Methods and Protocols in Food Science

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Andrea Gomez-Zavaglia *Editor*

Basic Protocols in Encapsulation of Food Ingredients

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Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

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Basic Protocols in Encapsulation of Food Ingredients

Edited by

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Series Preface

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated, and used by food and related scientists as well as theoretical basis is provided for each protocol. The aspects related to improvements in the protocols, adaptations, and further developments in the protocols may also be approached.

Methods and Protocols in Food Science series aims to bring the most recent developments in research protocols in the field as well as very well-established methods. As such the series targets undergraduates, graduates, and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by a comprehensive and step-by-step procedure on how to perform that experiment. The next section brings the do's and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions are addressed.

The *Methods and Protocols in Food Science* series will fill an important gap, addressing a common complaint of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has the potential to become a reference material in food science laboratories of research centers and universities throughout the world.

Preface

Encapsulation is a process to entrap active agents or biotechnologically relevant microorganisms (known as *nucleus*) within a carrier material. It provides a useful system for delivering sensitive molecules or living cells not only into food products but also into the gut, target organ for most of them. This strategy contributes to protect such compounds from technologically and/or storage undesirable conditions (e.g., high or low temperatures, dehydration, high osmolarity, pressures), as well as from the gastrointestinal conditions (e.g., low pH, exposure to digestive juices). Another benefit of encapsulation is a lesser evaporation and degradation of volatile actives (e.g., aroma). Likewise, encapsulation is used to mask unpleasant feelings for the consumer (e.g., bitter taste, astringency of polyphenols, fish flavor of polyunsaturated fatty acids). Another goal of encapsulation is to prevent reactions between the nucleus and other food components (e.g., water, oxygen). Finally, lactic acid bacteria and probiotics can be encapsulated to ensure high concentrations of viable cells at the moment of being consumed.

Besides the nucleus characteristics, the *carrier material* and the *encapsulation method* play a key role for a successful protection.

The selection of the *encapsulation material* is a challenging task because it has to fulfill technological (supporting the encapsulation process), regulative (regulations of international food-related organisms, like EFSA or FDA), and physiological (stability during gastrointestinal passage) requirements. Hence, the selected materials shall be biodegradable and food grade. Several encapsulating materials with such characteristics have been employed, including polysaccharides, proteins, and lipids, either isolated or associated to form complex delivery systems. Using such sources ensures the absence of cytotoxicity and the safety of the biodegradation products.

The *encapsulation methods* can be roughly classified into three categories:

(a) Extrusion based.

(b) Emulsion based.

(c) Dehydration based.

Extrusion- and emulsion-based ones usually refer to the cross-linking of a polymer solution after either dropping into cross-linker or suspension in oil, respectively. In turn, the dehydration techniques (e.g., spray-drying, freeze-drying) are usually complementary steps of extrusion- and emulsion-based methods, as they lead to a decrease in the particle size and the obtained powder can be much easily handled. In addition, they can also be employed without prior cross-linking or emulsification.

As a whole, defining protocols for encapsulation methods involves a deep knowledge about fundamental aspects of dehydration, including physico-chemical, physiological, microbiological, and structural ones, among many others. Hence, this is a process requiring a deep interdisciplinary knowledge where innovation plays an active role.

For these reasons, the *purpose of this book* is to give a comprehensive introduction into methods and procedures related to the encapsulation of *sensitive food nucleus,* especially considering the *carrier materials* and the *encapsulation techniques.* All chapters include introductions to the corresponding topic, lists of all necessary materials and reagents,

step-by-step, readily reproducible laboratory protocols, and a large number of notes giving tips on troubleshooting and for avoiding known pitfalls.

The following nucleus were considered: carotenoids, sunflower, olive and fish oils (Chapters 1, 8, 9, 12, 16), polyphenols and other antioxidants (Chapters 2, 3, 10, 14), lactic acid bacteria and other bacterial cells (Chapters 4, 5, 6, 7, 11), and enzymes (Chapters 15 and 17).

Regarding carrier materials, mono-, di-, oligo- and polysaccharides (Chapters 1, 3, 5, 8, 10, 11, 13, 14, 15, 17), proteins and lipids (Chapters 7, 9, 12) as well as liposomes (Chapter 16) were considered.

Finally, the encapsulation techniques include freeze-drying (Chapters 1, 5, 6, 9, 13, 14, 16), spray-drying (Chapters 2, 8, 12), ionic gelation (Chapters 3, 4, 15, 17), emulsification (Chapters 7 and 12), electrospraying (Chapter 10), and layer-by-layer encapsulation (Chapter 11).

La Plata, Argentina *Andrea Gomez-Zavaglia*

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Chapter 1

Encapsulation of Hydrophobic Compounds in Sugar Matrixes by Freeze-Drying

Nurys Tatiana Hoyos Merlano, Virginia Borroni, Rocío Belén Giménez, and María Lidia Herrera

Abstract

To encapsulate hydrophobic materials in sugar matrices, the initial system to prepare powders is an emulsion. The aqueous phase contains the sugar and the stabilizer selected, and the fat phase is a hydrophobic compound or a solution of the hydrophobic compound in a solvent. Preparation of emulsion is a three-stage process. The first part is the preparation of a coarse emulsion (average droplet size ranging from ~1 to 10 μm). The second stage is the preparation of a conventional emulsion or also called fine emulsion, starting from a coarse emulsion (fine emulsion average diameter from 0.2 to 0.6 μm). Finally, further treatment of the conventional emulsion leads to a nanoemulsion (radius < 100 nm). When the stabilizer is a small molecule such as Tween, a two-stage process may be suitable to achieve the nano range if a microfluidizer is used for the second stage. When using a protein stabilizer, the three stages are necessary for nano-based powders. The quality of powders and encapsulation efficiency will be very dependent on initial systems' average droplets size and physical stability. Stable systems containing nano range droplets will allow preparing better quality powders than coarse or fine emulsions.

Key words Encapsulation, Sugar matrices, Hydrophobic compounds, Protein stabilizers, Small molecule emulsifiers, Emulsion-based powder

1 Introduction

Microencapsulation is a technique whereby liquid droplets or solid particles are packed into continuous individual shells. In those shells, the encapsulated material is the core, and the matrix forms the walls. Important applications of microencapsulation in the food industry involve materials such as volatile compounds [1], essential oils and flavors [2], vitamins and precursors [3], and unsaturated lipids [4, 5], to name a few. The core is usually formed by hydrophobic compounds with carbon double bonds which make them labile substances. Encapsulation of these sensitive materials in sugar matrices makes it possible to incorporate them in dry form in a formulation so that they are protected by the walls against

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evaporation, oxidation, and chemical reaction. The encapsulated materials may also be released from the freeze-dried particles by different methods depending on application.

Spray-drying is the most widely used technique for encapsulation in food applications since it is inexpensive and has wide practical use. It is typically used in the preparation of dry, stable food additives, and flavors [6]. However, as it uses high temperatures, it is not the best method for thermosensitive substances. Freeze-drying is preferred for oxidizable samples since lipids on the surface of the microcapsules could be oxidized during drying process. For example, lipophilic carotenoids encapsulated by freeze-drying such as paprika pigment had higher color stability and better emulsion, and heating stabilities than powders encapsulated by spray-drying and the native pigment [7]. Strawberry flavor was encapsulated in order to produce high-quality products using different agents and different drying techniques. For those powders, the best drying yield was observed in the case of freeze-drying [8]. In addition to the mentioned physical and chemical properties, drying method selected affects dispersion of powder in water when it is used as a creamer or added as a powder to an aqueous-based food. OSA starch-linseed oil powders obtained by freeze-drying showed a much quicker dispersion when added to water than the ones obtained by spray-drying [9].

To encapsulate lipid materials, powders are prepared by freeze-drying emulsions. Thus, preparation of emulsions is the first step in powders production. Selecting the best methods for initial systems preparation is very important since droplet size distribution is very relevant to encapsulation efficiency as is emulsion stability [10]. For emulsions, the preparation method comprises three stages: the formation of a coarse emulsion (droplets in the microns, from 1 to 10), further homogenization to obtain a fine emulsion (droplets from 0.2 to 0.6 μm in diameter), and solvent evaporation under vacuum to give nanoemulsions (average radius < 100 nm). Figure 1 shows a scheme of preparation stages. When the stabilizer (emulsifier) is a small surfactant such as Tween, the nano range may be achieved with only two stages, using a microfluidizer for the second stage.

2 Materials

Wall materials: α,α-trehalose dihydrate (α-D-glucopyranosyl-(1–1)-α-D-glucopyranoside), sucrose (α-D-glucopyranosyl β-D-fructofuranoside), lactose (β-D-galactopyranosyl-(1–4)-α-D-glucopyranoside), food or analytical grade depending on intended use, industrial production, or scientific experiments, respectively.

Water: HPLC-grade water for scientific purposes, safe water for the food industry.

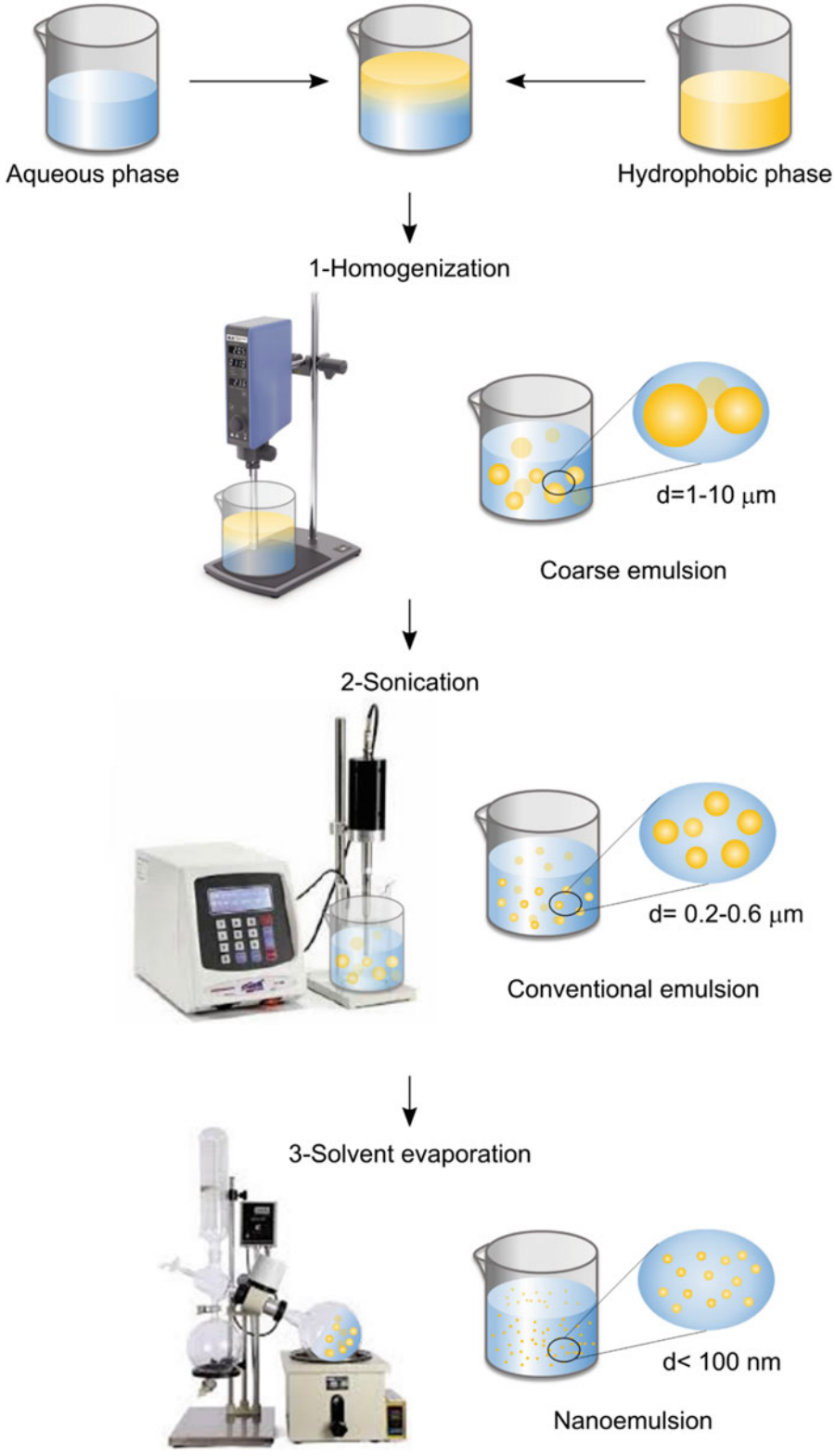

Fig. 1 Scheme of three-stage emulsion preparation method

Emulsifiers: Sodium caseinate (NaCas), commercial powdered whey protein concentrates WPC35 or WPC80 (WPC80 with the following contents in weight percent (wt%): 82.4% of protein (dry weight basis), 5.0% of lactose, 2.7% of fat, 4.3% of ashes, and 5.6% of moisture, and WPC35 with contents of 38.3%, 48.8%, 2.2%, 7.5%, and 3.2%, respectively), a sucrose ester mix of palmitic sucrose ester (SE) (P-170) with hydrophilic/lipophilic balance (HLB) = 1 and palmitic SE (P-1670) with HLB = 16 (the SE Mettler dropping points are 58.0 °C and 44.0 °C, respectively; P-170 has a monoester content of 1 wt%, with di-, tri-, and polyesters comprising 99 wt%. P-1670 had 80% monoester and 20 wt% di-, tri-, and polyester; the average HLB for the emulsifier blend should be 9), small molecules surfactants such as Tween 80, 40, or 20.

Core materials: Thermosensitive hydrophobic compounds such as sunflower oil (SFO), olive oil (OO), fish oils (CFO), β-carotene, low-melting milk fat fraction (melting point ~17 °C), blends of high-melting milk fat fraction (melting point 49.5 °C), and 40 wt.% sunflower oil.

To prepare powders from nanoemulsions: Ethyl acetate analytical grade or other purity depending on the purpose of powder preparation.

Encapsulation efficiency solvents: Hexane, anhydrous sodium sulfate (Na_2SO_4), and sulfuric ether (all reagents must be analytical grade).

3 Methods

3.1 Preparation of Course Emulsion

1. Weight accurately 20 g of wall material (trehalose, sucrose, or lactose) in aluminum foil (*see* **Note 1**).
2. Weight 5 g of emulsifier (sodium caseinate or whey protein) or 2 g of sucrose ester mix or Tween in aluminum foil (*see* **Note 2**).
3. Weight in a beaker of 250 mL the amount of water necessary to complete 100 g (75 g when the emulsifier is a protein or 78 g when using sucrose esters or Tween). Heat the water to 55 °C. Put into the beaker, a magnetic stirrer bar.
4. Place the beaker on a magnetic stirrer and add small amounts of wall material and wait until it dissolves. Then, add another small amount of the wall material and continue until all wall material weighted in **step 1** is dissolved.
5. Then add the emulsifier weighted in **step 2**, working in the same way as in **step 4** (*see* **Note 3**).

6. Weight 10 g of hydrophobic phase (core material). If the selected start system is a nanoemulsion, then go to step in Subheading 3.3.
7. Heat the two phases to 55 °C (*see* **Note 4**).
8. Mix 10 g of hydrophobic phase and 90 g of aqueous phase with a homogenizer Ultra-Turrax type, operating it at 20,000 rpm for 1 min. Repeat this procedure twice with an interval of 5 min to cool homogenizer equipment (*see* **Note 5**).

3.2 Preparation of Fine Emulsion

1. Place from 40 to 60 mL of coarse emulsion in a water-jacketed glass cell.
2. Connect the jacket to a water bath.
3. The sample-cell temperature must be controlled. Place a thermocouple inside the sample (*see* **Note 6**).
4. Further homogenize the resultant coarse emulsion for 20 min, using an ultrasonic liquid processing equipment, operating with a power of 750 W at an amplitude of 30% and a frequency of 20 kHz, using a dispersing tool of 13-mm-diameter and 136-mm-length tip (*see* **Note 7**).
5. Then, cool quiescently to ambient temperature (22.5 °C), (*see* **Note 8**).

