# Liposome systems for passion fruit seed oil encapsulation in view of cosmetic applications

**Gabriela Gomes da Silva**

Final dissertation submitted to **Escola Superior de Tecnologia e Gestão of Instituto Politécnico de Bragança** to obtain the Master's Degree in **Chemical Engineering** in the ambit of the double diploma with the **Universidade Tecnológica Federal do Paraná – Câmpus Apucarana.**

Supervisors:

**Prof. Doctor Maria Filomena Barreiro**

**Prof. Doctor Caroline Casagrande Sipoli**

**Doctor Isabel Patrícia Fernandes**

Bragança

2020

*"É preciso força pra sonhar e perceber*
*que a estrada vai além do que se vê..."*

*(Los Hermanos)*

**ACKNOWLEDGEMENTS**

I would like to extend thanks to many people, who contributed for making this thesis possible. I would like to start by thanking my supervisor and teacher Prof. Dr. Filomena Barreiro. I am very grateful for the opportunity to carry out this work, for all attention, experience, orientations, and advice. I also want to thank my co-supervisor, Dr. Isabel Fernandes, for the support, patience, attention, motivations, and friendship. I admire your work!

A special thank you to my co-supervisor Prof. Dr. Caroline Sipoli, first for the opportunity to work together again and for all support, teaching, patience, attention, and good friendship. It is always a pleasure to work with you.

I am grateful for the opportunity and experiences UTFPR has given me throughout these years, for my professional and personal growth. A thank you to IPB for having welcomed me so well and for all the resources provided to complete this work.

Thanks so much to all my friends who were present regardless the distance. Thank you also for the supporting of the friends I made here, you were so important for me to get here.

Finally, my special thanks to my family, mother, father, brother and, grandmother, for all sacrifices they made for me and all the support. I love you so much!

This work was financially supported by CIMO (UIDB/00690/2020) through Foundation for Science and Technology (FCT, Portugal) under national funds FCT/MCTES.

## ABSTRACT

The generation of waste is one of the greatest and most damaging environmental risks. In the passion fruit juice industry, where only pulp is used, seed residues represent 12% of the fruit. They contain a high oil content (28 to 30%) rich in unsaturated fatty acids, tocopherols, carotenoids, and phenolic compounds, being also reported as presenting antioxidant activity and health benefits. Liposomes are spherical vesicles formed by phospholipids that, due to their amphoteric nature, when placed in aqueous solution self-aggregate, exposing the hydrophilic part and hiding the hydrophobic part, thus being able to encapsulate substances. In this context, the application of the oil extracted from the passion fruit seed (obtained from UTFPR), its characterization and encapsulation in liposomes for cosmetic applications was studied. The oil was characterized in terms of fatty acid profile, antioxidant activity, and oxidative stability. The fatty acid profile showed a predominance of unsaturated fatty acids, especially linoleic and oleic acids. The antioxidant activity was measured through the reducing activity related to the presence of phenolic compounds and through the capture of DPPH free radicals, being verified an intermediate antioxidant activity. The study of the oxidative stability proved the low stability of the oil, justifying its encapsulation before application, to preserve properties. To prepare the liposomes, the phosphatidylcholine was used as the lipid and the ethanol injection method (scalable) selected as the productive process. The method consists of preparing an aqueous phase and an organic phase containing the active ingredient to be encapsulated. Then, the organic phase is pumped into the aqueous phase under controlled flow, temperature, and stirring. After the production of the vesicles, the final step corresponds to the evaporation of the solvent in a rotary evaporator. The influence of parameters such as stirring and solvent evaporation times, on the vesicle final sizes was studied, being verified that the increase in stirring and evaporation time favor the agglomeration of liposomes. Liposomes were characterized in terms of size, morphology, stability and encapsulation efficiency. Through optical microscopy (OM) it was verified the spherical morphology of the liposomes and the influence of the solvent in their behaviour. Encapsulation efficiency tests for different oil concentrations were done indicating that the one comprising 20% w/w of oil was the higher one. The zeta potential was measured (-28.23 mV), showing the low stability of the vesicles that were further subjected to coating with chitosan. Two methodologies for particle's drying were studied: lyophilization, with the use of a cryo-protector (maltodextrin), and spray-drying after chitosan coating. Dry liposomes were characterized in what concerns size, morphology and stability. Optical and scanning electron microscopy proved the sphericity of the vesicles. The laser diffraction technique allowed measuring the size of the particles, showing that the size increased after drying due, which was associated to particle agglomeration. Visual analyses allowed to check stability, being verified that lyophilized liposomes released part of the encapsulated oil after 2 months of storage, being spray-drying after chitosan coating the most suitable methodology to produce liposomes in the dry form.

**Keywords:** Passion fruit oil; liposomes; chitosan; spray-drying; cosmetics.

## RESUMO

A geração de resíduos constitui um dos maiores e mais danosos riscos ambientais. Na indústria de sumo de maracujá, por exemplo, apenas a polpa é utilizada. Entre estes resíduos, as sementes, que representam 12% do fruto, contêm um elevado teor de óleo (28 a 30%) rico em gorduras insaturadas, tocoferóis, carotenóides e compostos fenólicos, apresentando atividade antioxidante e benefícios para a saúde. Os lipossomas são vesículas esféricas formadas por fosfolipídios que devido à sua natureza anfotérica, quando colocados em solução aquosa, auto agregam-se expondo a parte hidrofílica e ocultando a parte hidrofóbica, podendo assim, encapsular substâncias. Neste contexto, a aplicação do óleo da semente de maracujá (obtido na UTFPR), sua caracterização e encapsulação em lipossomas visando aplicações cosméticas será estudado. A caracterização do óleo foi feita em termos do perfil de ácidos gordos, atividade antioxidante e estabilidade oxidativa. O perfil de ácidos gordos revelou a predominância de ácidos gordos insaturados, com destaque para os ácidos linoleico e oleico. A atividade antioxidante medida através da atividade redutora na presença de compostos fenólicos e através da captura de radicais livres DPPH, revelou uma atividade antioxidante intermédia. O estudo da estabilidade oxidativa comprovou a baixa estabilidade do óleo, justificando a sua encapsulação. Para a preparação dos lipossomas, utilizou-se a fosfotidilcolina como lípido e a metodolodia de injeção de etanol (factível de escalonamento) como o processo produtivo. O método consiste na preparação de uma fase aquosa e uma fase orgânica contendo o princípio ativo. Posteriormente, a fase orgânica é bombeada para a fase aquosa a caudal, temperatura e agitação controladas. Após produção das vesículas, procedeu-se à evaporação do solvente em evaporador rotativo. Estudou-se a influencia de parâmetros como tempo de agitação e de evaporação do solvente no tamanho das vesículas, verificando-se que o aumento destes favorecem a aglomeração dos lipossomas. Os lipossomas foram caracterizados quanto ao tamanho, morfologia, estabilidade e eficiência de encapsulação. Através de microscopia óptica (OM) comprovou-se a morfologia esférica dos lipossomas e a influência da presença de solvente no comportamento das mesmas. Os testes de eficiência de encapsulação para diferentes concentrações de óleo mostraram que a formulação contendo 20% m/m de óleo resultou no valor de eficiência mais elevado. O potencial zeta foi medido (-28,23 mV), evidenciando a baixa estabilidade das vesículas, tendo-se procedido ao revestimento com quitosano. Quanto à secagem estudou-se a liofilização, com o uso de um crioprotetor (maltrodextrina), e *spray-drying* após revestimento com quitosano. Os lipossomas secos foram caracterizadas quanto ao tamanho, morfologia e estabilidade. A microscopia óptica e eletrônica de varrimento comprovaram a esfericidade das vesículas. A técnica de difração a laser permitiu medir o tamanho de partícula, mostrando o aumento deste após o processo de secagem, em parte devido à aglomeração das partículas. A análise visual permitiu avaliar a estabilidade, sendo que os lipossomas liofilizados libertaram parte do óleo encapsulado após 2 meses de armazenamento, sendo a técnica de spray-drying após revestimento com quitosano a melhor técnica para obter lipossomas na forma seca.



**Palavras chave:** Óleo de maracujá; lipossomas; quitosano; *spray-drying*; cosméticos.

## LIST OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## GLOSSARY

**AA** – Acetic Acid

**ABS –** Absorbance

**AO** – Almond Oil

**AS** – Active substance

**CA** – Caffeic Acid

**CH** – Chitin

**CS** – Chitosan

**DMM –** Dispersion of Mixed Micelles

**DPPH** – 2-2-diphenyl-1-picrylhdrazyl

**EE** – Encapsulation Efficiency

**EIM –** Ethanol Injection Method

**FT-IR** – Fourier-transformed Infrared Spectroscopy

**GUV** – Giant Unilamellar Vesicles

**HPLC** – High performance liquid chromatography

**LC** – Loading capacity

**LD** – Laser Diffraction

**LFH** – Lipid Film Hydration

**LUV** – Large Unilamellar Vesicles

**MLV** – Multilamellar Vesicles

**MUFA** – Monounsaturated Fatty Acid

**OM** – Optical Microscopy

**PDI** – Polydispersity Index

**PEG** – Polyethylene Glycol

**PO** – Passion fruit oil

**PUFA** – Polyunsaturated Fatty Acid

**RPE** – Reverse Phase Evaporation

**SEM** – Scanning Electron Microscopy

**SFA** – Saturated Fatty Acid

**SFE –** Supercritical Fluid Extraction

**STD** – Standard Deviation

**SUV –** Small Unilamellar Vesicles

**TGA** – Thermogravimetric analysis

**TPP** – Tripolyphosphate

**UV-vis** – Visible Ultraviolet

**WPC** – Whey Protein Concentrate

**ZP** – Zeta Potential

# *Chapter 1 – INTRODUCTION*

## 1.1 Motivation and objectives

Waste generation is a significant and dangerous environmental problem. In the passion fruit juice industry, for example, only pulp is used, representing about 40% of the fruit's total weight. The remaining material (peels and seeds) are presently considered a waste leading to hazardous problems if no alternatives for their valorization are taken into consideration (Barbieri and Leimann, 2014).

The passion fruit seed represents 6 to 12% of the total fruit weight. It has a high oil content (28 to 30%), which is characterized by a high antioxidant power, when compared to oils extracted from other seeds; nevertheless, it can be variable depending on the used extraction process. This oil has potential to be used as a raw material for the production of biodiesel, finding also application in cosmetics, pharmaceuticals, among other industrial uses. The recovery and valorization of this waste from the fruit processing industries is a very desirable measure in the context of circular economy (Malacrida and Jorge, 2012).

In the last decades, products developed from natural bases have had their production expanded. This worldwide trend, especially in European countries, leads to an increase of this global market segment, which includes the cosmetics industry, among others (Miguel, 2011). Additionally, the global market has been passing major scientific and technological challenges, especially after nanotechnology and nanoscience have been recognized as important trends in the science and technology of the 21st century. The largest development level in nanotechnology occurred in countries such as the United States, European Union and Japan, which have invested about one billion dollars a year, concentrating approximately half of the total worldwide investment in this area (Baril *et al.*, 2012).

The combination of waste valorization with nanotechnology is part of the motivation for this work, namely the use of liposome systems has been attempted to protect the oil extracted from the passion fruit seed. In this respect, the work comprised the characterization of the obtained oil, the development of the process to prepare the liposome systems, and their characterization having in view their potential application in cosmetic formulations.

## 1.2 Layout

Within the described context, the objectives of the present work will be organized in the following sequential phases:

1. Characterization of the passion fruit oil: In this step the passion fruit oil will be characterized. This oil was extracted within the context of the "Extraction of total lipids and phenolic compounds from fruit seeds" project, conducted by a research group supervised by Prof. Dr. Rúbia Michele Suzuki, from UTFPR Campus Apucarana. It was extracted in mild conditions by testing less aggressive solvents, in this case, ethanol and hexane. The characterizations will include, fatty acids profile and antioxidant activity. In addition, the oxidative stability of the oil will be verified in order to evaluate further the advantages of using encapsulation strategies.
2. Preparation of liposomes: This part will involve the preparation of the liposomes through the ethanol injection method using the methodology proposed by Zômpero *et al.* (2015). From the adoption of the base formulation, different oil concentrations and stirring times will be tested, followed by liposome characterization in terms of particle size, zeta potential, encapsulation efficiency, morphology and stability studies.
3. Liposome drying: Spray drying and lyophilization methodologies will be tested. In the spray drying, the advantages of coating the liposomes with chitosan will be studied aiming at increasing particle stability. Concerning lyophilization, the use of a cryo-protectant use will be studied, namely maltodextrin. The particles will be characterized in terms of size, morphology and stability.

In order to achieve these objectives, the document was organized in five main chapters. Chapter 1 (Introduction) where the context and objectives of the present work are described highlighting the interest and novelty of the studied strategies; Chapter 2 (Bibliographic review) gives the most relevant concepts and information surveyed from literature, and organized in a coherent way to provide the state of the art in the thematic; Chapter 3 (Materials and Methods) describes the main experimental strategies, as well as the applied characterization procedures. Chapter 4 (Results and discussion) where the obtained results were compiled and discussed to draw the conclusions; Chapter 5 (Conclusions and future perspectives) summarizes the most relevant outputs of this work and provides some insights for future developments. Finally, a

chapter compiling all the surveyed literature (Chapter 6) was provided. Some appendices were added to provide complementary information to the performed experiments.

# *Chapter 2 – BIBLIOGRAPHIC REVIEW*

## 2.1 Nanotechnology applied to cosmetics

Along time, scientific development, and society needs have been modified and evolved according to the different epochs of history. Studies in the area of science and technology have provided the emergence of nanotechnology, a highly complex and transdisciplinary area, being considered a multidisciplinary science, since all areas of science contribute to its development, interfering directly with technology (Farkuh and Cuccovia, 2016).

Nanotechnology is an emerging area of science that is booming. It is dedicated to the research, development, and manipulation of materials, systems or devices of nanometric dimensions, between 1 and 1000 nm. Currently, an exponential development has been observed for all of their applications. Nanomaterials are used, for example, in the production of common products such as paints, batteries, automotive parts, as well as in the pharmaceutical industry, and in the formulation of cosmetic products (Gonçalves and Reis, 2014).

In the cosmetic sector, nanomaterials are present in several products, such as shampoos, conditioners, toothpastes, moisturizing creams, deodorants, soaps, sunscreens, makeup products, perfumes, and enamels. Renowned companies such as L'Oréal, Lancôme, Anna Pegova, Procter&Gamble and Christian Dior have already launched nanotechnology-based products on the market. These products promise greater hydration and penetration of active principles, which promote the improvement of the sensorial aspect of several products (Camargo and Filho, 2008).

## 2.2 Passion fruit oil

Passion fruit belongs to the *Passifloraceae* family and has more than 500 species worldwide. Among these, the fruits of only 20 varieties are edible, and the most cultivated species in the world are yellow passion fruit, sweet passion fruit, and purple passion fruit. Among these three, yellow passion fruit is the most cultivated one, being present in 95% of the orchards.

Yellow passion fruit is a fruit that grows in the tropical region. Currently, South America is the main exporter of concentrated juice, supplying the worldwide growing demand. Juice production generates a large amount of residues, such as the seeds, representing 6 to 12% of

the whole passion fruit composition. Seeds contain large amounts of fiber and oil, and the extraction of the oil can add value to this agroindustrial residue (Malacrida and Jorge, 2012).

Typically, the oil extraction process is initially done by drying the seeds at a temperature of about 60 ºC for 5 hours, followed by their gridding. The obtainment of the oil is done by extraction with an organic solvent. At a laboratorial level, the apparatus comprises the extraction vessel heated by a heating mantle, and a condenser maintained through a thermostatic bath at approximately 15 ºC. The crushed and dried seeds sample is inserted into an extraction cartridge, and refluxed for about 3 hours. Finally, the solvent is evaporated in a rotavapor, to obtain the oil (Barbieri and Leimann, 2014). At industrial level the oil is usually obtained by supercritical fluid extraction (SFE). According to Prado *et al.* (2011) SFE has proven to be technically and economically feasible, presenting several advantages when compared to traditional extraction methods. The SFE of the seeds biomass is carried out at a continuous solvent flow rate, with supercritical $CO_2$ as solvent (Coelho *et al.* 2018).

The extracted oil has a considerable amount of unsaturated fatty acids, phenolic compounds and antioxidant capacity. Due to this fact, the characterization is an important step to perform after the extraction. The unsaturated fatty acids, mainly the polyunsaturated, predominantly oleic and linoleic acids, are important for human nutrition, playing an important role in the regulation of a variety of physiological and biological functions in living organisms having a protective effect against many diseases (Pereira *et al.*, 2019).

The antioxidant activity is attributed to the presence of carotenoids, polyphenols and phytosterols, and its amount can vary with the used extraction method and processing conditions. This can be related with solvent's ability to extract different compounds, which may present variable antioxidant activity. Antioxidants act as health promoters, preventing diseases related to aging. In this context, the potential for re-using seeds to obtain functional ingredients, which can be applied to the pharmaceutical and cosmetic industry, is of high interest (Ferreira, 2006).

Exposure to light, heat, and oxygen can cause the loss and reduction of the biological activities of the oil. To protect and reduce the oxidative deterioration resulting from the reaction of the lipids with the atmospheric oxygen, the oil can be encapsulated. This strategy aims to protect the unsaturated fatty acids, and other potential bioactives, increasing the shelf life of the oil, guarantying the maintenance of bioactivity.

## 2.3 Liposomes

Liposomes, schematically represented in Figure 1 (A), are spherical vesicles of amphoteric nature, formed by phospholipids, and consisting of one or more concentric bilayers that isolate one or more aqueous compartments from the external environment. Because lipids, e.g. phospholipids, are amphiphilic molecules, that is they are composed by hydrophilic (water-soluble) and a hydrophobic (water-insoluble) groups, when placed in aqueous solution, they self-aggregate, exposing the hydrophilic part and hiding the hydrophobic one (Frezard *et al.*, 2005). These structures are designated by liposomes.

The lipids most used in liposome formulations are those that have a cylindrical shape such as phosphatidylcholine, phosphatidylserine, and phosphatidylglycerol since they tend to form a stable bilayer in aqueous solution. The phosphatidylcholine, whose structure is shown in Figure 1 (B), is the most used lipid, because of the great stability to pH variation, or used concentration. It has two long hydrocarbon chains (fatty acids) forming its hydrophobic part, and glycerol, and a phosphate group linked to choline (an amino-alcohol) forming the hydrophilic part (Mertins *et al.*, 2008).

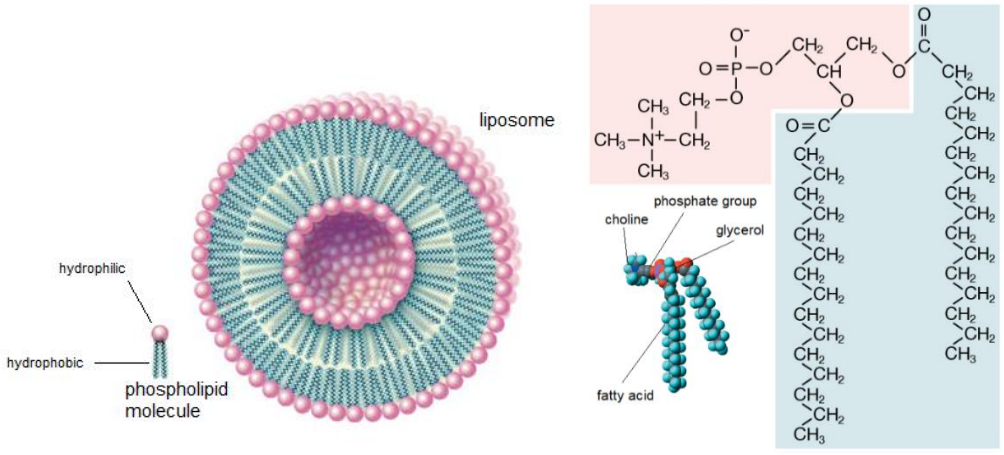



Figure 1 – Phospholipid structures: A. Liposome; B. Phosphatidylcholine.
Source: Adapted from Encyclopaedia Britannica (2007).

In general, liposomes are versatile systems that can be modified in size, lamellarity, surface area, lipid composition, volume and composition of the internal aqueous medium. Various processes can be used to prepare them: stirring, sonication, extrusion, lyophilization, freezing and thawing processes, reverse phase evaporation, among others. According to the

preparation method, many vesicular lipid forms can be obtained, with diameters ranging from 400 to 3500 nm (Machado *et al.*, 2018).

Liposomes are defined according to their size and amount of lamellae in their structure, as it is represented in Figure 2, being divided into the following categories (Chrysostomo, 2017):

- Small Unilamellar Vesicles - SUV: are the smallest, formed by a single bilayer, with a diameter varying from 20 to 80 nm;
- Large Unilamellar Vesicles - LUV: with intermediate size, are formed by a single lipid bilayer, with a diameter between 80 nm and 1 μm;
- Giant Unilamellar Vesicles - GUV: usually formed by a single lipid bilayer, having a diameter greater than 1 μm;
- Multilamellar Vesicles - MLV: with medium size, are formed by several lipid bilayers, with a diameter varying from 400 nm to few micrometers.

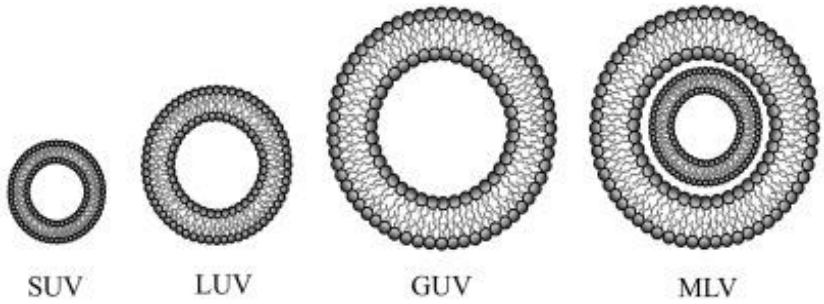



Figure 2 – Structuring of liposomes.
Source: Adapted from Torchilin (1998).

The steps of the productive process of liposomes are associated with the chemical composition, number of layers, size distribution, number of coverslips and encapsulation volume, which will define the structure of the final liposomes. In general, the preparation of the lipidic vesicles can be divided into three consecutive phases: (i) the production of the aqueous and lipid phase, (ii) the lipid hydration, and (iii) secondary processing for size reduction and homogenization (Santos and Castanho, 2002).

According to Batista *et al.* (2007), most methods include **Lipid Film Hydration (LFH)**, an older and known method, also called Bangham Method. In this process, firstly the lipids are dissolved in an organic solvent, followed by its evaporation with consequent formation of a lipid film. After formation, the film should be hydrated. This step is usually done with water or an aqueous buffer under vigorous magnetic stirring.

Due to the contact with the aqueous phase under stirring, the phospholipid film detaches from the balloon surface in the form of multilamellar vesicles (MLV) of high diameters, as shown in Figure 3 (Chrysostomo, 2017). According to Kozubek *et al.* (2000), in the phospholipid film hydration method, the substance to be encapsulated must be added to the buffer solution, if hydrophilic, or dissolved in the lipid mixture, if hydrophobic. According to Sharma and Sharma (1997), although the hydration of the lipid film is a simple technique, its major disadvantage is the low encapsulation efficiency, which typically ranges from 5 to 15%.

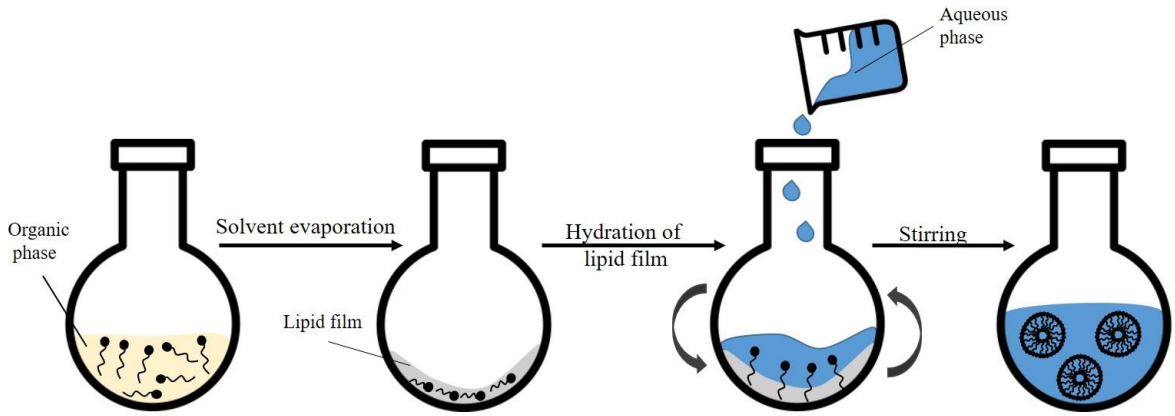



Figure 3 – Lipid film hydration method.

In an attempt to increase the encapsulation efficiency of the prepared vesicles, Woodle and Papahadjopoulos (1989) developed the **Reverse-Phase Evaporation** (RPE) technique. This method consists, firstly, in the preparation of an organic phase comprising the lipid dissolved in an organic solvent, which might be immiscible with water. Then this phase is added with an aqueous solution (containing or not the active principle to be encapsulated), forming two immiscible phases. The polar part of the phospholipids is attracted by the aqueous phase promoting lipids deposition at the organic/aqueous phase interface (Chrysostomo, 2017).

