioxidant against cell and tissue oxidation, and therefore a cellular defense against oxidative stress[129-131]. Some interesting works describe the supplementation with cell membrane forming lipids like PS (Lipid Replacement Therapy, LRT) in order to avoid the damage of mitochondrial membranes, and therefore, the loss of cellular functionality[74-76].

Defects on apoptosis regulation may lead to the development of diseases due to an excess of cell accumulation –as in cancer– or due to a cell loss –as in neurodegeneration and brain disorders or cerebrovascular accidents[130]–.

An alteration on the normal composition of phospholipids in brain may affect to neurological disorders[130]. Alzheimer's patients have been found to have alteration on membrane forming phospholipid's concentration, and positive results on an increase of brain glucose utilization, memory, information processing and the ability to perform activities were achieved after supplementation with PS[131-133].

Many clinical trials demonstrated an antidepressant effect, a reduction on anxiety, improvement on cognitive functions, and a more relaxed state after their treatment with PS[134]. Other observed functions significantly ameliorated were the ability to recall words by a 42 % in ≥ 60 year old people that complained of memory loss[135], or verbal learning and fluency, visual learning, face-recognition, attention, communication skills, initiative, socialization and self-sufficiency in many other clinical trials[136-138]. It is hypothesized that all these improvements are result to PS effects on membrane neuronal properties[136].

Lastly, an interesting review has been written about the supplementation of PS on healthy population for exercise-induced stress[129].

1.6 Vitamin C: ascorbic acid and ascorbyl palmitate

If the terminology "vitamin C" is looked out on an academic database website as for instance Web of Knowledge, the resulting number of published articles is as high as 94.140, by June 2020. Vitamin C is probably the most popular vitamin and the most widely employed one on human supplementation, with crucial importance on human health. It is an essential vitamin that cannot be synthetized by humans because of lack of L-gulono-1,4-lactone oxidase enzyme, and therefore need to be obtained through the diet[139].

Water-soluble vitamin C, also known as L-ascorbic acid, consists on a four-carbon lactone ring with a 2-en-2,3-diol-1-one moiety (Figure 8). Alkyl vitamin C derivatives are structured as a combination of the L-ascorbic acid nucleus with a lipophilic moiety, consequently exhibiting a much more fat-soluble behavior with the typical properties of surfactants. Indeed, this fat-soluble derivatives (such as ascorbyl palmitate) have been used for encapsulation due to its ability to form lamellar vesicles[140,141]. Additionally, alkyl derivatives have demonstrated an increased thermal and oxidative stability in comparison to L-ascorbic acid[142].

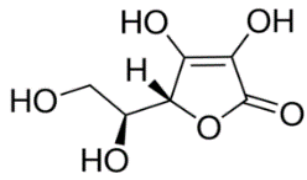

Figure 8. Detail of the chemical structure of ascorbic acid (AA).

At a vitamin C daily intake of 60-100 mg, plasma ascorbate concentrations quickly rise to around 50 μmol/l. Higher daily intakes increases levels no more than 70–80 μmol/l, when concentration reaches a plateau state limited by the reabsorption capacity on renal tubes. Too much vitamin C daily intake not only would not imply extra benefits, but also it may cause nausea or diarrhea. When plasma levels fall below 20 μmol/l are often associated with a deficit with signs of fatigue, muscle weakness, anemia, lethargy or even scurvy[143]. Scurvy is a pathological condition related to the deficit of vitamin C, where blood vessels and connective tissue get damaged due to the deficient production of collagen, leading to death in extreme situations[144].

The pleiotropic effect of vitamin C on every function it realizes is based on the key antioxidant activity, based on the donation of electrons. In brief, it has a fundamental function on acting as a cofactor on hydroxylation reactions for the biosynthesis of collagen, aminoacids, catecholamines, L-carnitine, Chol and some peptide hormones. Additionally, it has a demonstrated role on immune-supporting response, anti-inflammatory effect, and the most important, it acts as a potent reducing agent against oxidative stress by ROS species[144,145].

ROS are potential damaging radicals produced on metabolic cellular respiration. The antioxidant attributes of vitamin C make it a powerful radical scavenger that protects cells from oxidative damage reducing ROS to form an ascorbate free radical that may serve as an electron donator[192]. This fact collaterally affects many conditions directly related to oxidative stress as peroxidation of lipids, sepsis or DNA mutation. Since oxidation induced mutations on the DNA are key on cancer developments, anti-

cancer or cancer preventive potential of vitamin C should be considered[147].

Proline and lysine residues of pro-collagen need to be hydroxylated for a proper intracellular folding leading to collagen synthesis. Vitamin C as a cofactor, stimulates the specific required hydroxylase enzymes with the donation of electrons[148].

Vitamin notably has another important paper on the prevention of cardiovascular condition. Its influence on the synthesis of nitric oxide (NO) has been suggested to be the key aspect on its cardiovascular protective function, enhancing the activity of the NO synthase: the tetrahydrobiopterin cofactor is usually inhibited by ROS oxidation, so vitamin C may stabilize the reduced form. An increase of NO rate of production imply indirectly a protection of vascular endothelium due to the effects of NO, like smooth muscle cell relaxation, inhibition of proinflammatory cytokines and downstream vasodilatation. Studies have been carried out to investigate ascorbate effects on cardiovascular diseases with interesting results after vitamin C supplementation. All these results make plausible the preventive effect of vitamin C on endothelial dysfunction[149-158].

Lastly, other applications of vitamin C have been reported with apparent benefits on the field of ocular disease preventions, as cataracts, on the treatment of neuropathological diseases with improvements in memory or neurotransmission, or on the cellular response to low oxygen conditions[159-161].

1.7 Drug delivery systems

Encapsulation is the process by which one material or mixture of materials are coated with or entrapped within another material or system[162]. Encapsulation strategies are nowadays widely used in the pharmaceutical, cosmetics and the food supplement industry, although they have been reported since the middle 60s, or even earlier[163]. But going beyond the industrial encapsulation of actives, we can even find encapsulation examples on nature with bird'eggs, plant seeds or seashells.

Drug encapsulation strategies are of vital importance for the proper delivery and absorption of active substances, being an intelligent way to solve common problems like a poor solubility of actives in water, a reduced stability in front of temperature or humidity, or even for hiding adverse effects or unpleasant tastes. Additionally, the oral administration of drugs means an additional challenge because of the numerous adverse conditions the actives must overcome, including the pH of the stomach, low residence time of drugs on the body or the reduced absorption of actives, which can compromise their final bioavailability. Encapsulation may successfully address these problems as well, allowing the protection of the actives under potentially deleterious conditions.

1.7.1 Encapsulation materials

Polymers (starches, cellulose, sugars, gums, proteins, dextrins, alginates, chitosan, gellan, synthetic polymers, carrageenan…), lipid materials (fatty acids/alcohols, glycerides, waxes and phospholipids), proteins (caseins, gelatins, whey proteins), or a combination of substances can be employed as encapsulating materials[164-168]. They can be processed to form a simple

membrane, a multiwall structure with numerous layers of the same or different materials, or various cores on a simple capsule. Table 1 summarizes the most widely used encapsulation materials, further explained right after.

The polysaccharide starch is an α-D-glucose polymer with a chemical structure constituting of $(C_6H_{10}O_5)_n$, being in essence a composition of linear amylose/amylopectin. The higher the rate amylose/amylopectin content, the lower is the gel strength and the swelling power. Starch is in general a white powder, odorless and tasteless, insoluble in cold water. Functional derivatives of starch can be found on market, including cross-linked, oxidized, acetylated, hydroxypropylated and partially hydrolyzed molecules[150,154].

Table 1. Summarized encapsulation materials and its characteristics.

Group of Materials	Encapsulation Materials	Summarized Characteristics
Polymers	Polysaccharide Starch	White powder, odourless and tasteless, insoluble in cold water.
	Dextrin	Almost tasteless, solubility differs between species. Not absorbable.
	Celluloses	White powder, solubility differs between species.
	Xanthan Gum	High molar mass anionic polyelectrolyte.
	Gum Arabic	Soluble in cold water, causes viscosity on solution but does not gel.
	Gellan	Thermo-reversible gelation. Gradient on viscosity with acetylation.
	Pectines	White powder, solubility differs between species. Low viscosity.
	Alginates	Anionic hydrocolloids forming a stable gel reacting with ions.
	Chitosan	Different solubilities. Form gels with a good film-forming ability.
	Carrageenans	Different charact.; viscous solutions or thermally reversible gels.
	Polyvinylpyrrolidone	Differs between species.
	Shellac	Differs between species.
Proteins	Gelatin	Form stable solutions or form gel-states.
	Soy protein	Form stable solutions or form gel-states.
	Caseins	Extremely heat-stable proteins, good emulsifiers, viscosity or gel.
	Whey protein	Form thermally irreversible gels.
Lipid	Lipid components	Versatile materials. Form stable emulsions, encapsulations.

Dextrin comes from the heating of dry starch or its treatment with acid or base, until highly branched polymers are formed. Diverse dextrins are

used on encapsulation processes. Maltodextrins contain glucose polymers of various lengths and have no lipophilic groups, meaning therefore low emulsification properties. They are almost tasteless, which may make them an interesting option on some oral strategies. Cyclodextrins are cyclic derivatives composed of α-(1→4)D-glucopyranoside units that provide exceptional stability to oxidation and evaporative losses. Their solubility differs considerably between species and it is not absorbable by the intestinal tract [150,155,162].

Cellulose is a β-D-glucose polymer with a chemical structure constituting of poly(β-(1→4-glucopyranoses) oriented along the same molecular plane and forming long linear chains, although the majority contain a low percentage of glucose units. Due to this supramolecular structure, arranged cellulose molecules can form rigid structures. Cellulose hydroxyl groups can partially or completely react with diverse agents to form derivatives with different required or desired properties. Methylcellulose, ethyl methylcellulose and hydroxypropyl methyl cellulose are three examples, being a hydrophilic white powder which dissolves in cold water forming a clear viscous solution. They undergo reversible transformations upon heating and cooling passing from a solution state to gel state. Hydroxypropyl cellulose. In contrast, hydroxypropyl cellulose is soluble in cold water but does not form a gel, and ethylcellulose is non-soluble in water[150,156,157,169].

Gums are polysaccharide complex mixtures of polymers of different chemical structure/architecture. Xanthan gum is a high molar mass anionic polyelectrolyte composed by β-(1→4)-D-glucopyranosyl units with a trisaccharide side chain attached at the C-3 position of every second

unit. Xanthan is soluble in cold water, and although it does not gel, it causes viscosity on solution, which proportionally increases with the addition of salt over a concentration of 0.15 % xanthan. Gum Arabic is another polymer with a chemical structure constituting of β-(1→3)-linked D-galactopyranosyl units with 1,6-linked branches, containing also units of α-L-rhamnopyranosyl, α-L-arabinofuranosyl, 4-O-methyl-β-D-glucuronopyranosyl, and β-D-glucuronopyranosyl. It is odorless, tasteless and colorless, and does not alter odor, taste or color neither of other materials. It is very soluble in water independently of temperature, up to a concentration of the 50 % weight. Depending on the type of gum Arabic, pH and ionic strength viscosities on solutions variate (maximum viscosity at pH 6–7). It has also an excellent emulsifying ability, and can act as a protective film around oil droplets. The hydrophobic groups anchor the polysaccharide onto the oil droplet's surface, preventing its aggregation through the formation of a charged layer. Another example of widely used gum is gellan, a water-soluble anionic polyelectrolyte of high molar mass; it is a tetrasaccharide composed by a rhamnose unit, a glucuronic acid, and two glucose units. Depending on the degree of esterification, it can be commercially found a low acyl or high acyl variants with different characteristics, like a gradient on viscosity with the increase rate of acetylation. Thermo-reversible gelation happens upon heating and cooling solutions, with the need of added gelling cations in case of low acyl gellan, and the final texture widely depends on the degree of acetylation, from hard to elastic, transparent or flexible gels[150,151,158,162,170].

Pectines consist of negatively charged hetero-polymer chains of a very complex non-random structure, with linear blocks of at least a 65 % weight of (1→4)-linked D-galacturonic acid-based and highly branched

(1→2)-linked blocks. The carboxy groups of the galacturonic acid may be esterified on a range between the 20 and 80 %, distinguishing between high-esterified pectines (>50 % ester groups) and low-esterified pectines (<50 % ester groups). Pectines are soluble in water, in higher or lower degree depending on the pectin type, and produce low viscosity in comparison with other materials.

Alginates are anionic hydrocolloid materials that form a stable gel after reacting with calcium ions. They are solved in water solutions, which afterwards would be put in contact dropwise with a calcium chloride solution. Alginate polymers range between 12,000 and 180,000 molecular weight, formed by (1→4) glycosidic links between α-L-guluronic acid and β-D-mannuronic acid at different lengths. Viscosity increases exponentially with the molar mass. The formed gels can entrap any molecule with more than 5,000 Da. To notice that alginate is subject to rapid degradation, since many microorganisms digest it[150,162].

Chitosan is a linear copolymer which consists in a random mixture of β(1→4) linked D-glucosamine and N-acetyl-D-glucosamine with more or less degree of acetylation. Acetylation will variate the degree of solubility and final charge of chitosan, being only soluble in acidic media when acetylation is below 40 %. It may form gels with a very good film-forming ability[150,152,153,171].

Carrageenans are anionic sulphated polymeric chains alternating (1→3)-linked β-D-galactopyranosyl and (1→4)-linked α-D-galactopyranosyl units. There are three types of available commercial carrageenans, kappa (κ), iota (ι) and lambda (λ), with big variations on rheology and characteristics among them. They may form viscous solutions or

thermally reversible gels with both firm and elastic textures: both κ and ι are only soluble above 70 ºC and forms thermos-reversible gels, while λ is very soluble and does not form gels. On another hand, while κ gels are brittle and unstable, ι gels are elastic with no syneresis[150,172,173].

Polyvinylpyrrolidone and shellac materials are two synthetic polymers with very good film forming abilities, which makes them nice very suitable for coatings[150,171].

Protein-based materials such as gelatin o soy protein are composed by natural macromolecules consisting in long linear chains of aminoacids. They can form stable solutions or form gel-states, although have the inconvenience of low solubility in cold water or a potential reactivity with carbonyls. Caseins are the main milk phosphoproteins, characteristically forming large spherical micelles. Caseins are extremely heat-stable proteins, very good emulsifiers and generally insoluble but becoming soluble at pH above 5.5. Bellow pH 3.5, they are also soluble but with a high viscosity that become even gel[150]. Another type of protein-based materials are whey proteins, formed essentially by α-lactalbumin, immunoglobulins, β-lactoglobulin and serum albumin. They are globular soluble proteins, although become insoluble on pH ≈ 5 with very low ionic strength and with temperatures above 70 ºC because of whey protein denature, where they form thermally irreversible gels. By the addition of calcium to a preheated whey protein solution, cold gelation can be also achieved. Due to their flexible and amphiphilic nature, whey proteins form additionally homogeneous membranes on oil droplets allowing emulsification[150,175]. Gelatin is a widely used protein-based material formed by a heterogeneous combination of different polypeptides

containing between 300 and 4000 aminoacids. Generally, gelatins are translucent, colorless, tasteless, odorless and brittle materials, which dissolve in hot water and gel after cooling below 35 ºC approximately[150,176].

Lipid components have been also widely employed on encapsulation technology, including Solid Lipid Nanoparticles (SLNs), monolayered vesicles, double emulsions or liposomes. Depending on the final desired properties, many different lipids of different characteristics can be used: phosphatidylcholine, PS, phosphatidylethanolamine, Chol, peglicated lipids, glycerides, waxes…

In particular, phospholipids have acquired high importance on the pharmaceutical/food supplement/cosmetic industries due to their ability to form stable emulsions and their use on liposomal encapsulations. Phospholipids are the major component of human cell membranes. They structurally consist on a hydrophilic phosphate group, joined through a glycerol molecule to two fatty acid chains of very hydrophobic characteristics, conferring them very amphiphilic properties. The third hydroxyl group of the glycerol is linked with a base like choline or ethanolamine. The fatty acid chains can be saturated or unsaturated on one or other degree, which causes phospholipids with different transition temperatures and melting points.

Also, a blend of materials may be an option for the encapsulation of actives, like modified starches with maltodextrins, or guar gum with xanthan gum, which enhances synergistically the viscosity of a gel[151].

1.7.2 Encapsulation techniques

Diverse techniques are used to form the capsules and each of them is a unique procedure with different particularities, advantages and disadvantages to be considered. There are many different employed encapsulation techniques, including the spray drying, spray chilling or spray cooling, extrusion coating, fluidized bed coating, emulsion, liposome entrapment, coacervation, inclusion complexation, centrifugal extrusion and rotational suspension separation[167]. Table 2 recaps the different aforementioned encapsulation techniques.

Table 2. Summary of the aforementioned encapsulation techniques.

Encapsulation Techniques	Summarized Characteristics
Spray drying	A solution/suspension is atomized by a nozzle in the inside of the spray dryer, where solvent is evaporated.
Spray chilling/cooling	A solution/suspension is atomized by a nozzle, followed by a cooled or chilled air treatment with the aim of solidifying the capsule.
Extrusion	A pressurized chamber extrudes a dispersion into a dehydrating liquid, solidifying the material to small particles.
Fluidized bed coating	Particles are air-suspended while coating material agglomerates around them through atomization.
Coacervation	Emulsifying a material with a gelling/solidifying protein.
Inclusion complexation	Encapsulation method involving cyclodextrin.
Rotational suspension	Both core materials and coating materials are mixed and incorporated afterwards into a rotating disk.
Emulsification	Liquid-in-liquid dispersions of two immiscible liquids.
SLN	Submicron particles capable of incorporating both lipophilic and hydrophilic drugs in its solidified structure.
Liposomal technology	Spherical self-enclosing lipid bilayers with an aqueous core.

Spray drying is a widely used method of encapsulation and traditionally the most common method, because of its easy scaling-up, its effectivity while being considerably economic as well[162]. Apart of being used for encapsulation purposes, it has also been used as a dehydration method for

the production of powdered milk, for example. Usually, the encapsulation material is homogenized on a solution/suspension with the carrier material in a 1:4 ratio –although it needs to be optimized on every procedure– to be afterwards atomized by a nozzle in the inside of the spray dryer. Then, the solvent is evaporated by the hot air flowing on it and the capsules are recovered on a collector compartment.

Spray chilling and spray cooling are a variable of the spray drying; in this case, the solution/suspension is atomized and followed by cooled or chilled air treatment, so instead of a solvent evaporation, the objective in this case is to solidify the wall of the capsule around of the core. Both chilling and cooling methods differ only in the melting points of the selected materials[162,163].

Extrusion was developed by Swisher and patented by the same group in 1957[149]. Oils are dispersed on a heated mixture of glycerine and syrup solids at 125 ºC, and poured afterwards into a pressurized chamber to extrude them into a dehydrating liquid. This makes the material to solidify into small particles that would be collected after vacuum-drying of the solvent.

Fluidized bed coating is based on a temperature/humidity-controlled chamber where the particles to be encapsulated are suspended through high-velocity air while the coating material is atomized and thus agglomerating around them. The thickness of the wall will depend on the exposure time on the atomizing materials. It is a useful technique with coating materials like hydrogenated vegetable oils, fatty acids, emulsifiers, waxes or solvent-based coatings such as starches, gums and

maltodextrins, and depending on the material, it will be a solvent-evaporation based technique or to harden the coating[162].

Coacervation consists in the dissolution of a gelling protein as gelatin or gum acacia, followed by its emulsification with a material as an oil. The procedure is followed by the gelling protein coating the material and solidifying, and its collection afterwards by centrifuge or filtration. Coacervations can be simple or complex, with one gelling protein or more[167].

Inclusion complexation is an encapsulation method involving cyclodextrin. The core of the molecule has a hydrophobic behavior, while the outer part consists in seven glucose units thus conferring it a very hydrophilic behavior. When replacing the water molecules with less polar molecules, the complex precipitates and can be recovered and dried[113,167].

On the rotational or centrifugal suspension, both core materials and coating materials are firstly mixed and incorporated into a rotating disk. Then, while rotating the core materials to be encapsulated leave the disk with a coating in form of capsules[167].

It is estimated that nowadays, approximately the 90 % of drugs under development have very poor solubility in water, thus leading to bioavailability problems, uncertain absorption profiles, and consequently poor therapeutic effects[177-179]. Lipid-based formulations, like emulsions, SLN or liposomes, are promising formulations employed to improve the solubility of lipid substances, enhancing absorption and bioavailability together with some other possible benefits like an improve in dispersibility, stability, texture, appearance or tastes[79].

The emulsification process consists in the production of liquid-in-liquid dispersions of two immiscible liquids, usually water and an oil-based/organic solvent, stabilized with surfactants and emulsifiers that prevent aggregation and coalescence. Depending on the chemical affinity of the active substances to encapsulate, these would be incorporated into one or the other phase.

Various types of emulsions can be produced, differencing between water-in-oil (W/O) emulsions denoting the dispersion of water droplets in an oil medium, oil-in-water (O/W) emulsions signifying oil droplets dispersed in an aqueous medium, and more complex emulsions using more steps procedure to form water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) emulsions (Figure 9).

Emulsification processes are basically divided into two groups, the low-energy and the high-energy methods. High-energy methods, including high-pressure homogenization, ultrasonication or microfluidization processes, lead generally to smaller particle size and require lower quantities of surfactant, although instrumentation required is usually more complex and expensive. On the other hand, low-energy methods use less energy and are more efficient, and the procedures are simpler and less sophisticated, so they may be preferable when results may fit our requirements[180-182].

SLNs are submicron particles capable of incorporating both lipophilic and hydrophilic drugs in its solidified structure. They are formed by a lipid matrix, together with emulsifiers and sometimes co-emulsifiers, in a water media. The advantages of SLN are the limited mobility of entrapped drugs

with almost no leakage, a reduction on drug toxicity and an increase of bioavailability[183-185].

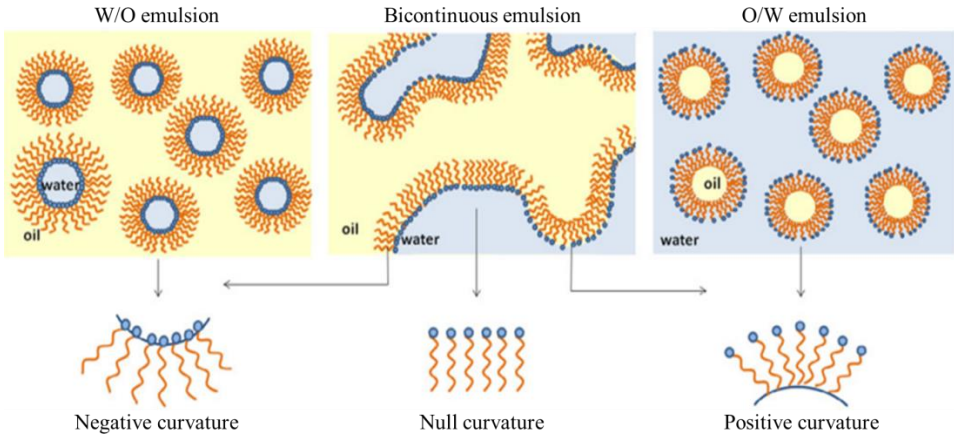

Figure 9. General structure of w/o, bicontinuous and o/w emulsions[182].

There are several preparation procedures for the synthesis of SLN. The most common used procedure may be the melt emulsification followed by hot or cold homogenization. Firstly, a pre-emulsion is prepared with the incorporation of the drug to the melted lipid. Then, on the hot homogenization, an emulsification with the aqueous emulsifying phase is carried out followed by a homogenization of the same, keeping always both phases above the melting temperature of the lipid. This emulsion will afterwards be cooled down until the solidification of the particles. The warmer the lipid, the less viscosity it will have, leading to smaller particles. However, high temperatures may result in degradation of the drug or the carrier. On the cold homogenization, this problem is avoided. Also, two other problems are overcome: the leak of the drug into the aqueous phase during homogenization and the complexity of the crystallization while cooling down the emulsion. In this case, before mixing the drug-loaded melted lipid with the aqueous phase, it is cooled

and the resulting solid is milled until obtaining microparticles. Then, it is mixed with the water phase to obtain a suspension that is afterwards subjected to high pressure homogenization. The solvent evaporation synthetic procedure consists in the preparation of an o/w emulsion, where the oil phase is dissolved with an organic solvent (as for example chloroform). The organic solvent of the emulsion is vacuum-evaporated, forming precipitation of the lipid and producing the SLN. Another synthetic procedure for the preparation of SLN is the microemulsion based SLN preparation technique: it would consist in the dispersion of a hot microemulsion (low melting fatty acid and co-emulsifiers with water) in cold water at 2-3 ºC, in ratio volumes of the range 1:25 – 1:50: This way, no energy is required to produce the nanoparticles.

Lastly, there is another very interesting and potential lipid-based encapsulation procedure that will further be described in the next section, the liposomal technology encapsulation.

1.7.3 Liposomal delivery systems

Since the discovery roughly 60 years ago that phospholipids can form closed structures in solution[186], liposomes have been evolving along the years becoming a pharmaceutical encapsulation technique of choice on multiple applications. Liposomes are now widely used biomaterials in encapsulation with the advantage of being easy to prepare, customize and administer[186]. Moreover, liposomes have the ability to incorporate hydrophilic and hydrophobic actives, making them a very versatile and useful vehicle. Additionally, they are highly biocompatible, have the capacity of protecting active substances from external conditions and

increase the retention time at the site of absorption thanks to their mucoadhesive properties, among others benefits like masking unpleasant flavors or undesired side-effects[187,188].

Benefits of drug load in liposomes are summed up in Table 3. All these advantages lead to an enhancement on the final bioavailability of the encapsulated actives, which is equal to a higher efficacy.

Table 3. Summarized benefits of drug loading onto liposomes[190].

Benefits	Examples
Improved solubility of lipophilic and amphiphilic drugs	Amphotericin B, porphyrins, minoxidil, some peptides, and anthracyclines, respectively; hydrophilic drugs, such as anticancer agent doxorubicin or acyclovir
Passive targeting to the cells of the immune System	Antimonials, amphotericin B, porphyrins, vaccines, immunomodulators
Sustained release System of systemically or locally administered liposomes	Doxorubicin, cytosine arabinoside, cortisones, biological proteins or peptides such as vasopressin
Site-avoidance mechanism	Doxorubicin andamphotericin B
Site-specific targeting	Anti-inflammatory drugs, anti-cancer, anti-infection
Improved transfer of hydrophilic, charged molècules	Antibiotics, chelators, plasmids, and genes
Improved penetration into tissues	Corticosteroids, anesthetics, and insulin
Increased efficacy and therapeutic effect	Actinomycin-D
Reduce the toxicity of the encapsulated agent	Amphotericin B, taxol
Others: Increased stability	

Liposomal composition is based in phospholipids that form spherical self-enclosing lipid bilayers with an aqueous core (Figure 7). Bilayers are formed in a thermodynamically favorable way because of phospholipid amphiphilic nature: the polar lipid heads are oriented such that they are at maximally exposure to the outer and inner surrounding aqueous phase, while hydrophobic acyl chains face one each other. Among the different phospholipids employed, the most common one is Phosphatidylcholine (PC): its chemical structure consists in a glycerol nucleus, with two acyl chains providing hydrophobicity at sn-1 and sn-2 positions, and a phosphate group attached to position sn-3, which is then linked to a choline (hydrophilic head)[188]. Other minor bilayer constituents may take

part in its formation, like Chol or hydrophilic polymer conjugated lipids. Chol stabilizes the bilayer and provides rigidity to the membrane through changing interactions between the hydrophobic and hydrophilic part of phospholipids[189].

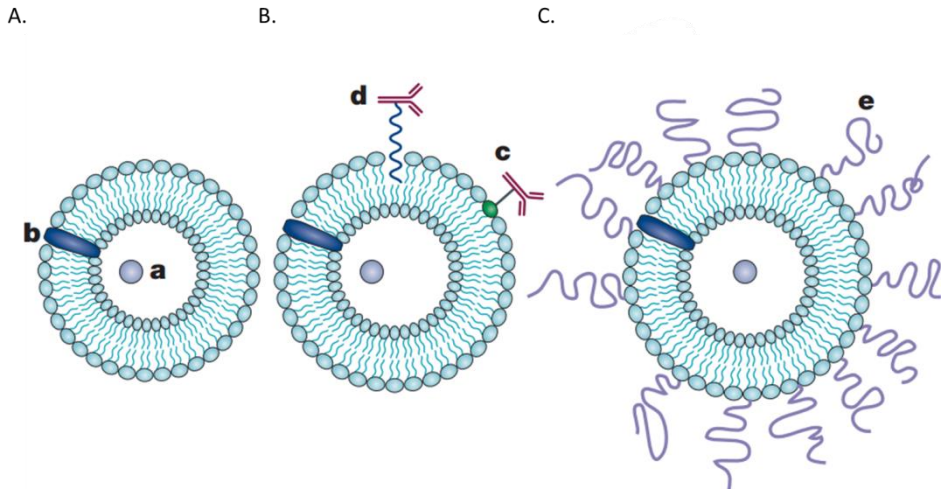

Figure 7. Graphical representation of the main types of liposomal structures[186]. A) Traditional structure consisting in a plain bilayer of phospholipids, with the polar heads facing the aqueous media, and the acyl chains touching ones with the adjacent ones. Lipid active substances (b) intercalate with the hydrophobic phospholipids being incorporated on the membrane, while hydrophilic active substances (a) get encapsulated in the core of the structure. B) Antibody-targeted immunoliposome with the covalent coupling of antibodies to (c) the polar head of phospholipids or (d) to the liposomal membrane using antibodies previously treated with a hydrophobic moiety. C) Long-circulating liposomes protected with an anchored polymer as PEG (e).

Additionally, the charge of liposomes plays a decisive role on final characteristics. Through modulating the liposomal composition, net charge of the membranes can be either positive, negative or neutral. On instance, the addition of PS induce to an increase on the negative charge of the bilayer, while 3ß-[N-(N',N'-dimethylaminoethane)-

carbamoyl]cholesterol hydrochloride (DC-Cholesterol·HCl) generates positively charged liposomes. When charged, the vesicles display electrostatic repulsion between them that prevents coalescence and aggregation, and thus making them suitable for longer storages[188,191,192].

Hydrophilic active substances get encapsulated in the core compartment, while lipophilic substances get entrapped on the lipid bilayer, acting as an integral part of the membrane or fully incorporated in the bilayer bridging the outside and inside of the membrane[189].

There are different types of liposomal vesicles. First, they can be differentiated on size and lamellae: multilamellar vesicles (MLVs) when consisting on several concentric lipid bilayers, generally in a range from 500 to 5.000 nm; small unilamellar vesicles (SUVs) for single bilayer liposomes around 100 nm in size; large unilamellar vesicles (LUVs) ranging from 200 to 800 nm; and giant unilamellar vesicles (GUVs) when they are bigger, sizing up to hundred microns[186,188].

On a different note, liposomes can be differentiated in by means of functionalization, delivery strategies or targets:

- Long-circulating liposomes are surface-modified liposomes as a strategy to avoid the reticuloendothelial system (RES) uptake. The RES capture liposomes in blood circulation to fast eliminate them from the body, so hiding liposomes from RES elongates the half-life ($t_{1/2}$) of the drug, and consequently enhances the efficacy. Hydrophilic polymers as PEG attached to membrane forming compounds are usually employed for this finality, which mechanism consist basically

on steric hindrance. Additionally, it was observed that SUVs have longer circulation times than larger ones[193-195].

- Surface attaching of ligands for a specific targeting. This strategy relies on the expression of ligands on the liposomal membrane, so they are focused to specifically interact with a determinate receptor that is expressed on the therapeutic target. Many research efforts have been carried out to find promising ligands overexpressed on target cells such as tumor cells. For instance, folate-modified liposomes target tumors selectively due to the frequent overexpression of folate receptors on tumor cells. Selectively targeting allows therapeutics with some highly cytotoxic anticancer agents that otherwise would not be viable on clinic because of side-effects[186,196,197]. Immunoliposomes, following the same line are liposomal vesicles carrying antibodies on their surfaces, so liposomes can selectively bond to the corresponding antigen.
- pH-sensitive liposomes are designed to degrade at pH $\leq$ 6.0 but stable at blood pH $\approx$ 7.4, constructed with pH-sensitive components. This way, on blood circulation they remain stable but when endocytosed, due to the lower pH of endosomes they fuse and release contents into cytoplasm. Other low pH areas as inflammation, ischemic myocardium or tumor, will also favorite the cellular uptake and cargo release of the liposomes[186,188,198,199].
- Thermosensitive liposomes use polymers on their structure that are water-soluble at a temperature of $\approx$ 37 ºC, but water-insoluble at warmer temperatures. This fact makes liposomal bilayers to disrupt when heat is applied, thus releasing the liposomal contents. Natural

polymers such as gelatin, chitosan, cellulose or some synthetic ones are widely used for their composition[200,201].

- Magnetic liposomes: apart from the drug loading of the vesicles, they can be produced co-encapsulating particles with ferromagnetic properties. Then, after the liposomal administration, vesicles are distributed towards the target directed by a magnetic field. Gold or silver particles can also be encapsulated with electron microscopy imaging purposes[186,202,203].

Currently used micro-encapsulation techniques are mostly based on sugars, alginates, gums, starches, dextrins or synthetics, although liposomes have been recently gaining more and more importance in the field of pharmaceutical, food and cosmetic industries[166,189,204]. There are many known methods to effectively produce liposomes, and depending on the needs of the production, one method may be more optimal and suitable than another one:

- The thin film method was the first described method to prepare liposomes: it starts with the dissolution in a round-bottom flask of the membrane forming lipids on an organic solution at the desired final molar ratio. Subsequently, the organic solvent is removed under vacuum and nitrogen to form a dry lipid film, which is afterwards hydrated with an aqueous phase above the transition temperature of the lipids. Under these conditions, the stacks of liquid crystalline lipid bilayers become fluid and swell, resulting in their detachment during agitation and their self-closure to form MLVs and GUVs. In order to homogenize the preparation, additional techniques could be required: sonication in a water bath or inserting a titanium probe into the

liposomal suspension, which has the drawback of enhancing temperature and possible metal contamination because of the probe; extruding multiple times through a polycarbonate membrane to reduce liposome's size; or using high pressure homogenizing systems[188,205].

- Reverse phase method is a procedure consisting in forming inverted micelles or w/o emulsions putting together the water phase (with the hydrophilic drugs on it) and the organic phase (with the hydrophobic drugs and the membrane forming lipids). This results in a biphasic system which through slow evaporation of the organic solvent, leads to the conversion of the w/o system into a gel-like solution, and right after to an aqueous liposomal suspension. The rests of organic solvent can be dialyzed, centrifuged or purified with a column[206].
- Organic injection technique is based on the injection of solubilized phospholipids from an organic solution into an aqueous phase. Depending on the employed organic solvent, liposomes would directly form (as with ethanol) or if the injected solvent is ether, the aqueous solution must be prewarmed at ≈ 60 ºC for inducing its evaporation[207].
- The use of microfluidic technologies are included among the more novel employed technologies. Microcapillary devices comprising hydrodynamic focusing of coaxial flow is an emerging field that allows the efficient formation in one-step of homogeneous liposomal vesicles. A particular feature of this methodology is its high capacity for controlling liposomal size, homogeneity and lamellarity, with also an approximate encapsulation efficiency of 100 % for hydrophilic drugs. All these makes them a unique tool on the field of liposomal drug encapsulation and delivery. Immiscible fluids pumped through different coaxial regions are forced through the collection tube and

form emulsion drops with controllable size and structure: some parameters such as size of microchannels, different employed flow rates or concentrations can be adjusted to regulate its characteristics[188,206,207]. However, the scalability on industrial production needs to be further investigated, although many studies are being undergone with promising results[208-211].