3.3 Preparation of Nanoemulsion

1. If powders need to be prepared from nanoemulsions, start preparation as indicated in Subheading 3.1 until **step 5**. Modify **step 6** as indicated below (*see* **Note 9**).
2. Dissolved 10 g of hydrophobic phase in 57 g of ethyl acetate (a concentration of 15 wt.% hydrophobic phase/ethyl acetate).
3. Continue working from Subheading 3.1 **step 7** under hood.
4. Then, place the emulsion obtained in Subheading 3.2 **step 5** in a rotary evaporator connected to a vacuum pump and a recirculating chiller to eliminate ethyl acetate.
5. Perform the process at 45 °C for around 20 min (*see* **Note 10**).

3.4 Powder Production

1. Freeze samples with liquid nitrogen (−190 °C) and stored overnight at −80 °C before freeze-drying to allow the highest amount of freezable water to crystallize (*see* **Note 11**).
2. After freezing, cover samples with 2 cm of liquid nitrogen and place them in the dryer.
3. Dry samples operating dryer at −110 °C and at a chamber pressure of 4.10^{-4} mbar for 48 h (*see* **Note 12**).
4. Place the dried samples in a dried box under nitrogen atmosphere.

Fig. 2 A photograph of a conventional emulsion and the corresponding powder

5. Then, break samples into powder using a mortar and pestle. Figure 2 shows an example of initial emulsion and resulting powder.

3.5 Encapsulation Efficiency

1. To determine encapsulation efficiency (retention), it is necessary to evaluate extractable and encapsulated fat.
2. To quantified extractable fat, weight 2 g of powder and disperse it in 15 mL hexane (analytical grade). Shake the dispersion for 15 min.
3. Filtered the sample and keep the soluble fraction.
4. Repeat the washing process twice.
5. Evaporate the solvent until constant weight. The obtained solid is the fat phase.
6. Weight the fat phase in an analytical balance.
7. The weight of the dried material representing the extractable fat is calculated as a percentage of the total fat in the dry powder.
8. Then, evaluate the powder, free of extractable fat. Mix 2 g of free-fat powder with 15 mL water and 15 mL ethanol. Extract the resulting solution with 40 mL sulfuric ether.
9. Collect the clear organic phase.
10. Dry the organic phase by filtering it with a filter paper containing Na_2SO_4.
11. Evaporate the solvent under N_2 stream.

12. Weight the solid in an analytical balance.
13. Calculate encapsulation efficiency as a percentage of the total fat in the dry powder (*see* **Note 13**).

4 Notes

1. These three sugars have been used as wall materials with very good encapsulation yields. A mix of trehalose with 30 wt.% sucrose is also very efficient [11]. The amount of sugar selected in this protocol is the one that leads to the highest encapsulation efficiency. Higher content of sugar makes the freeze-drying process more inefficient giving a powder with poor physical properties.
2. To prepare the emulsion, it is necessary to add an emulsifier to obtain stable systems. For this purpose, a protein stabilizer (such as sodium caseinate or whey protein) or small molecule emulsifiers (such as sucrose esters) may be used. Other emulsifiers such as gelatin or Tween are also successful [12]. For whey protein, 5 g of protein corresponds to different amounts of whey powder depending on protein concentration: WPC80 82.4 and WPC35 38.3 wt.% of protein (dry weight basis).
3. Sodium caseinate is hard to dissolve in water. It is very important to add sugar first and then protein. Sugar helps dissolution of sodium caseinate because it interacts with protein and improves the performance of water as a solvent. Besides, protein must be added to water. When water is added to protein, it is almost impossible to achieve dissolution.
4. When both phases are warm a better dispersion is obtained. This procedure leads to formation of emulsions with average droplet size from 1 to 10 μm. Depending on powder properties desired, a smaller particle size in starting emulsions could be required. If this is the case, follow the procedure in Subheading 3.2
5. After this step of preparation, the average droplet size obtained is between 1 and 10 μm. These emulsions are usually called coarse emulsions or pre-emulsions.
6. By setting 15 °C, the sample temperature will always be below 40 °C. This is especially important when the stabilizer selected is a protein. It is recommended to place a thermocouple to sense sample temperature and to set a temperature cutoff in the equipment of 40 °C since controlling temperature avoids protein denaturation.
7. For this step, it can also be used a high-pressure homogenizer or a microfluidizer. When using a high-pressure laboratory valve homogenizer, the working pressure must be set at

40 MPa, and the optimum results are obtained with four recirculations [11]. These are the conditions that allow obtaining the highest encapsulation efficiency. Higher pressure does not lead to a better performance. When using a microfluidizer, the coarse emulsion must be passed through the microfluidizer at 1000 bar for 7 cycles. It is advisable that all experiments are performed under cold condition (4 ± 1 °C) to prevent the heat generation during homogenization. In the case of a small molecule emulsifier such as Tween 40, this procedure leads to production of nanoemulsions. In this case, step in Subheading 3.3 is not necessary. However, for protein stabilized emulsions, it is not possible to reach nano range without performing step in Subheading 3.3 . Systems behave as conventional ones showing creaming and flocculation [13].

8. The pHs of the fine emulsions with these formulations are around 7. Usually, no buffer is added to emulsions. After this step of preparation, average droplet size is from 0.1 to 1 μm. The stability of these emulsions is approximately a week. They usually lead to good encapsulation yield. If more homogeneity of powder is needed, follow the procedure in Subheading 3.3
9. The proposed synthesis of nanoemulsions consists in a combination of a high-energy homogenization and evaporative ripening methods reported by Lee and McClements for whey protein-stabilized systems with some minor changes [14].
10. When the ethyl acetate evaporates, the droplets shrink and their size falls into the nanoscale (average radius < 100 nm). In this method, the degree of ethyl acetate evaporation is determined by carrying out a mass balance of emulsions before and after solvent evaporation. As a practical strategy, evaporation is performed until a constant weight is obtained. Some water may be evaporated too. After obtaining constant weight, it may be need to add water until the original formulation is restored. This preparation procedure is very good to obtain nanoemulsions when the stabilizer is a protein.
11. Freezing rate is very important since freeze-drying process is more efficient when crystals are small. By using liquid nitrogen, freezing rate is very fast, and as very small crystals are obtained, encapsulation efficiency is higher than the one achieved with slow cooling.
12. To obtain a powder with good physical properties, samples must be dried for 48 h. Sugar matrices are difficult to dry; therefore, drying time is very important.
13. The procedure in Subheading 3.5 allows confirming the success of powder preparation method. The quality of encapsulated fat powders is indicated by the amount of fat

not bounded in the structure of powder (the so-called "free" fat) and the size of fat globules. The third stage in the preparation process is very important since nano size droplet systems lead to powders with a more uniform structure and better physical properties.

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Chapter 2

Stabilization of Bioactive Molecules Through the Spray-Drying Technique: Current Applications and Challenges

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Abstract

Spray-drying is an old technology that is used in the cutting edge of science. From a drying technique used in the front lines of the Second World War to the most promising encapsulation techniques in drug delivery systems.

The most beneficiaries of this technique are usually the food and the pharmaceutical industry as the stability of active molecules is a key factor to the success of their application into the final formulations. Spray-drying provides a range of processes that can be optimized according to the final objective such as the operating conditions, the use of carrier agents, the drying gases, all of them having a direct impact in the final powders/particles.

This technology has changed over the years, it now takes advantage of mathematical modelling to optimize spray-drying of compounds. Countless extracts, molecules, drugs, and other compounds have been spray-dried over the last few decades, making the spray-drying technique one of the cornerstones of many industries.

In this chapter, the history, technical aspects, examples, and general usage are addressed, focusing on the food and pharmaceutical industries. Trends and challenges of this technology are also focused.

Key words Spray-drying, Operating conditions, Stabilization, Bioactive molecules, Industrial application

1 Introduction

1.1 Spray-Drying: What and Why?

Spray-drying is the transformation of a feed (that is pumpable) from a fluid state to a dried one by spraying the particles through a hot drying medium [1]. This technology's inception dates back to the 1870s, having been invented by Samuel Percy who filed a patent to the U.S. Patent Office named "Improvement in Drying

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and Concentrating Liquid Substances by Atomizing" [2]. Although the technology did suffer some improvements, its use was limited until World War II started. The military used the technology to reduce the weight of the food being transported to the front lines and distant battles. The major use was in powdering milk for soldiers, due to it being a reasonably complete nutritious and inexpensive food but had the drawback of its weight when in liquid state [3]. Since this war, improvements to the technology increased, and it disseminated into many industries, namely for the production or transformation of foods, pharmaceuticals, soaps, fertilizers, clays, ceramics, polymers, and many other products [3].

1.2 Featured Advantages and Disadvantages

In recent years, the food industry has become the major user of this technology, transforming millions of tons of ingredients and foods. According to Technavio, in 2016, the spray-drying market was valued at 1.2 billion dollars, and expected to rise to 1.5 billion by 2021, with just the spray-drying of milk representing 420 million dollars of the total amount. Still, by 2020, this global market had already reached a size of 4.5 billion dollars and expected to rise to 6 billion by 2025 [4].

Spray-drying represents considerable advantages when compared to other transformative technologies, namely the fact that it can be fully automized and work in a continuous manner with very low human intervention, reducing contamination of sensible products like food or pharmaceuticals.

This technology also has short residence times and is suitable for both heat-sensitive and heat-resistant foods and other products, with a wide variety of applications, provided they are pumpable. It allows a tailored approach and specific conditions for each of the products it is used with, accommodating the specific needs of a products. It can be used as an encapsulation technique, resulting in a homogenous product, resistant to thermal degradation and allowing a controlled release, being especially important for the pharma industry, and to encapsulate bioactive substances for foods [3, 5]. The disadvantages of this technology should not be overlooked, and one of the most significant is the price of a spray-drier, both for laboratorial and industrial use. Although the investment is quite high, a spray-drier, over time, will probably offset, but the initial investment might not be accessible to all. Furthermore, there are also considerable maintenance issues that increase the overall costs of the equipment. Another important drawback is the yield of particles, which can be as low as 20%, and tends to be lower in smaller, laboratorial sized spray-driers due to the particles remaining stuck to the walls of the drying chamber. In some cases, small particles, under 2 μm, can usually pass into the exhaust air and be removed. Another disadvantage, found especially for microencapsulation, is limited types of wall materials, and these must have a good solubility in water to be used [5, 6].

2 Spray-Drying Operating Processes

There are four primary steps, namely the atomization, followed by the droplet-to-particle stage, the moisture evaporation, and finally the particle collection (Fig. 1). The atomization stage is one of the most important, in which the liquid is atomized or divided into small droplets and become ready to undergo the next steps. In terms of atomization, there are several different atomizers that are adjusted to different products. Rotary atomizers, for instance, are used with low viscosity fluids and rely on centrifugal energy by discharging the fluids at a high velocity (200 m/s) from the edge of a wheel or disc. Two-fluid nozzle atomizers use kinetic energy and feature the impact of the droplets at high velocity with gaseous flows, allowing the production of particles with a relatively greater size. Hydraulic atomizers discharge the fluid under pressure through an orifice of variable sizes and can reach pressures of 250–10,000 PSI but produce less homogenous and coarser particles. The ultrasonic atomizers force the liquid through two piezoelectric disks that vibrate at high frequencies and ensure the vibration of the atoms of the droplets, reducing the surface tension. This type of atomization is intended for low-viscosity Newtonian

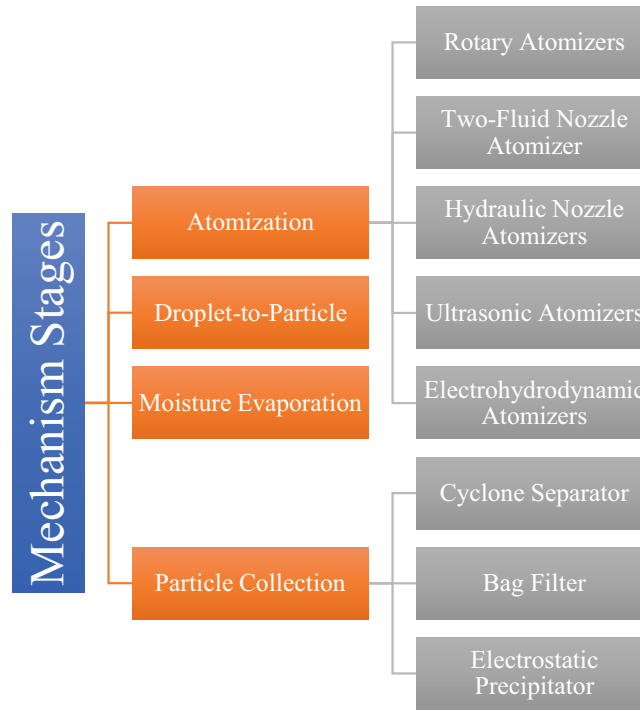

Fig. 1 Stages of the spray-drying procedure and different variations of the technology

fluids. Finally, the electrohydrodynamic atomizers pass an electric current through the fluid enabling the production of droplets of narrow particle sizes.

In terms of the particle collection, there are three types of collectors, namely the cyclone separators that use a centrifugal force to separate the solid particles from the carrier gas, bag filters that separate the particles by retaining them inside the bag and allowing the carrier gas through the bag material, and the electrostatic precipitators that retain the particles by using electrostatic forces to ionize the air and make the particles cling to collecting plates [1, 7].

3 Stabilization of Bioactive Molecules

Stability is a factor of great importance when it comes to the exploration of natural products [8]. Natural products have poor stability when compared to products obtained synthetically, namely by chemical synthesis. In order to enable their use by different industries, new approaches are constantly being researched to overcome these problems and make this possibility real [9]. There are several factors that affect the stability of molecules of natural origin, such as light, temperature, pH, the presence of oxygen, among others. This occours due to the recover the molecules it is necessary to remove them from their surroundings, leaving them unprotected and susceptible to various factors that can somehow affect their molecular structure and destabilize it, causing them to lose or modify properties of interest [10]. After the extraction process, the simple fact of removing the extractor solvent is in itself a process of stabilization, since a greater physical and chemical stability is achieved in the solid state [11]. Due to this there are several process to remove solvents, namely freeze drying, convective hot air drying, spray drying [12], among others, all with different costs, efficiencies, and sustainability.

In recent years, the pharmaceutical and food industries have expressed a preference for the spray-drying technique, as this is a methodology that, despite effectively removing the extractor solvent, is still capable of encapsulating the molecules of interest [13] and thus increase their stability in a fast and relatively cheap process.

During the spray-drying/encapsulation process, stability is achieved due by creation of a protective barrier against various factors that interfere with molecular stability. This is done in a fast process, where the small particles are formed while the solvent is extracted. For instance, dehydrated fruits, with a high content of organic acids and low molecular weight sugars, during the storage period tend to present a rubbery structure. To overcome this problem, some compounds can be added during the spray-drying process, known as carriers [14]. This example outlines the

versatility of the technology to adapt to various situations and different solvents and carriers, which is one of the strongest advantages. With spray drying-carriers, it is possible to increase stability and, depending on the proportion in which it is added, achieve the encapsulation, where the carrier becomes the actual wall material.

Some examples of carriers are high molecular weight biopolymers such as maltodextrins, modified starch or gums, or steric function biopolymers, such as fibers, proteins, or some inorganic compounds. The spray-drying process can be applied to a huge range of products for the food, pharmaceutical, and other industries [15–22]. Considering the food industry, spray-drying has been used in dairy products, essential oils, aromas, coloring compounds, phenolic compounds, probiotics, and others [23–28].

From Table 1, it is possible to see that the spray-drying technique is a highly inclusive technique regarding the type of samples that can be processed. It is possible to perform drying or encapsulation by spray-drying on natural compounds, bioactive molecules, color-holding molecules, probiotics, essential oils, molecules with pharmacological activity, among others [23–25, 28, 34]. Carrier molecules can be luteolin, maltodextrins, gum arabic, modified starches, and simple sugars such as trehalose and inulin [15, 29]. Oils are also an example of carrier agents [19, 22, 25, 31, 36, 37, 44, 53, 59, 64, 68, 78]. In the case of oils, the technique of spray-drying is of great importance, because, considering their composition, they are very susceptible to oxidative decomposition and have a volatile portion that besides having interesting biological activities can be protected. Encapsulation in the case of oils helps to provide a barrier against deterioration processes, increase stability, and suppress unpleasant aromas. The spray-drying technique is capable of much more than protecting molecules against deterioration processes and camouflaging unwanted aromas/flavors.