The system is then subjected to mechanical stirring, e.g. sonication, forming a water/oil emulsion, where reverse micelles with an aqueous core, dispersed in the organic phase, are formed. Thereafter, the organic solvent is evaporated at a temperature greater than the phase transition temperature of the used lipid, in order to cause the phase reversion. After complete elimination of the solvent, many micelles collapse and the formation of a high viscosity organogel occurs. Then, under stirring and addition of an aqueous solution, the liposomes are formed by the self-association of the double phospholipids layer, obtaining large unilamellar vesicles (LUV) (Souto *et al.*, 2011). The steps used in this method are shown in Figure 4.

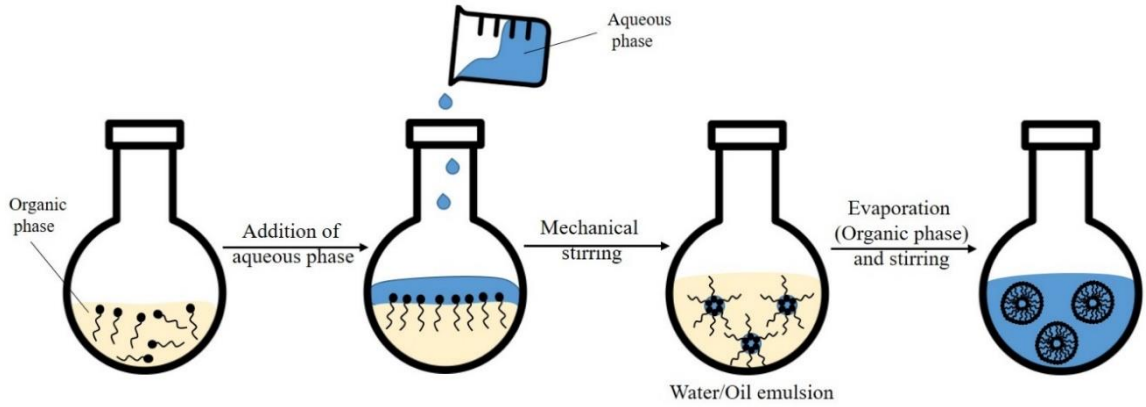



Figure 4 – Reversed phase evaporation.

For Lichtenberg (1988), the methodology was developer, this method involves the direct addition of the aqueous phase at three to six-fold larger volumes than the organic phase containing the phospholipid. The removal of the organic solvent by evaporation produces liposomes whose structural characteristics depend on the involved procedure. A rapid removal of the organic solvent leads to water evaporation, resulting in a lipid concentration increase, inducing the formation of MUV. On the other hand, slow evaporation will not affect the lipid-water ratio, resulting in LUV and SUV structures.

Maximiano and Cuccovia (2003) studied the production of liposomes through the **Dispersion of Mixed Micelles (DMM)** method. This method is based on the use of surfactants, substances that reduce surface tension, thus influencing the contact surface between the two liquids. Briefly, the lipids, with the aid of a surfactant, are dissolved in the aqueous medium, forming mixed micelles. As the surfactant is removed, the micelles become progressively richer in the phospholipid and, finally, combine to form LUV structures. The surfactant can be removed by dialysis. Enoch and Strittmatter (1979) indicate that the advantages of using surfactants have excellent reproducibility leading also to the production of liposome populations very homogenous in size. However, it points out that this technique presents as a disadvantage the retention of traces of surfactant inside the liposomes, which can compromise their applicability.

Batzri and Korn (1973) were pioneers in the work that involved the preparation of liposomes through the **Ethanol Injection Method (EIM)**. In this method, the lipids dissolved in a water-miscible organic solvent (containing or not the active substance) are brought into contact with the aqueous phase (containing or not a coating polymer) by direct injection. Upon in contact with the aqueous phase, the ethanol is diluted. Due to changes in the solubility

conditions of the lipids, these molecules precipitate, forming fragments of lipid bilayers (Zômpero *et al.*, 2015).

Through systemic energy dissipation (mechanical stirring, ultrasonication, etc.), the bilayer fragments tend to decrease the exposure of the hydrophobic part by rearrangement of the curvature in this region, thus resulting in the arching of the bilayer fragments, which form oval structures. The stirring and the injection force are sufficient to maintain a complete mixture so that the dilution of the solvent and the dispersion of the lipid molecules in the aerial phase are almost instantaneous (Justo and Moraes, 2003). This mechanism of vesicle formation through stirring is shown in Figure 5.

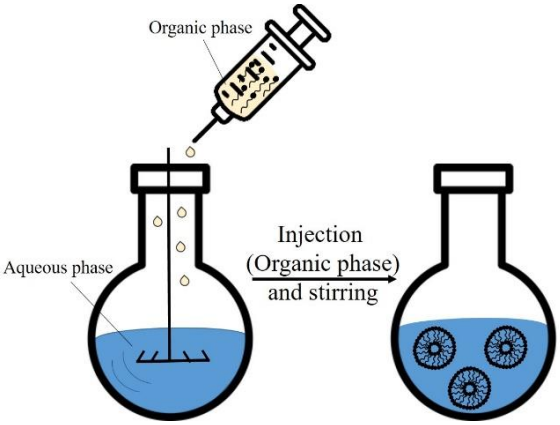



Figure 5 – Ethanol injection method.

Fan *et al.* (2008) established that the two most important variables in the EIM method are the concentration of the phospholipid in ethanol, and the ethanol injection rate. Results showed that rapid injection resulted in small vesicles (SUVs), while slow injection could be used to produce large vesicles (MLV). Justo and Morares (2003) also studied the processing variables of this method, namely by using an initial lipid concentration of 50 to 100 mmol/L, a stirring rate of the aqueous phase between150 to 650 rpm, and an ethanol injection flow rate of 60 to 300 mL/h. The aqueous phase temperature was in the range 30-60 ºC. Under these conditions, the size range of the produced liposomes varied between 200-800 nm.

Lichtenberg (1988) reported that the main disadvantage of the EIM method is the possible presence of residual ethanol within the formed vesicles. Pons *et al.* (1993) stated that this methodology has the advantage of being a simple procedure to obtain liposomes. It is a fast and easy procedure, not promoting degradation changes in lipids. Besides, this process represents one of the few alternatives that allow an easy transposition to industrial scale, being

a good option for encapsulation of active principles focused on cosmetics and pharmaceutical applications.

Zômpero *et al.* (2015), in their studies, obtained a high encapsulation efficiency of the studied active principle (β-carotene) using this method (about 100%). Also, they have shown that the residual presence of solvent can be easily solved, as it will be further explained next.

In order to summarize the most used methods to produce liposomes, as focused in this section, Table 1 presents the advantages and disadvantages of each one.

**Table 1** – Summary of liposome productive methods.

| Method | Advantages | Disadvantages |
|---|---|---|
| **Lipid film hydration** | Simple process | Small-scale production;<br>Low encapsulation efficiency |
| **Reversed phase evaporation** | High encapsulation efficiency | Organic solvent residue |
| **Dispersion of mixed micelles** | Simple process;<br>Easy control of particle's size | Surfactant residue;<br>Long-time process |
| **Ethanol injection method** | High encapsulation efficiency;<br>Easy control of particle's size;<br>Easy transposition to industrial scale | Organic solvent residue |

After analyzing the performed review on existing liposome preparation methodologies, and having in view the objectives of the present work, which is the encapsulation of passion fruit oil having in view cosmetic applications, the ethanol injection method was chosen. It gives rise to high encapsulation efficiency, besides being a simple method, easily transposed to industrial scale.

Regardless of the solvent used to form the vesicles, and the used preparation method employed, removal of the residual solvent is the last step of the liposome preparation process. According to Dua *et al.* (2012) & Rasti *et al.* (2012), for small volumes of organic solvent (<1 mL), the solvent can be evaporated using a stream of dry nitrogen or argon in a fume hood. For large volumes, it can be removed with a rotavapor at low pressure.

## 2.4 Liposomes coating process

In order to increase the stability and durability of the liposomes, the coating of the lipid bilayer can be made. The coating may be made using synthetic, natural and natural-derived biocompatible hydrophilic polymers, such as polyethylene glycol and chitosan. This superficial hydrophilic layer increases the durability since it prevents liposomes association and rupture (Fontes *et al.*, 2012). In an attempt to increase the encapsulation efficiency of the vesicles, one can also use proteins, peptides, carbohydrates or antibodies. These molecules are coupled to the surface of the liposomes, without compromising membrane integrity and properties (Lapenda *et al.*, 2010).

**Chitosan (CS)** is derived from chitin, a very abundant polymer in nature. It is obtained by chitin (CH) deacetylation reaction (Figure 6). According to Moghimipour and Handali (2014), this polymer is widely used in the coating of nanovesicles and its vast use is due to its easy manipulation and low cost. Liposomes coated with chitosan are an alternative to conventional liposomes since they have better stability and bioadhesiveness. Junior and Almeida (2012) developed and evaluated the stability of melatonin encapsulated in liposomes coated with chitosan. The obtained results demonstrated the better stability of the nanostructures, as well as the better organization, after the application of the coating. Hansen *et al.* (2015) who encapsulated curcumin, also shown that the addition of chitosan significantly increased the stability of liposomes.

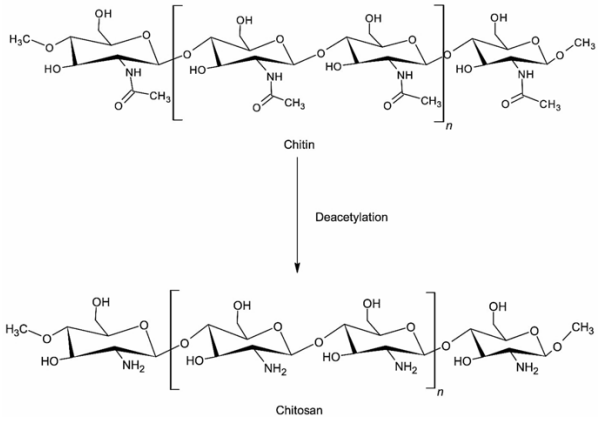



Figure 6 – Chitin and Chitosan structures.
Source: Adapted from Martins and Pessine (2007).

This coating step involves, initially, the preparation of a chitosan solution. Since chitosan is not soluble in water at physiological pH, a buffer solution with pH equal to 4.5 can be prepared, or oligosaccharides can be combined with chitosan molecule, allowing solubilization at neutral pH (Sá, 2015). The chitosan solution will receive the organic solution with the lipid, forming vesicles called chitosomes, which might contain polymer also inside the formed structures. Studies by Mertins *et al.* (2008) also demonstrate the preparation of the vesicles separately, followed by their dispersion in an aqueous solution containing the polymer.

Another important advance in this field of liposomes coating was the use of **polyethylene glycol** (PEG) as a coating medium, reaching the so-called long-circulation liposomes. Shukat and Relkin (2011) point that **whey proteins concentrate (WPC)** can be used as a coating, being natural and cheap sub-products of the cheese industry. Gómez-Mascaraque *et al.* (2015) also used WPC to coat liposomes showing that they have protective effects, increasing the encapsulation efficiency when used together with the encapsulation matrix.

Following the provided discussion considering the productive method, which might or not include a coating step, a summary of the conditions used by some authors is provided in Table A1 in the appendix section.

## 2.5 Liposomes drying process

The potential application of liposomes is still challenged by their physical and chemical instability in aqueous dispersion for long-term storage (Chen *et al.*, 2010). Particle aggregation and merging can occur after long storage periods, as well as degradation of the main constituents, and microbial contamination (Batista *et al.*, 2007). In order to reduce such instabilities, and facilitate the applicability of vesicles, drying techniques as lyophilization, freezing, spray-drying and supercritical fluid technology can be used.

**Lyophilization** is a process of freeze-drying and sublimation of ice under vacuum, which transforms solid water directly into vapor. According to Mohammed *et al.*, (2006) lyophilization offers the opportunity to remove water from the system thus preventing hydrolysis of phospholipids, and by producing a solid phase product with low molecular mobility, generally suppressing both chemical and physical instabilities. The lyophilization steps can damage the vesicles and to avoid deleterious effects it is necessary to add cryo-

protectants (Toniazzo *et al.*, 2017). The addition of cryo-protectants to the sample prevents damaging at low temperatures maintaining the structure and properties of the particles while also preventing aggregation and melting. The cryo-protectants used for liposome protection when applying lyophilization processes are normally oligosaccharides as sucrose, trehalose, maltose or lactose (Ferreira *et al.*, 2016). Table 2 presents a survey of some examples found in literature concerning liposomes lyophilization.

**Table 2** – Cryo-protectants used in lyophilization process

| Production method | Active substance (AS) | Cryo-protectant | Reference |
|---|---|---|---|
| Ethanol injection method | Quercetin | Sucrose | (Toniazzo *et al.*, 2015) |
| Ethanol injection method | Eugenol | Sucrose<br>Trehalose | (Sebaaly *et al.*, 2016) |
| Ethanol injection method<br>Film hydration method | Vitamin C | Sucrose | (Yang *et al.*, 2013) |
| Film hydration method | Ibuprofen | Amino acids | (Mohammed *et al.*, 2007) |
| Film hydration method | - | Trehalose<br>Glycerol<br>Sucrose<br>Glusoce<br>Raffinose<br>Inositol | (Crowe *et al.*, 1984) |
| Film hydration method | Quercetin | Sucrose | (Alexopoulou *et al.*, 2006) |
| Film hydration method | Essential oil | Trehalose<br>Sucrose | (Yoshida *et al.*, 2010) |
| Film hydration method | Casein hydrolysate | Trehalose<br>Sucrose | (Yokota *et al.*, 2012) |

Two theories can explain the effects of the cryo-protectants in the liposome membrane. The first one, proposed by Crowe *et al.* (1984), suggests that sugar molecules interacted with the polar group of phospholipids, keeping a space between them in its dehydrated state, as illustrated in Figure 7. This space is similar to the one existing in the hydrated form of liposomes, thus avoiding the total approximation between the hydrophilic groups, and the collapse of the liposome structure (Toniazzo *et al.*, 2015).

The second theory is the glazing theory, which appoints that dehydrated liposomes are stabilized due to the transformation of the concentrated solution with cryo-protectant to a glassy

state. During lyophilization, the sugar solution turns supersaturate, passing to a glassy state, holding the liposomes inside this amorphous matrix (Chen *et al.*, 2010).

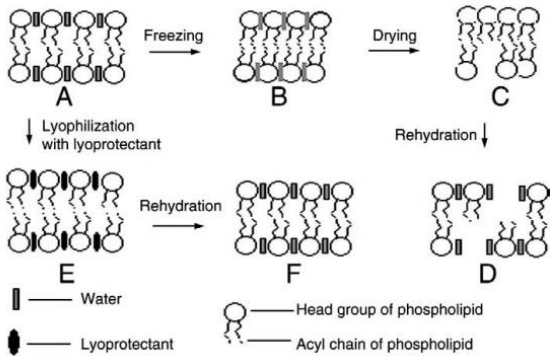



Figure 7 – Cryo-protectant effect.
Source: Adapted from Chen *et al.* (2010).

**Spray drying** is a widely used manufacturing process that uses the aerosol phase to dry particles, being utilized as an alternative to substitute other dry process such as rotavapor and lyophilization. According to Vehring *et al.* (2007), the technique has been applied in many areas, including food, pharmaceutical, ceramic, polymer, and chemical industry. It has been used to prepare powders, where suspensions are atomized into fine droplets, followed by a drying process that will result in solid particles (Saveyn *et al.*, 2007).

The method is basically divided in four steps: (1) atomization or division of the feed into a spray; (2) spray-air contact; (3) drying of the spray; (4) separation of the dried product from the drying gas (Luiz *et al.*, 2006). A schematic representation is shown in Figure 8. By spray drying, dry particles can be produced by atomization of different kinds of fluids, such as solutions, suspensions or emulsions (Peltonen *et al.*, 2010). The presence of drying adjuvants such as carbohydrates or polymers is necessary to increase the stability of nanoparticles, avoiding agglomeration and increasing the income of the process (Lo *et al.*, 2004). Table 3 presents some coaters used in spray drying process.

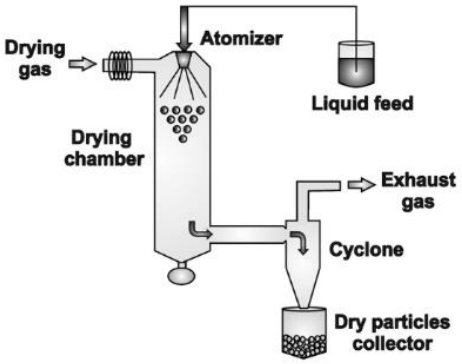



Figure 8 – Diagram of the equipment and process of conventional spray drying.
Source: Sosnik and Seremeta (2015).

The process has the advantages of being continuous, easy to scale-up, simple, cost-effective, so the use of spray drying might be preferable in the production of dry liposomal powders (Gültekin-Özgüven *et al.*, 2016).

**Table 3** – Coaters used in spray drying

| Production method | Active substance (AS) | Coating | Reference |
|---|---|---|---|
| Ethanol injection method | Curcumin | WPC | (Gómez-Mascaraque *et al.*, 2017) |
| Ethanol injection method | Superoxide dismutase | Sucrose<br>Trehalose<br>Lactose | (Lo *et al.*, 2004) |
| Film hydration method | Prednisolone disodium phosphate | PEG | (Van Den Hoven *et al.*, 2012) |
| Film hydration method | Dapsone | Lactose<br>Sucrose | (Chougule *et al.*, 2008) |
| Sonication method | Vitamin E | Chitosan | (Shin *et al.*, 2013) |
| Sonication method | Grape seed extract | Chitosan | (Gibis *et al.*, 2012) |
| Film hydration method | Hydroxyapatite | Chitosan<br>Alginate<br>Pectin | (Pistone *et al.*, 2017) |
| Film hydration method | Black mulberry extract | Chitosan | (Gültekin-Özgüven *et al.*, 2016) |

## 2.6 Typical characterization methods of liposome systems

The behavior of liposomes, both in relation to the stability of the encapsulated active principle and in the final media, is determined by factors such as particle size, surface charge,

sample concentration and the properties of the final medium itself. Thus, determining such parameters is essential to ensure the efficiency of the applied method to ensure the stability of the vesicle formulations (Lopes and Brandelli, 2018).

The **laser diffraction** method (LD) has been increasingly applied for quantifying of particle size distribution, owing to its advantages of rapid analysis, high reproducibility and continuous measurement for a wide range of size fractions (Yang *et al.*, 2019). The laser diffraction measures the particle size distribution by angular variation in the diffused light intensity as the laser beam interacts with the dispersed particle in the sample. The particle size is indicated as sphere diameter with an equivalent volume (Roggia *et al.*, 2019).

Another parameter calculated through this technique is the **polydispersity index** (PDI), which consists on analyzing the autocorrelation of light scattering intensity. According to Danaei *et al.* (2018), PDI values higher than 0.7 indicate that the sample has a very broad particle size distribution, so, PDI values can be used to describe the width of the particle size distribution and to know about the heterogeneity of the size distribution.

According to Mertins *et al.* (2008), colloidal systems such as liposomes in the aqueous medium may present different behaviors concerning aggregation phenomena. One way to study the colloidal stability of these particles is to determine the **Zeta Potential** (ZP) of the produced dispersions, which, depending on the intensity, can significantly contribute, to the aggregation processes. Most of the particles dispersed in the aqueous medium, due to the chemical nature of the constituents of the particle and the dispersion medium itself, tend to acquire a surface electric charge, as shown in Figure 9 (Lopes and Brandelli, 2018).

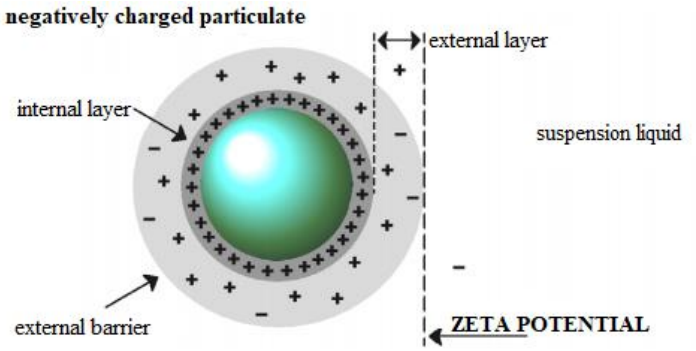



Figure 9 – Zeta Potential.
Source: Adapted from Mertins *et al.* (2008).

The ZP is, therefore, the potential difference between the dispersion medium and the outer layer. The magnitude of this potential allows the evaluation of the stability of the

suspended particles, considering that if all the particles present a highly positive or highly negative zeta potential value, they will repel each other, avoiding aggregation. Low values of zeta potential, less than +30 mV and greater than -30 mV, indicate the absence of surface charge, favoring interactions between the particles, promoting flocculation and aggregation processes. In general, suspensions with values above +30 mV, as well as values below -30 mV, are indicative of stability (Mertins *et al.*, 2008).

Lopes and Brandelli (2018) states that the characterization of liposomes through the determination of the **encapsulation efficiency** is of extreme importance since its physicochemical characteristics depend on the active compound incorporated into the nanostructures. Different methods can be used, among them: spectrophotometry, as UV-visible or infrared, fluorescence spectroscopy, enzymatic methods, electrochemical techniques and high-performance liquid chromatography (HPLC) (Pinilla and Brandelli, 2016). The method to be chosen depends on the specificities of the used active principle, namely the way it can be quantified.

Ghorbanzade *et al*. (2017), when studying fish oil charged liposomes for yogurt fortification, evaluated the encapsulation efficiency by centrifuging the liposomes in order to separate the non-encapsulated active compound (indirect method). Thus, considering the amount of oil initially used, the encapsulation efficiency (EE) was calculated through Equation (1) shown below, after quantifying the non-encapsulated oil, using an adequate analytical method, in this case, spectrophotometry.

$$\%EE = \frac{Total\ oil\ used\ in\ the\ formulation - Total\ non\ encapsulated\ oil}{Total\ oil\ used\ in\ the\ formulation} x100 \quad (1)$$

Taylor *et al*. (2007), in their study concerning liposome characterization, used the fluorescence spectroscopy technique, where the fluorescence of the samples with the encapsulated active principle was determined, based on the peaks reporting inherent fluorescence ($F_0$). To measure the fluorescence of the encapsulated active principle, a surfactant was added to cause liposome rupture and to register the fluorescence of the released tracer ($F_T$). Thus, the encapsulation efficiency was calculated according to Equation (2).

$$\%EE = 100\ x\left[1 - \left(\frac{F_0}{F_T}\right)\right] \quad (2)$$

Fish oil, similarly to passion fruit oil is also a triacylglycerol, so the methodology of Ghorbanzade *et al.*, (2017) offers the possibility to be adapted to the present study.

According to Bibi *et al.* (2011), for greater magnification, electron microscopy can be employed. Its use allowed insights into the bilayer characteristics and provided the visualization of small unilamellar vesicles. These systems work using an electron beam which is focused by various lenses onto the sample surface. Electrons are then scattered in two ways, with only changing their path, whilst others will collide and displace electrons around the nuclei of atoms of the sample. The electrons are then focused and magnified by a system of magnetic lenses to produce a projected image. The **scanning electron microscopy** (SEM) is an imaging technique used to characterize micro and nanostructures in powder form, providing the study of their morphology (Araújo and Mosqueira, 2009).

**Fourier transformed infrared spectroscopy** (FTIR) in recent years has become one of the methods used to study the behavior of lipid membrane systems. According to Blume, (1996), the method uses an empirical approach because the quantitative description obtained from the frequencies and band intensities of all the vibrational bands in a molecule like a phospholipid is difficult to achieve. Thus, most FTIR studies on bilayer systems deal with phenomenological aspects and in the mid-IR region, lipid aspects exhibit several characteristic marker bands that can be used to determine structural information on particle surfaces.

In their study, Yokota *et al.* (2012) obtained infrared spectra in a Spectrum One (FTIR with ATR), within a frequency range of 4000 $cm^{-1}$ to 600 $cm^{-1}$ at room temperature, using 4 $cm^{-1}$ of spectral resolution. Shashidhar and Manohar (2018) studied through FT-IR the iteration of the soluble compounds of the active principle in water, in the frequency between 4000 $cm^{-1}$ and 400 $cm^{-1}$.

Another technique that can be used to obtain information on the interactions between the active principle and the lipids, the physical state of the particles, and possible changes after processing is the **thermogravimetric analysis** (TGA). According to Meyagusku and Oliveira (2014), this technique provides weight variation information as a function of temperature or time at a constant temperature. The results provide information related to the composition of the sample and its thermal stability.

A more complete survey of liposome characterization and typical applications can be found in Table A2 and Table A3, respectively, in the Appendix section.

# *Chapter 3 – MATERIALS AND METHODS*

## 3.1 Materials

The chemical system used for liposome production is composed by distilled water, Phospholipon 90G obtained from Phospholipid GmbH (Germany), ethanol from Sigma-Aldrich (Germany), sweet almond Oil (AO) (LabChem, Portugal) and passion fruit oil (PO) obtained from UTFPR (Brazil). For liposome coating and drying processes, chitosan (CS) from BioLog Biotechnologie und Logistik GmbH (Germany), acetic acid from Panreac Applichem (Spain) (as medium acidifier), and maltodextrin (Cargill, France) were used.