- The supercritical reverse phase evaporation method (SRPE) consist in the use of CO_2 at its fluid state through holding it above its critical pressure and temperature (supercritical CO_2) in order to solve the phospholipids. A cell containing the phospholipids in an ethanol solution and CO_2 increases its pressure and temperature above supercritical values, with a temperature higher than the transition temperature (T_g) of the phospholipids. Afterwards, an aqueous solution is introduced on the cell and liposomes are spontaneously formed when pressure is back to normal[212,213].
- Spray drying is a single-step procedure that has been used in the pharmaceutical and food industry for different purposes like producing microcapsules, and lately has been used for the liposomes manufacturing. Spray drying can be used for drying already formed liposomal suspensions, or for the *in-situ* production of liposomes. For the last one, the procedure is based on the spraying of an organic solution with the forming membrane lipids solved on it. Then, when hydrated the obtained product with an aqueous solution, lipid vesicles are spontaneously formed[188,214,216].
- Membrane contactor technology consists in a pressurized solution of phospholipids in ethanol passing through a membrane into a column where an aqueous phase is flowing tangentially. Immediately, the

solution gets diluted and phospholipids self-assemble spontaneously in vesicles[217].

- The detergent depletion and the crossflow injection techniques are two methodologies with the same base, using a micellar-forming detergent starting material that would lately be removed to form the liposomal suspensions. While the conventional detergent depletion employs dialysis, tangential filtration subjected to pressure removes the detergent on the crossflow injection technique[188,218].

Applications on many different administration routes have been investigated and are currently being used on food, cosmetics and pharmaceutical industry. The most popular route of administration is the oral delivery since it is easy for drugs to be taken, although drugs need to undercome many physiological barriers before arriving to blood circulation. PEG-coated liposomes have been used for oral deliveries of vaccines and many other fields with significative improvements on therapeutic results[219,220]. Other administration routes include topical delivery[221], liposomal aerosols for delivering directly to lungs[222], intravenous administration or subcutaneous administration[223].

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CHAPTER 2. Objectives

Coencapsulation of different actives has been widely studied and reported on the last decades, and concretely liposomes have already been used for the encapsulation of the isolated antioxidant actives phosphatidylserine (PS), coenzyme Q10 (CoQ10), [redacted] nicotinamide adenine dinucleotide (NAD[redacted]) and vitamin C, but not as a combinatory oral food supplement therapy. Co-administration of these actives on a combinatory antioxidant therapy may provide a wide and complete supplementation with promising results on chronic fatigue syndrome (CFS) patients or people suffering from fatigue. The encapsulation on the same vehicle, although being challenging to achieve, would offer all the benefits of the liposomes on the protection of the actives and, consequently, the enhancement on bioavailability and effectiveness.

The main objectives of this doctoral thesis can be listed as follows:

- The development of a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of CoQ10, PS, and vitamin C, essential for the analysis and characterization of the selected drugs.
- Validation of the developed method following the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, to ensure system suitability, selectivity, linearity, robustness, precision, stability of the solution during the analyses, accuracy and limits of detection and quantification.

- The design, characterization and optimization of a novel liposomal-based food supplement against chronic fatigue syndrome with potential industrial scalability.
- Carry out effectivity studies on *in vitro* and *ex vivo* models to evaluate the potential of the previously optimized formula.
- Evaluate cytotoxicity and safety of the liposomal formulation on NIH-3T3 human cells
- Study the stability for long-term storages of the liposomal product at 4 ºC and 25 ºC, fact of vital importance for the industrial interests in the formula.
- The design and characterization of the previously developed and selected liposomal formula, on an adaptation to be produced within the state-of-the-art microfluidic techniques.
- Scaling up optimization of the most appropriate liposomal formula with views to industrial commercialization.

CHAPTER 3. Development and validation of a new high-performance liquid chromatography method for the simultaneous quantification of coenzyme Q10, phosphatidylserine, and vitamin C from a cutting-edge liposomal vehiculization.

ABSTRACT

A high-performance liquid chromatography (HPLC) method was developed to simultaneously quantify the three lipophilic active substances chosen for the liposomal encapsulation: coenzyme Q10 (CoQ10), the lipid vitamin C (6-O-Palmitoyl-L-ascorbic acid or PA) and phosphatidylserine (PS or DOPS). This new method optimizes current timing and costs in the analyses of these three active substances. Additionally, since the analyzed compounds were encapsulated on a cutting-edge liposomal formulation (further explained in detail in *Chapter 4*, together with its design and optimization), an additional processing of samples was necessary to be developed prior to HPLC analyses.

After the design and development of the method, a complete validation study was meticulously accomplished following the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH)[1] quality guidelines, thus obtaining data that demonstrates the sufficient reliability and reproducibility of results for quality controls and stability studies. It was also proved in different real circumstances like long-term stability studies at different conditions.

Support from the Analitical Chemistry Service of the Universitat Autònoma de Barcelona was hired for the part of the method development.

3.1 INTRODUCTION

Herein, a novel HPLC method of analysis has been developed for the simultaneous quantification of three co-encapsulated active substances with very similar characteristics of hydrophobicity in a liposomal matrix[2,3], and also a pre-treatment procedure for these liposomal samples prior to analyses. Specifically, the co-analyzed active substances are the CoQ10, PA and DOPS. These active substances are widely used in both basic research and in the pharmaceutical, cosmetics and food industry, thus having a high interest and a huge impact in the economic ambit[4-8]. In the last 10 years, 6898 scientific publications related with CoQ10 were divulged, 7479 related to PS and 85816 related with vitamin C, all of them with an exponential tendency of increase on each year[9]. Although many articles have been published describing analytical methodologies of each substance in separate, to the best of our knowledge there is no published method offering the simultaneous analysis of the three active substances, and even less co-encapsulated within the liposomal technology and the additional elements this means to be considered upon analysis.

For the proper determination and quantification of the active substances, it is important to have clear absorption peaks so we can extrapolate the areas to the actual amount we have of each active substance in the preparation. Since the chemical nature of all the three actives is very similar, it is actually very tricky to elucidate the compounds in separated peaks and avoid them to overlap. Additionally, both CoQ10 and vitamin C absorb at a wavelength of 275 nm, and all the three substances absorb at 200 nm wavelength as well, thus making it absolutely necessary to develop a methodology to separate the peaks for its quantification. With

combinations of the actives in separate, just one article of simultaneous analysis has been published, reported by Temova-Rakuša Ž *et al.*[10], with the co-analysis of CoQ10 and vitamin C.

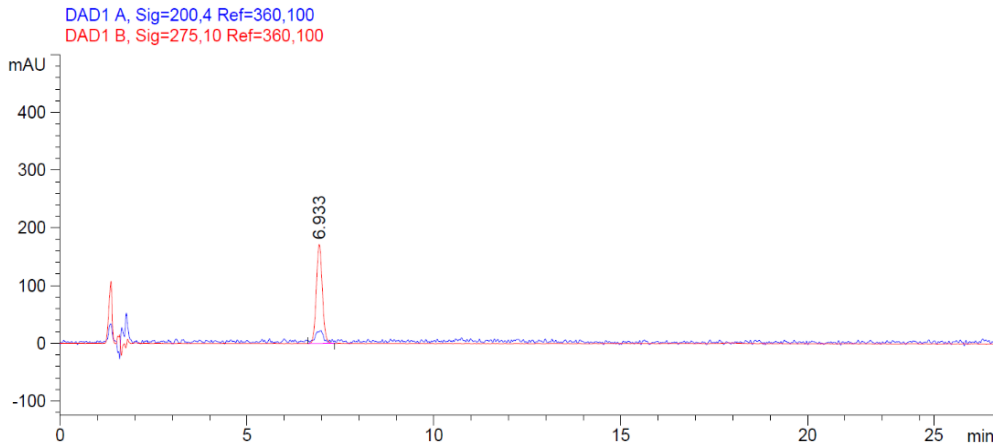

Figure 1. Chromatogram of the reported method by Temova-Rakuša Ž *et al.* with our three active substances. CoQ10 = 6.933 minutes, vitamin C = Not determined. DOPS = Not determined.

This article provides an interesting method for their quantification but not valid for the analysis of DOPS. This reported method was tried with our three active substances, and no peak was found for the DOPS nor the vitamin C, meaning it is not a suitable analytical method for this substance or for the simultaneous quantification of the three active substances (see Figure 1). Therefore, our method could be considered as a unique and interesting contribution with applications in pharmaceutical/cosmetic/food industry.

The technique was studied and adequately validated in accordance with the guidelines of the International Council for Harmonization of Technical

Requirements for Pharmaceuticals for Human Use (ICH)[1] regarding selectivity, linearity, accuracy, precision, and robustness. After data treatment of results, linear regressions for all active substances showed an optimal linearity with a correlation coefficient of >0.999 in the concentration range between 70 to 130 % of the liposomal formulation and less than a 3 % relative standard deviation (RSD) in accuracy and precision.

3.2 MATERIALS AND METHODS

3.2.1 Materials and chemical reagents

6-O-Palmitoyl-L-ascorbic acid (vitamin C, analytical standard ≥ 99.0 %), Cholesterol-(polyethylene glycol-600) (Chol-PEG) and cholesterol (Chol) was obtained from Sigma-Aldrich© (Darmstadt, Germany). Phosphatidylserine (PS, concretely 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt or DOPS, analytical standard ≥ 99.0 %) and Phosphatidylcholine (PC, concretely 1,2-dimyristoyl-sn-glycero-3-phosphocholine or DMPC) was purchased from Lipoid GmbH© (Switzerland). Coenzyme Q10 (CoQ10, United States Pharmacopeia (USP) reference standard quality ≥ 99.0 %) was obtained from Xiamen Kingdomway© (China). Ammonium acetate (NH4AcO) was acquired from Panreac Quimica (Barcelona, Spain).

3.2.2 Physicochemical properties of the drugs

Different mixtures and proportions between methanol, isopropanol, acetonitrile and water buffer were studied for the eluent choice on the analyses. Before starting the development of the method with trying

different conditions (diverse organic solvents, the combination of them and at different percentages, among others), it is necessary to understand the nature and the physicochemical properties of the targeted drugs to analyze, rationalizing and streamlining the experiments until a proper method is achieved, and avoiding in this way any problems and incompatibilities between the solvents and the active substances.

To choose an adequate buffer/solvent, it was necessary to look out to the acid dissociation constant (pKa) and the partition coefficient (log P) of the three substances. The pKa represents the pH at which the system is in equilibrium between the concentration of a protonated chemical specie (HA) and its conjugated base of the acid (A^-) plus a hydrogen ion (H^+)[11]. The equilibrium system can be represented as:

$$HA \rightleftharpoons H^+ + A^-$$

The log P is the ratio of concentrations of a compound in a mixture of two immiscible solvents at equilibrium[12], commonly measured with water and a hydrophobic solvent such as 1-octanol. Therefore, it is an indicator parameter of the hydrophobicity/hydrophobicity a chemical compound has. The log P may be represented as the equation:

$$\log P = \log \frac{[solute]octanol}{[solute]water}$$

Figure 2 shows the molecular structure of every active substance, while its corresponding pKa, partition coefficient and molecular weight are shown in Table 1[13].

Table 1. Chemical properties to be considered for the design of the method[13 – 15].

	DOPS	Vitamin C	CoQ10
Log P	10.55	6	10
MW (g/mol)	810.025	414.539	863.365
pKa	9.38 ; 1.47	4.36 ; -3	-4.7

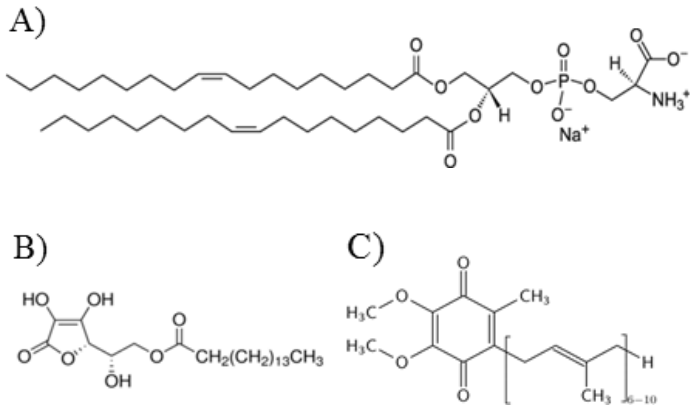

Figure 2. Chemical structure of the analyzed compounds. A) DOPS. B) Vitamin C. C) CoQ10

3.2.3 Instrumentation, column, mode of elution and mobile phase

Analyses were carried out with an HPLC system model 1100 HP from Agilent (Agilent technologies©, EEUU), composed by a quaternary pump, an automatic injector, a diode array detector and a column oven where our column was placed, all controlled by a Chemstation software version B04.02. For the freeze-drying of samples, a Telstar Cryodos–50 freeze dryer was used. The employed ultraviolet lamp was an UVATOM, and the IR apparatus was an infraTest Prüftechnik GmbH (Germany). The oven model was a HERAEUS t5028 (Heraeus Holding©, Germany).

For HPLC analyses, the employed column was a C18 Zorbax Eclipse Plus 2.1 x 150 mm 5-Micron, with a constant flow of 0.25 mL/minute and a controlled temperature of 25 ºC. The injection volume was 10 µL, and the detection wavelengths were λ = 275 nm for vitamin C and CoQ10, and λ = 200 nm for DOPS since no other absorbance was found after checking with the diode array accessory. After 19 minutes, all the compounds were already eluted, but the stop time was fixed at 28 minutes in order to return to the initial conditions and stabilize the equipment.

Table 2. Detailed gradient of the developed method. Solvent A in the table is isopropanol, while Solvent B is a buffer solution of 0.02M NH_4AcO.

Time (minutes)	Solvent A (%)	Solvent B (%)
0	40	60
5	40	60
10	100	0
18	100	0
19	40	60
28	40	60

The final elution mixture consists in a variable percentage on time of isopropanol and an aqueous buffer composed of 0.02 M NH_4AcO, adjusted until a pH = 6.5 is achieved. The detailed proportions of the two employed solvents during the time are shown in Table 2.

3.2.4 Sample preparation strategy

The treatment and preparation of samples prior to HPLC analyses was essential for allowing the quantification of the lipid active substances. Liposomal formulations have by definition the characteristic of including both water and water-insoluble compounds, and this could difficult any posterior analyses. Since the present of water is a very unfavorable

condition to dissolve and allow the flow of the substances through the column in an appropriate way, a procedure of analysis including the lyophilization of the samples was carried out: a scheme illustrating the preparation process of the samples can be observed in Figure 3.

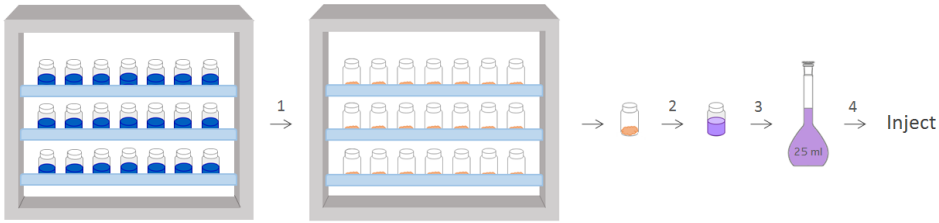

Figure 3. Graphical representation of the sample preparation strategy for its HPLC quantification. 1, lyophilization of samples; 2, addition of $CHCl_3$ to solve the lipids; 3, transfer the organic solution into a 25 mL volumetric flask, repeating 2 & 3 to ensure the complete transference of the lipids to the flask. Afterwards, make up to 25 mL with MeOH. 4, prepare HPLC vials after filtration and inject it for analyses.

For the lyophilization of samples, 1 mL of the liposomal solution was pipetted on each vial, and frozen afterwards at -80 ºC on an ultra-low temperature freezer for 12 hours. Once samples were completely frozen, they were freeze-dried for 4 days. Lyophilization conditions of pressure and temperature were 1 mbar and -50 ºC, respectively.

Once samples were completely dried, 2 mL of $CHCl_3$ were added to properly solubilize the very apolar active substances –DOPS, vitamin C and CoQ10 – under an extractor hood. After 5 minutes of slight shaking and only when the sample was completely dissolved, 10 mL of methanol were added, and the final solution was transferred into 25 mL volumetric flasks. The vials were afterwards cleaned with methanol, which was also transferred to the flasks, making up to 25 mL with more methanol. Lastly,

all solutions were filtered with 0.45 µm Polyvinylidene Fluoride (PVDF) filters and HPLC vials were prepared. The final concentration of each active compound at the moment of injection to the HPLC system was 776 µg/mL of DOPS, 240 µg/mL of CoQ10 and 200 µg/mL of vitamin C.

To validate the preparation procedure developed, 20 lyophilized vials were injected, establishing a RSD limit of 2.7 %.

3.2.5 Standard solution preparation strategy

For the preparation of a standard solution, 19.4 mg of DOPS, 6 mg of CoQ10 and 5 mg of vitamin C were accurately weighted, transferred to a 25 mL volumetric flask and dissolved in the same way than in the sample preparation procedure.

3.2.6 Calculations

The concentration of the detected and analyzed active substances is calculated with the following formula:

$$[\text{Active Ingredient}]_{\text{Test Solution}} = \frac{\text{Average Peak Area}_{\text{Test Solution}}}{\text{Response Factor}}$$

Where:

$$\text{Response Factor} = \frac{\text{Peak Area}}{[\text{Active Ingredient}]_{\text{Standard Solution}}}$$

For the calculation of the recovery between the theoretical concentration and the detected one on the analyses, it was employed the following formula:

$$\% \text{ Recuperation} = \frac{[\text{Active Ingredient}]_{\text{Test Solution}}}{[\text{Active Ingredient}]_{\text{Theoretical}}}$$

3.2.7 Method validation

The development of the method was followed by validation studies to ensure system suitability, selectivity, linearity, robustness, precision, stability of the solution during the analyses, accuracy and limits of detection and quantification of the method, following the ICH guidelines[1].

3.2.7.1 Suitability of the equipment

Prior to any other validation test, 6 injections of a standard solution were analyzed to ensure the correct resolution and reproducibility of the equipment for analyses. Retention times (RT) with less than 1.0 % variations, capacity numbers greater than 2 and symmetry of peaks between 0.7 and 1.2, theoretical plate numbers (N) above 2000 for any peak, and USP tailing factor smaller than 1.5 was checked out.

3.2.7.2 Selectivity

Selectivity is the ability to assess unequivocally the analyte in the presence of other components which may be expected to be present. For its study, both sample and standard solutions were compared, thus confirming the complete separation of all compounds with a purity peak test being carried out for each active substance.

3.2.7.3 Linearity

The linearity of an analytical procedure is its ability within a given range of concentrations to obtain test results, which are directly proportional to

the concentration of analyte in the sample. Linearity was tested preparing seven different concentrations per triplicate between 70 % and 130 % of the standard solution at the usual working concentration, studying if the response is directly proportional within this range. Response Factor (FR) was the analyzed parameter on linearity, being the result of dividing peak areas by the corresponding concentration.

3.2.7.4 Precision

Precision is the parameter that expresses the degree of scatter between a series of measures obtained from multiple measurements of the same homogeneous sample. In this case, the precision of the method and system was evaluated with the injection of 9 standard solutions at different concentrations, and in parallel with different analysts and different days of work, studying its repeatability.

3.2.7.5 Robustness

Robustness is a measure of the capacity to remain unaffected by small -but deliberated- variations in method parameters; as can be the temperature of the column (± 3 ºC), the analyzed wavelength (± 3 nm), the injection volume (± 5 µL) or modifications in the flow level of the pump (± 0.05 mL/minute). Its study is necessary to understand the importance of controlling a determinate parameter. Three liposomal samples were injected with the before mentioned modifications:

- Temperature of the column: 28 ºC.
- Temperature of the column: 22 ºC.
- Detector: Lower detection wavelength (197 nm and 272 nm).

- Detector: Higher detection wavelength (203 nm and 278 nm).
- Pump modification: Lower flow level (0.2 mL/minute).
- Pump modification: Higher flow level (0.3 mL/minute).
- Injection volume: Lower injection volume (5 µL).
- Injection volume: Higher injection volume: (15 µL).

Peak areas were analyzed in every chromatograph and results obtained were analyzed by means of a one-way Analysis of Variance (ANOVA) statistical test to check whether or not there are statistically significant differences.

3.2.7.6 Stability of the solutions

Stability of the active substances in the HPLC vials once prepared is also vital to understand for how long the solution is stable before injecting.

3.2.7.7 Accuracy

Lastly, the accuracy of the method was assessed to verify if any of the lipids of the liposome affect to the analyses of the active substances. To this end, three standard solutions plus the other lipids were prepared at 130 %, 100 % and 70 % of the usual working concentration, and real concentrations were compared with the theoretical ones. According to the American Association of Official Analytical Chemists (AOAC[16]), the limits for the RSD are established in consonance with the concentration of the analyte in the sample, so it was established a limit for the active substances of 2.7 % RSD. Every preparation was injected twice.

3.2.7.8 Limit of detection (LOD) and limit of quantification (LOQ)

The terms LOD and LOQ are terms used to describe the lowest concentration at which any measure can be a reliable analytical procedure. The LOD is the lowest concentration where the analyte can still be distinguished from the base line of the chromatogram, while the LOQ is the concentration where not only the active substances are detected but also with reliable analytical characteristics. Both parameters are studied based on the linearity curve of the three actives, with the standard deviation of the response and the slope[17].

A first calibration curve is formed with the concentrations and the obtained areas. The intersection with the Y axis (intercept) correspond theoretically to the response at a concentration of the substance equal to 0 or noise signal (Y_{bl}). The slope of this calibration curve will also be necessary on the final calculations (Slope). In the same way, to calculate the standard deviation produced because of the noise on the response, a calibration curve should be formed by using the standard deviation of the responses as intercept and the concentrations as x-axis. The intercept of the obtained curve will correspond to the standard deviation of the signal at a concentration of the substance equal to 0 (S_{bl}).

Afterwards, LOD and LOQ can be obtained following the subsequent calculations:

$$\mathrm{LOD} = \frac{\mathrm{Ybl} + (3 * \mathrm{Sbl})}{\mathrm{Slope} * \sqrt{\mathrm{n}}}$$

$$\mathrm{LOQ} = \frac{\mathrm{Ybl} + (10 * \mathrm{Sbl})}{\mathrm{Slope} * \sqrt{\mathrm{n}}}$$

3.2.7.9 Samples subjected to stress

Samples of CoQ10, vitamin C, and DOPS were prepared subjected to stress conditions, with some variations on time/concentration when needed to obtain 10-30 % degradation (approximately), up to 96 hours of exposition if no degradation was observed. The following assays were performed:

- Acid medium (hydrochloric acid 1 M / 0.1 M)
- Basic medium (sodium hydroxide 1 M / 0.1 M)
- Oxidizing medium (hydrogen peroxide 33 % / 7 %)
- Temperature (35 ºC)
- IR light
- UltraViolet light (UV light)
- Visible light (Vis-light)

Peak areas were analyzed in every chromatograph and results obtained were analyzed by means of a one-way ANOVA statistical test to check whether or not there are statistically significant differences.

3.3 RESULTS AND DISCUSSION

3.3.1 Sample preparation strategy

After some assays, results demonstrated that it is necessary to remove the water from the samples to allow the elution of substances at the chromatogram and to successfully analyze and quantify the peaks of the active substances. Firstly, with any water in the sample, it was almost impossible to correctly solve all the lipids. Secondly, being the active

substances hydrophobic, the peaks were not eluting in a satisfactory peak form or were not eluting at all. Freeze-drying of samples was carried out as previously described. Twenty different lyophilized vials with the liposomal formulation were injected and analyzed with the developed HPLC method to test the accuracy of the sample preparation strategy, and the RSD after the analyses of the all the vials was 2.35 % for CoQ10, 2.14 % for vitamin C and 2.74 % for DOPS, thus showing optimal results of repeatability.

3.3.2 Method development

The first chromatographic meticulous tested conditions showed us that for a favorable elution of CoQ10, a 100 % flow of pure methanol or isopropanol is needed, and any mixture with water made its elution impossible with an optimal peak shape. On the other hand, for the analysis of vitamin C, a mixture of organic solvent plus water was needed for its proper elution, and DOPS was not eluted in any isocratic condition with an optimal peak shape considering theoretical plate numbers (N) and USP tailing factor. At this point of the development, a gradient of NH4AcO buffer with isopropanol was extensively investigated at different proportions and with different gradient duration times until we found an adequate combination that allowed the optimal elution of all the compounds during the same analysis. An example of the obtained chromatogram after last optimization is shown in Figure 4.

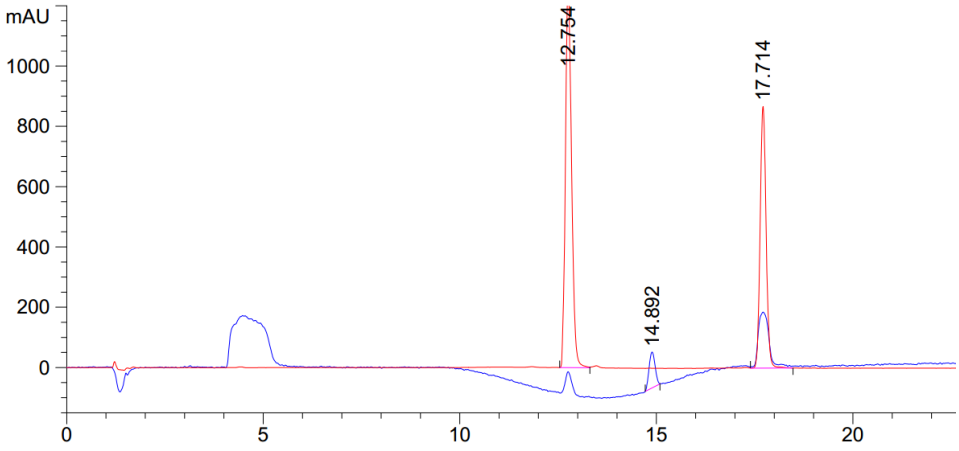

Figure 4. Chromatographic exemplification of the developed method with the three analyzed active substances. The peak with a RT of 12.754 minutes corresponds to vitamin C, RT of 14.892 corresponds to DOPS and RT of 17.714 to CoQ10. Red line is λ = 275 nm, while blue line is λ = 200 nm.

3.3.3 Method validation

3.3.3.1 Suitability of the equipment

Before carrying through the validation assays, the suitability of the equipment with the method was tested. Standard solutions were prepared as previously described, containing the active substances at the stablished concentrations (DOPS: 776 µg/mL; CoQ10: 240 µg/mL; vitamin C: 200 µg/mL), and injected afterwards. Table 3 shows RSD of retention times, symmetry of the peak, USP tailing factor, capacity factor and N, demonstrating consistence and appropriateness. The symmetry analyses on each peak of the active substances can be found in Figure 5, and the tabulated retention times of the six analyzed samples in Table 4.

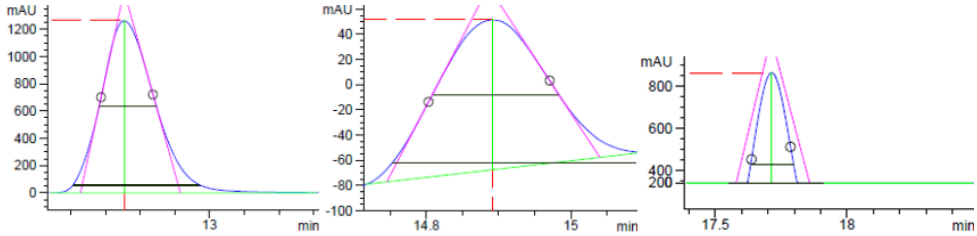

Figure 5. Vitamin C, DOPS, and CoQ10 peak symmetry analysis, respectively.

Table 3. Suitability of the equipment, data analyses.

	DOPS	Vitamin C	CoQ10
Retention times (minutes)	14.86	12.72	17.73
Retention times (RSD %)	0.09	0.25	0.14
Symmetry of peak	1.01	0.73	0.89
USP tailing factor	1.22	1.23	1.08
Capacity factor	3.08	2.49	3.85
Plate Numbers (N)	40891	33327	67904

Table 4. RSD analyses on retention times

	DOPS	Vitamin C	CoQ10
Sample 1 (minutes)	14.838	12.670	17.695
Sample 2 (minutes)	14.874	12.696	17.747
Sample 3 (minutes)	14.866	12.736	17.736
Sample 4 (minutes)	14.867	12.724	17.715
Sample 5 (minutes)	14.871	12.720	17.767
Sample 6 (minutes)	14.866	12.761	17.743
Mean	14.864	12.718	17.734
RSD (%)	0.09	0.25	0.14

3.3.3.2 Stability of the solution

After checking the suitability of the equipment and before starting any other validation step, it was necessary to understand for how long samples are stable above at least the 95 % of all active substances. Between injections, samples were maintained at room temperature until achieving at least the 5 % degradation on any active. It was determined that, in order to accomplish this requirement, liposomal samples could be injected up to 3 hours after its preparation in the HPLC vials without significant degradation of any samples, while for the standard solution the time was shortened to 1 hour. So, it is highly recommended to analyze samples right after its solubilization in organic solvents and preparation for HPLC analyses.

Table 5 and Table 6 reflect the percentage of active substances on the standard solution and on the test solution (respectively) at each time, taking time 0 of the solution as 100 %, while Figures 6-8 represent graphically the stability on time.

Table 5. Stabilities on time of the different active substances on the standard solution once prepared in HPLC vials, expressed in percentage.

Time	***Standard solution stability (%)***		
	DOPS	**Vitamin C**	**CoQ10**
0 hours	100.00	100	100.0
0.5 hours	99.7	99.9	100.1
1 hour	100.7	95.7	99.1
1.5 hours	100.3	93.8	98.3
2 hours	101.0	91.9	98.7
2.5 hours	101.6	89.9	98.1

Table 6. Stabilities on time of the different active substances on the liposomal samples once prepared in HPLC vials, expressed in percentage.

Time	***Liposomal samples stability (%)***		
	DOPS	**Vitamin C**	**CoQ10**
0 hours	100.0	100.0	100.0
0.5 hours	97.6	99.2	100.7
1 hour	98.5	98.6	100.2
1.5 hours	101.8	98.4	100.3
2 hours	98.9	97.1	100.1
2.5 hours	101.8	96.6	102.1
3 hours	98.6	95.8	99.3
3.5 hours	101.7	94.8	98.9

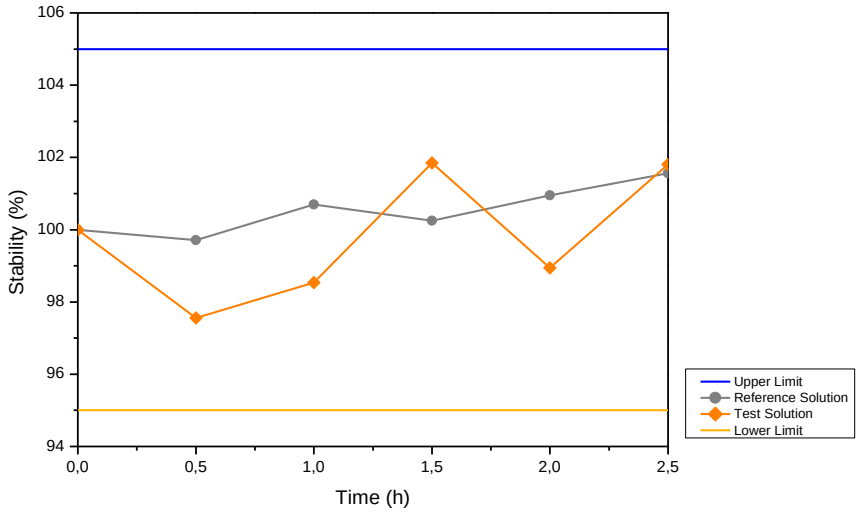

Figure 6. Graphical representation of the stability on time of DOPS, expressed in percentage.

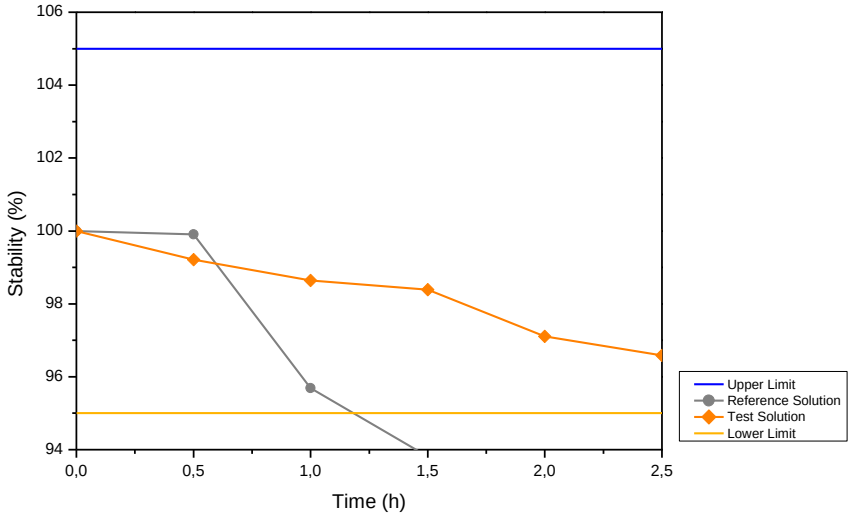

Figure 7. Graphical representation of the stability on time of vitamin C, expressed in percentage.

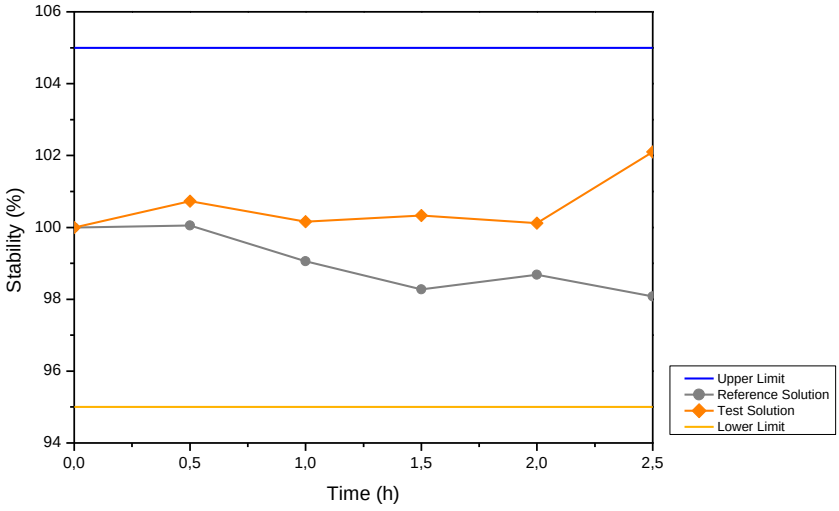

Figure 8. Graphical representation of the stability on time of CoQ10, expressed in percentage.

