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“ You never fail until you stop trying”

Albert Einstein

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ABSTRACT

Quercetin, the main flavonoid found in vegetables and fruits has been described to have wide range of health benefits with antioxidant/anti-inflammatory properties. Due to its low aqueous solubility and low bioavailability, this natural compound requires a drug delivery system. Liposomes, which are concentric lamellar vesicles mainly composed of phospholipids are well-known nanocarriers for drug delivery with non-toxic characteristics and biocompatible with cells, are very attractive for transporting quercetin. However, to be efficiently inhalable, an excipient that confers aerodynamic characteristics is necessary. Therefore, the objective of this thesis is to convert quercetin encapsulated in liposomes into quercetin-loaded liposomal dry powder formulations for the treatment of inflammatory lung diseases using a sustainable process – supercritical CO_2 - assisted spray drying (SASD).

In this way, liposomes were firstly prepared with different surface electric charges (neutral, positive, negative) varying the lipid-to-quercetin molar ratios (10:0.5, 10:1 and 10:1.5). Moreover, PEGylated liposomes were produced due to their well described prolonged time in blood circulation and their ability to significantly improve the delivery of drugs to the desired target. Herein, it was possible to prepare non-PEGylated positive charge formulations (10:0.5 (mol:mol) quercetin-to-lipid ratio) with 92 % lipid yield and 99 % incorporation efficiency. Next, the best formulations were selected considering the incorporation efficiency of quercetin, the size and polydispersion index of liposomes and the lipid yield, for further processing in the SASD. The non-PEGylated liposomal formulations with a positive superficial charge (10:0.5 mol:mol) were atomized using as excipient trehalose and leucine, producing nano-in-microparticles with an average diameter of 5.91 μm and a water content of 5 %. Physicochemical characterization suggested that liposomes are encapsulated in the powder. Further aerodynamic studies have shown that non-PEGylated positive charge formulations (10:0.5 mol:mol) of dry powders are suitable for inhalation because they have a fine particle fraction of 46 % and an average aerodynamic diameter of 1.37 μm. The powders produced by SASD, after the rehydration of excipients and resuspension of liposomes, were characterized to verify the incorporation efficiency of quercetin, as well as the size and polydispersion of liposomes. It was concluded that the resuspended liposomes presented an average size of 208 nm, with a lipid yield of 30 % and an incorporation efficiency of quercetin of 42 % (non-PEGylated liposomal formulation made with a positive charge lipid 10:0.5 mol:mol) having better incorporation efficiency results.

Keywords: Quercetin, Liposomes, Supercritical CO_2- assisted spray drying.

RESUMO

Quercetina é o principal flavonóide encontrado em vegetais e frutas e foi descrito como tendo ampla gama de benefícios para a saúde e é devido às suas propriedades antioxidantes/anti-inflamatórias. Por ter baixa solubilidade em água e baixa biodisponibilidade, este composto requer um sistema para a sua administração. Os lipossomas, que são vesículas lamelares concêntricas compostas principalmente por fosfolípidos, são os nanotransportadores mais conhecidos para entrega de fármacos e são por apresentarem biocompatibilidade com as células. Por este facto, são bastante atractivos para transporte da quercetina. Contudo, para serem eficientemente inaláveis é necessário um excipiente que confira características aerodinâmicas. Desta forma, o objetivo desta tese é converter quercetina encapsulada em lipossomas numa formulação lipossomal na forma de pó seco para o tratamento das doenças inflamatórias pulmonares usando uma processo sustentável – secagem assistida por dióxido de carbono supercrítico (SASD).

Desta forma, prepararam-se, primeiramente lipossomas com diferentes cargas elétricas de superfície (neutras, positivas e negativas) variandos as razões molares de lípido e quercetina de acordo com 10:0.5, 10:1 e 10:1.5. Além disso, produziram-se lipossomas PEGuilados, por forma a aumentar o tempo na circulação sanguínea e melhorar significativamente a entrega de fármacos para o alvo desejado. Foi possível preparar formulações não-PEGuiladas de carga positiva e rácio molar 10:0.5 com 92 % de rendimento lipídico e 99 % de eficiência de incorporação. De seguida, as melhores formulações foram selecionadas tendo em conta a eficiência de incorporação da quercetina, o tamanho e o índice de polidispersão dos lipossomas e o rendimento lipídico para posterior processamento no SASD. As formulações lipossomais não- PEGuiladas com carga superficial positiva e razões molares de lípido e quercetina de 10:0.5 foram atomizadas usando como excipiente trealose e leucina, produzindo-se nano-em-micropartículas com um diâmetro médio de 5.91 µm e uma massa de água residual de 5 %. A caracterização físico-química sugeriu que os lipossomas estão encapsulados no pó. Posteriores estudos aerodinâmicos demonstraram que formulações não-PEGuiladas de carga positiva (rácio molar 10:0.5) de pós secos são adequadas para inalação pois apresentam uma fração de partícula fina de 46 % e um diâmetro aerodinâmico médio de 1.37 µm. A partir dos pós produzidos no SASD, após a re-hidratação dos excipientes e ressuspensão dos lipossomas, estes foram caracterizados com o objectivo de verificar a eficiência da incorporação da quercetina, assim como os tamanhos e polidispersão dos lipossomas. Conclui-se que os lipossomas ressuspensos apresentaram um tamanho médio de 208 nm, com o rendimento lipídico de 30 % e uma eficiência de incorporação da quercetina de 42 % (formulação PEGuilada possuindo lípidos com carga positiva e com rácio molar 10:0.5) e que as formulações carregadas positivamente apresentam melhores resultados de eficiência de incorporação.

Palavras-chave: Quercetina, Lipossomas, Secagem assistida por CO_2 Supercrítico.

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LIST OF ABBREVIATIONS

ACI	Andersen Cascade Impactor
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infra-Red
Chol	Cholesterol
CO_2	Carbon Dioxide
COPD	Chronic obstructive pulmonary disease
DDS	Drug delivery system
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG	1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt
DoE	Design of Experiments
DPF	Dry Powder Formulation
DPI	Dry powder inhalers
DSC	Differential Scanning Calorimetry
DSPE-PEG	2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt 2000
D_V	Volume mean diameter
EPC	L-α-phosphatidylcholine
EtOH	Ethanol
FDA	Food and Drugs Administration
HPLC	High-Performance Liquid Chromatography
IE	Incorporation Efficiency
KF	Karl-Fischer coulometer
MDI	Pressurized metered-dose inhalers
MLV	Multilamellar Vesicles
MMAD	Mass Median Aerodynamic Diameter
NLC	Nanostructured lipid carriers
OLV	Oligo Lamellar Vesicles
PAT	Process Analytical Technology

PdI	Polydispersity index
QbD	Quality-by-Design
Q_Lip	Quercetin loaded liposomes formulations
Q_Lip-DPF	Quercetin loaded liposomes dry powder formulations
RA	Risk Assessment
SA	Stearylamine
SASD	Supercritical CO2 -Assisted Spray Drying
$ScCO_2$	Supercritical CO_2
SCF	Supercritical Fluid
SEM	**S**canning Electron Microscopy
T_g	Glass transition temperature
T_m	Phase transition temperature of the phospholipids
XRPD	X-Ray Powder Diffraction

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1. INTRODUCTION

1.1 Context and Motivation

Globally, more than 500 million people are suffering from inflammatory lung diseases and, as a consequence, an estimated 4.2 million people die each year worldwide [1-2]. The most well-known inflammatory lung diseases are pneumonia, asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, among others, with a vast majority appearing during childhood [3-4]. Currently, humanity is crossing a pandemic crisis (COVID-19) originated by the new coronavirus (SARS-CoV-2), where essentially who have chronic respiratory diseases are more vulnerable, at a higher risk of severe disease, or even their death [5]. For the treatment of the inflammatory process of these infections, anti-inflammatory drugs, such as corticosteroids, are often used. It has been reported that for situations where inflammation is not completely resolved, higher doses are required. Moreover, these treatments might be long-term, causing adverse effects such as osteoporosis, diabetes, adrenal suppression and cardiovascular disease. To overcome this issue it is urgent to produce more natural and safe medicines to replace this pulmonary therapy [2]. Such can be accomplished through the use of biopharmaceuticals, that have become very attractive in the pharmaceutical market [6].

This work purposes the development of newly drug delivery system (DDS) based on a natural origin anti-inflammatory drug, a flavonoid namely quercetin, which has a wide collection of pharmacological properties [7]. This formulation will be encapsulated into liposomes, one of the most successful nanoparticle DDS [8]. Furthermore, the so developed DDS will be encapsulated in a dry powder to allow inhalation therapy, that is very attractive to treat respiratory diseases [9].

1.2 Inflammatory lung diseases

The lung is considered the most vulnerable internal organ to infections and injuries since it is permanently exposed to particles, chemical compounds and organisms from the air, which can cause inflammatory lung diseases [10]. These diseases may be classified as chronic or acute [11]. Generally, they appears during childhood and essentially affects the airways, lung tissues, or blood circulation into and out of the lungs [3]. Regarding most well-known inflammatory lung diseases, pneumonia is characterized by an acute infection in the lungs. The inside of the alveoli has pus, making limited and painful breathing. The main causes of this infection are bacteria and viruses. Besides, pneumonia remains the leading infectious death of children worldwide [12-14]. Asthma is defined by inflammation and narrowing of the bronchi, compromising the air flow into the airways. This inflammatory disease is also associated with rhinitis, caused by an inflammation in the nasal mucous. Studies indicate that children with asthma may have abnormal lung growth at the risk of developing chronic obstructive pulmonary disease [10-11]. COPD, which includes emphysema and chronic bronchitis is characterized by the excessive production of mucus, fatigue and difficulty breathing. Alveoli become less efficient at bringing oxygen and transporting carbon dioxide out [11]. At last, cystic fibrosis is known as the most important lethal genetic and rare disease. This progressive disease is described by the modification of mucus in the body and may affect other organs besides the lungs, such as the liver, intestinal and pancreas [15-16]. The composition of mucus is thick and viscous, instead of being oily, resulting in the airway obstruction. Several reports claim that cystic fibrosis is caused by a genetic mutation that normally regulates the level of chlorine in cells, restricting the ability to breathe over time [16]. This mutation results in the malfunction of the gene, causing a change in mucous composition and increased chlorine in sweat [17].

Table 1.1 shows the number of infections and deaths worldwide in 2019 by inflammatory lung diseases. Regarding cystic fibrosis, this disease affects near 100 000 people worldwide and the numbers of 2019 only consider the United States of America [16,18]. Pneumonia is an inflammatory disease that affects more population. However, COPD is the one of the respiratory diseases that causes more deaths in the world.

Table 1.1- Number of infections and deaths in 2019 for Asthma, COPD, and Cystic Fibrosis [16,19-23].

Year	Disease	Nº of infections	Nº of deaths
2019	Asthma	262 000 000	461 000
	COPD	384 000 000	3 023 000
	Pneumonia	450 000 000	2 500 000
	Cystic fibrosis	31 199	373

Unfortunately, for these respiratory pathologies, a cure has not been found yet. Still, anti-inflammatory drugs are usually used to treat such conditions [11]. Anti-inflammatory drugs such as corticosteroids or glucocorticoids are a group of steroid hormones derived from the cortex of the adrenal gland. Cortisol or hydrocortisone is the natural hormone produced by humans. This hormone is essential for our well-being as it acts on glucose metabolism, the metabolic functions of the body, on healing and the immune and cardiovascular systems [24].

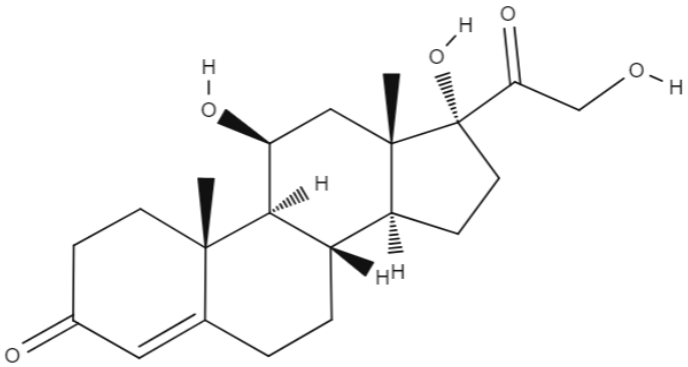

Figure 1.1 - Chemical structure of cortisol (Adapted from [25]).

The drugs currently used for such therapies have a synthetic origin. ***Table 1.2*** shows some of these corticosteroid with some of the pharmacological characteristics. Of all synthetic corticosteroids, these are more potent compared to natural corticosteroids, except hydrocortisone, which has an identical potential to the natural hormone [24].

Table 1.2- Principal pharmacological characteristics [26].

Glucocorticoid	Plasma half-life (Minutes)	Biological half-life (Hours)	Relative Power (Comparison with natural cortisol)		Equivalent dose (Mg)
			Anti Inflammatory	Mineralocorticoid	
Hydrocortisone	80-120	8-12	1	1	20
Prednisone	200-210	12-36	3.5-4.0	0.8	5
Prednisolone	120-300	12-36	4	0.8	5
Deflazacort	120	24-36	2.5-3.5	0.25	7.5
Methylprednisolone	200	12-36	5	0-0.5	4
Triamcinolone	0	12-36	5	0	4
Dexamatesone	300	36-72	30	0	0.75
Betamethasone	300	36-72	30	0	0.6

These anti-inflammatory drugs, though very useful, can be extremely harmful. In long-term treatment situations or by increased dose, they may cause unwanted adverse effects in our body. ***Table 1.3*** represents all the adverse effects caused by the excessive use of synthetic corticosteroids [2].

Table 1.3-Adverse effects caused by excess synthetic corticosteroids [26].

Regions	Effects
Adrenal gland	Adrenal atrophy, Cushing's syndrome
Skeletal muscle system	Myopathy, osteoporosis, osteonecrosis, growth retardation
Cardiovascular system	Thrombosis, hypertension
Central nervous system	Changes in behavior, cognition, mood and memory, brain atrophy
Digestive system	Gastrointestinal bleeding, peptic ulcer, pancreatitis
Immune system	Immunosuppression, latent virus binding
Skin	Cutanea atrophy, erythema, pectoral dermatitis, acne, stretch marks, delayed healing
Eyes	Cataract, glaucoma
Kidneys	Increased sodium retention and potassium excretion
Other	Hyperglycemia, increased risk of serious infections

To overcome these side effects, the scientific community has studied more natural and safer medicinal therapies for humans. The use of medicines from plants has acquired a lot of interest and consequently a growth in the market, due to its vast biological diversity and variety of chemical structures [1]. Phytoconstituents or phytochemicals are bioactive compounds, which play a protective role against infections or pathogens of the plants. Moreover, some of these compounds are responsible for some properties, such as color and smell [27]. At last, these compounds have anti-inflammatory/antioxidant benefits, playing a preventive role in cancer, cardiovascular and respiratory diseases, the main causes of death worldwide [1].

Many groups of phytoconstituents are categorized by polyphenols, alkaloids, terpenoids, organosulfur compounds and phytosterols [28]. Until now 8.000 polyphenolic compounds were identified with numerous health benefits, arousing interest in various industries, such as in food and pharmaceuticals [28-29]. Polyphenols are constituted with one or more aromatic rings and at least one hydroxyl group [28].

The main classes of polyphenols include phenolic acids, flavonoids, and non-flavonoids and these are classified by their chemical structure [29]. ***Figure 1.2*** shows the various classes and sub-classes of this phytochemical group.

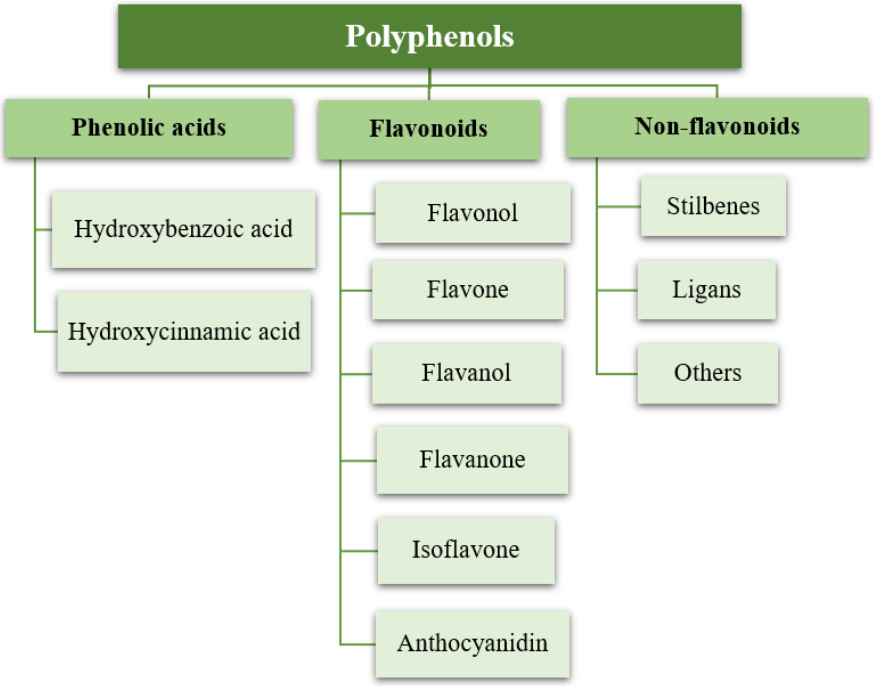

Figure 1.2 - Classification of polyphenols (Adapted from [29]).

The most therapeutic phytoconstituents are flavonoids, a group of natural compounds, which can be found in vegetables, fruits and plants. There are six different types of flavonoids, and each represents different structures. These are rich in antioxidant activities as well as engagement the toxins and stresses of the daily. Besides, flavonoids have anti-inflammatory action, helping the body to reject or decrease the existent inflammatory reaction [29].

1.3 Quercetin

Quercetin is the most abundant flavonoid in healthy food [30]. This hydrophobic flavonol is known to present unique biological properties and it has an appearance of yellowish color-bright lemon [31]. Furthermore, quercetin has a role in the anti-aging combat, anti-cancer properties and helps in the physical activity performance and blood sugar control. Since it has a wide range of health benefits, this flavonol is a strong bet for the development of new and more efficient medicines [32-33]. Nonetheless, quercetin easily decomposes when in contact with oxygen, temperature and ultraviolet light, decreasing the important health benefits [34]. Another limitation of this phytochemical is its low aqueous solubility, and a consequent low bioavailability and a reduced bioactivity, limiting its use in medicinal therapy.

Hence, it is necessary to resort to a drug delivery systems for quercetin's chemical and biological protection [35].

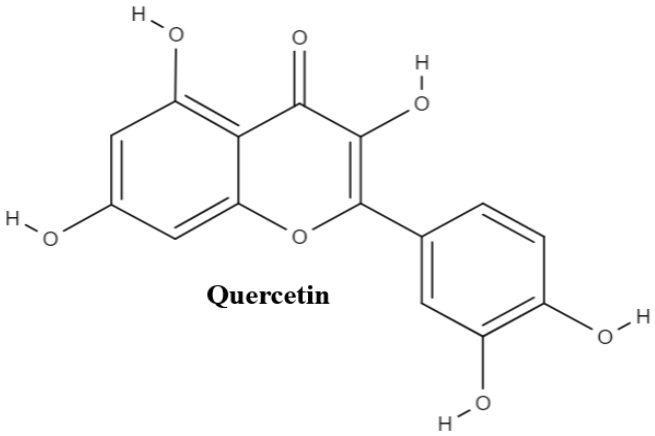

Figure 1.3 - Chemical structure of quercetin (Adapted from [36]).

1.3.1 Drug delivery systems for quercetin

There are many groups of DDS from a natural or synthetic origin. The DDS are developed to improve the therapeutic properties of pharmaceuticals [37]. Furthermore, the capacity of the DDS to transport and consequently release the drug after administration is an important parameter to regard [29]. ***Figure 1.4*** presents several examples of DDS at the nanoscale.