In oil characterization, for fatty acids analysis, methanol, sulphuric acid, toluene, diethyl ether, distilled water and standard mixture (47885-U, Sigma, USA) were used. For total phenolic compounds determination, distilled water, methanol, n-hexane, Folin-Ciocalteau, and sodium carbonate were used. Caffeic acid (CA) was for calibration purposes. To complete the antioxidant analysis, blocking effect of free radicals was studied using methanol, ethyl acetate and 2,2-diphenyl-1-picrylhydrazyl (DPPH), purchased from Sigma-Aldrich (Germany).

## 3.2 Methods

### 3.2.1. Oil characterization

**Fatty acids profile:** fatty acid methyl esters (FAME) were prepared by performing a trans-esterification procedure. Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 ºC and shaking at 180 rpm; then 3 mL of deionised water were added, to obtain phase separation. The FAME were recovered with 3 ml of diethyl ether by shaking in a vortex , and the upper phase was then passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water. The final sample was recovered in a vial with Teflon, and filtered with 0.2 µm nylon filter from Whatman before injection.

The fatty acid profile was determined by gas-liquid chromatography with flame ionization detection, using a Young In Crhomass 6500 GC System instrument equipped with a split/splitless injector set at 250 ºC with a split ratio of 1:80, a flame ionization detector (FID)

set at 260 ºC and a Zebron-Fame column (20 m × 0.18 mm ID × 0.15 µm df, Phenomenex, Portugal). The following oven temperature program was used: initial temperature of 80 ºC, held for 1.5 min, increase 40 ºC/min to 160 ºC, followed by a 5 ºC/min ramp to 185 ºC, 30 ºC/min ramp to 260 ºC and held for 4 min. The carrier gas (hydrogen) flow-rate was 0.6 mL/min, measured at 250 ºC. Fatty acids identification and quantification was performed by comparing the relative retention times of FAME peaks from samples with standards (standard mixture 47885-U, Sigma, USA). Results were recorded and processed using the Software Clarity DataApex 4.0 Software (Czech Republic) and expressed in relative percentage of each fatty acid.

**Total phenolic compounds:** the total phenolic compounds was determined by the Folin-Ciocalteau method, according to the methodology proposed by Alves *et al.* (2013). In a Falcon tube, 2.5 g of the oil were weight and dissolved with 2.5 mL of n-hexane and 2.5 mL of a methanol/water solution (80/20% v/v). The tube was left to ultrasound for 10 minutes and centrifuged at 5000 rpm for 5 minutes. After the extraction, 1 mL of supernatant was collected and combined with 1 mL of Folin-Ciocaliteau, 1 mL of $Na_2CO_3$ (7.5%) and 7 mL of water. The mixture was maintained in the refrigerator overnight (16 h) and the absorbance of the supernatant measured by spectroscopy using a UV/vis Genesys$^{TM}$ (Thermo Scientific, Germany) at 765 nm. A calibration curve with caffeic acid (CA) was prepared in the range 0.04 to 0.18 mg/mL, and the results were expressed in mg of CA/kg of oil.

**Antioxidant activity:** the antioxidant activity was measure by the DPPH method according to the work of Malacrida and Jorge (2012). Briefly, the oil was diluted with methanol at seven different concentrations (300 - 4.6875 mg/mL). As control sample, a methanol solution was used. Then, 270 µL of DPPH (2,2-diphenyl-1picrylhydrazil) in methanol solution ($6x10^{-5}$ mol/L) was mixed with 30 µL of the sample solutions and incubated in an incubator (Raypa, Spain) at 37 ºC for 1 hour in the dark. The absorbance of the reaction solution was measured at 517 nm with a microplate reader Epoch 2 (BioTek, USA). Each sample was analyzed in triplicate. The percentage of free radical scavenging (% Scavenging activity) was calculated according to Equation (3)

$$\%\, Scavenging\ activity = \frac{Abs(control) - Abs(sample)}{Abs(control)} x100 \quad (3)$$

where Abs(control) and Abs(sample) are the absorbance measurements for the control and the analyzed sample, respectively.

The results were expressed using the $EC_{50}$ value, defined as the concentration of antioxidant leading to a decrease of 50% in the DPPH absorbance, determined from a calibration curve representing the percentage of free radical scavenging *versus* concentration.

The methodology proposed by Alves *et al.* (2013) was also used for comparison purposes. For that, a solution of ethyl acetate and passion fruit oil (10% m/v) was prepared, thereafter 1 mL was added to a Falcon tube containing 4 mL of a DPPH solution ($1x10^{-4}$ M in ethyl acetate). The sample was mixed and kept in the dark conditions for 30 minutes. The absorbance was measured in a spectrophotometer UV/vis Genesys$^{TM}$ (Thermo Scientific, Germany) at 515 nm against a control (ethyl acetate). Each sample was analyzed in triplicate and the percentage of free radical scavenging was calculated according to Equation (3).

**Oxidative stability:** the oxidative stability of the oil was evaluated by a conductivity method using the Rancimat 743 (Methrom, Switzerland). This method consists in bubbling a dried, clean, and filtered air (flowrate of 20 L/h) through the sample (3 g of oil) heated to 120 ºC. The oxidation compounds formed over time, which are more polar than the triglycerides (e.g. hydroperoxides, alcohols and carbonyl compounds), are carried away by the air flow and bubbled in distilled water, whose conductivity increases. The device measures the conductivity continuously, and the time period that elapses from the starting of the experiment until the formation of the oxidation products (Induction period) translates the stability of the oil to oxidation. The higher this value is the less stable the oil is. This parameter is calculated by the software associated with the device, and corresponds to the time interval between the start of the record and the intersection point of the tangents to the curve.

### 3.2.2 Liposomes production

The liposomes were prepared using the ethanol injection method. The lipid (Phospholipon 90G) and the passion fruit oil were dispersed in ethanol at 60 ºC (above the phospholipid melting temperature), to obtain the organic phase. The organic phase was then pumped with a peristaltic pump (ISMATEC, Germany) at a flow rate of 30 mL/min into a

jacked reactor (Julabo F25 GmbH, Germany) containing water (aqueous phase) under controlled stirring speed (500 rpm) and temperature (60 ºC). The influence of stirring time was studied by testing 5, 10 and 15 minutes (500 rpm). The used experimental system is represented in Figure 10. In all experiments, the composition of the organic and aqueous phase was fixed according to Table 4, and the passion fruit oil concentration was changed to verify the one giving rise to the encapsulation efficiency.

**Table 4** – Composition of organic and aqueous phases.

| Phase | Composition | Amount |
|---|---|---|
| Organic | Ethanol | 20 mL |
| | Phospholipid | 1.6 g (80 g/L) |
| | Passion fruit oil | 0.32 g (20%, w/w phospholipid-basis) |
| Aqueous | Water | 180 mL |

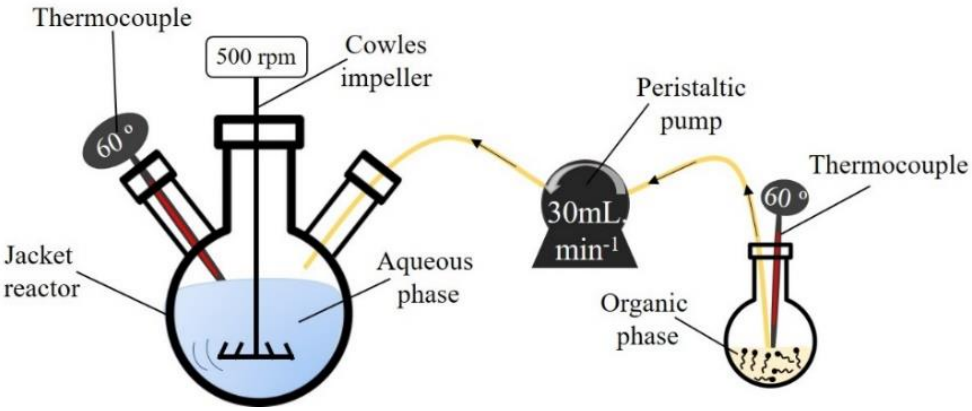



Figure 10 – Experimental system used for the preparation of liposomes.

After liposome production, a rotary evaporator (Rotavapor R-114, BUCHI, Switzerland) at 40 ºC and 175 mbar was used to evaporate the organic solvent (ethanol). For the evaporation time, 30 and 60 minutes were tested. The morphology of the particles, in the presence and absence of ethanol, was inspected by optical microscopy to look for signs of instability phenomena.

### 3.2.3 Liposomes coating

The liposomes coating was performed with chitosan (CS). For that, a solution of acetic acid (3 M) was prepared and used to acidify the liposome dispersion before adding the chitosan. Two pHs were tested (3.0 and 3.8) to verify the influence of pH in CS solubilization. The CS

was added to the liposomes solution at a concentration of 0.5% w/v (total solution volume basis) and the mixture allowed, under stirring, overnight at 40 ºC. This procedure was complemented with drying to check for the viability of obtaining coated liposomes in the dry form.

### 3.2.4 Liposomes drying

The liposomes can be dried by two methods: spray drying and lyophilization. In this work, both methodologies were tested and compared. The used apparatus is showed in Figure 11. The spray dryer was a Mini spray dryer B-290 (BUCHI, Switzerland) and the lyophilizer was a CoolSafe equipment (Labogene, Germany).

The procedure used in the spray dryer followed the methodology proposed by Gültekin-Özgüven *et al.* (2016). Briefly, a feed rate of 4 $cm^3$/min and an inlet temperature of 130-140 ºC, resulting in an outlet temperature of 90 ºC, were used. The atomizer was equipped with a 1.5-mm nozzle, operating at an atomizing flow rate of 5 $cm^3$/min. The obtained dried powders were placed in a desiccator at room temperature until analysis. This methodology was applied after the coating with chitosan.

The procedure for the lyophilization, i.e. the sublimation dehydration method, was carried out according to the methodology proposed by Cabral *et al.* (2004) with some modifications. A cryo-protector (maltodextrin) was added to the liposome solution at a proportion lipid:cryo-protector of 1:4 weight ratio. The chosen protector was maltodextrin. The solution was frozen for 24 hours, thereafter dehydrated by freeze-drying at -45 ºC during at least 36 hours.

A B

Figure 11 – Dryers: A. Spray-drying; B. Lyophilizer.

### 3.2.5 Liposomes characterization

**Optical microscopy (OM):** the morphology of the produced liposomes was analyzed using the optical microscope NiU (Nikon microscope Eclipse Ni, Nikon Corp., Japan) equipped with a digital camera. The images were made right after the preparation of each system and along the evaluated storage time, with a magnification of 400x, 200x and 100x. For the analysis, a drop of the liposome suspension was placed on a microscope slide and thereafter covered with a cover slip.

**Liposomes size determination:** liposomes size was determined using a laser diffraction (LD) equipment (Malvern, Mastersizer 3000, UK) equipped with red (650 nm) and blue (405 nm) solid-state laser diodes, which allowed the detection of particles in the size range of 10 nm and 3 mm. For the analysis, samples were added dropwise into approximately 300 mL of deionized water in the wet dispersion unit, using a refractive index of 1.336±0.001. The results were obtained from at least 10 measurements, and the software Malvern Access Controller was used to perform the particle size analysis, namely the size distributions and average sizes.

**Zeta potential (ZP) and polydispersity index (PDI):** the zeta potential and polydispersity index were determined by applying an electric field across the samples, with the ZP value obtained by measuring the velocity of the electrophoretic mobility of the particles using the laser Doppler anemometry technique with a dilution of 1:10. The measurements were performed with three samples, unloaded-liposomes, passion fruit oil-loaded liposomes and chitosan coated liposomes. The equipment was a Malvern Zetasizer Nano ZS (Malvern Instruments, WORCS, UK).

**Encapsulation efficiency (EE) and loading capacity (LC):** the encapsulation efficiency was determined by UV-vis spectroscopy (Jasco V-730 Spectrophotometer, Japan) according to the method described by Gómez-Mascaraque *et al.* (2017) and Ghorbanzade *et al*. (2017) with some modifications. First, passion fruit oil and phosphatidylcholine were analyzed to find the maximum of absorbance (215 nm for both). Thereafter, ethanol was used to prepare different PO concentrations to construct a calibration curve. For the EE analysis, the liposome dispersion

was centrifuged at 14800 rpm for 1 hour using the centrifuge Microfuge 16 (Beckman Coulter, EUA). Thereafter, the supernatant was collected and diluted 6x with ethanol to dissolve the non-encapsulated passion fruit oil. The empty liposomes were subjected to the same procedure and used as the blank sample. The oil concentration was then assessed by UV-vis spectroscopy at 215nm calculated from a previously prepared calibration curve (C=0.8722*Abs+0.0615). EE was calculated according to Equation (1) (see section 2.6). With the encapsulated mass values, it was possible to calculate the loading capacity (LC) as shown by Equation (4). LC refers to the maximum demand, stress or load that may be placed in a given system under normal or other specified conditions for an extended period of time.

$$\mathrm{LC} = \frac{Encapsulated\ mass\ (g)}{Total\ mass\ of\ lipid\ (g)\ in\ liposome\ formulation} x100 \quad (4)$$

**Stability evaluation:** the physical appearance of the liposomal dispersions was checked periodically along the storage time by visual inspection and photo capture at pre-defined time intervals was registered. The samples were stored at room temperature. These tests were used to verify the stability of liposomes along time.

**Morphology analysis:** the morphology of the dried liposomes was analyzed by Scanning electron microscopy (SEM) using the apparatus Phenon Pro microscope (Phenon World, The Netherlands).

**Fourier-transformed Infrared Spectroscopy (FTIR):** FTIR spectra were recorded in Attenuated Total Reflection (ATR) mode using an ABB Inc. FTIR spectrometer model MB 300 (Quebec, Canada). The used spectral range was 4000 and 550 $cm^{-1}$ at a resolution of 16 $cm^{-1}$ and cumulative acquisition of 32 scans. The used software for acquisition and data treatment was the Horizon MB v.3.4 software. This technique was used to check structural features of the liposomes, including the ones arising from the coating and drying processes.

# *Chapter 4 – RESULTS AND DISCUSSION*

## 4.1 Oil characterization

### 4.1.1 Fatty acids profile

The full fatty acid composition of the passion fruit oil is shown in Table 5. From the preformed analysis, it was concluded that it is a linoleic acid (C18:2) rich oil (67.92%). Other abundant fatty acids were oleic acid (C18:1) and palmitic acid (C16:0), with contents of 15.69% and 12.08%, respectively.

**Table 5** – Fatty acids profile of passion fruit oil (Average±STD).

| | **Passion fruit oil** |
|---|---|
| C14:0 (myristic acid)* | 0.086±0.004 |
| C15:0 (pentadecylic acid)* | 0.027±0.001 |
| C16:0 (palmitic acid)* | **12.08±0.01** |
| C16:1 (palmitoleic acid)** | 0.189±0.005 |
| C17:0 (margaric acid)* | 0.092±0.001 |
| C18:0 (stearic acid)* | 2.87±0.01 |
| C18:1n9c (oleic acid)** | **15.69±0.01** |
| C18:2n6c (linoleic acid)*** | **67.97±0.02** |
| C18:3n3 (gamma linoleic acid)*** | 0.482±0.003 |
| C20:0 (arachidic acid)* | 0.20±0.01 |
| C20:1 (paullinic acid)** | 0.122±0.001 |
| C22:0 (behenic acid)* | 0.087±0.004 |
| C24:0 (lignoceric acid)* | 0.116±0.004 |
| SFA* | 15.55±0.02 |
| MUFA** | 16.00±0.01 |
| PUFA*** | 68.45±0.03 |

*SFA: saturated fatty acids; **MUFA: monounsaturated fatty acids; ***PUFA: polyunsaturated fatty acids

The obtained fatty acid profile was similar to the ones reported in other studies dealing with the analysis of passion fruit seed oil, namely in what concerns the prevalence of linoleic acid. For example, a value of 65.72% was reported by Surlehan *et al.* (2019), and a value of 73.14% by Malacrida and Jorge (2012). The high amount of linoleic acid in the passion fruit seed oils indicates that the oil is a light-oil, and thus can easily penetrate human skin, comparatively with oils with higher saturated fatty acid contents. According to Calder (2015), linoleic acid is an important constituent of ceramides, particularly those found in the skin, and is an essential fatty acid whose deficiency results in the breakdown of skin integrity and inability to prevent transdermal water loss.

According to Pereira *et al.* (2019), a higher amount of linoleic acid relatively to oleic, acid indicates a better quality of the vegetable oil, namely by avoiding the formation of bad cholesterol. Moreover, the presence of high amounts of this essential fatty acid suggests that the oil is highly nutritious. Therefore, yellow passion fruit seed oil could be used as a promising source of oil with high value for food, pharmaceutical and cosmetic sectors (Lucarini *et al.*, 2019).

The relationship between the contents of polyunsaturated and saturated fatty acids is expressed as the PUFA/SFA index. This value is another important parameter for the determination of the nutritional value of oils; namely, oils with a PUFA/SFA index value higher than 1 are considered of high nutritional value (Kostik, Memeti and Bauer, 2013). According to the studies of Lawton *et al.* (2000), a higher value of PUFA/SFA index means a lower deposition of lipids in the body, which is regarded as favorable for the prevention of heart diseases. In this work, the PUFA/SFA index is higher than 1 (4.40) corroborating the good nutritional value of this oil.

### 4.1.2 Antioxidant activity

According to Malacrida and Jorge (2012) in passion fruit oil, the antioxidant activity is influenced by the amount of phenolic compounds and tocopherols composition. In this context, the total phenolic compounds was measured by the Folin-Ciocalteau method, as described in section 3.2.1. However, since the Folin-Ciocalteau reagent is not specific for phenolic compounds, being able to quantify reducing compounds also (which accelerate self-oxidation), the term "total phenolic compounds" might be inappropriate, unless the interfering species are removed. Thus, the results in this work will be termed as "total reducing capacity", instead of "total phenolic compounds". Figure 12 shows the Falcon tubes after the extraction procedure and before the absorbance measurement.

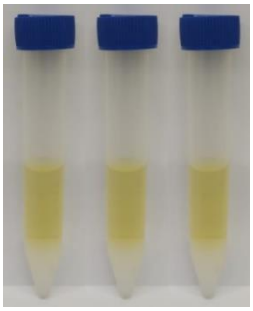


Figure 12 – Falcon tubes used for the extraction of the phenolic compounds.

A calibration curve made with caffeic acid (C=0.1511*Abs+0.0172) was used, and the results were expressed in mg of CA/kg of oil. The obtained results for each triplicate are represented in Figure 13, and the average value of the total reducing capacity for the analyzed passion fruit oil was 54.04±4.83 mg of CA/kg. In comparison with the value reported by Alves *et al.* (2013) (23.20 mg/kg), a higher value was obtained in this work. Nevertheless, in the work of Malacrida and Jorge (2012) a much higher value (1441.28 mg/kg) was obtained. According to Martínez *et al.* (2012) the concentration and type of phenolic substances depend on several factors, namely the differences in varieties, ripeness and season. Also, environmental factors such as soil type and climate, genetic factors and used processing (as the chosen solvent) and extraction methods can influence this value. In Martínez *et al.* (2012) work, the used passion fruit seed oil was obtained by extraction with ethanol and methanol-acetone solvents, thus proving the influence of the used solvent on the total reducing capacity, which was 50 mg/kg of oil and 170 mg/kg, respectively.

Machetti and Keneni (2017) studied different oil extraction methods and their influence in the final product. They point out that the principal disadvantage of using a solvent extraction method is the possibility to promote the degradation of phenolic compounds. According to Cvetanović (2019), the traditional extraction with organic solvent, can lead to the presence of unwanted components in the obtained extracts, reducing their biological potential. Thus, the difference between the achieved results of this work in comparison with the analyzed references can be associated with the used method, in particular the used solvent.

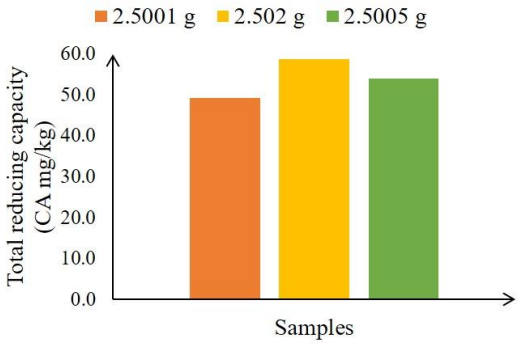
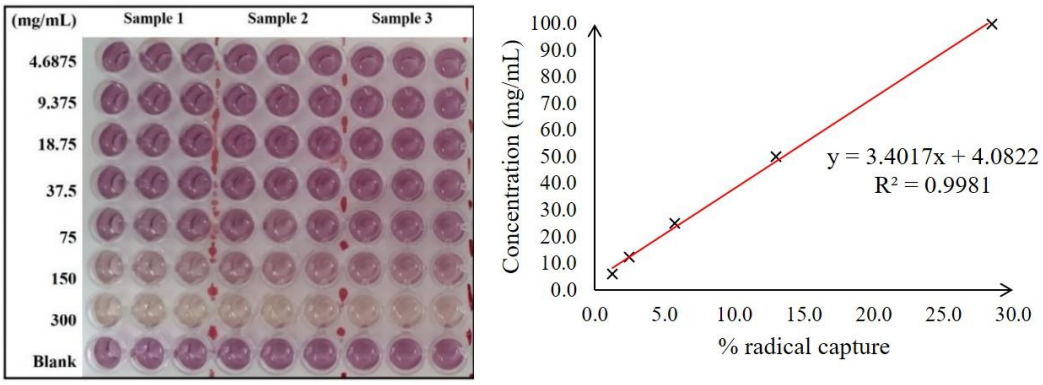



Figure 13 – Total reducing capacity.

The antioxidant activity was also evaluated by the DPPH method. The progress of the color from purple to yellow, observed for the samples that presented antioxidant activity indicate the reduction of the DPPH radical into hydrazine. Figure 14 (A) shows the microplate used for each sample when applying this methodology using methanol as the solvent. Through

the observation of the microplate, the evolution of purple to yellow occurs between the concentrations 150 and 300 mg/mL (mg of passion fruit oil/ mL solvent). The measured absorbance gave a value of EC50% equal to 174 mg/mL according to the produced calibration curve (Figure 14 B). So, to decrease of 50% in the DPPH absorbance is necessary at least a oil concentration of 174 mg/mL.




A B

Figure 14 – DDPH antioxidant activity assay: A. Photo of the microplate; B. Calibration curve.

The results with ethyl acetate are represented in Figure 15**,** showing the percentage of radical capture for a 100mg/mL oil-in-solvent concentration (in triplicate). They indicate that this oil has an intermediary DPPH radical scavenging activity (around 34%), below 50%, to concentrations up to 100 mg/mL. Other authors obtained 29.94% and 44.1% for an oil extracted from purple passion fruit (Alves *et al.*, 2013; Barbieri and Leimann, 2014). Oliveira *et al.* (2012) justify this result due to the extraction with organic solvents and the use of high temperatures. Some authors have studied the correlation between the antioxidant activity and the total phenolic compounds, so the low concentration of phenolic compounds, which can be related with the extraction with organic solvents, can justify the values obtained by the DPPH method.

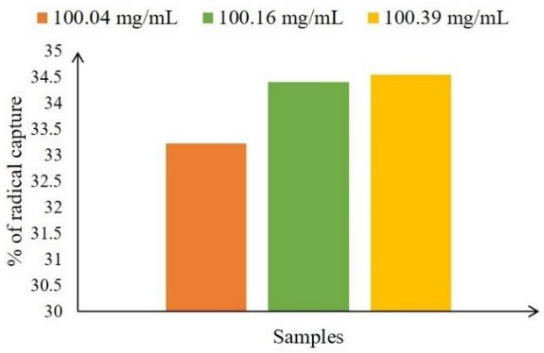



Figure 15 – Percentage of radical capture to 100 mg/mL of oil concentration.

### 4.1.3 Oxidative stability

The oxidative stability index is an important parameter, indicating the susceptibility to oxidation, which can be related with the shelf life. The methodology described in section 3.2.1 was applied in duplicate and the obtained results are showed in Figure 16**.**

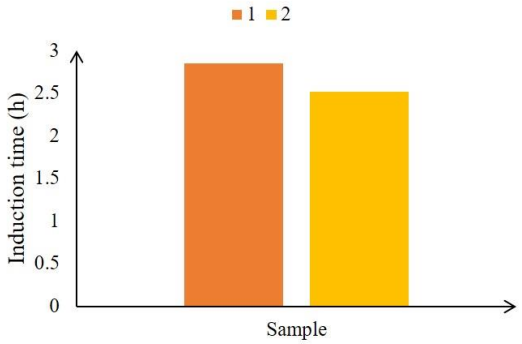



Figure 16 – Oxidative stability of passion fruit seeds oil.

The average stability index was 2.69 h. The loss of oxidative stability can be related to lipid oxidation. The oxidation is a degradative process when the oxygen reacts with the unsaturated fatty acids present in the oil. The results of the fatty acid profile indicated the prevalence of unsaturated fatty acids in the composition, factor that can promote low oxidative stability. Other authors determined the oxidative stability of the passion fruit seed oil, obtaining similar values, namely values varying from 2.46 h to 2.68 h (Giuffrè, 2007; Pardauil *et al.*, 2011). Malacrida and Jorge (2012) obtained values around 7.89 h, while Alves *et al.* (2013) obtained 0.78h. The difference between the values can be related with the conditions used in the oil extraction method.