3.3.3.3 Selectivity

Identification of the active substances

Although high purity (*ca.* 99 %) was confirmed on the provided certificates, the identity of the active substances was proved with the realization of Infrared Spectra of the raw materials, and by injecting the actives separately to identify their peaks. Signals detected on the spectra correspond to the expected for each molecule (Figures 9 – 14).

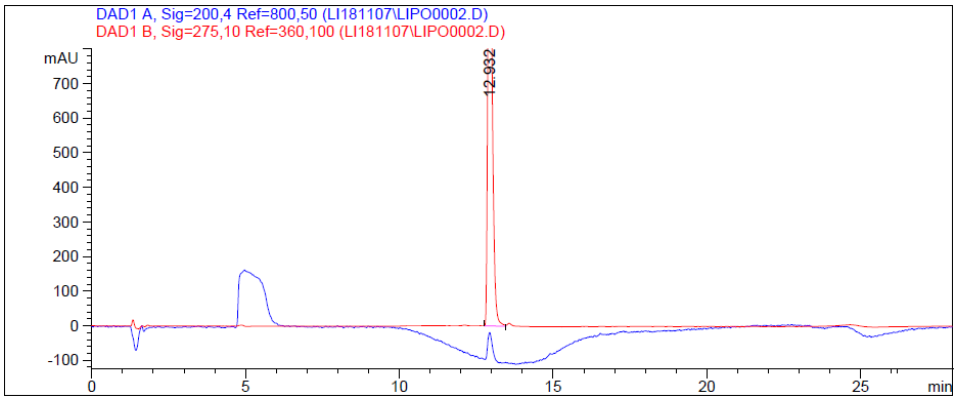

Figure 9. Chromatogram corresponding to the supplied vitamin C raw material.

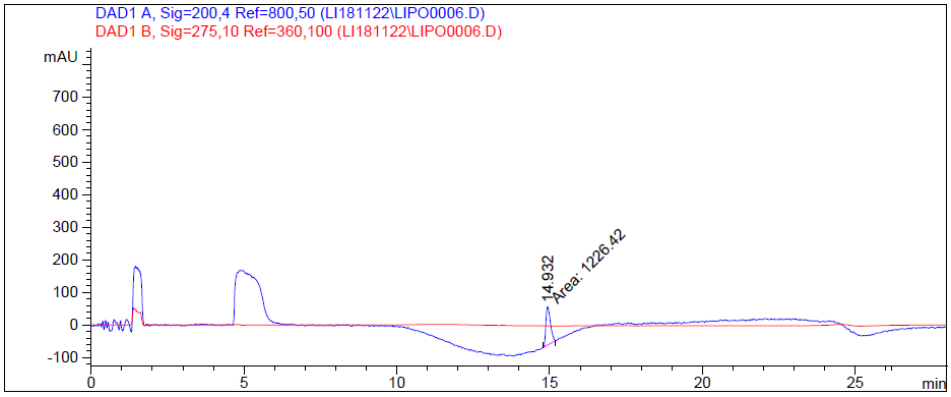

Figure 10. Chromatogram corresponding to the supplied DOPS raw material.

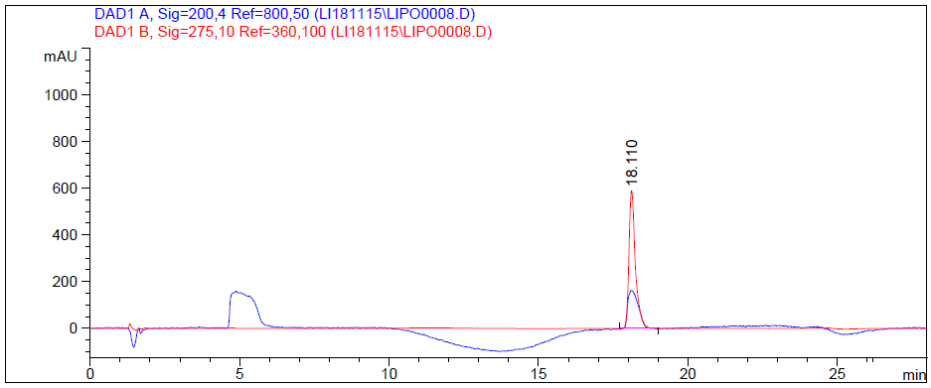

Figure 11. Chromatogram corresponding to the supplied CoQ10 raw material.

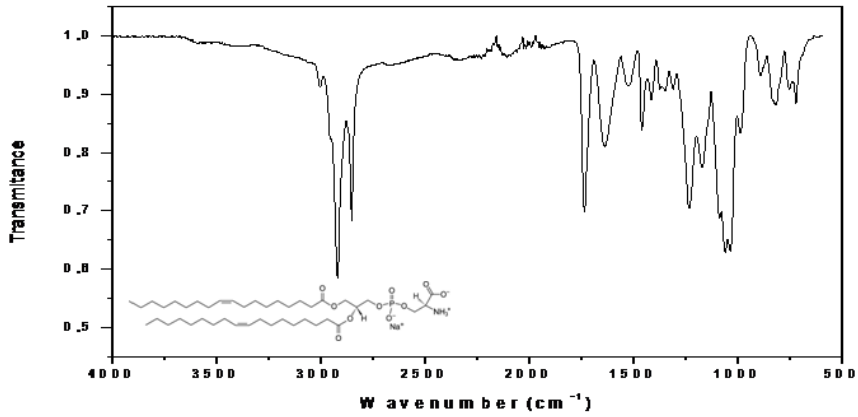

Figure 12. Infrared spectrum of DOPS.

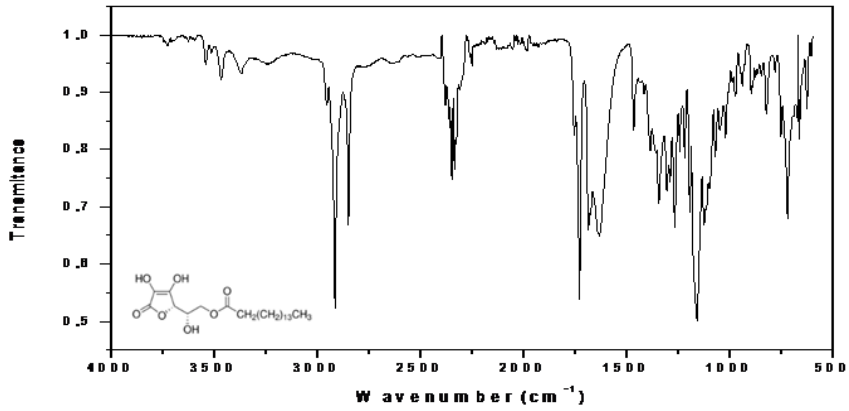

Figure 13. Infrared spectrum of vitamin C (L-Ascorbyl palmitate).

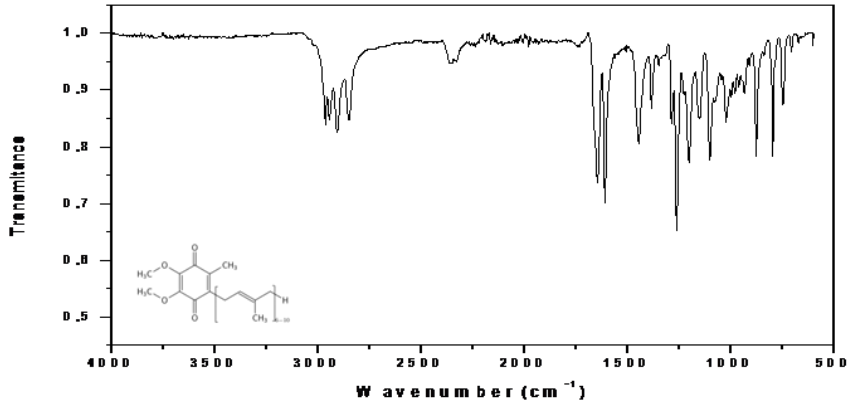

Figure 14. Infrared spectrum of CoQ10.

Additionally, the purity factor of peaks was studied. In all cases, purity was above the threshold limit, set at 990.000 (Figure 15).

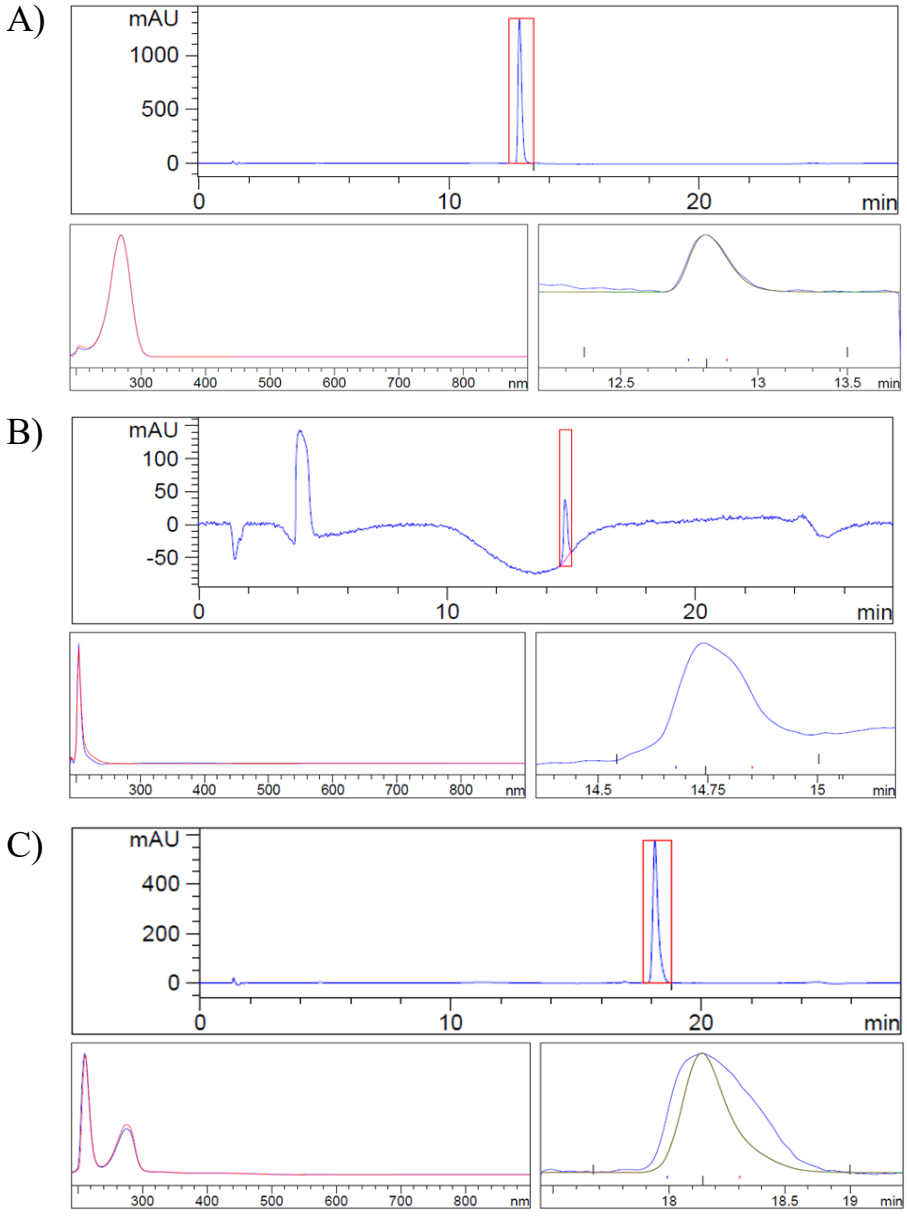

Figure 15. Peak purity analysis of: A) DOPS. B) Vitamin C. C) CoQ10.

Absence of interference of the formulation excipients

To demonstrate the absence of interferences by excipients and the active substance of the formulation, it was prepared -as indicated previously- a placebo solution (basically, a solution with PC, Chol and Chol-PEG) and a solution containing both placebo and the active substances at the usual established concentration. Afterwards, they were injected and compared, with no interferences observed between the peaks of the excipients and the ones of the active substances (Figure 16).

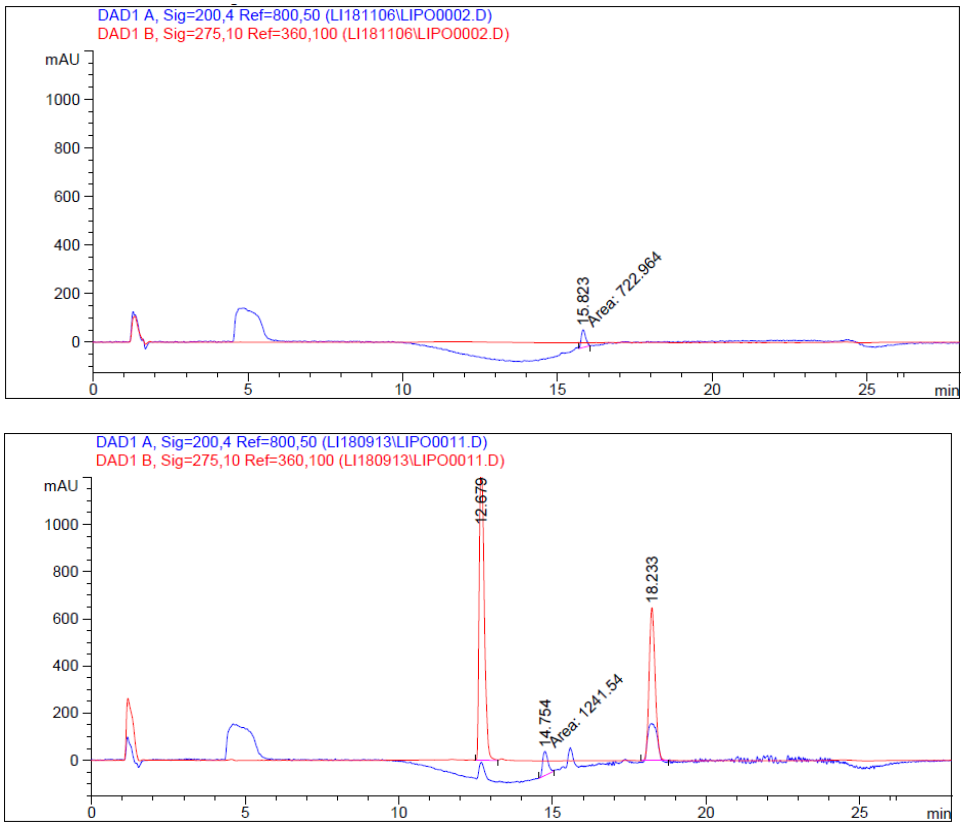

Figure 16. Chromatograms of the two prepared solutions after been analyzed, to check there are no interferences between them. The first chromatogram attached corresponds to the placebo solution and the second to the placebo plus active substances solution.

Absence of interference of the extraction solvents

To demonstrate the absence of interferences by any extraction solvents, a solution containing only the solvents was also injected (Figure 17). No peak close to any active's retention time was detected (12.932 minutes, 14.932 minutes, and 18.110 minutes for vitamin C, DOPS and CoQ10, respectively).

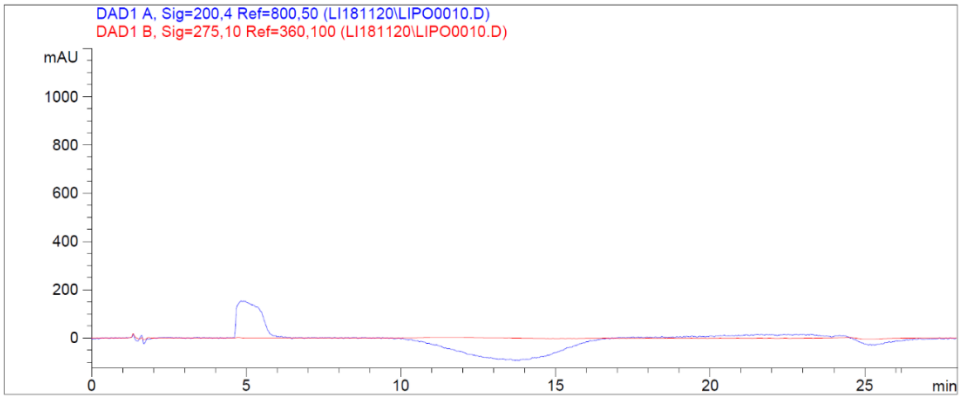

Figure 17. Chromatogram corresponding to the analysis of an injection with only the employed solvents.

3.3.3.4 Precision

Precision of the standard solution

To demonstrate the precision of the standard solution, 9 individual samples of standard solution were prepared as previously described and at different concentrations: 3 solutions at 70 % concentration of the established concentration, 3 solutions at 100 % and 3 more solutions corresponding to the 130 % of the established concentration. Each solution was injected and analyzed twice. The obtained results are expressed as a response factor, which calculation formula is expressed as:

$$Response\ factor = \frac{Concentration}{Area}$$

In all cases, response factor resulted in less than a 2 % RSD (Tables 7–9).

Table 7. Tabulated data after the analysis of 9 individual samples of standard solutions of DOPS. RSD (%) analyses of the different response factors (Concentration/Area) are 1.97 %.

	Concentration (mg/mL)	Area 1 (mAU*s)	Area 2 (mAU*s)	Mean (mAU*s)	Response Factor
130 %	1.025	1645.672	1639.429	1642.550	1603.427
	1.010	1624.076	1654.223	1639.150	1622.278
	1.005	1676.242	1669.125	1672.683	1664.030
100 %	0.793	1277.566	1339.628	1308.596	1650.601
	0.784	1312.706	1279.859	1296.282	1654.265
	0.784	1262.614	1295.579	1279.097	1630.669
70 %	0.54	891.355	922.356	906.856	1679.362
	0.558	952.389	957.161	954.775	1712.294
	0.554	931.829	917.495	924.661	1667.860
				RSD (%)	1.97

Table 8. Tabulated data after the analysis of nine individual samples of standard solutions of vitamin C. RSD (%) analyses of the response factors (Concentration/Area) are 1.96 %.

	Concentration (mg/mL)	Area 1 (mAU*s)	Area 2 (mAU*s)	Mean (mAU*s)	Response Factor
130 %	0.265	1.85E+04	1.82E+04	18366.85	69361.216
	0.256	1.80E+04	1.78E+04	17898.05	69805.187
	0.242	1.64E+04	1.59E+04	16127.9	66644.215
100 %	0.198	1.41E+04	1.37E+04	13879.75	70099.748
	0.202	1.38E+04	1.37E+04	13725.55	68083.085
	0.199	1.37E+04	1.34E+04	13562.0	68219.316
70 %	0.15	1.07E+04	1.05E+04	10577.8	70518.667
	0.146	1.01E+04	1.02E+04	10177.85	69711.301
	0.155	1.11E+04	1.09E+04	11003.9	70901.418
				RSD (%)	1.96

Table 9. Tabulated data after the analysis of nine individual samples of standard solutions of CoQ10. RSD (%) analyses of the response factors (Concentration/Area) are 0.97 %.

	Concentration (mg/mL)	Area 1 (mAU*s)	Area 2 (mAU*s)	Mean (mAU*s)	Response Factor
130 %	0.313	1.13E+04	1.13E+04	11345.9	36225.734
	0.318	1.14E+04	1.19E+04	11621.1	36544.339
	0.311	1.13E+04	1.11E+04	11174.35	35999.839
100 %	0.243	8939.382	8700.855	8820.119	36326.683
	0.247	9156.167	9197.435	9176.80	37122.981
	0.246	8876.351	8836.884	8856.617	35944.063
70 %	0.173	6308.201	6301.602	6304.902	36486.699
	0.182	6650.624	6655.685	6653.154	36475.627
	0.178	6525.241	6509.279	6517.260	36613.820
				RSD (%)	0.97

Intermediate precision of the method

The intermediate precision of the analytical method is determined by analyzing the content of active substances (DOPS, vitamin C and CoQ10) on seven standard solutions in different conditions: different days and different analysts. All solutions were analyzed according to the previously described analytical method, and the obtained results are expressed as a response factor, for which it was stablished a limit of 2.8 % RSD and 3.7 % RSD for repeatability between days and analysts, respectively (Tables 10 – 15).

Table 10. Tabulated data after the analysis of seven individual samples of DOPS standard solutions on different days.

	Day 1		Day 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	1255.229	1615.066	1186.518	1526.658
2	1309.171	1684.472	1207.772	1554.004
3	1224.968	1576.129	1215.797	1564.329
4	1229.403	1581.837	1172.156	1508.178
5	1246.416	1603.726	1203.002	1547.866
6	1235.216	1589.315	1189.64	1530.674
7	1213.662	1561.583	1237.668	1592.471
	Mean	1601.733	**Mean**	1546.311
	RSD (%)	2.53	**RSD (%)**	1.79
	RSD (%)	2.80		

Table 11. Tabulated data after the analysis of seven individual samples of vitamin C standard solutions on different days.

	Day 1		Day 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	1,28E+04	64850.202	13312.2	67369.433
2	1,36E+04	68703.441	1.33E+04	67419.0283
3	1,30E+04	65830.971	13435.6	67993.927
4	1,27E+04	64169.028	13237.4	66990.891
5	1,31E+04	66309.716	13107.9	66335.526
6	1,35E+04	68517.712	12915.4	65361.336
7	1,35E+04	68370.951	12824.5	64901.315
	Mean	66678.860	**Mean**	66624.494
	RSD (%)	2.80	**RSD (%)**	1.72
	RSD (%)	2.80		

Table 12. Tabulated data after the analysis of seven individual samples of CoQ10 standard solutions on different days.

	Day 1		Day 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	7640.364	31729.087	7970.835	33101.476
2	8138.434	33797.483	8044.810	33408.681
3	7697.995	31968.418	8134.951	33783.019
4	7546.931	31341.078	8018.291	33298.552
5	7813.575	32448.403	7904.912	32827.710
6	8093.278	33609.961	7787.136	32338.607
7	8064.421	33490.122	8064.579	33490.777
	Mean	32626.365	**Mean**	33178.403
	RSD (%)	3.07	**RSD (%)**	1.44
	RSD (%)	2.45		

Table 13. Tabulated data after the analysis of seven individual samples of standard solutions of DOPS, carried out by different analysts.

	Analyst 1		Analyst 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	1186.518	1526.657	1289.146	1658.706
2	1207.771	1554.003	1219.341	1568.889
3	1215.796	1564.329	1205.528	1551.117
4	1172.156	1508.178	1206.213	1551.999
5	1203.001	1547.866	1195.667	1538.430
6	1189.64	1530.674	1311.967	1688.067
7	1237.668	1592.470	1204.401	1549.667
	Mean	1546.311	**Mean**	1586.696
	RSD (%)	1.79	**RSD (%)**	3.81
	RSD (%)	3.18		

Table 14. Tabulated data after the analysis of seven individual samples of standard solutions of vitamin C, carried out by different analysts.

	Analyst 1		Analyst 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	1.33E+04	67369.433	13310.1	67358.805
2	1.33E+04	67419.028	13549.2	68568.825
3	1.34E+04	67993.927	13055	66067.813
4	1.32E+04	66990.890	13339.7	67508.603
5	1.31E+04	66335.526	12747.2	64510.121
6	1.29E+04	65361.336	13508.4	68362.348
7	1.28E+04	64901.315	13073.8	66162.955
	Mean	66624.493	**Mean**	66934.210
	RSD (%)	1.72	**RSD (%)**	2.15
	RSD (%)	1.89		

Table 15. Tabulated data after the analysis of seven individual samples of standard solutions of CoQ10, carried out by different analysts.

	Analyst 1		Analyst 2	
	Area (mAU*s)	**Response Factor**	**Area (mAU*s)**	**Response Factor**
1	7970.835	33101.476	7941.036	32977.726
2	8044.810	33408.681	8075.083	33534.399
3	8134.951	33783.019	7874.905	32703.097
4	8018.295	33298.552	7998.403	33215.962
5	7904.912	32827.710	7566.583	31422.690
6	7787.136	32338.607	8058.132	33464.004
7	8064.579	33490.776	7806.073	32417.247
	Mean	33178.403	**Mean**	32819.304
	RSD (%)	1.44	**RSD (%)**	2.24
	RSD (%)	1.89		

3.3.3.5 Linearity

Through analyzing all seven concentrations between 130 % and 70 % of the standard solution, results demonstrated an adequate linearity in this concentration range with correlation coefficients of 0.999 for DOPS and CoQ10, and an excellent correlation coefficient of 1.000 for vitamin C (Tables 16 – 18, Figures 18 – 20).

Table 16. Tabulated data after the analysis of seven concentrations ranging from 130 % to 70 % of DOPS.

Concentration (%)	Concentration (mg/mL)	Area (mAU*s)	Response Factor
130	1.008	1581.143	1620.308
120	0.934	1498.337	1604.445
110	0.845	1352.268	1600.444
100	0.776	1241.914	1600.955
90	0.697	1128.440	1633.499
80	0.620	1009.606	1676.424
70	0.543	889.442	1656.228
		Mean	1627.472
		RSD (%)	1.82

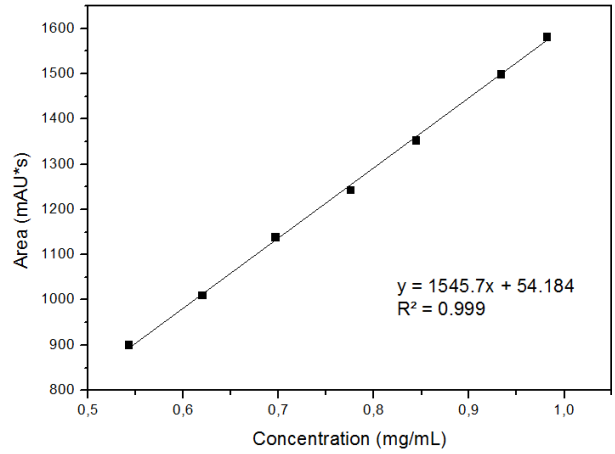

Figure 18. Graphical representation of the linearity data obtained from analyzing DOPS, with the corresponding regression line.

Table 17. Tabulated data after the analysis of seven concentrations ranging from 130 % to 70 % of vitamin C.

Concentration (%)	Concentration (mg/mL)	Area (mAU*s)	Response Factor
130	0.260	17964.7	70101.587
120	0.259	18178.6	70061.408
110	0.219	15483.6	70465.716
100	0.205	14243.8	69459.282
90	0.181	12838.3	70903.903
80	0.160	11409.8	71252.186
70	0.140	10005.6	70928.092
		Mean	70453.167
		RSD (%)	0.89

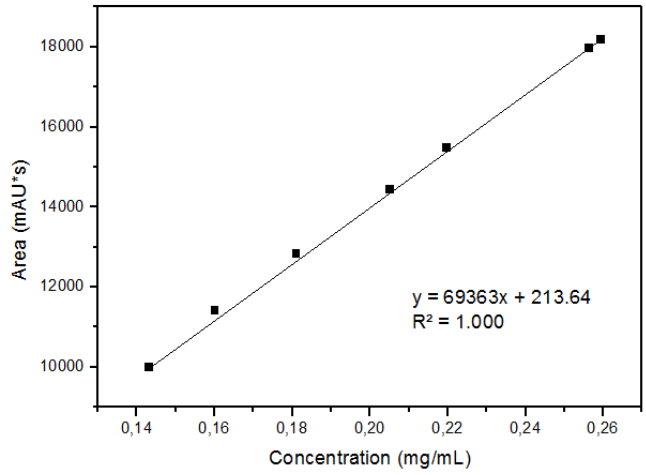

Figure 19. Graphical representation of the linearity data obtained from analyzing vitamin C, with the corresponding regression line.

Table 18. Tabulated data after the analysis of seven concentrations ranging from 130 % to 70 % of CoQ10.

Concentration (%)	Concentration (mg/Ml)	Area (Mau*s)	Response Factor
130	0.312	11307.734	36492.257
120	0.290	10420.616	35637.489
110	0.274	9902.178	36174.543
100	0.242	8817.799	36477.381
90	0.216	7956.511	36767.611
80	0.193	6941.157	36051.716
70	0.168	6023.511	36315.379
		Mean	36273.768
		RSD (%)	1.01

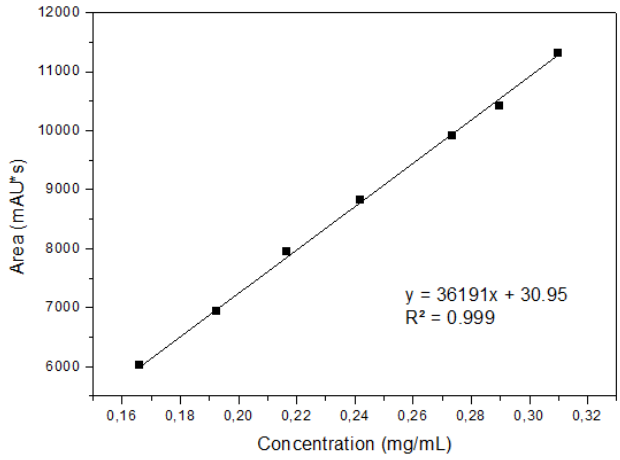

Figure 20. Graphical representation of the linearity data obtained from analyzing CoQ10, with the corresponding regression line.

3.3.3.6 Accuracy

Table 19 details the percentage of recovery on the nine analyses at different concentrations for evaluating accuracy. Every concentration resulted in less than a 2 % RSD, and ANOVA test showed non-significant differences on any active substance at any concentration.

Table 19. Detailed results of studying the accuracy.

	DOPS	**Vitamin C**	**CoQ10**
Recovery at 130 % (%)	100.21 ± 1.61	101.20 ± 0.93	100.21 ± 1.82
Recovery at 100 % (%)	98.42 ± 0.23	100.28 ± 1.83	100.17 ± 1.01
Recovery at 70 % (%)	98.48 ± 1.26	100.98 ± 0.80	99.71 ± 0.93
ANOVA test (p – value)	0.98	0.67	0.88

All data regarding linearity, precision and accuracy is summarized on Table 20, with RSD and correlation coefficients accomplishing our stablished limits.

Table 20. Summarized data of linearity, precision and accuracy during the validation of the method.

Analytes	**Linearity**			**Precision**			**Accuracy**
	Range (mg/Ml)	Correlation Coefficient	FR RSD (%)	Standard solution – RSD (%)	Different days – RSD (%)	Different analysts – RSD (%)	Recovery (% – Max/min value)
DOPS	1.008 – 0.543	0.999	1.82	1.97	2.80	3.18	101.35 – 98.25
CoQ10	0.312 – 0.168	0.999	1.01	0.97	2.45	1.89	101.92 – 98.30
Vitamin C	0.260 – 0.140	1.000	0.89	1.96	2.80	1.89	101.89 – 98.28

3.3.3.7 Robustness

Robustness studies revealed the method must be controlled in terms of temperature, injection volume, wavelength detection and flow, since otherwise results could slightly variate. Being a method with three analyzed compounds, each parameter resulted in some degree of retention time's differences or peak areas for at least one compound.

Table 21 indicates which parameter showed significant or non-significant differences after deliberated changes upon its analysis with a one-way ANOVA test. On this table, although significant differences were shown in many of the deliberated changes, RSD were not in any case over 5 % but with wavelength for DOPS. Since λ = 200 nm is a wavelength were also solvents have certain absorbance, it is a critical parameter to be controlled. Specifically, a two-tailed T-student test was carried out to compare wavelengths = 197 nm vs. 200 nm and 200 nm vs. 203 nm. The first T-test contained high significantly differences with a p–value of >0.01, but non-significant differences were observed with a p–value of 0.80 between 200 and 203 nm, thus being 203 nm also a suitable wavelength to be used.

Table 21. Summarized statistics from ANOVA test on robustness. * indicates that there are significant statistical differences.

	DOPS		**Vitamin C**		**CoQ10**	
	p-value	RSD (%)	p-value	RSD (%)	p-value	RSD (%)
Temperature	0.239	1.72	> 0.01*	4.47	> 0.01*	1.35
Injection volume	0.225	3.34	0.792	1.15	0.032*	0.70
Wavelength	> 0.01*	14.88	0.013*	1.85	0.135	0.75
Flow	0.695	2.17	> 0.01*	7.22	0.341	0.92

3.3.3.8 Limit of detection (LOD) and limit of quantification (LOQ)

Based on the standard deviation of the response and the slope, based on the calibration curve obtained from linearity, LOD and LOQ were calculated for three analytes. Results are presented in Table 22.

Table 22. Tabulated LOD and LOQ results.

Parameter	DOPS	Vitamin C	CoQ10
LOD (mg/mL)	0.082	0.018	0.021
LOQ (mg/mL)	0.249	0.054	0.063

3.3.3.9 Samples subjected to stress

DOPS, vitamin C and CoQ10 treatment with hydrochloric acid.

In Tables 23, 24 and 25 it is represented the course of degradation of the actives at the different times. All samples were treated as previously described to find degradations up to 30 % of the initial concentration, in order to find possible primary degradation products. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

Table 23. Degradation of DOPS under acid conditions.

Acid Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
HCl 1 M, 0.5 hours	102.23	0
HCl 1 M, 1 hours	102.52	0
HCl 1 M, 3.5 hours	94.68	0
HCl 1 M, 5 hours	86.67	3

After studying the acidic degradation of DOPS, the formation of three new peaks at the wavelength of 200 nm was detected as primary degradation products. The retention times of these products were:

- 1st peak retention time (λ = 200 nm) = 12.697 minutes
- 2nd peak retention time (λ = 200 nm) = 13.466 minutes
- 3rd peak retention time (λ = 200 nm) = 15.396 minutes

The peak purity of DOPS was also studied at HCl 1 M 5 h, with a purity factor of peak = 996.881 and thus showing great purity (threshold limit set at 990.000).

Table 24. Degradation of vitamin C under acid conditions.

Acid Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
HCl 1 M, 0.5 hours	74.40	0

After studying the acidic degradation of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.596 and thus showing great purity (threshold limit set at 990.000).

Table 25. Degradation of CoQ10 under acid conditions.

Acid Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
HCl 1 M, 1 hours	97.7	0
HCl 1 M, 3 hours	94.7	0
HCl 1 M, 6 hours	89.8	0

After studying the acidic degradation of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied at HCl 1 M, 6 h, with a purity factor of peak = 999.204 and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 treatment with sodium hydroxide.

The samples were subjected to basic treatment as previously described with little variations on concentrations and time for an appropriate analysis, since quick degradation was observed in some of the samples.

In Tables 26, 27 and 28 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find degradations up to 55% of the initial concentration, in order to find possible primary degradation products. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

Table 26. Degradation of DOPS under basic conditions.

Basic Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
NaOH 1 M, 0.5 hours	97.7	2

After studying the basic degradation of DOPS, the formation of two new peaks at the wavelength of 200 nm was detected as primary degradation products. The retention times of these products were:

- 1^{st} peak retention time (λ = 200 nm) = 12.668 minutes

- 2nd peak retention time (λ = 200 nm) = 15.375 minutes

The peak purity of DOPS was also studied, with a purity factor of peak = 993.535 and thus showing great purity (threshold limit set at 990.000).

Table 27. Degradation of vitamin C under basic conditions.