The added value of this technique is to increase the stability of the molecules of interest, and thus, enable different industries to resort to new alternatives to incorporate in their products. It allows to make products more natural and to develop new products for the food industry, since it offers a whole new panoply of substances, for example with regard to molecules to be explored as colorants of natural origin [14, 15, 34, 39, 45, 61, 66, 80], probiotics, and bioactives [12, 23, 24, 49, 54, 57].

In the pharmaceutical industry, this technique is used to increase molecular stability, producing small-sized particles, which are easier to transport and thus allow the use and approach of new therapies. With spray-drying, it is possible to encapsulate active ingredients that have some difficulty in expressing their beneficial effect, such as solubility problems. The pharmaceutical industry takes advantage of all the possibilities that the spray-drying technique offers, such as encapsulating the active ingredients, increasing

Table 1
Molecules stabilized through the spray-drying technique

Sample	Auxiliary molecules		Operating conditions				Stability	Destination	References
	Molecule	Acting as	Air flow	Feed rate	Inlet	Outlet			
Citrus paradisi var. star ruby juice	Gum arabic Maltodextrin Whey protein isolate	Carrier agent	473 l/h	9 ml/min	148 °C		Relative humidity, exposure to light, and time	Food industry	[14]
Lutein	Maltodextrin Gum arabic Modified starch	Encapsulating agent	50 m^3/h	4 ml/min	185 ± 5 °C	100 ± 5	Temperature and relative humidity	Food industry	[15]
	Trehalose Inulin Modified starch	Encapsulating agent	–	7 ml/min	180–200 °C	75 and 85 °C	Temperature degradation kinetics		[29]
Flaxseed-peptide fractions	Chitosan	Encapsulating agent	0.54 m^3/h	5 ml/min	130 ± 1 °C	73 ± 2 °C	Thermal stress and dehydration	Food industry	[21]
Bioactive compounds of *Bidens pilosa* L.	Microcrystalline cellulose MC102: Aerosil:	Encapsulating agent	1.67×10^{-2} m^3/s	1.49×10^{-4} kg/s	155 °C	–	Relative humidity temperature sampling time	Food and pharmaceutical industry	[24]
Juglans regia L. and *Salvia hispanica* L. oils	Maltodextrin: hydroxypropyl methylcellulose	Encapsulating agent	–	279 l/h	163 °C	–	Oxidative stability	Food industry	[25]
Salvia hispanica L.	Maltodextrins DE10, DE20, whey proteins Gum arabic	Encapsulating agent	–	7 ml/min	160 ± 2 °C	60 ± 5 °C	Oxidative stability	Food industry	[30]
Sour cherry seed oil	Gum arabic Maltodextrin	Encapsulating agent	600 ml/h	8 ml/min	195 °C	–	Thermal and oxidative stability.	Food industry	[31]
Enzalutamide	Hydroxypropyl methylcellulose Acetate succinate	Carrier agent	6.83 l/min	3 ml/min	100 °C	70 °C	Physical stability	Pharmaceutical industry	[32]
Cocoa hull waste phenolic extract	Chitosan Maltodextrin	Coating agent Encapsulating agent	5 cm^3/min	2.5 cm^3/min	170 °C	75–80 °C	Physic-chemical stability	Food industry	[33]
Probiotics (*Lactobacillus acidophilus*)	Maltodextrin: Gum arabic	Encapsulating agent	–	8 ml/min	–	55 ± 3 °C	Physic-chemical stability	Food industry	[23]

Probiotic (*Lactiplantibacillus plantarum* 299v; *Pediococcus acidilactici* HA-6111- 2)	Maltodextrin (in the orange powder)	Encapsulating agent	25 ml/min	–	150 °C	70 °C	Cell viability over time	Food industry	[12]
Beta vulgaris L. extract	Maltodextrin Inulin Whey protein isolate	Carrier agents	35 l/min	0.8 l/h	150 °C	96 ± 4 °C	Adsorption isotherms, thermogravimetric analysis, and accelerated storage	Food industry	[34]
Moringa stenopetala extract bioactive compounds	Maltodextrin: Dextrose: high methoxul pectin	Encapsulating agents	–	–	–	–	Temperature, time	Food and pharmaceutical industry	[16]
Immunoglobulin G	Threalose + (leucine, phenylalanine, arginine, cysteine, and glycine)	Carrier agent	–	–	−50 °C	–	Temperature and relative humidity	Pharmaceutical industry	[35]
Euphausia superba oil	Whey protein concentrate, maltodextrin, and gum arabic (8:2:0.5)	Encapsulating agent	2.5 m^3/min	5 cm^3/min	130 °C	71–75 °C	chemical stability of krill oil in terms of oxidative stability	Food industry	[36]
Fish-oil	Hydroxy propyl cellulose	Encapsulating agent	600 l/h	1 ml/min	200 °C	–	Temperature, time light and oxidative stability	Food industry	[37]
Physalis peruviana (carotenoids) juice	Maltodextrin Modified starch Inulin Gum arabic Alginate	Encapsulating agents	473 l/h	–	140 °C	70 ± 4°C	Time, temperature, relative humidity.	Food industry	[38]
Blackberry pulp	Arrowroot starch: gum arabic (1:1.78)	Encapsulating agent	0.6 m^3/h	–	143 °C	105.43 ± 3.13 °C	pH	Food industry	[39]
L-Lactic dehydrogenase	Trehalose	Carrier agent	150 l/min	0.2 ml/min	120 °C	–	Temperature and shear stress	Pharmaceutical industry	[40]
Conjugated linoleic acid	octenyl-succinic anhydride:starch: xanthan gum	Encapsulating agent	–	–	160 ± 5 °C	75 ± 5 °C	Oxidative stability	Food and pharmaceutical industry	[17]

(continued)

Table 1
(continued)

Sample	Auxiliary molecules		Operating conditions				Stability	Destination	References
	Molecule	Acting as	Air flow	Feed rate	Inlet	Outlet			
Nilotinib hydrochloride	–	–	–	–	120 ° C	75 °C	Thermal	Pharmaceutical industry	[41]
L-ascorbic acid	Taro starch spherical aggregates	Encapsulating agent	–	19.5 g/min	145 °C	80 °C	Relative humidity	Food, pharmaceutical, and cosmetic industry	[18]
Vanillin	Whey protein isolate β-cyclodextrin	Encapsulating agents	–	20 ml/min	110 ± 2 °C	60 ± 2 °C	Temperature	Food industry	[42]
Lysozyme	–	–	35 m^3/h	2.3–2.6 ml/min	130 °C	69–75 °C	Protein	Pharmaceutical industry	[43]
Red palm oil	Sodium caseinate: maltodextrin: sou lecithin	Encapsulating agents	150 l/h	15 ml/min	165 ± 2 °C	60 ± 2 °C	Storage and oxidative	Food industry	[44]
Beta vulgaris juice	Maltodextrin	Encapsulating agent	450 l/h	6 ml/min	170 ± 5 °C	65 ± 5 °C	Temperature	Food industry	[45]
Novel structured lipids enriched with medium- and long-chain triacylglycerols	Whey protein isolate: maltodextrin: inulin	Encapsulating agent	300 nl/min	14–15 ml/min	180 ± 5 °C	80 ± 5 °C	Oxidative stability	Food industry	[46]
Pseudomonas phages, (PEV2 (Podovirus) and PEV40 (Myovirus))	Threhalose:leucine	Encapsulating agent	0.742 m^3/h	1.8 ml/min	–	40–45 °C	Temperature, time	Pharmaceutical industry	[47]
Oil / milkfat	Sodium caseinate: lactose	Encapsulating agent	–	–	180 °C	80 °C	pH, physical	Food industry	[48]
Lacticaseibacillus casei LK-1	Skim milk Trehalose Maltodextrin	Encapsulating agent	–	320 ml/h	–	70 °C	Temperature, physical	Food industry	[49]
Therapeutic (or monoclonal) antibodies (mAbs)	Trehalose: amino acids	Carrier agents	10 l/min	3 ml/min	120 °C	55–60 °C	Temperature, time	Pharmaceutical industry	[50]

Infant milk formula	Lactose: maltodextrin	Carrier agents	–	–	180 °C	90 °C	Physic-chemical stability	Food industry	[51]
Lime and rosemary essential oils and α-tocopherol	Maltodextrin	Encapsulating agent	40 l/h	600 ml/h	140 °C	–	Light, temperature	Food industry	[52]
Vitamin D2	Casein micelles	Encapsulating agent	6.7 kg/min	203 g/min	180 °C	80 °C	Storage	Food industry	[53]
Polyphenols-rich grape marc extract	Maltodextrin: whey protein isolate: pea protein isolate	Encapsulating agent	–	21.5 g/min	140 °C	–	Temperature, time	Food industry	[54]
Tadalafil	Glycyrrhizin	Carrier agent	–	3 ml/min	80 °C	60 °C	Storage	Pharmaceutical industry	[55]
Curcuma longa	Skim milk powder	Encapsulating agent	–	–	160–165 °C	88–93 °C	Temperature, time	Food industry	[26]
Cinnamomum zeylanicum proanthocyanidins	Maltodextrin	Carrier agent	–	40 ml/min	130 or 160 °C	–	Temperature, time	Food industry	[56]
Lactiplantibacillus plantarum B21 and A6	Whey protein isolate	Encapsulating agent	70 m^3/h	6.6 ml/min	110 °C	68 °C	Temperature, time	Food industry	[57]
Allium cepa extract	Maltodextrin	Encapsulating agent	–	0.39 l/h	160 ± 2 °C	90 ± 2 °C	Storage	Food industry	[58]
Strawberry flavour	Maltodextrin Gum arabic Modified starches Xanthan Cyclodextrins	Encapsulating agents	–	2.5 ml/min	180 ± 2 °C	90 ± 2 °C	Temperature, time	Food industry	[27]
Moringa oleifera oil	Maltodextrin Gum arabic Whey protein concentrate	Encapsulating agent	73 m^3/h	10 g/min	180 °C	85 °C	Oxidative	Food industry	[59]
Tamarillo juice	Maltodextrin n-octenyl succinic anhydride modified starch Low viscosity gum arabic Resistant maltodextrin Gum arabic	Encapsulating agent	–	6–10 rpm	140–170 °C	60–90 °C	Physical and storge	Food industry	[60]

(continued)

Table 1
(continued)

Sample	Auxiliary molecules		Operating conditions				Stability	Destination	References
	Molecule	Acting as	Air flow	Feed rate	Inlet	Outlet			
Vaccinium spp. (anthocyanin extract)	Maltodextrin DE20: hi-maize	Encapsulating agent	–	–	120, 140 and 160 °C	79.75 °C, 100 °C and 108.25 °C	Time	Food industry	[61]
Lactiplantibacillus plantarum CIDCA 83114	Amorphous and crystalline inulins	Carrier agent	–	–	160 °C	65 ± 5 °C	Temperature	Food industry	[62]
Food-grade solid lipid particles and nanostructured lipid carriers containing ω-3 fish oil	Maltodextrin types (DE 6 and DE 21)	Encapsulating agents	–	6 cm^3/min	140 to 170 °C	65 to 95 °C	Storage, temperature, time	Food and pharmaceutical industry	[19]
Peptide fractions (flaxseed protein)	Polysorbate 80 (Tween-80): maltodextrin (MD)	Encapsulating agent	0.54 m^3/h	5 ml/min	110 ± 1 °C	60 ± 2 °C	Physicochemical	Food and pharmaceutical industry	[20]
Emulsion containing ultrahigh oil content	Whey protein	Encapsulating agent	–	10 l/h	105 ± 2 °C	65 ± 2 °C	Mechanical and oxidative	Food, pharmaceutical, personal care and home care industries	[22]
Ciprofloxacin hydrochloride	Sucrose Lactose Trehalose Mannitol L-leucine	Carrier agent	700 l/h	2 ml/min	120 ± 2 °C	60 ± 2 °C	Relative humidity, protein physical stability	Pharmaceutical industry	[63]
Matcha-tuna oil and matcha-maltodextrin-tuna oil emulsions	Maltodextrin	Encapsulating agent	–	12 g emulsion/min	185 °C	80 °C	Oxidative	Food industry	[64]
Alginate-coated chitosan-stabilized Pickering emulsion	Maltodextrin	Encapsulating agent	–	120 ml/h	150 ± 2 °C	95 ± 2 °C	Oxidative	Food industry	[65]

Lycopene	Maltodextrin Whey protein isolate Modified starch	Encapsulating agent	700 l/h	34 ml/min	160 ± 2 °C	80 ± 2 °C	Storage time	Food industry	[66]
D-limonene ethyl hexanoate	Yeast cells (*Saccharomyces cerevisiae*) Maltodextrin	Encapsulating agents	110 kg/h	25 ml/min	160 °C	97 to 108 °C	Oxidative	Food industry	[67]
Anguilla anguilla oil	European eel protein isolate	Encapsulating agent	35 l/min		175 °C	80 °C	Oxidative	Food industry	[68]
Sulforaphene	Hydroxypropyl-β-cyclodextrin Maltodextrin Isolated soybean protein Chitosan	Encapsulating agent	–	–	180 °C	–	Temperature	Food industry	[69]
Lutein *Quillaja saponaria Molina* saponin extract	Glucose syrup	Encapsulating agent	–	50 ml/min	180 °C	68–70 °C	Colour	Food industry	[70]
Norbixin	Gum arabic: Maltodextrin	Encapsulating agent	600 l/h	0.39 l/h	150 °C	75 °C	Temperature	Food industry	[71]
Donkey milk	–	–	–	–	190 °C	90 °C	Temperature	Food industry	[28]
Liposomes	Calcium alginate	Encapsulating agent	–	–	–	–	–	Pharmaceutical industry	[72]
Paeonia sect *Moutan* DC seed oil	Whey protein isolate: corn syrup: soy lecithin	Encapsulating agent	500 l/h	5 ml/min	180 ± 5 °C	90 ± 5 °C	Oxidative	Food industry	[73]
Lactococcus lactis ssp. cremoris and Lactobacillus acidophilus NCFM	Reconstituted skim milk	Protectant/ encapsulating agent	–	0.85 ± 0.08 g/min	97 ± 5 °C	58 ± 2 °C	Temperature	Food industry	[74]
Small interfering RNAs	Mannitol	Encapsulating agent	35 m^3/h	473–742 l/h	80 to 200 °C	60 to 125 °C	Temperature	Pharmaceutical industry	[75]
Antidesma puncticulatum Miq. anthocyanin-rich extract	Maltodextrin	Encapsulating agent	473 l/h	6 ml/min	140 °C	–	Temperature	Food industry	[76]

(continued)

Table 1
(continued)

Sample	Auxiliary molecules		Operating conditions				Stability	Destination	References
	Molecule	Acting as	Air flow	Feed rate	Inlet	Outlet			
Chicorium intibus and *Brassica oleracea* var. capitata f.rubra poliphenols	Modified starch	Encapsulating agent	40 m^3/h	5 ml/min	140 ± 3 °C	70 ± 3 °C	Temperature	Food industry	[77]
Trypsin	Trehalose	Encapsulating agent	260 ± 5 l/min	–	180 ± 3 °C	90 ± 3 °C	Temperature	Pharmaceutical industry	[78]
Vaccinium macrocarpon Ait. Phenolic compounds	Gum arabic: maltodextrins (M1, 10–13 DE; M3, 17–20 DE)	Encapsulating agents	600 l/h	–	185 °C	105 °C	Storage, temperature	Food industry	[79]
Grape skin anthocyanins extract	Sodium alginate	Encapsulating agent	4.2 m^3/h	0.85 ml/min	120	70	Light, temperature	Food industry	[80]

its stability, and altering its bioavailability in the organism. Through this technique, it is possible to obtain particles with a gradual and controlled release of the active principle. This is of great importance as it allows a gradual dosing of the same and that it remains constant during therapy [63, 75, 78].