## 4.2 Liposomes characterization

Following the ethanol injection method proposed by Zômpero *et al.* (2015) for liposome production, the influence of parameters such as the stirring time (rate fixed as 500 rpm) and the ethanol evaporation time were evaluated. Moreover, the presence of organic solvent on the morphology and particle size of the obtained liposomes was also studied. Oil-loaded liposomes

were characterized and compared with the unloaded liposomes. In this session these results are presented and discussed.

### 4.2.1 Influence of stirring and evaporation times

According to Zômpero *et al.* (2015), one of the most influential variables of the ethanol injection process is the stirring time during liposomes production. Moreover, the ethanol evaporation time is another important variable. Thus, to check the influence of these variables in the production of the liposomes, experiments with sweet almond oil, used as a model oil, were conducted. The objective was to determine the best stirring and evaporation time conditions to subsequently use with the passion fruit oil.

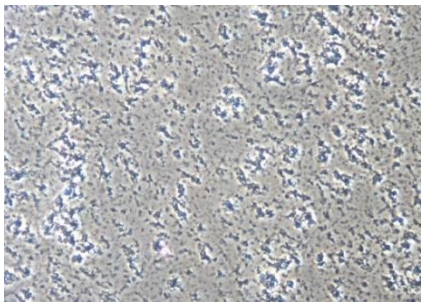
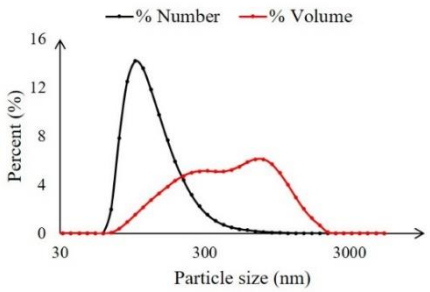
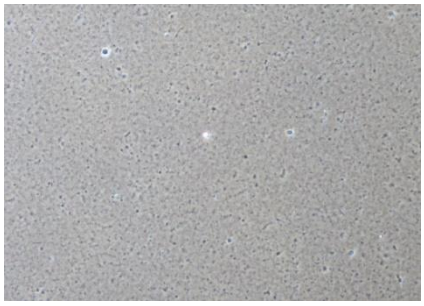
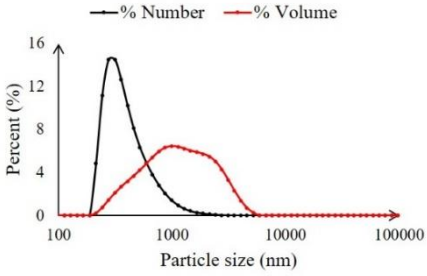
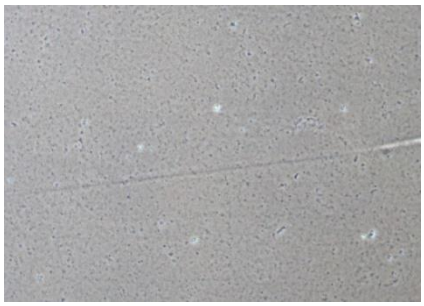
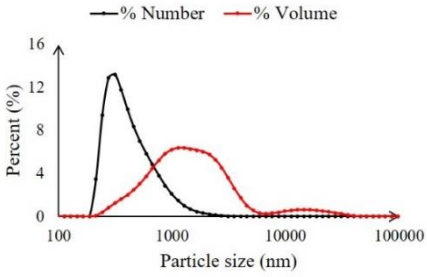
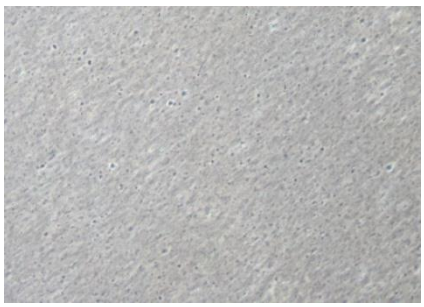
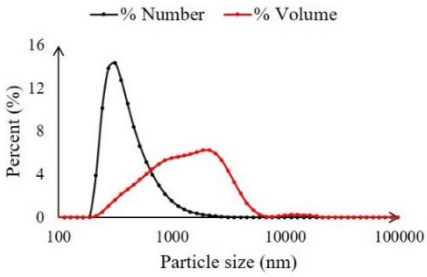
To determine the influence of stirring time, a Cowles impeller was used and samples were prepared with different stirring times, namely 5, 10 and 15 minutes. To study the influence of evaporation time, 30 minutes and 60 minutes were tested. Each of the produced samples were characterized by optical microscopy to verify the morphology of the formed vesicles and by laser diffraction to access size distributions. The obtained results are listed in Table 6.

Analyzing the OM images present in Table 6 it is possible to observe that sample SAOL_1 has particle clusters, indicating that the used conditions (15 minutes of stirring and 60 minutes in the rotary evaporator) were not adequate. Probably the used stirring time was too long for this system leading to agglomeration. For the other samples (SAOL_2 to SAOL_4), the OM results were inconclusive since the images were quite similar. According to the results obtained by LD, also present in Table 6, the size distribution for SAOL_2 shows that the distribution was the narrower one among all the produced samples. For SAOL_3 and SAOL_4 the distribution in volume evidenced a second peak around 10000 nm (bimodal distribution), indicating the formation of agglomerates.

Table 7 presents the statistical analysis of the size distributions in volume in terms of D10, D50 and D90. The distribution in volume was used since for spherical particles it is more representative (Singh *et al.,* 2019). The parameter D90 represents the particle size corresponding to 90% cumulative volume or number (from 0 to 100%) undersize particle size distribution. D50 and D10 have the same meaning for 50% and 10%, respectively. So, from these definitions, D50 value corresponds to the average diameter (Jillavenkatesa *et al.*, 2001).

The results show that SAOL_2 sample has an average size in volume of 1190 nm, the lowest among the analyzed samples, as well as the lowest standard deviation. Still, the range of sizes obtained for this formulation is within the sizes indicated by the method, around 1µm. Thus, after this analysis, 5 minutes of stirring and 30 minutes of evaporation time were considered to be adequate to proceed with the production of the liposomes with the passion fruit oil.

**Table 6** – Influence of stirring and evaporation times represented by the OM analysis and size distributions.

| Sample | OM (Magnification = 400x) | LD |
|---|---|---|
| **SAOL_1**<br>**15min, 60min** |  |  |
| **SAOL_2**<br>**5min, 30min** |  |  |
| **SAOL_3**<br>**10min, 30min** |  |  |
| **SAOL_4**<br>**15min, 30min** |  |  |

(*) the first value in minutes indicates the stirring time and the second one the evaporation time.

**Table 7** – Statistical analysis (D10, D50, and D90) of size distributions (in volume).

| Sample | D10 | | D50 | | D90 | |
|---|---|---|---|---|---|---|
| | Size (nm) | (±) Std | Size (nm) | (±) Std | Size (nm) | (±) Std |
| **SAOL_1** | 501 | 2.37 | 1470 | 3.43 | 3670 | 3.14 |
| **SAOL_2** | 454 | 1.52 | 1190 | 1.29 | 2940 | 2.38 |
| **SAOL_3** | 553 | 2.56 | 1460 | 1.73 | 4020 | 23.1 |
| **SAOL_4** | 497 | 0.17 | 1440 | 2.33 | 3980 | 8.13 |

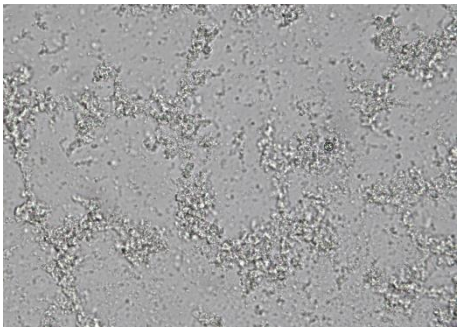
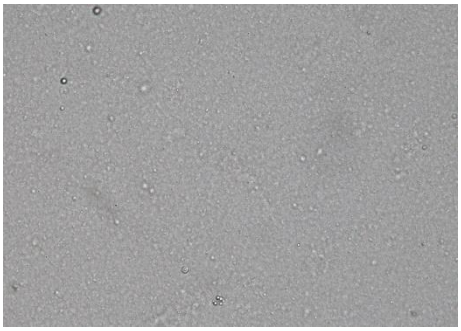
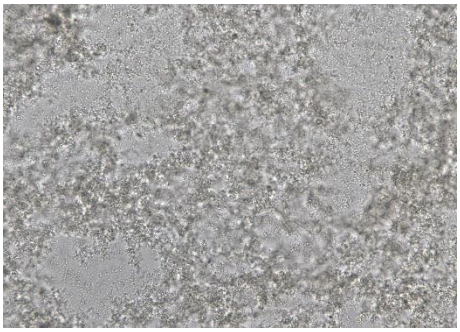
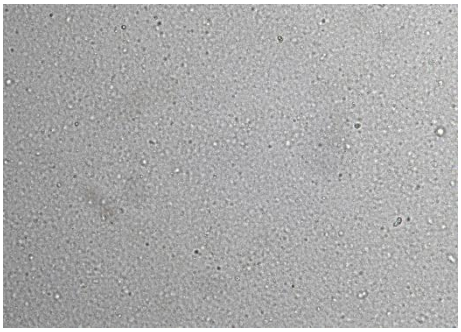
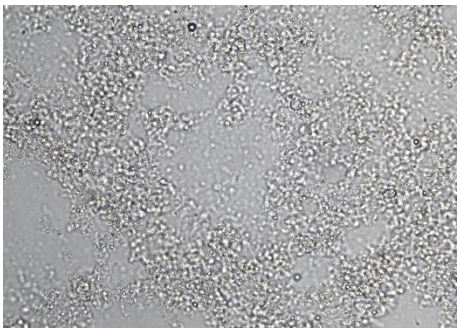
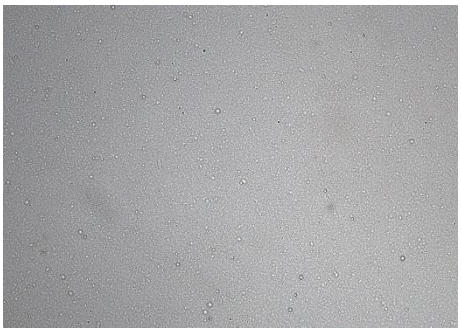
### 4.2.2 Influence of the solvent evaporation step on liposomes morphology

The presence of the organic solvent in the final liposomal dispersion, according to Sebaaly *et al.*, (2016), can influence the stability of the vesicles. In order to verify the influence of the used ethanol evaporation step in the morphology of the formed liposomes, OM was used. The samples were analyzed before and after the ethanol evaporation step and the results obtained for a set of selected samples is presented in Table 8.

According to the images collected in Table 8 it is possible to observe that the presence of ethanol promotes the agglomeration of the vesicles, which after the evaporation step assume a scattered pattern. The agglomeration occurs due to the affinity between the lipid and the ethanol solvent, resulting in an attractive effect. Therefore, the presence of ethanol can impact product stability and safety as it could cause the bilayer to be leaky, tending to increase the liposome size, and reduce the entrapment efficiency. The OM images obtained for the other analyzed samples are showed in Table B1 presented in appendix.

In the work of Bnyan *et al.* (2020), the same effect was observed. They proved that the ethanol removing step resulted in the liposomal size reduction and net charge increase, which in turn helped to maintain the uniform size distribution due to the repulsion effect between the liposomes. The repulsion effect is related to the zeta potential of liposomes, which is directly related to the dielectric constant of the solvent. As dielectric constant of water is greater than that of ethanol, when removing ethanol from the liposome solution an increase in the dielectric constant of media will result, and, consequently, an increase in zeta potential. The high net charge after ethanol evaporation could increase the degree of stabilization, reducing the agglomeration among the particles.

**Table 8** – OM analysis of selected samples before and after ethanol evaporation.

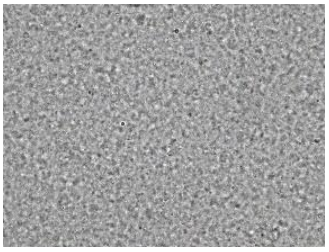
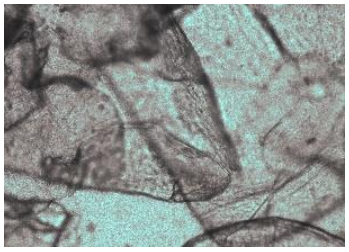
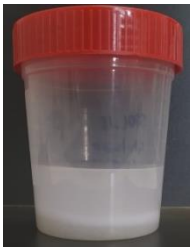
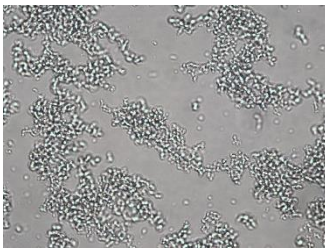
| | **Magnification** | |
|---|---|---|
| | 400x | |
| **Sample** | **Before solvent evaporation** | **After solvent evaporation** |
| **SPOL_4**<br>**5min, 30min** |  |  |
| **SPOL_7**<br>**5min, 30min** |  |  |
| **SPOL_17**<br>**5min, 30min** |  |  |

### 4.2.3 Liposomes coating with chitosan

In order to increase the stability of the passion fruit oil liposomes, chitosan can be added to the dispersion to promote the formation of a coating around the particles. Moreover, this step can be followed by a drying step using e.g. the spray drying technique to obtain the dry form of the liposomes, an interesting feature from the point of view of the final product. In order to conduct this step, it is needed to solubilize the chitosan, and the pH of the solution must be adjusted.

According to Gültekin-Özgüven *et al.* (2016), the pH needed for chitosan dissolution must be acidic, namely they used a value of 3.8. Moreover, they tested different concentrations of chitosan (0.5–1%, w/v). Gibis *et al.*, (2012) in a study dealing with the encapsulation of grape seed extract used a pH of 3.0. In the present work a concentration of 0.5% was used, and the influence of pH was done by testing the pHs 3.0 and 3.8 using an acetic acid solution (3M) for the adjustment. Optical microscopy and visual analysis were used to conduct this study. A selection of the obtained results are listed in Table 9.

**Table 9** – Influence of pH: OM analysis and visual inspection.

| pH | Sample | OM: Supernatant | OM: Precipitate | Visual inspection |
|---|---|---|---|---|
| | | Magnification | | |
| | | 400x | 100x | |
| 3.8 | SPOL_5<br>5min, 30min |  |  |  |
| 3.0 | SPOL_18<br>5min, 30min |  | * |  |

(*) Image not acquired due to the absence of precipitate formation.

Analyzing the results of Table 9 it is possible to conclude, by visual analysis, that pH 3.8 did not lead to complete dissolution of chitosan, while a pH of 3.0 render chitosan completed dissolved thus forming a stable dispersion. By OM analysis, at pH 3.8 it is possible to observe agglomerated vesicles involved by chitosan. Moreover, the sample separated into two phases, a supernatant where the vesicles are uncoated, and a precipitate where a chitosan film was formed.

The observed phenomenon can be explained due to the charge attraction between the phosphate group of phosphatidylcholine (negatively charged), and the amine group of chitosan (positively charged). According to Naumowicz and Figaszewski (2014), at pHs ranging between 1 and 3 ionization of the phosphate group occurs, allowing the attraction with the

chitosan, which in acid medium has a positive charge. At higher pHs the ionization of the amino group of the phosphatidylcholine, which has a positive charge, promotes the repulsion with chitosan. Thus, it is possible to conclude that an ideal pH, in this case is 3.0, needs to be used. This analysis was made in triplicate and the same pattern was verified for the other samples (Table B2 in appendix).

### 4.2.4 Zeta potential and polydispersity index

To verify the stability of the liposome dispersion and check the homogeneity of liposome size distribution, zeta potential and polydispersity index (PDI) were determined. Three samples were studied: unloaded liposomes (SEL_3), passion fruit oil-loaded liposomes (SPOL_18) and oil-loaded liposomes coated with chitosan (SPOL_18_CS). Each analysis was done in triplicate in order to study the reproducibility. Table 10 summarizes the obtained results.

**Table 10** – Zeta potential and polydispersity index.

| **Sample** | **Zeta potential (mV)** | | **PDI** | |
|---|---|---|---|---|
| | Average | Std (±) | Average | Std (±) |
| **SEL_3** | -19.7667 | 0.4889 | 0.6710 | 0.0193 |
| **SPOL_18** | -28.2333 | 0.4222 | 0.6065 | 0.0075 |
| **SPOL_18_CS** | 46.5333 | 0.5111 | 0.9317 | 0.0911 |

Values of zeta potential above +30mV and below -30mV pointed out for a good stability of the suspension. The uncoated liposomes have a negative zeta potential. After chitosan coating, zeta potential shifted from negative to positive values. This fact can be attributed to the more cationic nature of the polymers adsorbed at liposome's surface. In this case, since chitosan carries a high positive charge, the adsorption of chitosan appears to have increased the density of positive charge and hence turn zeta potential to a positive value. To note also that this value is higher than 30, thus pointed out a high stability of the coated liposomes.

According to Mady and Darwish (2010), chitosan coated-liposomes have been used as mucoadhesive delivery systems because their positively charged surface favors the adhesion to the cells membrane, which are normally negatively charged. In his work, the same behavior was obtained, the uncoated liposome had a negative zeta potential, and the addition of chitosan promoted a change in the charge and an increase of its absolute value.

Danaei *et al.* (2018) stated that the polydispersity index (PDI) is the better parameter to define the size range of liposomes, giving information about the heterogeneity of the system. This index is dimensionless; values smaller than 0.3 are mainly observed for highly monodisperse standards, while PDI values bigger than 0.3 indicate that the sample has a broad particle size distribution, i.e. the sample has a heterogeneous distribution (Roggia *et al.*, 2019).

Analyzing the results listed in Table 10**,** the PDI for all samples is higher than 0.3, indicating that the systems have a heterogeneous size distribution. For the uncoated samples this result can be attributed to phospholipid concentration, which for SEL_3 and SPOL_18 is higher than the active principle. To minimize this effect, the concentration of phospholipid must be reduced. For the coated liposomes, SPOL_18_CS, the higher PDI can be justified by some agglomeration deriving from the presence of chitosan. This effect might be minimized by reducing chitosan concentration or by using a chitosan of lower molecular weight.

### 4.2.5 Size analysis

According to Singh *et al.* (2019), for a heterogeneous population (PDI > 0.3), LD is the most suitable technique to analyze the size distribution. Beginning by the empty liposomes, a comparison between the size distribution of three assays is presented in Figure 17. The samples SEL_1 and SEL_3 were analyzed in the same week of the preparation, while the sample SEL_2 was analyzed after 1 month of storage.

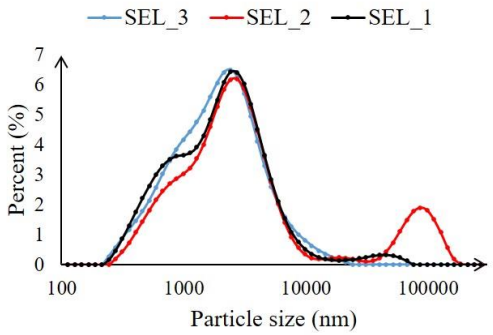



Figure 17 – Size distribution of three assays conducted with the empty liposomes.

Analyzing Figure 17 it is possible to note that the size distribution among the analyzed samples is not too much different considering the principal population, however, for the sample

SEL_2 a peak centered around 100000 nm appeared. This fact can be justified by the low stability of the unloaded-liposomes that leaded to vesicle agglomeration with time, as already stated in the section 4.2.4, in the discussion about zeta potential. The individual size distributions are presented in Table C1 in appendix section.

The same study was done with the passion fruit oil loaded-liposomes, by analyzing three samples. The samples SPOL_8 and SPOL_18 were characterized in the same week they were prepared, while the sample SPOL_17 was only characterized after 1 month under storage. Figure 18 presents the obtained results. According to Figure 18, and in comparison with the empty liposomes, it is possible to conclude that the profiles remain very similar. Only a slight increase in the peak located at 100000 (related to the agglomeration phenomena), was perceived in sample SPOL_17, meaning that the oil-loaded samples are more stable than the empty ones. Moreover the profile of sample SPOL_8 was displaced for slight lower sizes.

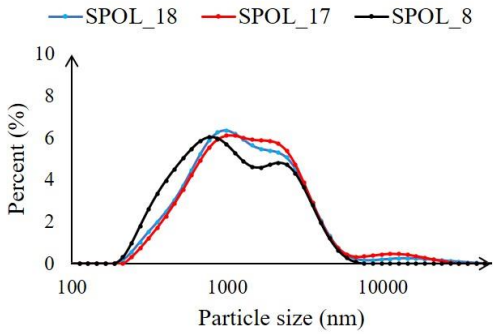



Figure 18 – Size distribution of three assays conducted with the loaded liposomes.

A comparison between the unloaded and loaded-liposomes was also performed. The samples SEL_1 and SPOL_8 were prepared in the same week, as well as samples SEL_2 and SPOL_17, and samples SEL_3 and SPOL_18. In Table 11 and Figure 19 this comparison is provided.

**Table 11** – Comparison between unloaded and loaded liposomes.

| **Sample** | **D10** | | **D50** | | **D90** | |
|---|---|---|---|---|---|---|
| | Size (nm) | (±) Std | Size (nm) | (±) Std | Size (nm) | (±) Std |
| **SEL_1** | 609 | 0.70 | 2180 | 4.53 | 5830 | 46.6 |
| **SPOL_8** | 418 | 2.31 | 1090 | 9.33 | 3180 | 9.68 |
| **SEL_2** | 754 | 8.34 | 2660 | 39.9 | 6970 | 13.5 |
| **SPOL_17** | 540 | 5.94 | 1410 | 1.02 | 3740 | 2.92 |
| **SEL_3** | 648 | 10.7 | 2070 | 2.30 | 5550 | 8.33 |
| **SPOL_18** | 507 | 2.30 | 1310 | 3.24 | 3550 | 12.5 |

Analyzing the results provided in Table 11 it can be perceived that both samples analyzed 1 month after being produced (SEL_2 and SPOL_17), have the higher sizes suggesting that agglomeration or swelling might occur with time. Moreover, and as previously discussed, this phenomenon is much more evident in the unloaded samples. Another point to note is the fact that the loaded samples have always a smaller size when compared with the respective unloaded samples. This effect can be explained by the sensitivity of the liposomes to the ionic strength and osmolarity of the medium, determining a variation on liposomal size. According to Perinelli *et al.* (2019), unloaded liposomes tend to increase their size due to swelling of the vesicles with water, while for loaded liposomes, this swelling is hindered by the presence of the oil.

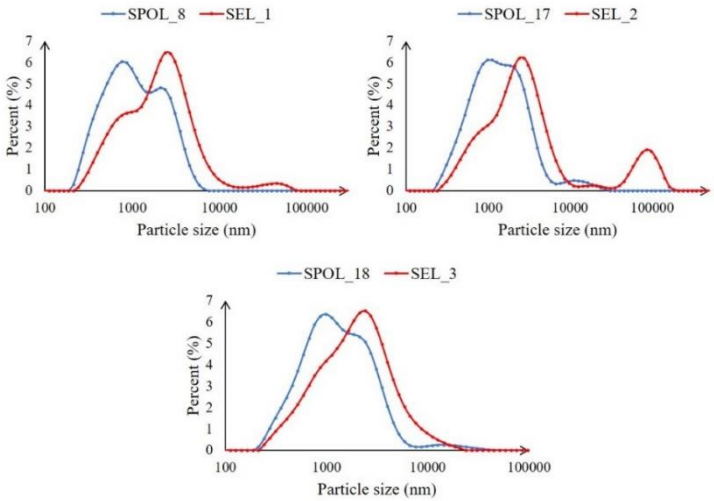



Figure 19 – Comparison between unloaded and loaded liposomes.

### 4.2.5 Encapsulation efficiency

According to Lopes and Brandelli, (2018) the determination of the encapsulation efficiency is an important step in liposome characterization. In this work, the chosen method was based on UV-vis spectroscopy and the used methodology adopted from the works of Ghorbanzade *et al.* (2017) and Gómez-Mascaraque *et al.* (2017), with some modifications.

First, to produce the calibration curve it was necessary to determine the maximum of absorptivity for the passion fruit oil, which was determined as 215 nm. Ethanol was used as solvent, and different oil concentration solutions were prepared in order to build the calibration

curve measuring the absorbance at 215 nm. The obtained calibration curve is represented in Figure 20.

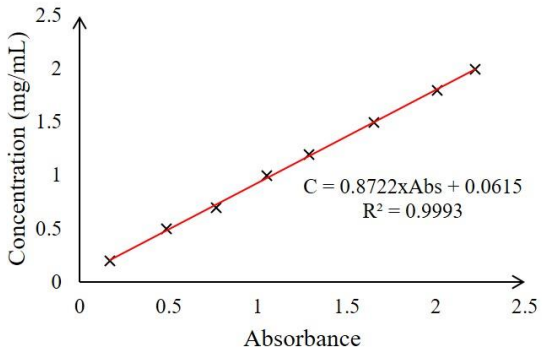



Figure 20 – Calibration curve to passion fruit oil (215nm).

To verify the best formulation to prepare oil-loaded liposomes, four different concentrations of passion fruit oil were tested, namely 20%, 40%, 60% and 80%. All samples, including the unloaded liposomes (blank; used for correction proposes), were centrifuged, thereafter the recovered supernatant diluted to guaranty that the measurement is within the limits of the produced calibration curve. The absorbance of each sample was then measured by UV-vis at 215 nm. The obtained results are listed in Table 12. Knowing that the used volume of dispersion was 2 mL, the oil mass entrapment inside the liposomes was calculated and related with the initially used oil to calculate the encapsulation efficiency. The obtained results are presented in Table 13.