Basic Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
NaOH 1 M, 1 hour	0	0
NaOH 0.1 M, 1 hour	89.1	0

After studying the basic degradation of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.951 and thus showing great purity (threshold limit set at 990.000).

Table 28. Degradation of CoQ10 under basic conditions.

Basic Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
NaOH 1 M, 1 hour	54.7	1
NaOH 0.1 M, 1 hour	95.2	0
NaOH 0.1 M, 2 hours	89.6	0

After studying the acidic degradation of CoQ10, the formation of one new peak at the wavelength of 200 nm was detected as primary degradation product. The retention time of this product was:

- Peak retention time (λ = 200 nm) = 16.567 minutes

The peak purity of CoQ10 was also studied at NaOH 1 M 1 h, with a purity factor of peak = 999.506 and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 treatment with hydrogen peroxide

The samples were subjected to oxidant treatment as previously described with little variations on concentrations and time for an appropriate analysis, since quick degradation was observed in some of the samples.

In Tables 29, 30 and 31 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find degradations up to 55 % of the initial concentration, in order to find possible primary degradation products, or treated until a maximum time of 96h when no degradation was yet observed. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

Table 29. Degradation of DOPS under oxidant conditions.

Oxidant Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
H_2O_2 33 %, 1 hour	100.0	0
H_2O_2 33 %, 3.5 hours	99.8	0
H_2O_2 33 %, 10 hours	97.5	0
H_2O_2 33 %, 96 hours	68.7	1

After studying the oxidant degradation of DOPS, the formation of one new peak at the wavelength of 200 nm was detected as primary degradation product. The retention time of this product was:

- Peak retention time (λ = 200 nm) = 14.489 minutes

The peak purity of DOPS was also studied, with a purity factor of peak = 1000.000 and thus showing great purity (threshold limit set at 990.000).

Table 30. Degradation of vitamin C under oxidant conditions.

Oxidant Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
H_2O_2 33 %, 1 hour	18.2	0
H_2O_2 7 %, 1 hour	38.0	0

After studying the oxidant degradation of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 1000.000 at H_2O_2 33 % for 1 hour exposure, and = 999.454 at H2O2 7% for 1 hour, and thus showing great purity (threshold limit set at 990.000).

Table 31. Degradation of CoQ10 under oxidant conditions.

Oxidant Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
H_2O_2 33 %, 1 hour	68.2	0

After studying the oxidant degradation of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied, with a purity factor of peak = 998.023 at H_2O_2 33 % for 1 hour exposure, and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 exposure to 35 ºC temperature.

The samples were subjected to exposure of 35 ºC as previously described. In Tables 32, 33 and 34 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find possible primary degradation products or until a maximum time of 96 hours when no degradation was yet observed. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

Table 32. Degradation of DOPS under 35 ºC exposure.

35 ºC Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
Oven at 35 ºC, 24 hours	101.1	0
Oven at 35 ºC, 96 hours	100.4	1

After studying the degradation at temperature of DOPS, the formation of one new peak at the wavelength of 200 nm was detected as primary degradation product. The retention time of this product was:

- Peak retention time (λ = 200 nm) = 15.251 minutes

The peak purity of DOPS was also studied, with a purity factor of peak = 1000.000 and thus showing great purity (threshold limit set at 990.000).

Table 33. Degradation of vitamin C under 35 ºC exposure.

35 ºC Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
Oven at 35 ºC, 24 hours	104.0	0
Oven at 35 ºC, 96 hours	103.8	0

After studying the degradation at temperature of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.962 and thus showing great purity (threshold limit set at 990.000).

Table 34. Degradation of CoQ10 under 35 ºC exposure.

35 ºC Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
Oven at 35 ºC, 24 hours	101.4	0
Oven at 35 ºC, 96 hours	103.7	0

After studying the degradation at temperature of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied, with a purity factor of peak = 995.408 and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 exposure to Infrared Radiation (IR).

The samples were subjected to exposure of IR as previously described.

In Tables 35, 36 and 37 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find degradations up to 30 % of the initial concentration, in order to find possible primary degradation products, or until a maximum time of 96h when no degradation was yet observed. Also, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap

between degradation products and the ones corresponding to the active substances.

Table 35. Degradation of DOPS under IR exposure.

IR Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
IR Exposure, 24 hours	103.7	0
IR Exposure, 96 hours	74.5	0

After studying the degradation at IR exposure of DOPS, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of DOPS was also studied, with a purity factor of peak = 1000.000 and thus showing great purity (threshold limit set at 996.869).

Table 36. Degradation of vitamin C under IR exposure.

IR Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
IR Exposure, 24 hours	104.3	0
IR Exposure, 96 hours	104.8	0

After studying the degradation at IR exposure of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.950 and thus showing great purity (threshold limit set at 990.000).

Table 37. Degradation of CoQ10 under IR exposure.

IR Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
IR Exposure, 24 hours	97.9	0
IR Exposure, 96 hours	97.2	0

After studying the degradation at IR exposure of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied, with a purity factor of peak = 995.691 and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 exposure to Ultraviolet Radiation.

The samples were subjected to exposure of ultraviolet radiation as previously described.

In Tables 38, 39 and 40 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find degradations up to 20 % of the initial concentration, in order to find possible primary degradation products, or until a maximum time of 96h when no degradation was yet observed. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

After studying the degradation at UV exposure of DOPS, no new peaks formation was detected as primary degradation products at any wavelength.

Table 38. Degradation of DOPS under UV exposure.

UV Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
UV Exposure, 24 hours	99.1	0
UV Exposure, 96 hours	81.3	0

The peak purity of DOPS was also studied, with a purity factor of peak = 995.083 and thus showing great purity (threshold limit set at 996.869).

Table 39. Degradation of vitamin C under UV exposure.

UV Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
UV Exposure, 24 hours	102.3	0
UV Exposure, 96 hours	104.8	0

After studying the degradation at UV exposure of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.968 and thus showing great purity (threshold limit set at 990.000).

Table 40. Degradation of CoQ10 under UV exposure.

UV Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
UV Exposure, 24 hours	97.1	0
UV Exposure, 96 hours	102.9	0

After studying the degradation at UV exposure of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied, with a purity factor of peak = 998.365 and thus showing great purity (threshold limit set at 990.000).

DOPS, vitamin C and CoQ10 exposure to visible light.

The samples were subjected to exposure of visible light as previously described.

In Tables 41, 42 and 43 it is represented the course of degradation of the actives at the different conditions. All samples were treated to find degradations up to 15 % of the initial concentration, in order to find possible primary degradation products, or until a maximum time of 96h when no degradation was yet observed. In addition, at the last times of every degradation series, purity of peaks was analyzed to avoid any peak overlap between degradation products and the ones corresponding to the active substances.

Table 41. Degradation of DOPS under Vis-light exposure.

Vis-light Exposure of DOPS	Concentration (% from initial)	Degradation Peaks (Number)
Vis-light Exposure, 24 hours	100.6	0
Vis-light Exposure, 96 hours	86.7	0

After studying the degradation at Vis-light exposure of DOPS, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of DOPS was also studied, with a purity factor of peak = 994.347 and thus showing great purity (threshold limit set at 996.869).

Table 42. Degradation of vitamin C under Vis-light exposure.

Vis-light Exposure of Vitamin C	Concentration (% from initial)	Degradation Peaks (Number)
Vis-light Exposure, 24 hours	98.9	0
Vis-light Exposure, 96 hours	101.2	0

After studying the degradation at Vis-light exposure of vitamin C, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of vitamin C was also studied, with a purity factor of peak = 999.923 and thus showing great purity (threshold limit set at 990.000).

Table 43. Degradation of coenzyme Q10 under Vis-light exposure.

Vis-light Exposure of CoQ10	Concentration (% from initial)	Degradation Peaks (Number)
Vis-light Exposure, 24 hours	98.6	0
Vis-light Exposure, 96 hours	98.0	0

After studying the degradation at Vis-light exposure of CoQ10, no new peaks formation was detected as primary degradation products at any wavelength.

The peak purity of CoQ10 was also studied, with a purity factor of peak = 994.875 and thus showing great purity (threshold limit set at 990.000).

Table 44. Summary of primary degradation products found on DOPS, vitamin C and CoQ10.

Summary table of primary degradation products			
Active substance	**Conditions**	**Retention time (minutes)**	**Wavelength λ (nm)**
DOPS	Acid	12.697	200
DOPS	Acid	13.466	200
DOPS	Acid	15.396	200
DOPS	Basic	12.668	200
DOPS	Basic	15.375	200
DOPS	Oxidant	14.489	200
DOPS	Temperature	15.251	200
CoQ10	Basic	16.567	200

3.3.4 Applications of the Method

Once validated, the proposed method was used to accurately evaluate the stability of the three active substances encapsulated on the mentioned liposomal formula after 3 months of storage at 4 °C and at 25 °C. This study verifies the applicability of the method in a real case study. Results are presented in Table 45, while an illustrative chromatogram of each temperature is included in Figure 21.

Table 45. Results of stability studies with the developed method – 3 months.

	DOPS	**Vitamin C**	**CoQ10**
4 °C (%)	100.09 ± 0.31	95.27 ± 0.58	97.56 ± 0.20
25 °C (%)	96.19 ± 0.22	85.57 ± 0.15	87.27 ± 0.23

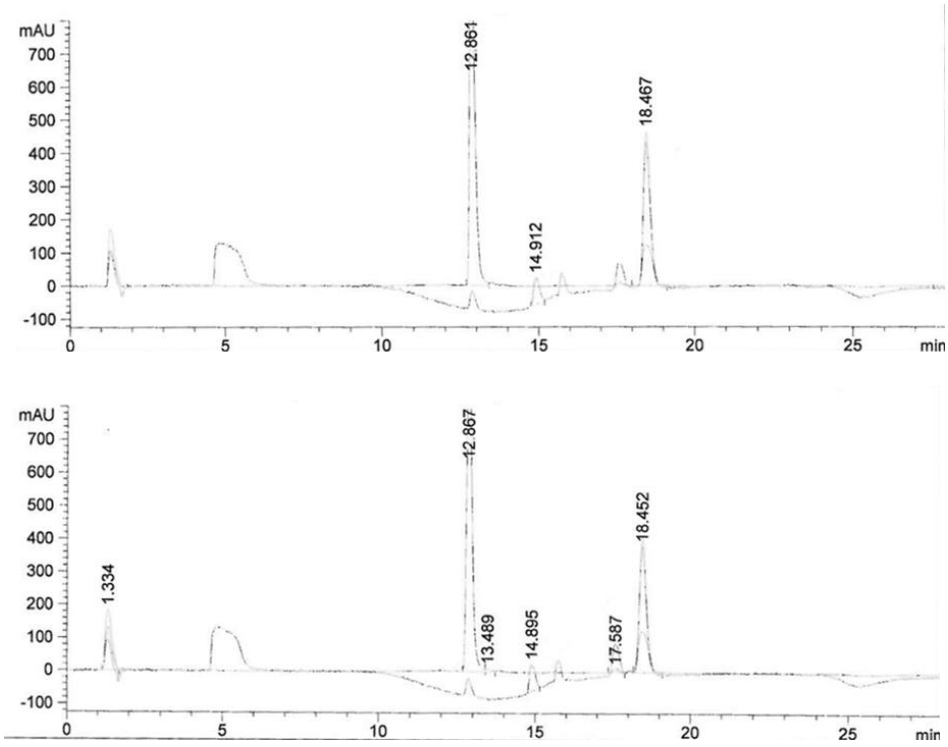

Figure 21. Exemplification chromatogram of an analyzed sample after 3 months' stability at 4 ºC and 25 ºC. The peaks did not show any difference on retention time and could be properly analyzed and quantified, as a result of an adequate validated method. Both CoQ10 (RT = 18.4 minutes) and vitamin C (RT = 12.8 minutes) showed a significant decrease on signal absorption at 25 ºC, denoting degradation.

3.4 CONCLUSION

The developed method demonstrated promising results on the simultaneous analyses of CoQ10, vitamin C and DOPS. To the best of our knowledge, this is the first reported method for the determination of these widely used active substances at the same time. Thus, this method could result an ideal analytic method for the cosmetics, pharmaceutical and food supplement industry.

Additionally, the formulation involving the liposomal technology may be a tricky challenge to allow the correct quantification and analyses. Especially for very lipophilic active substances, to which the presence of water can signify a complicated limitation for the analyses. The performed methodology of sample treatment prior to its analyses may be as well an additional step of valuable interest. The concept may be applied to any other possible liposomal formulations with highly hydrophobic active substances.

In terms of validation of the method, following the ICH guidelines, it was tested the suitability of the equipment, selectivity of the method, linearity, robustness, precision, accuracy and stability of the solution during the analyses, with positive and encouraging results.

Lastly, with the purpose of verifying the applicability of the method, a testing of the method with a real case was carried out. Samples subjected to stability studies were analyzed, and results were studied and interpreted, with excellent results.

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CHAPTER 4. Design, characterization and optimization of a novel liposomal-based food supplement against chronic fatigue syndrome.

ABSTRACT

In this chapter, we develope a novel nanocarrier based on the coencapsulation of coenzyme Q10 (CoQ10), [redacted] nicotinamide adenine dinucleotide (NAD[redacted]), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS) and vitamin C into liposomes as a combinatory therapy against Chronic Fatigue Syndrome (CFS), coencapsulating. This prototype, which is based on the simultaneous encapsulation of the above mentioned actives into a homogeneous unilamellar liposomal suspension, shows improvements on *in vitro* experiments of stomach resistance to digestion and an increase on the retention time in the intestinal tract. In addition, *ex vivo* experiments using Franz diffusion cells demonstrated that liposomes enhance the absorption velocity and rate of the studied actives. Overall, the liposomal prototype may be considered as a potential formulation to mitigate symptomatology in CFS patients.

4.1 INTRODUCTION

Chronic fatigue syndrome (CFS), also named myalgic encephalomyelitis, is a cluster of clinical symptoms which is defined as the presence of unexplainable fatigue lasting more than 6 months accompanied by substantial impairment in short-term memory, tender lymph nodes, sore throat, muscle pain, multiple joint pain without swelling or redness, headache and unrefreshing sleep, among others[1-3]. Additionally, CFS majorly affects productivity, since CFS patients had discontinued employment because of fatigue-related symptoms. It is estimated that CFS will have a prevalence of 857/100,000 habitants (2.8 M and 6.4 M people in USA and Europe, respectively) by July 2020[4,5]. Thus, identifying the potential CFS population is crucial for early intervention. To date, the cause of CFS is unknown, although some theories have been suggested. The symptoms may be related to infection with effects on the immune system. Several viruses and bacteria have been studied as possible causes of CFS, but no cause-and-effect relationship has been discovered[6]. Alternatively, other authors have described the dysfunction of the mitochondria as a key factor for the development of CFS, since mitochondria are responsible of producing up to 90 % of the human body energy in form of ATP[7–10]. Dysfunction of mitochondria is defined as the sum of deleterious changes occurred in the cell due to oxidative metabolism.

To date, few therapies have been developed for the treatment of CFS. Among them, some interesting results have been obtained in CFS patients treated with mitochondrial antioxidant therapy based on oral supplementation with CoQ10, NAD and PS[11–20]. CoQ10 is a strong

antioxidant and an essential nutrient of the mitochondrial respiratory chain that also has been described as an anti-inflammatory agent[11]. Supplementation with CoQ10 has been tested in double-blinded clinical studies in CFS patients, and several CFS symptoms like depression, tiredness, weakness and sleep quality seemed to be significantly improved[11,12]. On other hand, NAD is a coenzyme known for triggering ATP production in mitochondria that has also been reported to reduce fatigue in clinical assays with CFS patients[13,14]. Recent studies have tested the co-administration of CoQ10 and NAD as mitochondrial antioxidant therapy in double-blind trials, obtaining an impressive recovery on biochemical parameters in CFS and a significant improvement in fatigue impact scale total score[15,16]. Additionally, it has been proven that PS is able to reduce muscle soreness and induce well-being and exercise capacity[17], and some interesting works described the supplementation with cell membrane forming lipids like PS (Lipid Replacement Therapy, LRT) in order to avoid the damage of mitochondrial membranes and therefore, the loss of cellular functionality[18,19]. Recently, in another clinical trial, 58 patients were administered with a cocktail of CoQ10, NAD and PS, resulting in a 30.7 % statistically significant reduction of the fatigue symptomatology after 60 days of study[20].

Lastly, strong and widely known antioxidant activity has been used and reported on vitamin C, which has been very used on both pharmaceutical, cosmetic and food supplement industry[21,22].

However, a challenge of this oral mitochondrial antioxidant therapy is that the above-mentioned actives must overcome numerous adverse conditions, including the low pH of the stomach or the reduced absorption

of actives, which can compromise their bioavailability. These problems can be successfully addressed by encapsulation, which would be able to protect the actives under potentially deleterious conditions. Among the many diverse biomaterials used for encapsulation, liposome, alginate, gelatin, lecithin, starch are the most used in pharmaceutical and the food oral supplement industry[23–25]. Liposomes are widely used biomaterials in nanoencapsulation with the advantage of being easy to prepare, customize and administer[27]. Moreover, liposomes are biocompatible, have the ability to incorporate hydrophilic and hydrophobic actives, to enhance the absorption rate of the encapsulated actives, to increase the retention time at the site of absorption, and have the capacity of protecting active substances from external conditions, among others benefits[28,29]. Coencapsulation of different actives has been widely studied and reported on the last years[30-37], and concretely liposomes have already been used for the encapsulation of the isolated antioxidant actives PS, CoQ10, NAD[redacted] and vitamin C, but not as a combinatory oral food supplement therapy. Co-administration of these actives on a combinatory antioxidant therapy may provide a wide and complete supplementation with promising results on CFS patients or people suffering from fatigue. The encapsulation on the same vehicle, although being challenging to achieve, would offer all the benefits of the liposomes on the protection of all the actives and the enhancement on bioavailability and effectiveness.

For example, CoQ10 has been encapsulated in liposomes for early theranostics of diabetic nephropathy[38], and as dermal antioxidant carrier for *in vivo* topical applications[39,40]. On the other hand, NAD[redacted] has been mainly encapsulated in liposomes for the stabilization of enzymes like the formate dehydrogenase[41], and for studying the consumption of oxygen in

Human Epithelial Colorectal Adenocarcinoma (Caco-2) cells[42]. Likewise, vitamin C has also been entrapped in liposomes for improving doxorubicin, docetaxel or paclitaxel based anticancer therapies[43-45] and for anti-aging therapy of the skin[46]. Finally, PS has been used in the preparation of antigen-loaded liposomes for the treatment of autoimmune diseases like Type 1 diabetes[47] or sclerosis[48].

Here, our hypothesis is that the bioavailability and effectivity of mitochondrial antioxidant actives can be enhanced when they are encapsulated into liposomes, optimizing the opportunities to reach the target and increasing their antioxidant effect, while making the active safer. For this purpose, on this study, we have explored and characterized the encapsulation of a cocktail of antioxidant actives formed by hydrophilic (NAD[redacted]) and hydrophobic (CoQ10, PS and vitamin C) actives in liposomes as an improved mitochondrial antioxidant therapy against CFS.

4.2 MATERIALS AND METHODS

4.2.1 Materials and chemical reagents

Phosphatidylserine (PS, concretely [redacted] PS) and phosphatidylcholine (PC, concretely [redacted] PC) were purchased from [redacted] Cholesterol-(polyethylene glycol-600) (Chol-PEG), cholesterol (Chol), 6-O-palmitoyl-L-ascorbic acid (PA), trehalose, polaxamer 407 (Pluronic f-127, BASF corporation, New Jersey, U.S.A.), alginic acid sodium salt (alginate) and mucin type III were acquired from [redacted]. Vybrant DiI was

acquired from [redacted]. 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Cholesterol·HCl) was purchased from [redacted]. β-nicotinamide-adenine dinucleotide disodium salt (NAD[redacted]), sodium ascorbate (NaA) and CoQ10 was obtained from [redacted].

4.2.2 Preparation and characterization of liposomes

Liposomal formulations were prepared using [redacted] Particle size distributions of the liposomes were determined using a Dynamic Light Scattering (DLS) analyzer combined with noninvasive backscatter technology (Malvern Zetasizer; Malvern Instruments, United Kingdom). The stability of the liposomes was examined by measuring their Zeta Potential (ZP) with an electrophoretic mobility and light scattering analyzer (Malvern Zetasizer). Liposome

morphology was examined using cryo-transmission electron microscopy (Cryo-TEM) in a Tecnai F20 EM microscope (FEI, Eindhoven, The Netherlands). Visual evaluation of the NAD[redacted] encapsulation was also carried out in liposomes with Vibrant DiI labeled bilayers thanks to the autofluorescence of NAD[redacted], with excitation at λ = 340 nm and emission on the blue field at λ = 460 nm[51], on a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Germany).

4.2.2.1. Encapsulation yield

Encapsulation efficiencies (EE) were calculated according to the following equation[52]:

$$EE(\%) = \frac{C_{Active\ Total} - C_{Active\ Out}}{C_{Active\ Total}}\ x\ 100$$

where $C_{Active\ Total}$ is the initial concentration of the hydrophilic active substance (NAD[redacted]/NaA) and $C_{Active\ out}$ is the concentration of non-encapsulated active substance. To measure $C_{Active\ out}$, liposome suspensions were centrifuged at 7000 rpm for 10 minutes at 5 ºC in 30 kDa Molecular Weight Cut-Off (MWCO) Amicon Ultra-4 Centrifugal filters (Millipore). Supernatant aliquots were taken to quantify the concentration of non-encapsulated active by UV-Vis spectroscopy using a Nanodrop 2000c (Thermo Scientific, USA). NAD[redacted] was linearly detected in a range from 0.1 mg/mL to 1.65 mg/mL ([redacted], R^2 = 0.999), while sodium ascorbate was linearly detected in a range from 0.1 mg/mL to 1.9 mg/mL ([redacted], R^2 = 0.999). To measure $C_{Active\ Total}$, breakage of liposomes was necessary, and blight and dyer extraction method[53] was performed. At lower NAD[redacted] concentrations than 0.1

mg/mL, supernatants were analyzed by fluorimetry (Cory Eclipse Fluorescence Spectrophotometer, Agilent Technologies, United States). NAD[redacted] was linearly detected in a range from 0.24 μg/ml to 16.63 μg/ml (Abs at 475 nm, r2= 0.999).

To calculate the EE of the hydrophobic actives ([redacted]PS, CoQ10 and fat-soluble vitamin C), we followed the already reported procedure with high-performance liquid chromatography (HPLC) quantification[54], extensively explained in *Chapter 3*. Briefly, liposomes were first lyophilized, and then they were dissolved in chloroform. The resulting solution was analyzed by liquid chromatography with ultraviolet detection (Agilent 1100 Series HPLC System with Diode-Array Detection (DAD) detector, Agilent Technologies, United States).

4.2.3 *In vitro* encapsulated NAD[redacted] release

The kinetics of NAD[redacted] release from liposomes incubated with 0.2 M sodium chloride media were also assessed. Experimentally, cleaned liposomes were submitted to extensive dialysis against a 10-fold excess of media at 37 ºC for [redacted] under agitation in 2K MWCO dialysis tubing membranes (Thermo Fisher, Walthman, MA, USA). For each time, supernatant was collected and replaced to maintain a constant volume. NADH concentration was calculated as described above.

4.2.4 Study of the liposomal protection conferred to the core-encapsulated NAD[redacted] at acidic pHs

A series of experiments were designed to test the degree of protection of the lipid-bilayer provides to the core-encapsulated active substance

NAD[redacted]. Briefly, 2 mL of liposomal suspensions were introduced against a 10-fold excess of media during one hour – various pH were assayed in order to accurately evaluate the protection against different acid media –. For each time and pH, 300 μL aliquots were collected and replaced with the same volume of media. NAD[redacted] concentration was calculated as described above. All the experiments were undergone on triplicate, with cleaned and non-cleaned liposomal suspensions, together with the non-encapsulated active, so the differences could clearly be studied and liposomal barrier protection could be analyzed. For cleaning liposomal suspensions and removing the non-encapsulated NAD[redacted], suspensions were extensively dialyzed against a 10-fold excess of media at 4 ºC under agitation in 2K MWCO dialysis tubing membranes (Thermo Fisher, Walthman, MA, USA).

4.2.5 *In vitro* NAD[redacted] stability in simulated gastric fluids

Active loaded liposomes were *in vitro* tested for their stability in simulated gastric fluid (SFG) at 37 ºC[55,56]. Briefly, 2 mL of liposomal suspensions were introduced against a 10-fold excess of media at 37 ºC under agitation at pH 5.2 for one hour to mimic the conditions of human stomach at 50 % of gastric emptying. Afterwards, pH was increased to 7.2 for 6 hours to mimic the conditions of intestinal tract. For each time and pH, 300 μL aliquots were collected and replaced with the same volume of media. NAD[redacted] concentration was calculated as described above. The same methodology was used to test the non-encapsulated active. Stability of the lipid active substances was also studied and quantified.

4.2.6 *In vitro* cytotoxicity of liposomes to NIH-3T3 cells

Mouse fibroblasts (NIH-3T3) cell line was incubated with the final liposomal formulation loaded with the actives and then, the effect of liposomes on cell viability was assessed by using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 H-tetrazolium-5-carboxanilide) cell viability assay after 24 hours incubation time[57]. The cells were seeded into 96-well plates, incubated for 24 hours and the exposed to fresh media containing a PBS suspension of the liposomal formulation (total lipid concentrations: 0.1 mM to 10 mM). At 24 hours incubation, aliquots of 20 mL of XXT solution were added to each well, and the resulting colour was quantified (λ=450 nm) in a spectrophotometric plate-reader (PerkinElmer Victor3V). Cell viability was expressed as a percentage of the control level. All the measurements were performed in triplicate, in three independent experiments. The same procedure was employed to evaluate the cytotoxicity of NAD[redacted], and empty liposomes to NIH-3T3 cells. Liposomal formulations in both liquid state and lyophilized with trehalose were tested.

4.2.7 *In vitro* bioadsorption and mucoadhesion study

The amount of mucin adsorbed by liposomes in a certain period of time was quantified to determine mucoadhesive properties[58-60]. Briefly, liposomes were mixed and incubated with mucin type III from porcine stomach at 37 ºC for 18 hours. Then, the suspension was centrifuged at 10000 rpm at 4 ºC for 15 minutes, and the concentration of mucin in the supernatant was determined by the periodic acid schiff (PAS) colorimetric method[61]. Concentration of mucin in solution before and after absorption by liposome particles was measured to determine mucoadhesion. Mucin

standards were measured by the same procedure to obtain a mucin calibration curve.

4.2.8 *Ex vivo* permeation studies

4.2.8.1 Biological tissues

Abdominal skin samples were extracted from 3-4 month-old female pigs (weight 30-40 kg, n=3) following the protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona (Spain) and the Committee of Animal Experimentation of the regional autonomous government of Catalonia (Spain). The surgical procedure was performed by a veterinarian at the Animal Facility (Bellvitge Campus, University of Barcelona, Spain) after the animals were sacrificed using an overdose of sodium thiopental anesthesia.

4.2.8.2 Frank diffusion cells

Permeation study of liposome formulation was performed in vertical Franz diffusion cells[62] with diffusion area of 0.64 cm^2. Fresh biological tissues were mounted in the membrane holders between the donor and the receptor compartment, which was filled with water or water:transcutol® P (40:60, v:v) and kept under stirring at 600 rpm. Sink conditions were guaranteed and the temperature was controlled at 37 ± 0.5 °C by a circulating water bath. Testing samples were placed in the donor compartment (and covered with paraffin to avoid evaporation). 300 µL aliquots were withdrawn from the receptor compartment for 6 hours and replaced by an equivalent volume of receptor medium at the same temperature. The amount of NAD permeated from liposomes through

biological tissues was analyzed using water as the receptor compartment, while the permeation of [redacted]PS, CoQ10 and vitamin C was studied using the mixture of water:transcutol® P (40:60). For each time, active concentrations were quantified as described above. Liposome permeability through biological tissues was then detected by Cryo-TEM analysis in the receptor compartment.

4.2.8.3 Active recovery

After permeation test was finished, tissue membranes were cleaned with gauze soaked in 0.05 % solution of sodium dodecyl sulfate (SDS) and washed with distilled water. The permeation areas were then excised and weighted. Actives retained in the tissues were extracted using the corresponding receptor media under sonication for 20 minutes. The resulting solutions were centrifuged at 15000 rpm for 10 minutes, and the supernatants were analyzed. Results are shown as the median and range of three triplicates.

4.2.8.4 Permeation parameters

The cumulative amount of active permeated (Q_t) through biological membrane was plotted as a function of time. The slope and intercept of the linear portion of the plot was derived by regression using the Prism® software, v 3.o (GraphPad Sofware Inc., San Diego, CA, USA). Active fluxes (*J, µg/h/cm*$^{-2}$*)* through tissues were calculated from the slope of the linear portion of the cumulative amount permeated per unit surface area *vs.* time plot. The permeability coefficient (K_p, *cm/h*) was obtained by dividing the *J* by the initial active concentration (*Co*) in the donor compartment. Results are shown as the median and range of three

triplicates. Statistical significance of experimental results was studied with Student's t-test. It was considered statistically different when $p<0.05$, high statistically different when $p<0.01$ and very high statistically different when $p<0.001$.

4.2.9 Lyophilization and stability of liposomes

For the long-term storage of the liposomes loaded with antioxidant actives, the cryoprotectant trehalose was added during their synthesis, and the encapsulated prototype was subjected to lyophilization as follows. The dried lipid film was hydrated with a suspension containing the NAD[redacted] and trehalose at different proportions to study the necessary amount for the perfect reconstitution at rehydration.

Glass transition temperature of encapsulated prototype was determined by Differential Scanning Calorimetry (DSC) measurements to ensure an optimal freeze-drying of samples[63,64].

The analysis of DSC was realized with a DSC821 (Mettles Toledo®, U.S.A.) equipment and STARe SW V9.30 software. 30 µL of sample were placed on a 40 µL aluminum melting pot were the measurement is performed. The method consisted in one cycle of three phases, being the first one a transition from 25 ºC to -80 ºC at 10 ºC/minutes, followed by one minute where temperature gets stable and afterwards it starts getting warm again until achieving 25 ºC at a velocity of 10 ºC/minutes. On a heat flow vs. temperature graphic, there is a moment where a slightly change on linearity – not a sharp peak – appear, giving information about the Tm of the liposomal bilayer.

The resulting cryo-protected liposomes were frozen at -80°C for 12 hours and then, lyophilized at -50°C for 96 hours. When needed, the lyophilized samples were re-suspended in Milli-Q water and the morphology and size of the reconstituted liposomes were assessed by Cryo-TEM and DLS. To quantify the degradation of antioxidant actives on time, liposomes encapsulating NAD[redacted], [redacted]PS, CoQ10 and vitamin C were prepared and lyophilized, and finally stored at 4 ºC. At 1 month, 3 months, 6 months, 9 months and 15 months, the dried liposomal cakes were re-suspended with Milli-Q water and the concentration of NAD[redacted], [redacted]PS, CoQ10 and vitamin C was separately quantified by UV detection.

4.2.10 Potential dosage forms

After the galenic design and optimization of the liposomal encapsulation for an oral administration of the selected active substances, the final potential dosage forms for the product were analyzed. Additionally, two variants of the product were studied for a final sublingual alternative: the addition of polaxamer 407 and the use of sodium alginate.

Briefly, a 50 % liposomal solution and 50 % polaxamer 407 solution 40 % m/v were mixed until a homogeneous solution was achieved. Afterwards, 0.6 – 1 mL of the resultant solution was placed on a rheometer (RheoStress RS600, Thermo Scientific) and viscosity was measured at 25 ºC and 37 ºC for 30 seconds.

The performed assay with the use of sodium alginate consisted in adding alginate directly to the final liposomal formula, so when in contact with saliva on its sublingual administration, the solution gels because of the divalent cations content of human saliva. With this purpose, 5mg/mL of

sodium alginate was added to the liposomal formula, and the resulting solution was dropwise added to a saliva simulation solution[65] with 4 mmol/L of Ca^{2+} and 0.2 mmol/L of Mg^{2+}.

4.3 RESULTS AND DISCUSSION

4.3.1 Preparation and characterization of liposomes

On the design of the formula for the production of the oral liposomal food supplement against CFS, the promoter company Vitae Health Innovation, S. L. decided that the chosen four active substances to encapsulate into the liposomes would be: Serine, CoQ10, NAD[redacted] and vitamin C. Regarding the active substances, in the case of serine, it was decided to use as far as possible its lipid variant, phosphatidylserine ([redacted]PS), since it is also a commonly used phospholipid on the composition of liposomal membranes for encapsulation. In the choice of the other phospholipids to make the liposomes, different combinations with neutral, positive and negative lipids were assayed to achieve the better possible results (see below). We ideally targeted a 50-200 nm mean size liposomes, with good homogeneity, stability and the highest encapsulation efficiency possible. Following the phospholipids, the next predominant component would be the Chol (Figure 1A). Chol is widely reported to provide flexibility and stability to the membrane, abolishing the appearance of a phase transition on transforming the membrane into a smooth ordered fluid phase and also reducing the leakage of encapsulated material, making membranes impermeable due to an increase on the packing density of the phospholipids[66,67,68]. Lastly, in all formulas, a small percentage of peglicated lipids was added; in this case, a 5 % of Chol-PEG was always

maintained (Figure 1B). Peglication of liposomal membranes is of great utility to enhance the stability of the suspensions due to the steric hindrance caused by the long carbon chains, thus avoiding the agglomeration of the vesicles. Additionally, peglicated lipids allow the vesicles to remain in blood-circulation for longer time extending in this way their half-life in the body. Peglication renders liposomes invisible to macrophages, making them to evade the immune system[66].

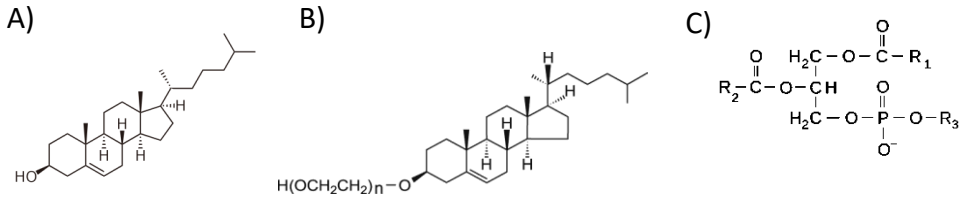


Figure 1. Representation of the chemical structure of the membrane forming molecules. A) Chol. B) Chol-PEG. C) Chemical representation of a phospholipid structure in general. R_1 and R_2 = Saturated or unsaturated carbonated chains with undefined number of carbons. Depending on the chains, chemical properties of the phospholipids variate, like phase transition temperatures. R_3= phosphate attached chemical group, like choline, ethanolamine or serine.

In the case of NAD[redacted], vitamin C and CoQ10, encapsulation studies and their optimizations were firstly made separately, with little variations on the compositions of the membrane ingredients (phospholipids, Chol, Chol-PEG), maintaining in all cases a majoritarian proportion of the zwitterionic phospholipid [redacted]PC as a main structural membrane lipid (Figure 2A). Here, we tested different charged membranes to evaluate eventual differences on encapsulation efficiencies. In these testings, the use of [redacted]PS caused a final negative ZP on the vesicular suspensions (Figure 2B), whereas the addition of DC-Cholesterol·HCl caused a final

positive charge (Figure 2C). All the prototypes were prepared using an extrusion pore membrane of 400 nm.