Mainly due to these characteristics, the spray-drying technique is progressively a viable resource for different industries, which is constantly being improved, by varying the molecules, the carriers, and the operating conditions, allowing to explore a whole new range of molecules and products. This technique allows to explore and develop the industries, more precisely the food and pharmaceutical industry.

3.1 Spray-Drying Operational Conditions

The spray-drying operating conditions must be chosen considering the chemical features of the material that will be spray-dried. The use of the spray-drying is increasing due to its ability to protect molecules from deterioration and volatile losses, allowing the protection of the target compounds from adverse factors such as light, moisture, oxygen, among others [81].

Furthermore, this technology also allows the encapsulation of bioactive compounds, leading to the increase in their solubility, their affinity with the destination matrix, or to allow a controlled release [81]. Bearing this in mind, the deep knowledge regarding the chemical features of the material to be spray-dried, as well as the final desirable particles, are crucial to establish the most efficient operating conditions. The secret to a successful spray-drying operation is the choice of the operating conditions, namely inlet/outlet temperature, drying air flow rate, feed flow rate, speed of the atomizer, carrier agent, and respective concentration [82, 83].

3.1.1 Temperature

One of the most important parameters to be considered is the temperature. This technology needs high temperatures that can cause thermal degradation of the target molecules. In fact, the material to spray-dry has a very short contact with high temperatures, namely the inlet temperature that is commonly in the range of 150–220 °C and the outlet temperature between 50 and 80 °C. In general, there are several factors directly affected by the inlet temperature such as the final size of the produced particles which is directly related with the inlet temperature; high temperatures lead to faster solvent evaporation, causing the faster production of spheres without the ideal shrinkage, thus producing larger particles [84].

Also, the solubility of the final powders is affected by the inlet temperature. Daza et al. [84] described that an increase in the inlet temperature from 120 to 160 °C improved the solubility of samples. For instance, the outlet temperature is a crucial parameter and must be controlled to assure that this temperature is lower than the thermal degradation temperature of the constituents, to avoid the powder degradation by high temperatures [85, 86].

Several studies suggest that the outlet temperature is the most relevant to control the droplet drying temperature or droplet drying speed. The outlet temperature is directly related and increases with the increase of the inlet temperature and drying flow rate and decreases with the decrease in the feed flow rate and atomizing air flow rate [87]. High outlet temperatures cause the reduction in moisture contents, increasing the process yields, while low outlet temperatures improve the sphericity of particles, causing higher retention of some compounds such as anthocyanins, thus being a key factor on the physicochemical properties of the final powders [87]. Nevertheless, this temperature cannot be too low, as it can lead to water accumulation in the final product, resulting in a significant decrease in the product stability and shelf life.

The direct contact of the material with inlet temperature causes significant heat and mass transfer during the droplet process and affects the particle formation caused by the high rate of solvent evaporation. Singh et al. [85] analyzed these effects and described that this leads to a pressure gradient inside, but also outside the droplet, causing morphologic alterations in the final powder, namely surface roughness.

Thus, a thermal equilibrium must be found in order to maintain the particles stability during the process, without compromising the final stability.

3.1.2 Carrier Agents

Carrier agents are very important to overcome some drawbacks of spray-drying. For instance, samples with high concentrations in sugars mostly cannot be spray-dried without a carrier agent, due to their stickiness, leading to serious drying problems and consequent low yields [86]. The use of carrier agents decreases the stickiness of samples and their hygroscopicity allowing the obtention of dried powders.

Arabic gum, maltodextrins, starches, pectin, alginates, and combinations of these agents are the most used carriers [88], used for their high solubility, low viscosity, high molecular weight, capacity to decrease stickiness, and protect the material from external factors such as heat, oxygen, humidity, pH, among others [86].

Regarding the concentrations of carrier agents and analyzing the results from the literature (Table 1), significant different amounts of carrier agents are applied even for the same samples; thus the concentration of the carrier agents must be applied accordingly to each different sample.

3.1.3 Feed Concentration and Rate

The feed flow rate corresponds to the atomizer speed. Specifically, it relies on the concentration of the feed solutions; higher concentrations have high solid contents and lead to the presence of less solvent in the droplets, causing short evaporation times and the formation of agglomerates constituted of porous final particles with low density [89].

Another important factor is the feed rate that basically corresponds to the speed of the atomizer system. If the feed rate is high, the systems will need more energy to evaporate the solvent from the droplets, and it does not allow an ideal interaction between the feed droplets and the hot air, producing wet particles that stick on the wall of the drying chamber and leading to a less effective heat and mass transfer, corresponding to high moisture contents in the final particles, and low processed yields [87, 90].

According to the available literature, high feed rates lead to lower yields in the spray-drying process, and they increase the particle size and bulk density [91]. Nevertheless, there are also some exceptions such is the case of the same authors [91] thar described that the spray-drying process of orange juice with high feed rates lead to less moisture in the final particles and less bulk density.

3.1.4 Atomization Parameters and Drying Gases

Probably the most important parameter of the spray-drying process is the atomization step; it is crucial to the final particles size, density, velocity, among other important characteristics of the final powders. The main objective of the atomizer is the maximization of the surface volume of liquid area of the feed solution for an efficient drying step. Therefore, choosing the ideal atomizer system is crucial to the final particles and to their physicochemical parameters, since their properties are directly related with the atomizer design and performance features [92].

Concerning the atomizer conditions, the pressure is also an important parameter and also influences the final product features. According to the available literature, higher pressures in the range of 1–2.5 bar create smaller particles and larger surface areas, increase the total solid percentage and bulk density, in turn increasing the drying process efficiency [86, 93]. In another study, Tee et al. [92] reported that increasing the atomizer pressure from 80 to 100% produced smaller particles and decreased the moisture content, while also increasing the process yield and hygroscopicity.

Nevertheless, the use of excessive pressures also leads to an enormous energy consumption without bringing additional benefits regarding the particle size and yields of the process.

Particular attention must be given to the atomizer choice; the most commonly used are the rotary atomizer, pneumatic, ultrasonic, and hydraulic nozzles. When comparing the efficiency of these different atomizers, the literature describes that rotary atomizers create larger particles when comparing with nozzle atomization, and two-fluid nozzle atomizer usually produces the smaller particles [86].

The speed of the atomization is another parameter that directly influences the final product characteristics. For instance, higher atomization speeds (10,000 to 25,000 rpm) usually lead to a

decrease in the moisture contents and a reduction in the final size. As the increase in the atomization speed results in the increase in the flow rate, creating tiny particles, resulting in a higher area of contact that allows for a faster drying procedure, and an increase in the yields [86–94].

Different gases are commonly used in the spray-drying process, being compressed air, CO_2, and N_2 the most common ones. These gases and their properties also represent key factors to the success of the spray-drying products. For instance, the use of low-density gases such as nitrogen, an inert gas, which is commonly used in solutions with high concentrations of organic solvents and in solutions with easily oxidable compounds, produces smaller particles with different surface morphologies [85]. On the other hand, CO_2 that presents higher density properties produces larger particles. Several authors reported the effect of the atomization gas type and concluded that the crystallinity of the final particles is directly influenced by the type of gas, describing that N_2 allows the production of higher crystallin particles than CO_2 and compressed air [95]. On the other hand, the atomization with CO_2 means higher temperature and mass transfer during the process, obtaining higher efficiency in the drying process, resulting in 20% faster drying, which offers 4% energy savings on the heat input according to Kudra and Poirier [96].

As stated above, all the spray-drying parameters are strongly related with the final particle's characteristics, namely in terms of particle distribution, moisture, yields, particle size, and morphology. According to the final applications, the operating conditions can be adjusted and optimized to target different morphologies, yields, particle sizes, and distribution.

Furthermore, these parameters can be optimized using mathematical models such as the response surface methodology that can predict the ideal operating conditions to the desirable target particle characteristics, a technology based on reduced experimental data that is already applied to the spray-drying processes, namely by the pharmaceutical industry to increase particle yields [86, 97].

4 Challenges, Trends, and Conclusions

Considering that published papers in 2003 with the keywords "spray-drying" were about 200 and had risen to over 1000 in 2017, it is clear that this technology has improved over time, and pondering the consistent growth until today, it is expected to continue into the next decade [98]. Although spray-drying technology is centuries old, it is still developing, and important developments have been introduced in the past years, with some important ones that are previewed for the near future. With

globalization, the cost of the equipment has seen some important reduction in their price, allowing more businesses and research centers to use them, thus creating a bigger potential audience for improvements which in turn encourages more improvements and breakthroughs. Sosnik and Seremeta [5] stated that the trends in spray-drying would include the production of finer particles, narrowing of the size distributions, and the improvement of yields by reduction of product loss on the walls of the drying chambers. The improvements in polymer chemistry and nanotechnology are also important for the miniaturization of encapsulation techniques, allowing for the encapsulation of smaller and smaller products, which result in finer powders that are suitable for broader applications. Another improvement that is envisioned for the near future is the stability of the dried or encapsulated products over a longer period, as well as the improvement of nano-spray driers. Special emphasis is also considered in the food industry, due to the higher need for encapsulation of natural food additives. These natural compounds have seen increasing demand by consumers due to the concomitant increase of distrust of synthetic additives and the need of higher stabilization from the natural additives [86, 98, 99].

Overall, the contribution of spray-dryers to the food and pharmaceutical industries is undeniable. From a simple method of drying and encapsulating food during the end of the twentieth century to a technology that allows controlled release of ingredients and active compounds in drugs, spray-drying is an essential technology in the twenty-first century. With the previewed enhancements to the technique, longer shelf-lives are expected in foods, better efficacy in drugs, nano-spray driers, and an overall reduction in the cost of the equipment which will democratize its use, leading to a broader use.

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Chapter 3

Calcium Alginate Capsules: Particularities of Natural Antioxidants and Plant Germplasm Systems

Lorena Deladino and Aline Schneider-Teixeira

Abstract

Calcium alginate beads have been extensively used in microencapsulation because they are extremely easy to prepare on a lab-scale, the process is very mild, it can be conducted in sterile environments, and virtually any ingredient can be encapsulated, whether it is hydrophobic or hydrophilic, sensitive to temperature, liquid or viscous oil or solid, and even shoot tips. However, for each specific active compound, fundamental understanding of the physical and chemical phenomena determining stability, release, perception, and final use must be taken into account.

Natural antioxidants from *Ilex paraguariensis* were encapsulated for food applications, as a practical solution to protect them from the surrounding medium (other food constituents or gastric juices) or the processing conditions during food production. Moreover, controlled release of the active compound can be also a desired objective. Whereas, in the case of explants, encapsulation allows the subsequent application of very drastic treatments including preculture with high sucrose concentrations and desiccation to low moisture contents which would be highly damaging or lethal to nonencapsulated samples. Thus, the same technique, with identical encapsulating materials, can be used for these two very different purposes of research in agri-food.

Alginate beads with shoot tips of a many number of species had been successfully cryopreserved. An encapsulation-dehydration or encapsulation-vitrification protocol comprises the following steps: pretreatment, encapsulation, preculture, desiccation, freezing and storage, warming, and regrowth.

Key words Microcapsules, Ionic gelation, *Ilex paraguariensis*, Cryopreservation, Encapsulation-dehydration, Encapsulation-vitrification

1 Introduction

Alginate is a linear polysaccharide composed of two monosaccharide residues, (1,4)-β-D-mannuronic acid (M) and (1,4)-α-L-guluronic acid (G). The particularity of alginate is that it can form a physical hydrogel (insoluble form) in the presence of divalent cations such as Ca^{2+} and Ba^{2+}, which form an ionic cross-linking between the G monomers of two adjacent polymer chains [1]. It

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can be extracted from marine brown algae (*Phaeophyceae*) and as capsular polysaccharides in soil bacteria [2].

This gelling ability, almost independent of temperature, is took in advantage to produce calcium alginate microcapsules; when a sodium alginate solution is drop wised into a gelling bath, instantaneous gelification occurs turning each drop into a potential carrier system. Active compound can be solved in the alginate solution, in the gelling bath or in other solution and get into the porous matrix of the microcapsule later, by diffusion.

Depending on processing conditions (concentrations of alginate and calcium solution, immersion times, collecting distance, etc.), the microcapsules are more or less porous and allow fast and easy diffusion of water and other fluids in and out of the alginate matrix. This might be an advantage for the immobilization of live cells and enzymes that are meant to be accessible to their environment but is a drawback when trying to protect an ingredient from the environment. In this occasion, additional layers of calcium alginate or chitosan over formed beads have been applied to increase protection or delay releasing rate. In the case of chitosan, an additional factor like the formation of a chitosan–alginate complex should also be considered [3].

Polyphenols are the major plant compounds with antioxidant activity, although they are not the only ones. In addition, other biological properties such as anticarcinogenicity, antimutagenicity, antiallergenicity, and antiaging activity have been reported for natural and synthetic antioxidants [4]. As a case of study, yerba mate (*Ilex paraguariensis*) is a traditional beverage highly consumed in South America rich in polyphenols [5, 6], and its aqueous extract has been encapsulated in our lab in different calcium alginate-based systems with applications in food industry [7–12]. Obtaining spherical and monodispersed particles is crucial to develop highly reproducible reaction or controlled release rates, to produce free-flowing particles during dosing and handling in order to ensure dosage consistency and dust-free environment (due to particle abrasion), and to improve the aesthetic quality [13].

In this sense, Chan et al. [13] developed prediction models for shape and size of ca–alginate beads; the main conclusions that they achieved were that the minimum alginate concentration required to enable the formation of spherical bead was 1.5% (w/v). Sphericity factor was found to be an efficient dimensionless shape indicator to study the shape of the alginate drops which could be distinguished into four basic types: tear-shaped, egg-shaped, pear-shaped, and spherical. Spherical form is directly related with the collection distance whereas bead size was affected by the tip size and the M/G ratio of alginate. In this sense, an increase in either the molecular weight or the M/G ratio of alginates resulted in an increase in the size of the microbeads, due to an increase in the viscosity of the solutions or the swelling capacity of the hydrogel

networks, respectively. At the nanostructural level, the alginate with the highest molecular weight gave rise to more densely packed and ordered features, with smaller junction zones, whereas M/G ratio had a greater effect on the density of the hydrogel networks, which was lower for the high-M alginate [14]. However, the high viscosity of higher concentration solution maybe a limitation for the peristaltic bomb or dripping device selected.

Calcium chloride concentration determines the porosity of the matrix, and Pathak et al. [15] studied this effect with calcium concentrations between 0.2 and 1.7 M and found that porosity decreased with ion content.

Deeping into the wide spectrum of calcium alginate beads, encapsulation-dehydration [16, 17] or encapsulation-vitrification [18] procedures arise as cryopreservation techniques. Cryopreservation methods and in vitro culture of plant germplasm are tools used to conserve the genetic diversity of rare cultivars, landraces, and threatened plant species from around the world. Species that are on the brink of extinction because of the rapid loss of genetic diversity and habitat come mainly from resource-poor areas of the world and from global biodiversity hotspots and island countries. These species are unique because they are endemic, and only a few small populations or sometimes only a few individuals remain in the wild [19–21]. Cryopreservation is also employed for uses other than germplasm conservation, and it has been assessed as a means of eliminating viruses from infected plants (cryotherapy), as a substitute or in complement to classical virus eradication techniques such as meristem culture and thermotherapy [22].

Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra-low temperatures, preferably at that of liquid nitrogen (LN). The development of cryopreservation strategy for plant cells and organs has followed the advances made with mammalian systems, albeit several decades later [23].

Encapsulation (dehydration or vitrification) is based on the technology developed for producing synthetic seeds, through the encapsulation of explants in calcium alginate beads [24] that generate protection against ice-crystal formation, and improving survival after direct immersion in liquid nitrogen (−196 °C) [25].

These procedures replace freeze-induced cell dehydration at very low temperature by removal of a major part of freezable cell water through exposure to air-drying (encapsulation-dehydration protocol) at nonfreezing temperature before freezing or to a highly concentrated vitrification solution (vitrification protocol). Different plant vitrification solutions (PVS) have been developed by various research teams worldwide [26]. However, the most commonly used solutions are the glycerol-based vitrification solutions designated PVS number 2 (PVS2) [27–29] and number 3 (PVS3) [30, 31].