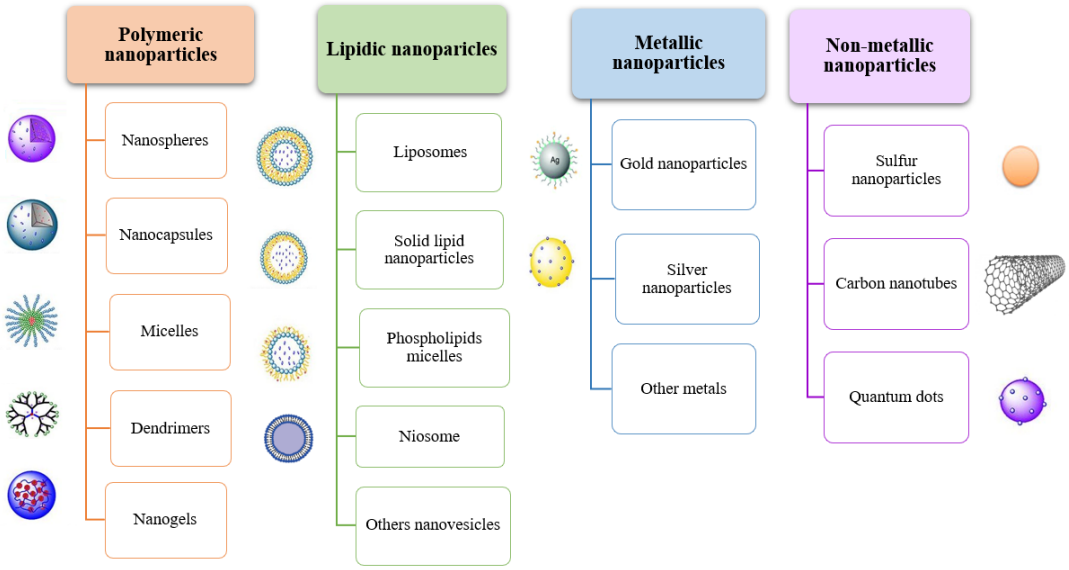

Figure 1.4 - Different groups of nanocarriers used for drug delivery systems (Adapted from [38]).

Recent studies report how polymers and lipids may be considered essential components to produce improved therapeutic formulations. In the 19th century, these compounds started to be used for the preparation of pills and capsules. Nowadays, with the advancement of medicine, there is an open range of natural, synthetic, or semi-synthetic lipids and polymers produced in various pharmaceutical grades [39]. Although these components have different physicochemical properties, they are used in a similar application [40]. Regarding lipids, carriers such as micelles, liposomes, phytosomes and ethosomes can be used [2]. For quercetin delivery, a few types of lipid-based carriers have been proposed - ***Figure 1.5***. Despite being composed of lipids, they present different physical states and compositions [41]. Recently, lipid-based nanoparticles have been used to develop vaccines against **COVID-19** [59].

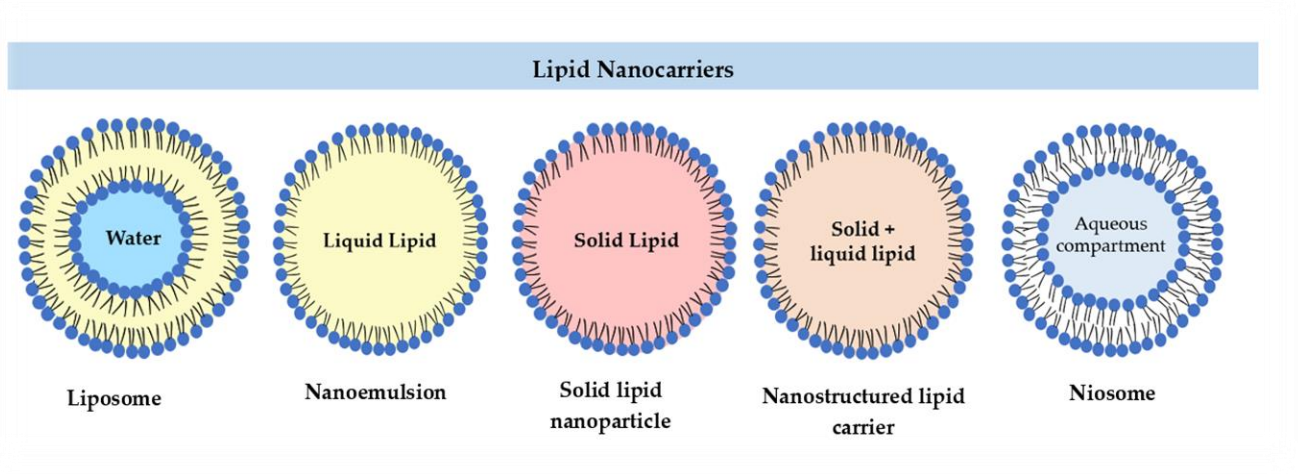

Figure 1.5- Types of lipid carriers used for quercetin (Adapted from [38]).

In the last years, there has been a greater interest in exploring proteins and their interactions with the different biologically active compounds to develop new wellness products [42]. Proteins and peptides have a lot of important roles in several biochemical processes in the body and as result, they are frequently used to combat various diseases like cancer and diabetes [43]. Moreover, recent studies propose quercetin-protein formulations to improve the performance of flavonoids. Stefania *et al.* [42], Zahra *et al.* [37] and Ho-Kyung Ha *et al.* [44], made a study with β-lactoglobulin peptide as novel drug delivery systems for flavonoids. This peptide performs important roles in binding and transportation to target places of different ligands, like fatty acids, cholesterol, among others. Therefore, the results obtained were positive and give some benefits to discovering the bioactive potential of both flavonoids and bioactive peptides, for the development of formulations with improved functional properties [42].

1.3.2 Liposomes

Liposomes, discovered in the 1960s by Bangham, are vesicles composed essentially by phospholipids. Phospholipids, constituted by a hydrophilic head and hydrophobic tails, form a liposomal structure characterized by an inner aqueous core involved by one or more phospholipids layer [2,45]. Besides, the hydrophobic interactions of these lipid bilayers are defined by the Van der Waals forces (the attraction and repulsions between molecules), which hold apolar tails jointly [46-47]. Meanwhile, hydrogen bonds and polar interactions between water molecules in an aqueous environment and the hydrophilic interactions stabilize the organization. ***Figure 1.6*** represents the chemical structure of a phospholipid molecule.

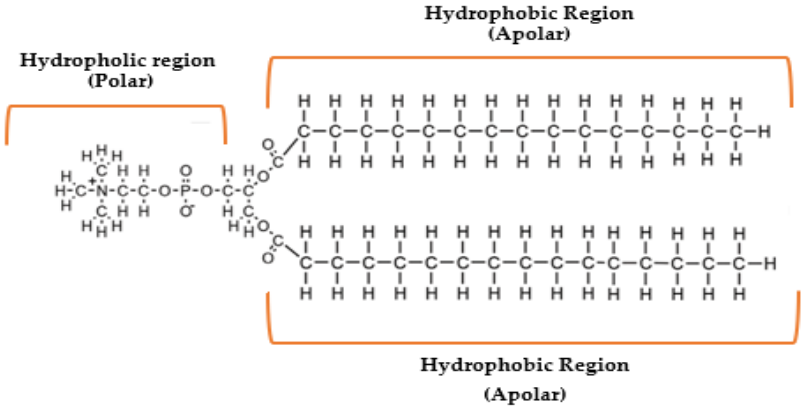

Figure 1.6 - Chemical structure of phospholipid molecule (Adapted from [48]).

Liposomes have a high level of biodegradability, biocompatibility and non-toxicity with cells since the cell membrane is also constituted by a lipid bilayer, having attracted a huge interest as DDS/carriers for vaccines, nutrients, cosmetics and other bioactive agents [49-51]. Moreover, the liposomal formulations improve the drug availability, increasing the drug distribution and penetration into cells [40]. Furthermore, these systems may enhance the therapeutic efficacy, preserving the minimum side-effects for a prolonged time [52]. Due to their physical and chemical properties, liposomes can encapsulate hydrophilic compounds inside the inner aqueous core and incorporate hydrophobic drugs into the lipid bilayers, as represented in ***Figure 1.7*** [48].

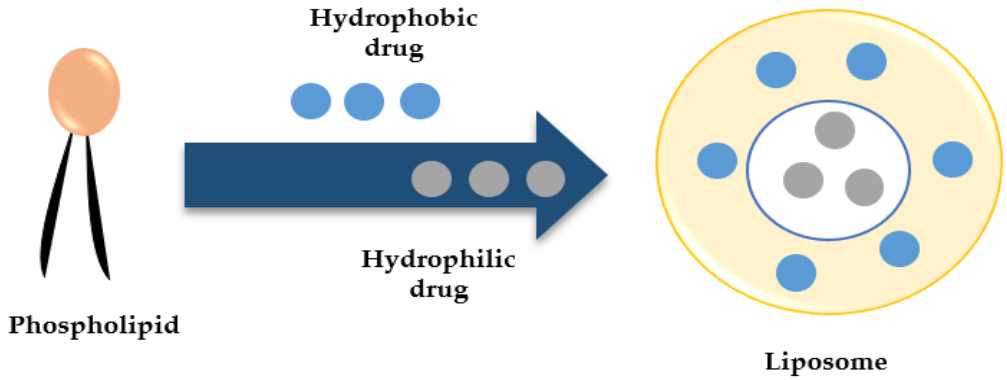

Figure 1.7 - Liposome formation and encapsulation of drug molecules (Adapted from [48]).

The phospholipids used to prepare liposomes may be obtained from natural sources like egg yolk, soya bean and olive oil, or by synthetic sources. Moreover, synthetic phospholipids present more rigidity. The most common synthetic and natural phospholipids used in the formulation of liposomes are present in ***Table 1.4*** [45].

Table 1.4 – Group of phospholipids used in the formulation of liposomes [45,53-54].

Phospholipids
Dioleoyl phosphatidylethanolamine (DOPE)- synthetic
Phosphatidylethanolamine (PE)
Phosphatidylserine (PS)
Phosphatidylinositol (PI)
Dipalmitoyl phosphatidylcholine (DPPC)- synthetic
Phosphatidylglycerol (PG)
L-α-phosphatidylcholine (EPC)
Di-stearoyl phosphatidylcholine (DSPC) - synthetic
Phosphatidic acid (PA)
1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) - synthetic
1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG)- synthetic

Cholesterol can be included in the formulation of liposomes, as it increases the stability of liposomal systems while diminish *in vitro* drug release by enhancing the rigidity of the fluid membrane and also reducing lipid permeability [55-56]. Furthermore, the content of cholesterol could control the stoutness in the liposomal composition [54]. Cholesterol, named in 1815 by Michel Chevruel, is used in mammals to produce cell membranes and hormones [57-58].

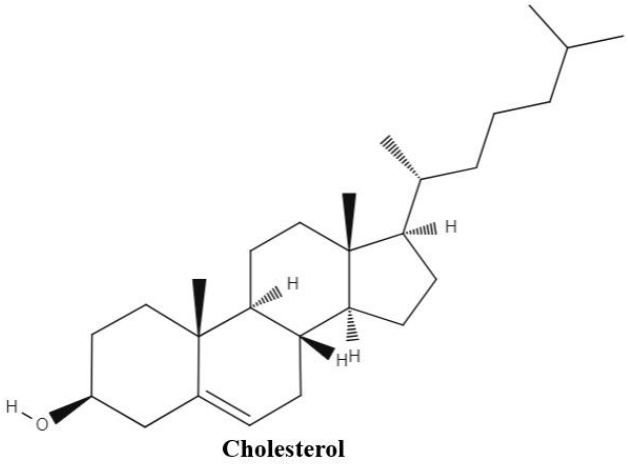

Figure 1.8 - Chemical structure of cholesterol (Adapted from [59]).

Regarding the size of the liposomes, these can be produced from nano to micro scale, being a fundamental parameter to take into account in different applications used [49, 60]. Yet, liposomes up to 150 nm have many advantages as they are capable of escaping from circulation and target the sites of inflammation and can accumulate on inflamed regions, taking benefit with the permeability and retention effect (EPR) [60-61]. Besides, the liposomes may present different surface charges and lamellarity, which is defined by the number of double layers of phospholipids near the inner aqueous core. ***Table 1.5*** shows the classification of liposomes regarding their size and lamellarity.

Table 1.5- Classification of liposomes regarding their size and lamellarity [49].

Dimensions		**Lamellarity**	
Name	**Size Range (µm)**	**Name**	**Number of Layers**
Single Unilamellar Vesicles (SUV)	0.02-0.20	*Oligo Lamellar Vesicles (OLV)*	< 5
Medium Unilamellar Vesicles (MUV)	0.20-0.50	*Multi Lamellar Vesicles (MLV)*	5-20
Large Unilamellar Vesicles (LUV)	0.50-10	*Multi Vesicular Vesicles (MVV)*	>50
Giant Unilamellar Vesicles (GLV)	100-200		

The permeability of the liposomes is a major physical characteristic on liposomal technology since this has a higher influence on the action target of the compounds. Then, their permeability is enhanced when the lipid bilayer is converted into a fluid phase, designated as the phase transition temperature of the phospholipids (T_m) [62]. This phase transition temperature has a significant effect on the drug encapsulation efficiency and stability [63]. Moreover, when the temperature is close to T_m, the phospholipids become loose and consequently resulting in more free spaces. Meanwhile, when the temperature is below**,** the phospholipids are nearly packed and expanded, being unable to form liposomes [48,62]. Thus, the length and saturation of the lipid chain have an impact on the value of T_m**.** Therefore, different composite membranes by different lipids may exhibit different levels of fluidity at the same temperature [64].

Nowadays, liposomes can be prepared by diversifying methods. These methods may be divided into two categories, **conventional methods**, which are easier to use and **novel methods,** more advantageous on large scale, but that require special equipment. Nonetheless, the generation of liposomes is not spontaneous, requiring always an external force, likewise an incorporation of buffer solution or water under constant agitation, resulting in these vesicles [48,65]. Besides, the major advantages and disadvantages of the most common liposomes production are presented in ***Table 1.6***.

Conventional Methods:

- **Thin-film hydration (Alex Bangham, 1965) -**This technique also named the Bangham method, is the simplest and oldest one used in the liposome technology [63]. The process consists of the dissolution of a lipid in an organic phase such as chloroform, followed by removal generally using evaporation. Afterward, a thin lipid layer is formed on the walls of the volumetric balloon. However, in this method, the encapsulation efficiency is low, especially in water-soluble active agents [49, 66-67].

- **Ethanol/ Ether Injection Method (Batzi and Korn, 1973)-**The ether and ethanol process consists of the dissolution of the lipid into an organic phase and the injection of the lipid solution into an aqueous media, creating liposomes. It is used a needle to inject the lipidic solution. Besides being a simple, if an homogeneous mixture is not reached, it is generally associated with the formation of heterogenic liposomes, due to the low solubility of some lipids in ethanol. Furthermore, in the method of ether injection is different, since the ether is immiscible with the aqueous phase. The ether injection technique has more advantages since the liposomal product is more concentrated and has a greater encapsulation efficiency [49,66].

- **Detergent Removal Method-** This technique consists in the formation of a lipid detergent and consequently the removal of the detergent, forming a wide variety and homogeneous liposomes. However, in this process, the concentration of liposomes in the solution and the entrapment of some hydrophobic compounds are low. In addition, the homogeneity and size of liposomes are related to the percentage of detergent removed [66].

- **Reverse Phase Evaporation Method (Szoka and Papahadjopoulo,1978)-** This process consists of dissolving lipids in an organic solvent adding an amount of water, to form an emulsion of water into the oil. Since the phospholipids are constituted by a hydrophilic and hydrophobic layer, they install at the interface between oil and water, forming inverted micelles. Then the solvent is eliminated through a rotary evaporator and the micelles are converted into a layer of gel, where the liposomes will form. In this technique, the compound to be encapsulated will be in contact with the organic solvent, which presents a disadvantage for more fragile molecules such as peptides [49, 66].

Novel Methods:

- **Microfluid Channel Method (Jahn et al, 2007) -**This technique was developed to control the formation of liposomes. This process involves a chain of lipid dissolved in alcohol, where it passes between two aqueous currents in a microfluidic canal, forming liposomes. The size of liposomes can be controlled through laminar flow in the canals [49, 66].

- **Supercritical Fluid Injection and Decompression Method (Castor and Chun, 1994)-** In the injection method, the lipid compound, organic solvent and compressed gas are injected with the aid of a nozzle in an aqueous solution. However, the decompression method consists of the junction of the lipid compound, an organic solvent in an aqueous solution, where they are decompressed through a spout, thus forming the liposomes. In addition, in the injection method, the compressed phase is sprayed into the water and in the decompression method, the aqueous phase is incorporated into the compressed phase, where it is sprayed into the air. Finally, through these processes, it is possible to produce sterile liposomes with the recommended size [49, 65].

- **Supercritical Reverse Evaporation Process (Otake et al,2001) -**This method where the lipid mixture, organic co-solvent and compressed gas are stirred at a lipid phase transition temperature. Liposomes are produced by low pressure and consequently release of compressed gas. However, the efficiency of imprisonment released is low. Recently,

Otake et al improved this technique in order to avoid the use of organic solvents and increase their stability and performance [49, 66].

Table 1.6.- The principal advantages and disadvantages of the most common liposomes production methods [66].

Methods	Advantages	Disadvantages
Thin-film hydration	Simple process	Contains the organic solvent, Large vesicles without controlling on particle size
Ethanol/Ether injection	Simple process	Organic solvent residue
Detergent Removal	Simple design Homogenous product Control of particle size	Contains the organic solvent Detergent residue Poor entrapment efficiency Low yield
Reverse phase evaporation	Simple design, Suitable encapsulation efficiency	Not suitable for encapsulation of fragile molecule
Microfluidic Channel	Control of particle size	Not suitable for bulk production Organic solvent use
Supercritical fluid injection and decompression	Control of particle size, Low organic solvent consumption	High cost Low yield High pressure
Supercritical reverse-phase evaporation	No need for using nozzles, Low organic solvent consumption Enhance stability	High cost High pressure

i) PEGylated liposomes

To enhance the delivery capacity of the liposomal systems, compounds with hydrophilic properties namely 2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt 2000 (DSPE-PEG) are added to the lipidic bilayer. DSPE-PEG, which has a polymer (PEG) covalently bound to lipid (DSPE), has proven to have a prolonged long-time in blood circulation and can improve significantly the delivery of active molecules to the desired target. Moreover, characteristics such as higher biocompatibility and amphiphilic properties can be applied in diversified nanoparticles [68]. In fact, the presence of PEG renders some advantage to the liposomes in escaping the mononuclear phagocytic system. This system is composed of macrophages, dynamic cells, with a huge role in the protection of the body against foreign bodies and inflammation resolution supplying cells phagocytic in inflammatory processes followed by cytokines or proteins [64,69]. Yet, it is reported that when PEG is shielding the liposomes, macrophages do not interact with the liposomes as represented in ***Figure 1.9*** [70].

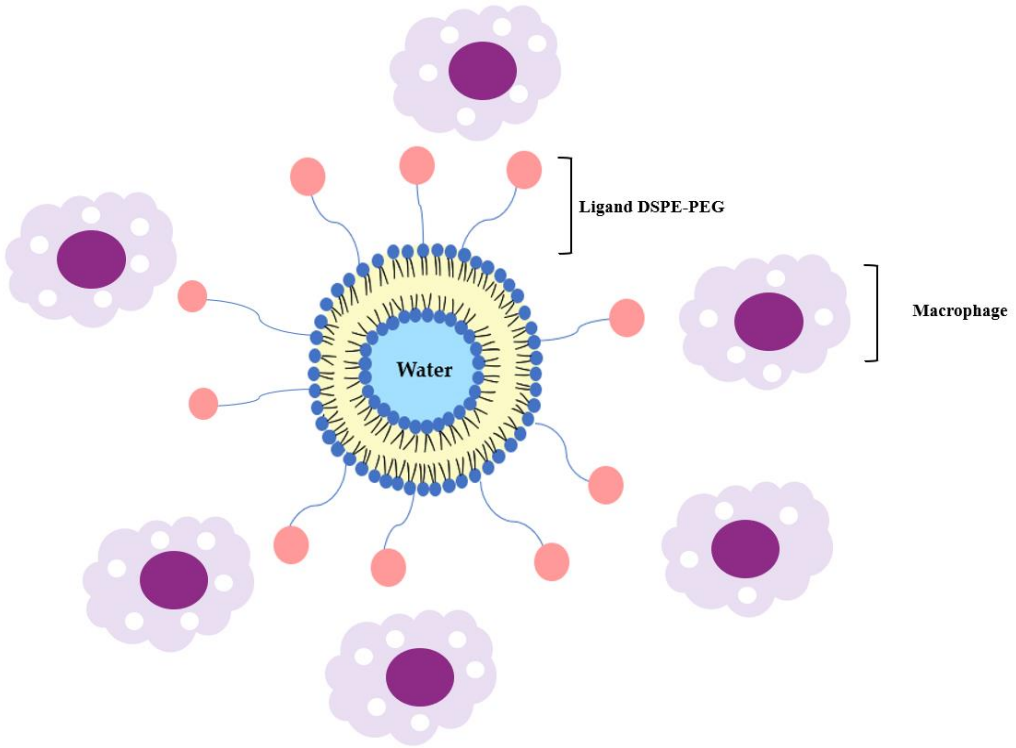

Figure 1.9- Structure of PEGylated ligand-targeted liposomes and macrophages (Adapted from [68]).