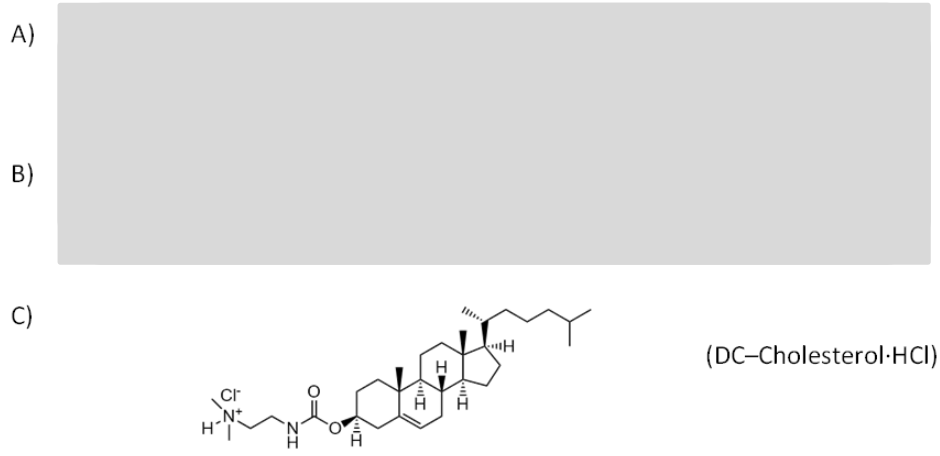


Figure 2. Representation of the chemical structure of the membrane forming molecules. A) Phosphatidylcholine, concretely [redacted]. B) Phosphatidylserine, concretely [redacted]. C) DC-Cholesterol·HCl or 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride, a positive charged membrane forming lipid.

Initially, we start evaluating the encapsulation of NAD[redacted] on different lipid mixtures to determine its highest encapsulation efficiency (Table 1). The first assayed formula (prototype 1.1 or P1.1) was based on a non-charged [redacted]

[redacted]. This prototype was imaged using Cryo-TEM microscopy, confirming the formation of unilamellar liposomes (Figure 3).

Table 1. Composition and characterization of the assayed liposomal formulations to optimize NADH encapsulation.

Lipid composition	Mean Size (nm)	PdI	ζ Potential (mV)	NAD EE (%)

Each value represents the average from three independent experiments ± the standard deviation.

On a second batch of experiments, liposomal encapsulation of vitamin C was studied. For the vitamin C, two variants of the active substance were contemplated. On one hand, sodium ascorbate (NaA), the hydrosoluble form of vitamin C (Figure 4A), which would be encapsulated on the core of the liposome (its aqueous part). On the other hand, the liposoluble form of the vitamin C, ascorbyl palmitate (ascorbic acid attached through an ester bond to palmitic acid; Figure 4B) which would be basically inserted

in the lipidic membrane. Accordingly, the encapsulation of both variants of vitamin C, NaA and PA, was evaluated. All encapsulation experiments of NaA are summarized on Table 2, while PA assays are compilated on Table 3.

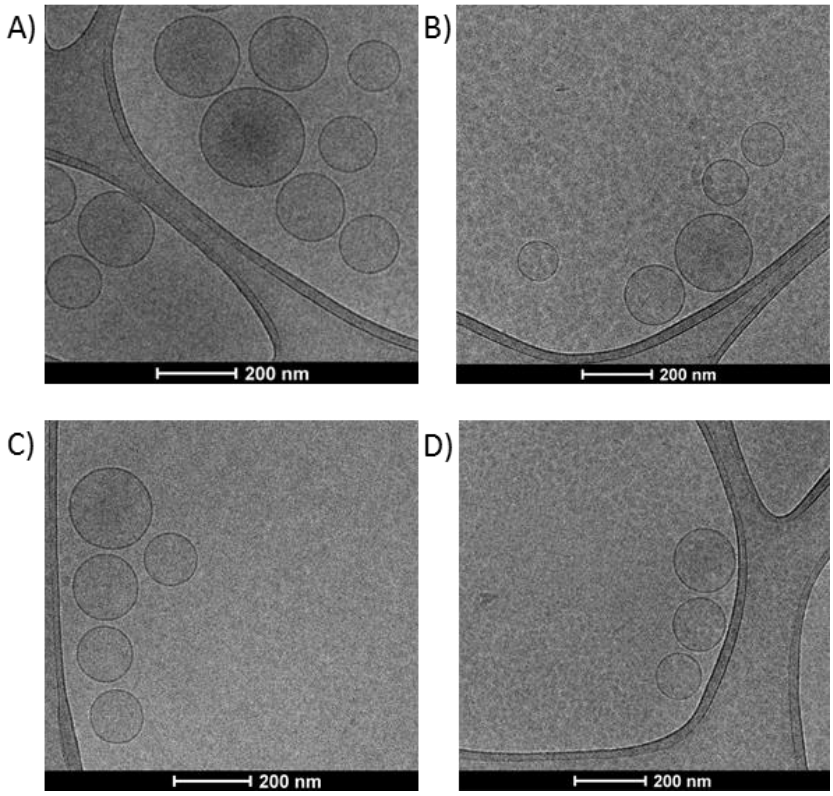


Figure 3. A-D) Cryo-TEM imaging of the P1.3 formula on the optimization of NAD encapsulation.

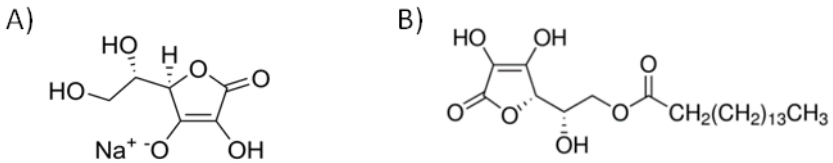


Figure 4. Representation of the chemical structure of: A) Sodium Ascorbate. B) Palmitoyl Ascorbate.

Table 2. Composition and characterization of the assayed liposomal formulations to optimize NaA encapsulation.

Name	Lipid composition	[NaA] (mg/mL)	Mean Size (nm)	PdI	ζ Potential (mV)	NaA EE (%)

Each value represents the average from three independent experiments ± the standard deviation.

[redacted]

Table 3. Composition and characterization of the two assayed liposomal formulations to study PA encapsulation.

Name	Lipid composition	Mean Size (nm)	PdI	ζ Potential (mV)	PA EE (%)

Each value represents the average from three independent experiments ± the standard deviation.

[redacted]

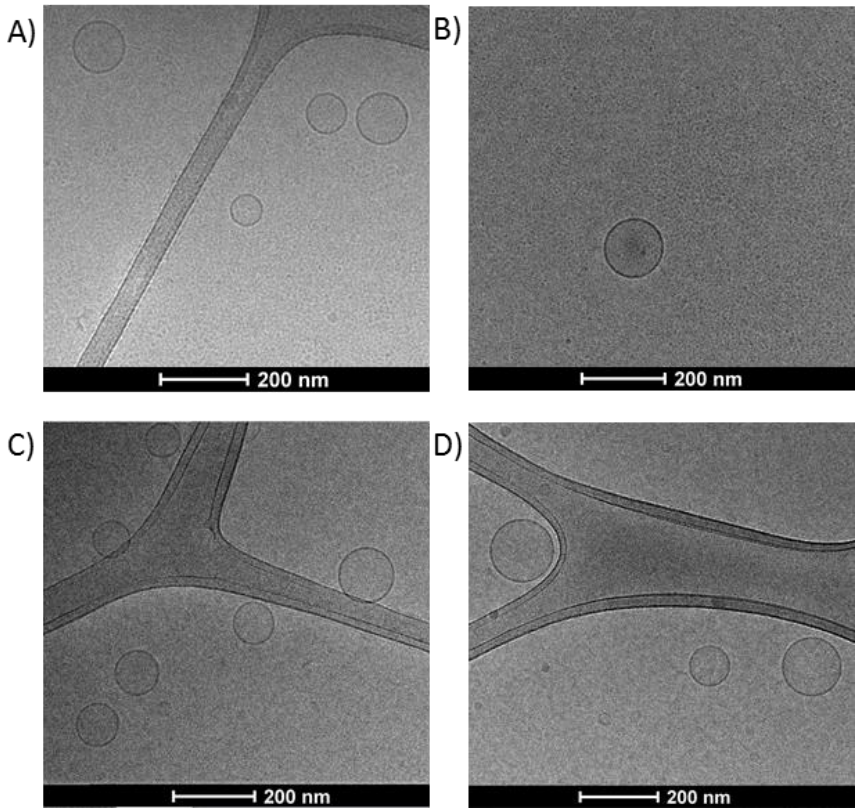


Figure 5. Cryo-TEM imaging of the optimization of vitamin C encapsulation. A – B) liposomes encapsulating NaA from P2.8. C – D) Liposomes encapsulating PA from the prototype P3.1.

Lastly, for CoQ10, and based on the high fat-soluble nature (log P = 10)[69] that would make it an ideal active to be incorporated into the lipid bilayer, it was decided to add it on each formulation assay as if it was one structural ingredient more. [redacted]

Table 4. Composition and characterization of the assayed liposomal formulations to study CoQ10 encapsulation.

Name	Lipid composition	Mean Size (nm)	PdI	ζ Potential (mV)	CoQ10 EE (%)

Each value represents the average from three independent experiments ± the standard deviation.

Once the individual encapsulation of the different actives was proved, we used the optimized formulation to co-encapsulate the four species (Figure 7).

[redacted]

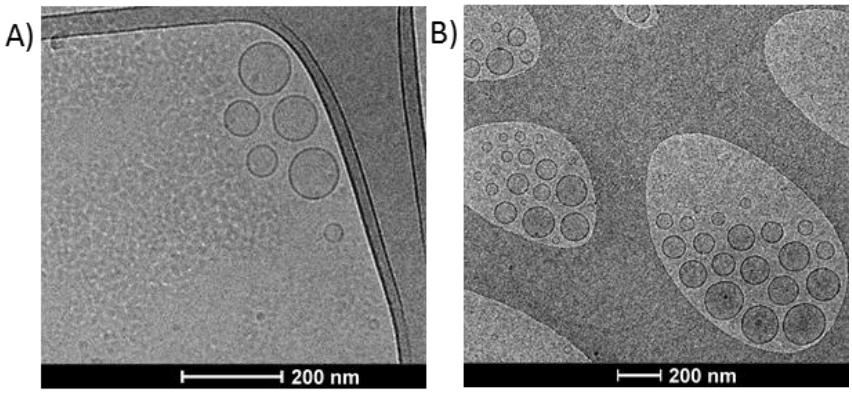


Figure 6. A – B) Cryo-TEM imaging of formulation P4.2.

Using these optimized conditions, the final liposomal formulation [redacted]. Mean size was maintained (164.3 ± 2.7 nm) but PdI was highly improved, with a PdI of 0.07. With the aim of characterizing the physico-chemical structure, this last liposomal cocktail was analyzed by Cryo-TEM and confocal microscopy (CLSM). As shown in Figure 8, the liposomal cocktail was formed by small unilamellar vesicles (SUVs) with a particle size in agreement with the observed by DLS. In addition, natural autofluorescent NAD[redacted] was encapsulated into DiI-labeled liposomes.

Confocal microscope images of non-extruded liposomes demonstrated that NAD[redacted] is entrapped in their aqueous core (Figure 9).

Figure 7. Schematic representation of the liposomes containing the four actives (NAD[redacted], [redacted]PS, PA and CoQ10).

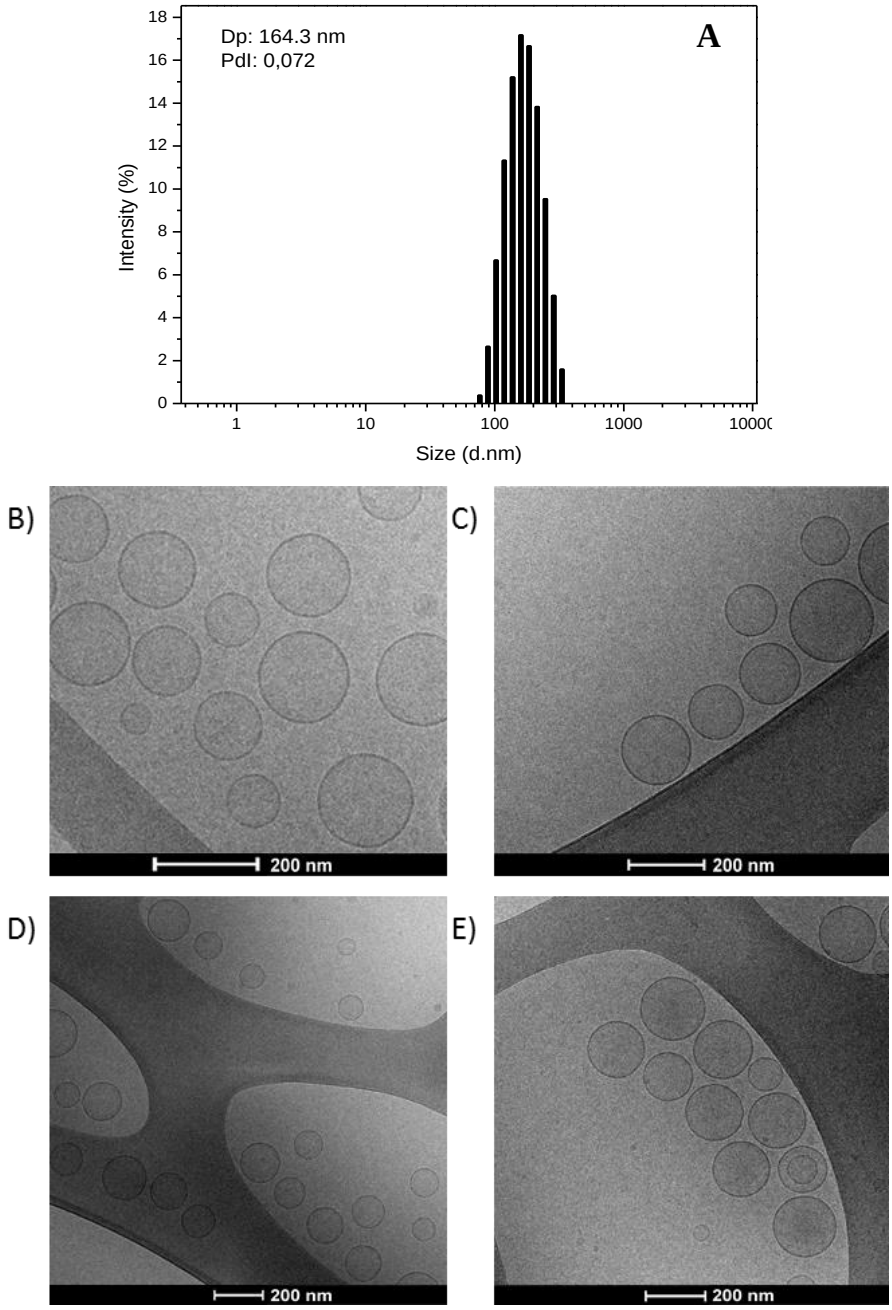


Figure 8. A) Particle size distribution of the final liposomal prototype. B – D) Cryo-TEM images of the final liposomal prototype. Scale bars = 200 nm.

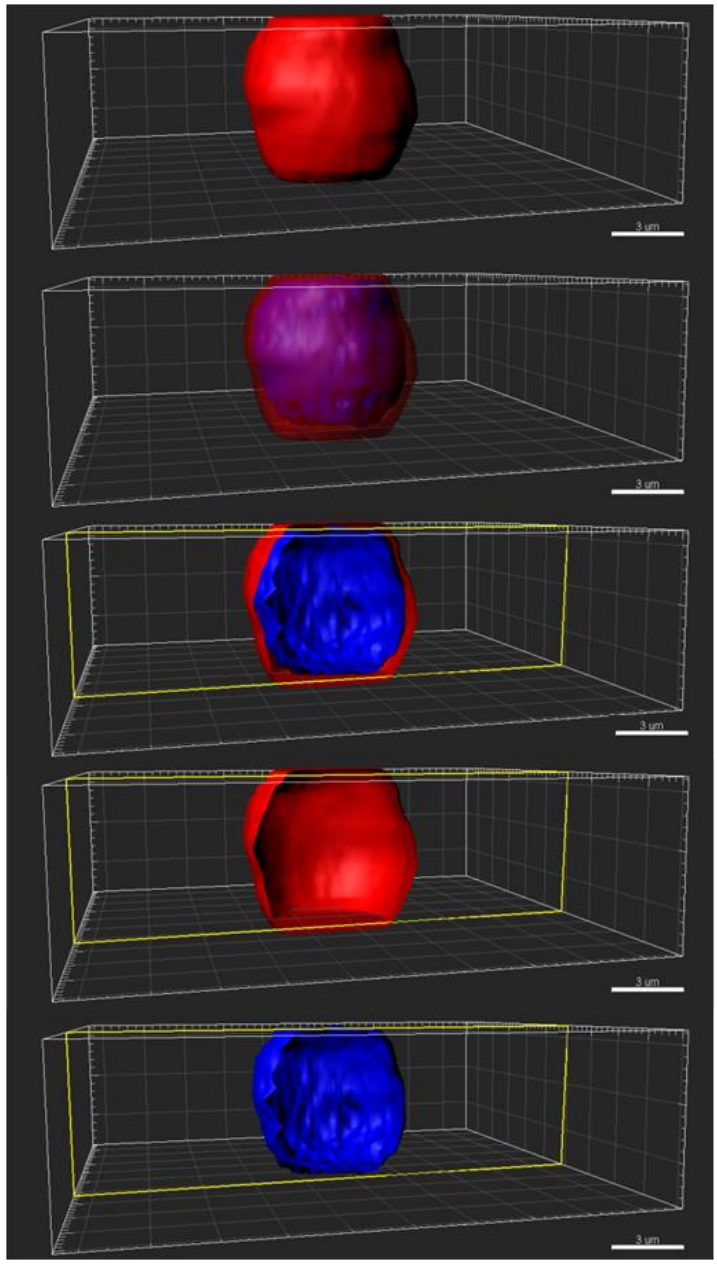


Figure 9. 3D confocal laser microscopy images of autofluorecent NAD (blue) encapsulated into fluorescent DiI-labeled liposomes (red). 3D images of the liposome surface is shown on the top two images and the corresponding cross-sectional images on the right. Scale bars = 3 µm.

4.3.2 *In vitro* encapsulated NAD release

A modified-release oral pharmaceutical form is designed in a way that alters the velocity or place of release of the active substance in respect to what would be in the case of an immediate administration of the drugs. Liposomes are a modified-release kind of controlled and sustained release pharmaceutical form based on a semipermeable membrane: the delayed drug release can contribute to an enhancement on the bioavailability of actives, elongating the therapeutic time, reducing the fluctuation on plasmatic concentrations and minimizing drug release in gastric fluid but increasing drug release in intestinal fluid[70-72].

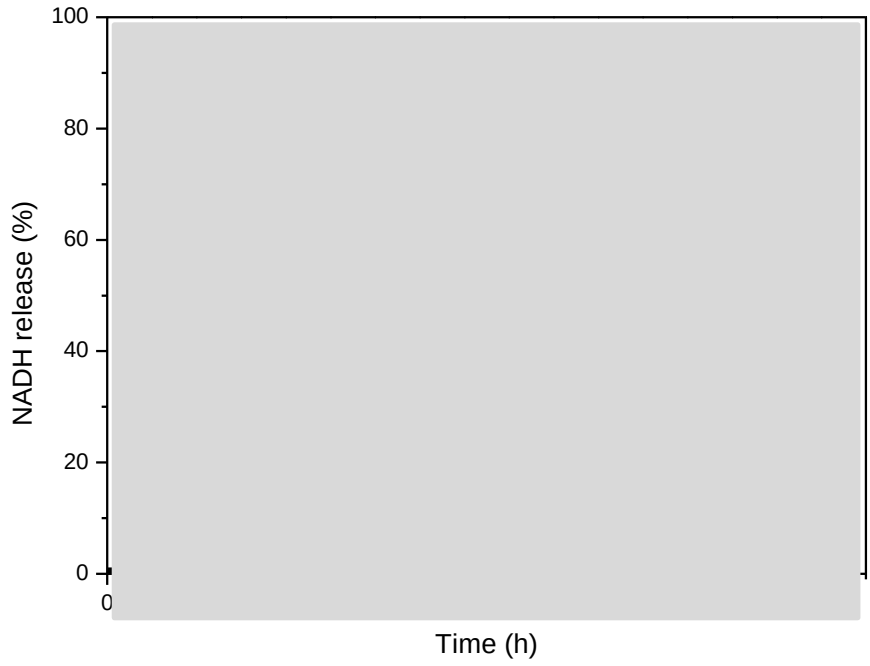


Figure 10. *In vitro* release of liposome-encapsualted NAD incubated in sodium chloride media at 37 ºC. Each value is the average of three independent experiments ± standard deviation.

The kinetics of NAD[redacted] release from liposomes was studied by incubating them for up to [redacted] at 37 ºC with sodium chloride media fluid. Figure 10 shows the evolution of NAD[redacted] released during this period. From it, we found out that [redacted] (Figure 10). This fact confirms the capacity of the vehicle to modify and delay the release of the hydrophilic NAD[redacted] in a sustained manner to the body on an adequate range of time [redacted].

4.3.3 Study of the liposomal protection conferred to the core-encapsulated NAD[redacted] at acidic pHs

Among all the benefits that liposomes may confer to the active substances, there is the fact that encapsulated substances get a protective barrier that separates the nucleus of the vesicle from the external media, and this may improve stabilities along the digestion[24]. Since the core-encapsulated NAD[redacted] is the most sensitive active to extreme conditions as acid or basic media, some experiments were planned to evaluate if NAD[redacted] was stabilized inside the liposomes. To test how much the lipid barrier protects the core-encapsulated NAD[redacted] on the designed final liposomal prototype, different experiments comparing the liposomal NAD[redacted] and the non-encapsulated NAD[redacted] were undergone exposing them to various acid media.

To start this study, the non-encapsulated actives (mainly NAD) were first removed from the liposomal suspension through extensive dialysis against in 2K MWCO dialysis tubing membranes. The selected pH for the acid media were 2.8, 3.8 and 4.8 for 1 hour, followed by 6 hours of pH 7.2 basic media. Table 6 summarizes the percentages and standard deviations after the acid and the basic media exposure, while Figure 11 represents it graphically in a much more visual comparison. At pH 2.8, a 28.3 % more resistance was significantly observed after 1 hour; at pH 3.8, a 36.0 % more resistance was significantly observed after 1 hour, and at pH 4.8, a 16.7 % more resistance was significantly observed after 1 hour. These results demonstrated once more the protective behavior that liposomes confer to the core-encapsulated actives.

Table 6. Data showing resistance percentages of the core-encapsulated NAD after 1 hour of acid media exposure of the dialyzed liposomal solution.

	Liposomal NAD			Non-encapsulated NAD		
Time (hours)	**pH = 2.8 (%)**	**pH = 3.8 (%)**	**pH = 4.8 (%)**	**pH = 2.8 (%)**	**pH = 3.8 (%)**	**pH = 4.8 (%)**
0	100.0 ± 0.4	100.0 ± 0.4	100.0 ± 0.4	100.0 ± 4.4	100.0 ± 4.4	100.0 ± 4.4
1	33.7 ± 4.0	72.5 ± 4.2	101.4 ± 3.3	4.7 ± 0.1	32.3 ± 2.8	83.1 ± 2.2

On another hand, the liposomal suspension without dialysis – closer to what the final product would be after the industrial scale-up – was exposed to different acid media for 1 hour right after its production, and compared with the non-encapsulated NAD stability on the same conditions. The selected pHs for the acid media were again 2.8, 3.8 and 4.8. Table 7 summarizes the percentages and standard deviations after the acid and the basic media exposure, while Figure 12 represents it graphically in a much more visual comparison.

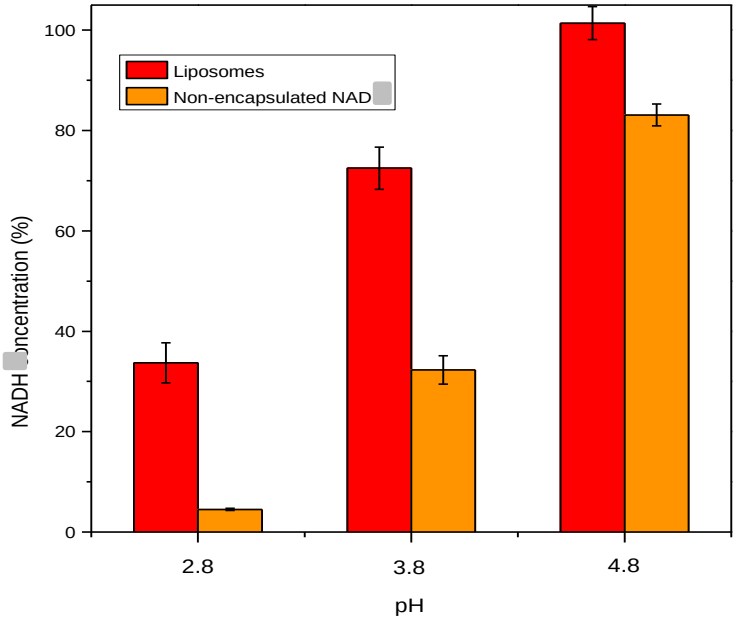


Figure 11. Graphical representation of NAD stability on dialyzed liposomal formulations and without encapsulation, during the exposition to 1 hour of acid media at different pHs. Each value is the average of three independent experiments ± standard deviation.

Significant differences were observed in all the studied pHs comparing the liposomal solution and the non-encapsulated NAD. At pH 2.8, a 14.7 % more resistance was significantly observed after 1 hour; at pH 3.8, a 25.0 % more resistance is significantly observed after 1 hour, and at pH 4.8, a 12.68 % more resistance was significantly observed after 1 hour. These differences demonstrate the protective effect of the lipid bilayer, which shows a semi-permeable behavior due to the fact that NAD gets a protective effect with significant improvement on stability, but not a complete impermeable protection.

Table 7. Data showing resistance percentages of the core-encapsulated NAD after 1 hour of acid media and 6 hours of basic media exposure of the non-cleaned liposomal solution.

	Liposomal NAD			Non-encapsulated NAD		
Time (hours)	**pH = 2.8 (%)**	**pH = 3.8 (%)**	**pH = 4.8 (%)**	**pH = 2.8 (%)**	**pH = 3.8 (%)**	**pH = 4.8 (%)**
0	100.0 ± 0.2	100.0 ± 0.2	100.0 ± 0.2	100.0 ± 4.4	100.0 ± 4.4	100.0 ± 4.4
1	19.4 ± 0.6	57.3 ± 3.4	95.7 ± 7.7	4.7 ± 0.1	32.3 ± 2.8	83.1 ± 2.2

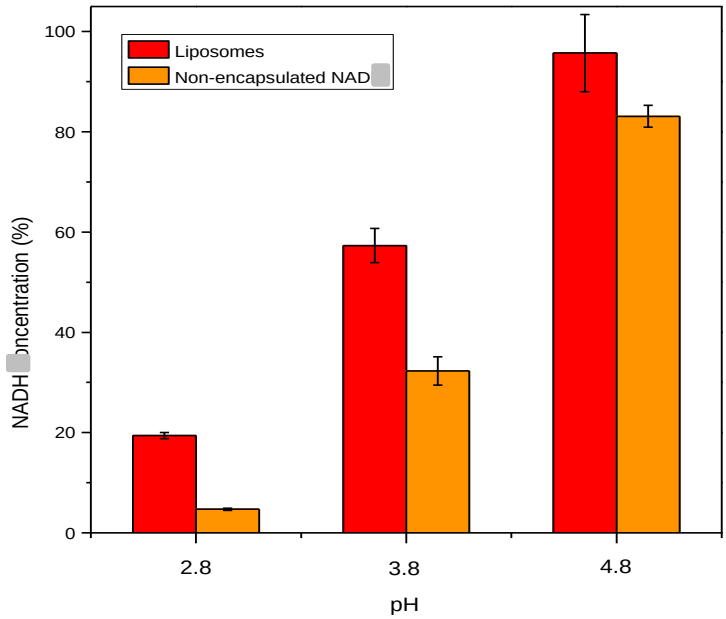


Figure 12. Graphical representation of NAD stability on non-dialyzed liposomal formulations and without encapsulation, during the exposition to 1 hour of acid media at different pHs. Each value is the average of three independent experiments ± standard deviation.

4.3.4 *In vitro* NAD stability in simulated gastric fluids

The acid stability of the liposome-encapsulated NAD was tested by incubating the liposomal suspension (and also the non-encapsulated NAD for comparison purposes) at 37 ºC in simulated gastric fluid (pH 5.2) for 1 hour to mimic the conditions of human stomach at 50 % of

gastric emptying, and then, at pH 7.2 for 6 hours to mimic the conditions of intestinal tract. Figure 13 shows the comparison of the NAD concentration after exposing both samples to the incubation conditions.

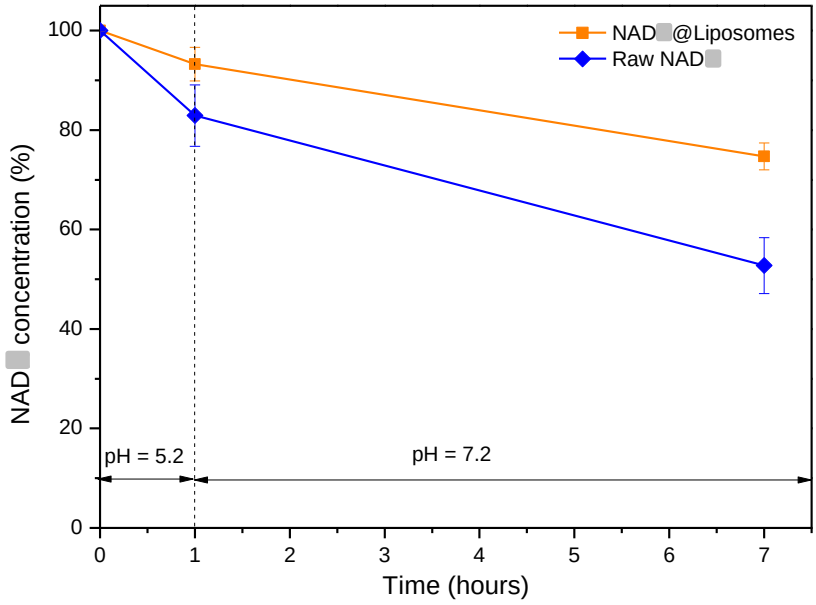


Figure 13. Graphical representation of the NAD stability during the *in vitro* simulation of gastric digestion, encapsulated on the core of the liposomes and without encapsulation. Each value is the average of three independent experiments ± standard deviation.

We observed that, the concentration of encapsulated NAD and non-encapsulated NAD were 93.25 ± 3.36 % and 82.94 ± 6.17 % after 1 hour of incubation, respectively. Remarkably, liposomes exhibited a significant improvement in NAD stability in front of the simulated human stomach conditions. Indeed, this fact was getting even more accentuated at pH 7.2 with time, where the concentration of encapsulated NAD and raw NAD reached values of 74.74 ± 2.7 % and 52.75 ± 5.61 % after 6 hours of incubation, respectively. Stability of the membrane-encapsulated lipid actives was also quantified and graphically represented on Figure 14. Note

here that the quantification of the other three actives after incubating in simulated gastric fluids showed that, after one hour, CoQ10 and PA concentrations were 89.46 ± 3.42 % and 71.07 ± 2.17 %, respectively, and after 6 hours of basic pH incubation, their concentrations were 66.51 ± 3. 48 % and 56.44 ± 2.11 % respectively. In contrast, [redacted]PS showed stability on time with no significant differences.

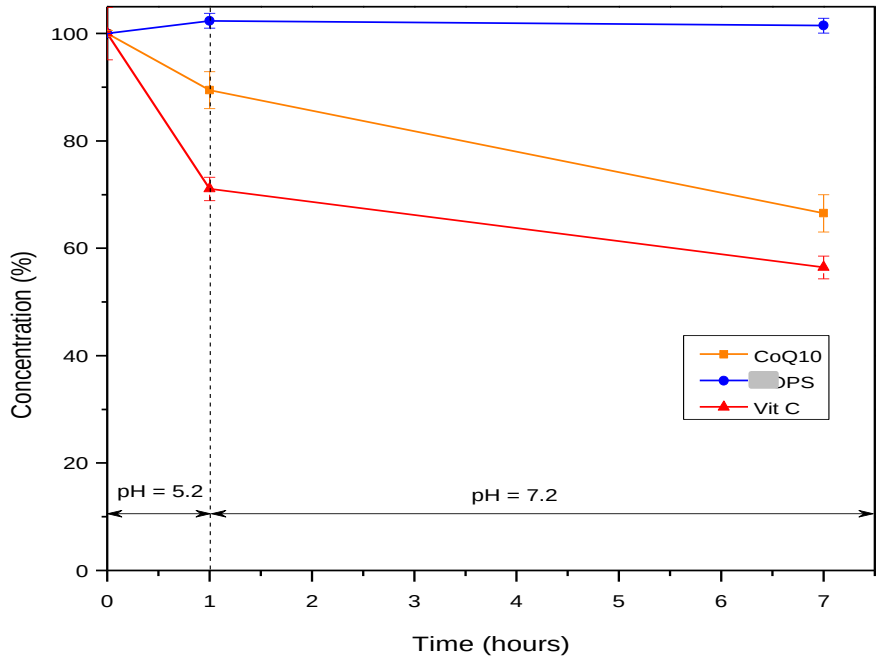


Figure 14. Stability of the different lipophilic actives encapsulated on the liposomal bilayer during the *in vitro* simulation of gastric digestion (n = 3).

4.3.5 *In vitro* cytotoxicity of liposomes to NIH-3T3 cells

The effect of active-loaded liposomes and empty liposomes on cell viability was test in NIH-3T3 human cells using XTT assay, at lipid doses ranging from 0.1 to 5 mM, for 24 hours (Figure 15).