**Table 12** – Absorbance of diluted supernatant.

| Sample | Absorbance |
|---|---|
| SEL_Blank | 1.294 |
| SPOL_20% | 1.390 |
| SPOL_40% | 1.675 |
| SPOL_60% | 2.331 |
| SPOL_80% | 2.894 |

**Table 13** – Calculated oil loaded masses and corresponding encapsulation efficiency.

| SPOL | Abs. | Conc. (mg/mL) | Unencapsulated mass (g) | Added mass (g) | Encapsulated mass (g) | EE (%) |
|---|---|---|---|---|---|---|
| **20%** | 0.096 | 0.1452 | 0.0261 | 0.3226 | 0.2965 | 91.8987 |
| **40%** | 0.381 | 0.3938 | 0.0709 | 0.6428 | 0.5719 | 88.9732 |
| **60%** | 1.037 | 0.9660 | 0.1739 | 0.9620 | 0.7881 | 81.9258 |
| **80%** | 1.600 | 1.4570 | 0.2623 | 1.2894 | 1.0271 | 79.6599 |

The results showed that the encapsulation efficiency decreases as the oil content increases. Thus, the best formulation to avoid oil losses during the process was the formulation using 20% of oil, which lead to an encapsulation efficiency of 91.9%. Even so, the obtained values are high for all the tested formulations (roughly over 80%). These high values for the encapsulation efficiency were expected since the encapsulated active principle is hydrophobic having a low affinity with the aqueous phase, which facilitates their encapsulation, and protection by the hydrophobic part of the phospholipid. Comparing the obtained results with the published ones, it is possible to conclude that similar values were obtained, namely in the work of Ghorbanzade *et al.* (2017), for example, the encapsulation of fish oil led to a value of encapsulation efficiency of 92%.

The samples containing different amounts of oil were also examined by optical microscopy being possible to note that the liposome size increase as the content of oil increased. The OM of these samples are in Table B4 presented in the appendix section. This effect is related with the loading capacity (LC), a parameter that describes the encapsulated oil in the carrier. The LC was calculated according to Equation (4), and it can be observed that it increased as the oil content in the formulation increased. Table 14 presents the calculated loading capacity of each analyzed sample.

**Table 14** – Loading capacity.

| Sample | Encapsulated mass (g) | Lipid mass (g) | LC (%) |
|---|---|---|---|
| **SPOL_20%** | 0.2965 | 1.6035 | 18.4886 |
| **SPOL_40%** | 0.5719 | 1.6053 | 35.6270 |
| **SPOL_60%** | 0.7881 | 1.6047 | 49.1136 |
| **SPOL_80%** | 1.0271 | 1.6027 | 64.0877 |

The maximum loading capacity reached a value close to 64%, corresponding to the formulation SPOL_80%. The high values of LC can be attributed to the fact that the hydrophobic domains of the liposomes make up a relatively large proportion of their total volume, ensuring a high loading. However, studies carried out by Cheng *et al.* (2019) showed that high levels of active principle may promote a change in the structure of the liposomes disturbing the packing of the phospholipid bilayer making the vesicles more unstable. Thus, a more in-depth study on the structure of liposomes with different formulations should be done to obtain further conclusions.

## 4.3 Liposomes drying

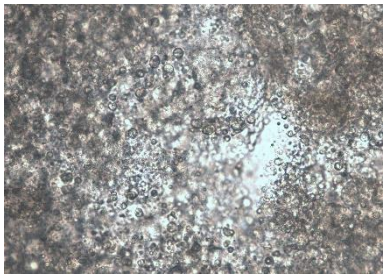
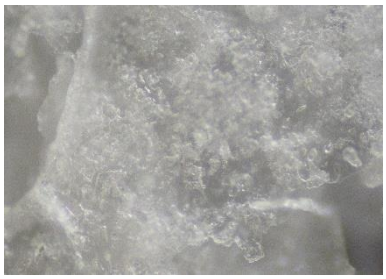
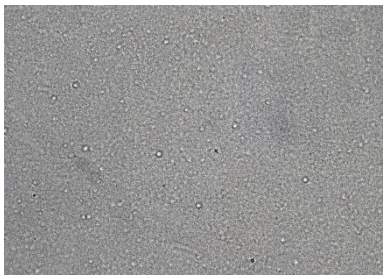
One important issue when dealing with liposomes is to be able to produce their solid form, since at industrial level this form is often preferred. Having this in view, in this section the liposomes drying process was studied. Liposomes were dried by two different methods, namely lyophilization and spray drying. In this section the obtained results for each method will be discussed.

### 4.3.1 Morphology analysis

Morphology analysis was performed by OM and SEM. For OM analysis, the dried samples were analyzed after dispersed in water. The used magnifications were 100 and 400x as shown in Table 15. According to OM images, it is possible to distinguish, in lyophilized liposomes, the presence of a film surrounding the particles. This might be, probably, due to the presence of the cryo-protector. Moreover, the liposomes seem to be non-spherical in the dried state, but assuming a shape very close to the one before drying when dispersed in water. For the liposomes dried by spray drying, the vesicles look more spherical after dispersed in water, even a higher size was noticed, fact associated with agglomeration phenomena due to the presence of chitosan, which is soluble only at acidic pH. The imagens acquired for all produced samples are shown in Table B3 present in appendix.

It is important to emphasize that OM is not the most suitable method to characterize the liposome systems due to the small size of these particles. Thus, to get more insights into the morphology of these systems, scanning electron microscopy (SEM) was also used.

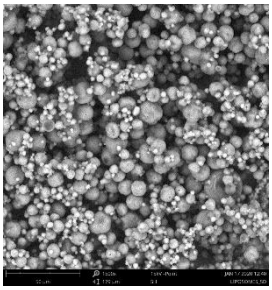
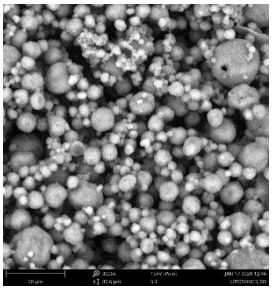
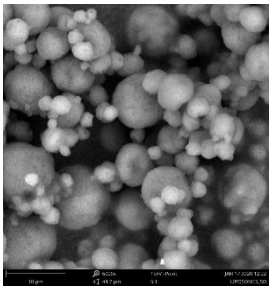
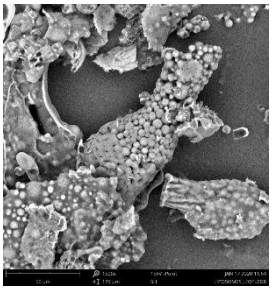
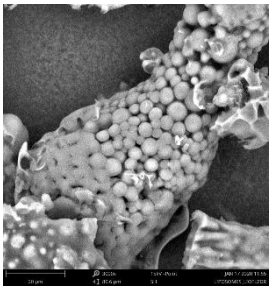
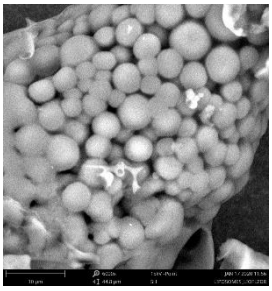
**Table 15** – OM analysis of dry liposomes (as such or dispersed in water).

| | **Magnification** | |
|---|---|---|
| | 100x | 400x |
| **Method** | **Dried** | **Dispersed in water** |
| **Spray drying** |  |  |
| **Lyophilization** |  |  |

The acquired SEM images are organized in Table 16 reporting three magnifications (1500, 3000 and 6000x). From the observation of the images it is possible to conclude that the spray-dried liposomes present spherical shape, smooth surface, and a non-uniform particle size distribution. Moreover, some particle clusters (agglomeration) can be perceived even the vesicles remain intact indicating an efficient protection of the oil inside them.

Concerning the lyophilized liposomes, which were dried in the presence of maltodextrin, a film surrounding the particles is perceptible. Nevertheless, a spherical shape and smooth surface was observed, as in the case of spray drying, with the individual vesicles presenting a more uniform particle size in this last case. The observed uniformity in size can be explained due to the cryo-protectant effect as previously illustrated in Figure 7 (see section 2.5), which hinders agglomeration and fusion of the phospholipid compounds. The SEM images of lyophilized and spray-dried liposomes, acquired at other magnifications can be consulted in Table D1 in appendix.

**Table 16** – SEM images of the dried liposomes at different magnifications (1500, 3000 and 6000x).

| **Method** | **Magnification** | | |
|---|---|---|---|
| | 1500x | 3000x | 6000x |
| **Spray drying** |  |  |  |
| **Lyophilization** |  |  |  |

Comparing both methods, the lyophilized liposomes present smaller size comparatively with the spray-dried liposomes. The first ones were in a range lower than 5µm, while for the spray-dried samples, a size range between 5µm and 20µm was achieved. Although SEM images are important to have information about morphology, the most feasible technique is LD, and the determinations according to this method will be discussed further in next section.

### 4.3.3 Size analysis

The dried samples, according to the two methodologies, were analyzed in the same week where they were prepared. Figure 21 and Table 17 show the size distribution and respective analysis.

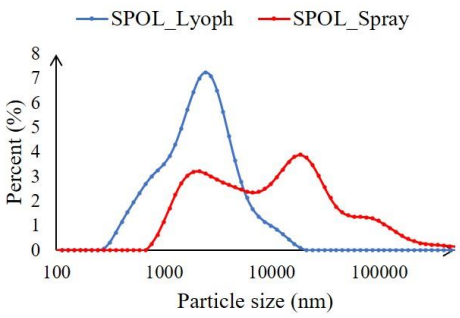



Figure 21 – Comparison between spray-dried and lyophilized liposomes.

**Table 17** – Comparison between spray-dried and lyophilized liposomes.

| Sample | D10 | | D50 | | D90 | |
|---|---|---|---|---|---|---|
| | Size (nm) | (±) Std | Size (nm) | (±) Std | Size (nm) | (±) Std |
| **SPOL_Spray** | 1740 | 3.76 | 11100 | 75.6 | 74500 | 610 |
| **SPOL_Lyoph** | 739 | 0.49 | 2300 | 1.48 | 5820 | 16.6 |

As previously concluded by SEM, Figure 21 corroborates the fact that the spray-dried liposomes presented a more non-uniform size distribution. These can be proved by the presence of several peaks in the distribution, indicating different size populations. Moreover, it is also possible to conclude that lyophilized liposomes have smaller sizes than the spray-dried ones. The same can be checked in Table 17, where the average size of spray-dried liposomes is around 11100 nm, while for lyophilized ones is 2300 nm.

Comparing the sizes of the dried liposomes with the ones before drying (Table 11, section 4.2.5), both methods promoted a size increase. Concerning the lyophilized liposomes, and according to Araújo *et al.* (2009), the increase in size can be related to the freeze conditions. If a fast freezing (liquid nitrogen) is done, the increase in size is negligible, while in the case of slow freezing, the higher the temperature is, the greater is the increase in size.

Sipoli *et al.* (2015) investigated the influence of the nanoparticle size as the CHI/TPP (chitosan/tripolyphosphate) weight ratio increases, showing that the higher is this ratio, the larger is the nanoparticle size. Polexe and Delair (2013) studied the influence of the molecular weight of chitosan in the nanoparticle size, showing that an increase in CS molecular weight leads to an average particle size increase. Umerska *et al.* (2012) showed the same effect, which is the formation of particles of different sizes, mainly large size particles were formed when a high molecular weight chitosan was used. For low CS molecular weight, the particles had a more compact and uniform structure, resulting from the higher mobility of the polymer chains, which facilitates the achievement of the right conformation and needed adaptation to improve charge matching. Thus, the large size obtained in this work can be associate with the CS molar weight or with the used CS concentration. Since the molecular weight of the used CS was not determined, and only the degree of deacetylation (85%), viscosity (10 mPas), and ash content (<1%) were known, a study deeper study can be done.

### 4.3.4 FTIR analysis

In order to disclose the structural features caused by the presence of a chitosan coating, the interactions between the lipids and chitosan were studied by FTIR. FTIR was used to infer possible changes in the structure of phospholipid by the presence of the polysaccharide by analysing the frequency of different functional groups of the lipid molecules in their absence and presence.

#### 4.3.4.1 Analysis of the base materials

To get information about the chemical structure of the liposomes coated with chitosan, it is necessary, firstly, to study the individual materials separately. Concerning the Phospholipon 90H spectrum it was characterized by a C–H stretching band, ascribed to the long fatty acid chain in the range 2918-2850 $cm^{-1}$. In addition the following assignments can be registered: a C=O stretching band at 1738 $cm^{-1}$ associated to the fatty acid ester group, a P=O stretching band at 1239 $cm^{-1}$, a P–O–C stretching band at 1091 $cm^{-1}$, and a $–N^{+}(CH_3)^3$ stretching at 970 $cm^{-1}$. These assignments were collected based on the work of Saoji *et al.* (2016).

The infrared spectrum of chitosan shows a strong band in the region 3291-3340 $cm^{-1}$ corresponding to the N–H and O–H stretching, as well as to the intramolecular hydrogen bonds. The absorption bands around 2921 and 2877 $cm^{-1}$ were attributed to the C–H symmetric and asymmetric stretching, respectively. The presence of residual N-acetyl groups was confirmed by the bands located around 1645 $cm^{-1}$ (C=O stretching of amide I), 1550 $cm^{-1}$ (N-H bending of amide II) and 1325 $cm^{-1}$ (C–N stretching of amide III). The $CH_2$ bending and $CH_3$ symmetrical deformation appear around 1423 and 1375 $cm^{-1}$. The absorption band at 1153 $cm^{-1}$ can be attributed to the asymmetric stretching of the C–O–C bridge, and the band at 1050 $cm^{-1}$ was assigned to the C–O stretching. These assignments were made based in the work of Queiroz *et al.* (2015).

4.3.4.2 Analysis of the liposomes

The liposomes (loaded and unloaded), both coated with chitosan were analysed. Figure 22 presents the respective spectra in the range 3700-2700 $cm^{-1}$, and Figure 23 in the range 1800-1200 $cm^{-1}$. Figure E1 in the appendix presents the overall spectra and complete analysis. In this section, only the most important features will be discussed. Comparatively with the base coating material (analysis provided in the last section), the formation of a complex between the liposome's material and chitosan resulted in a considerable change observed in the absorption bands of the O–H groups (3340 to 3355 $cm^{-1}$). This is due to the formation of intermolecular hydrogen bonds. The presence of chitosan in the liposomes caused also changes in the absorption bands corresponding to the acyl chains. For example, the symmetric and asymmetric stretching of the $CH_2$ and $CH_3$ groups, with bands located at 2918 and 2850 $cm^{-1}$, respectively, were changed to 2923 and 2854 $cm^{-1}$.

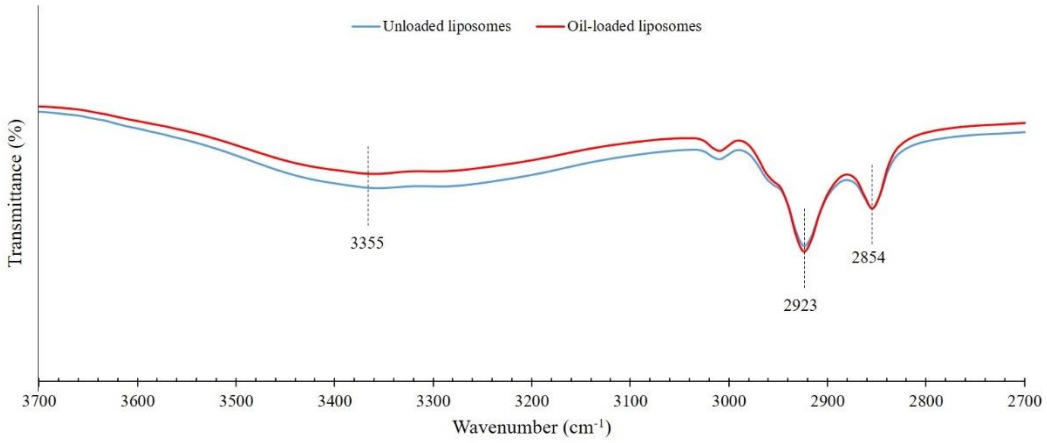



Figure 22 – FTIR spectra of the unloaded and loaded chitosan coated liposomes (region 3700-2700 $cm^{-1}$).

The symmetrical stretching vibration absorption of the phospholipid C=O shifted from 1738 to 1735 $cm^{-1}$, with the intensity reduced after coating, indicating hydrogen bonding was formed between the carbonyl of lipid bilayer and chitosan hydroxyls. Moreover, the peak at 1645 $cm^{-1}$ was shifted to 1650 $cm^{-1}$, which may support the formation of electrostatic interactions between the amine groups of chitosan and the phosphoric groups of the phosphatidylcholine. The characteristic band of chitosan at 1550 $cm^{-1}$, corresponding to the scissoring vibration of the protonated amine groups ($NH_3^+$), was shifted to higher frequencies (1581 $cm^{-1}$). The absorption band of liposomal phosphate groups also shifted to a higher frequency (from 1239 to 1242 $cm^{-1}$), meaning that the interaction with polymer ligands leads

to dehydration of phosphate groups. Apparently, most hydrogen bonds of the phosphate group are disrupted due to the electrostatic interactions with the amino groups of chitosan.

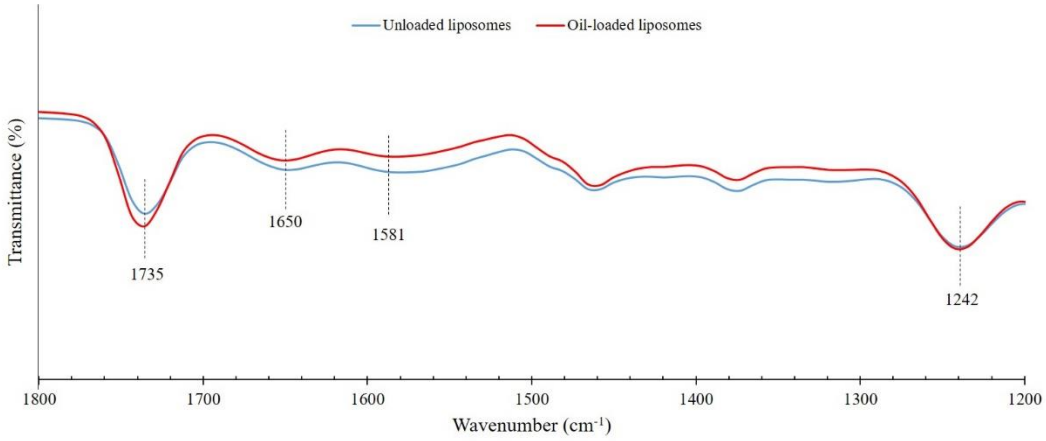



Figure 23 – FTIR spectra of the unloaded and loaded chitosan coated liposomes (region 1800-1200 $cm^{-1}$).

Summarizing, the results of FTIR spectroscopy indicated that the chitosan successfully coated the liposomes. Moreover, the results show that the load with passion fruit oil didn't modify the chitosan-coating structure, because the samples presented similar peaks in the performed spectroscopy analysis.

### 4.3.5 Visual inspection analysis

The stability of the dried liposomes is an important parameter to conclude about the best methodology. For that, each sample was monitored for two months after the drying stage. Figure 24 shows the samples treated with maltodextrin and chitosan after the storage. At the end of theses months, the lyophilized liposomes showed to be unstable by presenting signs of the release of the encapsulated oil. Appositively, spray-dried liposomes maintained the integrity over the two months. According to Terroni *et al.* (2013), lyophilization is not the most adequate method to dry particles with encapsulated oil because these products are more susceptible to oxidation, due to the presence of the lipids, limiting their conservation, thus, vacuum packaging under inert atmosphere are often required. In the present case, spray-drying revealed to be a more adequate technology to dry the liposomes.

A B

Figure 24 – Visual analysis of dried liposomes by: A. Lyophilization; B. Spray drying.

In the work performed by Ribeiro *et al.* (2016), the release of an active principle was studied. They observed a slower release of the active principle when dried particles prepared by spray-drying were used. Namely, release time of 10.13 h was observed while the dried product obtained by lyophilization showed lower values, 3.65 h. This fact was justified by the structural influence regarding the formation of ice crystals between the nanostructures, which might have contributed to the increase speed of active principle release.

# *Chapter 5 – CONCLUSIONS AND FUTURE PERSPECTIVES*

## 5.1 Conclusions

The present work was devoted to the development of a liposome system to be used in the encapsulation of an active principle, in this case, passion fruit oil, aiming at a future application in the cosmetic sector. For this purpose, passion fruit seed oil was extracted and supplied by the UTFPR Campus Apucarana research group, under the project "Extraction of total lipids and phenolic compounds from fruit seeds" supervised by Prof. Dr. Rúbia Michele Suzuki, utilizing industrial residues from Polpa Norte Company (Japurá-PR, Brazil). This oil was characterized concerning the fatty acid profile, verifying the major presence of unsaturated fatty acids where the PUFAs represent 68.45% and MUFAs 16%. Linoleic and oleic acids are the unsaturated fatty acids present at higher amounts, indicating that the oil is a light-oil and can easily penetrated the human skin.

Antioxidant activity of the oil was studied by determining the total phenols and the capture of DPPH radicals. The presence of phenolic compounds proved to be modest, with a total reducing capacity of 54 mg of CA/kg oil. The method of capture of the DPPH radicals was done by testing two different solvents: methanol and ethyl acetate but achieving similar results. This oil presented intermediary radical capture, where 100 mg/mL (oil/solvent) can reduce the free radicals in 34%, corresponding to an EC50% of 174 mg/mL. The achieved low antioxidant activity can be related to the used extraction methodology to obtain the oil, namely the use of organic solvents. Oxidative stability was also measured and the samples presented, in average, a stability index of 2.69 h. The low stability can be related with the presence of unsaturated fatty acids.

The liposomes were produced following the methodology proposed by Zômpero *et al.* (2015). Different stirring (5, 10 and 15 minutes) and evaporation times (30 minutes and 60 minutes) were studied to find the best production conditions. Based on the achieved results, the best production conditions were 5 minutes of stirring and 30 minutes in the rotary evaporator, being applied to all samples. The produced liposomes were characterized in terms of particle size, zeta potential, encapsulation efficiency, morphology and stability. Different concentrations (20%, 40%, 60% and 80% w/w) of oil were tested to analyze the influence in the encapsulation efficiency and load. Concerning encapsulation efficiency, the formulation using 20% gives rise to the higher values (91.9%). For the load the value increased as the oil

concentration increased. Thus the best solution must find a compromise between these two parameters.

The OM analysis used to monitor the morphology of the liposomes allowed to conclude that liposomes agglomerate in the presence of solvent. The size distribution was measured by laser diffraction and the average size was around 1μm. Comparisons between unloaded liposomes and oil-loaded liposomes showed that the empty vesicles present higher size, fact explained by the swelling of vesicles with water. Samples with 1 month of storage were also analyzed being verified that the sizes increased, indicating the low stability of the system.

To surpass the reported stability issues, coating with chitosan was done to increase stability. The zeta potential showed an increase in the stability with chitosan addition, since zeta potential changed from -28.23 to 46.53 mV, respectively from the uncoated to the coated liposomes. The PDI indicated an heterogeneous size distribution (PDI > 0.3) where the high PDI achieved for the coated liposomes can be justified by the agglomeration of samples in the presence of CS together with the large size of liposomes.

The liposomes were dried by two methods: lyophilization and spray drying, and the particles were characterized in terms of size, morphology and stability. The sizes of dried-liposomes increased in relation to the dispersed ones. In lyophilized liposomes, this effect was related to the presence of the cryo-protectant used and the applied freeze conditions. The effects in spray-dried liposomes can be explained by the concentration and molar mass of the used chitosan.

The morphology was studied by optical and scanning electron microscopy. The results showed that lyophilized liposomes presented a maltodextrin film coating around the particles, however they presented spherical shape and an uniform particle size. The spray-dried liposomes also presented a spherical morphology but a more uniform size distribution.

The analysis by FTIR of the chitosan-coated liposomes showed changes in the chemical structure comparatively to the uncoated ones. The principal interactions can be associated with the phosphate group of the phospholipid and the amino group of chitosan. The peak at 1650 $cm^{-1}$ indicates the electrostatic interaction between amine groups of chitosan and the phosphoric groups of phosphatidylcholine. The one at 1581 $cm^{-1}$ represents the vibration of protonated amine group $NH_3^+$ and the one at 1242 $cm^{-1}$ shows the dehydration of phosphate groups due to the interaction with polymer ligands. It is important to note that the peaks of the base materials used were based on references. For a better analysis of the results, it important to perform the

analysis of these materials. In addition, for a better comparison, it would be important to analyze also the uncoated liposomes.

The stability of the dried liposomes was verified by visual inspection. After 2 months under storage, the lyophilized liposomes released part of the encapsulated oil, concluding that this methodology is not the most adequate for this system. The ones coated with chitosan remained stable.

In conclusion, the passion fruit seeds oil presented a low oxidative stability being encapsulation needed to preserve the properties before the application. The liposomes coated with chitosan can be proposed for the encapsulation of active principles, e.g. in view of cosmetic applications.