1.3.3 Liposomes based systems for quercetin delivery

Previous studies with liposomal systems incorporated with quercetin were performed. ***Table 1.7*** represents the characterization of the Q_loaded liposomes obtained in the different studies. It can be reported that the previous work used the same techniques of production of liposomes and also, the values obtained were promising results, essentially in the incorporation of efficiency.

Table 1.7 - Liposomal systems for quercetin delivery [36, 71-74].

Lipid Constitution	Methods	Size (nm)	Zeta Potential (mV)	PdI	*IE*(%)
Soy lecithin	Thin-film hydration Sonication Extruser	134.2 ± 3.4	-23.1 ± 2.1	0.219 ± 0.03	70.4 ± 2.9
Phosphatidylcholine (SPC)	Thin-film hydration Sonication	194.4 ± 28.5	-4.9 ± 0.7	0.35 ± 0.05	85.1 ± 4.6
Lipoid S 75 :70% phosphatidylcholine, 10% phosphatidylethanolamine	Thin-film hydration Sonication	94.4 ± 6.5	-43.4 ± 4.1	0.14	67.2 ± 4.1
Lipoid S 75	Thin-film hydration Sonication	116 ± 5.3	-9 ± 0.4	0.35	52 ± 4.4
Lipoid S 75	Thin-film hydration Sonication	80	-38	≤ 0.10	72.5 ± 7.3
Lipoid S 75	Thin-film hydration Sonication	80	-38	≤ 0.10	~70

1.3.4. Stability of liposomes-based systems

Although liposomes are a strong bet, they present some obstacles with their stability and the needing for a cold chain during distribution [75]. Liposomes can present instabilities, especially in aqueous dispersions, diminishing the benefit condition or even their degradation. These may suffer chemical degradation, likewise oxidation and hydrolysis, physical and also biological degradation [64,75].

Chemical degradation

Oxidation - Despite all types of fatty acids are vulnerable to oxidation, unsaturated fatty acids are more susceptible due to the presence of double bonds in the lipid tails. Nonetheless, saturated fatty acids can also be oxidized at high temperatures. The unsaturated fatty acids are the most susceptible ones to radical generation, since the unsaturation allows the movement of the unpaired electron across the lipid chain [53,65]. Besides, the presence of oxygen develops more the generation of hydroperoxides fission of the fatty acids. This process can be decelerated using appropriate antioxidants or stored from protection light, low temperatures or oxygen atmosphere [75]. The outstanding materials more common are hydroperoxides and EDTA to form a complex with transition metal ions. Moreover, the antioxidants used are butylhydroxytoluene (BHT) and a-tocopherol [53].

Hydrolysis – The hydrolysis of ester bonds results in lysophospholipids, free fatty acids and phosphoglycerols compounds. It can be catalysed by base or acid. Besides, hydrolysis most occurs at low levels of pH (around pH= 6). Plus, the presence of water in liposomes formulations and the required refrigeration storage [65]. This reaction may affect several properties likewise, temperature, ionic strength, among others [53]. The use of lipids ester-linked is can be a useful solution [65].

Physical degradation:

The physical stability includes the changes in the average particle size and size distribution, aggregation and fusion of membrane bilayers or even loss of the encapsulated drug [75]. Changes in the average particle size and size distribution of liposome dispersion occur due to aggregation and fusion and these affect the pH, medium and lipid composition. Uncharged Liposomes have less stability than charged liposomes. So, the addition of a charge-carrying lipid into the liposomal formulation may prevent or decelerate the aggregation [53, 65]. Moreover, the medium hydration should not contain polyvalent cations, which induce aggregation and consequently fusion of the liposomes. This effect can be overcome by the use of chelating agents likewise EDTA, as reported by Lichtenberg *et al*. [53]. The loss of the encapsulated water-soluble drugs depends on the composition of the liposome as well as the size and the physical state of the bilayer-forming lipids (a gel or liquid crystalline). Generally, in a gel state, the leak is slower than from a liquid crystalline state [53].

Biological degradation:

One of the most requirements for the use of liposomes as drug carriers is that they must circulate and keep the drug for sufficient time for effective access and interaction with the target, normally in the blood, in the capillary walls. The major disadvantage of liposomes use is their quick removal from the blood circulation due to adsorption to plasma proteins by phospholipids and triggering recognition and capture of liposomes by the mononuclear phagocytic system [64].

All these aspects affect the quality of formulations and can cause unwanted side effects. In response to this challenge, converting into a dry powder formulation (DPF) overcome this problem. Thus, the stabilization is achieved, its shelf-life increases and consequently the need for a cold chain is eliminated [75].

1.4 Drying processes and strategies used for stabilization

Pulmonary administration has been growing in the pharmaceutical industry [75]. Even though oral administration still has a massive market share, around 60 %, the inhalation way has become more desirable due to the evolution of nanotechnology and also the growth of people with respiratory pathologies [9,76-77]. Pulmonary administration accrues many advantages such as the potential utility of the lung as a gateway for the entry of peptides and proteins due to the large surface area available for absorption. As a result, the lungs have low metabolic activity, fast action, high bioavailability and may cause fewer unwanted side effects [78]. Besides that, the pulmonary administration avoids the effect of the first passage to the liver, escaping the liver degradation and improving the therapeutic effect [79]. Due to the many advantages of the pulmonary route, the development of dry powder systems for this privileged organ has acquired a great investment [80]. There are 3 types of inhalation systems that have been developed which include nebulizers, pressurized metered-dose inhalers (MDI) and dry powder inhalers (DPI). Nebulizers were the first devices developed for the inhalation therapy market. The MDI have been the pillar of pillar of pulmonary therapy and were developed in the 50s to improve the drug efficiency and the shorten time. However, the MDI is not environmentally friendly and only a little fraction of the drug passes the lung. So, DPI devices were developed with the aim to disperse dry particles as an aerosol. The mechanism works as follows, inhalers are triggered by breathing detect inhalation of the patient through the actuator and therefore trigger the inhaler automatically. These are elected for their stability and processing, as they are usually formulated with mixtures of solid particles of a phase [76,78]. Nowadays, there are essentially two types of these inhalers, which include those where the drug is packaged in individual doses, namely single dose or unit-dose devices and those that cover multiple doses, namely multi-unit and multi-dose devices. ***Table 1.8***, displays a few types of inhalers attended in the market and ***Table 1.9*** represents the advantages and disadvantages of these types of inhalers [78].

Table 1.8- Examples of market inhalers [76].

Types of inhalers						
	MDI			**DPI**		
Nebulizers	*Press-and breath products*		*Breath-actuated products*	*Single Dose*	*Multiple Unit Dose*	*Multidose*
	Bronchodilators	*Steroids*				
Atrovent	Maxair	Beclazone	AeroBec	Spinhaler	Diskhaler	Turbuhaler
Duovent	Proventil	Beclovent	Beclazone	Rota-haler	Diskus	Easyhaler
Duo-Medihaler	Ventolin	Pulmicort	Maxair	Aerolizer	Aerohaler	Novolizer

Table 1.9- Advantages and Disadvantages of the inhalers [76].

Types of inhaler	**Advantages**	**Disadvantages**
Nebulizers	Alveolar deposition can be easily achieved	Limited portability Difficult to use
MDI	Easy portable Tamper Proof Multidose	Difficult to use Use of propellants
DPI	Propellant-free Easy to use Formulation stability	Complex development and manufacture Dose uniformity problems

The scientific community has been investigating strategies for the stabilization of liposome-loaded formulations during drying operations such as stabilizers agents to combat liposomes aggregation and fusion or even the leak of the drug [60,75]. It has been reported the use of diverse excipients as stabilizers in dry powder formulations for liposome membrane protection. In fact, few theories may explain the mechanisms behind the stabilization activity, including the presence of the excipients in drying processes which may increases the stability of the formulation [75]. Water replacement, vitrification and kosmotropic effects are the major theories implemented on liposomal technology. Water replacement theory, the oldest theory, explains that when the formulations convert into a solid state, the water is remove and replaced with hydrogen chains, which establish hydrogen bonds with phosphates. These links can hold the liposomes, due to the excipient action. The vitrification theory addresses that excipients create a highly viscous glassy matrix surrounding the liposome, which reduces

the molecular mobility during the process. Moreover, the physical properties of the excipients influence the formation of a glassy matrix, which is around the T_g. Meanwhile, above the T_g, the viscosity decreases and consequently the molecular mobility raises, resulting in disruption of the glassy matrix. At last, the kosmotropic effects indicate that excipients bind bulk water, interrupting the structure of the water, reducing the water content [75]. Therefore, groups of carbohydrates are mainly used. Regarding pulmonary delivery, it is necessary to select the best excipients for inhalation already approved by the Food and Drug Administration for respiratory drug delivery [78]. ***Table 1.10*** represents the list of excipients already approved or alternative suggestions for pulmonary delivery [78].

Table 1.10- Excipients already approved or alternative suggestions for pulmonary delivery [78].

Excipients	Description	Status
Sugars:	Coarse/ fine carrier	
Lactose		Approved and commonly used
Glucose		Approved (Bronchodual
Mannitol		Approved(Exubera)
Trehalose		Promising alternative
Amino acids:	Improved aerosol efficiency	Endogenous substance but no data on lung toxicity
Leucine		
Trileucine		
Polymer:	Absorption for proteins and peptides	Pro-inflammatory effect observed
Chitosan,Trimethyl chitosan		
Biodegradable polymers	Used in sustained-release formulations	Immunogenicity effect observed
PLGA		

Regarding carbohydrates, trehalose is a promising excipient, approved by FDA for the pulmonary route for systematic drug delivery selected in most studies [9]. This sugar is defined as such a non-reducer and also a higher glass transition temperature *Tg*, which presents a favorable lipid bilayer through hydrogen bonding [75]. Moreover, the addition of an-amino acids, such as l-leucine may protect against moisture [60]. Yet, l-leucine requires appreciative properties such as the formation of the

crystalline hydrophobic shell, enhancing the aerodynamic action and consequently the reduction of the cohesion of the particles [9].

Several drying technologies have been developed such as freeze-drying, spray-drying, spray-freeze-drying and finally supercritical CO_2 assisted spray drying. Besides that, these techniques are also available for the stabilization of liposomes.

1.4.1 Freeze-drying

It is the most common operation used in dry liposomal dispersions. This process consists of three phases such as freezing and also primary and secondary drying. The freezing phase is characterized by cooling, where most of the solvent is removed from liposomes and consequently forms a block of ice. The primary drying is associated with the reduction of pressure and the increase in temperature to offer enough heat for the liposomal suspension for water sublimation. In the secondary drying, water is desorbed from the frozen formulation at a high temperature and a low pressure. This technique ensures the stability of liposomes as they can influence their structure. As a result, this freezing may cause an increase in the concentration of liposomes, leading to rupture of the liposomal bilayer, in aggregation or fusion. These causes can be diminished by optimization of the process parameters such as freezing temperature, freezing rate and operation time. Moreover, a quick-freezing rate usually results in the formation of thin ice crystals and a homogeneous distribution of the lyoprotector, reducing the rupture of the structure of the liposomal layer. In contrast, when the freezing rate is slow, it can bring benefits, particularly in the prevention of liposome leaks during the freeze-dried process. Since this reduces the osmotic pressure caused by the frozen concentrates generated. The primary and secondary drying phases do not have as much influence regarding the composition of liposomes. In drying processes it is identified what the residual content of the final dry liposome [75].

1.4.2 Spray-drying

This process has been widely used in the production of dry powder formulations, due to its ease of operation, simplicity and ability to produce compound materials. Gil *et al*. [81] reported that this method in 2010 was elected as one of the most captivating in pharmaceutical technology. A large range of products can be achieved, such as very fine powders for inhalation. Spray drying technology is a step in the process in which it is defined by atomizing the feeding of the solution in a spray. The solvent in the atomized is thermally removed in the drying of the particles, which are separated from the air by a filter or cyclone. However, this technique has some disadvantages, notably in the possible degradation of heat-sensitive thin particles [76].

1.4.3 Spray-freeze-drying

Spray freeze-drying is a method recently used in the pharmaceutical industry. This is characterized by the combination of lyophilization and spray drying stations involving the atomization of a feeding solution in a cryogenic medium followed by lyophilization of dispersion. Bi *et al*. [82] applied this technique to the insulin-laden dry liposomal formulation for a pulmonary administration. Lyoprotectors were used as stabilizers of this formulation and the influence of the type and concentration of these stabilizers on the preservation of the drug was specifically studied. Besides, Swweenet *et al.* [83] also used this technique in order to develop a powder formulation containing ciprofloxacin.

1.4.4 Supercritical CO_2 -assisted spray drying

It is possible to make drying processes using a supercritical fluid, in this case, CO_2. These processes have appeared as a green alternative since the environment is a huge concern in present days [9]. Besides that, the $ScCO_2$ processes combined with nanotechnologies give some advantages on the development of essentially wellness products and are also modern and green alternatives for an extensive variety of processes such as the solubility improvement of low-soluble drugs, polymer plasticization, surface modification and chromatographic extraction [76,84]. Supercritical fluids (SCFs) are characterized as compressed liquids or gases that are above critical temperatures and pressures [76], which results in liquid-like and gas-like viscosity, density and diffusivity properties, represented in ***Table 1.11*** [85]. Moreover, SCFs present many advantages as solvents or anti solvents. Due to the low supercritical properties of $SCCO_2$, presented in ***Figure 1.10***, this supercritical fluid is environmentally friendly, inert, safe and low costs associated and is the most used on pharmaceutical technology [76,85]. Furthermore, the SCO_2 application as a co-solvent allows the minimization of the use of organic solvents, reduces the drying temperature process and the production of small particles [9]. In addition, the use of low temperatures enables processing materials such as biological particles [86].

Some of the methods for atomization of particles using supercritical CO_2 technology are:

i) the rapid expansion of supercritical solutions (RESS), where the solid material is dissolved in supercritical CO_2 and rapidly expanded leading to the formation of the particles; it has an important limitation related with the poor solubility of the drugs in $scCO_2$;

ii) supercritical antisolvent (SAS), where the particles are produced when the solution is in contact with supercritical CO_2, through a semicontinuous method. In this process there is the advantage of being possible to control the physical structure of the powders, changing the operating parameters such as pressure, temperature; in this process the drug

must have very low solubility in $scCO_2$ which happens for most of the drugs and the solvents used must be miscible with $scCO_2$;

iii) particles gas saturated solutions (PGSS), which is based on two main steps; the first is characterized by the saturation of a solution containing solute with CO_2 in a static mixer [76] and the second step is characterized by the expansion of the mixture through a nozzle in a spray tower.

iv) particle formation via phase induced by polymerization separation (PIPS) which consists of producing particles through the synthesis in a supercritical fluid medium, such as polymerization and each drop of emulsion results in precipitation, encapsulation and production of uniform particles.

v) supercritical fluid extraction of emulsions (SFEE) uses $scCO_2$ in order to extract the organic phase of emulsions; as the sc CO_2 extracts the organic solvent, the drugs precipitate from the solution;

vi) supercritical assisted atomization (SAA) or supercritical CO_2-assisted spray drying (SASD), one of the most recent methods is associated by CO_2 solubilization and takes benefits in the ability to use aqueous solutions and organic solvents, offering adequate control over particle size and distribution [76].

Table 1.11- Physical-chemical properties of gases, liquids and SCFs (Adapted from [85]).

	Density (kg/m^3)	**Viscosity (mPa.s)**	**Diffusivity (cm^2/s)**
Gas	0.8-1.3	0.01- 0.03	0.1-0.2
Liquid	800-1200	0.4-1.1	0.00001-0.0001
SCF	300-1000	0.05-0.01	0.0001-0.001

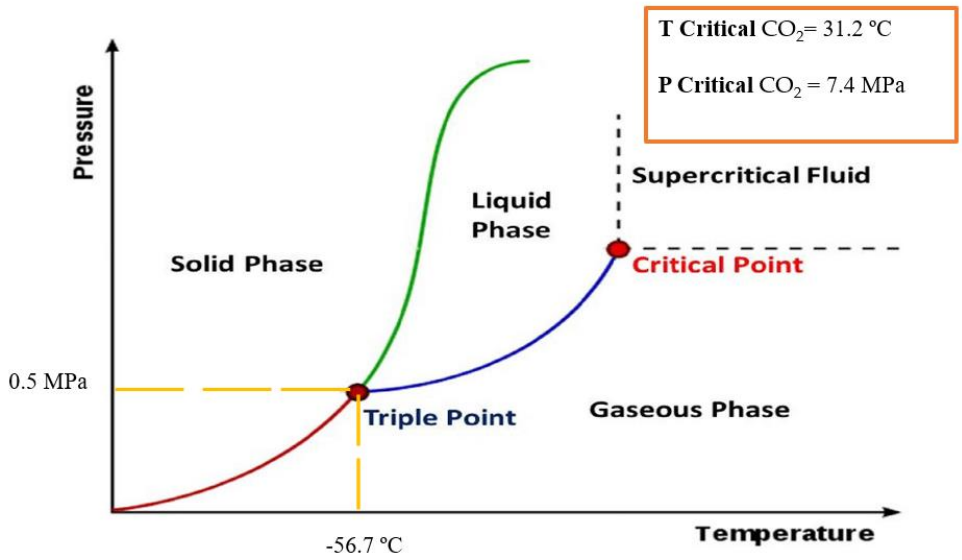

Figure 1.10- Schematic phase of CO_2 Supercritical (Adapted from [85,87]).

1.5 Quality by design

The pharmaceutical industry is increasing exponentially. For the development of medicines, there are production standards that ensure their efficacy, safety and quality [88]. Quality by design (QbD) is a concept developed by the engineer Joseph Moses Juran, in 1950 and was adopted for the first time by the Food and Drug Administration (FDA) [89]. Then, the pharmaceutical industry has implemented this concept in all different products [90]. The engineer believed that problems related to the quality of a product were associated with the way it was developed, so it was necessary to control quality at each stage of the process toward maximizing the probability of achieving the established specifications [88]. Pharmaceutical quality is defined as a product free of contamination, in which the therapeutic benefit promised to the consumer complies with the label [91]. Quality by design goals are to ensure the quality of medicines through statistical, analytical and risk management methods in the design, development and manufacture [92]. Moreover, the implementation of this concept combines with new technologies and low costs [88]. The implementation of this methodology involves elements and tools. The elements aim to direct the objectives of the process, define the attributes that allow experimental search and finally demonstrate how it should be described. Meanwhile, the tools have the purpose of monitoring quality through systematic control of the variables associated with the process. ***Figure 1.11***, shows the many elements and ***Table 1.12*** the tools of QbD. Furthermore, ***Figure 1.12*** represents how the production process works.

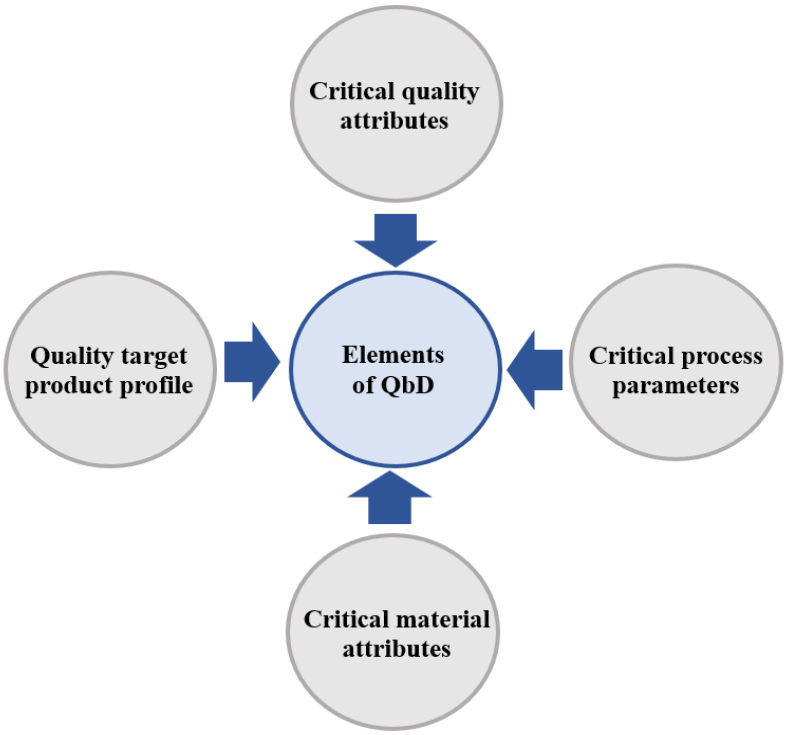

Figure 1.11 - The elements of Quality by Design. [89-90].