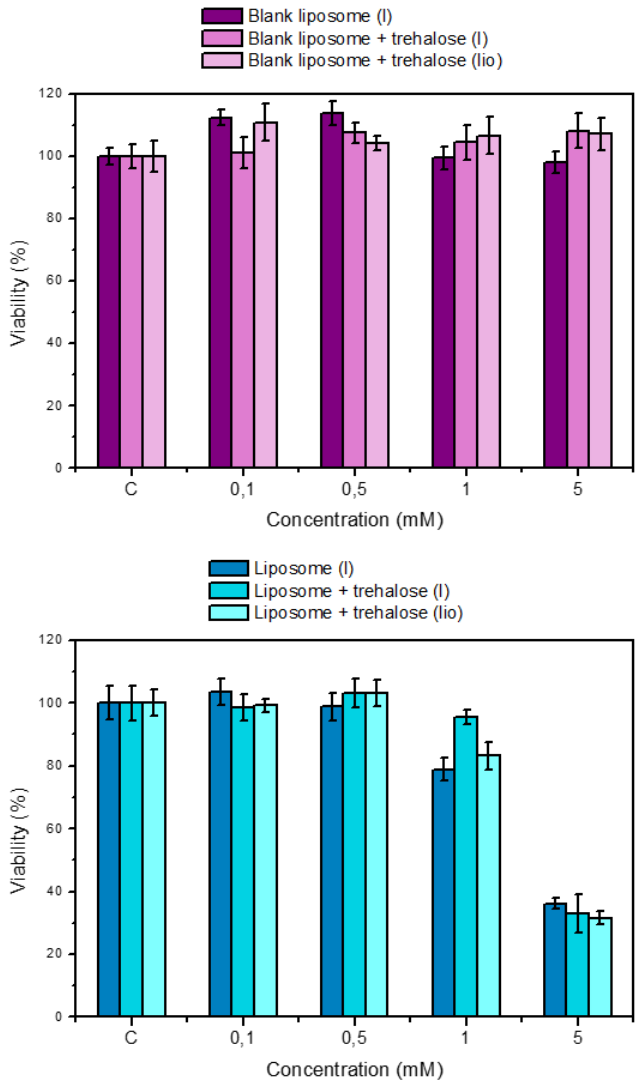


Figure 15. *In vitro* cytotoxicity of liposomes to human cells. Cell viability of NIH-3T3 cells after 24 hours incubation with empty and active loaded liposomes at various concentrations (0.1 to 5 mM). Untreated cells were used as controls (marked as C). Data represent the mean standard error of the mean (SEM) of three independent experiments. The darkest tonality corresponds to the liposomal solution without trehalose, middle dark tonality corresponds to the liposomal solution with trehalose in liquid state, and the lightest tonality corresponds to the liposomal solution with trehalose and lyophilized.

Empty liposomes showed little or no cytotoxicity, even at the highest dose (5 mM). On the other hand, the cytotoxicity of the active-loaded liposomes depended on the lipid concentration. Active-loaded liposomes exhibited little cytotoxicity except for the highest dose (5 mM) at 24 hours incubation, since this sample showed moderate toxicity to NIH-3T3 cells (36.12 ± 1.71 viability). Moreover, it was observed that the active concentrations do not interfere in the cell viability until 1 mM of lipid concentration. Similar results were obtained when the samples (empty or loaded) were in liquid state, with 9 % of trehalose cryoprotectant, or lyophilized with 9 % of trehalose.

4.3.6 *In vitro* bioadsorption and mucoadhesion study

Oral drug delivery needs to surpass an important challenge, which would be – among many others – the low residence time of the active substances at the site of action. From this point of view, liposomal delivery systems can be particularly useful, since they are reported to have the ability to adhere to the oral mucosa on the intestinal tract, providing a significant advantage as they may increase the effectiveness and bioavailability of the therapy[59]. Prolonged retention on the mucus surface in the gastrointestinal tract is of great value to oral delivery systems, and thanks to this, active-loaded liposomes fully interact with intestinal epithelial cells to then be transported into blood circulation. Therefore, adhesion to intestinal mucus is a key step for prolonging the residence time of drugs at the absorption site, thereby resulting in improved oral active delivery and intestinal absorption.

Here, the amount of mucin adsorbed by liposomes in a certain period of time was quantified to determine mucoadhesive properties[58-60]. Mucoadhesion efficacy of active-loaded liposomes was evaluated quantitatively by assaying the mucin absorbed amount at 37 ºC for 18 hours. Results showed that the 40.61 ± 6.23 % of the mucin in solution (0.24 mg/mL) was adhered to the liposomal bilayer, and that therefore, liposomes could theoretically bind to the intestinal mucosa *via* covalent bonds with the glycoproteins present in the mucus layer. In addition, it was observed that liposomes remain adhered to the intestinal mucus at least 18 hours, thus proving a prolonged residential time of liposomes in the intestinal track, and offering an efficient approach for active oral delivery.

4.3.7 *Ex vivo* permeation studies

Permeation study of liposome formulation was performed in vertical Franz diffusion cells, with fresh biological tissues mounted in 0.64 cm^2 diffusion membrane holders between the donor and the receptor compartment. [redacted]

[redacted]

A)

B)

Figure 16. Cumulative amounts of NAD[redacted] (A) and [redacted]PS (B) permeated through biological tissues from samples. Data represent mean ± SD (n= 5).

[redacted]

[redacted]

Figure 17. [redacted]

A)

C)

[redacted]

Table 8. Median values of retained amount (Q_T), flux (J) and permeability coefficient (K_p) from samples of raw active and active-loaded liposomes through abdominal pig tissues.

Parameters	Raw NADH	NADH@liposomes	Raw PS	PS@liposomes	Raw CoQ_{10}	CoQ_{10} @liposome

[a] Stadistically significant differents ($p<0.05$); [b] high statistically different ($p<0.01$); and [c] very high statistically different ($p<0.001$).

[redacted]

4.3.8 Lyophilization and stability of liposomes

With the goal of obtaining a stable product suitable for long-term storage, we evaluated the lyophilization of the liposome-encapsulated actives. Prior to lyophilization, different concentrations of cryoprotectant trehalose were added to the NADH aqueous media during the synthesis hydration step. Liposomes without cryoprotectant were also lyophilized as control. Lyophilization process was carried out at temperatures below the glass transition temperature (T_g) of liposomes, with or without cryoprotectant[74]. T_g values were previously determined by differential scanning calorimetry (DSC), obtaining a T_g [redacted]. [redacted]. Afterwards, each resulting powder was reconstituted by resuspension in aqueous media, and the size and ZP of th eliposomes were determined. Table 9 shows the mean particle size, PdI and ZP of both liposomal formulations after and before lyophilization at different trehalose concentrations, while their corresponding particle size distributions are represented in Figure 18. As seen, the values of mean particle size and PdI were consistently much lower when trehalose concentration was increasing, obtaining an optimal recovery of liposome morphology after rehydration. Under these conditions, the minimal particle size of liposomes (200.8 nm) and lower PdI (PdI = 0.183) were obtained at [redacted] % of trehalose, and no significant improvements were

achieved when higher concentration of cryoprotectant was used. A T_g value of [redacted] ºC of the liposomal suspension with [redacted] % trehalose was determined, indicating a [redacted] lyophilization temperature should be always kept below this value. Moreover, the overall morphology of the rehydrated liposomes was evaluated by Cryo-TEM. Figure 19B show that the unilamellarity of liposome morphology was maintained, although some degree of homogeneity was lost during lyophilization as confirmed by the PdI value (Table 9).

Table 9. Liposome characterization before and after lyophilization at different cryoprotectant concentrations.

Trehalose concentration (% w/v)	Before lyophilization			After lyophilization		
	Mean size (nm)	PdI	ζ Potential (mV)	Mean size (nm)	PdI	ζ Potential (mV)

Each value represents the average from three independent experiments ± the standard deviation.

Once the liposomes were lyophilized, the stability of the encapsulated and isolated actives was also studied (Table 10 and 11). To this end, they were stored at 4ºC and at room temperature, and their degradation was determined at different periods of time. As can be observed in Table 10, [redacted]

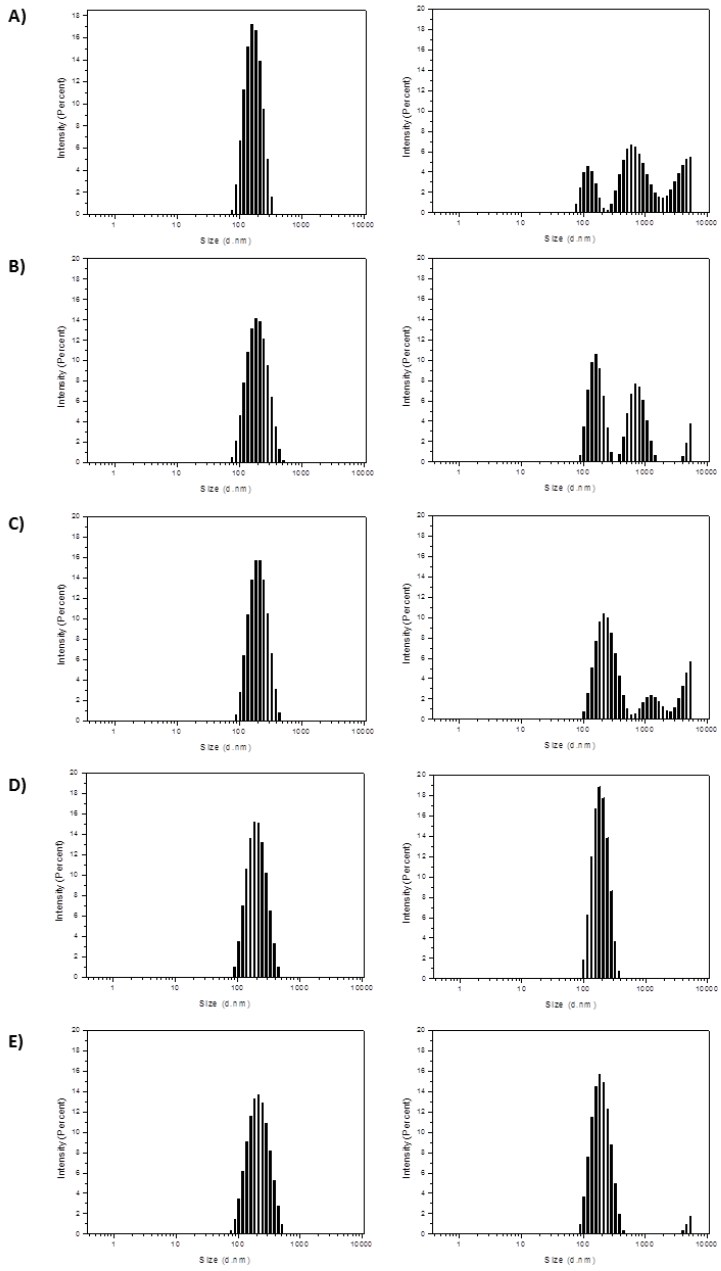


Figure 18. Particle size distributions of the final liposomal prototype before and after lyophilization plus rehydration at the different cryoprotectant concentrations. A) [redacted] % Trehalose. B) [redacted]% Trehalose. C) [redacted] % Trehalose. D)[redacted]% Trehalose. E) [redacted]% Trehalose.

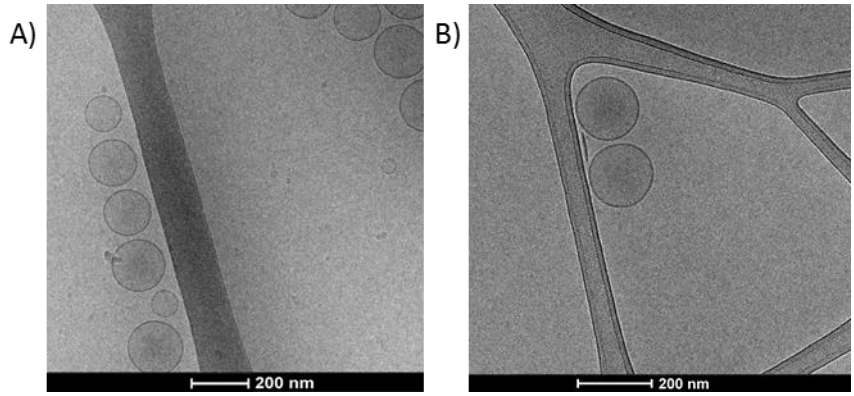


Figure 19. Cryo-TEM images of liposomes at [redacted] % w/v trehalose concentration: while A corresponds to the liposomal formulation right after its homogenization, B corresponds to the liposomes after their lyophilization and rehydration with water. Scale bars = 200 nm.

[redacted]

[redacted]

Table 10. Stability of encapsulated actives after lyophilization upon time on the liposomal suspension with [redacted] % trehalose (n = 3).

		1 month	3 months	6 months	9 months	15 months
NADH	4ºC					
	25ºC					
VITAMIN C	4ºC					
	25ºC					
COQ10	4ºC					
	25ºC					
DOPS	4ºC					
	25ºC					

Table 11. Stability of isolated actives upon time (n = 3).

		1 month	3 months	6 months	9 months	15 months
NADH	4ºC					
	25ºC					
VITAMIN C	4ºC					
	25ºC					
COQ10	4ºC					
	25ºC					
DOPS	4ºC					
	25ºC					

[redacted]

Table 12. Stability of encapsulated actives after lyophilization upon time on the liposomal suspension without any addition of trehalose (n = 3).

		1 month	3 months	6 months	9 months	15 months
NADH	4ºC					
	25ºC					
VITAMIN C	4ºC					
	25ºC					
COQ10	4ºC					
	25ºC					
DOPS	4ºC					
	25ºC					

[redacted]

Table 13. Stability of encapsulated actives after lyophilization upon time on the liposomal suspension with [redacted] % trehalose but without PA (n = 3).

		1 month	3 months	6 months	9 months	15 months
NADH	**4ºC**					
	25ºC					
COQ10	**4ºC**					
	25ºC					
DOPS	**4ºC**					
	25ºC					

[redacted]

[redacted]

Table 14. Stability of encapsulated actives after lyophilization upon time on the liposomal suspension with [redacted] % trehalose but without PA nor CoQ10 (n = 3).

		1 month	3 months	6 months	9 months	15 months
NADH	4ºC					
	25ºC					
DOPS	4ºC					
	25ºC					

According to this, this last liposomal prototype was also characterized in terms of size, ZP and encapsulation efficiency of the three active substances (Table 15). As expected, Cryo-TEM images confirmed the formation of small unilamellar vesicles (Figure 20). Similarly, DLS measurements demonstrated that there are not relevant changes on the size of the liposomes (mean size: 185.1 ± 2.9 nm), and on the negative charge (-45 ± 0.8 mV). [redacted].

Table 15. Liposomal characterization on the final product formula without PA.

Mean Size (d.nm)	PdI	ζ Potential (mV)	
185.1 ± 2.9	0.09 ± 0.0	- 45.5 ± 0.8	

Each value represents the average from three independent experiments ± the standard deviation.

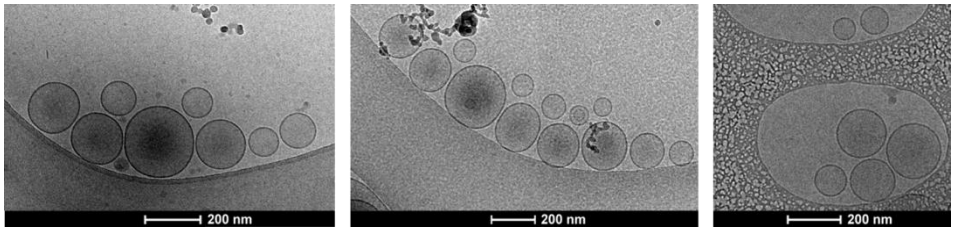


Figure 20. Cryo-TEM images of liposomes at [redacted] % w/v trehalose concentration without PA.

4.3.9 Potential dosage forms

Once the final formulation was decided, we also analyzed the potential final dosage forms. To this end, we end up with three possible forms that would directly use the lyophilized liposomal powder. This three potential usage forms are:

- Sachets: Small bag containing the lyophilized liposomal powder that should be opened and mixed with water right before its intake (Figure 21A).
- Hard capsules: Dosage form consisting in two parts (composed by, for example, gelatin manufactured from collagen, or hypromellose), where the two halves get separated to fill them with powder and closed back afterwards (Figure 21B). The lyophilized liposomal powder may directly fill the capsules in the desired concentration for the final product, and one or more pills could be taken at the same time.
- Extemporaneous solutions: Glass vial where the lyophilized liposomal powder is entrapped at the desired concentration. Right before its intake, the vial should be opened and filled with a determined quantity of water (Figure 21C). An

alternative from the standard extemporaneous solutions would be a vial where the powder and the aqueous solutions are both contained, but separately. The powder would be entrapped on the vial cap, and when unscrewing the vial, the separation breaks and gets mixed with the aqueous solution (Figure 21D).

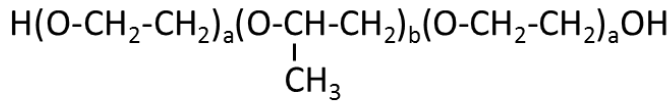
Figure 21. Potential final dosage forms. A) Sachets. B) Hard capsules. C) Glass vial for extemporaneous solution. A mark should be printed on the glass to know until where should be filled with water. D) Extemporaneous solution flask. The powder is contained on the cap, and at its opening breaks and gets mixed with the aqueous solution.

In addition to these three potential usage forms, there is also the possibility to fabricate a sublingual dosage formulation. To this end, the liposomal formula should be mixed with polaxamer 407 or alginate. Both copolymers have the common ability of converting liquid in gel solutions, but with a very different mechanism.

Polaxamer 407 is a thermoreversible non-ionic triblock copolymer consisting on a central block of propylene glycol surrounded by two blocks of polyethylene glycol[82] (Figure 22A). Depending on the added concentration of the polymer to the solution, and the temperature of that solution, the viscosity will highly variate (Figure 22B). Therefore, a 20 % polaxamer 407 liposomal solution was formulated, expecting it to be very

liquid at 25 ºC, and solid/gelled at its contact with the sublingual cavity at 37 ºC (corporal body temperature). To study this expected behavior, viscosities were measured by triplicate at 25 ºC and at 37 ºC on the same sample during 30 seconds, with the aim of understanding if there was an increase on viscosity and if this increase was reversible on a reduction of temperature. Results showed a perfectly reversible increase on almost the double of viscosities, from 0.126 ± 0.001 Pa·S at 25 ºC with a very liquid appearance, to 0.206 ± 0.005 Pa·S at 37 ºC with gelled characteristics (Figure 23). Almost inappreciable standard deviations showed no significant differences on viscosities from the first and the third measurement, demonstrating the reversible behavior from liquid to gel.

A



B

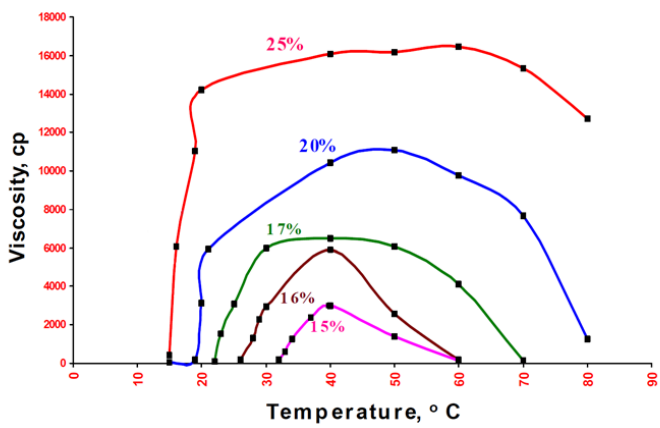


Figure 22. Polaxamer 407. A) Chemical structure of polaxamer 407 (a, ethylene oxide; b, propylene oxide). B) Graphical representation of polaxamer 407 viscosity in function of temperature at different concentrations, retrieved from BASF with some modifications.

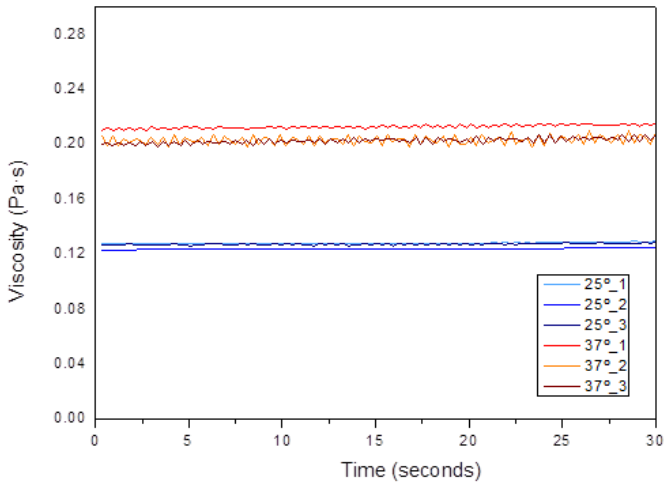


Figure 23. Graphical representation of viscosities on time on the two tested temperatures, 25 ºC and 37 ºC.

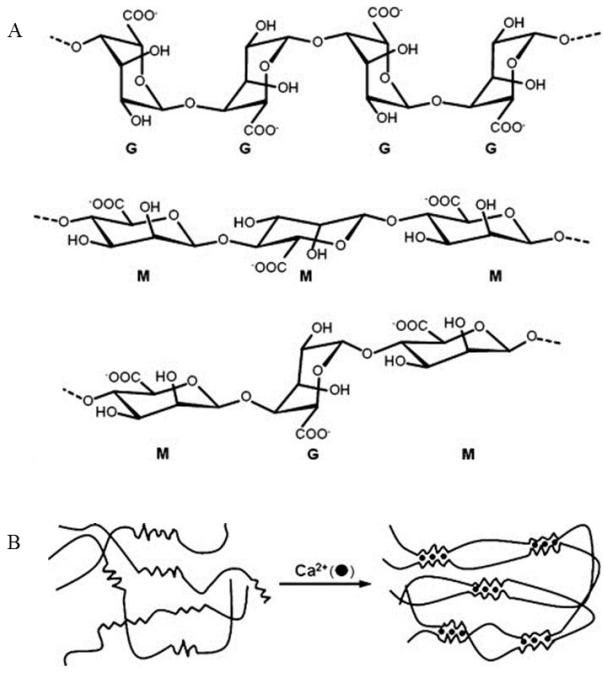


Figure 24. Sodium alginate. A) Chemical structures of a guluronate-block, a mannuronate-block, and alternating block in alginate[77]. B) Egg-box-like structure formation.

Alginate is an anionic block copolymer extensively used in biomedicine, which is composed mostly of L-guluronate to D-mannuronate[83] (Figure 24A). This polymer has the particularity of forming hydrogel solutions when in contact with divalent cations such as Ca^{2+}. The divalent cations solely bind to the guluronate blocks, which ionically cross-links with guluronate blocks of an adjacent chain, thus forming the termed egg-box model of cross-linking (Figure 24B).

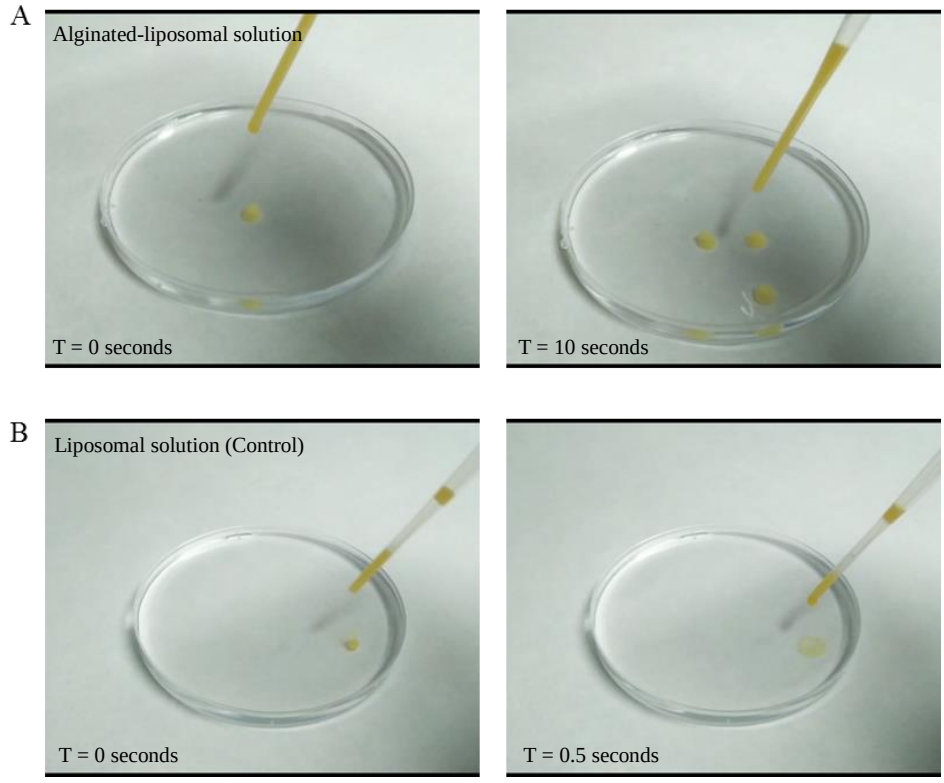


Figure 25. A) Screenshots from the dropwise addition of the alginated-liposomal solution to the saliva simulation. The first screenshot was taken right after the drop touched the solution, while the second screenshot is 10 seconds later. B) Screenshots from the dropwise addition of the normal liposomal solution to the saliva simulation. The first screenshot was taken right after the drop touched the solution, while the second screenshot is half a second later, approximately.

Alginate-liposomal solutions were prepared and added dropwise to the simulated saliva solution rich in Ca^{2+} and Mg^{2+}. A normal liposomal solution was also added dropwise as a control and videos were recorded. In Figure 25 different screenshots from the video can be observed, where the different evolution of the alginate-liposomal drops and the normal liposomal drops can be compared. A geling behavior can be appreciated with the addition of alginate, thus confirming the potential for a sublingual dosage form.

4.4 CONCLUSION

It has been designed an optimal vehicle for oral delivery of antioxidant actives (NAD[redacted], CoQ10, [redacted]PS and vitamin C) based on negatively charged and nanosized liposomes. The liposomal formulation was formed by small unilamellar vesicles with high encapsulation yield of hydrophobic antioxidants (CoQ10, [redacted]PS and vitamin C) and the hydrophilic NAD[redacted] actives. The *in vitro* release studies confirmed the capacity of the vehicle to release the hydrophilic NAD[redacted] efficaciously to the body in an adequate range of time. In this line, liposomal protection towards the core-encapsulated actives was tested and the resistance conferred by the bilayer was demonstrated. Additionally, in front of the simulated human stomach conditions, NAD[redacted] exhibited a significant improvement in stability in comparison to the non-encapsulated NAD[redacted]. *In vitro* bioadsorption studies with mucin showed that liposomes provide prolonged residential time upon oral administration and may remain adhered to the intestinal mucus at least 18 hours, thus offering an efficient approach for an optimal active oral delivery. [redacted]

[redacted]. Active-loaded liposomes exhibited little cytotoxicity [redacted] In addition, this last prototype was characterized and showed very equivalent characteristics with no relevant changes. Lastly, some potential dosage forms were suggested for the final human administration of the liposomal cocktail.

Thus, given the fact that these liposomes, once lyophilized, can be easily stored for long periods of time without losing their efficacy, and the promising results obtained with liposomes on the different experiments, we are confident that this approach will ultimately provide fruitful improvements in CFS treatment.

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CHAPTER 5. Coencapsulation of NAD[redacted], vitamin C, phosphatidylserine and coenzyme Q10 in homogeneous giant unilamellar vesicle liposomes using microfluidics.

ABSTRACT

In this chapter, we show the straightforward and effective production of a Giant Unilamellar Vesicle (GUV) liposomal cocktail using microfluidics. This cocktail contains the four actives, reduced nicotinamide adenine dinucleotide (NAD[redacted]), phosphatidylserine ([redacted]PS), vitamin C and coenzyme Q10 (CoQ10), against chronic fatigue syndrome (CFS). Starting from the formation of double emulsions, homogeneous and stable GUVs are efficiently formed and characterized.

5.1 INTRODUCTION

Liposomal formulations have been widely studied and commercialized during the past decades due to the multiple benefits they provide to drugs[1-4]. It has been extensively reported that they can improve effectiveness and bioavailability on encapsulated drugs. In addition, they can protect from degradation and enhance the retention time on the body[5-10]. However, liposomal production has still some weak points that have the necessity to be improved and remain unsolved until the date. Usually, low encapsulation efficiencies are reported for the hydrophilic drugs on its core, being rarely above the 30 %[11-14]. Also, after their formation with traditional methods like the thin-film or the ethanol injection, additional steps need to be carried out to allow the homogenization of the vesicles and to control their final desired size[15-17].

In this context, glass microcapillary devices comprising hydrodynamic focusing of coaxial flow is an emerging field that allows the efficient, one-step formation of homogeneous liposomal vesicles, also with an approximate encapsulation efficiency of 100 % for hydrophilic drugs. Thus, this method is a promising tool on the field of drug encapsulation and delivery[18-21]. Immiscible fluids pumped through different coaxial regions are forced through the collection tube and form emulsion drops with controllable size and structure. Due to the chemical resistance of glass to most acids, bases, and organic solvents, microcapillary devices are particularly suitable for producing a variety of emulsion drops that act as templates for micro-materials, such as particles, capsules, and vesicles[22-27]. These materials have wide applications in many fields

including pharmaceutics[28,29], cosmetics[30,31], food industry[32,33], agriculture[34,34], and tissue engineering[36,37].

Although quick advances are being undergone to scale-up this technique[38-40], reported liposomes generated by microfluidics are to date usually very simple in formula and very few encapsulation cocktails of drugs have been described. In this chapter, we successfully optimized the production of a GUV liposomal cocktail of four active substances (NAD[redacted], CoQ10, [redacted]PS and vitamin C) against chronic fatigue syndrome (CFS) with the use of microfluidic techniques. The optimized procedure allows the simultaneous and complete encapsulation of the four actives in homogeneous GUVs.

5.2 MATERIALS AND METHODS

5.2.1 Materials and chemical reagents

6-O-palmitoyl-L-ascorbic acid (PA or vitamin C, analytical standard ≥ 99.0 %), cholesterol-(polyethylene glycol-600) (Chol-PEG), cholesterol (Chol), α-Hemolysin from Staphilococcus aureus (α-HL), and trimethoxy(octadecyl)silane were obtained from [redacted]. Phosphatidylserine (PS, concretely [redacted]PS, analytical standard ≥ 99.0 %) and phosphatidylcholine (PC, concretely [redacted]PC) were purchased from [redacted]. Coenzyme Q10 (CoQ10, United States Pharmacopeia (USP) reference standard quality ≥ 99.0 %) was obtained from [redacted]. 2-[methoxypoly(ethylenoxy)6-9propyl]dimethylmethoxysilane was purchased from [redacted]

[redacted]. Lissamine™ Rhodamine B (rhodamine DHPE) and Invitrogen™ Dil Stain (DiI) were purchased at [redacted], while naphto[2,3-a]pyrene was obtained from [redacted].

5.2.2 Glass capillary devices

Two different microcapillary devices were fabricated and used to produce the GUV liposomes. The structure of the first fabricated glass capillary devices is schematized in Figure 1. Essentially, it is formed by three cylindrical tapered capillaries with an outer diameter (OD) of 1.00 mm; all three of them stretched with a Flaming-brown micropipette puller (model P-1000, Sutter Instrument, U.S.A.) and sanded on the tip with sand paper or ceramic tile (Sutter Instrument, U.S.A.) to crop the taper tip and achieve the desired size. One of them was inserted into another (approximately 120 μm OD tip) to allow the flow of both the inner and middle phase, and inserted on one end of a borosilicated square tube with an inner diameter (ID) and OD of 1.05 mm and 1.5 mm through where the outer phase would pass by. On the other end of the borosilicated square tube, the third capillary of OD approximately 350 μm tip was inserted to allow the collection of the double emulsions. All the capillaries were carefully fixed onto a glass slide with 5-minutes epoxy, while making sure the tips were aligned and separated around 100 μm from each other. The round capillary tubes were coaxially assembled and aligned very carefully under a microscope.

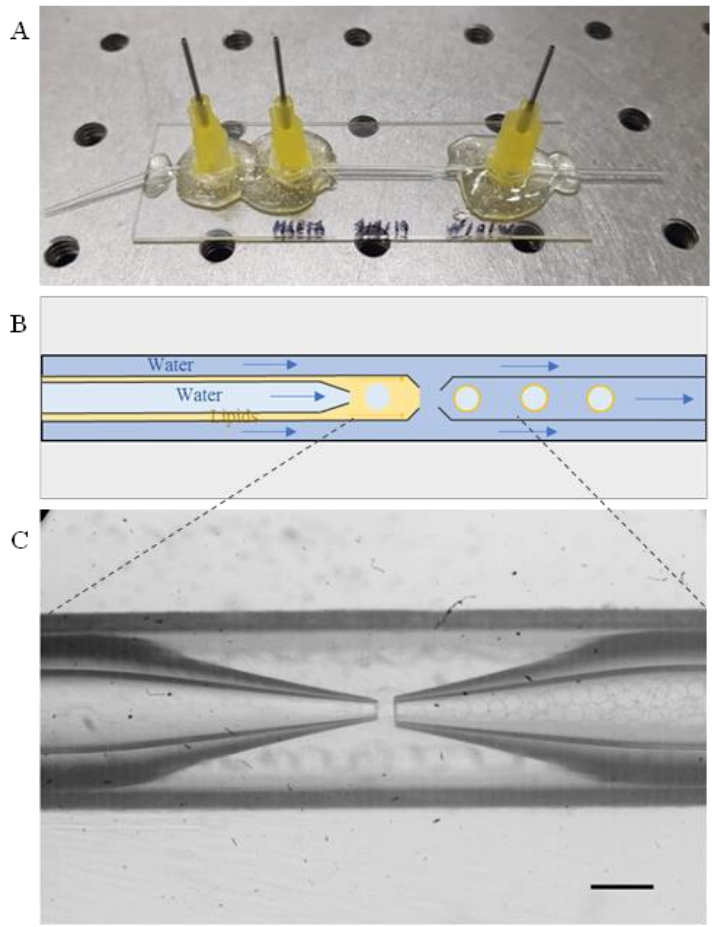

Figure 1. A) Photography of the first fabricated glass capillary device. B) Corresponding graphical representation of this microfluidics device. C) Detail of the double emulsion formation. Scale bar = 500 µm.

The inner and collecting tubes were treated with 2-[methoxypoly(ethylenoxy)6-9propyl]dimethylmethoxysilane to render the surface of the capillary hydrophilic, and the middle-phase tube was treated with trimethoxy(octadecyl)silane in toluene at 300 mM to make the surface hydrophobic.

Once the structure of the first device was assembled, three needles were attached to the connections of each capillary and fixed with 5-minutes epoxy, through which the different phases would be connected to make the flows possible.

For the second type of device, we employed a design developed by Jiang K. *et al.*[41] (Figure 2). The main innovation of the design is the assembly of the tapered capillary tubes, in a coaxially structure of different cross-sectional shapes and with 45º of rotation between the squared tubes. During the assembling, the tubes were automatically locked on the touch of the immediate outer one without blocking the flow channel in between them, since there were only four touch points between the capillaries. Fluids flow through the different coaxial regions towards the same direction in an organized and effective way, without the need of assembling with a microscope as with the previous device in a very patient and meticulous way. This fact makes this capillary-based technology a tremendous potential for a scaling production.

In this second device, a squared capillary with an ID of 0.4 mm and an OD of 0.8 mm was inserted onto another squared capillary (ID of 1.05 mm and an OD of 1.5mm) with a 45º rotation after burning, stretching and sanding their tips, where inner and middle phase would flow through (Figure 2). Then, both capillaries were inserted on one end of a squared borosilicated tube of OD 2.7 µm and ID 2.1 µm, a tube which previously had been stretched on its central part with a Flaming-brown micropipette puller. The constrictive throat on the outermost tube had a more or less 350 µm dimension, and the orifices of the middle and innermost tubes were approximately 350 µm and 150 µm, respectively. Every capillary

was meticulously assembled with 5-minutes Epoxy so the tips were aligned and separated of 200 µm from each other, close to the central stretched part of the squared tube.