2 Materials

2.1 Reagent Preparation

Prepare all solutions using distilled water at 25 °C and analytical grade reagents. Store all the solutions at 4 °C but put them under ambient temperature before encapsulating procedure.

- Sodium alginate: Dissolve the salt in distilled water, under continuous stirring in a hot plate agitator with temperature on (50–100 °C) (*see* **Notes 1** and **2**). Let the solution rest before using it until no visible bubbles exist, at ambient temperature (25 °C).
- Calcium chloride solution ($CaCl_2$): Dissolve the salt in distilled water at desired concentration.
- Whatman #1 paper.

2.2 Natural Antioxidants Encapsulation

- Sodium alginate solution (2% w/v): Medium-viscosity alginic acid and distilled water.
- Calcium chloride solution ($CaCl_2$, 0.5 M): Distilled water and calcium chloride dihydrate.
- Buffer solution (acetic acetate, pH 5.5): Distilled water, sodium acetate, and acetic acid.
- Sodium citrate solution (10% w/v): Distilled water and sodium citrate.
- Peristaltic pump with a silicone tube (3 mm diameter).
- Hypodermic needles of outer diameter of 1 mm to be used as dripping tips (*see* **Note 3**).
- Forced-air convection oven.
- Antioxidant extract: It was obtained from yerba mate (*Ilex paraguariensis*) by mixing 1 g of commercial yerba mate with 100 ml of distilled water in a glass vessel, heated in a thermostatic bath at 100 °C for 40 min. Then, the extract was filtered, transferred to dark flasks, and cooled in an ice bath. Once the liquid extract is at ambient temperature, there are two options: (1) to solve the alginate salt directly in the liquid extract and get ready for bead obtention or (2) to lyophilize the aqueous extract and keep it in tightly closed flasks in a desiccator until using it. Then, before bead preparation, the desired amount of lyophilized extract can be easily solved in the alginate solution.

2.3 Shoot Tips Encapsulation

All the material preparation and procedures for plant germplasm encapsulation must be performed in sterility.

- Preculture medium: Solid medium (agar or phytagel) supplemented with solid medium Murashige and Skoog (MS) [32] and 0.3 M sucrose.

- Load solution: Liquid medium containing MS and 0.75 M sucrose.
- PVS2: 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO, and 0.4 M sucrose in MS medium.
- PVS3: 40% (w/v) glycerol and 40% (w/v) sucrose in MS medium.
- Sodium alginate solution (3% w/v): Low-viscosity alginic acid in liquid MS, at pH 5.7.
- Calcium chloride solution: MS, 1 M $CaCl_2$, and 0.4 M sucrose.
- Pasteur pipette.
- Horizontal air laminar flow hood (0.5 m/s, *see* **Note 4**).
- Petri dishes.
- Shoot tips (1.5–3 mm) in vitro (*see* **Note 5**).

3 Methods

Beads formation must be achieved always at the same temperature conditions (recommended 20–25 °C).

3.1 Natural Antioxidants

1. Prepare the experiment setup as shown in Fig. 1, the bomb with the desired extrusion tip, a glass beaker or graduated cylinder with the natural antioxidant extract dissolved in the alginate solution at 2% w/v, and a glass beaker with the gelling bath of calcium chloride 0.5 M (*see* **Notes 6–9**).
2. Turn on the bomb in fast mode until the alginate solution reaches the needle or tip.
3. Set the bomb at the usual speed (we used 45 rpm, *see* **Note 10**).
4. Let the alginate solution drop into the gelling bath during a determined time (i.e., 5 min, *see* **Note 11**).
5. Beads should stay at least 15 min in the $CaCl_2$ solution [7]. When calcium and alginate solutions get in contact, a gel is formed immediately at the interface, thus matrix homogeneity depends on the calcium diffusion through the gel network [33].
6. Filter the beads through a Whatman #1 paper and wash them with buffer solution (acetic acetate, pH 5.5).
7. Allow beads stabilizing in air for 15 min (calcium chloride still migrates in the interior of the matrix), and the size of the beads is 4 mm (Fig. 1).
8. Dry the beads in a convection oven at 6°°C for 3 h, or until constant weight depending of your system (*see* **Note 12**), and the final size of the capsules is 1.5 mm.

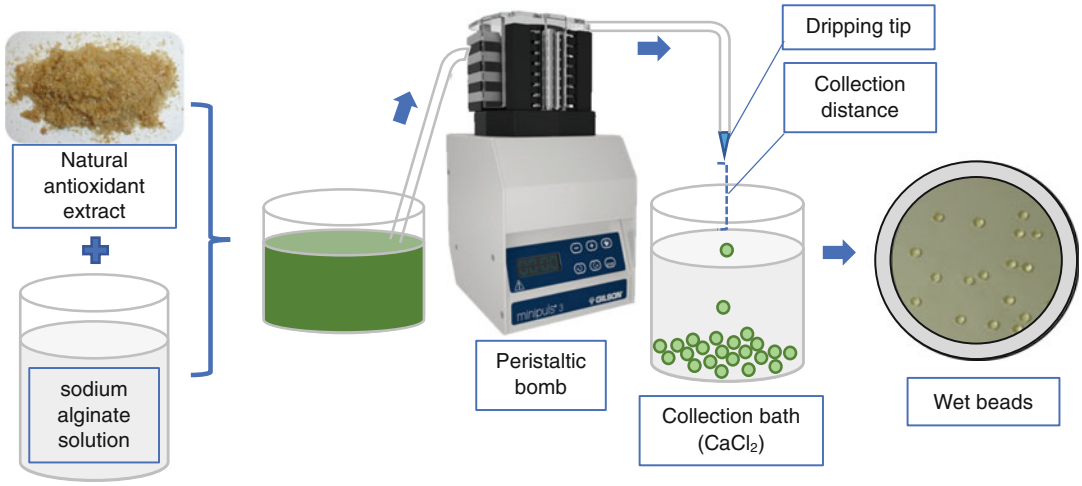

Fig. 1 Scheme of the process to obtain calcium alginate beads loaded with yerba mate natural antioxidants

9. Store the dry capsules in a dark hermetic glass container.
10. The amount of extract in the beads (E_∞) can be performed adding a known mass/number of beads to a sodium citrate solution (5% w/v). Place beads on an orbital shaker at 37 °C and 180 rpm for 3 h. Once beads are totally disintegrated, quantify the polyphenols concentration by Folin–Ciocalteu method [34].
11. Calculate encapsulation efficiency of hydrogels beads as follows:

$$\mathrm{EE}\% = \frac{E_\infty}{E_0} * 100 \qquad (1)$$

where E_∞ is the amount of extract determined after disintegrating the beads with sodium citrate (*see* **Note 13**), and E_0 is the initial amount of extract dissolved in the alginate solution called theoretical load [7].

3.2 Shoot Tips Encapsulation

3.2.1 Plant Material and Shoot Tips Extraction

1. Use one-nodal segments of in vitro plants of selected varieties (Fig. 2). The physiological state of the cells and tissues to be cryopreserved must be optimal for the acquisition of maximum possible dehydration tolerance and for producing vigorous recovery growth.
2. Transfer the one-nodal segments to a preculture medium. Incubate with temperature, photoperiod, and an irradiance controlled for 4 weeks (*see* **Note 14**).
3. Isolate shoot tips with the help of a magnifying glass, moving the axis to the posterior laminar flow.

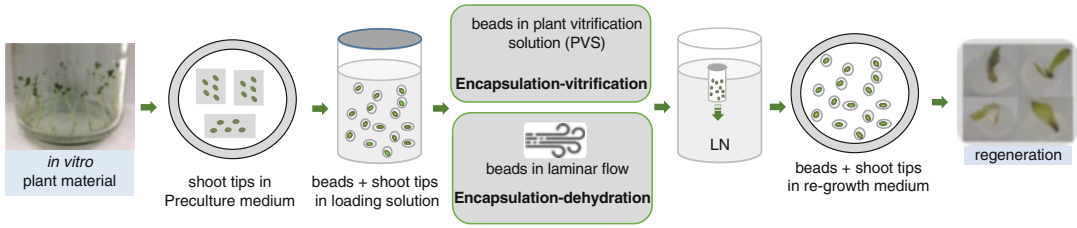

Fig. 2 Scheme of the process for encapsulation-dehydration or encapsulation-vitrification techniques

3.2.2 Encapsulation Protocol: Dehydration and Vitrification

1. Place the shoot tips in a petri dish with preculture medium overnight (Fig. 2).
2. Submerge the explants in an alginate solution for 2 min (*see* **Note 15**).
3. Suck the shoot tips suspended in the alginate solution with the help of a Pasteur pipette, one by one.
4. Drip the alginate-covered shoot tip into a calcium chloride solution.
5. Repeat **steps 3** and **4** until the total amount of shoot tips are encapsulated (*see* **Note 16**).
6. Allow beads to polymerize in $CaCl_2$ solution for 30 min, and discard the solution afterwards by filtering, blotting dry the beads on sterile filter paper.
7. Culture the encapsulated shoot tips overnight in loading solution (Fig. 2) on a rotary shaker (130 rpm) (approx. 10 beads per 50 mL). Filter the solution and blot dry the beads on sterile filter paper.
8. This step requires choosing the type of dehydration to be used. The petri dish must be positioned in the anterior half of the laminar flow.
   (a) Encapsulation-dehydration protocol: Blot dry the beads on filter paper, transfer them to an open glass petri dish, separated enough to do not being in contact between, and dehydrate in a laminar flow hood for different times (*see* **Note 17**).
   (b) Encapsulation-vitrification protocol: Transfer the beads to an open glass petri dish, and dehydrate osmotically with PVS2 or PVS3 in a laminar flow hood at 25 °C or 0 °C for different lengths of time (*see* **Note 18**).
9. Place the beads in a 2 mL cryotube and then directly plug in LN (−196 °C).
10. Transfer the cryotubes rapidly to sterile distilled water in a water bath at 40 °C. Shake vigorously the cryotubes during rewarming for 2 min (*see* **Note 19**).

11. Place the beads on the regrowth medium. After 1 day, transfer the shoot tips to a petri dish containing the same medium (Fig. 2) (*see* **Note 20**).
12. Shoot formation is recorded as the percentage of the total number of shoot tips forming normal shoot about 3–4 weeks after plating, depending on the responsivity of the treated material (*see* **Note 21**).

4 Notes

1. Add the alginate salt little by little in the water to prevent lumping up. If lumps do occur, disarm them slowly with a glass stick.
2. Alginate solubilization can take between 2 and 4 h.
3. Needle size can vary from 0.40 to 1.65 mm, for example [13], or a pipette tip can be used as well, and you may try some options with your own system (it will be also related to the viscosity of the alginate solution).
4. Horizontal laminar flow is preferred due to working comfort and reduced contamination.
5. Studies showed that smaller shoot tips (1.5 or 3.0 mm in diameter) displayed higher regeneration than larger ones (4.5 mm in diameter).
6. Secure the joint of the silicone tube and the needle with a security seal, to avoid disconnecting during the dripping process due to high pressure.
7. Place the dripping tip always at the same height from the gelling bath container, this will give you the exact collection distance of your assay (Fig. 1), and the beads will show the same shape.
8. Make sure you know precisely the amount of extract present per alginate volume, and this date will be useful to calculate the encapsulation technique efficiency.
9. Fill the beaker always with the same calcium chloride volume. It will make your efficiency calculus more precise and you will obtain a more homogeneous product between different batches.
10. Set the bomb always at the same flow (mL of extract alginate solution/min), and make sure the drops fall with a certain time interval between them, so they do not collapse before touching the gelling bath (approx. 1 drop/s).
11. Avoid long dripping times, with short times (i.e., 5 min) the capsules will present uniformity in their polymerization time.

12. For drying, place a single layer of beads in aluminum trails with same space between each one (they tend to stick together) to obtain homogeneous shape.
13. Sodium citrate is a chelating agent which disintegrates the calcium alginate gel [2].
14. Preconditioning of samples includes acclimation (in the case of temperate plant species to the cold) and culture on medium with high sugar level for various durations (in the case of tropical plant species). Samples to be cryopreserved by encapsulation may be submitted to one or both of these treatments, applied successively or concomitantly, depending on the responsivity of the treated material.
15. The shoot tips must be submerged with the help of a forceps, remaining completely immersed in the alginate solution. The presence of hairs on the surface of the plant material makes it difficult to adhere to the alginate solution, also not desired air bubbles can be generated.
16. The shoot tips encapsulation may take time, and it is advisable to make small batches every 4–5 min, so the capsules will present uniformity in their polymerization time.
17. Water content in beads decreases with drying time. The final drying time depends on the responsivity of the treated material, and each plant germplasm species is different.
18. The control of time exposure helps preventing injury by chemical toxicity or excessive osmotic stress during treatment with the PVS2 solution.
19. Rapid freezing and rewarming are essential to achieve vitrification of samples, internal solutes and of the surrounding cryoprotective medium (during freezing), and to avoid potentially damaging devitrification and recrystallization events (during rewarming).
20. The success of an encapsulation protocol is acquired by optimizing the preconditioning and loading treatments, as well as the duration and temperature of exposure to the dehydration. Warming and post-LN handling of the samples are also essential steps to achieve successful procedure.
21. Survival and regrowth can be calculated as percentages over the total number of beads used, the time depending on the responsivity of the treated material. Survival is defined including all forms of visible viability (evidence of green structures or callus). Regrowth is defined as the formation of small plantlets. Use at least six replicates (of at least five beads each) per treatment. Apply arcsine transformation prior to analysis of variance and Duncan's Multiple Range Test ($\alpha = 0.05$) [35].

Check that your transformed data follows (more or less) a normal distribution and homoscedasticity of the variances, as these are the ANOVA assumptions. Use an appropriate statistical test, e.g., for normality, Shapiro-Wilk test, Kolmogorov-Smirnov test, etc. For homoscedasticity, Bartlett, residual plot etc. In some cases, you can use ANOVA for non-normal data, and you should add a reference for this.

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Chapter 4

Pectin-Iron Capsules: A Nontraditional Delivery System Based on Ionic Gelation

Esteban Gerbino and Andrea Gómez-Zavaglia

Abstract

Encapsulation is one of the strategies to protect lactic acid bacteria from technological and physiological adverse environments. This chapter provides experimental conditions to produce iron-pectin microcapsules by ionic gelation, as matrices to encapsulate *Lactiplantibacillus plantarum* CIDCA 83114. Low degree of methylation pectins (from citrus) and $FeSO_4$ can be used to generate the beads, and the obtained beads can be physicochemically, structurally, and thermically characterized using scanning electron microscopy (SEM), swelling, texture and thermogravimetric assays, as well as porosimetry, X-ray fluorescence analysis (XRF), particle size, ζ potential, and Mössbauer and infrared spectroscopy. The experimental details have been provided.

Key words Ionic gelation, Delivery system, Pectin, Iron, Physicochemical characterization

1 Introduction

Ionic gelation is one of the most extended techniques to encapsulate bioactive compounds into polymeric based-particles, with several applications in the food, pharmaceutical, and nutraceutical industries. It allows the production of nanoparticles and microparticles by electrostatic interactions between two ionic species, one of them being a polymer and the other one being an ionic salt. The chemistry of both the polymer (e.g., pK_a values) and the salt determines the capacity of the polymer–ion interaction to encapsulate the compound under study, and also the experimental conditions to carry out the experiments (e.g., pH, ionic strength).

Chitosan and alginate are, by far, the most commonly used polymers in ionic gelation [1]. Other polymers, including gellan gum, fibrin, collagen, gelatin, hyaluronic acid, dextran, carboxymethyl cellulose, and pectins, have also been used. Among these latter, dietary oligo and polysaccharides are particularly interesting for this purpose because of their good biocompatibility,

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nontoxicity, and controlled release properties [2]. This chapter will be focused on pectins, polymers naturally occurring in fruits represented by alternate units of D-galactose, and 3.6 anhydro-galactose bound by α-1,3 and β-1,4-glycosidic linkages and containing within 15% and 40% of ester-sulfates, carrageenans. Those of low degree of esterification (<50%) are useful for ionotropic gelation [3]. Besides that, pectins belong to dietary fiber, so that they are not hydrolyzed in the upper part of the gastrointestinal tract and are able to deliver bioactive compounds whose target is the gut (e.g., intestinal drugs, probiotics) [4, 5].