Quality target product profile (QTPP) is defined by a set of features that correspond to the product to be ideally achieved. Besides that, these will define the security and therapeutical efficacy. Critical Quality attributes (CQA), represent the physical, chemical, biological and microbiological properties of the product and certify if this has the desired quality. Critical material attributes (CMA) are characterized by the raw materials and these control the product quality assurance. Moreover, CMA highlights the importance of raw materials in the development of the formulation. At last, Critical Process Parameters, CPP has a significant role in the quality of the manufactured material and should be assessed as to the risk that its variation represents in the quality of the final product [88-89].

Table 1.12- Description of the tools of Quality by design [88-89].

Tools of Quality by design	
Risk Assessment, RA	✓ Risk assessment in product development ✓ Quality risk management ✓ Identify high-risk formulations and variations that may influence the quality of the medicinal product
Design of Experiments, DoE	✓ Aims to collect information from the experimental data ✓ Plan and investigate the most important factors is constituted by two phases: 1st identification of the main sources of variation and 2nd this data are used in the studies of interactions between the factors of the system ✓ Helps determine the model that best represents the object of search through statistical treatment
Process Analytical Technology, PAT	✓ Set of tools to analyze and reduce the risks associated with the development and production of the product ✓ Expands the capacity of process monitoring and drastically changes the way quality control is performed

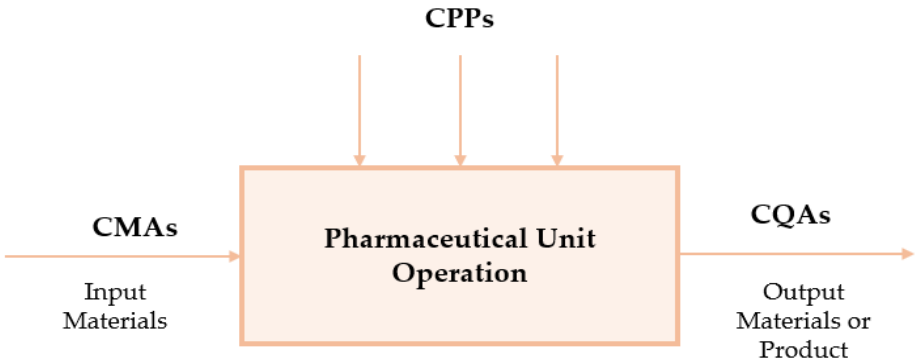

Figure 1.12 - Representation of the production process. (Adapted from [89]).

The application of this method takes many advantages for the pharmaceutical industry since this sector is highly pressured by competitiveness, leading for the need to develop new medicines and evaluate the cost associated with the operation. ***Table 1.13*** stands for the advantages of QbD in the general industry and particularly in the pharmaceutical industry [88].

Table 1.13-Advantages of Quality by design in the industry, specifically in the pharmaceutical industry [91].

Advantages of Quality by design	
Benefits for Industry	**Benefits for Pharmaceutical Industry**
✓ Better understanding of the process ✓ Less batch failure ✓ More efficient and effective control of change ✓ More profit	✓ Innovative process validation approaches ✓ Realistic risk perceptions ✓ Less validation burden ✓ Real-time controls ✓ For the consumer, greater drug consistency ✓ More efficient technology transfer to manufacturing

1.5.1 Design of Experiments

Design of Experiments, DoE is characterized by being a systemic and efficient method that allows engineers and the scientific community to study and relate between input variables (factors) and various output variables (responses) [93]. Furthermore, this mathematical tool is of major importance on a specific processing and to control them to optimize the system performance. Yet, DoE determines which variable affects the product performance [94]. The DoE process can be divided into five steps, represented in ***Figure 1.13*** and in ***Table 1.14*** represented the parameters of DoE process.

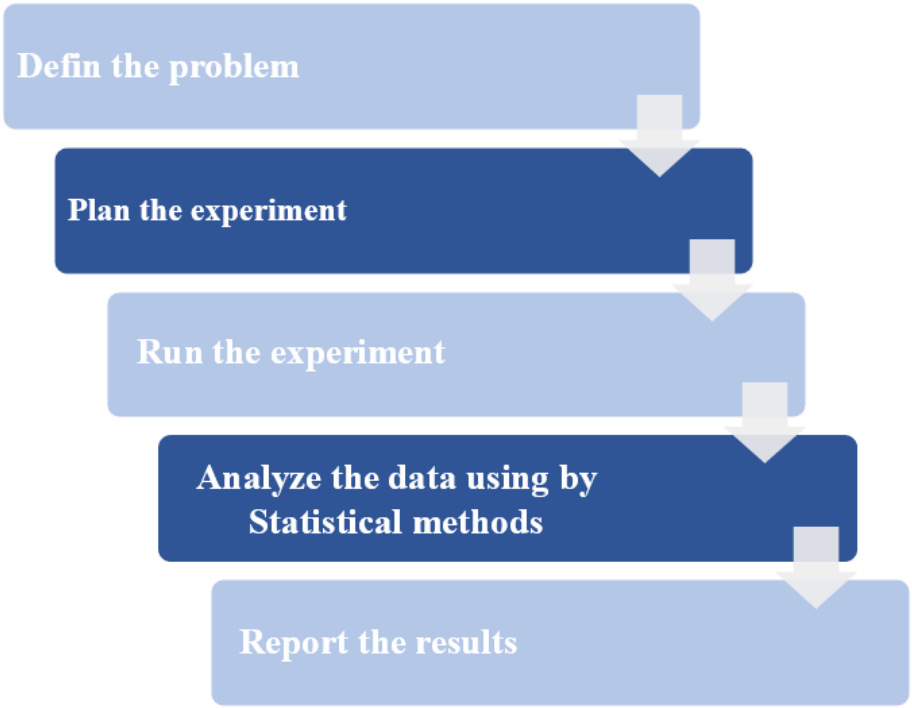

Figure 1.13 - DoE process.

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Table 1.14- Parameters of DoE [95].

Parameters of DoE	
Answer Variable	An observed or measured variable. It can be more than one response variables
Factor	An independent variable, that varies to observe which effects on response are significant. It can be considered qualitative/ quantitative and controllable /non-controllable
Level	Given the value of a factor in the experience
Effect	The change in the response variable that occurs when a factor is changed from a level to the other
Replication	Is essential in the design of experiments, since it allows get the experimental error and a more accurate estimate
Randomness	The experiments must be carried out randomly, to ensure an even distribution of all factors not considered
Analyses of Variance (ANOVA)	Evaluate the importance of one or more factors.
Residue Analysis	To confirm the assumptions considered in the Analysis of Variances are valid. It is possible to detect if the residues are independent or not
Transformed Answer	Data transformation for the analysis dispersion effects with the aim to guaranteeing the reduction of variability

1.6 Goals and Thesis Outline

The main goal of this dissertation is to convert quercetin-loaded liposomes into quercetin-loaded liposomal dry powder formulations, using a sustainable process – drying assisted by supercritical carbon dioxide. Plus, the several secondary goals of this work are:

1. Production of quercetin-loaded liposomal systems with different lipid-to-quercetin molar ratios (10:0.5, 10:1 and 10:1.5), in triplicate with the neutral, positive and negative surface electric charges;
2. Development of PEGylated Q_Lip in the same conditions and compare the results with the non - PEGylated Q_Lip formulations;
3. Characterization of the liposomal properties determining the zeta potential, size, the yield of lipid and incorporation efficiency of the drug in liposomes;
4. Select the better Non- PEGylated Q_Lip and PEGylated Q_Lip formulations to process in SASD;
5. Evaluate if quercetin remains incorporated in liposomes after the SASD process;
6. Characterization of the dry powder formulations through numerous methods;
7. Select the best dry powder formulations for future work.

The thesis includes 4 chapters, describing the work developed during this dissertation project.

Chapter 1: In this chapter, the contextualization and motivation of this work are explained. Furthermore, it presents an introduction of the theme, which includes the concept of inflammatory lung diseases, the development of liposomal-quercetin systems, drying technologies existing and finally it described the main goals of this work.

Chapter 2: In this chapter, the entire experimental procedure is explained by describing the materials used as well as the characterization of the techniques chosen in this study.

Chapter 3: In this chapter, the results obtained are presented and discussed.

Chapter 4: In this chapter, the main conclusions of this study are presented and also the proposals for future work.

2

2. MATERIALS AND METHODS

2.1 Materials

Quercetin dihydrate (97 % purity) was purchased from Alfa Aesar (Haverhill, EUA). L-α-phosphatidylcholine (EPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt (DMPG) and 2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt 2000 (DSPE-PEG) were obtained from Lipoid GmbH (Frigenstrasse, Germany). Cholesterol (Chol, 95-98 % purity), Stearylamine (SA ≥ 99 % purity) and L-leucine (Leu ≥ 98 % purity) were purchased from Sigma-Aldrich (St.Louis, EUA). Methanol 99.9 % (v/v), Acid perchloric 70-72 % (v/v),Hexa-Ammoniumheptamolybdat-4-hydrate (ammonium molybdate, 99 % purity) and Ethanol (EtOH) ≥ 99.8 % (v/v) were obtained from Honeywell (Charlotte, EUA). MiliQ water was prepared by reverse osmosis from Millipore (Burlington, EUA). Sodium Chloride (NaCl, 99.5 % purity), Citric Acid 1-hydrate (99.5 -102. 0 % purity), Trifluoroacetic acid (TFA) 99.8 % (v/v), Sodium dihydrogen phosphate 1 -hydrate (98-102 % purity) were purchased from PanReac (Barcelona, Spain). Chloroform ≥ 99 % (v/v), L (+) Ascorbic acid (≥ 99 % purity) were purchased from Carlo Erba Reagents (Barcelona, Spain). D-(+)-Trehalose dihydrate (Tre >98 % purity) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Industrial nitrogen and Industrial carbon dioxide (>99.93 % purity) were obtained from Air Liquide (Paris, France).

2.2 Development of Q_loaded liposomes (Q_Lip)

In this study, several systems were developed in order to study the incorporation of quercetin depending on the liposomes' surface electric charge (neutral, positive and negative surface electric charges). EPC lipid was used in liposomal systems with neutral charges since this presents neutralizing properties[97]. In Q_Lip with positive charges, the cationic molecule Stearylamine (SA) was combined [98]. At last, Q_Lip systems negative charges were produced with synthetic lipid. To acquire a negative surface charge an anionic lipid (DMPG) was mixed with DMPC lipid [98]. Moreover, liposomal systems were developed with different lipid-to-quercetin molar ratios (0.5, 1 and 1.5) which corresponds to F1, F2 and F3 respectively. Furthermore, PEGylated liposomes were produced, since the incorporation of this molecule can enhance significantly drug delivery, active molecules to the desired target [68].

Besides, the development of PEGylated and non-PEGylated systems were produced by the same method, further described in this section. ***Tables 2.1*** represent all the liposomal systems produced and their respective molar ratios.

Table 2.1-Q_Lip formulations produced.

Formulations	Neutral charged phospholipids	Positive charged phospholipid	Negative charged phospholipid
Non- PEGylated Q_Lip	EPC:Chol (8:2)	EPC:Chol:SA (7:2:1)	DMPC:DMPG (7:3)
PEGylated Q_Lip	EPC:Chol:DSPE-PEG (7.6:2:0.4)	EPC:Chol:SA:DSPE-PEG (6.65:2:1:0.35)	DMPC:DMPG:DSPE-PEG (6.65:2.85:0.5)

2.3 Q_loaded liposomes Preparation (Q_Lip)

The formulations Q_Lip were prepared by thin-film hydration, as previously described. Briefly, quercetin was dissolved in methanol (F1- 0.5 mL, F2- 1 mL and F3- 1.5 mL) and this solution was added to the other compounds (total lipid concentration of 32 µmol/mL). Afterward, the formulations were dissolved in chloroform and then dried by a rotary evaporator (Buchi Rotavapor RE-111, Switzerland), under a vacuum for 45 minutes -1 hour at 37ºC, resulting in lipid film. The lipid films (Q_Lip) were hydrated by 6 mL trehalose buffer solution (10mM acid citric,280mM trehalose, pH 6.0) and agitated at room temperature for 45 min to form multi-lamellar vesicles. Furthermore, glass balls were used in the hydration to improve liposomes arrangement. Due to the higher rigidity of DMPC and DMPG, Q_Lip with negative surface charges were hydrated in a bath at 25ºC.

2.4 Size reduction of liposomes

The extruder technique was used to obtain liposomes with homogenizing and desirable size [60]. The extruder used was a Lipex ™ Thermobarrel Extruder, Biomembranes Inc., Vancouver, BC, Canada) with a Nucleopore® Track-Etched Membranes (Whatman®, USA) with pore sizes ranging from 800 to 100 nm and under nitrogen pressure (10–500 lb/in^2). In this study pore sizes from 600, 400 and 200 or 100 nm, two times each were applied. Besides, all the liposomal formulations were prepared at room temperature, nonetheless, Q_Lip with negative charges were thermostated extruded at 25ºC.

2.5 Separation of non-corporated quercetin

Size exclusion chromatography (with 1000 D Dalton cut-off (Econo-Pac®, USA) was used to remove the unincorporated quercetin. This technique aims to separate components from a sample based on their size. This method is achieved by filtering through a gel containing spherical granules. These granules have the pores in order to exclude or include molecules of different sizes [100].

2.6 Dry powder liposomal formulations (Q_Lip-DPFs)

The liposomal formulations were dried through SASD technology. Briefly, a casting solution (50 mL) of excipients trehalose and L-leucine (total concentration of 300 mM) with 85:15 % (v/v) MiliQ water: ethanol was prepared. L-leucine was dissolved in 7.5 mL of ethanol and trehalose was dissolved in MiliQ water. The solution was added to a volumetric flask (without reaching the meniscus) and then dissolved on an agitation plate. Regarding SASD operation, firstly the CO_2 provided industrial bottle was cooled in a cryogenic bath around -15 ºC (Flow rate CO_2= 25 mL/min) and then pumped by a high-pressure pump (HPLC pump K-501, Knauer). Afterward, the dioxide carbon was heated to achieve the supercritical properties ($T_{bath\ CO_2}$ ~ 80 ºC) and then go to the static mixer. Besides, the compressed air stream is opened (Flow rate ~ 30 m ^{3}/h, T_{in} ~ 100 ºC). Secondly, when the casting solution was completely dissolved, was added 2 mL of Q_Lip and consequently reached the meniscus. The liquid solution was placed in a high-pressure pump Smartline pump 1000, Knauer (Flow rate = 3.5 mL/min), into a heated static mixer (3/16 model 37-03-075 Chemieer) comprising a high-pressure column of 4.8 mm diameter, 191 mm length and 27 helical mixing elements and heated by a serpentine. Then, the solution was atomized by a drying chamber, incorporated with a nozzle (150 µm internal diameter). Finally, the particles were formed and transferred to a glass vessel. Moreover, the

solvent/anti-solvent were evaporated through cyclone equipment. The simplified schematic representation of SASD is presented in ***Figure 2.1***.

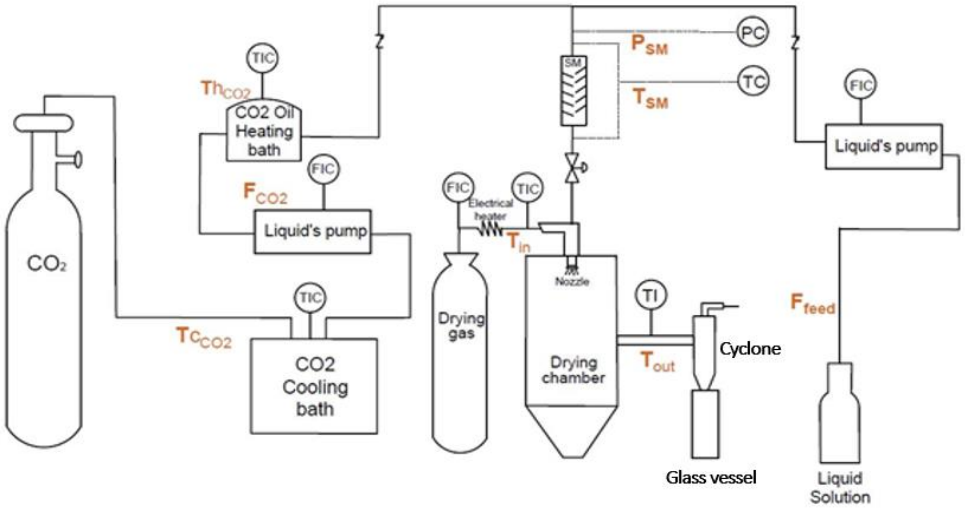

Figure 2.1- Simplified schematic representation of the SASD (Adapted from [98]) Legend of SASD conditions: $\mathbf{TC_{CO2}}$ – CO_2 cooling bath temperature, $\mathbf{F_{CO2}}$- CO_2 flow rate, $\mathbf{Th_{CO2}}$- CO_2 oil bath temperature, $\mathbf{T_{in}}$-inlet air temperature, $\mathbf{T_{out}}$- outlet air temperature, $\mathbf{F_{feed}}$ – liquid feed solution flow rate, $\mathbf{P_{SM}}$- pressure at the static mixer and $\mathbf{T_{SM}}$- temperature at the static mixer

2.7 Resuspension of Q_Lip-DPFs

This method aims to analyze if the formulations preserve the same structure and evaluate the lipid concentration and incorporation efficiency after the drying process. Then, is necessary to remove the trehalose and L-leucine added in preparation for the casting solution for the SASD process. Yet, ultracentrifugation was used with 49 000 rpm, at 300 000 g, 2 h, at 15 °C in Optima™ XL-90 Ultracentrifuge (Beckman Coulter, USA). Thereafter, the supernatant was removed, and the pellets were resuspended at 500 µ*L* of NaCl buffer solution (10mM acid citric,145mM NaCl, pH 6.0), obtained 1 mL of Q_Lip formulations after SASD.

2.8 Liposomes Characterization

2.8.1 Lipid quantification

This technique determines the lipid yield on the liposomal formulations and was performed by the Rouser method [100]. Briefly, the samples were placed in tubes and evaporated at 180ºC. Afterward, perchloric acid was added and acid hydrolysis at 180ºC for 45 min. Then, 1 mL MiliQ water,400 µL of ammonium molybdate and 400 µL of ascorbic acid were added and incubated in a bath at 100ºC for 5

min. At last, the absorbance was read (797 nm) in the Spectrophotometer UV-mini 1240 spectrophotometer, Shimadzu. ***Equation 2.1*** represents the lipid yield.

$$\eta_{\textbf{lipid}}\,(\%) = \frac{mass\ \ of\ lipid\ _{\ after\ SASD}\ (\mu g)}{mass\ \ of\ lipid\ _{\ before\ SASD}\ (\mu g)} \times 100 \qquad \textbf{Equation 2.1}$$

2.8.2 Quercetin quantification

This method was made to determine the incorporation efficiency of quercetin. The drug quantification was analyzed by high-performance liquid chromatography (HPLC) in a Purospher® STAR RP-18 endcapped (5 µm) HPLC column, 4.6 x 250 mm (Merck, Germany) using a Beckman System Gold HPLC (Beckman Coulter, Inc., USA) with a mobile phase consisting of methanol: water acidified with 1 mM trifluoroacetic acid (70:30, v/v). Before the quantification solutions of Q_Lip and methanol were made. This solvent was used to destroy liposomes, returning to their initial form (phospholipids). Besides, these dilutions were prepared in the day before and placed in the refrigerator. In the next day were filtered through a PTFE membrane with 0.2 µm pore diameter at 360 nm. ***Equation 2.2*** represents the ratio (quercetin/lipid after SASD and the ratio (quercetin/lipid) before SASD.

$$\boldsymbol{IE}(\%) = \frac{\left[\frac{quercetin\ (\mu g)}{lipid(\mu mol)}\right]_{after\ SASD}}{\left[\frac{quercetin(\mu g)}{lipid(\mu mol)}\right]_{before\ SASD}} \times 100 \qquad \textbf{Equation 2.2}$$

2.8.3 Size and polydispersion distribution

The particle size and polydispersity (PdI) of liposomal systems were determined by Zetasizer Nano S (Malvern Panalytical Ltd., Malvern Instruments, UK). In this method was used 10 µL of formulation and 1mL of NaCl buffer solution (10mM acid citric,145mM NaCl, pH 6.0). All measurements were run at 25ºC.