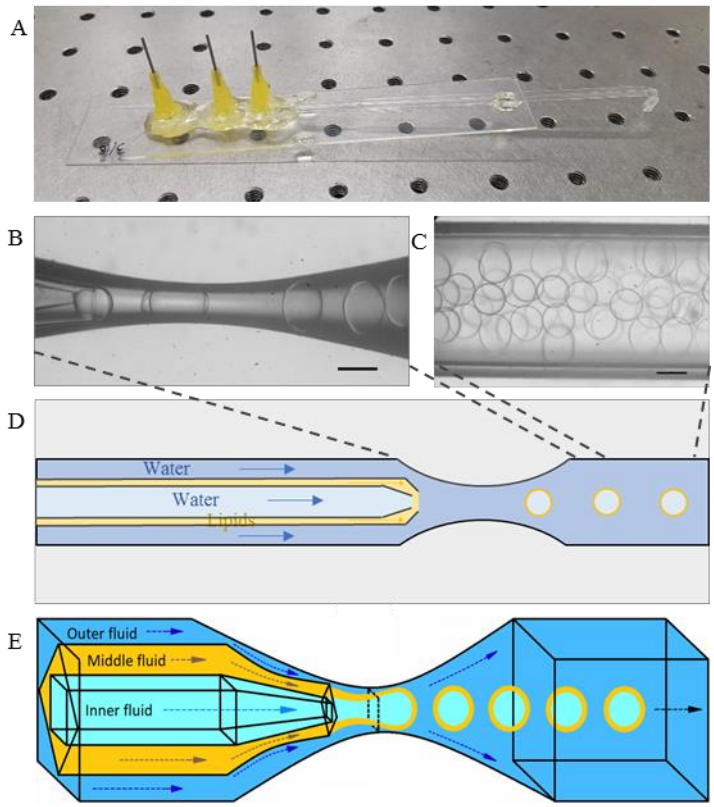

Figure 2. A) Photograph of the second glass capillary device. B) Detail of the double emulsion formation: Screenshot from a recorded video. C) Already formed double emulsions eluding through the glass capillary. D) Graphical representation of the microfluidics device used to form the water-in-oil-in-water double emulsions. E) 3-D graphical representation of the double-emulsion device, where the rotational angles of the three squared capillaries can be appreciated[47]. Scale bars = 500 µm.

The inner and squared tubes were treated with 2-[methoxypoly(ethylenoxy)6-9propyl]dimethylmethoxysilane to render the surface of the capillary hydrophilic, and the middle-phase tube was

treated with trimethoxy(octadecyl)silane to make the surface hydrophobic. Afterwards, three needles were attached to the connections of each capillary and fixed with more 5-minutes epoxy, through which the different phases would be connected to make the flows possible.

5.2.3 Phase compositions

For the formation of the double emulsions, the employed formula was the final cocktail liposomal formula against chronic fatigue syndrome, previously described in *Chapter 4*, but with little changes. The inner phase used was a solution of the active ingredient NAD[redacted] at 2.5 mg/mL, with an 8.5 % w/w of polyvinyl alcohol (PVA) to enhance the stability of the vesicles. The middle organic phase was a 4:7 ratio of chloroform and hexane respectively. The molar ratio of lipids and lipid active substances was adjusted at a 7.7 mM concentration: the employed structural lipids were [redacted] mg/mL of [redacted]PS, [redacted] mg/mL of [redacted]PC, [redacted] mg/mL of Chol and [redacted] mg/mL of Chol-PEG, while 0.5 mg/mL of vitamin C and 0.6 mg/mL of CoQ10 were the concentrations of both active substances. Different fluorescent dyes were also added to the middle phase depending on the experiment. Lastly, the outer phase was a 10 % w/w PVA solution.

5.2.4 Characterization and imaging of GUVs

The formation of the double emulsions was recorded using a high-speed camera (Miro 320, Phantom Vision Research Inc., U.S.A.) connected to an inverted microscope (Nikon Eclipse Ts2, Nikon Instruments Inc., Japan). Once formed the double emulsions, a collector chamber with a sucrose solution of 34.2 mg/mL was used to collect the vesicles for its observation with microscopic techniques. Osmolality on the collector

solution was measured and adjusted to be equal to the one on the inner phase, hence ensuring the stability of the collected vesicles. Both optical and confocal fluorescent observation was carried out. For confocal observation (ZEISS LSM 800, Carl Zeiss AG, Germany), a 10x objective was employed and different lasers were selected for different imaging depending on the dye. The NAD[redacted] active ingredient on the inner phase conferred fluorescence on DAPI laser excitation at 405 nm. For the middle phase, three different dyes were employed with different lasers: DiI and DHPE Rhodamine dyes were observed using a 543 nm laser, whereas a 458 nm laser was employed for naphto[2,3-a]pyrene.

5.2.5 Phase separation of lipids

To detect phase separation of lipids upon the formation of lipid bilayers, 30 µg/mL of a rhodamine DHPE were added at the middle phase lipid blend. After the GUVs formation and collection, fluorescent confocal imaging was performed looking for sections with asymmetrical fluorescence in the membrane.

5.2.6 Unilamellarity of GUVs

An α-hemolysin (α-HL) concentration of 2 µM was added to the inner phase and the double emulsion formation was carried out as previously described. Liposomes were collected on the sucrose solution and the fluorescence variations of the core-encapsulated active ingredient NAD[redacted] was followed on time during 80 minutes on both vesicles with and without α-HL.

5.3 RESULTS AND DISCUSSION

5.3.1 Phase compositions

After the meticulous assembling of both devices, GUV formation were assayed with successful results using both devices, although significant differences were observed in the resulting vesicular suspensions collected. On the first device, although the vesicles seemed to form in a very stable and homogeneous way once the correct flows were selected, the formation of double emulsions was less efficient since single emulsions were also formed in an intermittent way. This would additionally entail the need of one more step for separating single and double emulsions from the collected suspension.

On the contrary, with the second device, the formation of double emulsions was seen to be continuous and homogeneous under optimized conditions. Table 2 shows some of the studied ratios for this optimization.

Table 2. Summary of the applied inner, middle and outer flow rates.

Inner Flow Rate (µL/minute)	**Middle Flow Rate (µL/minute)**	**Outer Flow Rate (µL/minute)**	**Result**
800	200	3000	Homogeneous
1500	200	3000	Breakage and non-correct formation
800	500	3000	Middle phase too thick
200	200	3000	Formation of both single and double emulsions
500	200	3000	Middle phase too thick
800	200	1000	Breakage and non-correct formation

We found that the optimum flow ratio was 800/200/3000 µL/minute for inner, middle and outer phase, respectively. On the contrary, other flow

ratio combinations produced a bad formation of the vesicles, insufficient homogeneity, or the vesicles were not formed at all. Specifically, when the middle flow rate was higher to 200 µL/minute, too thick vesicular walls were being formed due to an excessively large flux of middle phase per second. In a similar way, when the inner flow rate was inferior to 800 µL/minute, too thick vesicular walls were also being formed or even single emulsion drops were being produced. If the selected inner flow rate was much higher than 800 µL/minute, the formed vesicles broke, were formed in an unappropriated way or not formed at all. This happens due to the lack of enough middle phase to cover the inner phase, or to the formation of a continuous flow of the inner phase that produces a jet-like injection of the solution, disabling the possibility of forming double emulsions. Therefore, a correct inner/middle flow ratio is an essential parameter for the optimal formation of double emulsions. Additionally, different outer flow rates were studied and assayed. When the outer flow was too slow (< 2500 µL/minute, approximately), vesicles were not getting properly formed. On the contrary, it was found out that a higher (inner/outer)/outer ratio (quick outer flow rate) could be employed without affecting significantly the formation of the vesicles.

Due to the large dimension of the outer tube and the relatively small flow rates, the produced drops could stay within the channel for 5 – 10 minutes before leaving the channel. During this period, chloroform diffuses away from the middle phase, triggering the dewetting of the thin shell and thus, the formation of liposomal vesicles.

5.3.2 Characterization and imaging of GUVs

GUVs synthesized under optimized conditions were characterized by both inverted and confocal microscopy. To this end, using the optimized conditions, the vesicles were formed adding DiI red fluorophore (30 μg/mL) in the middle phase to integrate it in the membrane. In this sample, NAD was encapsulated in the core of the GUVs as the active ingredient. Figure 3 shows both inverted and confocal microscopy of the resulting vesicles. In these images, we clearly observed the successful formation of the GUVs and the encapsulation of the blue fluorescent NAD[67] into them.

Additionally, the size distribution of the formed GUVs was calculated with the diameter measurement of n = 50 collected liposomes, resulting in a mean average size of 289.0 ± 5 r. μm (Figure 3D). Finally, stability of these GUVs was evaluated after storing them one month. Remarkably, optical images of stored GUVs did not show significant differences, either on the shape or size distribution.

5.3.3 Phase separation of lipids

Once the organic solvents on the middle phase of the double emulsions evaporate, the GUVs are formed and the lipids get distributed along the vesicles forming a bilayer membrane. It is expected that lipids on the membrane get localized asymmetrically after its assembling, with the formation of different sections on the bilayer depending on the molecular structure and composition[42-44].

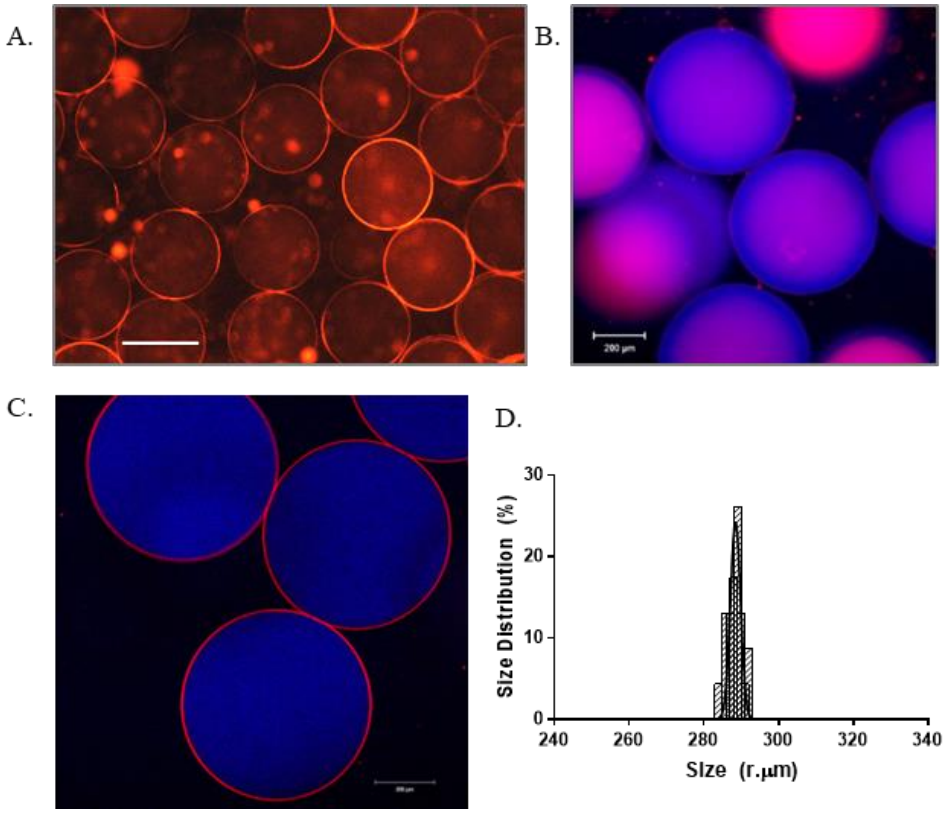

Figure 3. A) Optical microscopy imaging of the resulting liposomal suspension: fluorescent bilayers can be observed in red (DiI dye). Scale bar = 500 µm. B) and C) Confocal microscopy imaging of the liposomal suspension: bilayers can be observed in red (DiI), while the NAD[redacted] active substance perfectly encapsulated can be appreciated in the blue fluorescence. Scale bar = 200 µm. C) Particle size distribution of the liposomal vesicles, with a mean size of 289.0 ± 5 r. µm.

To confirm this hypothesis, we added the green naphto[2,3-a]pyrene dye in the middle phase to integrate it in the membrane of GUVs. Green naphto[2,3-a]pyrene is a fluorescent dye that selectively associates with the lipid ordered domains[45]. As the solvent of the double emulsion shell evaporates and the GUVs get formed, the lipids adsorbed to the interfaces between the inner core and the outer phase organize in ordered and

disordered lipid microdomains over the GUV surface. Figure 4 shows the imaging of one GUV on different perspectives, where the asymmetries on lipid distribution are appreciated. This phase separation demonstrated our proposed bilayer assembly and therefore, the correct GUV formation after the evaporation of organic solvents from the middle phase.

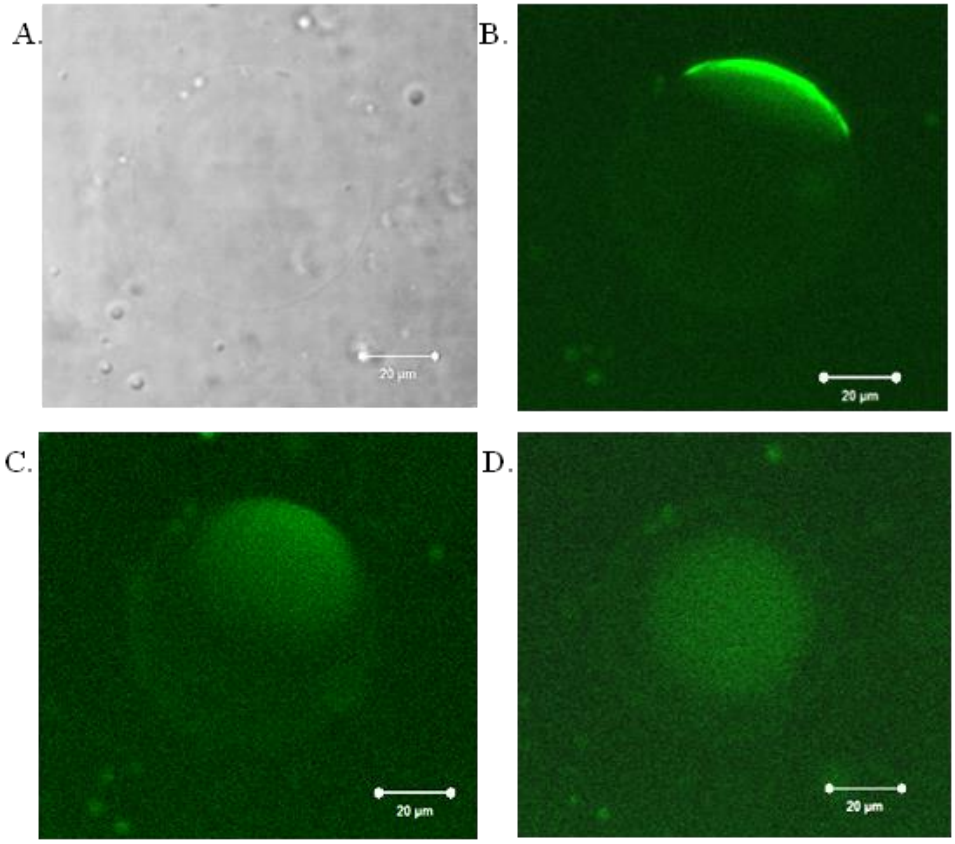

Figure 4. Confocal imaging evidencing the phase separation of the lipids in already dewetted liposomes, as shown by the naphto[2,3-a]pyrene green dye. The first image (A) shows the liposome in the brightfield, while the rest (B – D) show the fluorescence of the same liposome observed from different perspectives. Scale bars = 20 µm.

5.3.4 Unilamellarity of GUVs

After the double emulsion formation, the organic solvent of the middle phase would slowly experiment dewetting until the formation of a unilamellar bilayer of lipids on the vesicle, thus converting the double emulsions in GUVs. To demonstrate the unilamellarity of the vesicles, the pore-forming protein α-Hemolysin (α-HL) was incorporated in the bilayer as it only gets attached to bilayered membranes[46,47]. Therefore, if the double emulsion drops transform to unilamellar vesicles, the α-HL in the core will be incorporated in the bilayer and form nanopores serving as transport channels of relatively small molecules, such as NAD, across the membrane.

Once GUVs were formed with the addition of pore-forming protein α-HL, the relative fluorescence on the core of the vesicles was measured and imaged on every minute during 80 minutes (Figure 5). The same procedure was carried out on a control liposome without the protein, and the differences were graphically represented. After 80 minutes, NAD fluorescence decreased a 60.4 ± 3.9 % on the α-HL GUV, while no significant differences were observed after the 80 minutes on the control in comparison with the time = 0. This indicates the existence of nanopores on the vesicle membranes with α-HL added, thus confirming the structure of a single bilayer of lipids and the perfect formation of GUV vesicles.

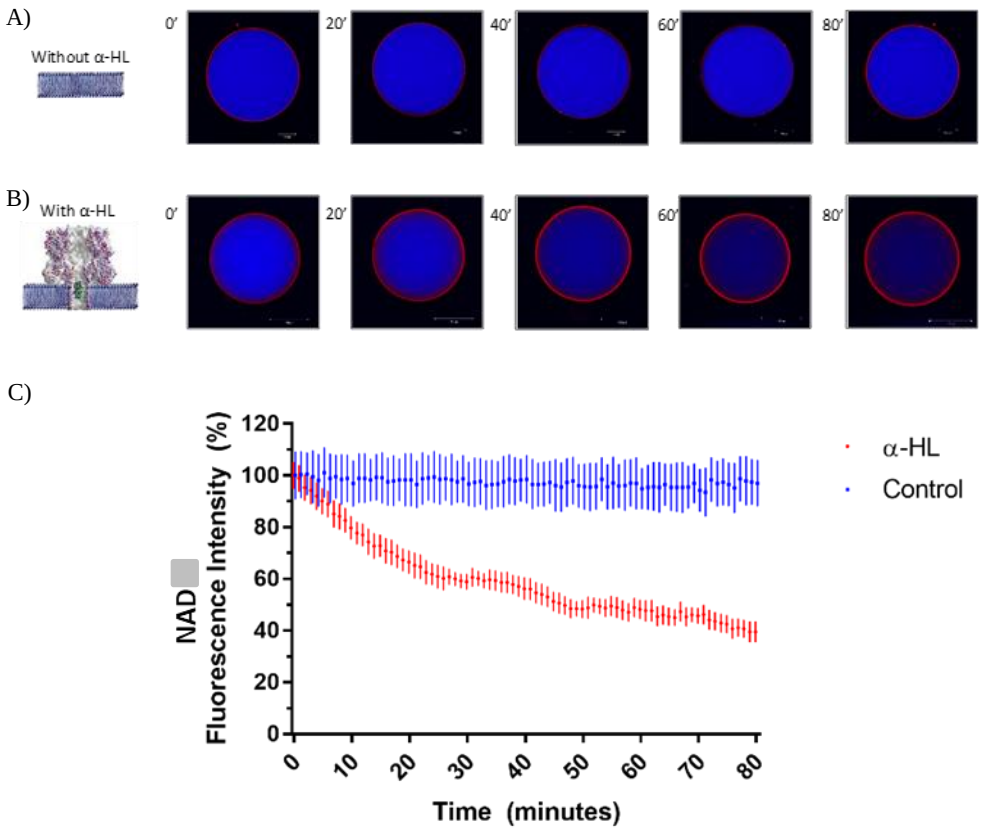

Figure 5. A) Confocal images of a liposome without α-HL on the bilayer (control) through 80 minutes of time, with the bilayer in red (DiI dye) and the core in blue (NAD[redacted]): the inner fluorescence of the NAD[redacted] can be appreciated being constant on the time. B) Confocal images of a liposome with α-HL incorporated on the bilayer through 80 minutes of time: inner fluorescence of NAD[redacted] is appreciatively decreased on each time. Scale bars = 100 µm. C) Graphic representation of the relative NAD[redacted] fluorescence on the core of the liposome through time in presence or absence of α-HL protein: NAD[redacted] fluorescence decreased a 60.4 ± 3.9 % after 80 minutes with α-HL, while no significant differences are observed without it.

5.4 CONCLUSIONS

We successfully produced a stable GUV liposomal cocktail of NAD[redacted], vitamin C, [redacted]PS and CoQ10 tuning up microfluidic techniques. The

synthesized GUVs have a homogenous size distribution and high encapsulation yield of the active substances, thus giving interesting insights towards the drug delivery of active ingredients against CFS. Liposomes were efficiently formed and characterized with microscopical imaging. Additionally, we demonstrated its unilamellarity by the proper incorporation of α-HL protein on its membrane, and lipid separation sections on the membrane were also imaged and reported. We believe this approach may be useful in constructing functional delivery systems for therapeutic applications on CFS.

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CHAPTER 6. Scale-up of the liposomal formula containing coenzyme Q10, NAD and phosphatidylserine for commercial purposes on the food supplement's industry

ABSTRACT

Scale-up studies were carried out on the final optimized liposomal formulation selected and studied in *Chapter 4*. The proposed protocol for a future scale-up production of the formulation includes three steps: i) the formation of multilamellar vesicles (MLV) through the thin-film method with the help of an industrial-scale rotary evaporator; ii) homogenization step with the use of Microfluidizer® processors; and iii) lyophilization for long-term stabilities with an industrial-scale freeze dryer.

6.1 INTRODUCTION

Although liposomes have been known since it discovery in the 60s, it was not until the late nineties when the commercialization of this vesicular encapsulation system started. The first liposomal encapsulated drug introduced in the market was distributed under the name "AmBisome" by Vestar[1]. Nowadays, several drugs are available under a liposomal encapsulated presentation[2,3], and the number of commercialized products is only increasing, with also its lately appearance in other fields such as the cosmetic industry or food supplement industry.

The most widely employed and traditional methodologies for the production of liposomes have been reviewed in *Chapter 1.7.3*, although novel strategies have been reported and proposed during the last years. A very complete review by C. Has *et al*.[4] summarizes the most recent described methodologies. It is important to understand that every methodology has key parameters that may be decisive for a correct selection of the most suitable methodology on any particular encapsulation. Among others, the exposition to high temperatures, rapid and extreme pH changes or the employment of organic solvents are differences between the methodologies that would make one or other process more appropriate for a particular case.

In *Chapter 4*, an optimized liposomal prototype was selected as a great candidate for its scaling-up with industrial purposes. The main aim of this chapter is to study the viability for the industrial production in bigger batches of the designed and optimized prototype of the liposomal cocktail.

Due to the known sensitivity of the actives on this final prototype ([redacted] nicotinamide adenine dinucleotide (NAD[redacted]) is particularly sensitive to high temperatures or pH conditions), some methodologies can be discarded from the very beginning. [redacted]

6.2 METHODS, RESULTS AND DISCUSSION

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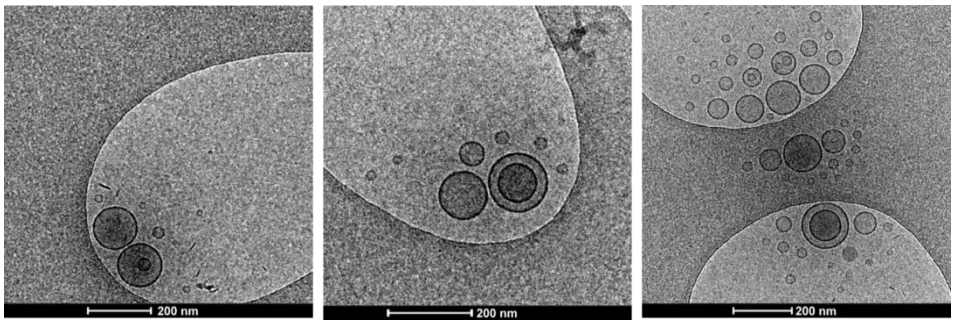
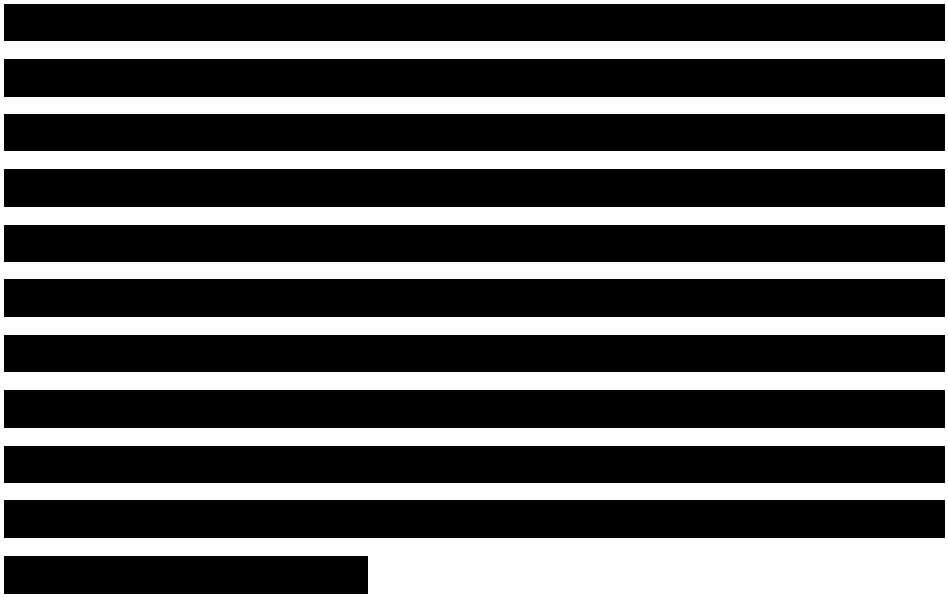


Figure 6. Cryo-TEM images of the liposomal suspension [redacted]. Scale bar = 200 nm



[redacted]

[redacted]

[redacted]

6.2.4 Equipment Qualification

To ensure the obtained product and data from a new equipment is consistent and reliable, an equipment qualification (EQ) must be performed before its normal use[12-15]. The EQ is the recompilation of

documented evidences to demonstrate the suitability of an instrument for its normal use, therefore providing robustness to the obtained data/product.

The EQ is formed by four phases: the design qualification (DQ), the installation qualification (IQ), the operational qualification (OQ) and the performance qualification (PQ). Some of the phases can be combined or executed simultaneously (it particularly happens frequently with OQ and PQ), but some others need a specific order. In any case, it always needs to be defined and documented (Table 5).

	Design Qualification	**Installation Qualification**	**Operational Qualification**	**Performance Qualification**
Timing and Applicability	Prior to purchase of a new model of instrument	At installation of each instrument	After installation or major repair of each instrument	Periodically at specified intervals for each instrument
Activities	Assurance of manufacturer's DQ	Description ↔	Fixed parameters ↔	Preventive maintenance and repairs
	Assurance of adequate support availability from manufacturer	Instrument delivery		Establish practices to address operation, calibration, maintenance, and change control
	User requirement specifications	Utilities / facility ↔	Environment	
		Assembly and installation		
		Network and data storage ↔	Secure data storage, backup, and archive	
		Installation verification ↔	Instrument function tests ↔	Performance checks

Table 5. Timing, applicability and activities for each phase of analytical instrument qualification[12-14]. Arrows indicate the activities that may be combined on the different phases.

DQ is the documented verification of adequateness of the equipment for the use to which it is designed, so it fulfils all the requirements of the user

and follows the User Requirement Specifications (URS). It must include the assurance of manufacturer's DQ, the assurance of adequate support availability from the manufacturer and the instrument's fitness for use in the laboratory. In the DQ it is additionally included all the plans, classification of the different zones, environmental conditions, flow of personnel, materials, conditions to prevent cross-contamination, dimensions of the areas, etc.

IQ is the documented verification that the equipment installation meets all the stablished requirements with the approved design, the specifications of the user and the recommendations of the manufacturer. The objective is to verify the correct installation of the equipment. The protocol usually includes:

- The results of the FAT proves (Factory Acceptance Test) from the manufacturer.
- The protocol of installation on the site or SAT (Site Acceptance Test).
- Some additional checks such as the verification of a correct installation, a compilation of the performance and safety instructions, together with maintenance requisites.
- Calibrations.
- Verification of the materials.

OQ is the documented verification certifying that the installed equipment works correctly inside specifications. The objective is to demonstrate the correct operation or performance on the work environment. It comes right after the IQ, and it is based on the verification of functionality assays. The protocol includes:

- The revision of the procedures of use, maintenance, calibration and cleaning.
- Alarm tests.
- The assay of all instrumentation
- Verification of the right operation of the start and stop sequences.

Lastly, the PQ is the documented verification certifying that the equipment works adequately once integrated with the fabrication procedure, and it is able to produce efficiently and continuously the product as the expected by the specifications. The objective of the protocol is to demonstrate the effectivity and reproducibility of the equipment to obtain the desired product. The PQ assays are normally based on the manufacturing routine.

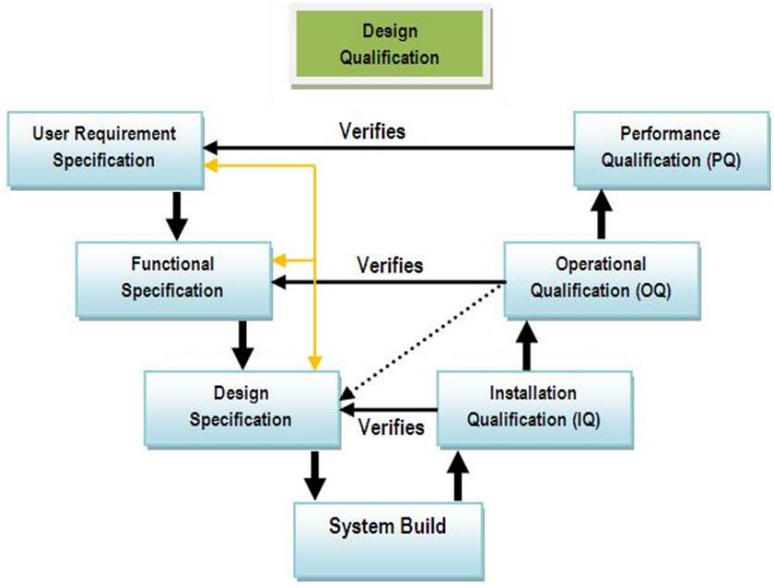


Figure 4. V model concept for system validation. The procedures on the right verify the protocols on the left[14].

All these phases are usually schematized in a graphical representation with "V" shape, where the procedures on the right verify the protocols on the left (Figure 4).

6.2.5 Finished product

Finally, to prove that a finalized product could be fabricated, we prepared a sachet's final dosage form. Figure 5 ilustrates how would the product look like once produced, on a photography of a finished product's prototype.

Figure 5. Photography of the proposal for a final dosage's form prototype of the finished product.

6.3 CONCLUSION

Scale-up options and solutions have been studied, with successful results and positive options for bringing the liposomal formulation to industrial scale. Every instrumentation used on the formation and optimization of the formula achieved in *Chapter 4* has an equivalent to satisfy the needs of a bigger batch production, [redacted] Resulting solutions from this alternative procedure were characterized, obtaining positive results on encapsulation rates, size distribution of vesicles and both physical and chemical stabilities on time. Therefore, it can be concluded that the designed liposomal formulation could be easily scaled-up for industrial purposes.

6.4 REFERENCES

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CHAPTER 7. Overall Discussion

Food supplements are products of increasing interest on today's society where people are everyday gaining more interest towards a wholesome life. One third part of FDA-approved drugs over the past 20 years are based on natural products or their derivatives, so food extracts and derivatives certainly have a great potential towards wealthiness, on both prevention or treatment of health conditions.

Chronic fatigue syndrome, characterized by an unexplainable fatigue accompanied by headaches and many other conditions, although being an idiopathic disease of unknown etiology, has been reported on many clinical assays to show improvements on their symptomatology when patients were treated with a food supplementation of [redacted] nicotinamide adenine dinucleotide (NAD[redacted]), coenzyme Q10 (CoQ10) or Phosphatidylserine (PS).

On another hand, food supplement industry is everyday getting more oriented in the same direction than the pharmaceutical industry, with further research for achieving the best therapeutic effect possible. In this sense, encapsulation of actives for enhancing stabilities, bioavailability or effectivity, among others, is an interesting approach towards this motivation.

With the aim of developing a liposomal-based formula for the encapsulation of the active ingredients NAD[redacted], CoQ10, PS and vitamin C, in a first place, a High-performance liquid chromatography (HPLC) method was developed. NAD[redacted] has a very hydrophilic nature, and it can be separated from the others and quantified. However, to detect and

quantify the other three encapsulated hydrophobic actives, it was necessary to develop the HPLC method. First, since the matrix would be an aqueous vesicular suspension of very hydrophobic actives, a treatment of samples to remove water was carried out. Liposomal solutions were lyophilized for the preparation of samples. Afterwards, the method was developed consisting on a gradient of isopropanol and an NH_4AcO buffer, which was carefully optimized until optimal peak shapes, theoretical plate numbers and United States Pharmacopeia (USP) tailing factors were achieved. Many articles describe analyses of the actives separately, but just one article reported by Temova-Rakuša Ž *et al*.[1] described the co-analysis of CoQ10 and vitamin C.

The validation was successfully carried out following the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines in terms of selectivity, linearity, accuracy, precision and robustness, with Relative Standard Deviation (RSD) values on all the parameter within the stablished < 3 % deviation except for quantifications with different analysts with a 3.18 %. The stability of the prepared solutions for HPLC analyses stated that analyses needed to be done right after the digestion of the samples with organic solvents to avoid possible degradations. Additionally, robustness pointed out that any parameter such as temperature or wavelength should not be changed from the original method to avoid deviation on results.

On the design of the formula for the production of the oral liposomal food supplement, diverse lipid combinations and multiple assays were performed until the final formula was defined. The best results were achieved on a combination of [redacted] % [redacted]PS, [redacted] % [redacted]PC, [redacted] % Chol and

[redacted] % Cholesterol-(polyethylene glycol-600) (Chol-PEG), together with 6 mg/mL of CoQ10, 5 mg/mL of PA and an aqueous phase with a 2.5 mg/mL of NAD[redacted]. Obtained homogeneous vesicles sized in a mean of 164.3 ± 2.7 nm, with PdI (Polydispersity Index) as low as 0.07 and a negatively charge of -48.1 ± 2.9 mV. According to many previously reported works[2,3], these characteristics correspond to SUVs in a range perfectly adjusted for active delivery purposes. [redacted]

While entrapment of hydrophobic active substances was expected to be high for being attached to the lipid bilayer, the obtained encapsulation efficiency of NAD[redacted] can be considered high according to other many publications of encapsulating hydrophilic actives[4-7]. Liposomes were also successfully imaged with both cryogenic electron microscopy (Cryo-TEM) and confocal microscopies.

In vitro evaluation of NAD[redacted] release kinetics showed an adequate release with [redacted]. Release velocity is important to understand if actives will remain encapsulated on an adequate range of time, not too fast and neither too slow, as may happen with other formulas[8].

The lipid barrier protection against acid pHs conferred to the core-encapsulated NAD[redacted] by liposomes was also tested with the exposure of different external pH media. In accordance to bibliography[2,8], liposomes demonstrated a semipermeable behavior with a decrease on degradation of NAD[redacted] when exposed to unfavorable conditions but no completely

impermeable protection. Resistance was characterized to improve up to 36.0 % when liposomally encapsulated.