## 5.2 Future perspectives

Regarding suggestions for future works, the following points can be considered relevant as a continuity of the present study:

- Verify the anti-inflammatory and antimicrobial activity of the passion fruit oil;
- Deepen the study of chitosan's influence in the spray-dried liposomes to find an optimized concentration to use.
- Completion of the characterization of the dried liposomes, to better understand their properties and structure, namely by using SEM of high resolution;
- Monitor the preservation of the properties of the encapsulated active principle;
- To follow with incorporation studies, namely to use the produced liposomes in moisturizing creams and make a comparative study with the use of the free oil forms.

# *Chapter 6 – BIBLIOGRAPHIC REFERENCES*

Alexopoulou, E., Georgopoulos, A., Kagkadis, K. A. and Demetzos, C. (2006) Preparation and characterization of lyophilized liposomes with incorporated quercetin'. *Journal of Liposome Research*, vol. 16, pp. 17–25.

Alves, A. I. P., Pereira, J. A. C. and Silva, M. F. L. (2013) 'Contributo para a caraterização química e atividade antioxidante de diferentes partes de Passiflora edulis Sims edulis'. Thesis (Master's degree). *Instituto Politécnico de Bragança*.

Araújo, R. S. and Mosqueira, V. C. F. (2009) 'Desenvolvimento, caracterização e liofilização de nanopartículas e encapsulamento de antibiótico de uso veterinário'. Thesis (Master's degree). *Universidade Federal de Ouro Preto*.

Barbieri, J. C. and Leimann, F. V. (2014) 'Extração de Óleo da Semente do Maracujá e Microencapsulação em Poli (ε-Caprolactona)'. *Revistra Brasileira de Pesquisa em Alimentos*, vol. 5, pp. 1–9.

Baril, M. B., Franco, G. F., Viana, R. S. and Zanin, S. M. W. (2012) 'Nanotechnology Applied To Cosmetics'. Thesis (Master's degree). *Universidade Federal do Paraná*, pp. 45–54.

Batista, C. M., Carvalho, C. M. B. and Magalhães, N. S. S. (2007) 'Lipossomas e suas aplicações terapêuticas'. *Revista Brasileira de Ciências Farmacêuticas*, vol. 43, pp. 167–179.

Batzri, S. and Korn, E. D. (1973) 'Single bilayer liposomes prepared without sonication'. *Biochimica et Biophysica Acta*, vol. 298, pp. 1015–1019.

Bibi, S., Kaur, R., Henriksen-Lacey, M., McNeil, S. E., Wilkhu, J., Lattmann, E., Christensen, D. and Mohammed, A. R. (2011) Microscopy imaging of liposomes: From coverslips to environmental SEM'. *International Journal of Pharmaceutics*, 417, pp. 138–150.

Blume, A. (1996) 'Properties of lipid vesicles: FT-IR spectroscopy and fluorescence probe studies'. *Current Opinion in Colloid & Interface Science*. Current Science Ltd., vol. 1, pp. 64–77.

Bnyan, R., Cesarini, L., Khan, I., Roberts, M. and Ehtezazi, T. (2020) 'The effect of ethanol evaporation on the properties of inkjet produced liposomes'. *DARU, Journal of Pharmaceutical Sciences*, vol. 28, pp. 271–280.

Cabral, E. C. M., Zollner, R. L. and Santana, M. H. A. (2004) 'Preparation and characterization of liposomes entrapping allergenic proteins'. *Brazilian Journal of Chemical Engineering*, vol. 21, pp. 137–146.

Calder, P. C. (2015) 'Functional Roles of Fatty Acids and Their Effects on Human Health'. *Journal of Parenteral and Enteral Nutrition*, vol. 39, pp. 18S-32S.

Camargo, M. F. P. and Filho, P. A. R. (2008) 'Desenvolvimento de nanoemulsões à base de óleo de maracujá e óleo essencial de lavanda e avaliação da atividade antiinflamatória tópica'. Thesis (Master's degree). *Universidade de São Paulo*, pp. 1–119.

Charcosset, C., Juban, A., Valour, J., Urbaniak, S. and Fessi, H. (2014) 'Chemical Engineering Research and Design Preparation of liposomes at large scale using the ethanol injection method: Effect of scale-up and injection devices'. *Chemical Engineering Research and Design. Institution of Chemical Engineers*, vol. 94, pp. 508–515.

Chen, C., Han, D., Cai, C. and Tang, X. (2010) 'An overview of liposome lyophilization and its future potential'. *Journal of Controlled Release,* vol. 142, pp. 299–311.

Cheng, C. Wu, Z., McClements, D. J., Zou, L., Peng, S., Zhou, W. and Liu, W. (2019) 'Improvement on stability, loading capacity and sustained release of rhamnolipids modified curcumin liposomes'. *Colloids and Surfaces B: Biointerfaces*, vol. 183, pp. 11–46.

Chrysostomo, T. N. (2017) 'Estudo in vitro do efeito da ativação do Sistema Complemento na estabilidade de lipossomas de diferentes composições'. Thesis (Master's degree). *Universidade de São Paulo*, pp. 1–38.

Coelho, J. P., Filipe, R. M., Robalo, M. P. and Stateva, R. P. (2018). 'Recovering value from organic waste materials: Supercritical fluid extraction of oil from industrial grape seeds'. *Journal of Supercritical Fluids*, vol. 141, pp. 68–77.

Crowe, J. H., Whittan, M. A, Chapman, D. and Crowe, L. M. (1984) 'Interations of phospholipid monolayers with carbohydrates', *Biochimica et Biophysica Acta*, vol. 769, pp. 151–159.

Cvetanović, A. (2019) 'Extractions Without Organic Solvents: Advantages and Disadvantages'. *Chemistry Africa.* vol. 2, pp. 342–349.

Danaei, M., Dehghankhold, M., Ataei, S., Hasanzadeh Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S. and Mozafari, M. R. (2018) 'Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems'. *Pharmaceutics*, vol. 10, pp. 1–17.

Dua, J. S., Rana, A. C. and Bhandari, A. K. (2012) 'Liposome: Methods Of Preparation And Applications'. *International Journal of Pharmaceutics Studies and Research*, pp. 1–7.

Dubey, V., Mishra, D. and Jain, N. K. (2007) 'Melatonin loaded ethanolic liposomes: Physicochemical characterization and enhanced transdermal delivery'. *Science Direct*, vol. 67, pp. 398–405.

Enoch, H. G. and Strittmatter, P. (1979) 'Formation and properties of 1000-A-diameter, single bilayer phospolipid vesicles'. *Biochemistry,* vol. 76, pp. 145–149.

Fan, M., Xu, S. and Xia, S. (2008) 'Preparation of salidroside nano-liposomes by ethanol injection method and in vitro release study'. *Eur Food Res Technol*, pp. 167–174.

Farkuh, L. and Cuccovia, I. M (2016) 'Estudo e desenvolvimento de lipossomas com potencial para aplicação em base cosmética'. Thesis (Master's degree). *Universidade de São Paulo*, pp. 1–89.

Ferreira, H. S. C. M. (2006) 'Utilização de lipossomas como modelos de biomembranas na avaliação e quantificação da actividade de anti-inflamatórios'. Thesis (Master's degree). *Faculdade de Farmácia da Universidade de Porto*, pp. 1–351.

Ferreira, L. S., Jange, C. G. and Pinho, S. C. (2016) 'Caracterização De Formulações Aquosas E Curcumina'. Thesis (Master's degree). *Universidade de São Paulo*.

Filipovic-Grcïicâ, J., Sïkalko-Basnet, N. And Jalsïenjak, I. (2001) 'Mucoadhesive chitosan-coated liposomes: characteristics and stability'. *Journal of Microencapsulation*, vol. 18, pp. 3–12.

Fontes, F. S. P. S., Corvo, M. L and Marinho, H. S. P. C. (2012) 'Efeito da Administração de Enzissomas de Superóxido Dismutase na Lesão de Reperfusão do Fígado'. Thesis (Master's degree). *Universidade de Lisboa*, pp. 1–154.

Frezard, F., Schettini, D. A., Rocha, O. G. F. and Demicheli, C. (2005) 'Liposomes: Physicochemical and pharmacological properties, applications in antimony-based chemotherapy'. *Quimica Nova*, vol. 28, pp. 511–518.

Ghorbanzade, T. Jafari, S. M., Akhavan, S. and Hadavi, R. (2017) 'Nano-encapsulation of fish oil in nano-liposomes and its application in fortification of yogurt'. *Food Chemistry*, vol. 216, pp. 146–152.

Gibis, M., Vogt, E. and Weiss, J. (2012) 'Encapsulation of polyphenolic grape seed extract in polymer-coated'. *Food Function*, vol. 3, pp. 246–254.

Giuffrè, A. M. (2007) 'Chemical composition of passion fruit (Passiflora Edulis, Sims.)'. *La rivista italiana delle sostanze grasse*, vol. 84, pp. 188–192.

Gómez-Mascaraque, L. G., Sipoli, C. C., De La Torre, L. G. and López-Rubio, A. (2017) 'Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin'. *Food Chemistry*, vol. 233, pp. 343–350.

Gómez-Mascaraque, L. G., Lagaron, J. M. and López-Rubio, A. (2015) 'Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods'. *Food Hydrocolloids*, vol. 49, pp. 42–52.

Gonçalves, J. C. and Reis, C. P. (2014) 'Nanotecnologia Aplicada à Pele'. *Universidade Lusófona de Humanidades e Tecnologias*, pp. 1–73.

Gültekin-Özgüven, M., Karadaɨ, A., Duman, Ş., Özkal, B., Özçelik, B. (2016) 'Fortification of dark chocolate with spray dried black mulberry (Morus nigra) waste extract encapsulated in chitosan-coated liposomes and bioaccessability studies'. *Food Chemistry*, vol. 201, pp. 205–212.

Hansen, A. H., Mouritsen, O. G. and Arouri, A. (2015) 'Enzymatic action of phospholipase A 2 on liposomal drug delivery systems'. *International Journal of Pharmaceutics*, vol. 491, pp. 49–57.

Jillavenkatesa, A., Dapkunas, S. J. and Lum L. H. (2001) 'Particle Size Characterization'. *Materials Science and Engineering Laboratory.* Special Publication 960-1.

Junior, R. G. de O. and Almeida, J. R. G. S. (2012) 'Prospecção tecnológica de fotoprotetores derivativos de produtos naturais'. *Geintec: Gestão, Inovação e Tecnologias*, vol. 3, pp. 32–40.

Justo, O. R. and Moraes, A. M. (2003) 'Produção de lipossomas pelo método de injeção de etanol encapsulando agentes tubérculos e avaliação do potencial de escalonamento do processo'. Thesis (Master's degree). *Universidade de Campinas*, pp. 1–225.

Kostik, V., Memeti, S. and Bauer, B. (2013) 'Fatty acid composition of edible oils and fats'. *Journal of Hygienic Engineering and Design*, vol. 4, pp. 112–116.

Kozubek, A., Gubernator, J., Przeworska, E. and Stasiuk, M. (2000) 'Liposomal drug delivery, a novel approach: PLARosomes'. *Acta Biochimica Polonica*, vol. 47, pp. 639–649.

Lapenda, T. L. S., Magalhães, N. S. S. and Maciel, M. A. M. (2010) 'Desenvolvimento e caracterização de lipossomas contento desidrocrotonina e avaliação da atividade antitumoral'. Thesis (Master's degree). *Universidade Federal de Pernambuco*, pp. 1–115.

Lawton, C. L., Delargy, H. J., Brockman, J., Smith, F. C. and Blundell, J. E. (2000) 'The degree of saturation of fatty acids influences post-ingestive satiety'. *British Journal of Nutrition*, vol. 83, pp. 473–482.

Lichtenberg, D. (1988) 'Liposomes: Preparation, Characterization, and Preservation'. *Methods of Biochemical Analysis*, vol. 33, pp. 1–126.

Lo, Y. L., Tsai, J. C. and Kuo, J. H. (2004) 'Liposomes and disaccharides as carriers in spray-dried powder formulations of superoxide dismutase'. *Journal of Controlled Release*, vol. 94, pp. 259–272.

Lopes, N. A. and Brandelli, A. (2018) 'Desenvolvimento de lipossomas nanométricos para armazenamento e liberação controlada de peptídeos antimicrobianos'. Thesis (Master's degree). *Universidade Federal do Rio Grande do Sul*, pp. 1–84.

Lucarini, M., Durazzo, A., Raffo, A., Giovannini, A. and Kiefer, J. (2019) 'Fruit Oils: Chemistry and Functionality'. *Fruit Oils: Chemistry and Functionality*, pp. 577–603.

Luiz, A., Martins, L., Taveira, S. F. and Furquim, A. (2006) 'Desenvolvimento de micropartículas contendo nanopartículas lipídicas por spray drying'. Thesis (Master's degree). *Faculdade de Farmácia da Universidade Federal de Goiás*.

Machado, L. C., Gnoatto, S. A. and Klüppel, M. L. W. (2018) 'Lipossomes applied in pharmacology: a review' *Estudos de Biologia*, vol. 29, pp. 215–224.

Mady, M. M. and Darwish, M. M. (2010) 'Effect of chitosan coating on the characteristics of DPPC liposomes'. *Journal of Advanced Research*. University of Cairo, vol. 1, pp. 187–191.

Malacrida, C. R. and Jorge, N. (2012) 'Yellow Passion Fruit Seed Oil (*Passiflora edulis f. flavicarp* ): Physical and Chemical Characteristics'. *Brazilian Archives of Biology and Technology*, vol. 55, pp. 127–134.

Marchetti, J. M. and Keneni, Y. G. (2017) 'Oil extraction from plant seeds for biodiesel production'. *AIMS Energy*, vol. 5, pp.316-340.

Martínez, R., Torres, P., Meneses, M. A., Figueroa, J. G., Pérez-Álvarez, J. A. and Viuda-Martos, M. (2012) 'Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate'. *Food Chemistry*, vol. 135, pp. 1520–1526.

Martins, M. H. and Pessine, F. B. T. (2007) 'Encapsulação e caracterização de lipossomas contendo isotretina para aplicação dermatológica'. Thesis (Master's degree). *Universidade de Campinas*, pp. 1–189.

Maximiano, F. A. and Cuccovia, I. M. (2003) 'Micelas mistas de surfactantes zwiteriônicos e catiônicos'. Thesis (Master's degree). *Universidade de São Paulo*, pp. 97–185.

Mertins, O., Pohlmann, A. R., Silveria, N. P. and Marques, C. M. (2008) 'Estudo físico-químicos e estruturais de lipossomas compósitos de fosfatidilcolina e quitosana'. Thesis (Master's degree). *Universidade Federal do Rio Grande do Sul*, pp. 1–206.

Meyagusku, V. M. and Oliveira, A. G. (2014) 'Desenvolvimento E Caracterização De Ciprofloxacino Ciprofloxacino'. Thesis (Master's degree). *Universidade Estadual Paulista*, pp. 1–99.

Miguel, L. M. (2011) 'Tendências do uso de produtos naturais nas indústrias de cosméticos da França'. *Revista Geográfica da América Central*, pp. 1–15.

Moghimipour, E. and Handali, S. (2013) 'Liposomes as Drug Delivery Systems: Properties and Applications'. *Journal of Pharmaceutical, Biological and Chemical Sciences*, vol. 4, pp. 1–169.

Mohammed, A. R., Bramwell, V. W., Coombes, A. G.A. and Perrie, Y. (2006) 'Lyophilisation and sterilisation of liposomal vaccines to produce stable and sterile products', *Methods*, vol. 40, pp. 30–38.

Mohammed, A. R., Coombes, A. G. A. and Perrie, Y. (2007) 'Amino acids as cryoprotectants for liposomal delivery systems', *European Journal of Pharmaceutical Sciences*, vol. 30, pp. 406–413.

Naumowicz, M. and Figaszewski, Z. A. (2014) 'The effect of pH on the electrical capacitance of phosphatidylcholine- phosphatidylserine system in bilayer lipid membrane'. *Journal of Membrane Biology*, vol. 247, pp. 361–369.

Oliveira, D. A., Mezzomo, N., Gomes, C. and Ferreira, R. S. S. (2012) 'Encapsulation of passion fruit seed oil by means of supercritical antisolvent process'. *Journal of Supercritical Fluids*, vol. 51, pp. 322–323.

Pardauil, J. J. R., Souza, L. K.C., Molfetta, F. A., Zamian, J. R., Rocha Filho, G. N. and Da Costa, C. E. F. (2011) 'Determination of the oxidative stability by DSC of vegetable oils from the Amazonian area'. *Bioresource Technology*, vol. 102, pp. 5873–5877.

Patel, V. B., Misra, A. N. and Marfatia, Y. S. (2001) 'Preparation and Comparative Clinical Evaluation of Liposomal Gel of Benzoyl Peroxide for Acne'. *Drug Development and Industrial Pharmacy*, vol. 27, pp. 863–870.

Peltonen, L., Valo, H., Kolakovic, R., Laaksonen, T. and Hirvonen, J. (2010) 'Electrospraying, spray drying and related techniques for production and formulation of drug nanoparticles'. *Expert Opinion on Drug Delivery*, vol. 7, pp. 705–719.

Perinelli, D. R., Cespi, M., Rendina, F., Bonacucina, G. and Palmieri, G. F (2019). 'Effect of the concentration process on unloaded and doxorubicin loaded liposomal dispersions'. *International Journal of Pharmaceutics*, vol. 560, pp. 385–393.

Pereira, M. G., Maciel, G. M., Haminiuk, C. W. I., Bach, F., Hamerski, F. P., Scheer, A. and Corazza, M. L. (2019) 'Effect of Extraction Process on Composition, Antioxidant and Antibacterial Activity of Oil from Yellow Passion Fruit (Passiflora edulis Var. Flavicarpa) Seeds'. *Waste and Biomass Valorization*, vol. 10, pp. 2611–2625.

Pinilla, B. and Brandelli, A. (2016) 'Antimicrobial activity of nanoliposomes co-encapsulating nisin and garlic extract against Gram-positive and Gram-negative bacteria in milk'. *Innovative Food Science and Emerging Technologies*, vol. 36, pp. 287–293.

Polexe, R. C. and Delair, T. (2013) 'Elaboration of stable and antibody functionalized positively charged colloids by polyelectrolyte complexation between chitosan and hyaluronic acid'. *Molecules*, vol. 18, pp. 8563–8578.

Pons, M., Merc, F. and Estelrich, J. (1993) 'Liposomes obtained by the ethanol injection method'. *International Journal of Pharmaceutics*, vol. 95, pp. 51–56.

Prado, J. M., Prado, G. H.C. and Meireles, M. A. A. (2011). 'Scale-up study of supercritical fluid extraction process for clove and sugarcane residue'. *Journal of Supercritical Fluids*, vol. 56, pp. 231–237.

Queiroz, M. F., Melo, K. R. T., Sabry, D. A., Sassaki, G. L. and Rocha, H. A. O. (2015) 'Does the use of chitosan contribute to oxalate kidney stone formation?'. *Mar. Drugs*, vol. 13, pp. 141–158.

Rasti, B., Jinap, S., Mozafari, M. R. and Yazid, A. M. (2012) 'Comparative study of the oxidative and physical stability of liposomal and nanoliposomal polyunsaturated fatty acids prepared with conventional and Mozafari methods'. *Food Chemistry*, vol. 135, pp. 2761–2770.

Ribeiro, R., F., Motta, M. H., Härter, A. P. G., Flores, F. C., Beck, R. C. R., Schaffazick, S. R. and De Bona Da Silva, C. (2016). 'Spray-dried powders improve the controlled release of antifungal tioconazole-loaded polymeric nanocapsules compared to with lyophilized products'. *Materials Science and Engineering C*, vol. 59, pp. 585-594.

Roggia, I., Dalcin, A. J. F., Ourique, A. F., Da Cruz, I. B.M., Ribeiro, E.E., Mitjans, M., Vinardell, M. P. and Gomes, P. (2019) 'Protective effect of guarana-loaded liposomes on hemolytic activity'. *Colloids and Surfaces B: Biointerfaces*, (April), p. 110636.

Sá, F. A. P. (2015) 'Desenvolvimento e Caracterização de lipossomas mucoadesios para liberação ocular de fármacos'. Thesis (Master's degree). *Universidade de Brasilia*, pp. 1–62.

Santos, N. C. and Castanho, M. A. R. B. (2002) 'Lipossomas: a bala mágica acertou?'. *Quimica Nova*, vol. 25, pp. 1–10.

Saoji, S. D., Raut, N. A., Dhore, P. W., Borkar, C. D., Popielarczyk, M. D. and Vivek S. (2016). 'Preparation and Evaluation of Phospholipid-Based Complex of Standardized Centella Extract (SCE) for the Enhanced Delivery of Phytoconstituents'. *AAPS Journal*, vol. 18, pp. 102–114.

Saveyn, P., Cocquyt, J., Bomans, P., Frederik, P., Cuyper, M. and Meeren, P. V. (2007) 'Osmotically Induced Morphological Changes of Extruded Dioctadecyldimethylammonium Chloride (DODAC) Dispersions'. *Langmuir*, vol. 23, pp. 4775–4781.

Sebaaly, C., Greige-Gerges, H., Stainmesse, S., Fessi, H. and Charcosset, C. (2016) 'Clove essential oil-in-cyclodextrin-in-liposomes in the aqueous and lyophilized states: From laboratory to large scale using a membrane contactor'. *Carbohydrate Polymers*, vol. 138, pp. 75–85.

Sebaaly, C., Greige-Gerges, H., Stainmesse, S., Fessi, H. and Charcosset, C. (2016) 'Effect of composition, hydrogenation of phospholipids and lyophilization on the characteristics of eugenol-loaded liposomes prepared by ethanol injection method'. *Food Bioscience*, vol. 15, pp. 1–10.

Sharma, A. and Sharma, U. S. (1997) 'Liposomes in drug delivery: progress and limitations'. *International Journal of Pharmaceutics,* vol. 154, pp. 123–140.

Shashidhar, G. M. and Manohar, B. (2018) 'Nanocharacterization of liposomes for the encapsulation of water-soluble compounds from Cordyceps sinensis CS1197 by a supercritical gas'. *Royal Society of Chemistry*, vol. 8, pp. 34634–34649.

Shin, G. H., Chung, S. K., Kim, J. T, Joung, H. J. and Park, H. J. (2013) 'Preparation of chitosan-coated nanoliposomes for improving the mucoadhesive property of curcumin using ethanol injection method'. *Journal of Agricultural and Food Chemistry,* vol. 61, pp. 11119–111126.

Shukat, R. and Relkin, P. (2011) 'Lipid nanoparticles as vitamin matrix carriers in liquid food systems: On the role of high-pressure homogenization, droplet size and adsorbed materials'. *Colloids and Surfaces B: Biointerfaces,* vol. 86, pp. 119–124.

Singh, P., Bodycomb, J., Travers, B., Tatarkiewicz, K., Travers, S., Matyas and G. R., Beck, Z. (2019) 'Particle size analyses of polydisperse liposome formulations with a novel multispectral advanced nanoparticle tracking technology'. *International Journal of Pharmaceutics,* vol. 566, pp. 680–686.

Sipoli, C. C., Santana, N., Shimojo, A. A. M., Azzoni, A. and Torre, L. G. (2015) 'Scalable production of highly concentrated chitosan/TPP nanoparticles in different pHs and evaluation of the in vitro transfection efficiency'. *Biochemical Engineering Journal*., vol. 94, pp. 65–73.

Skalko, N. and Jalsenjak, I. (1992) 'Liposomes with clindamycin hydrochloride of Acne vulgaris in the therapy'. *International Journal of Pharmaceutics,* vol. 85, pp. 97–101.

Sosnik, A. and Seremeta, K. P. (2015) 'Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers'. *Advances in Colloid and Interface Science,* vol. 223, pp. 40–54.

Souto, E. B., Severino, P., Santana, M. H. A. and Pinho, S. C. (2011) 'Nanopartículas de lipídios sólidos: métodos clássicos de produção laboratorial'. *Quimica Nova,* vol. 34, pp. 1443–1449.

Surlehan, H. F., Noor Azman, N. A., Zakaria, R. and Mohd Amin, N. A. (2019) 'Extraction of oil from passion fruit seeds using surfactant-assisted aqueous extraction'. *Food Research*, vol. 3, pp. 348–356.

Takahashi, M., Kitamoto, D., Asikin, Y., Takara, K. and Wada, K. (2009) 'Liposomes Encapsulating Aloe vera Leaf Gel Extract Significantly Enhance Proliferation and Collagen Synthesis in Human Skin Cell Lines'. *Journal of Oleo Science*, vol. 650, pp. 643–650.

Taylor, T. M., Gaysinsky, S., Davidson, P. M., Bruce, B. D. and Weiss, J. (2007) 'Characterization of Antimicrobial-bearing Liposomes by zeta-Potential, Vesicle Size, and Encapsulation Efficiency'. *Food Biophysics*, vol. 2, pp. 1–9.

Terroni, H. C., Jesus, J. M., Artuzo, L. T., Ventura, L. V., Santos, R. F. and Damy-Benedetti, P. de C. (2013) 'Liofilização', *Revista Científica Unilago*, pp. 271–284.

Toniazzo, T., Pinho, S. C. and Dacanal, G. C. (2017) 'Produção de pós alimentícios enriquecidos com quercetina encapsulada com lipossomas liofilizados'. *Universidade de Campinas.*

Toniazzo, T., Galeskas, H. and Pinho, S. C. de (2015) 'Produção E Caracterização De Lipossomas Liofilizados Encapsulando Quercetina'. Thesis (Master's degree). *Universidade Federal de São Carlos.* pp. 1666–1675.