2.8.4 Zeta potential

Zeta Potential aims to determine the surface charge of the formulations. The zeta potential analysis was determined by Zetasizer Nano Z (Malvern Panalytical Ltd., Malvern Instruments, UK). The measurement cells were run with 50 µL of formulation and NaCl buffer solution (10mM acid citric,145mM NaCl, pH 6.0).

2.9 Characterization of Q_Lip-DPFs

2.9.1 ATR-FTIR spectroscopy

ATR-FTIR purpose is to acquire an infrared spectrum of the liposomal dry powder formulations. The analysis was achieved by a Spectrum Two FTIR spectrometer (Perki-nElmer, Inc.). Briefly, a portion of each Q_LIP-DPFs were placed in a crystal diamond plate, completely covering the prism surface and was recorded with 16 scans from 400 to 4000 cm^{-1}.

2.9.2 Differential scanning calorimetry (DSC)

The thermoanalytical technique aims to determine the thermal behavior of the powders. The powder was assessed in a DSC - Model Q200 from TA Instruments (Focus Scientific, Ireland) equipped with a Refrigerated Cooling System (RCS). Briefly, were weighted around 5 mg of Q_Lip-DPFs into an aluminum capsule (Tzero Hermetic Lid, TA) and heated from temperature range -90 ºC to 175 ºC, for 10ºC /min, in two cycles for 2 hours. Plus, the capsule was holed to remove the water content of each sample. After the measured technique, the capsule was overweighted to determine the weight loss and consequently the percentage of water content presented in Q_Lip DPF.

2.9.3 Karl-Fischer coulometer (KF)

In this method was determined the amount of water content of the Q_Lip-DPFs by Karl Fischer coulometric technique. This equipment only accesses liquid samples, so were necessary to dissolve the Q_Lip-DPF into a dried solvent. The solvent selected was EtOH ≥ 99.8 % (v/v) and firstly was dried by activated molecular sieves in a glass bottle for at least 3 days. Posteriorly, 15 mg of Q_Lip -DPF were dissolved in 5 mL of ethanol. The lecture of each powder was read in triplicate and using a syringe (B|Braun, Injekt®-F 0.01 mL–1 mL) around 0.5 mL. The equipment used was T831 KF Coulometer (Metrohm Ltd., Antwerp, Belgium) coupled with a 728 Stirrer (Metrohm Ltd, Antwerp, Belgium) where the reagent solution was Hydranal® Coulomat AG (Sigma -Aldrich, Bornem, Belgium).

2.9.4 Particle size distribution and aerodynamic particle size

The particle size (PS) and the particle size distribution (PSD) of the powder formulations were performed by Morphologi G3 equipment (Malvern Instruments, UK), by analysing 30,000 particles For each Q_Lip-DPF, enough amount was analyzed. Moreover, to assist the technique, industrial nitrogen was used (4 bar) to disperse the powder. This method has an automatic sample dispersion unit to a microscope with 5x, 10x, 20x and 50x objectives. For each Q_Lip-DPF, were obtained the particle

diameter in volume (D_v) to 10, 50 and 90 % of the total population. Thus, the particle size distribution (span) was determined in **Equation 2.3**.

$$\boldsymbol{span = \frac{D_{v,90} - D_{v,10}}{D_{v,50}}} \qquad \boldsymbol{Equation\ 2.3}$$

2.9.5 Particles morphology

The shape and surface morphology of the powder formulations were measured in Scanning Electron Microscopy (SEM). Firstly, the Q_Lip -DPFs were prepared into adhesive carbon tapes and the excessive powder was removed with a jet of compressed air. Afterward, the samples were available to analyze through Thermo Scientific Phenom ProX G6.

2.9.6 Process yield

The process yield was determined by the ratio between the mass of the excipients used (trehalose and l-leucine) after SASD and before SASD, expressed in ***Equation 2.4***.

$$\eta(\%) = \frac{mass\ of\ excipients\ _{after\ SASD}\ (g)}{mass\ of\ excipients\ _{before\ SASD}\ (g)} \times 100 \qquad \boldsymbol{Equation\ 2.4}$$

2.9.7 X-ray powder diffraction (XRD)

This method purpose to assess the powder amorphicity and crystallinity structure of the powders. The measurements were performed in RIGAKU X-ray diffractometer (Benchtop RIGAKU - MiniFlex II), using Cu radiation (30 kV/15 mA) with a 2θ angle ranging between 5º and 40º and a step size of 0.015º.

2.9.8 *In-vitro* aerodynamic performance

Andersen Cascade Impactor (ACI) is the most usually test used for aerosol particles inhalation [101]. The Q_Lip-DPFs were evaluated gravimetrically using 8–stages of Andersen Cascade Impactor (Copley Scientific). Firstly, the airflow of the ACI was regulated on a critical flow controller model TPK (Copley), to adjust the flow rate 60 mL /min during 4s, according to the European pharmacopeia [102]. Secondly, glass microfiber filters (MFV1 80 mm, Filter Lab) were weighed and emplaced at all stages. Moreover, the DPI was counted too. Afterward, for each Q_Lip-DPF were approximately counted 30 mg for 3 capsules (HPMC, Aerovaus), placed in a handheld breath-activated inhaler device (Aerolizer Plastique 60 LPM–Model 7 DPI) and deposited into the inlet of the ACI, as

represented in ***Figure 2.2.*** At last, after the three capsules were released, the total mass of powder deposited in each stage filter was determined and the amount of residual powder in DPI too. Furthermore, were calculated the mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD) and fine particle fraction (FPF). The FPF was defined by the interpolation of the percentage f the particles with less than 5 mm diameter. Regarding MMAD, this corresponds to the diameter of 50 % of the particles. Finally, the GSD of the powder formulations was determined by ***Equation 2.5*** :

$$GSD = \sqrt{\frac{d_{84}}{d_{16}}} \qquad \textbf{Equation 2.5}$$

where d_{84} and d_{16} is the diameter corresponding to 84 and 16 % of the cumulative distribution, respectively.

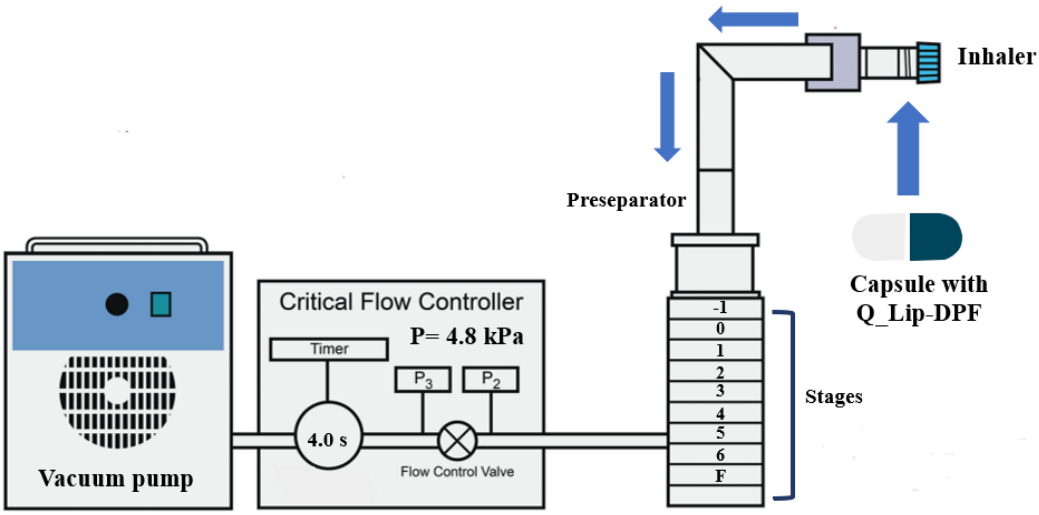

Figure 2.2 - Schematic representation of ACI (Adapted from [103]).

3

3. RESULTS AND DISCUSSION

3.1 Liposomes Characterization

Prior to converting quercetin-loaded liposomes into liposomal dry powders formulations using SASD process, it was important to select the best Q_Lip formulations taking in account the liposome size, PdI, zeta potential, yield of lipid and the incorporation efficiency of the drug (*IE*). In this way, the first step of this work was to study the incorporation of quercetin depending on the liposomes' surface electric charge. Therefore, quercetin-loaded liposomal formulations with different zeta potential (positive, neutral, and negative) were studied (***Table 3.1***).

Table 3.1 - Liposomal characteristics of non- PEGylated Q_Lip formulations with a neutral, positive and negative charged phospholipids (mean ± SD,n = 3).

Q_Lip with neutral charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	150 ± 5	0.113 ± 0.029	-1.1 ± 0.2
F2	153 ± 7	0.152 ± 0.023	-1.0 ± 0.1
F3	148 ± 2	0.226 ± 0.104	-0.9 ± 0.1
Q_Lip with positive charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	194.8 ± 0.2	0.093 ± 0.016	14.3 ± 1.5
F2	188 ± 10	0.133 ± 0.057	13.6 ± 1.5
F3	163 ± 1	0.203 ± 0.026	9.6 ± 3.0
Q_Lip with negative charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	137 ± 14	0.089 ± 0.048	-24.2 ± 1.8
F2	147 ± 1	0.114 ± 0.017	-28.0 ± 0.6
F3	132 ± 2	0.073 ± 0.007	-28.4 ± 0.5

From the results obtained it was observed that the size of the liposomes was in line with the targeted size (in the range of 100- 200 nm). In fact, the liposome with sizes up to 150 nm can escape from blood circulation and can target the inflammation region, taking benefit with the permeability and retention effect (EPR) [60]. Over the polydispersion index (PdI), in which 0 represents a homogeny formulation and 1 is a heterogeny formulation, liposomes must present a value lower than 0.2. It was observed that liposomal formulations without DSPE-PEG have a PdI below 0.2, except for neutral F3 formulation, which has a PdI slightly superior to 0.2. It might be related with the value of the potential zeta. In fact, Singhvi *et al.* [104] have reported that liposomal formulations with a zeta potential, lower than that -30 mV and higher than + 30 mV is usually have better colloidal stability. The repulsive forces avoid the aggregation and flocculation of the particles.

Another study was the importance of the presence of PEG in liposomal formulation. Corvo *et al.* [61] have reported that PEGylated liposomes might increase the liposomes circulation in the bloodstream and their hindering from macrophages. Then, taking in account the advantages of the PEGylated liposomes, DSPE-PEG was added to each formulation as described in the Materials and Methods section. The results of the PEGylated Q_Lip formulations are presented in ***Table 3.2***.

Table 3.2 - Liposomal characteristics of PEGylated Q_Lip formulations with a neutral, positive and negative charged phospholipids (mean ± SD,n = 3).

PEGylated Q_Lip with neutral charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	179 ± 3	0.095 ± 0.024	-1.2 ± 0.4
F2	174 ± 8	0.098 ± 0.012	-1.0 ± 1.1
F3	172 ± 9	0.181 ± 0.092	-0.9 ± 0.1
PEGylated Q_Lip with positive charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	163 ± 12	0.107 ± 0.006	-0.8 ± 0.8
F2	148 ± 5	0.135 ± 0.045	-0.7 ± 0.6
F3	135 ± 8	0.142 ± 0.016	-0.6 ± 0.8
PEGylated Q_Lip with negative charged phospholipids			
	Size (nm)	**PdI**	**Zeta potential (mV)**
F1	65 ± 26	0.219 ± 0.013	-3.7 ± 0.5
F2	137 ± 8	0.147 ± 0.017	-8.1 ± 1.6
F3	164 ± 17	0.151 ± 0.005	-10.5 ± 1.0

Regarding the PEGylated Q_Lip, the PdIs were below 0.2, proving the homogeneity of the formulation. Even for formulations with neutral charges, those are stabilized by PEG. The PEGylation of liposomes creates a steric barrier, forming a hydration cloud, preventing liposomes aggregation and further fusion [105]. However, the zeta potential in positive formulations presents lower values compared to non-PEGylated liposomes. It might be explained by the presence of the PEG since the zeta potential measures the surface charge and the PEG has a slightly negative electric charge [106].

3.1.1 Lipid and quercetin quantification

After the quercetin incorporation in liposomes (film formation and extrusion) and the removal of remove the non-incorporate quercetin by molecular exclusion chromatography, quercetin-loaded liposomes were characterized. In this way. the lipid and quercetin were be quantified. ***Table 3.3*** shows the lipid yield and incorporation efficiency of quercetin for the liposomal formulations with different surface electric charges.

Table 3.3 - Lipid yield (η_{Lipid}) and incorporation efficiency (*IE*) of Q_Lip formulations with a neutral, positive and negative charged phospholipids (mean ± SD, n = 3).

Q_Lip with neutral charged phospholipids

	PEGylated Q_Lip			Non- PEGylated Q_Lip		
	η_{Lipid}(%)	**$(Quer/Lipid)_{final}$**	***IE*(%)**	**η(%)**	**$(Quer/Lipid)_{final}$**	***IE*(%)**
F1	97 ± 11	14 ± 1	88 ± 10	95 ± 3	19 ± 4	99 ± 15
F2	95 ± 9	25 ± 1	73 ± 4	96 ± 5	34 ± 5	72 ± 5
F3	97 ± 3	24 ± 4	51 ± 1	96 ± 8	45 ± 14	72 ± 14

Q_Lip with positive charged phospholipids

	PEGylated Q_Lip			Non-PEGylated Q_Lip		
	η_{Lipid}(%)	**$(Quer/Lipid)_{final}$**	***IE*(%)**	**η(%)**	**$(Quer/Lipid_{)final}$**	***IE*(%)**
F1	77 ± 8	8 ± 1	85 ± 12	92 ± 7	15 ± 3	99 ± 5
F2	91 ± 3	19 ± 8	78 ± 25	108 ± 4	28 ± 1	88 ± 10
F3	108 ± 4	20 ± 4	84 ± 21	78 ± 6	31 ± 9	93 ± 8

Q_Lip with negative charged phospholipids						
	PEGylated Q_Lip			Non-PEGylated Q_Lip		
	η_{Lipid}(%)	$(Quer/Lipid)_{final}$	*IE*(%)	η(%)	$(Quer/Lipid)_{final}$	*IE*(%)
F1	86 ± 4	17 ± 4	87 ± 16	101 ± 6	12 ± 5	92 ± 3
F2	107 ± 3	28 ± 7	66 ± 14	105 ± 2	35 ± 6	89 ± 1
F3	114 ± 2	47 ± 1	146 ± 51	93 ± 3	48 ± 3	90 ± 1

As expected, the lipid yield of the formulations was high, meaning no substantial lipid losses throughout the quercetin exclusion in the chromatographic column. Regarding the incorporation efficiency, it was observed that quercetin was incorporated successfully taking in account the *IE* values. Yet, yields greater than 100 % were observed. There are different hypotheses to justify this phenomenon: i) liposomes might not be well distributed; ii) inefficient vortex before the HPLC iii) non-efficient removal of non-incorporated quercetin.

Analysing ***Table 3.3***, it is possible to observe that the non-pegylated formulations have a higher or similar lipid yield as well as the *IE* compared to the pegylated liposomes. In fact, Man-Yi Wong and Gigi [108] have inferred that the presence of DSPE-PEG prevents the aggregation of quercetin in liposomes. Moreover, it was also reported that DSPE-PEG had not influenced in incorporation efficiency of quercetin into liposomes. Appraising the results, the formulations selected for processing in the SASD, are represented in ***Table 3.4***. Although all formulations showed similar results, the F3 formulations presented some higher standard deviations.

Table 3.4 – The formulations selected for process in SASD.

Q_Lip Formulations	Non- PEGylated	PEGylated
Neutral charged phospholipids	(10:0.5), (10:1)	(10:0.5), (10:1)
Positive charged phospholipids	(10:0.5), (10:1)	(10:0.5), (10:1)
Negative charged phospholipids	(10:0.5), (10:1)	(10:0.5), (10:1)

3.2 Production of quercetin-loaded dry powder formulations

After characterization and the selection of the better formulations, the Q_Lip was converted in quercetin-loaded liposomal dry powder formulations (Q_Lip-DPFs), using the green process SASD. Under our knowledge, this is the first time that Q_Lip-DPFs are processed using SASD. For this reason, the main goal of this section is to evaluate if quercetin remained incorporated in liposomes after the supercritical process. This will be verified through the *IE* after the SASD processing. Furthermore, in this section was only made a screening study of each formulation.

3.2.1 Supercritical CO_2-assisted spray-drying process

Before processing in SASD, the Q_Lips were sterilized and mixed into a casting solution composed by trehalose and leucine, with an osmolarity of 300 mOsm. It is important to highlight that the casting solution should have the same osmolarity that the aqueous space of liposomes, to avoid their disruption. The process parameters applied in this process were the same as in previous work [60]. According to the production of Q_Lip-DPFs, the process parameters of SASD and more characterizations results after SASD are shown in ***Table 3.5.***

Table 3.5- Characterization of Q_Lip-DFPs with neutral, positive and negative charged phospholipids in the SASD process. The parameters of SASD are expressed in mean ± SD (n=3).

Q_Lip-DPFs with neutral charged phospholipids	**P_{SM} (bar)**	**T_{SM} (ºC)**	**T_{in} (ºC)**	**T_{out} (ºC)**	**Yield Process (%)**	**Water content(%)**
Q_Lip_F1_ Non- PEGylated	121 ± 1	92 ± 3	93 ± 2	63± 0.2	66	6
Q_Lip_F1_ PEGylated	121 ± 1	86 ± 2	101± 2	64 ± 6	63	6
Q_Lip_F2_ Non- PEGylated	121 ± 2	83 ± 7	98± 4	66 ± 5	48	7
Q_Lip_F2_ PEGylated	121 ± 1	92 ± 4	106 ± 5	69 ± 4	62	7
Q_Lip-DPFs with positive charged phospholipids	**P_{SM} (bar)**	**T_{SM} (ºC)**	**T_{in} (ºC)**	**T_{out} (ºC)**	**Yield Process (%)**	**Water content(%)**
Q_Lip_F1_ Non- PEGylated	120 ± 1	83 ± 7	102 ± 7	68 ± 1	65	5

Q_Lip_F1_ PEGylated	120 ± 1	84 ± 9	102 ± 8	66 ± 3	66	6
Q_Lip_F2_ Non- PEGylated	121 ± 1	80 ± 5	101 ± 8	66 ± 1	56	6
Q_Lip_F2_ PEGylated	121 ± 1	87 ± 4	101 ± 1	70 ± 2	52	6

Q_Lip-DPFs with negative charged phospholipids	**P_{SM} (bar)**	**T_{SM} (ºC)**	**T_{in} (ºC)**	**T_{out} (ºC)**	**Yield Process (%)**	**Water content(%)**
Q_Lip_F1_ Non- PEGylated	121 ± 1	91 ± 4	99 ± 7	66 ± 5	58	6
Q_Lip_F1_ PEGylated	120 ± 1	85 ± 7	100 ± 8	60 ± 2	62	6
Q_Lip_F2_ Non- PEGylated	120 ± 1	84 ± 10	101 ± 8	66 ± 5	65	6
Q_Lip_F2_ PEGylated	121 ± 2	93 ± 3	105 ± 4	66 ± 3	52	6

In all Q_Lip -DPFs, despite being different batches, the process parameters factors were kept the same, decreasing the batch-to-batch variations of the SASD process. Regarding the yield process, the powder formulations obtained approximately 60%. This value is consistent with the yields of previous works (REF). Furthermore, the water content of all Q_Lip-DPFs was in the range of 5-7 %, meaning the high drying efficiency of the SASD process. Costa *et al.* [60] have reported similar water content values (water content of 6%) for the same parameters. Then, all the dry powder formulations were stored in a desiccator with liquid nitrogen to protect the Q_Lip-DPFs against humidity, for further characterization.