Calcium and zinc have been majorly employed as counterions for ionotropic gelation. Although the technological efficiency of these ions is out of question, other counterions have been scarcely explored [6, 7]. Among them, iron is particularly relevant because of its important nutritional and physiological function (e.g., binding oxygen to hemoglobin, catalytic center of enzymes). In fact, iron deficiency is the most common nutritional deficit worldwide and represents a public health problem in both industrialized and nonindustrialized countries.

Using iron and pectins to produce micro or nanocapsules can provide a useful strategy for both protecting bioactive compounds from adverse technological and physiological environments and as a source of iron.

Lactic acid bacteria and probiotics have an important role in the food and pharmaceutical industries, as they are extensively used as starters in the development of food and pharmaceutical products. To exert their action, viable bacteria must arrive at suitable levels to the colon. However, technological processes and digestion conditions expose them to adverse environments. For this reason, using pectin-iron complexes to encapsulate probiotics can provide a smart strategy to simultaneously provide iron, dietary fiber, and viable probiotic bacteria. In this chapter, the protocols involved to prepare those systems will be presented.

2 Materials and Equipment

2.1 Materials

- *Lactiplantibacillus plantarum* CIDCA 83114, isolated from kefir grains [8].
- Nonfat milk solids (Difco, MA, USA).
- de Man, Rogosa, Sharpe broth (MRS) (Difco, MA, USA) [9].
- K_2HPO_4, NaCl, Na_2HPO_4 (Sigma Aldrich, Buenos Aires, Argentina).
- Pectin from citrus peel (galacturonic acid ≥74.0%, Sigma Aldrich, Buenos Aires, Argentina).
- Acetic acid (Sigma Aldrich, Buenos Aires, Argentina).

- Sodium acetate (Sigma Aldrich, Buenos Aires, Argentina).
- $FeSO_4$ (Sigma Aldrich, Buenos Aires, Argentina).
- 0.3 mm needle (~10 μL/drop).
- Stainless-steel mesh of 0.10 mm.

2.2 Equipment

- Freeze-dryer Heto FD4 equipment (Heto Lab Equipment, Denmark).
- Sorvall centrifuge (Thermo Fisher, MA, USA).
- Scanning electronic microscope (FEI La B6, Eindhoven, Netherlands).
- Sputter coater (Polaron Thermo VGScientific, East Grinstead, Sussex, UK).
- Analytical balance (Adventurer Ohaus, Parsippany, NJ, USA balance sensitivity: 0.1 mg).
- High-Resolution–Modulated Thermogravimetry Hi-Res-MTGA (TA Instruments Q500, USA; balance sensitivity: 0.1 μg).
- AutoPore IV 9500 (Micromeritics, Atlanta, USA).
- TA-XT2i Texture Analyser (Stable Microsystems, UK).
- X-ray fluorescence analyzer (XRF), benchtop high-sensitivity with an X-ray tube with a tungsten target (Hitachi SEA6000VX, Japan).
- Nano-SZ 100 analyzer (Horiba, Japan).
- Mössbauer spectrometer (WissEL) (GMBH, Germany).
- ATR-FTIR Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA).

3 Methods

3.1 Encapsulation

3.1.1 Microorganisms

- Grow *L. plantarum* CIDCA 83114 in MRS broth at 37 °C in aerobic conditions and monitor the growth by plate counting every 3 h (Subheading 3.2.1).
- Once arrived to the stationary phase (~2×10^8 CFU/mL, overnight), harvest cells by centrifugation, wash them twice with phosphate buffer saline (PBS; K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L, pH 7), and collect the pellets for further encapsulation.

3.1.2 Pectin and Iron Solutions

- Prepare pectin from citrus peel in 0.060 M acetic acid-sodium acetate pH 5.0 (final concentration of pectin: from 1 to 4% w/v) (*see* **Note 1**).

- Prepare $FeSO_4$ solutions in milli Q water in concentrations within 75 and 500 mM (*see* **Note 2**).

3.1.3 Procedure

The following steps should be followed to obtain the iron-pectin capsules (beads):

- Suspend the bacterial pellets (Subheading 3.1.1) in the pectin solution (*see* **Note 3**).
- Drip the obtained suspension into the $FeSO_4$ solutions using a 0.3 mm needle (~10 μL/drop) under continuous agitation for 30 min (Fig. 1a) (*see* **Note 4**).
- Filter the obtained capsules through a stainless-steel mesh of 0.10 mm, and rinse three times with distilled water (Fig. 1b).
- Freeze the capsules at −80 °C and freeze-dry for 48 h (temperature of the condenser: −45 °C; pressure of the chamber: 0.04 mbar) (Fig. 1c).
- Store the samples at 4 °C.

3.2 Microbiological, Thermal, Mechanical, and Structural Characterization of the Beads

3.2.1 Bacterial Plate Counts

- For fresh microorganisms (growth kinetics, Subheading 3.1.1; and controls—non-encapsulated bacteria), dilute 100 μL bacterial of suspensions into 1 mL phosphate saline buffer (PBS) (K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L, pH 7).
- For encapsulated microorganisms, solubilize 10 beads (representing ca. 100 μL of fresh culture) in 1 mL PBS under continuous stirring (*see* **Note 5**).
- Dilute serially bacterial suspensions arising from both encapsulated and non-encapsulated microorganisms and plate them on MRS agar, and incubate at 37 °C for 48 h in aerobic conditions (*see* **Note 6**).
- Carry out this procedure before and after freeze-drying and along storage.

3.2.2 Scanning Electron Microscopy (SEM)

The morphological features and size of resulting microcapsules should be evaluated by SEM as follows:

- Mount the beads obtained in Subheading 3.1.3 (Fig. 1b) on metal stubs with double-sided adhesive tape.
- Coat the stubs with gold using a sputter coater under vacuum and 18 mA at room temperature [10].
- Observe the beads' morphology on a SEM at 14 kV (electron detector for low vacuum conditions), focusing both on the surface aspect and on the inner structure of the capsules.

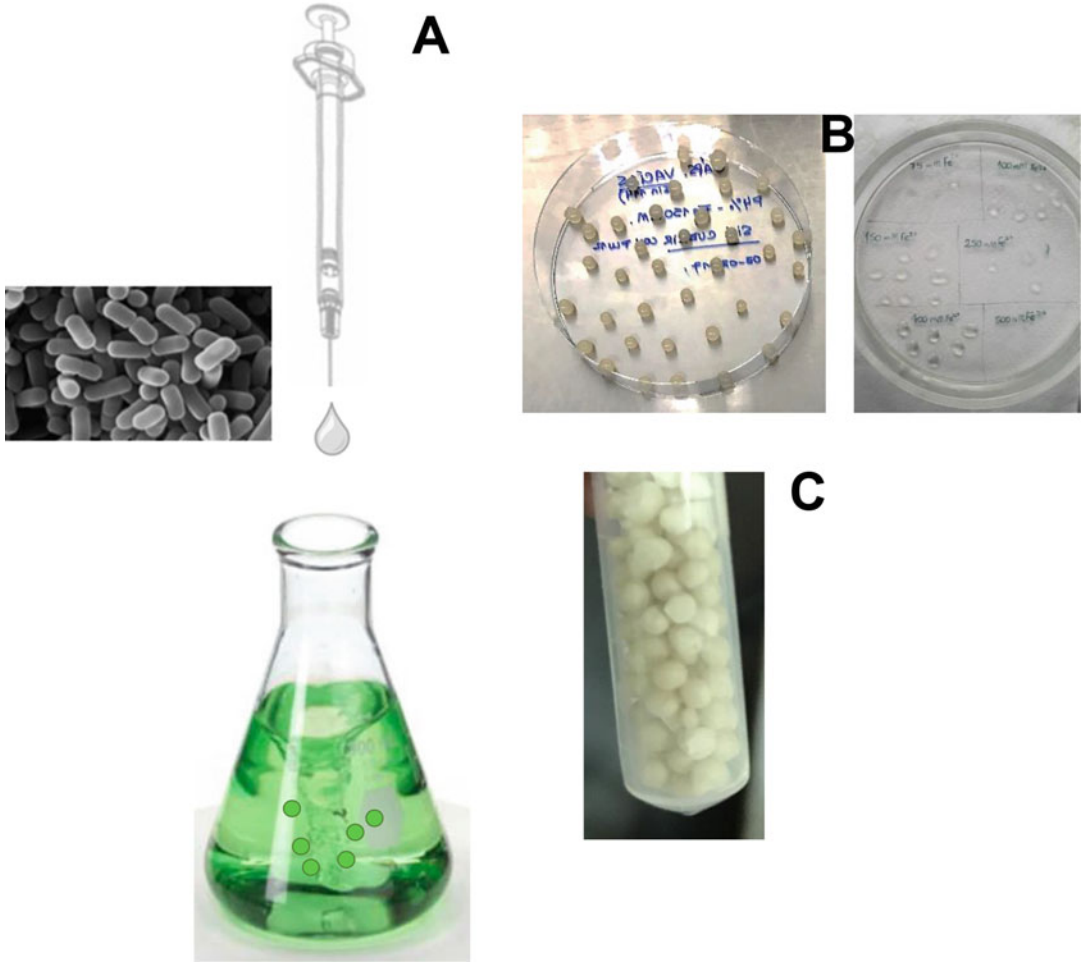

Fig. 1 (**a**) Dripping of pectin solution containing *L. plantarum* CIDCA 83114 into $FeSO_4$ solutions. (**b**) Beads obtained after filtering and rinse the obtained capsules. (**c**) Freeze-dried capsules

- For examination of the inner structure, cut the beads in half with a steel blade.

3.2.3 Swelling of the Beads

- Weigh the freeze-dried beads (Subheading 3.1.3, Fig. 1c) on an analytical balance.
- Hydrate them for 15 min in water for 60 min.
- Dry the beads were dried for 5 min at 20 °C on cellulose paper (Whatman no 1) and weigh them again.
- Calculate the degree of swelling (ability to absorb water in the interstices of the microspheres) as:

$$\frac{Wh - Wd}{Wd} \times 100 \quad (1)$$

where *Wh* is the weight of hydrated beads, and *Wd*, the weight of the dehydrated ones.

3.2.4 Mechanical Characterization of Microcapsules

Analyzing the tensile properties of the capsules is important to determine their stiffness and flexibility. Tensile tests should be performed according to ASTM D638-94b method (1994).

- Prior to running the mechanical tests, condition the beads at 65 ± 5% relative humidity for 72 h at 20 °C.
- Then, cut them into strips of 5 × 25 mm.
- Fix strips between the grips with an initial separation of 50 mm in a texture analyzer.
- Set the crosshead speed at 10 mm/min.
- Calculate the ultimate strength (σ_u), elongation at break (ε_u), and elastic modulus (E).

3.2.5 Thermogravimetric Analysis (TGA)

The thermal stability of the beads should be determined by performing thermogravimetric analysis as follows:

- Weigh 10 mg of beads.
- Calibrate the High-Resolution–Modulated Thermogravimetry Hi-Res-MTGA equipment by measuring the Curie point of nickel standard in open platinum crucibles, under a dry nitrogen purge flow of 100 mL/min (heating rate: 2 °C/min; modulation period: 200 s; amplitude temperature: ±5 °C).
- Determine the weight loss of the capsules within 20 and 600 °C.
- Plot results as weight loss vs temperature and, if necessary, determine the first derivative of the weight loss.

3.2.6 Mercury Intrusion Porosimetry

To determine the porosity of iron-pectin beads, the mercury intrusion should be assessed as follows:

- Quantify the porosity and the pore size distribution of the freeze-dried beads (Fig. 1c) by mercury intrusion porosimetry at a pressure of 0.5 and 30 psi.

3.2.7 X-Ray Fluorescence Analysis (XRF)

- Determine the chemical composition of the iron-pectin beads in an XRF equipment. Use commercial pectins as controls not containing iron.
- Undertake measurements in air at atmospheric pressure, operating at potentials of 15 and 50 kV and a current of 1000 mA, and a 3-mm-wide primary beam collimator (energy-dispersive Vortex Si semiconductor detector positioned at a scattering angle of 135° and a distance of 19 mm from the sample, with no additional slits to restrict its acceptance angle).

- Express results as percentage of the normalized detected elements (e.g., S, Cl, Ca, Cr, Mn, Fe, Cu, Zn, Br, K) or in ppm.

3.2.8 Particle Size and Zeta (ζ) Potential Measurement

- Place samples in sample holders of a Nano-SZ analyzer, at 37 °C.
- Determine the average hydrodynamic particle size (Z-average) using dynamic light scattering at backward scattering (173°) using the equipment software.
- Determine the ζ potential by measuring at least 10 determinations for each sample.

3.2.9 Mössbauer Spectroscopy

- Smash the beads and register ^{57}Fe Mössbauer measurements in transmission geometry at 20 °C (^{57}Co/Rh source with ca. 10 mCi).
- Fit the obtained spectra using least square fitting of several lines using NORMOS program [11].
- Determine the isomer shifts relatively to α-Fe.

3.2.10 Fourier Transform Infrared (FTIR) Spectroscopy

The molecular interactions of pectins, iron, and microorganisms can be analyzed by ATR-FTIR spectroscopy (*see* **Note 7**) as follows:

- Smash the capsules and place 5 mg on the sample holder of an ATR-FTIR equipment.
- Register at least 10 spectra for each sample in the 4000–500 cm^{-1} spectral range by co-adding 64 scans with 4 cm^{-1} spectral resolution.
- Use OMNIC software (version 8.3, Thermo Scientific, MA, USA) for spectra analysis.
- Investigate changes in the bands at ~1740 cm^{-1} (arising from the νC=O from ester group) and at ~1630–1600 cm^{-1} (due to the νCOO^- from the carboxylate group) to determine the degree of carboxylate methylation (%DM, Eq. 2) [12] and to understand the interaction of iron ions with carboxylate groups.

$$\%DM = \frac{A_{1740}}{A_{1740} + A_{1630}} \times 100 \quad (2)$$

where A_{1740} is the area of the band at 1740 cm^{-1}, and A_{1630}, that of the band at 1630 cm^{-1}.

4 Notes

1. The concentration of pectin to be used will depend on the consistence of the generated capsules. Pectins with low degree of methoxylation are preferable because they have a greater number of free carboxylate groups to interact with cations.

2. The concentration of $FeSO_4$ to be employed for the production of the beads should be defined considering the maximal inhibitory concentration (MIC), that is, the lowest concentration of $FeSO_4$ that inhibits the growth of *L. plantarum* CIDCA 83114.
3. The selected concentrations of $FeSO_4$ and pectin will determine the physicochemical, structural, and mechanical characteristics of the obtained beads.
4. As the size of the capsules will determine the applications of the beads, using needles with diameters lower than 0.3 mm enables a reduction of the droplets and thus, a decrease in the capsules' sizes.
5. For plate counting encapsulated microorganisms, the solubilization of capsules in PBS should be thoroughly setup, to enable a complete release of the encapsulated microorganisms. Instead of PBS, distilled water or NaCl 0.85% w/v can also be used.
6. As lactobacilli are microaerophilic bacteria, incubation in anaerobic conditions is also possible.
7. In order to avoid the spectral interference of environmental water vapor and/or CO_2, FTIR equipment should be appropriately purged.

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Chapter 5

Encapsulation of Lactic Acid Bacteria in Sugar Matrices To Be Used as Starters in the Food Industry

Natalia S. Brizuela, Liliana C. Semorile, Bárbara M. Bravo-Ferrada, and Emma Elizabeth Tymczyszyn

Abstract

Encapsulation of bacterial cultures by freeze-drying enables the storage and distribution of microorganisms in a reduced volume, avoiding the necessity of using freezing temperatures. Here we describe the encapsulation by freeze-drying process of lactic acid bacteria in different sugar matrices (sucrose, trehalose, or maltodextrin) to be used in the food industry.