In vitro simulation of NAD[redacted] stability in gastric fluids showed that the concentration of encapsulated NAD[redacted] and raw NAD[redacted] were 93.25 ± 3.36 % and 82.94 ± 6.17 % after 1 hours of incubation, respectively. Remarkably, liposomes exhibit a significant improvement in NAD[redacted] stability in front of the simulated human stomach conditions, in accordance with other studies in literature[9,10]. Indeed, this fact was getting even more accentuated at pH 7.2 with time, where the concentration of encapsulated NAD[redacted] and raw NAD[redacted] reached values of 74.74 ± 2.7 % and 52.75 ± 5.61 % after 6 hours of incubation, respectively.

Mucoadhesion efficacy of active-loaded liposomes was evaluated quantitatively by assaying the mucin absorbed amount at 37 ºC for 18 hours. Mucoadhesion efficacy has been widely reported and our results coincide with other mucoadhesion studies[11], demonstrating the valuable feature of phospholipid membranes to get attached to mucosa and prolong residential time of liposomes in the intestinal track.

The effect of active-loaded liposomes and empty liposomes on cell viability was tested in NIH-3T3 human cells using XTT assay, and a non-cytotoxic behavior was exhibited until very high doses of 5 mM. Usually, concentrations used for the evaluation of toxicity are on the order of µM or nM. Therefore, our formula showed high safety and non-interference with cell viability[12].

Interesting results can be discussed from the *ex vivo* permeation studies carried out with excised samples of pig abdominal tissues. [redacted]

[redacted]

[redacted].

Lyophilization of the samples was optimized until physical stability upon rehydration was achieved. A [redacted] % trehalose was determined to be the optimal amount needed for maintaining properly the size and homogeneity, with the better results among the studied batches on a particle size of 191.1 ± 0.8 nm and a PdI of 0.105 before lyophilizing, and

200.8 ± 1.6 nm and a low PdI of 0.183 after lyophilizing and its rehydration. [redacted]. Many different cryoprotectants have been used to protect the stability of liposomes during freeze-drying for long-term stabilities, but trehalose is described to be a cryoprotectant of preference due to the avoidance of Maillard reaction[16]. It has been additionally reported to have interesting properties on the enhancement of chemical stabilities, as observed in our results[16-18]: in addition to the physical preservation of liposomes on the lyophilization process, stabilities on the prototype without trehalose showed a surprisingly high significant accelerated degradation in comparisson with the prototype with the cryoprotectant trehalose. [redacted].

[redacted]

[redacted]. In this sense, hard capsules, sachets, and two different types of extemporaneous solutions were proposed as possible final dosage forms for the formulation. Obtained results on long-term stability are remarkably positives: the encapsulated active substances are very unstable and sensitive, and here an industrial viable option with enough stability on time was achieved.

In addition, possible secundary formulations were studied with the addition of polaxamer 407 and alginate[20-22]. Thermoreversible gels with polaxamer were successfully formed, and alginate showed a cross-linking behaviour on the formula when in contact with divalent cations.

On the use of a different method for the production of liposomes, microfluidic techniques were studied for the straightforward formation of GUVs and satisfactory results were achieved. Two different devices were fabricated and assayed, and the best results were obtained with a novel coaxially structured capillary device developed by Jiang K. *et al.*[23]. The formation of double emulsions was achieved to be continuous and homogeneous with 800 µL/minute, 200 µL/minute and 3000 µL/minute for the inner, middle and outer flow rate respectively. The inner phase used was a solution of the active ingredient Nicotinamide Adenine Dinucleotide (NAD[redacted]) at 2.5 mg/mL, with an 8.5 % p/p of Polyvinyl Alcohol (PVA) to enhance the stability of the vesicles. The middle organic

phase was a 4:7 ratio of chloroform and hexane respectively, and the outer phase was a 10 % p/p PVA solution. The molar ratio of lipids and lipid active substances on the middle phase was adjusted at a 7.7 mM concentration with the same ratio than the used [redacted]. Since no other studies of liposomal formation have been reported with this device previously, the production cannot be accurately compared with other works.

Confocal fluorescent imaging of the resultant vesicles showed a perfect encapsulation with a bright blue fluorescent in the core of vesicles. Size distribution of the formed GUVs had a mean average size of 289.0 r. μm.

Unilamellarity of the vesicles was studied by the incorporation of pore-forming protein α-Hemolysin (α-HL) on its membrane. Results, in accordance with the expected by other reported studies[24], showed a reduction of fluorescence on time on the vesicles with the pore-forming protein incorporated at the middle phase solution, thus demonstrating its unillamelarity.

Lipid separation sections on the membrane are expected depending on molecular structure and composition[25]. Asymmetrical fluorescent dye was observed on imaging the resulting vesicles, demonstrating GUVs formation and the arrangement of the bilayer membrane.

Lastly, a scale-up optimization was investigated, and positive options for bringing the liposomal formulation to industrial scale were studied. In addition to the 100 % scalable procedure and instrumentation described in *Chapter 4*, [redacted]. Resulting solutions from this

alternative procedure were characterized, obtaining positive results on encapsulation rates, size distribution of vesicles and both physical and chemical stabilities on time.

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CHAPTER 8. Conclusions

Based on all the work carried out during the study and realization of the present thesis, the following conclusions can be made:

- A high-performance liquid chromatography method was developed, thus allowing the simultaneous quantification of coenzyme Q10 (CoQ10), Phosphatidylserine ([redacted]PS), and vitamin C. The method, consisting on a gradient of isopropanol with an aqueous buffered solution, was also tuned up to proper analyze the active substances from liposomal samples.
- The developed method was validated according to the ICH guidelines, and positive and encouraging results were obtained from testing system suitability, selectivity of peaks, absence of interferences, stability of the solution during the analyses and limits of detection and quantification. Linear regression for all the active substances showed an optimal correlation coefficient of >0.999 in the concentration range between 70 to 130% of the liposomal formulation and less than a 3% relative standard deviation (RSD) in accuracy, repeatability and precision. Only a 3.18% RSD was obtained when analyses were carried out by different analysts. Robustness testing revealed that attention must be paid on controlling analyses conditions such as temperature and wavelength.
- It has been designed an optimal vehicle for oral delivery of antioxidant actives (NAD[redacted], CoQ10, [redacted]PS and vitamin C) based on negatively charged and nanosized liposomes. The liposomal formulation is formed by small unilamellar vesicles with high

encapsulation yield of hydrophobic actives (CoQ10, [redacted]PS and vitamin C) and the hydrophilic NAD[redacted].

- *In vitro* studies of release, gastrointestinal digestion, resistance conferred by the liposomal bilayer and bioadhesion to mucosa were carried out to evaluate the potential of the liposomal vehiculization. Release confirmed the capacity of the vehicle to release the hydrophilic NAD[redacted] efficaciously in an adequate range of time; bioadhesion studies demonstrated the ability of liposomes to get attached to mucosa, thus elongating the residence time in the human body; and gastrointestinal digestion and resistance studies showed an improvement on the core encapsulated NAD[redacted] stability due to the bilayer protection. Additionally, *ex vivo* permeation studies have revealed that active-loaded liposomes have a remarkable ability to enhance skin absorption of actives after oral administration.
- Active-loaded liposomes were incubated on NIH-3T3 human cell cultures, exhibiting little cytotoxicity and consequently safety on the intake of the formulation.
- After long-term stability studies, it was concluded that the product is adequately stable [redacted].

- [redacted].
- A stable GUV liposomal cocktail of NAD[redacted], vitamin C, [redacted]PS and CoQ10 was tuned up with the use of microfluidic techniques. Liposomes were efficiently formed, and unilamellarity was demonstrated by the proper incorporation of α-HL protein on its membrane.
- Scale-up options and solutions have been studied, with successful results and positive options for bringing the liposomal formulation to industrial scale.

To sum up, the main goal of this Thesis has been met, providing to Vitae Health Innovation, S.L. a new comercializable product within the concept of liposomal encapsulation technology.

ANNEX: Publications

1. Articles

1. **Title:** Development and Validation of a New High-Performance Liquid Chromatography Method for the Simultaneous Quantification of Coenzyme Q10, Phosphatidylserine, and Vitamin C from a Cutting-Edge Liposomal Vehiculization.
   Authors: Ruiz-Garcia, M., Pérez-Lozano, P., Mercadé-Frutós, D., Nardi-Ricart, A., Suñé-Pou, M., Cano-Sarabia, M., Garcia-Jimeno, S., Suñé-Negre, J. M., Maspoch, D. & García-Montoya, E.
   Journal: ACS Omega
   DOI: 10.1021/acsomega.9b02456
   Status: Published (November 11th, 2019).
   Impact Factor: The 2019 impact factor of ACS Omega is 2.870. Scimago Journal Ranking: Q1 in Chemical Engineering (miscellaneous) and Chemistry (miscellaneous).
2. **Title:** Current Processing in Micro- and Nanoencapsulation of Coenzyme-Q10 for Oral Delivery System. (Provisional)
   Authors: Ruiz-Garcia, M., Arenas-Jal, M., García-Montoya, E. & Tapia-Hernandez, J. A.
   Journal: Trends and Food Science and Technology
   DOI: -
   Status: In submission.
   Impact Factor: The 2020 impact factor of Trends and Food Science and Technology is 8.519. Scimago Journal Ranking: Q1

in Agricultural and Biological Sciences (Food Science) and Biochemistry, Genetics and Molecular Biology (Biotechnology).

2. Patent-pending research results

1. **Title:** Composition comprising a liposome including NAD, ubiquinone or ubiquinol, a phospholipid and lyophilizate thereof.
   Authors: Ruiz-Garcia, M., Cano-Sarabia, M., Garcia-Jimeno, S., García-Montoya, E. & Maspoch, D.
   Status: The industrial property agency Torner, Juncosa i Associats, S.L. is currently working on the patent submission.

3. Oral Communications

1. ***Invited seminar*** **- Levich Institute seminar series**
   Title: Food Supplementation within Liposomal Encapsulation Technology against Chronic Fatigue Syndrome
   Speaker: Ruiz-Garcia, M.
   Date: Tuesday, August 27, 2019, 2:00 PM
   Place: Steinman Hall, #312
   (Chemical Engineering Conference Room)
   City College of CUNY
   140th Street & Convent Avenue
   New York, NY 10031

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Article

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Development and Validation of a New High-Performance Liquid Chromatography Method for the Simultaneous Quantification of Coenzyme Q10, Phosphatidylserine, and Vitamin C from a Cutting-Edge Liposomal Vehiculization

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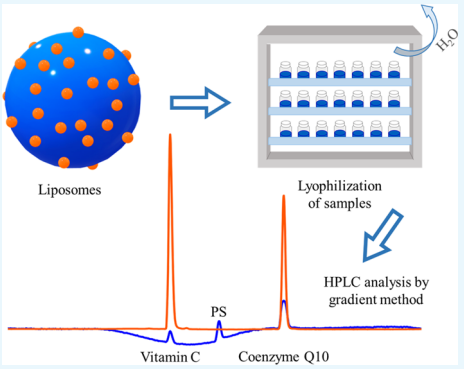
Supporting Information

ABSTRACT: A high-performance liquid chromatography (HPLC) method was developed to simultaneously quantify three widely used active substances such as coenzyme Q10, phosphatidylserine, and vitamin C. This new method optimizes current timing and costs in the analyses of these three active substances. Additionally, since the analyzed compounds were encapsulated on a cutting-edge liposomal formulation, further processing was necessary to be developed prior to HPLC analyses. The technique was studied and adequately validated in accordance with the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) regarding selectivity, linearity, accuracy, precision, and robustness. After data treatment of results, linear regressions for all active substances showed an optimal linearity with a correlation coefficient of >0.999 in the concentration range between 70 to 130% of the liposomal formulation and less than a 3% relative standard deviation (RSD) in accuracy and precision.



INTRODUCTION

During the past years, science is accomplishing huge advances in drug discovery through modeling and synthesis toward the treatment of idiopathic diseases.[1−4] However, even if promising molecules are identified and synthesized, its effectiveness will be reduced or equal to zero if they are unable to achieve the therapeutic target because of a poor absorption. It is estimated that, nowadays, 90% of the developed molecules on investigation are chemically lipophilic drugs or, in other words, insoluble drugs in water and unable to cross physiological barriers on oral administration.[5,6]

To enhance drug bioavailability of lipophilic actives, the liposomal technology of encapsulation has been a broadly reported vehicle during the past decades on the literature. Discovered by Bangham and Horne in 1964,[7] this lipid spherical structure allows the insoluble active substances in water to get intertwined with the forming phospholipids of the membrane, while hydrophilic actives can also be encapsulated in its aqueous core. Among many other benefits, this has converted them in an extensively used and very versatile drug delivery technology.[8−12]

Herein, we describe a novel method of analysis for the simultaneous quantification of three coencapsulated active substances with very similar characteristics of hydrophobicity in a liposomal matrix, and also, we explain the pretreatment procedure for these liposomal samples prior to analyses. Specifically, the coanalyzed active substances are the coenzyme Q10 (CoQ10), 6-*O*-palmitoyl-L-ascorbic acid (the lipid-soluble vitamin C), and phosphatidylserine (PS). These active substances are widely used in both basic research and in the

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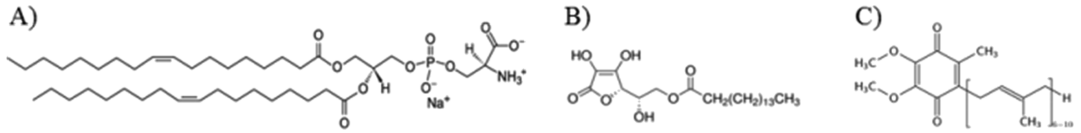

Figure 1. Chemical structure of the analyzed compounds. (A) PS. (B) Vitamin C. (C) CoQ10.

pharmaceutical, cosmetics, and food industry, thus having a high interest and also a huge impact in the economic ambit. In the past 10 years, 5651 articles related with CoQ10 were published in scientific journals, 6969 articles related to PS, and 46,079 articles related with vitamin C, all of them with an exponential tendency of increase on each year.[13] Although many articles have been published describing analytical methodologies of each substance in separate, to the best of our knowledge, there is no published method offering the simultaneous analysis of the three active substances, and even less coencapsulated within the liposomal technology and the additional elements, this means to be considered upon analysis. To the proper determination and quantification of the active substances, it is important to have clear absorption peaks, so we can extrapolate the areas to the actual amount we have of each active substance in the preparation. Since the chemical nature of all the three actives is very similar, it is actually very tricky to elucidate the compounds in separated peaks and avoid them to overlap. Additionally, both coenzyme Q10 and vitamin C absorb at a wavelength of 275 nm, and all the three substances absorb at 200 nm wavelength as well, thus making it absolutely necessary to develop a methodology to separate the peaks for its quantification. With combinations of the actives in separate, just one article of simultaneous analysis has been published, reported by Temova-Rakuša et al.,[14] with the coanalysis of coenzyme Q10 and vitamin C. This article provides an interesting method for their quantification but not valid for the analysis of phosphatidylserine. In the Supporting Information (Figure S1), we attach a chromatogram of this reported method with our three active substances, where any absorption peak can be observed for our third active substance, the phosphatidylserine, meaning that it is not a suitable analytical method for this substance or for the simultaneous quantification of the three active substances. Therefore, our method could be considered as a unique and interesting contribution with applications in pharmaceutical/cosmetic/food industry.

After the design and development of the method, a complete validation study was meticulously accomplished following the ICH quality guidelines. It was also used and proved in different real circumstances such as long-term stability studies at different conditions.

RESULTS AND DISCUSSION

Sample Preparation Strategy. After some assays, results demonstrated that it is necessary to remove the water from the samples to allow the elution of substances at the chromatogram and to successfully analyze and quantify the peaks of the active substances. First, with any water in the sample, it was almost impossible to correctly solve all the lipids. Second, being hydrophobic active substances with water, the peaks were not eluting in a satisfactory peak form or were not eluting at all. Freeze-drying of samples was carried out. Afterwards, twenty different lyophilized vials with the liposomal formulation were injected and analyzed with the developed HPLC method to test the accuracy of the sample preparation strategy, and the RSD after the analyses of the all the vials was 2.35% for CoQ10, 2.14% for vitamin C, and 2.74% for PS, thus showing optimal results of repeatability.

Method Development. The designed HPLC method was meant to properly analyze the content of CoQ10, vitamin C, and PS from a liposomal formulation. First, we assayed different mixtures and proportions between methanol, isopropanol, acetonitrile, and water buffer. To choose an adequate buffer, we looked out to the pK_a of the three substances and selected buffer at a pH where all compounds would be in an undissociated form, such as 0.02 M NH_4AcO at pH = 6.5. Figure 1 shows the molecular structure of every active substance, while its corresponding pK_a, partition coefficient, and molecular weight are shown in Table 1. The

Table 1. Chemical Properties To Be Considered for the Design of the Method[15−17]

parameter	PS	vitamin C	CoQ10
log *P*	10.55	6	10
MW (g/mol)	810.025	414.539	863.365
pK_a	9.38; 1.47	4.36; −3	−4.7

first chromatographic meticulously tested conditions showed us that for a favorable elution of CoQ10, a 100% flow of pure methanol or isopropanol is needed, and any mixture with water made its elution impossible with an optimal peak shape. On the other hand, for the analysis of vitamin C, a mixture of organic solvent and water was needed for its proper elution, and PS was not eluted in any isocratic condition with an optimal peak shape, considering theoretical plate numbers (*N*) and USP tailing factor.

At this point of development, a gradient of NH_4AcO buffer with isopropanol was extensively investigated at different proportions and with different gradient duration times until we found an adequate combination that allowed the optimal elution of all the compounds during the same analysis. An example of the obtained chromatogram after last optimization is shown in Figure 2.

Method Validation. Before carrying through the validation assays, the suitability of the equipment with the method was tested. Table 2 shows RSD of retention times (RT), symmetry of the peak, USP tailing factor, capacity factor, and *N*, demonstrating consistency and appropriateness. The tabulated retention times of the six analyzed samples and symmetry analyses on each peak of the active substances can be found in the Supporting Information (Scheme S2 and Figures S3−S5).

After checking the suitability of the equipment and before starting any other validation step, it was necessary to understand for how long samples are stable above at least 95% of all active substances. It was determined that, to accomplish this requirement, liposomal samples could be injected up to 3 h after its preparation in the HPLC vials without significant degradation of any sample, while for the standard solution, the time was shortened to 1 h. So, it is

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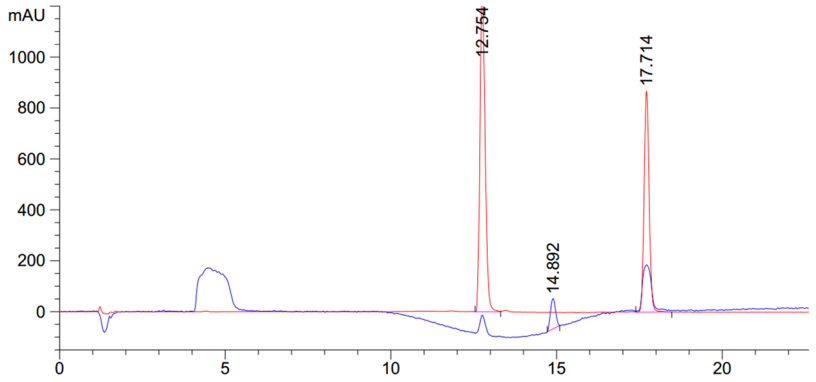

Figure 2. Chromatographic exemplification of the developed method with the three analyzed active substances. The peak with an RT of 12.754 min corresponds to vitamin C, an RT of 14.892 min corresponds to PS, and an RT of 17.714 corresponds to CoQ10. The red line is λ = 275 nm, while the blue line is λ = 200 nm.

Table 2. Suitability of the Equipment and Data Analyses

parameter	PS	vitamin C	CoQ10
retention times (min)	14.86	12.72	17.73
retention times (RSD %)	0.09	0.25	0.14
symmetry of peak	1.01	0.73	0.89
USP tailing factor	1.22	1.23	1.08
capacity factor	3.08	2.49	3.85
plate numbers (N)	40,891	33,327	67,904

highly recommended to analyze samples right after its solubilization in organic solvents and preparation for HPLC quantification.

The precision of the system was also checked before any other validation step. After five consecutive injections of a 100% concentration of PS, the area scattered only in a 0.59%, being inside our acceptance criteria of ≤1.0%, thus confirming its reliability (see the Supporting Information).

Selectivity was confirmed by overlapping the chromatograms of an analyzed sample with a standard solution and a third one with the rest of the substances (Figure 3). Additionally, the purity factor of peaks was studied. In all cases, purity was above the threshold limit, set at 990.000 (see the Supporting Information, Table S6 and FiguresS7 and S8).

Through analyzing all seven concentrations between 130 and 70% of the standard solution, results demonstrated an adequate linearity in this concentration range. Data is summarized on Table 3, with RSD and correlation coefficients accomplishing our established limits. Table 3 also shows data regarding precision and accuracy.

For the precision of the method evaluated in different days, we established a 2.8% RSD and a 3.7% for repeatability (intra-laboratory). Regarding accuracy, every recovery percentage after the study was included in our quality specifications. All this data demonstrated excellent results with respect to the validation of the method and indicated that this method is precise and accurate.

Table 4 details the percentage of recovery on the nine analyses at different concentrations for evaluating accuracy. Every concentration resulted in less than a 2% RSD, and ANOVA test showed nonsignificant differences on any active substance at any concentration.

Lastly, robustness studies revealed that the method must be controlled in terms of temperature, injection volume, wavelength detection, and flow since otherwise results could slightly variate. Being a method with three analyzed compounds, each

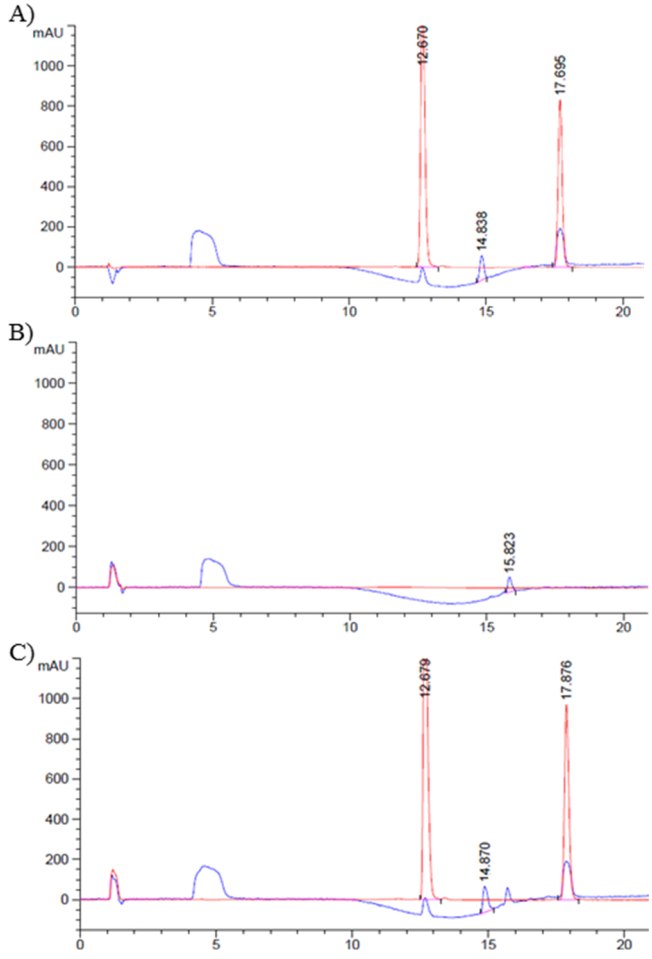

Figure 3. Representative chromatograms. (A) Standard solution. (B) Mixture of the other lipids: PC, Chol, and Chol-PEG. (C) Liposomal sample.

parameter resulted in some degree of retention time differences or peak areas.

Table 5 indicates which parameter showed significant or nonsignificant differences after deliberated changes upon its analysis with a one-way ANOVA test. On this table, although significant differences were shown in many of the deliberated changes, RSD values were not in any case over 5% but with wavelength for PS. Since λ = 200 nm is a wavelength where also solvents have certain absorbance, it is a critical parameter

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Table 3. Summarized Data of Linearity, Precision, and Accuracy during the Validation of the Method

	linearity			precision			accuracy
analytes	range (mg/mL)	correlation coefficient	RF RSD (%)	standard solution, RSD (%)	different days, RSD (%)	different analysts, RSD (%)	recovery (%, max and min values)
PS	1.008−0.543	0.999	1.82	1.97	2.80	3.18	101.35−98.25
CoQ10	0.312−0.168	0.999	1.01	0.97	2.45	1.89	101.92−98.30
vitamin C	0.260−0.140	1.000	0.89	1.96	2.80	1.89	101.89−98.28

Table 4. Detailed Results of Studying the Accuracy

parameter	PS	vitamin C	CoQ10
recovery at 130% (%)	100.21 ± 1.61	101.20 ± 0.93	100.21 ± 1.82
recovery at 100% (%)	98.42 ± 0.23	100.28 ± 1.83	100.17 ± 1.01
recovery at 70% (%)	98.48 ± 1.26	100.98 ± 0.80	99.71 ± 0.93
ANOVA test (*p*-value)	0.98	0.67	0.88

Table 5. Summarized Statistics from ANOVA Test on Robustness[a]

	PS		vitamin C		CoQ10	
parameter	*p*-value	RSD (%)	*p*-value	RSD (%)	*p*-value	RSD (%)
temperature	0.239	1.72	>0.01*	4.47	>0.01*	1.35
injection volume	0.225	3.34	0.792	1.15	0.032*	0.70
wavelength	>0.01*	14.88	0.013*	1.85	0.135	0.75
flow	0.695	2.17	>0.01*	7.22	0.341	0.92

[a]* indicates that there are significant statistical differences.

to be controlled. Specifically, a two-tailed T-student test was carried out to compare wavelengths 197 nm versus 200 nm and 200 nm versus 203 nm. The first T-test contained highly significant differences with a *p*−value of >0.01, but non-significant differences were observed with a *p*−value of 0.80 between 200 and 203 nm, thus being 203 nm also a suitable wavelength to be used.

Based on the standard deviation of the response and the slope, based on the calibration curve, LOD and LOQ were calculated for three analytes. Results are presented in Table 6.

Table 6. LOD and LOQ Results

parameter	PS	vitamin C	CoQ10
LOD (mg/mL)	0.082	0.018	0.021
LOQ (mg/mL)	0.249	0.054	0.063

Applications of the Method. Once validated, the proposed method was used to accurately evaluate the stability of the three active substances encapsulated on the mentioned liposomal formula after 3 months of storage at 4 and 25 °C. This study verifies the applicability of the method in a real case study. Results are presented in Table 7, while an illustrative chromatogram of each temperature is included in the Supporting Information (Figures S9 and S10).

CONCLUSIONS

The developed method demonstrated promising results on the simultaneous analyses of CoQ10, vitamin C, and PS. To the best of our knowledge, this is the first reported method for the determination of these widely used active substances at the same time. Thus, this method could result in an ideal analytic method for the cosmetics, pharmaceutical, and food supplement industry.

Table 7. Results of Stability Studies with the Developed Method after 3 Months

temperature condition	PS	vitamin C	CoQ10
4 °C (%)	100.09 ± 0.31	95.27 ± 0.58	97.56 ± 0.20
25 °C (%)	96.19 ± 0.22	85.57 ± 0.15	87.27 ± 0.23

Additionally, the formulation involving the liposomal technology may be a tricky challenge to allow a correct quantification and analyses, especially for very lipophilic active substances to which the presence of water can signify a complicated limitation for the analyses. The performed methodology of sample treatment prior to its analyses may be as well an additional step of valuable interest. The concept may be applied to any other possible liposomal formulations with highly hydrophobic active substances.

In terms of validation of the method, following the ICH guidelines, the suitability of the equipment, selectivity of the method, linearity, robustness, precision, accuracy, and stability of the solution during the analyses were tested with positive and encouraging results.

Lastly, with the purpose of verifying the applicability of the method, a testing of the method with a real case was carried out. Samples subjected to stability studies were analyzed, and results were studied and interpreted, with excellent results.

EXPERIMENTAL SECTION

Materials and Chemical Reagents. 6-*O*-Palmitoyl-L-ascorbic acid (vitamin C; analytical standard, ≥99.0%), cholesterol-(polyethylene glycol-600) (Chol-PEG), and cholesterol (Chol) were obtained from Sigma-Aldrich (Darmstadt, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (phosphatidylserine or PS; analytical standard, ≥99.0%) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine or PC) were purchased from Lipoid GmbH (Switzerland). Coenzyme Q10 (CoQ10; United States Pharmacopeia (USP) reference standard quality, ≥99.0%) was obtained from Vitae Health Innovation S.L (Spain). Ammonium acetate (NH_4AcO) was acquired from Panreac Quimica (Barcelona, Spain).

Instrumentation. Analyses were carried out with an HPLC system model 1100 HP from Agilent (Agilent Technologies, Santa Clara, EEUU), composed of a quaternary pump, an automatic injector, a diode array detector, and a column oven where our column was placed, all controlled by a ChemStation software version B04.02. For the freeze-drying of samples, a Telstar Cryodos−50 lyophilizator was used.

Preparation of Liposomes. Liposomal formulations were prepared using the thin-film hydration method with some modifications.[18] Briefly, PC, PS, Chol, Chol-PEG, and CoQ10 were dissolved in chloroform solutions (100 mg/mL) or chloroform/ethanol 7:3 for vitamin C and mixed afterward at

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the desired molar. In this case, the molar concentration was 79 mM, with 30.4% PS, 22.8% PC, 19.0% Chol, 15.3% vitamin C, 8.8% CoQ10, and 3.8% Chol-PEG. The organic solvent was removed under vacuum and nitrogen to afford a dry lipid film, which was hydrated with an aqueous solution under vigorous stirring for 15 min at 40 °C. Under these conditions, the stacks of liquid crystalline lipid bilayers become fluid and swell, resulting in their detachment during agitation and their self-closure to form multilamellar large vesicles (MLVs). Small unilamellar vesicles (SUVs) were obtained by homogenizing the MLV suspension using an extruder (Lipex Biomembranes, Canada) and a polycarbonate membrane (pore size, 200 nm).

Sample Preparation Strategy. The treatment and preparation of samples prior to HPLC analyses were essential for allowing the quantification of the lipidic active substances. Liposomal formulations have by definition the characteristic of including both water and water-insoluble compounds, and this could make any posterior analyses difficult. Since the presence of water is a very unfavorable condition to dissolve and allow the flow of the substances through the column in an appropriate way, a procedure of analysis including the lyophilization of the samples was carried out: an scheme illustrating the preparation process of the samples can be found in the Supporting Information (Scheme S2). For the lyophilization of samples, 1 mL of the liposomal solution was pipetted on each vial and frozen afterward at −80 °C on an ultralow temperature freezer for 12 h. Once samples were completely frozen, they were freeze-dried for 4 days. Lyophilization conditions of pressure and temperature were 1 mbar and −50 °C, respectively.

Once samples were completely dried, 2 mL of $CHCl_3$ was added to properly solubilize the very apolar active substances: PS, vitamin C, and CoQ10. After 5 min of slight shaking and only when the sample was completely dissolved, 10 mL of methanol was added, and the final solution was transferred into 25 mL volumetric flasks. The vials were afterward cleaned with methanol, which was also transferred to the flasks, making up to 25 mL with more methanol. Lastly, all solutions were filtered with 0.45 μm PVDF filters, and HPLC vials were prepared. The final concentration of each active compound at the moment of injection to the HPLC system was 776 μg mL^{-1} PS, 240 μg mL^{-1} CoQ10, and 200 μg mL^{-1} vitamin C.

To validate the preparation procedure developed, 20 lyophilized vials were injected, establishing an RSD limit of 2.7%.

Standard Solution Preparation. For the preparation of a standard solution, 19.4 mg of PS, 6 mg of CoQ10, and 5 mg of vitamin C were accurately weighed and dissolved in the same way than in the sample preparation procedure, so the final peak areas would be also the same both in standard solutions and sample solutions.

Chromatographic Conditions. For HPLC analyses, the employed column was a C18 Zorbax Eclipse Plus 2.1 × 150 mm (5 μm), with a constant flow of 0.25 mL min^{-1} and a controlled temperature of 25 °C. The injection volume was 10 μL, and the detection wavelengths were λ = 275 nm for vitamin C and CoQ10 and λ = 200 nm for PS since no other absorbance was found after checking with the diode array accessory. After 19 min, all the compounds were already eluted, but the stop time was fixed at 28 min to return to the initial conditions and stabilize the equipment. The detailed proportions of the two employed solvents during the time are shown in Table 8.

Table 8. Detailed Gradient of the Developed Method

time (min)	solvent B (%)	solvent A (%)
0	60	40
5	60	40
10	0	100
18	0	100
19	60	40
28	60	40

A gradient method was developed and is detailed in Table 8. Solvent A in the table is isopropanol, while Solvent B is a buffer solution of 0.02 M NH_4AcO.

Method Validation. The development of the method was followed by validation studies to ensure selectivity, linearity, robustness, precision, stability of the solution during the analyses, and accuracy of the method, following the ICH guidelines.[19] Prior to any validation test, six injections of a standard solution were analyzed to ensure the suitability of the equipment. Retention times with less than 1.0% variations, capacity numbers greater than 2, and symmetry of peaks between 0.7 and 1.2, theoretical plate numbers (N) above 2000 for any peak, and USP tailing factor smaller than 1.5 were checked out.

Selectivity is the ability to assess unequivocally the analyte in the presence of other components, which may be expected to be present. For this study, both sample and standard solutions were compared, thus confirming the complete separation of all compounds with a purity peak test being carried out for each active substance. Linearity was tested by preparing seven different concentrations per triplicate between 70 and 30% of the standard solution at the usual working concentration, studying if the response is directly proportional within this range. Response factor (RF) was the analyzed parameter on linearity, being the result of dividing peak areas by the corresponding concentration. Precision is the parameter that expresses the degree of scatter between a series of measures. In this case, the precision of the method and system was evaluated with the injection of nine standard solutions at different concentrations and with different analysts and different days of work, studying its repeatability. Robustness is a measure of the capacity to remain unaffected by small but deliberated variations in method parameters such as the temperature of the column (± 3 °C), the analyzed wavelength (± 3 nm), the injection volume (± 5 μL), or modifications in the flow level of the pump (± 0.05 mL/min). This study is necessary to understand the importance of controlling a determinate parameter. Stability of the active substances in the HPLC vials once prepared is also vital to understand for how long the solution is stable before injecting. Lastly, the accuracy of the method was assessed to verify if any of the lipids of the liposome affect the analyses of the active substances. To this end, three standard solutions and the other lipids were prepared at 130, 100, and 70% of the usual working concentration, and real concentrations were compared with the theoretical ones. According to the American Association of Official Analytical Chemists (AOAC[20]), the limits for the RSD (coefficient of variation) are established in consonance with the concentration of the analyte in the sample, so it established a limit for the active substances of 2.7% RSD.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02456.

Instrumentation details, statistical parameters explained in the text, graphical chromatograms from validation, and graphical examples of real analyses (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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