Torchilin, V. P. (1998) 'Polymer-coated long-circulating microparticulate pharmaceuticals'. *Journal of Microencapsulation*, vol. 15, pp. 1–19.

Umerska, A., Paluch, K. J., Inkielewicz-Stepniak, I., Santos-Martinez, M. J., Corrigan, O. I. and Medina, C., Tajber, L. (2012) 'Exploring the assembly process and properties of novel crosslinker-free hyaluronate-based polyelectrolyte complex nanocarriers'. *International Journal of Pharmaceutics*, vol. 436, pp. 75–87.

Vehring, R., Foss, W. R. and Lechuga-Ballesteros, D. (2007) 'Particle formation in spray drying'. *Journal of Aerosol Science*, vol. 38, pp. 728–746.

Viriyaroj, A., Ngawhirunpat, T., Sukma, M., Akkaramongkolporn, P., Ruktanonchai, U. and Opanasopit, P. (2009) 'Physicochemical properties and antioxidant activity of gamma-oryzanol-loaded liposome formulations for topical use'. *Pharmaceutical Development and Technology*, vol. 14, pp. 665–671.

Woodle, M. C. and Papahadjopoulos, D. (1989) 'Liposome Preparation and Size Characterization'. *Methods in enzymology*, vol. 171, pp. 193–217.

Yang, S. B., Liu, C. M., Liu, W., Yu, H., Zheng, H. J., Zhou, W. and Hu, Y. (2013) 'Preparation and characterization of nanoliposomes entrapping medium-chain fatty acids and vitamin C by lyophilization'. *International Journal of Molecular Sciences*, vol. 14, pp. 19763–19773.

Yang, Y., Wang, L., Wendroth, O., Liu, B., Cheng, C., Huang, T. and Shi, Y. (2019) 'Is the Laser Diffraction Method Reliable for Soil Particle Size Distribution Analysis?'. *Soil Science Society of America Journal*, vol. 83, pp. 276–287.

Yokota, D., Moraes, M. and Pinho, S. C. (2012) 'Characterization Of Lyophilized Liposomes Produced With Non-Purified Soy Lecithin: A Case Study Of Casein Hydrolysate Microencapsulation'. *Brazilian Journal of Chemical Engineering*, vol. 29, pp. 325–335.

Yoshida, P. A., Yokota, D., Foglio, M. A., Rodrigues, R. A.F. and Pinho, S. C. (2010) 'Liposomes incorporating essential oil of Brazilian cherry (Eugenia uniflora L.): Characterization of aqueous dispersions and lyophilized formulations'. *Journal of Microencapsulation*, vol. 27, pp. 416–425.

Zômpero, R. H. de F., López-Rubio, A., Pinho, S. C., Lagaron, J. M. and Torre, L. G. (2015) 'Hybrid Encapsulation Structures based on beta-carotene-loaded nanoliposomes within electrospum fibers'. *Colloids and Surfaces B: Biointerfaces*, pp. 1–28.

# *Appendix*

## Appendix A – Bibliographic review

**Table A1** – Conditions used for the production of liposomes.

| O/W(*) Ratio | Lipid Concentration (g/L) | Active substance (AS) | Polymer concentration | Solvent Injection Flow Rate | Temperature (°C) | | Stirring system/ rate | Ethanol removal | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | AS phase | Aqueous phase | | | |
| 10% | 20 – 80 | 1 – 8% (Curcumin) | 20% (WPC) | Manual injection | 25 | 25 | Cowles type impeller (1336 rpm) | - | (Gómez-Mascaraque *et al.*, 2017) |
| 13% | 30 | 250 μM (FITC-dextran) | 20 (Chitosan) | Manual injection | - | - | Magnetic stirring | - | (Filipovic-Grcïic *et al.*, 2001) |
| 10% | 15.6 | 0.054 g/L (β-Carotene) | 20 (PVOH) | 30 mL/min | 51 | 60 | Ultra-Turrax and Cowles propeller type (1336 rpm) | Evaporator or Molecular sive systems | (Zômpero *et al.* 2013) |
| - | 30 | 7.5% (Flurbiprofen) | 0.1 – 0.4% (Chitosan) | Manual injection | 25 | 55 | Magnetic stirring | Under nitrogen | (Chen *et al.*, 2016) |
| - | 30 | 10% (Curcumin) | 0.1 – 0.5% (Chitosan) | Manual injection | - | - | Mild and ultrasonic stirring | Under reduced pressure | (Shin *et al.*, 2013) |

*(O/W) - Ratio between organic phase (O) and aqueous phase (W);

**Table A2** – Characterization methodologies conventionally used for liposomes analysis.

| Reference | Characterization methodologies |
|---|---|
| (Gómez-Mascaraque *et al.*, 2017) | **Size distribution:** Via dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Corp.) with lipid concentration of 20 μg/mL.<br><br>**Morphology:** Via transmission electron microscopy (TEM) using a LEO 906E microcope (Zeiss) with lipomes concentration of 1 mg/mL in 400-mesh copper grids carbon film and via Negative Staining with 1% uranyl acetate.<br><br>**Encapsulation Efficiency (%EE):** Via centrifugation at 4 ºC using a Heal Force Neofuge 23R centrifuge (Thanes Science), to precipitate the non-encapsulated AS. The concentration of AS was assessed by UV-vis spectroscopy at 425 nm using a Thermo spectrophotometer model Genesys 6.<br><br>$$\%EE = \frac{Mass_{experimental}\ of\ entraped}{Total\ mass_{theoretical}\ added} x100$$ |
| (Ghorbanzade *et al.*, 2017) | **Size distribution:** Via DLS, with Nano ZS90 (Malvern Instruments) at 25 ºC, using a He-Ne laser of 633 nm and a detector angle of 173º. An optical microscope (phase contrast) was used to confirm liposome sizes. Samples were analysed 24 h after preparation.<br><br>**Encapsulation Efficiency (%EE):** Via centrifugation (Hettich Lab Technology) at 4200 rpm for 15 minutes to separate non-encapsulated oil. The absorbance was measured by spectrophotometer (PG-Instrument) at 280 nm.<br><br>$$\%EE = \frac{Total\ oil\ within\ liposomes - non\ encapsulation\ oil}{Total\ oil}\ x\ 100$$<br><br>**Stability (LS):** Via centrifugation (15 minutes at 3500 rpm)<br><br>$$LS = \frac{V_f}{V_i}\ x\ 100$$<br><br>*Vf is the final volume and Vi is the initial volume of liposomal dispersion |

| | |
|---|---|
| (Taylor *et al.*, 2007) | **Size distribution:** Via (Zetasizer Nanoseries ZS, Malvern Instruments), varying the temperature of samples from 25ºC to 75 ºC, in increments of 5 ºC.<br><br>**Zeta Potential:** Via particle electrophoresis instrument (Zetasizer Nanoseries ZS, Malvern Instruments), varying the temperature of samples from 25ºC to 75 ºC, in increments of 5 ºC.<br><br>**Encapsulation Efficiency:** Via LS-50B fluorescence spectrophotometer (PerkinElmer, Wellesley), when the vesicles were diluted to 100 μM. Fluorescence of incubated samples (F0) was scanned from 500 to 550 nm with excitation of 495 nm. Triton X-100 solution was added to induce rupturinf of liposomes and record the fluorescence of the released tracer (FT).<br>$$\%EE = 100\,x\,\left[1 - \left(\frac{F_0}{F_T}\right)\right]$$ |
| (Sipoli *et al.*, 2015) | **Size distribution:** Via DLS**,** with Nano ZS90 (Malvern Instruments), using a He-Ne laser of 633 nm and a detector angle of 173º.<br><br>**Zeta Potential:** Using the laser Doppler anemometry technique, with Zetasizer Nano ZS (Malvern Instruments) at 25 ºC, measuring the velocity of the electrophoretic mobility of the particles.<br><br>**Morphology:** By TEM and the negative staining method. The sample was diluted and added to the Carbon-coated 200 mesh copper grids with collodion film for 5 minutes at 25 ºC. The staining was performed with uranyl acetate solution. A Carl Zeiss CEM 902 Microscope (Zeiss, Jena) equipped with a Castaing-Henry-Ottensmeyer energy filter was used.<br><br>**Stability:** Via stiring at 200 rpm using a cowles impeller, with vesicles concentration of 833 μg/mL, evaluated the average diameter, PDI and zeta potential for a period of 4h. |
| (Charcosset *et al.*, 2014) | **Size distribution:** By DLS using a Zetasizer Zen 3600 (Malvern Instruments) for mean particle size and polydispersity index (PDI), analysed at 25 ºC.<br><br>**Zeta Potential:** Using a Malvern Zetasizer Zen 3600 (Malvern Instruments), calculated from electrophoretic mobility.<br><br>**Morphology:** By TEM, with images using a CM 120 microscope (Philips, Eindhoven).<br><br>**Stability:** Evaluated by measuring the particle size and PDI after liposomes suspensions were stored at 8 ºC during a period of 1 month. |

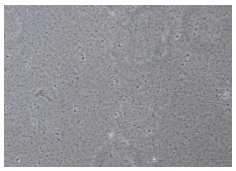
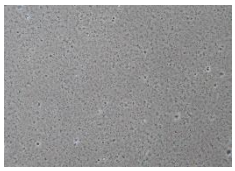
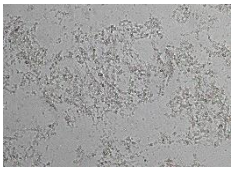
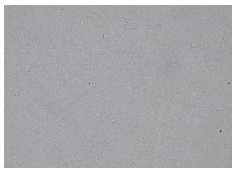
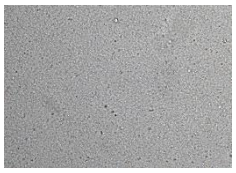
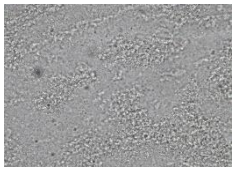
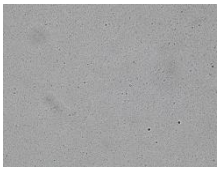
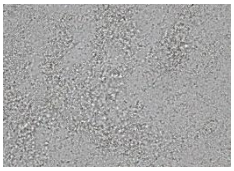
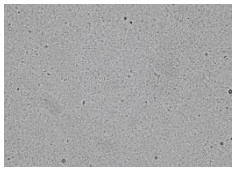
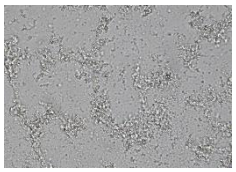
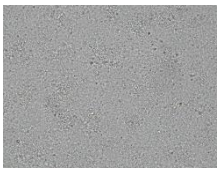
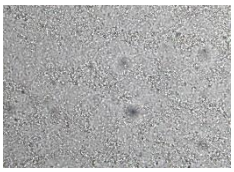
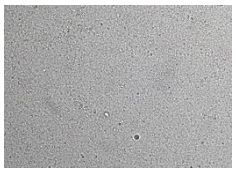
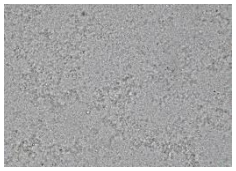
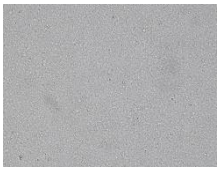
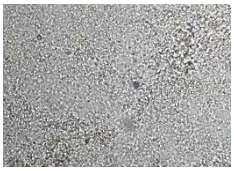
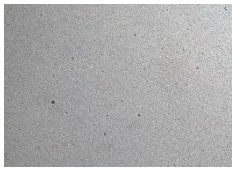
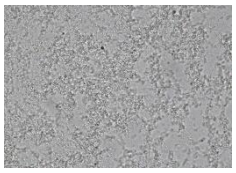
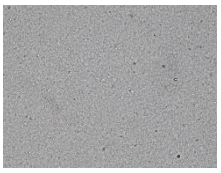
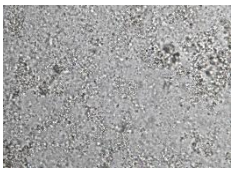
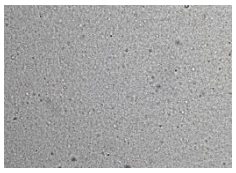
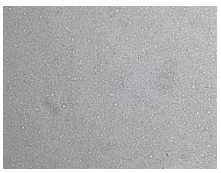
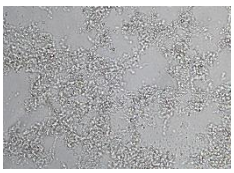
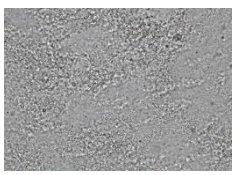
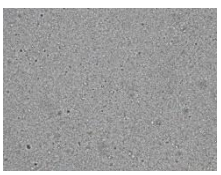
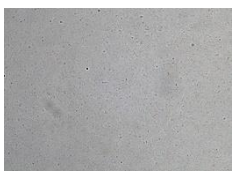
**Table A3** – Examples of liposomes application in cosmetic products and characterization.

| Reference | Cosmetic product | | Cosmetic product characterization |
|---|---|---|---|
| | **Applications** | **Formulation** | |
| (Skalko and Jalsenjak, 1992) | Acne | Soya lecithin (lipid)<br>Clindamycin hydrochloride (AS)<br>PEG (polymer) | Drug content (%): 1<br>Average diameter size (μm): 1.7<br>Range of sizes (μm): 0.77 – 6.78<br>$t_{50\%}$ in vitro (days)(*): 0.1<br>$t_{100\%}$ in vitro (days)(*): 2 |
| (Patel *et al.*, 2001) | Acne | Phosphatidylcholine (lipid)<br>Benzoyl peroxide (AS)<br>Carbopol (polymer) | Entrapment efficiency (%): 33 – 76<br>Vesicle diameter (μm): 2.52 (**) and 2.55 at 2 – 8 ºC<br>2.52 (**) and 2.59 at 25 ºC |
| (Takahashi *et al.*, 2009) | Antioxidant | Soybean lecithin (lipid)<br>Aloe vera leaf gel extract (AS) | Particle size (nm): 684 (mixed by homogenization) and 185.5 (mixed by homogenization and microfluidization)<br>Stability: 0.25% (AS) > 0.5% (AS) (14 days after preparation) |
| (Dubey *et al.*, 2007) | Antioxidant | Soya phosphatidylcholine (lipid)<br>Melatonin (AS) | Size (nm): 122<br>Polydispersity index: 0.032<br>Entrapment efficiency (%): 70<br>Release rate: 62.3<br>Transdermal flux (μg/cm²h): 59.2<br>Lag time (h): 0.9<br>Skin drug deposition (%): 9.46 |
| (Viriyaroj *et al.*, 2009) | Antioxidant | Phosphatidylcholine (lipid)<br>Gama-oryzanol (AS)<br>Triton-X100 (Surfactant) | Size (nm): 97.4<br>Zeta Potential (mV): -2.5<br>Entrapment efficiency (%): 72 – 100<br>Scavenging activity (μg/mL): 38.72<br>Antioxidant activity (μM): 0.01 - 10 |

(*) Time needed for 50% or 100% release of the drug from liposomes as measured by dialysis.
(**) Immediately after preparations

## Appendix B – Optical microscopy

**Table B1** – Influence of solvent evaporation in liposome agglomeration.

| SPOL | Before solvent evaporation | After solvent evaporation | SPOL | Before solvent evaporation | After solvent evaporation |
|---|---|---|---|---|---|
| **1** |  |  | **10** |  |  |
| **2** |  |  | **11** |  |  |
| **3** |  |  | **12** |  |  |
| **4** |  |  | **13** |  |  |
| **5** |  |  | **14** |  |  |
| **6** |  |  | **15** |  |  |
| **7** |  |  | **16** |  |  |
| **8** |  |  | **17** |  |  |

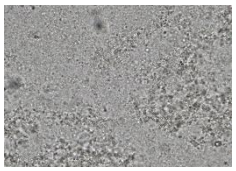
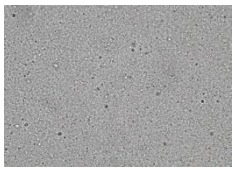
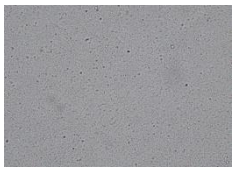
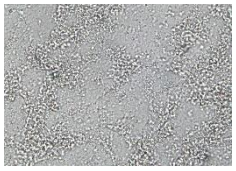
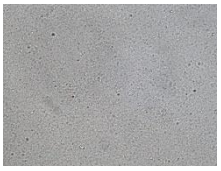
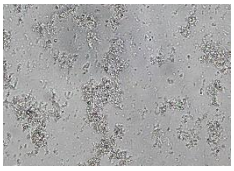
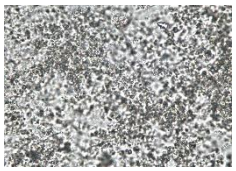
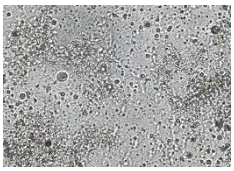
| | | | | |
|---|---|---|---|---|
| **9** |  |  | **18** |   |
| **19** |  |  | **20** |   |
| **21** |  |  | **22** |   |

**Table B2** – Influence of pH in chitosan coating.

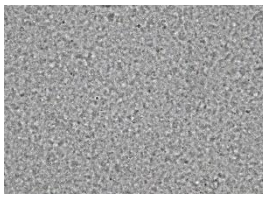
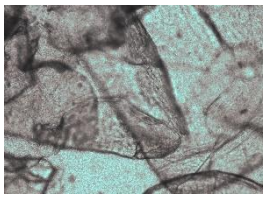
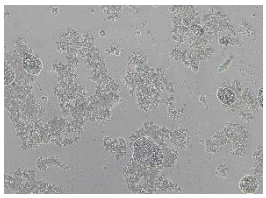
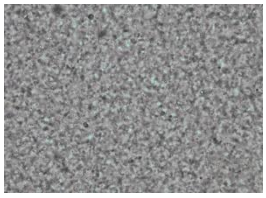
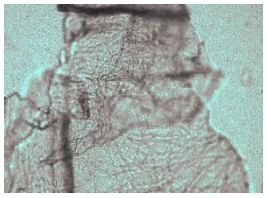
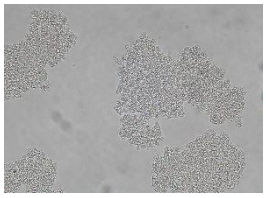
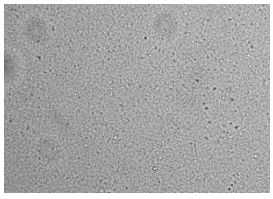
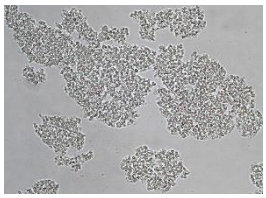
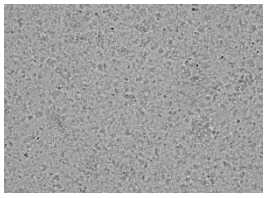
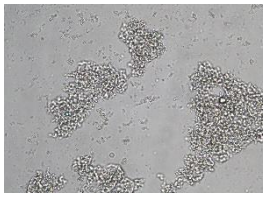
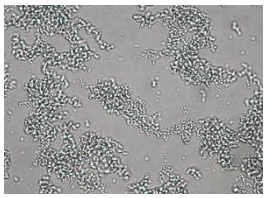
| **Specif** | **Magnification** | | **Specif** | **Magnification** |
|---|---|---|---|---|
| pH = 3.8 | 400x | 100x | pH = 3.0 | 400x |
| **Sample** | OM: Supernatant | OM: Precipitate | **Sample** | OM |
| **SPOL_5** |  |  | **SPOL_2** |  |
| **SPOL_7** |  |  | **SPOL_13** |  |
| **SPOL_8** |  |  | **SPOL_14** |  |
| **SPOL_12** |  |  | **SPOL_15** |  |
| **-** | **-** | **-** | **SPOL_18** |  |

**Table B3** – Dried liposomes.

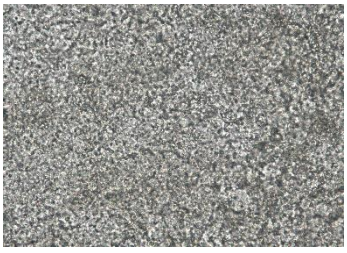
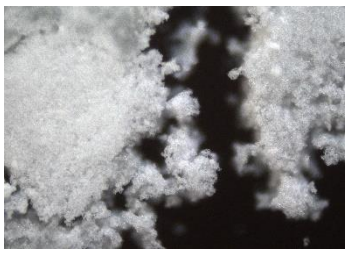
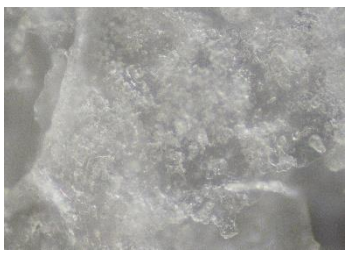
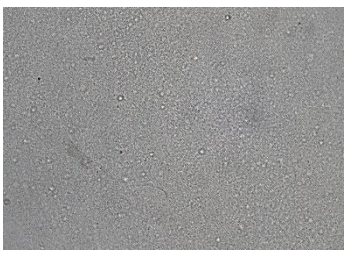
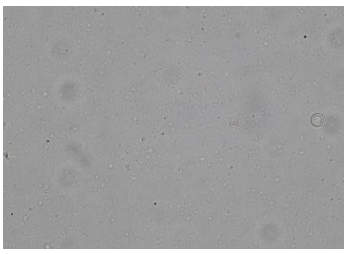
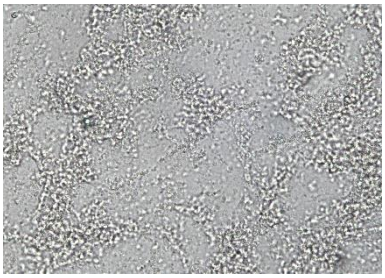
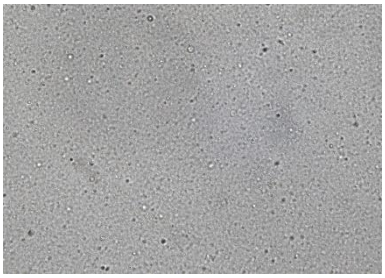
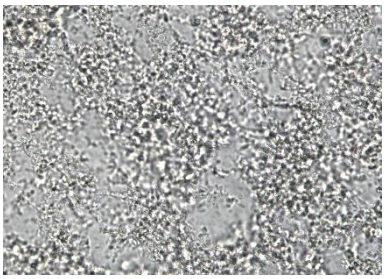
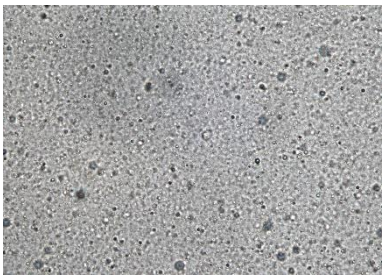
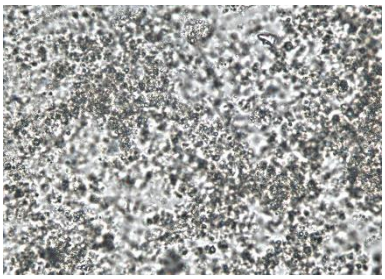
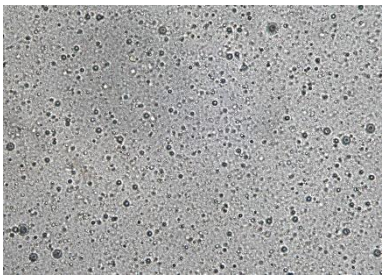
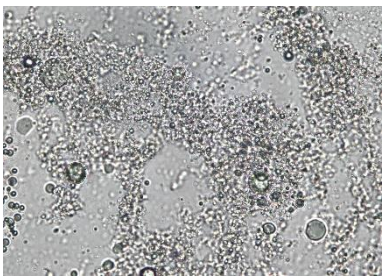
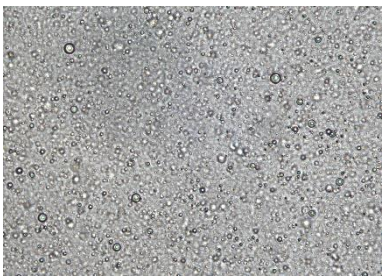
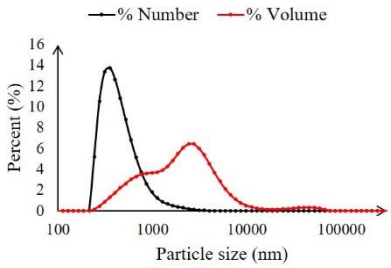
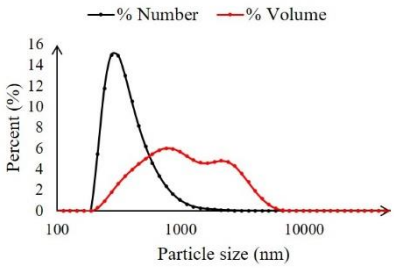
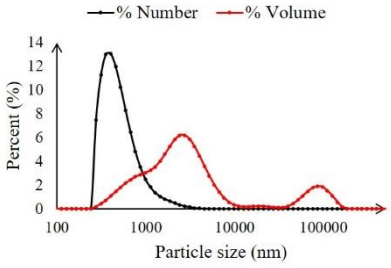
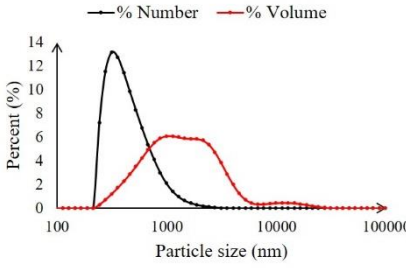
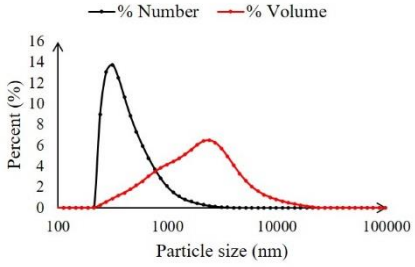
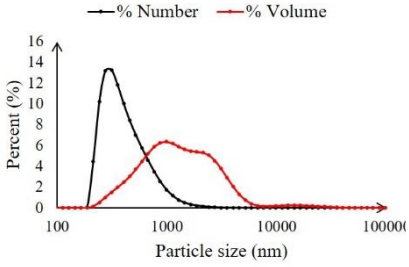
| | | Magnification | |
|---|---|---|---|
| | | 100x | 400x |
| **Method** | Sample | Dried | Dispersed in water |
| **Spray drying** | SPOL_2 |  |  |
| | |  |  |
| **Spray drying** | SPOL_13 |  |  |
| | |  |  |
| **Lyophilization** | SPOL_16 |  |  |
| | |  |  |