Key words Freeze-drying, Sugar, Storage, Viability, Lactic acid bacteria, Food, Fermentation

1 Introduction

The industrial exploitation of lactic acid bacteria (LAB) as starter cultures strongly depends on the preservation technologies used, which are required to guarantee long-term delivery of stable cultures in terms of viability and activity [1]. Production of lactic acid bacteria starters requires the application of successive steps: fermentation, concentration, stabilization, and storage. These steps lead to environmental stresses on the bacterial cells, because they involve freezing and drying, long-term exposure to low water activity, and changes of pH [1]. The most widely used technique for LAB preservation is freeze-drying [1–3]. The most important factors determining cell survival include the intrinsic tolerance of strains, the initial bacterial concentration, the growth conditions, the drying medium and protective agents used, the freezing rate, and the storage (temperature, atmosphere, relative humidity) and rehydration conditions [1, 4, 5]. Freeze-dried cultures should ideally have

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the same functionality properties than before being preserved. However, during these processes, the reduction of water activity produces damages on the cell structures and thus, microorganisms may die [6, 7]. To avoid these damages, sugars are usually added to the preservation medium as cryo or lyoprotectants, trehalose, sucrose, and maltodextrin being the most frequently used [8–10]. From a chemical point of view, sugars have hydroxyl groups that can replace water molecules on macromolecules during drying. This replacement of water molecules explains the proteins and lipid membranes protection during preservation and thus, the capacity of cells to recover after such processes [7].

In this chapter, the protocols for the encapsulation of lactic acid bacteria in sugar matrices (sucrose, maltodextrin, trehalose) will be presented (Fig. 1).

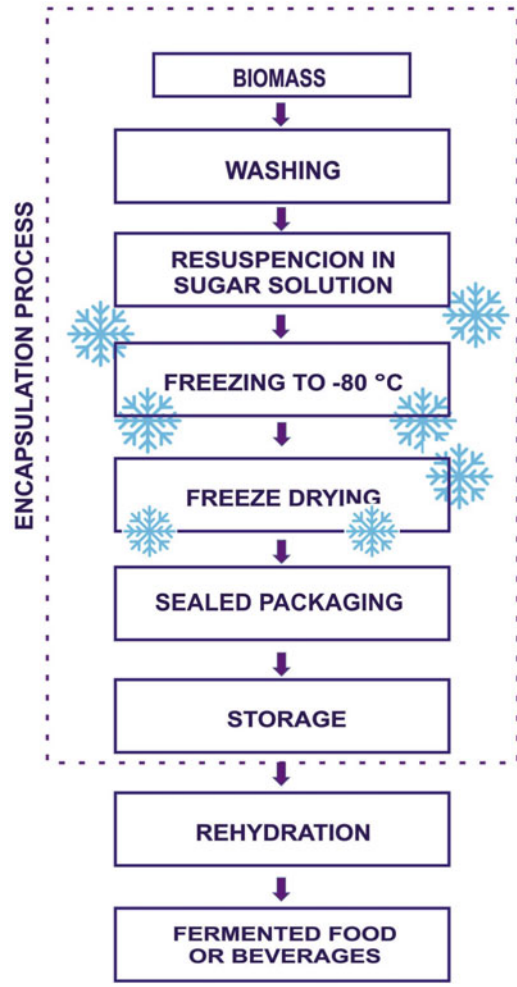

Fig. 1 Schematic representation of encapsulation process

2 Materials

2.1 Reagents

- Distilled water.
- Trehalose for biochemistry (Merck KGaA, Darmstadt, Alemania).
- MRS broth (Biokar, France).
- Sodium chloride (ACS reagent, ≥99.0%) (Sigma-Aldrich).

2.2 Equipment

- Laboratory culture stove (Biotec, model GSA, Argentina).
- Electric autoclave (BioLabs, model SXS16L, Argentina).
- Vacuum filtration equipment (Isolab, Germany).
- Laboratory centrifuge (Mellth, model TDL 80-2B, Argentina).
- Freeze-dryer equipment: Freeze-dryer (Labconco freeze-dryer system/Freezone 4.4, Kansas City, MO, USA). Condenser temperature: −50 °C; chamber pressure: 0.06 mbar.
- Laboratory Ultra freezer (Righi, model UF386-86LV, Argentina).
- Vacuum-packed: Foodsaver® OSTER tumble dryer (model V204).

2.3 Solutions and Media

1. Prepare MRS broth according to product specifications, and autoclave it at 121 °C for 15 min.
2. Dissolve 10 g of trehalose in distilled water to obtain 10% w/v solutions. Sterilize it using a 0.22μm membrane filter.
3. Dissolve 0.9 g of NaCl in distilled water to obtain 0.9% w/v solutions, and then, sterilize it using a 0.22μm membrane filter.
4. Aliquot the solutions in 15-ml sterile tubes and MRS broth into glass bottles.

3 Methods

3.1 Biomass

1. Inoculate 1 ml of the stock bacterial sample into 10 ml of previously sterilized MRS broth (work bacterial suspension).
2. Incubate the bacterial culture at 28 °C for 48 h or until the exponential phase, without shaking (*see* **Note 1**).
3. To encapsulate the bacterial biomass, inoculate 10 ml of the bacterial working suspension into 250 ml of MRS broth, and incubate it at 28 °C for 48 h or until the exponential phase (Fig. 2).

Fig. 2 Scheme of biomass production

3.2 Washing the Bacterial Biomass

1. Fraction the bacterial biomass in 50-ml tubes, and centrifuge them for 15 min at 8000 × *g*.
2. After that remove the supernatant and add 10 ml of 0.85% w/v NaCl solution.
3. Shake the mixture for 1 min, centrifuge it again (5000 × *g*), and discard the supernatant (Fig. 2). This operation shall be repeated twice (*see* **Note 2**).

3.3 Encapsulation

1. Resuspend the bacterial biomass obtained in Subheading 3.2 in 5 ml of a 10% w/v trehalose solution (*see* **Note 3**).
2. Freeze samples at −80 °C for 48 h. Twist the tubes to enlarge the exposure surface (*see* **Note 4**).
3. Freeze-dry the samples at <0.06 mBar ($T < -50$ °C) for 48 h.

3.4 Storage Conditions

1. Store the freeze-dried powders at 4, 20, and 30 °C in sealed package, protected from humidity and light for at least 60 days. Two conditions should be considered: under vacuum and without vacuum (*see* **Notes 5** and **6**).

3.5 Moisture Content

Desiccate freeze-dried samples in an oven at 100–105 °C until constant weight. Calculate the moisture content (MC) on a wet-weight basis according to Eq. 1:

$$\mathrm{MC}\% = \frac{W1 - W2}{W1} \times 100 \qquad (1)$$

where $W1$ and $W2$ are the weights of sample before and after drying, respectively (*see* **Note 7**).

3.6 Cellular Rehydration

Rehydrate freeze-dried cultures in 0.85% w/v NaCl solution at 20 °C for at least 15 min to the original volume (*see* **Note 8**).

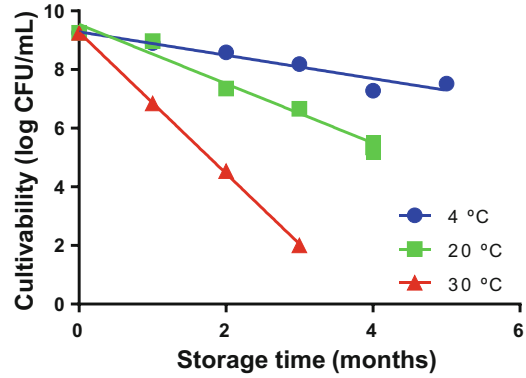

Fig. 3 Cultivability of samples stored at 4, 20, and 30 °C for different periods

3.7 Cultivability of Bacterial Cells

1. Serially dilute rehydrated cells in 0.85% w/v NaCl solution.
2. Plate count cultivable cells on MRS agar plates.
3. Incubate plates at 28 °C for 48 h.
4. Express results as Log CFU/ml.

3.8 Survival Analysis

The analysis of CFU/ml after storage time could help us to determine the deterioration process in dried samples, and so, choose the best condition to stabilize dried samples for prolonged periods, maintaining their activity for food application.

Figure 3 shows the possible values of Log CFU/ml for samples stored at different temperatures and storage periods.

In this representation, we can apply a linear regression and obtain the constant rate of inactivation *"k"* according to Eq. 2:

$$\text{Log CFU/ml} = -k.t \tag{2}$$

where Log CFU/ml is the survival at a given time, t is the storage time (days, weeks, or months), and k, the inactivation constant.

At higher temperatures, the loss of viability is faster, so the k values are higher. Storage at low temperatures improves the shelf life of dried cultures.

In the same way that dried food and pharmaceutical products, the correlation of k values of dried lactic acid bacteria and temperature is given by the Arrhenius model as follows:

$$\text{Ln } k = -\frac{\text{Ea}}{RT} + \text{Ln } A \tag{3}$$

where k is the bacterial inactivation rate constant, A is a frequency factor in units of time^{-1}, Ea is the apparent Energy of activation in kJ/mol, R is the gas constant (8.314 J/Kmol), and T is the storage temperature in K [10, 11].

Figure 4 shows the Ln k for dried cultures stored at different relative humidities (11, 22, and 33%). The estimated value of Ea is important to determinate the type of process occurring in dried

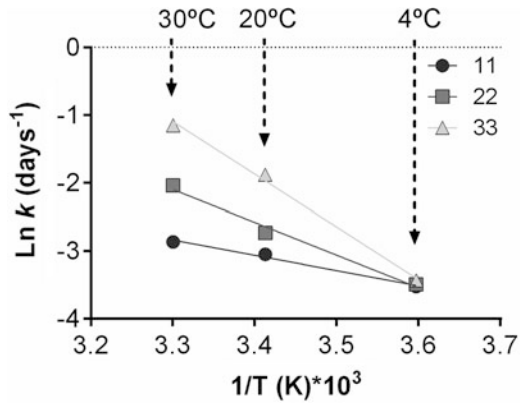

Fig. 4 Arrhenius representation for dried culture. k values were obtained from Fig. 3

samples (e.g., diffusion or chemical reaction). The magnitude of Ea is relevant to compare different encapsulation processes or storage conditions and to determine the best condition (in terms of viability and/or activity) to be applied in food and beverages fermentation processes [12].

4 Notes

1. Incubation temperature: The growth temperature of the cultures depends on each bacterial species.
2. Biomass washing: The biomass washing step can be avoided, but previously we must measure the pH of the growth medium and evaluate the cell death of the bacterial species under these conditions.
3. Encapsulation: Depending on the genus or species of lactic acid bacteria, sugars such as sucrose (purity ≥99.5%, Sigma-Aldrich, solution 20% w/v), maltodextrin DE 12 (Food grade (dextrose equivalent: 12) Ingredion, Buenos Aires, Argentina, solution 10% w/v), fructose (D-(−) fructose, purity ≥99.5%, Sigma-Aldrich, solution 20% w/v) as well as sodium glutamate (mono-sodium glutamate, purity ≥99.0%, solution 20% w/v) can be used. All solutions must be sterilized by filtration using 0.22μm filters.
4. Freezing prior to freeze-drying: Biomass can be frozen at −20 °C. Cell survival results will depend on the bacterial species and the cryoprotectant used. For susceptible bacteria, low freezing rate (such as −20 °C) could be deleterious and high freezing rates (−80 °C) could be used.
5. The dried sample must be stored at temperature under the glass transition temperature (*Tg*), to ensure that samples are in amorphous or vitreous state [13].

6. Storage with and without vacuum: Samples can be stored without vacuum, but in that case, the oxidation of membrane lipids should be also evaluated [14].
7. Water content (Karl Fisher titration), relative humidity % (RH %), or water activity could be measured instead of moisture content (MC) [10].
8. Rehydration: In the case that dried culture must be inoculated in a harsh medium, dried samples should be rehydrated in MRS broth for 24 h, to enhance the bacterial survival.

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Chapter 6

Measurements of Viability in Microencapsulated Bacterial Cells with Flow Cytometry

Karoliny Brito Sampaio, Thatyane Mariano Rodrigues de Albuquerque, and Evandro Leite de Souza

Abstract

Microencapsulation process can affect negatively the viability of microencapsulated cells in different extent. Flow cytometry (FC) is a technique receiving increasing attention in last years as a method to measure viability of bacterial cells. Principle of FC relies on fact that each cell passes a point within the flow path, being intercepted by a laser, which scatters light in two major directions known as forward angle light scatter or side angle light scatter. Cell detection and enumeration involves collection of fluorescence signals emitted at distinct wavelengths from labeled/stained cells as they pass lasers. Data is collected from individual stained cells, and degree of uptake of a particular stain allows differentiation of cells into discrete subpopulations. FC data can reflect distinct physiological conditions and viability of bacterial cells. Therefore, a combination of dyes, such as propidium iodide and thiazole orange, has been successfully used to assess viability in bacterial cells.

Key words Propidium iodide, Thiazole orange, Membrane integrity, Flow cytometer, Cell damage

1 Introduction

Microencapsulation process can affect several physiological functions in bacterial cells, which can result in loss of viability in part of population of microencapsulated cells [1]. Flow cytometry (FC) is a technique receiving increasing attention as a method to measure viability and physiological functions of bacterial cells [2, 3]. FC was developed to make rapid measurements on individual illuminated particles or cells when they flow in a fluid stream passing a sensing point [4]. FC involves the generation of a liquid suspension of bacterial cells from different samples (e.g., food, water, and biological fluids), which are moved within a liquid stream known as sheath fluid. Principle of FC relies on the fact that each cell passes a point within the flow path, being intercepted by a laser, which scatters light in two major directions known as forward angle light

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scatter (forward scatter/FSC) or side angle light scatter (side scatter/SSC). Cell detection and enumeration involves collection of fluorescence signals emitted at distinct wavelengths from labeled/stained cells as they pass lasers. Data is collected from individual stained cells, and degree of uptake of a particular stain allows differentiation of cells into discrete subpopulations. FC data can reflect distinct physiological conditions, such as extent of cell membrane integrity and membrane potential functionality, intracellular enzyme activity, and DNA base composition [5–8]. Additional data can be obtained regarding individual cell physiology and structure by staining cells with specific fluorescent dyes. Combinations of dyes are commonly used to generate multiparametric data from individual cells and subpopulations [9].

Additionally, FC enables fast and reliable detection of multiple parameters in both cultivable and noncultivable cells [2, 3]. Conventional methods for cell population analysis are limited due to need of determining a single value for each cell parameter, which is considered representative of a whole cell population. In contrast, FC provides segregated data for different cell subpopulations [10]. Some advantages of using FC as a method to measure cell viability are: (a) a very large number of particles can be measured, commonly 5000 cells per second and even up to 100,000 in specialized instruments; (b) measure of multiple cellular parameters on each cell simultaneously; (c) rapid assay time and data generation (1–2 min); (d) use of minimal sample volume (from 5 μL); (e) potential high throughput; (f) multiplicity of stains available to examine various aspects related to cell viability, structure, and/or metabolism (multiparametric); and (g) less laborious and space required compared with conventional plating techniques [8, 11]. Combination of dyes, such as propidium iodide (PI) and thiazole orange (TO), has been used to assess cell viability, through to the differentiation of bacterial cell physiological states on the basis of membrane integrity [12, 13].

2 Materials

Prepare all solutions with sterile utensils. Ultrapure filtered water (prepared by purifying deionized water, such as Milli-Q® water) is ideal for FC analysis. Prepare and store all solutions at room temperature (unless otherwise indicated).

2.1 Dye Dilution

1. Thiazole orange (TO): Use a work concentration of 10 μg/mL in dimethyl sulfoxide (DMSO) for molecular biology. Weigh 0.00001 g of TO and add 1 mL of DMSO (*see* **Note 1**).
2. Propidium iodide (PI): Use the stock solution as commercially purchased (1 mg/mL diluted in distilled water) stored under refrigeration (*see* **Note 2**).