3.2.2 Characterization of Q_Lip-DPFs

i) Characterization of Q_Lip before and after SASD

After the SASD processing, it was important to evaluate if the presence of ethanol and the pressure of $scCO_2$ during the process affected the structure of the liposome and, consequently, the incorporation of the drug. Thus, the Q_Lip-DPFs were resuspended in Mili-Q water (in order to maintain the osmolarity of the liposomes) and ultracentrifuged. This was an important step to remove the excipients

and recover the resuspended liposomes. Then, Q-Lip with neutral surface electric charge after SASD were characterized in terms of their size, PdI, lipid yield and *IE* (***Table 3.6***).

Table 3.6 - Characterization of Q_Lip with neutral charged phospholipids before and after SASD.

Q_Lip with a neutral charged phospholipids						
		Size (nm)	**PdI**	**Zeta potential (mV)**	**Ƞ lipid (%)**	***IE*(%)**
Non-PEGylated liposomes	F1 (Before SASD)	168	0.183	-1.8	-	-
	F1 (After SASD)	145	0.303	-2.3	22	34
	F2 (Before SASD)	150	0.184	-1.6	-	-
	F2 **(**After SASD)	162	0.401	-2.3	56	16
PEGylated liposomes	F1 (Before SASD)	174	0.097	-2.5	-	-
	F1 **(**After SASD)	160	0.231	-2.7	31	26
	F2 (Before SASD)	170	0.097	-1.7	-	-
	F2 **(**After SASD)	184	0.239	-2.3	50	21

The ***Table 3.6*** shows that the size of the Non- PEGylated and PEGylated liposomes with a 0.5 ratio of quercetin is lower after SASD, as expected. This decrease might be related with the presence of L-leucine. Deber and Stone [108] have reported that the hydrophobic interaction between de leucine and lipids, via Wan der Waals forces, might lead to packing and consequently liposomes size decrease. Nevertheless, the size of the remaining liposomes increased, as not expected. It might be an error associated since it was only a screening study. However, the increase of the PdI was not expected, as well as the low lipid yield. It might suggest a loss of liposomes during the process. Regarding to quercetin incorporation, it was observed an *IE* increase with the decrease of the molar ratio of quercetin, for both for pegylated and non-pegylated formulations. This suggests that the presence of less quercetin may result in more compact liposomes. Then, during the process the $scCO_2$ cannot diffuse easier into the lipidic bilayer, dragging the quercetin.

Table 3.7 - Characterization of Q_Lip with positive charged phospholipids before and after SASD.

Q_Lip with positive charged phospholipids						
		Size (nm)	**PdI**	**Zeta potential (mV)**	**ɳ lipid (%)**	***IE*(%)**
Non-PEGylated liposomes	F1 (Before SASD)	128	0.163	14.2	-	-
	F1 (After SASD)	208	0.353	1.9	30	42
	F2 (Before SASD)	176	0.153	8.71	-	-
	F2 **(**After SASD)	262	0.533	6.6	32	37
PEGylated liposomes	F1 (Before SASD)	183	0.094	0.11	-	-
	F1 **(**After SASD)	195	0.298	-2.0	35	34
	F2 (Before SASD)	173	0.183	2.10	-	-
	F2 **(**After SASD)	251	0.275	-0.86	77	16

Analyzing the ***Table 3.7*** it is possible to observe that the size of the Q_Lip with positive charge increased after the SASD process. This fact might be explained by by the instability caused by Stearylamine. Then adjustments in the ratios may be necessary.Regarding the zeta potential, it can be observed that during the drying process there was a loss of charged lipid since the zeta potential decreased drastically after SASD. It might also explain the values of *IE*. The *IE* of the non- PEGylated liposomes and PEGylated liposomes were similar since the addition of DSPE-PEG does not affect the incorporation of the drug [107]. In contrast, the Q_Lip _F2 PEGylated liposomes had the lowest *IE*, which may be an error associated with the production of the formulation. According to the PdI, it is also possible to see the increase of the heterogeneity of the formulations after the SASD process, which does not fit with the expected results.

Table 3.8 - Characterization of Q_Lip with negative charged phospholipids before and after SASD.

Q_Lip with negative charged phospholipids						
		Size (nm)	**PdI**	**Zeta potential (mV)**	**Ƞ lipid (%)**	***IE*(%)**
Non-PEGylated liposomes	F1 (Before SASD)	131	0.187	-24.8	-	-
	F1 (After SASD)	117	0.139	-7.2	71	14
	F2 (Before SASD)	150	0.146	-23.8	-	-
	F2 **(**After SASD)	150	0.240	-12.2	65	13
PEGylated liposomes	F1 (Before SASD)	120	0.048	-1.8	-	-
	F1 **(**After SASD)	180	0.176	-3.23	54	8
	F2 (Before SASD)	143	0.130	-15.6	-	-
	F2 **(**After SASD)	181	0.248	-2.8	41	11

Regarding the Q_Lip with negative charges (***Table 3.8***), it can be observed that the size of the non-PEGylated liposomes decreases or was maintained after the drying process, which was expectable due to the L-leucine action [108]. In contrast, in PEGylated liposomes, this was not observed since the size difference between both formulations was not as the previous results. Such can be justified with: i) deviations error in the measurement characterizations performed after SASD, ii) only made a screening test for each formulation, requiring increasing the number of assays. Furthermore, it is also observed a loss of charged lipid during the Supercritical CO_2 process since the zeta potential decreased drastically after SASD and might also explain the values of *IE* obtained.

In sum, after evaluating if quercetin remained incorporated in liposomes after the supercritical process, it was shown that quercetin, apart from some losses due to the drying process, manages to maintain the incorporation into the liposome system. Besides, the Q_Lip with positive charge presented the better *IE*. Nonetheless, it was expected higher yields of *IE* of the drug in all Q_Lip-DPFs. For this reason, further studies are required.

ii) Characterization of Q_Lip -DPFs

After the SASD processing, Q_Lip-DpFs were characterized in terms of powder properties. In this way, Q_Lip-DPFs were characterized using ATR-FTIR spectroscopy, differential scanning calorimetry (DSC) and Karl-Fischer coulometer (KF). Their morphology was characterized using Morpologi G3 and scanning electron microscopy (SEM) and aerodynamic studies were performed using the Anderson Cascade Impactor (ACI).

- *ATR-FTIR spectroscopy*

The ATR-FTIR technique indicated the chemical structure and composition of the dry powder formulations. The ***Figure 3.1***, represents the structure of different liposomal dry powder formulations. It can be observed that the liposomes might be not on the surface of the liposomes since there is no difference between the spectrum with different formulations. Moreover, this statement is in line with work studied previously by Costa *et al*. [60]. Dry powder formulations with different surface-charged liposomes were characterized. All the powders showed the same spectrum. According to the chemical bonds, it was possible to see peaks between 3550-3200 cm^{-1} correspond to the O-H (O-H stretching) connection and between 3000-2840 cm^{-1} these peaks correspond to the C-H (C-H stretching) connection. At 1818 cm^{-1} it is possible to visualize the functional carbonyl group (C=O), between 1300-1200 cm^{-1} the C=C group and between 1000-900 cm^{-1} the C-O group (C-O stretching) [109]. Therefore, this observation suggested that liposomes are encapsulated in the powder rather in the powder's surface.

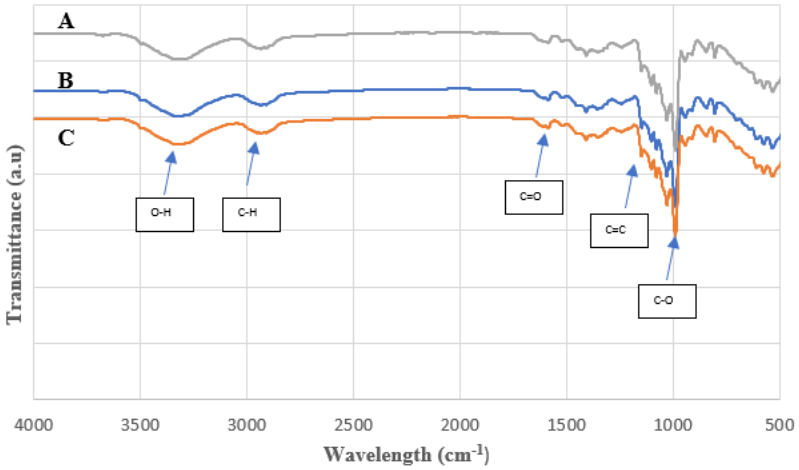

Figure 3.1 - ATR-FTIR spectrum analysis of Q_Lip-DPFs after SASD . **A** – Positive _F1_ Non- PEGylate Q_Lip-DPFs, **B**- Neutral_F1_ Non- PEGylated Q_Lip-DPFs, **C**- Negative _F1_ Non- PEGylate Q_Lip-DPFs

- *Differential scanning calorimetry*

DSC was performed to evaluate the thermal behavior of the powders. The ***Figure 3.2*** shows the glass transition temperature (T_g) of dry powder formulations after the SASD process. This graph represents the second cycle while the first cycle aims to remove the water content measurement of the formulations. It is possible to observe that all the Q_Lip-DPFs have the same T_g of 119.2 ºC, reinforcing that liposomes do not influence the powder's properties. In fact, in a previous study, C. Costa *et al*. [60] reported an equal T_g. In the previous study, a different drug was loaded in liposomes, but the same system trehalose/ l-leucine was used as excipient.

XRD analyses of a similar system [60] has shown that these powders have an amorphous structure after SASD processing. In fact, the amorphous structure of the powders is an advantage for pulmonary drug delivery. Khaled *et al*. [110] reported that amorphous particles enhance the bioavailability of poorly water-soluble drugs.

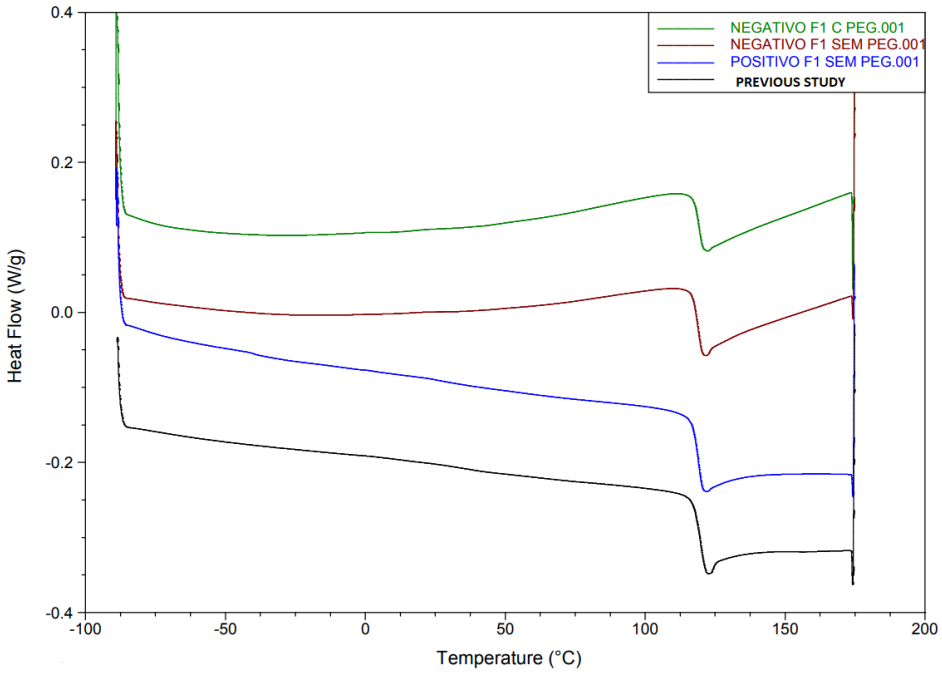

Figure 3.2 -DSC analysis of Q_Lip.DPFs after SASD.

- Particle Size and Particle Size Distribution

The morphology of the particles after the SASD process was investigated using Morphologi G3, and the images obtained are shown in ***Figure 3.3.***

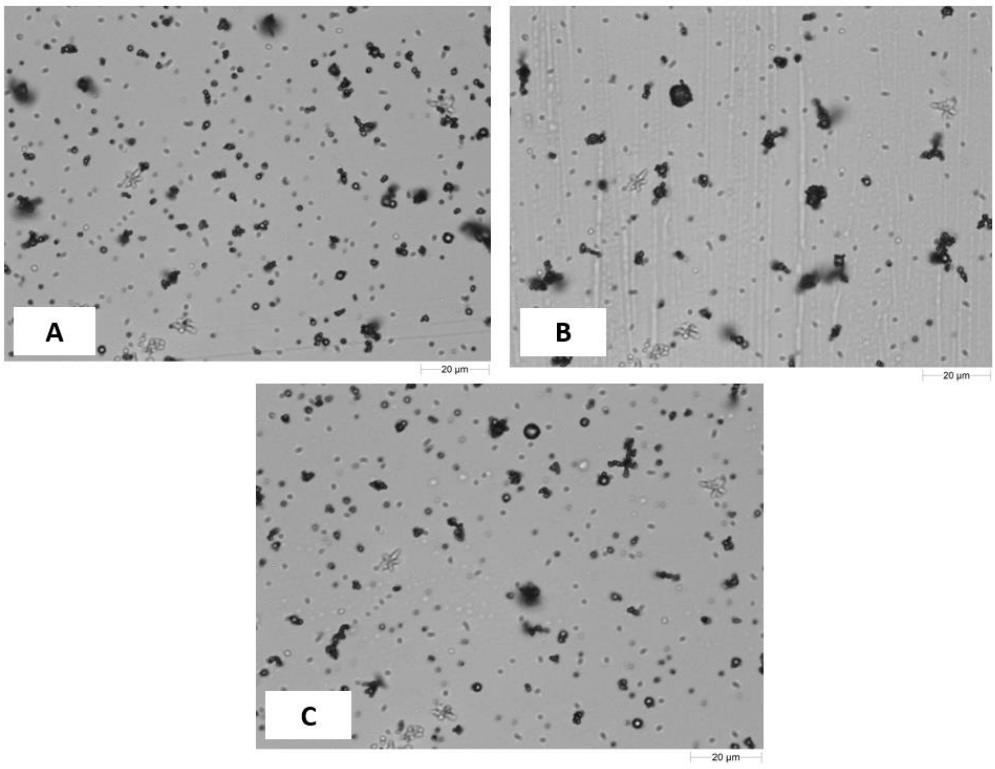

Figure 3.3 **-** Morphologi G3 images of Q_Lip-DPFs with 50 000 x magnification and scale bar 20 µm.

A- Neutral_F1_ PEGylated Q_Lip-DPFs , **B-** Negative_ F2_ PEGylate Q_Lip-DPFs , **C-** Positive _F2_PEGylate Q_Lip-DPFs

The volume mean diameter for 50% of the particles sample population, (Dv_{50}), and for 10% and 90% of the sample population, (Dv_{10}) and (Dv_{90}), respectively, and span are shown in ***Table 3.9*** for all Q_Lip-DPFs obtained. Regarding the D_{v50}, the volume mean diameter adequate for pulmonary delivery should be in the 0.5 -5 µm range [111]. It can be observed that mostly Q_Lip-DPFs presented the desired range. Nonetheless, the Q_LIP-DPFs with negative charge showed a higher value than expected, 10.64 and 10.45 µm, which may be an error associated with the formulation. All the Q_Lip-DPFs showed span close to 1, indicating that the dry powder formulations have narrow particle size distributions. The analysis of the morphology of the particles, by scanning electron microscopy (SEM), represented in ***Figure 3.4***, revealed the sphericity of the microparticles with a narrow size distribution.

Table 3.9- Particles size and distribution of Q_Lip-DPFs with neutral, positive and negative charged phospholipids.

Q_LIP-DPFs with neutral charged phospholipids	**$D_{v\,10}$ (µm)**	**$D_{v,50}$ (µm)**	**$D_{v,90}$ (µm)**	**span**
Q_Lip_F1_ Non- PEGylated	2.92	5.99	10.10	1.20
Q_Lip_F1_ PEGylated	2.43	4.54	8.68	1.38
Q_Lip_F2_ Non- PEGylated	3.06	6.87	11.78	1.27
Q_Lip_F2_ PEGylated	1.86	3.82	6.99	1.35
Q_LIP-DPFs with positive charged phospholipids	**$D_{v\,10}$ (µm)**	**$D_{v,50}$ (µm)**	**$D_{v,90}$ (µm)**	**span**
Q_Lip_F1_ Non- PEGylated	3.10	5.91	11.48	1.42
Q_Lip_F1_ PEGylated	2.29	4.73	8.81	1.38
Q_Lip_F2_ Non- PEGylated	3.25	6.57	12.89	1.47
Q_Lip_F2_ PEGylated	2.11	3.87	6.61	1.16
Q_LIP-DPFs with negative charged phospholipids	**$D_{v\,10}$ (µm)**	**$D_{v,50}$ (µm)**	**$D_{v,90}$ (µm)**	**span**
Q_Lip_F1_ Non- PEGylated	4.82	8.80	14.90	1.15
Q_Lip_F1_ PEGylated	5.36	10.64	17.87	1.18
Q_Lip_F2_ Non- PEGylated	5.42	10.45	16.92	1.10
Q_Lip_F2_ PEGylated	3.34	6.56	18.77	2.35

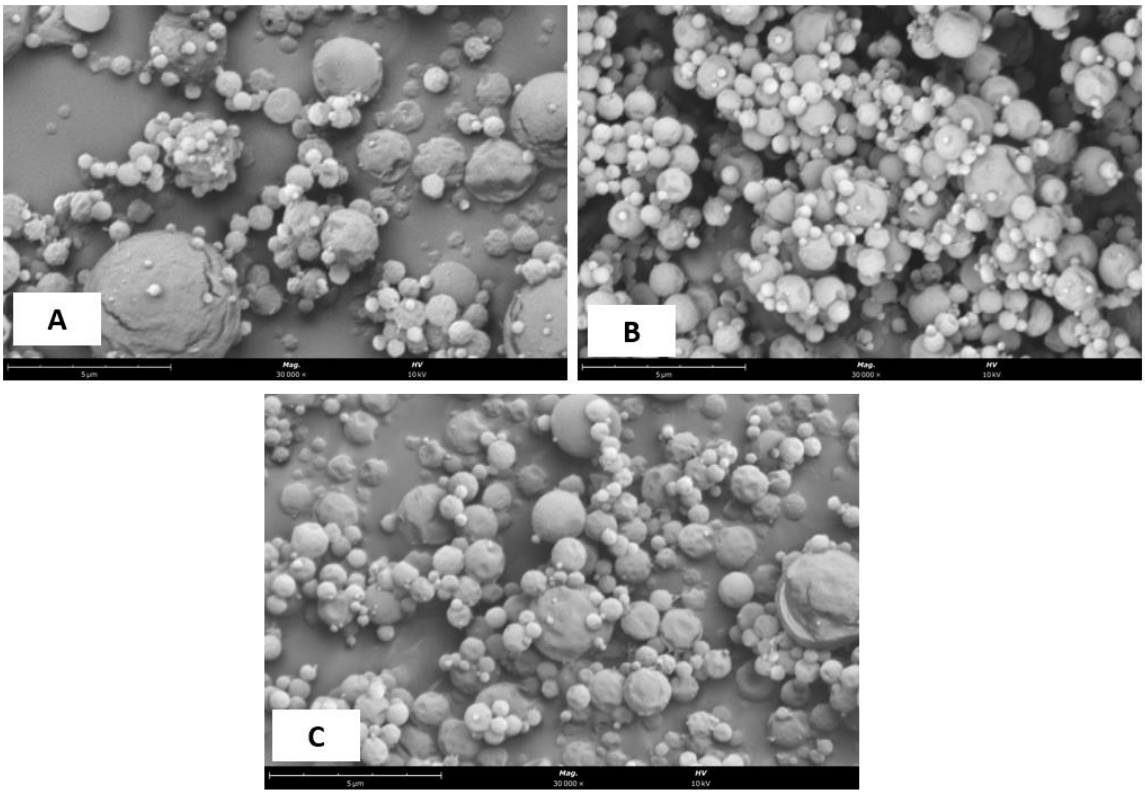

Figure 3.4 - SEM images of Q_Lip-DPFs (scale bar : 5 µm , magnitude: 30 000x and high-voltage:10 kV).