**Table B4** – Liposomes with different concentrations.

| | **Magnification** | |
|---|---|---|
| | 400x | |
| **Sample** | **With ethanol** | **Without ethanol** |
| **SPOL_20%** |  |  |
| **SPOL_40%** |  |  |
| **SPOL_60%** |  |  |
| **SPOL_80%** |  |  |

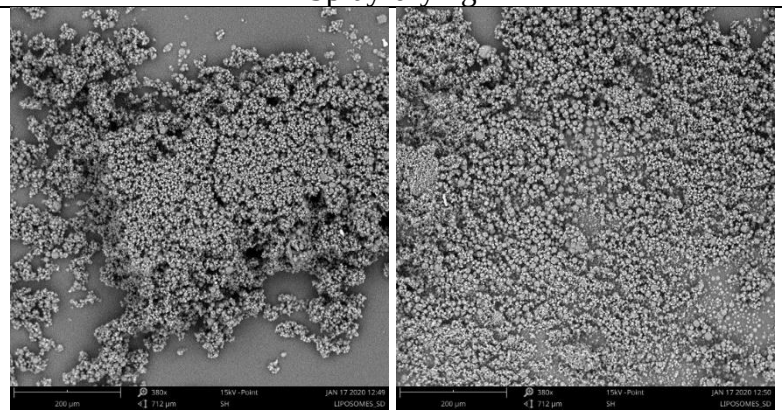
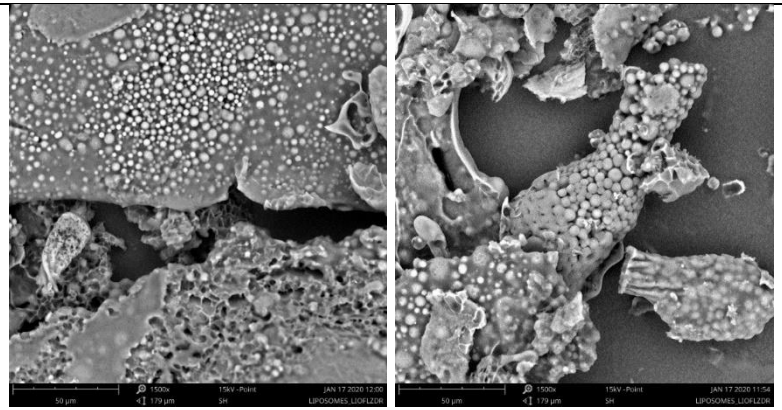
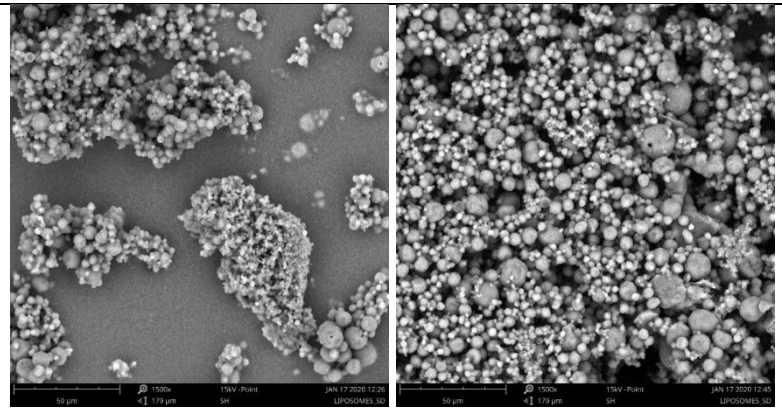
## Appendix C – Size distribution

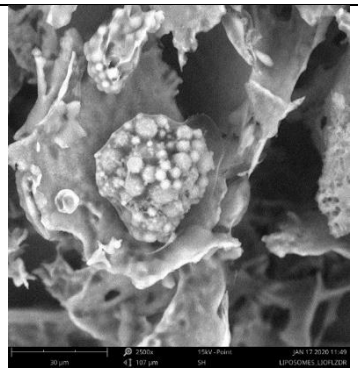
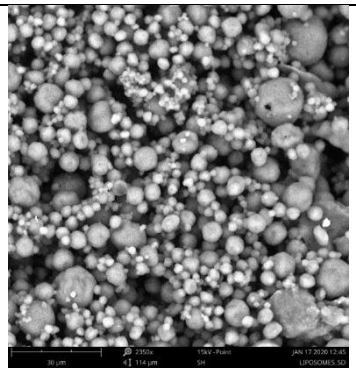
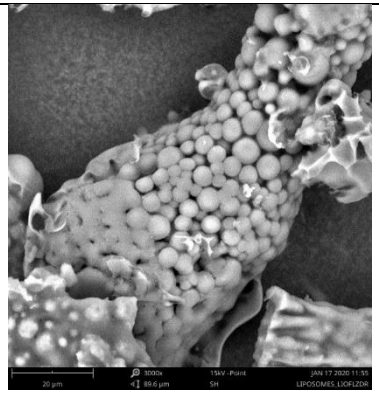
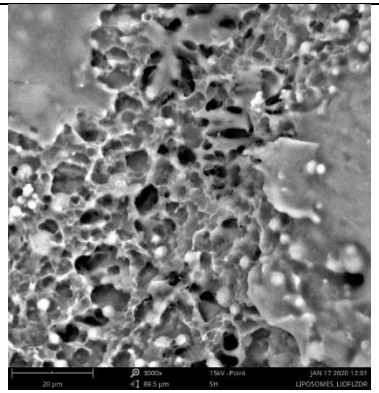
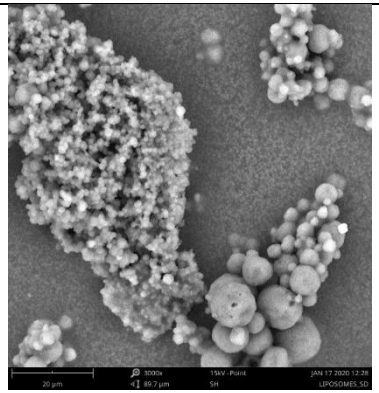
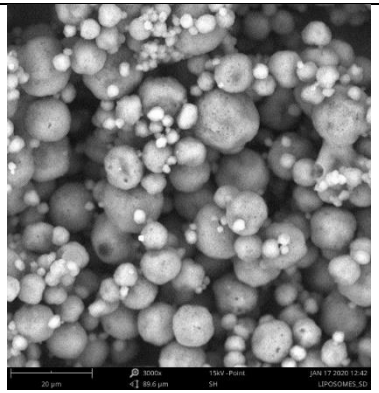
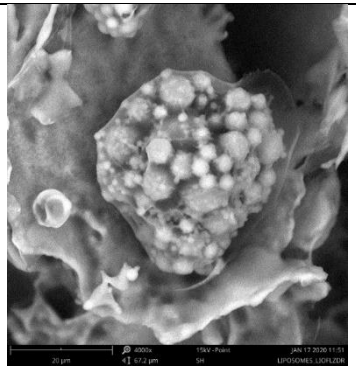
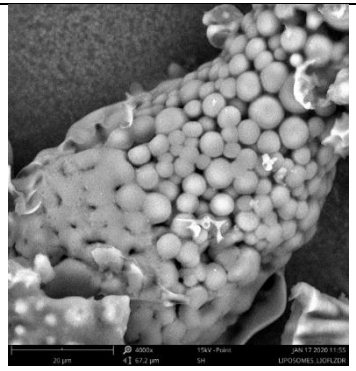
**Table C1** – Size distribution by laser diffraction.

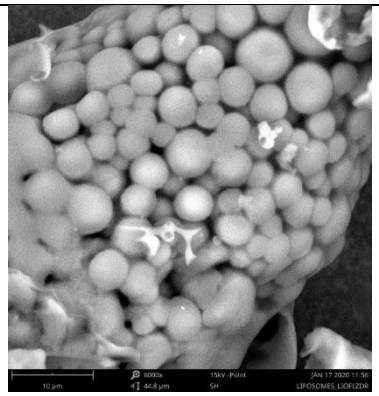
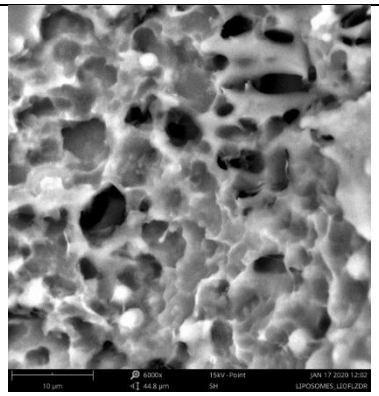
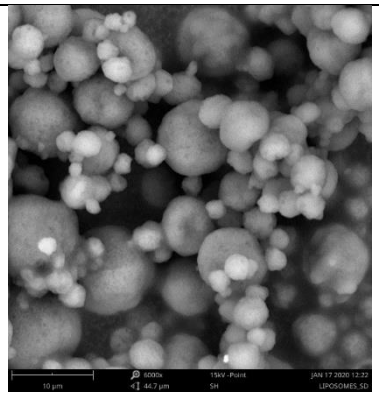
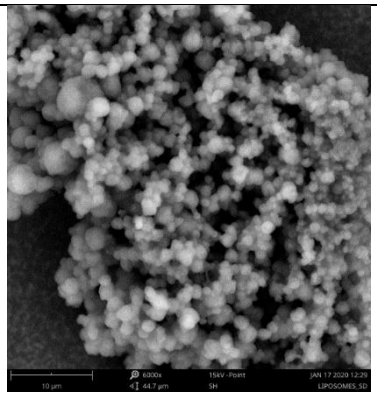
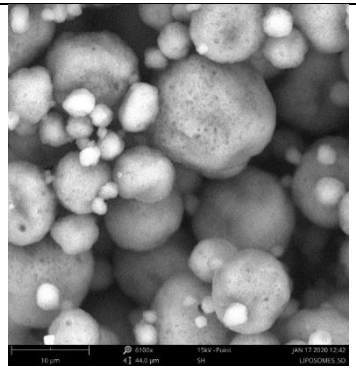
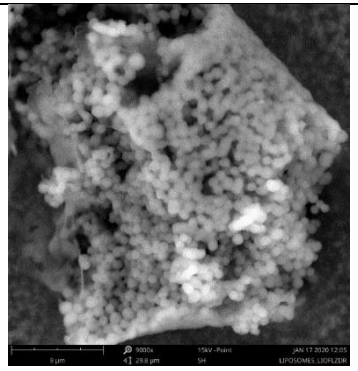
| Unloaded liposomes | | Loaded liposomes | |
|---|---|---|---|
| **Sample** | Size distribution | **Samples** | Size distribution |
| **SEL_1** |  | **SPOL_8** |  |
| **SEL_2** |  | **SPOL_17** |  |
| **SEL_3** |  | **SPOL_18** |  |

## Appendix D – Scanning electronic microscopy

**Table D1** – SEM images of the dried liposomes at different magnifications.

| | **Method** | |
|---|---|---|
| **Magnification** | Lyophilization | Spray-drying |
| **380x** | - |  |
| **1500x** |  |  |

| | | |
|---|---|---|
| **2500x** |  |  |
| **3000x** |   |   |
| **4000x** |   | - |

| | | | | |
|---|---|---|---|---|
| **6000x** |  |  |  |  |
| **6100x** | - | |  | |
| **9000x** |  | | - | |

**Appendix E – FTIR spectra**

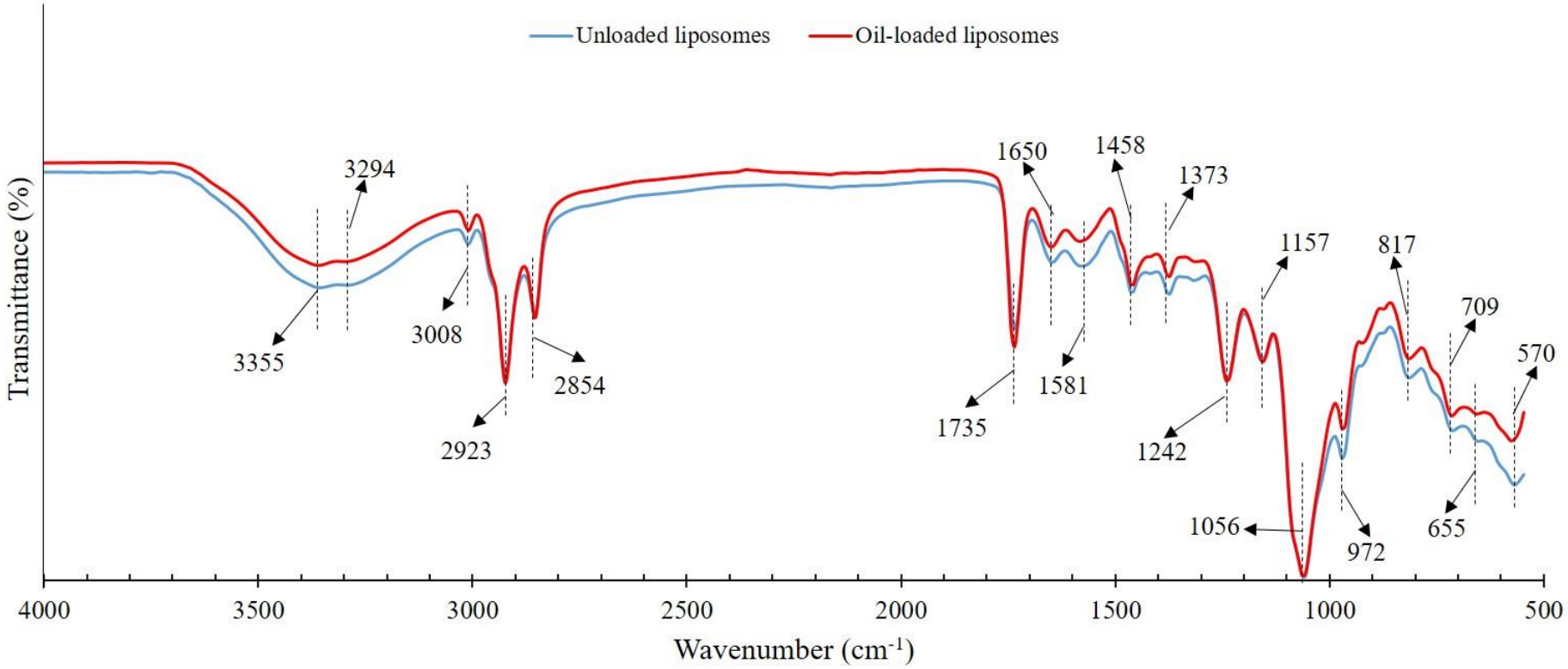



Figure E1 - Complete FTIR spectra of coated liposomes (4000 – 500 $cm^{-1}$).

## Appendix F – Presentations

F1 – 2nd Double Diploma Summer School & Symposium DD 2019

# ENCAPSULAÇÃO DE ÓLEO DE MARACUJÁ EM LIPOSSOMAS PARA APLICAÇÃO COSMÉTICA

**(✉) G.G. Silva, C.C. Sipoli (Supervisor UTFPR), I. P. Fernandes (Supervisor IPB), M.F. Barreiro (Supervisor IPB)**

**[1]Mestrado em Engenharia Química, Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Bragança, Portugal; [2]Graduação em Engenharia Química, Campus Apucarana, Universidade Tecnológica Federal do Paraná, Brasil**

**gabrielagomes652@gmail.com**

**[3]Centro de Investigação da Montanha (CIMO), Instituto Politécnico de Bragança, Portugal; [4]Laboratory of Separation and Reaction Engineering, Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Portugal**

**ipmf@ipb.pt, barreiro@ipb.pt**

**[5]Programa de Pós-Graduação em Engenharia Química (PPGEQ), Campus Apucarana, Universidade Federal Tecnologica do Paraná, Brasil**

**carolinesipoli@utfpr.edu.br**

## Resumo

A geração de resíduos constitui um dos maiores e mais danosos riscos ambientais. Na indústria de suco de maracujá, por exemplo, apenas a polpa é utilizada, representando cerca de 40% do peso da fruta. O resto são cascas e sementes, desperdício para o qual não existe alternativa para o seu aproveitamento. Entre estes resíduos, a semente representa 12% da massa do fruto que contém um elevado teor de óleo (28 a 30%). Este é rico em gorduras insaturadas, ácidos gordos, tocoferóis, carotenóides e compostos fenólicos, apresentando atividade antioxidante e benefícios para a saúde, prevenindo, por exemplo, doenças relacionadas com o envelhecimento. Assim, a extração do óleo a partir da utilização de sementes de maracujá para obtenção de ingredientes funcionais torna-se uma grande vantagem para a indústria de processamento de fruta, que vê os resíduos valorizados, e para a indústria farmacêutica e cosmética, cujo interesse em princípios ativos de origem natural tem aumentado.

Os lipossomas são vesículas esféricas de natureza anfotérica, formadas por fosfolipídios, sendo constituídos por uma ou mais bicamadas concêntricas que isolam um ou mais compartimentos internos do ambiente externo. Por serem anfifílicos, isto é serem compostos por um grupo hidrofílico e outro hidrofóbico, quando colocados em solução aquosa auto agregam-se, expondo a parte hidrofílica e ocultando a parte hidrofóbica, podendo assim, encapsular substâncias hidrofóbicas. Em geral, a preparação de vesículas lipídicas pode ser dividida em três fases consecutivas: (i) produção da fase aquosa e lipídica; (ii) hidratação da fase lipídica e (iii) processamento secundário, que consiste nas estapas de redução de tamanho e remoção do solvente. Neste contexto, o presente trabalho tem por objetivo a valorização do óleo extraído da semente de maracujá (a ser obtido na UTFPR), sua caracterização e encapsulação em lipossomas visando aplicações cosméticas. O método de preparação consiste no método da injeção de etanol (factível de escalonamento), e o lípido a utilizar é a fosfatidilcolina. Resumidamente, a fosfatidilcolina é dissolvida em etanol e adicionada com o óleo de maracujá a ser encapsulado. Esta solução é injetada rapidamente numa solução aquosa, originando os lipossomas. Com o objetivo de aumentar a eficiência de encapsulação será testado o revestimento com polímeros hidrofílicos, nomeadamente o quitosano. Como última etapa, faz-se a remoção do etanol em evaporador evaporação rotativo. Os sistemas de lipossomas obtidos serão caracterizados quanto ao tamanho de partícula e estabilidade, eficiência de encapsulação e posteriormente incorporados num cosmético base (creme hidratante) em diferentes etapas do processo produtivo visando obter a máxima eficácia e benefícios do óleo de maracujá encapsulado.

Agradecimentos: CIMO (UID/AGR/00690/2019) através do FEDER/PT2020 e LSRE-LCM (UID/EQU/50020/2019) através da FCT/MCTES (PIDDAC).

F2 – Encontro de Jovens Investigadores

# Encapsulação do óleo de semente de maracujá em lipossomas visando aplicações cosméticas

**G. G. Silva[1,2]; I. P. Fernandes[3,4*]; C. C. Sipoli[2**]; M. F. Barreiro[3,4***]**

[1]Mestrado em Engenharia Química, Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Bragança, Portugal; [2]Engenharia Química, Universidade Tecnológica Federal do Paraná, Câmpus Apucarana, Brasil; [3]Centro de Investigação de Montanha (CIMO); [4]Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), Instituto Politécnico de Bragança, Câmpus de Santa Apolonia, 5300-253, Bragança, Portugal.

*ipmf@ipb.pt; ** carolinesipoli@utfpr.edu.br; ***barreiro@ipb.pt

## Resumo

A geração de resíduos constitui um dos maiores e mais danosos riscos ambientais. Na indústria de suco de maracujá, por exemplo, apenas a polpa é utilizada. Dentre seus resíduos as sementes, que representam 12% do fruto, contém um elevado teor de óleo (28 a 30%) rico em gorduras insaturadas, ácidos gordos, tocoferóis, carotenóides e compostos fenólicos, apresentando atividade antioxidante e benefícios para a saúde. Os lipossomas são vesículas esféricas de natureza anfotérica, formadas por fosfolipídios, capazes de proteger substâncias do ambiente externo. Por serem anfifílicos, isto é, compostos por um grupo hidrofílico e outro hidrofóbico, quando colocados em solução aquosa auto agregam-se, expondo a parte hidrofílica e ocultando a parte hidrofóbica, podendo assim, encapsular substâncias hidrofóbicas. Neste contexto, o presente trabalho tem por objetivo a valorização do óleo extraído da semente de maracujá (obtido na UTFPR), sua caracterização e encapsulação em lipossomas visando aplicações cosméticas. O método de preparação consiste no método da injeção de etanol (factível de escalonamento), e o lípido a utilizar é a fosfatidilcolina. Como última etapa, faz-se a remoção do etanol em evaporador rotativo. Os lipossomas são caracterizados quanto ao tamanho, eficiência de encapsulação e posteriormente serão incorporados num cosmético base (creme hidratante). Foi verificada a influencia de parâmetros como tempo de agitação e de evaporação do solvente nos tamanhos das vesículas, verificado-se que o aumento do tempo de agitação e de evaporação favorecem a aglomeração dos lipossomas. Com o objetivo de aumentar a eficiência de encapsulação está sendo testado o revestimento dos lipossomas com quitosano.

**Palavras-chave:** óleo de maracujá; lipossomas; injeção de etanol; quitosano; cosméticos.

**Financiamento:** Este trabalho foi financiado pelo Laboratório Associado LSRE-LCM (UID/EQU/50020/2019), financiado por fundos nacionais através da FCT/MCTES (PIDDAC) e da Fundação para a Ciência e Tecnologia (FCT, Portugal) e CIMO (UID/AGR/00690/2019) através do FEDER no âmbito do Programa PT2020.

# Passion fruit seed oil encapsulation in liposomes in view of cosmetic applications

**G. G. Silva[1,2]; I. P. Fernandes[3,4*]; C. C. Sipoli[2**]; M. F. Barreiro[3,4***]**

[1]Chemical Engineering Master Degree, School of Technology and Management, Polytechnic Institute of Bragança, Portugal; [2]Chemical Engineering, Federal University of Technology – Parana, campus Apucarana, Brazil; [3]Mountain Reserach Centre (CIMO); [4]Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Campus de Santa Apolonia, 5300-253, Bragança, Portugal.

*ipmf@ipb.pt; ** carolinesipoli@utfpr.edu.br; ***barreiro@ipb.pt

## Abstract

Waste generation is one of the biggest and most harmful environmental problems. In the passion fruit juice industry, for example, only pulp is used. Among its residues are seeds, which represent 12% of the fruit and contain a high oil content (28 to 30%) rich in unsaturated fats, fatty acids, tocopherols, carotenoids and phenolic compounds, presenting antioxidant activity and health benefits. Liposomes are spherical vesicles of amphoteric nature, formed by phospholipids, capable of protecting substances from the external environment. They are amphiphilic, i.e. they are composed of one hydrophilic and one hydrophobic group. When placed in aqueous solution they self-aggregate, exposing the hydrophilic part and hiding the hydrophobic part, being able to encapsulate hydrophobic substances. In this context, the present work has as objective the valorisation of the oil extracted from the passion fruit seed (obtained at UTFPR), its characterization and encapsulation in liposomes in view of cosmetic applications. The method of preparation is the ethanol injection method (scalable) and the lipid to be used is phosphatidylcholine. As a last step, ethanol is removed by rotary evaporation. Liposomes are characterized in size, encapsulation efficiency and later incorporated in a base cosmetic (moisturizing cream). The influence of parameters such as stirring and solvent evaporation time on vesicle sizes was verified, being found that increasing stirring time and evaporation time favour liposome agglomeration. In order to increase encapsulation efficiency, the coating of chitosan liposomes is being tested.

**Keywords:** passion fruit oil; liposomes; ethanol injection; chitosan; cosmetics.

**Funding:** This work was financially supported by Associate Laboratory LSRE-LCM (UID/EQU/50020/2019) funded by national funds through FCT/MCTES (PIDDAC), and Foundation for Science and Technology (FCT, Portugal) and CIMO (UID/AGR/00690/2019) through FEDER under Program PT2020.