A- Negative_F1_ PEGylated Q_Lip-DPFs , **B-** Positive_ F1_ PEGylate Q_Lip-DPFs , **C-** Neutral_ F2_PEGylate Q_Lip-DPFs

- *In-vitro* aerodynamic performance

The Anderson Cascade Impactor (ACI) tests were performed to evaluate the aerodynamic properties of the Q_Lip-DPFs. Regarding the MMAD parameter, the particles should have aerodynamics in a range between 0.5 – 5 µm, for the success of the inhalation route [111]. The main goal for the powder is to achieve bronchi, if the particles have diameters lower than 0.5 µm will be exhaled and fail to deposit in the deep lung. Moreover, particles with sizes above 5 µm may be freely captured by macrophages [111]. ***Table 3.10*** summarizes the aerodynamic properties for the overall dry powder formulations.

Table 3.10- Characterization of Q_Lip-DPFs with neutral, positive and negative charged phospholipids on ACI.

Q_LIP-DPFs with neutral charged phospholipids	**MMAD (µm)**	**GSD**	**FPF(%)**
Q_Lip_F1_ Non- PEGylated	2.00	3.13	35
Q_Lip_F1_ PEGylated	1.09	3.04	24
Q_Lip_F2_ Non- PEGylated	1.78	2.41	47
Q_Lip_F2_ PEGylated	2.22	2.35	41
Q_LIP-DPFs with positive charged phospholipids	**MMAD (µm)**	**GSD**	**FPF(%)**
Q_Lip_F1_ Non- PEGylated	1.37	2.45	46
Q_Lip_F1_ PEGylated	1.94	2.65	36
Q_Lip_F2_ Non- PEGylated	1.47	2.32	48
Q_Lip_F2_ PEGylated	2.04	2.72	29
Q_LIP-DPFs with negative charged phospholipids	**MMAD (µm)**	**GSD**	**FPF(%)**
Q_Lip_F1_ Non- PEGylated	3.73	2.30	39
Q_Lip_F1_ PEGylated	2.55	2.30	49
Q_Lip_F2_ Non- PEGylated	1.46	2.43	53
Q_Lip_F2_ PEGylated	1.76	2.27	46

It can be observed that the average mass median aerodynamic diameter (MMAD), the breathable fraction, varied between 1.09 µm and 3.73 µm. The geometric standard deviations (GSD) ranged from 2.27 to 3.13, values that are suitable for inhalation. The FPF, the respirable fraction that is most likely to deposit in the deep lung, varied from 24% to 53%. Moreover, high FPF values mean promising results for inhalation.

4

4. CONCLUSIONS AND FUTURE WORK

In this work, the objective was to convert quercetin-loaded liposomes into quercetin-loaded liposomal dry powder formulations, using the SASD technology. Additionally, liposomes should keep the incorporation efficiency of the drug similar to what it had before processing. In this way, first, quercetin was encapsulated in liposomes with different surface electric charge. It was observed that incorporation efficiency and lipid yield of the results of all formulations produced (neutral, positive and negative charge) were above 70 %. Then, the best formulations were processed in SASD. The powder formulations were obtained with approximately 60% of yield and with 5-7 % of water content. Physicochemical characterizations such as the ATR-FTIR spectrum, suggested that liposomes were successfully encapsulated in the powder. Besides, the powders present an amorphous structure, which is a great advantage for pulmonary delivery. Regarding aerodynamic properties, ACI studies showed a MMAD within the desired range (0.5 –5 µm) for some formulations and FPFs ranging from 24 % to 53 % demonstrating promising results for inhalation. Liposomes were obtained after the rehydration of excipients and further ultracentrifugation. Overall, it was observed that liposomes' size increased and that the incorporation efficiency of the quercetin after the SASD process decreased. Moreover, Q_Lip-DPFs with positive charged phospholipids having better incorporation efficiency results, being the most promising formulations to study in the future.

Taking all the results in account, for the future a process of optimization is required, applying Quality by Design using the Design of Experiments tool to study the effects of L-leucine, trehalose and ethanol percentages in the casting solution and the SASD operating parameters. Moreover, it would be interesting to perform *in vitro* toxicity studies, using lung cell models to verify if the developed micronized powers comprising quercetin loaded liposomes affect the metabolic activity of the cells, and are able to combat lung inflammation. Additional optimization of the liposomes composition (ratio between lipids/stearylamine/quercetin) taking in account their properties after SASD would be also interesting to perform.

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5. REFERENCES

[1] P. Mehta, C. Bothiraja, K. Mahadik, S. Kadam, A. Pawar, "Phytoconstituent based dry powder inhalers as biomedicine for the management of pulmonary diseases," *Biomed. Pharmacother.*, vol. 108, no. July, pp. 828–837, 2018, doi: 10.1016/j.biopha.2018.09.094.

[2] V. Komalla , V. S. R. R. Allam, P. C. L. Kwok, B. Sheikholeslami, L. Owen, A. Jaffe, S. A. Waters, S. Mohammad, B.G. Oliver, H. Chen, M. Haghi "A phospholipid-based formulation for the treatment of airway inflammation in chronic respiratory diseases," *Eur. J. Pharm. Biopharm.*, vol. 157, no. May, pp. 47–58, 2020, doi: 10.1016/j.ejpb.2020.09.017.

[3] H. J. ANDERSON and G. M. STOREY, "Chronic respiratory disease.", *Physiotherapy*, vol. 41, no. 12, pp. 389–93, Dec. 1955, doi: 10.1136/jech.47.1.4.

[4] "Inflammatory Lung Diseases - Pharma Models." https://www.pharmamodels.net/indications/inflammatory-lung-diseases/ (accessed Mar. 17, 2021).

[5] "COVID-19 - Página Inicial." https://covid19.min-saude.pt/ (accessed Mar. 17, 2021).

[6] K. S. A. Okada, H. Todo, I. Hijikuro, S. Itakura, K. Sugibayashi, "Controlled release of a model hydrophilic high molecular weight compound from injectable non-lamellar liquid crystal formulations containing different types of phospholipids," vol. 8502, no. 94, pp. 761–766, 1994.

[7] L. F. C. Silva, G. Kasten, C. E. M. de Campos, A. L. Chinelatto, E. Lemos-Senna, "Preparation and characterization of quercetin-loaded solid lipid microparticles for pulmonary delivery," *Powder Technol.*, vol. 239, pp. 183–192, 2013, doi: 10.1016/j.powtec.2013.01.037.

[8] L. Bungener, A. Huckriede, J. Wilschut, T. Daemen, "Virosomes as a vaccine delivery system," in *Micro- and Nanotechnology in Vaccine Development*, Elsevier Inc., 2000, pp. 845-850 BT-EFIS 2000: 14TH EUROPEAN IMMUNOLOGY, doi: 10.1016/B978-0-323-39981-4/00012-9

[9] C. Moura, T. Casimiro, E. Costa, A. Aguiar-Ricardo, "Optimization of supercritical CO 2 -assisted spray drying technology for the production of inhalable composite particles using quality-by- design principles," *Powder Technol.*, vol. 357, pp. 387–397, 2019, doi: 10.1016/j.powtec.2019.08.090.

[10] "Forum of International Respiratory Societies The Global Impact of Respiratory Disease," 2012, Accessed: Mar. 17, 2021. [Online]. Available: https://www.who.int/.

[11] "Lung Inflammation: Symptoms, Causes, Treatment, More." https://www.verywellhealth.com/chest-pain-common-potential-causes-1745274 (accessed Mar. 17, 2021).

[12] I. Europe, “6.22 Pneumonia,” in *Priority diseases and reasons for inclusion*, vol. 22, 2013, pp. 1–4

[13] A. S. Ginsburg, R. Izadnegahdar, K. P. Klugman, “World Pneumonia Day 2016: pulse oximetry and oxygen,” *Lancet Glob. Heal.*, vol. 4, no. 12, pp. e893–e894, 2016, doi: 10.1016/S2214-109X(16)30296-0.

[14] “Pneumonia - NHS.” https://www.nhs.uk/conditions/pneumonia/ (accessed Nov. 22, 2021).

[15] F. R. Rosa, F. G. Dias, L. N. Nobre, H. A. Morais, “Fibrose cística: Uma abordagem clínica e nutricional,” *Rev. Nutr.*, vol. 21, no. 6, pp. 725–737, 2008, doi: 10.1590/S1415-52732008000600011.

[16] C. Fibrosis Foundation, “2019 PATIENT REGISTRY ANNUAL DATA REPORT.”

[17] “Chronic Lung Diseases: Causes and Risk Factors.” https://www.healthline.com/health/understanding-idiopathic-pulmonary-fibrosis/chronic-lung-diseases-causes-and-risk-factors (accessed Mar. 17, 2021).

[18] “FAQs.” https://www.cysticfibrosis.org.uk/what-is-cystic-fibrosis/faqs (accessed Dec. 01, 2021).

[19] “Asthma.” https://www.who.int/news-room/fact-sheets/detail/asthma (accessed Nov. 24, 2021).

[20] “Chronic obstructive pulmonary disease (COPD).” https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd) (accessed Nov. 24, 2021).

[21] “APSR: 2018 World Lung Day.” https://www.apsresp.org/archive/world-days/2019-lung.html (accessed Nov. 24, 2021).

[22] “World Pneumonia Day - Stop Pneumonia / Every Breath Counts.” https://stoppneumonia.org/latest/world-pneumonia-day/ (accessed Dec. 01, 2021).

[23] O. Ruuskanen, E. Lahti, L. C. Jennings, D. R. Murdoch, “Viral pneumonia,” *Lancet*, vol. 377, no. 9773, pp. 1264–1275, 2011, doi: 10.1016/S0140-6736(10)61459-6.

[24] “CORTICOIDES - Tipos, indicações e efeitos • MD.Saúde.” https://www.mdsaude.com/endocrinologia/glicocorticoides/ (accessed Mar. 17, 2021).

[25] “O que é fórmula estrutural? - Brasil Escola.” https://brasilescola.uol.com.br/o-que-e/quimica/o-que-e-formula-estrutural.htm (accessed Mar. 17, 2021).

[26] S. M. A. Anti, R. D. N. Giorgi, W. H. Chahade, “Antiinflamatórios hormonais : Glicocorticóides Steroidal antiinfl ammatory drugs : glucocorticoids,” *Einstein*, vol. 6, no. Supl 1, pp. 159–165, 2008.

[27] A. N. M. Alamgir, *Phytoconstituents—Active and inert constituents, metabolic pathways, chemistry and application of phytoconstituents, primary metabolic products, and bioactive compounds of primary metabolic origin*, vol. 74. Springer International Publishing, 2018, doi: 10.1007/978-3-319-92387-1_2

[28] A. G. Bayir, H. S. Kiziltan and A. Kocyigit, “Plant Family, Carvacrol, and Putative Protection in Gastric Cancer,” *Diet. Interv. Gastrointest. Dis. Foods, Nutr. Diet. Suppl.*, pp. 3–18, 2019, doi: 10.1016/B978-0-12-814468-8.00001-6.

[29] T. F. Rambaran, "Nanopolyphenols: a review of their encapsulation and anti-diabetic effects," *SN Appl. Sci.*, vol. 2, no. 8, 2020, doi: 10.1007/s42452-020-3110-8.

[30] "What Is Quercetin? Benefits, Foods, Dosage, and Side Effects." https://www.healthline.com/nutrition/quercetin (accessed Apr. 04, 2021).

[31] F. J. Pérez-Cano, M. Castell, "Flavonoids, inflammation and immune system," *Nutrients*, vol. 8, no. 10, pp. 8–11, 2016, doi: 10.3390/nu8100659.

[32] "Everything You Need to Know About Flavonoids." https://www.healthline.com/health/what-are-flavonoids-everything-you-need-to-know (accessed Mar. 17, 2021).

[33] Viafarma, "Silibin Phytosome ®."

[34] M. Román-Aguirre, C. Leyva-Porras, P. Cruz-Alcantar, A. Aguilar-Elguézabal, M. Z. Saavedra-Leos, "Comparison of polysaccharides as coatings for quercetin-loaded liposomes (Qll) and their effect as antioxidants on radical scavenging activity," *Polymers (Basel).*, vol. 12, no. 12, pp. 1–15, 2020, doi: 10.3390/polym12122793.

[35] M. Huang, E. Su, F. Zheng, C. Tan, "Encapsulation of flavonoids in liposomal delivery systems: The case of quercetin, kaempferol and luteolin," *Food Funct.*, vol. 8, no. 9, pp. 3198–3208, 2017, doi: 10.1039/c7fo00508c.

[36] C. Caddeo, O. Díez-Sales, R. Pons, X. Fernàndez-Busquets, A. M. Fadda M. Manconi, "Topical anti-inflammatory potential of quercetin in lipid-based nanosystems: In vivo and in vitro evaluation," *Pharm. Res.*, vol. 31, no. 4, pp. 959–968, 2014, doi: 10.1007/s11095-013-1215-0.

[37] Z. Chavoshpour-Natanzi, M. Sahihi, "Encapsulation of quercetin-loaded β-lactoglobulin for drug delivery using modified anti-solvent method," *Food Hydrocoll.*, vol. 96, no. December 2018, pp. 493–502, 2019, doi: 10.1016/j.foodhyd.2019.05.051.

[38] S. A. Fereig, G. M. El-Zaafarany, M. G. Arafa, M. M. A. Abdel-Mottaleb, "Tackling the various classes of nano-therapeutics employed in topical therapy of psoriasis," *Drug Deliv.*, vol. 27, no. 1, pp. 662–680, 2020, doi: 10.1080/10717544.2020.1754527.

[39] J. Siepmann, A. Faham, S. D. Clas, B. J. Boyd, V. Jannin, A. Bernkop-Schnürch, H. Zhao, S. Lecommandoux, J. C. Evans, C. Allen, O. M. Merkel, G. Costabilei, M. R. Alexander, R. D. Wildman, C. J. Roberts, J. C. Leroux, "Lipids and polymers in pharmaceutical technology: Lifelong companions," *Int. J. Pharm.*, vol. 558, no. October 2018, pp. 128–142, 2019, doi: 10.1016/j.ijpharm.2018.12.080.

[40] V. De Simone, A. Dalmoro, S. Bochicchio, D. Caccavo, G. Lamberti, P. Bertoncin, A. A. Barba "Nanoliposomes in polymeric granules: Novel process strategy to produce stable and versatile delivery systems," *J. Drug Deliv. Sci. Technol.*, vol. 59, no. June, p. 101878, 2020, doi: 10.1016/j.jddst.2020.101878.

[41] N. P. Aditya, A. S. Macedo, S. Doktorovova, E. B. Souto, S. Kim, P. S. Chang, S. Ko, "Development and evaluation of lipid nanocarriers for quercetin delivery: A comparative study of solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and lipid nanoemulsions (LNE)," *LWT - Food Sci. Technol.*, vol. 59, no. 1, pp. 115–121, 2014, doi: 10.1016/j.lwt.2014.04.058.

[42] Ștefania A. Milea, I. Aprodu, L. Mihalcea, E. Enachi, C.A. Bolea, G. Râpeanu, G. E. Bahrim, N. Stˇanciuc, "Bovine β-lactoglobulin peptides as novel carriers for flavonoids extracted with supercritical fluids from yellow onion skins," *J. Food Sci.*, vol. 85, no. 12, pp. 4290–4299, 2020, doi: 10.1111/1750-3841.15513.

[43] P. K. Deb, O. Al-Attraqchi, B. Chandrasekaran, A. Paradkar, R. K. Tekade, "Protein/peptide drug delivery systems: Practical considerations in pharmaceutical product development," *Basic Fundam. Drug Deliv.*, pp. 651–684, 2018, doi: 10.1016/B978-0-12-817909-3.00016-9.

[44] H. K. Ha, J. W. Kim, M. R. Lee, W. J. Lee, "Formation and characterization of quercetin-loaded chitosan oligosaccharide/β-lactoglobulin nanoparticle," *Food Res. Int.*, vol. 52, no. 1, pp. 82–90, 2013, doi: 10.1016/j.foodres.2013.02.021.

[45] M. Sharma, J. Joshi, N. Kumar Chouhan, M. N. Talati, S. Vaidya, and A. Kumar, "Liposome-A Comprehensive Approach for Researchers," *Molecular Pharmacology*, IntechOpen, Dec. 16, 2020, doi: 10.5772/intechopen.93256

[46] C. T. de Barros , A.C. Rios, T.F.R. Alves, F. Batain, K. M. M. Crescencio , L. J. Lopes, A. Zieli´nska, P. Severino, P. G. Mazzola, E. B. Souto, M. V. Chaud, "Cachexia: Pathophysiology and ghrelin liposomes for nose-to-brain delivery," *Int. J. Mol. Sci.*, vol. 21, no. 17, pp. 1–16, 2020, doi: 10.3390/ijms21175974.

[47] "Van der Waals Forces - Chemistry LibreTexts." https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Physical_Properties_of_Matter/Atomic_and_Molecular_Properties/Intermolecular_Forces/Van_der_Waals_Forces (accessed Dec. 03, 2021).

[48] C. Isalomboto Nkanga, A. Murhimalika Bapolisi, N. Ikemefuna Okafor, and R. Werner Maçedo Krause, "General Perception of Liposomes: Formation, Manufacturing and Applications," in *Liposomes - Advances and Perspectives*, 2019, doi: 10.5772/intechopen.84255.

[49] P. Trucillo, R. Campardelli, E. Reverchon, "Liposomes: From bangham to supercritical fluids," *Processes*, vol. 8, no. 9, pp. 1–15, 2020, doi: 10.3390/pr8091022.

[50] M. R. Mozafari, "Liposomes: An overview of manufacturing techniques," *Cell. Mol. Biol. Lett.*, vol. 10, no. 4, pp. 711–719, 2005.

[51] "Lipossomas: a nova tecnologia dos suplementos - blog." https://www.sundt.pt/blog/lipossomas/ (accessed Jan. 27, 2022).

[52] A. Nagalingam, "Drug Delivery Aspects of Herbal Medicines," in *Japanese Kampo Medicines for the Treatment of Common Diseases: Focus on Inflammation*, Elsevier Inc., 2017, pp. 143–164, doi: 10.1016/B978-0-12-809398-6.00015-9

[53] M. Grit and D. J. A. Crommelin, "Chemical-stability-of-liposomes-implications-for-their-physical-stability_1993_Chemistry-and-Physics-of-Lipids," *Chem. Phys. Lipids*, vol. 64, pp. 3–18, 1993, doi: 10.1016/0009-3084(93)90053-6

[54] M. L. Briuglia, C. Rotella, A. McFarlane, D. A. Lamprou, "Influence of cholesterol on liposome stability and on in vitro drug release," *Drug Deliv. Transl. Res.*, vol. 5, no. 3, pp. 231–242, 2015, doi: 10.1007/s13346-015-0220-8.

[55] H. C. Huang, S. Mallidi, G. Obaid, B. Sears, S. Tangutoori, T. Hasan, "Advancing photodynamic therapy with biochemically tuned liposomal nanotechnologies," in *Applications of Nanoscience in Photomedicine*, Woodhead Publishing Limited, 2015, pp. 487–510, doi: 10.1533/9781908818782.487

[56] D. N. Fakult, "Development of Proliposomal and Liposomal Formulations with Poorly Water-soluble Drugs," p. 195, 2019.

[57] "Cholesterol in Liposomes." https://avantilipids.com/news/cholesterol-in-liposomes (accessed Sep. 15, 2021).

[58] "Colesterol." https://www.pfizer.com.br/sua-saude/coracao/colesterol (accessed Sep. 15, 2021).

[59] Sigma-Aldrich, "Cholesterol Sigma Grade," 2014. https://www.sigmaaldrich.com/PT/en/product/sigma/c8667 (accessed Sep. 23, 2021).

[60] C. Costa, B. Nobre, A.S. Matos, A.S. Silva, T. Casimiro, M.L. Corvo, A. Aguiar-Ricardo "Inhalable hydrophilic molecule-loaded liposomal dry powder formulations using supercritical CO2 – assisted spray-drying," *J. CO2 Util.*, vol. 53, no. May, p. 101709, 2021, doi: 10.1016/j.jcou.2021.101709.

[61] M. Luisa Corvo, J. C. S. Jorge, R. Van't Hof, M. E. M. Cruz, D. J. A. Crommelin, G. Storm, "Superoxide dismutase entrapped in long-circulating liposomes: Formulation design and therapeutic activity in rat adjuvant arthritis," *Biochim. Biophys. Acta - Biomembr.*, vol. 1564, no. 1, pp. 227–236, 2002, doi: 10.1016/S0005-2736(02)00457-1.

[62] W. Chen, F. Duša, J. Witos, S. K. Ruokonen, S. K. Wiedmer, "Determination of the Main Phase Transition Temperature of Phospholipids by Nanoplasmonic Sensing," *Scientific Reports*, vol. 8, no. 1, pp. 1–11, 2018, doi: 10.1038/s41598-018-33107-5.

[63] H. Zhang, "Thin-film hydration followed by extrusion method for liposome preparation," *Methods Mol. Biol.*, vol. 1522, pp. 17–22, 2017, doi: 10.1007/978-1-4939-6591-5_2.

[64] C. M. Batista, C. M. B. De Carvalho, N. S. S. Magalhães, "Lipossomas e suas aplicações terapêuticas: Estado da arte," *Rev. Bras. Ciencias Farm. J. Pharm. Sci.*, vol. 43, no. 2, pp. 167–179, 2007, doi: 10.1590/S1516-93322007000200003.

[65] M. Çağdaş, A. D. Sezer, S. Bucak, "Liposomes as Potential Drug Carrier Systems for Drug Delivery," in *Application of Nanotechnology in Drug Delivery*, InTech, 2014, doi:10.5772/58459.

[66] B. Maherani, E. Arab-Tehrany, M. R. Mozafari, C. Gaiani, M. Linder, "Liposomes: A Review of Manufacturing Techniques and Targeting Strategies," *Curr. Nanosci.*, vol. 7, no. 3, pp. 436–452, 2011, doi: 10.2174/157341311795542453.

[67] R. Shukla, M. Handa, N. Vasdev, D. P. Singh, P. Kesharwani, *Nanomedicine in pain management*, no. 4. Elsevier Inc., 2021.

[68] "New: dPEG-Phospholipids for superior liposomes." https://www.iris-biotech.de/en/blog/dPEG-Phospholipids-for-superior-liposomes/ (accessed Dec. 03, 2021).

[69] E. A. Ross, A. Devitt, J. R. Johnson, "Macrophages: The Good, the Bad, and the Gluttony," *Front. Immunol.*, vol. 12, p. 3234, Aug. 2021, doi: 10.3389/FIMMU.2021.708186/BIBTEX.

[70] M. L. Corvo, O. C. Boerman, W. J. G. Oyen, L.V. Bloois, M. E. M. Cruz, D. J. A. Crommelin,G. Storm, "Intravenous administration of superoxide dismutase entrapped in long circulating liposomesII. In vivo fate in a rat model of adjuvant arthritis," *Biochim. Biophys. Acta - Biomembr.*, vol. 1419, no. 2, pp. 325–334, 1999, doi: 10.1016/S0005-2736(99)00081-4.

[71] J. Zhang, Y. Luo, X. Zhao, X. Li, K.Li, D. Chen, M. Qiao, H. Hu, X. Zhao, "Co-delivery of doxorubicin and the traditional Chinese medicine quercetin using biotin-PEG2000-DSPE modified liposomes for the treatment of multidrug resistant breast cancer," *RSC Adv.*, vol. 6, no. 114, pp. 113173–113184, 2016, doi: 10.1039/c6ra24173e.

[72] B. Giordani, P. Basnet, E. Mishchenko, B. Luppi, N. Škalko-Basnet, "Utilizing liposomal quercetin and gallic acid in localized treatment of vaginal Candida infections," *Pharmaceutics*, vol. 12, no. 1, pp. 1–21, 2020, doi: 10.3390/pharmaceutics12010009.

[73] C. Caddeo, A. Nacher, A. Vassallo, M. F. Armentano, R. Pons, X. Fernàndez-Busquets, C. Carbone, D. Valenti, A. M. Fadda, M. Manconi, "Effect of quercetin and resveratrol co-incorporated in liposomes against inflammatory/oxidative response associated with skin cancer," *Int. J. Pharm.*, vol. 513, no. 1–2, pp. 153–163, 2016, doi: 10.1016/j.ijpharm.2016.09.014.

[74] C. Caddeo, R. Pons, C. Carbone ,X. Fern`andez-Busquets, C. Cardia, A. M. Fadda, M. Manconi, "Physico-chemical characterization of succinyl chitosan-stabilized liposomes for the oral co-delivery of quercetin and resveratrol," *Carbohydr. Polym.*, vol. 157, pp. 1853–1861, 2017, doi: 10.1016/j.carbpol.2016.11.072.

[75] P. T. Ingvarsson, M. Yang, H. Mø. Nielsen, J. Rantanen, C. Foged, "Stabilization of liposomes during drying," *Expert Opin. Drug Deliv.*, vol. 8, no. 3, pp. 375–388, 2011, doi: 10.1517/17425247.2011.553219.

[76] T. Tavares, A. S. S. Ma, A. Aguiar-Ricardo, "Sustainable strategies for nano-in-micro particle engineering for pulmonary delivery," 2014, doi: 10.1007/s11051-014-2602-0.

[77] I. Duarte, "Development of new screening methodologies and preparation methods with application in amorphous solid dispersions and pharmaceutical cocrystals," pp. 1–189, 2016.

[78] G. Pilcer, K. Amighi, "Formulation strategy and use of excipients in pulmonary drug delivery," *Int. J. Pharm.*, vol. 392, no. 1–2, pp. 1–19, 2010, doi: 10.1016/j.ijpharm.2010.03.017.

[79] A. F. dos S. Fadista, “Insulinoterapia: vias e sistemas de administração alternativos,” p. 4, 2011.

[80] R. P. Cabral., A.M.L. Sousa, A.S. Silva, A.I. Paninho, M. Temtem, E. Costa, T. Casimiro, A. Aguiar-Ricardo, “Design of experiments approach on the preparation of dry inhaler chitosan composite formulations by supercritical CO_2-assisted spray-drying,” *J. Supercrit. Fluids*, vol. 116, pp. 26–35, 2016, doi: 10.1016/j.supflu.2016.04.001.

[81] G. I. L. Marco, J. Vicente, F. Gaspar, “Scale-up methodology for pharmaceutical spray drying,” *Chim. Oggi*, vol. 28, no. 4, pp. 18–22, 2010.

[82] R. Bi, W. Shao, Q. Wang, N. Zhang, “Spray-freeze-dried dry powder inhalation of insulin-loaded liposomes for enhanced pulmonary delivery,” *J. Drug Target.*, vol. 16, no. 9, pp. 639–648, 2008, doi: 10.1080/10611860802201134.

[83] L. G. Sweeney, Z. Wang, R. Loebenberg, J. P. Wong, C. F. Lange, W. H. Finlay, “Spray-freeze-dried liposomal ciprofloxacin powder for inhaled aerosol drug delivery,” *Int. J. Pharm.*, vol. 305, no. 1–2, pp. 180–185, 2005, doi: 10.1016/j.ijpharm.2005.09.010.

[84] A. Aguiar-Ricardo, “Building dry powder formulations using supercritical CO2 spray drying,” *Curr. Opin. Green Sustain. Chem.*, vol. 5, pp. 12–16, 2017, doi: 10.1016/j.cogsc.2017.03.005.

[85] M. Temtem, C. Moura, T. Casimiro, E. Costa A. Aguiar-Ricardo, “Benchmarking Supercritical CO2-assisted Spray Drying with Conventional Spray Drying for the Manufacture of Inhalation Formulations,” *Respir. Drug Deliv. Eur.*, pp. 1–14, 2017.

[86] C. Moura, E. Costa, A. Aguiar-Ricardo, “Optimization of Supercritical-CO_2 Assisted Spray Drying for the Production of Inhalable composite particles,” *Ddl 2016*, p. 2829, 2016.

[87] “(PDF) Supercritical Fluid technology a boon for pharmaceutical Particle Manufacturing.” https://www.researchgate.net/publication/301887983_Supercritical_Fluid_technology_a_boon_for_pharmaceutical_Particle_Manufacturing (accessed Dec. 03, 2021).

[88] M. P. Bezerra L. N. C. Rodrigues, “Quality By Design (Qbd) Como Ferramenta Para Otimização Dos Processos Farmacêuticos,” *Infarma - Ciências Farm.*, vol. 29, no. 1, pp. 5–12, 2017, doi: 10.14450/2318-9312.v29.e1.a2017.pp5-12.

[89] L. X. Yu, G. Amidon, M.A. Khan, S.W. Hoag, J. Polli, G. K. Raju, J. Woodcock “Understanding pharmaceutical quality by design,” *AAPS J.*, vol. 16, no. 4, pp. 771–783, 2014, doi: 10.1208/s12248-014-9598-3.

[90] “Blog: Introduction to Quality by Design.” https://www.alcaminow.com/blog/introduction-to-quality-by-design (accessed Apr. 05, 2021).

[91] “Pharmaceutical ‘Quality by Design’ (QbD): An Introduction, Process Development and Applications - Learnaboutgmp: Accredited Online Life Science Training Courses.” https://learnaboutgmp.com/good-validation-practices/pharmaceutical-quality-by-design-qbd-an-introduction-process-development-and-applications/ (accessed Apr. 05, 2021).

[92] "Quality by design | European Medicines Agency." https://www.ema.europa.eu/en/human-regulatory/research-development/quality-design (accessed Apr. 05, 2021).

[93] "Desenho de experimentos | Introdução às estatísticas | JMP." https://www-jmp-com.translate.goog/en_au/statistics-knowledge-portal/what-is-design-of-experiments.html?_x_tr_sl=en&_x_tr_tl=pt&_x_tr_hl=pt-PT (accessed Dec. 04, 2021).

[94] H. F. Wagner, J. R., Mount, E. M., & Giles, "Design of Factorial Experiments," *Extrusion*, pp. 291–308, 2014, doi: 10.1016/B978-1-4377-3481-2.00025-9.

[95] "O que é um Planejamento de Experimentos? Como aplicar na indústria?" https://www.fm2s.com.br/planejamento-de-experimentos/ (accessed Dec. 04, 2021).

[96] P. C. Soema, G. J. Willems, W. Jiskoot, J. P. Amorij, G. F. Kersten, "Predicting the influence of liposomal lipid composition on liposome size, zeta potential and liposome-induced dendritic cell maturation using a design of experiments approach," *Eur. J. Pharm. Biopharm.*, vol. 94, pp. 427–435, 2015, doi: 10.1016/j.ejpb.2015.06.026.

[97] M. S. Webb, J. J. Wheeler, M. B. Bally, L. D. Mayer, "The cationic lipid stearylamine reduces the permeability of the cationic drugs verapamil and prochlorperazine to lipid bilayers: Implications for drug delivery," *BBA - Biomembr.*, vol. 1238, no. 2, pp. 147–155, 1995, doi: 10.1016/0005-2736(95)00121-I.

[98] M. A. Voinov, I. Rivera-Rivera, A. I. Smirnov, "Surface electrostatics of lipid bilayers by EPR of a pH-sensitive spin-labeled lipid," *Biophys. J.*, vol. 104, no. 1, pp. 106–116, 2013, doi: 10.1016/j.bpj.2012.11.3806.

[99] "Cromatografia da exclusão do tamanho (filtragem de gel)." https://www.news-medical.net/life-sciences/Size-Exclusion-Chromatography-(Gel-Filtration)-(Portuguese).aspx (accessed Sep. 24, 2021).

[100] G. Rouser, S. Fleischer, A. Yamamoto, "Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots," *Lipids*, vol. 5, no. 5, pp. 494–496, 1970, doi: 10.1007/BF02531316.

[101] "Andersen Cascade Impactor-ACI - Westech Scientific Instruments." https://www.westechinstruments.com/pharmaceutical/andersen-cascade-impactor-aci/ (accessed Dec. 06, 2021).

[102] A. S. Silva, A. M. Sousa, R. P. Cabral, M. C. Silva, C.Costa, S. P. Miguel, V. D. B Bonifácio, T. Casimiro, I. J. Correia, A. Aguiar-Ricardo,"Aerosolizable gold nano-in-micro dry powder formulations for theragnosis and lung delivery," *Int. J. Pharm.*, vol. 519, no. 1–2, pp. 240–249, 2017, doi: 10.1016/j.ijpharm.2017.01.032.

[103] Copley Scientific, "Andersen Cascade Impactor (ACI)," *Andersen Cascade Impactor (ACI)*, vol. 12, no. 4, pp. 584–588, 2016, [Online]. Available: http://www.copleyscientific.com/home/inhaler-testing/aerodynamic-particle-size/andersen-cascade-impactor-aci.

[104] E. Joseph, G. Singhvi, *Chapter 4. Multifunctional nanocrystals for cancer therapy: a potential nanocarrier*. Elsevier Inc., 2019.

[105] W. L. J. Hinrichs, F. A. Mance˜nido , N. N. Sanders, K. Braeckmans , S.C. De Smedt, J. Demeester, "The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying," *Int. J. Pharm.*, vol. 311, no. 1–2, pp. 237–244, 2006, doi: 10.1016/j.ijpharm.2005.12.032.

[106] "Polyethylene Glycol (PEG) Surface – nanoComposix." https://nanocomposix.com/pages/polyethylene-glycol-peg-surface (accessed Jan. 27, 2022).

[107] M. Wong, G. N. C. Chiu, "DEVELOPMENT AND CHARACTERIZATION OF A," vol. 8, pp. 175–179, 2009.

[108] C. M. Deber, T. A. Stone, "Relative role(S) of leucine versus isoleucine in the folding of membrane proteins," *Pept. Sci.*, vol. 111, no. 1, 2019, doi: 10.1002/pep2.24075.

[109] H.Anselmo, "Preparation of Encapsulated Add-Value Bioactive Phenolic Compounds by Supercritical CO 2 -Assisted Spray Drying," 2020.

[110] K. AboulFotouh, Y. Zhang, M. Maniruzzaman, R. O. Williams, Z. Cui, "Amorphous solid dispersion dry powder for pulmonary drug delivery: Advantages and challenges," *Int. J. Pharm.*, vol. 587, p. 119711, 2020, doi: 10.1016/j.ijpharm.2020.119711.

[111] M. C. Silva, A. S. Silva, J. Fernandez-Lodeiro, T. Casimiro, C. Lodeiro, A. Aguiar-Ricardo, "Supercritical CO_2-assisted spray drying of strawberry-like gold-coated magnetite nanocomposites in chitosan powders for inhalation," *Materials (Basel).*, vol. 10, no. 1, 2017, doi: 10.3390/ma10010074.

A

A.APPENDIX

APPENDIX A

***A.1*-**Process conditions of Q_Lip with neutral, positive and negative charged phospholipids.

CODE	PRESSÃO Static mixer(PSI)	PRESSÃO Static mixer (BAR)	TEMP Static mixer (ºC)	TOUT(GÁS) ºC	TIN(Gás) ºC
Neutro_F2_ Non-PEGylated	1730	119	82	68,2	100
	1767	122	90	59,6	94
	1780	123	76	69,4	100
Negativo_F1_ Non-PEGylated	1738	120	88	70,8	107
	1769	122	96	62,5	93
	1745	120	90	63,8	98
Neutro_F2_ PEGylated	1766	122	88	73,1	110
	1772	122	95	65,9	108
	1750	121	92	69,3	100
Negativo_F2_ PEGylated	1772	122	95	65,9	108
	1727	119	93	63,7	107
	1750	121	90	69	100
Neutro_F1_ Non-PEGylated	1737	120	94	63,2	91
	1743	120	89	62,9	95
	1767	122	94	63,0	92
Positivo_F2_ PEGylated	1746	120	83	71,8	102
	1750	121	90	69	100
	1760	121	88	68	100
Neutro_F1_ PEGylated	1765	122	84	64,5	103
	1742	120	87	56,9	100
	1750	121	88	69	101
Positivo_F1_ Non-PEGylated	1728	119	84	67,7	110
	1740	120	76	68,6	98
	1764	122	89	68,1	97
Negativo_F1_ PEGylated	1733	119	93	61,3	107
	1732	119	81	59,1	92
	1750	121	80	60	100
Positivo_F1_ PEGylated	1736	120	80	69,6	108
	1738	120	94	64,6	105
	1762	121	78	63,5	93
Positivo_F2_ Non-PEGylated	1748	120	85	65,0	105
	1784	123	76	66,9	100
	1750	121	80	65	100
Negativo_F2_ Non-PEGylated	1739	120	75	60,7	93
	1739	120	95	67	100
	1765	122	83	70,6	109

APPENDIX B

B.1- Quercetin quantification in Q_Lip with neutral charged phospholipids before and after SASD.

Q_Lip with neutral charged phospholipids	**Time**	**Area**
Q_Lip_F1_ Non- PEGylated (Before SASD)	4.717	461 129
Q_Lip_F1_ Non- PEGylated (After SASD)	4.633	30 926
Q_Lip_F1_ PEGylated **(** Before SASD)	4.700	424 022
Q_Lip_F1_ PEGylated (After SASD)	4.617	31 193
Q_Lip_F2 _ Non- PEGylated (Before SASD)	4.700	1 015 154
Q_Lip_F2_ Non- PEGylated (After SASD)	4.617	89 077
Q_Lip_F2_ PEGylated (Before SASD)	4.683	1 194 159
Q_Lip_F2_ PEGylated (After SASD)	4.617	124 366

B.2- Quercetin quantification in Q_Lip with positive charged phospholipids before and after SASD.

Q_Lip with positive charged phospholipids	**Time**	**Area**
Q_Lip_F1_ Non- PEGylated (Before SASD)	4.667	247 294
Q_Lip_F1_ Non- PEGylated (After SASD)	4.617	28 729
Q_Lip_F1_ PEGylated **(** Before SASD)	4.667	323 015
Q_Lip_F1_ PEGylated (After SASD)	4.617	35 889
Q_Lip_F2_ Non- PEGylated (Before SASD)	4.667	565 323
Q_Lip_F2_ Non- PEGylated (After SASD)	4.617	64 883
Q_Lip_F2_ PEGylated (Before SASD)	4. 667	1 047 655
Q_Lip_F2_ PEGylated (After SASD)	4.617	64 883

B.3- Quercetin quantification in Q_Lip with negative charged phospholipids before and after SASD

Q_Lip with negative charged phospholipids	**Time**	**Area**
Q_Lip_F1_ Non- PEGylated (Before SASD)	4.650	847 148
Q_Lip_F1_ Non- PEGylated (After SASD)	4.617	79 782
Q_Lip_F1_ PEGylated **(**Before SASD)	4.650	647 857
Q_Lip_F1_ PEGylated (A**f**ter SASD)	4.617	33 773
Q_Lip_F2_ Non- PEGylated (Before SASD)	4.650	1 610 391
Q_Lip_F2_ Non- PEGylated (After SASD)	4.617	171 670
Q_Lip_F2_ PEGylated (Before SASD)	4.633	156 4876
Q_Lip_F2_ PEGylated (After SASD)	4.633	83 255

B.4- Ratio of (quercetin/ lipid) before SASD process in liposomal formulations.

Q_Lip with neutral charged phospholipids	**PEGylated Q_Lip**	**Non- PEGylated Q_Lip**
	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$
F1	8	8
F2	25	25
Q_Lip with positive charged phospholipids	PEGylated Q_Lip	Non- PEGylated Q_Lip
	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$
F1	9	6
F2	20	13
Q_Lip with positive charged phospholipids	PEGylated Q_Lip	Non- PEGylated Q_Lip
	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$	$(Quer/Lipid)_{Before\ SASD}$ $(\mu g/\mu mol)$
F1	22	21
F2	44	43

Appendix C

C.1- Total lipid concentration before and after SASD in liposomal formulations.

Q_Lip with neutral charged phospholipids	**PEGylated Q_Lip**	**Non- PEGylated Q_Lip**	**PEGylated Q_Lip**	**Non- PEGylated Q_Lip**
	C lipid $_{\text{Before SASD}}$ (µmol/ml)		C lipid $_{\text{After SASD}}$ (µmol/ml)	
F1	24	25	7	5
F2	21	18	10	10
Q_Lip with positive charged phospholipids	PEGylated Q_Lip	Non- PEGylated Q_Lip	PEGylated Q_Lip	Non- PEGylated Q_Lip
	C lipid $_{\text{Before SASD}}$ (µmol/ml)		C lipid $_{\text{After SASD}}$ (µmol/ml)	
F1	15	18	5	5
F2	23	20	18	6
Q_Lip with negative charged phospholipids	PEGylated Q_Lip	Non- PEGylated Q_Lip	PEGylated Q_Lip	Non- PEGylated Q_Lip
	C lipid $_{\text{Before SASD}}$ (µmol/ml)		C lipid $_{\text{After SASD}}$ (µmol/ml)	
F1	16	18	9	13
F2	18	20	8	13