3. Phosphate-buffered saline (PBS): Put 800 mL of distilled water in a suitable container. Add the components in the following order: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.245 g KH_2PO_4. Check if the prepared buffer has reached pH 7.4. Add distilled water to 1 L (*see* **Note 3**).

3 Methods

Carry out all procedures in the dark and at room temperature unless otherwise specified.

3.1 Staining Procedure

1. Sample preparation: Harvest by centrifugation (7000 × *g*, 10 min, 20 °C) and wash twice the microencapsulated bacterial cells with PBS and resuspend in PBS to reach 10^6–10^7 cells per mL (*see* **Note 4**).
2. For each 1 mL of bacterial cell suspension in PBS, add 1 μL of TO and incubate for 15 min at 37 °C. Afterwards, centrifuge (8000 × *g*, 10 min, 4 °C) the sample and discard the supernatant (*see* **Note 5**).
3. Resuspend with 1 mL of PBS to add 10 μL of PI, being followed by incubation for 15 min at 37 °C (*see* **Note 6**). Afterwards, centrifuge (8000 × *g*, 10 min, 4 °C) the sample, discard the supernatant, and resuspend with 1 mL of PBS.
4. Positive control sample: Referred to untreated/non-encapsulated bacterial cells. Preparation of bacterial cells must follow the same procedure used to preparation of microencapsulated bacterial cells, being followed by staining with TO/PI (*see* **Note 7**).
5. Negative control sample: Microencapsulated bacterial cells should be suspended in 70% ethanol for 30 min at room temperature. Ethanol-killed cells must be centrifuged again (7000 × *g*, 10 min, 20 °C), washed twice, and resuspend in sterile PBS, being followed by staining with TO/PI (*see* **Note 8**).
6. Before reading on flow cytometer, each sample must be vortexed for approximately 3 s.

3.2 Flow Cytometer Settings

1. Template: Collect the signs of TO in FL1 and of PI in FL3 band-pass filters. Configure the scatter and fluorescence signals of individual cells passing through the laser zone as logarithmic signals. Use a threshold level adjusted for FSC of 12,000. Operate on sample acquisition at the low flow rate setting (12 μL/min), acquiring a total of 10,000 events for each sample (*see* **Note 9**).

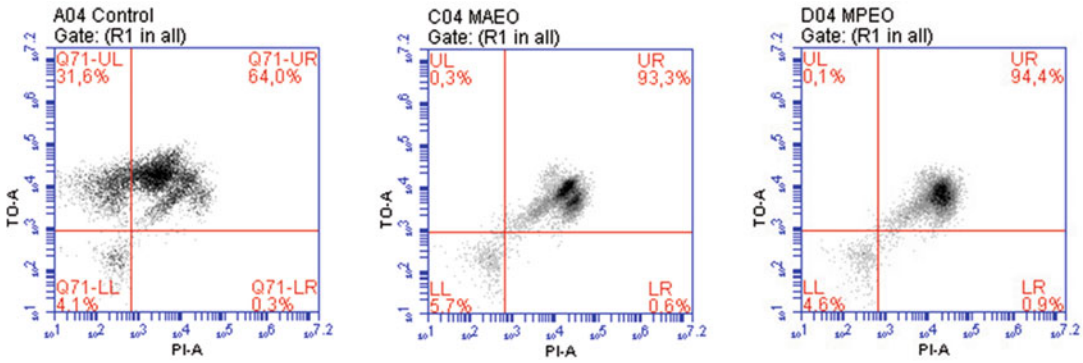

Fig. 1 Fluorescence density plots of bacterial cells in response to staining with TO and PI after exposure to a stressing condition. Control represents bacterial cells without exposure to examine stressing condition. Vertical axis indicates the fluorescence intensity of TO; horizontal axis indicates the fluorescence intensity of PI. Percentage of cell populations that fell in each gate is displayed in four edges of each plot [13]

3.3 Flow Cytometer Reading

1. Pass an unmarked sample before marked sampled in order to define the parameters of cytometer gates (*see* **Note 10**).
2. Pass positive and negative control samples (*see* **Note 11**).
3. Pass samples to be evaluated (*see* **Note 12**).
4. Evaluate graphs generated (Fig. 1) with data acquired for each evaluated sample (*see* **Note 13**).

4 Notes

1. TO is a permeant dye that enters into all cells (live and dead) to varying degrees. TO is dissolved in DMSO to a work concentration of 4.2 mM. After addition to the sample, the final concentration is 100 μg/mL.
2. PI is used for measuring membrane integrity. PI is dissolved in distilled water to a stock concentration of 4.3 mM. After addition to the sample, the final concentration is 10 μg/mL.
3. PBS is a very sensitive solution that needs precision in weighing all components to reach pH 7.4. Shake manually to dissolve all components.
4. Centrifugation and washing the samples prior to staining procedure should help to remove debris and substances that could interfere with analysis. Speed, time, and temperature of centrifugation should be variable according to sample type. Standardization of bacterial cell suspension can be done with measurements of optical density.

5. Proceed with experiment even if you do not see sediment at the bottom of tube. This can happen when working with small sample volumes (<1 mL).
6. The two nucleic acid dyes TO and PI are used in combination to discriminate between live and dead cell subpopulations, respectively, according to membrane integrity.
7. Positive and negative control samples can be adjusted according to characteristics of each experiment.
8. Exposure time used in negative control samples, needed to cause sharp damage in measured bacterial cell functions, is used to direct the selection of exposure time of microencapsulated bacterial cells used in FC analysis.
9. For each cell crossing the laser focus point, two light-scattering signals, forward scatter (FSC) and side scatter (SSC) and two fluorescence signals for red and green fluorescence, should be recorded. Fluorescence of TO is collected in FL1 photomultiplier with a band-pass filter of 525 nm. Fluorescence of PI is recorded in FL3 photomultiplier with a short-pass filter of 620 nm. For each sample, 10,000 events are measured at a flow rate of approximately 800 events per second (eps). Recorded light-scattering and fluorescence signals are collected as logarithmic signals.
10. Blank (unmarked) samples are needed to eliminate any potential auto-flowering bacteria or environment in which they should be found. Controls consisting of live unstained, live TO stained, and dead PI-stained cells are used for fluorescence compensation in order to avoid overlap in emission spectra of these two fluorochromes.
11. Gates representing total (TO-positive) and dead (PI-positive) cells are drawn using control samples consisting of live and dead ethanol treated TO/PI-stained cells. Dead cell subpopulation (nonviable) is defined as cells with a combined high fluorescence of both stains (TO-positive and PI-positive). In order to exclude cell doublets and clumps, a plot of FSC-H versus FSC-A should be created for every analysis.
12. Backflush for backwashing should be done between reading samples to avoid overlap in emission spectra of the two fluorochromes. Remove Eppendorf with sample and place an empty Eppendorf.
13. Results are presented as density dot plots of red versus green fluorescence. Percentage of cells for each subpopulation (viable and dead/nonviable) is determined as the number of events in each gate as a proportion of total number of events.

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Chapter 7

Microfluidic Glass Capillary Devices: An Innovative Tool to Encapsulate *Lactiplantibacillus plantarum*

Gabriel Quintana, Esteban Gerbino, and Andrea Gómez-Zavaglia

Abstract

In this chapter, a detailed preparation of W/O emulsions using microfluidic devices is presented. Soybean oil is proposed as continuous phase, and two hydrophilic polymers, sodium caseinate and alginate, are proposed as dispersed phases. This approach enables the controlled encapsulation of different cells and/or bioactive compounds. The encapsulation of the probiotic strain *Lactiplantibacillus plantarum* CIDCA 83114 is presented.

Key words Microfluidic device, *Lactiplantibacillus plantarum*, Emulsification

1 Introduction

An emulsion is a colloid of two or more immiscible liquids where one liquid contains a dispersion of the other liquids. The process of turning a liquid mixture into an emulsion is known as "emulsification." Generating emulsions by manual or mechanical agitation leads to nonuniform droplets whose formation cannot be precisely controlled. To overcome this problem, and have a better control over the size and polydispersity of the droplets, various methods have been developed. Among them, microfluidics is a kind of microfluidics emulsification method.

Microfluidic technology allows the control of small amounts of fluids through micrometer-scale channels, enabling the production of droplet-based biomaterials [1]. It offers a versatile tool to obtain emulsions, enabling an exquisite control over the size, number, and properties of the droplets, which are formed one-by-one [2, 3]. Microfluidics has been used for the preparation of monodispersed vesicles constituted of particles with different internal structures or for precisely incorporating specific materials inside emulsions [4, 5]. As microfluidic devices have micrometrical

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dimensions comparable to the size of cells, this approach represents an invaluable tool to investigate complex cellular systems [6].

For all these reasons, microfluidics offers the following advantages:

Size reduction: the size of the system is reduced when working with microfluidic devices that have micrometric dimensions, avoiding the use of large workspaces.

Portability: allows an easy transport of the devices from the workplace to the analysis site, without losing the properties of the fluid.

Efficiency: offers a higher analysis speed, greater sensitivity, and greater effectiveness due to short response times.

Cost: low manufacturing costs.

Taking advantage of microfluidic devices, using them for encapsulating probiotic bacteria would not only allow their incorporation into the droplets with greater accuracy, but it would also offer the possibility of incorporating materials having a protective effect on microorganisms from processing and storage conditions. For this reason, in this chapter, the strain *Lactiplantibacillus plantarum* CIDCA 83114 will be encapsulated using microfluidic devices. Different microfluidic devices architectures, enabling the possibility of using more than one dispersed phase, will be presented. As continuous phase, soybean oil will be used.

2 Materials and Equipment

2.1 Materials

- *Lactiplantibacillus plantarum* CIDCA 83114, isolated from kefir grains [7].
- Nonfat milk solids (Difco, MA, USA).
- de Man, Rogosa, Sharpe broth (MRS) (Difco, MA, USA) [8].
- K_2HPO_4, NaCl, KCl, $CaCl_2$, Na_2HPO_4 (Sigma-Aldrich, Buenos Aires, Argentina).
- Sodium caseinate (Sigma Chemical, St. Louis, MO, USA).
- Acetic acid (Sigma-Aldrich, Buenos Aires, Argentina).
- Sodium acetate (Sigma-Aldrich, Buenos Aires, Argentina).
- Soybean oil (Sigma-Aldrich, Buenos Aires, Argentina).
- Elastomer [poly(dimethylsiloxane) (PDMS)] (Sylgard™ 184 Silicone Elastomer Base, Midland, MI, USA).
- Curing agent (Sylgard™ 184 Silicone Elastomer Curing Agent, Midland, MI, USA).
- Trichloro (1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich, St. Louis, MO, USA).

- Lecithin (Sigma-Aldrich, Buenos Aires, Argentina).
- Sodium alginate (Alfa Aesar, Tewksbury, MA, USA).
- 6-carboxyfluorescein (Fluka, BioChemika, Barcelona, Spain).
- Fine-caliber polyethylene tubing (Portex, Smiths medical, Keene, NH, USA).

2.2 Equipment

- Microfluidic devices (chips).
- Freeze-dryer Heto FD4 equipment (Heto Lab Equipment, Denmark).
- Sorvall centrifuge (Thermo Fisher, MA, USA).
- Optical microscope (Optika, Ponteranica, Italy).

3 Methods

3.1 Preparation of the Device

Microfluidic chips are micro-channels etched or molded into an elastomer, connected together to achieve the desired features of the fluids taking part of the emulsions (Fig. 1). For the fabrication of the devices, follow the following steps:

1. Weigh 15 g of PDMS and 1.5 g of curing agent, maintaining an elastomer/curing agent ratio of 10:1 (*see* **Note 1**). Mix thoroughly for 5 min to obtain a total integration of both. Then, connect the mixture to a vacuum pump to degas the material (elimination of the burbles).
2. Pour the degassed preparation over Petri dishes containing a sealed master with the desired channel structure (Fig. 1a).
3. After a second degassing, cover the mold and place it overnight in a stove at 65 °C for the curing process.
4. Cool down the molds to 25 °C and peel off the area surrounding the quadrant (where the chips were located) with a scalpel (Fig. 1b, c).
5. Drill 1 mm diameter holes with a retractable hole puncher (e.g., 50, 200 μm) to define the inlet and outlet points (Fig. 1d) (*see* **Note 2**).
6. Join the chips to a microscope glass slide and seal them with oxygen plasma in a clean room, and flow with trichloro (1H,1H,2H,2H-perfluorooctyl)silane in fluorinated oil HFE-7500 (1%, w/w; 3 M) to provide a channel with hydrophobic surfaces [9] (Fig. 1e). The treatment with oxygen plasma should be carried out as follows:
   (a) Turn on the vacuum pump and plasma machine;
   (b) Set up the power (100 W) and gas flow (150 mL/min) conditions;

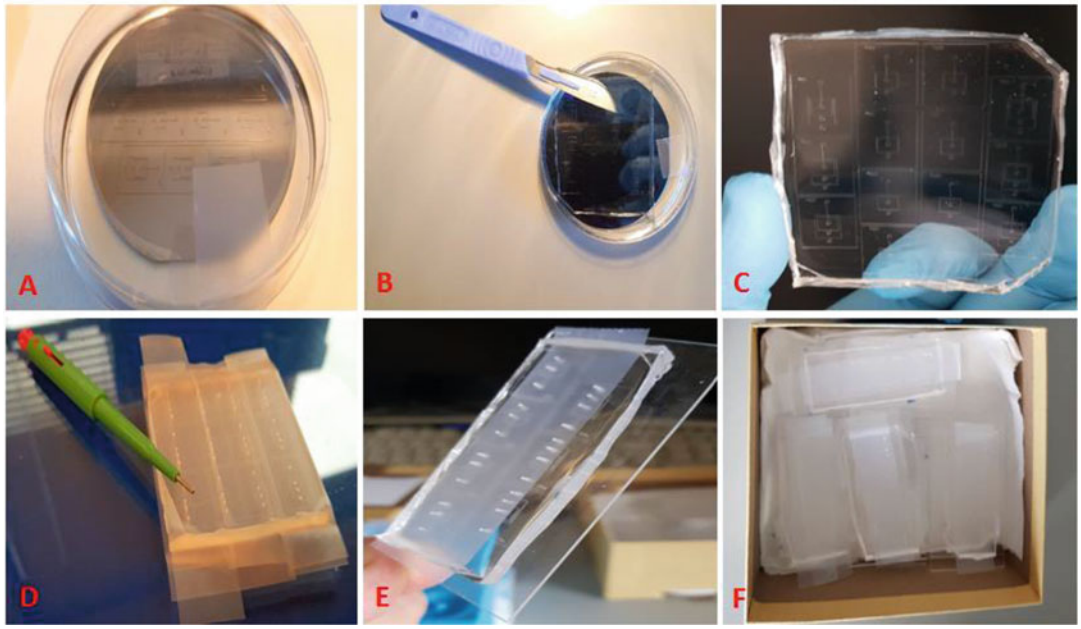

Fig. 1 (**a**) Mold on a Petri dish containing the print of the working chips. (**b**) Plate with the prepared chips, ready to be cut. (**c**) Plate with the printed chips. (**d**) Drill to make the holes at the entry and exit points of the chips. (**e**) Plate with chips sealed on glass plate (slide) and ready to be used. (**f**) Chips taped and stored to prevent the deposition of powder in the microfluidic channels

(c) Place the chips and glasses in the sealing chamber, exposing the side where they will be joined;

(d) Close the chamber and purge it until stabilization (approximately 0.3–0.5 bar);

(e) Open the oxygen tube;

(f) Activate the plasma once the oxygen flow is stabilized (approximately 2 min);

(g) Turn-off the plasma and the gas, and break the vacuum;

(h) Open the chamber, remove the chips and glasses and join them quickly, slightly pressing and checking that no bubbles are present;

(i) Heat on a hot plate at 100 °C for 5–10 min, to strengthen the sealing;

(j) Close the oxygen tube and the gas inlet valve. Close the chamber and turn-off the pump and plasma generator.

7. After that the chips are ready-to-use (Fig. 1f).

3.2 Assembling the Equipment

Microfluidic devices assemblies can be constructed with different architectures, namely devices with two inlet and one outlet (Fig. 2a), or devices with three inlet and one outlet points (Fig. 2b).

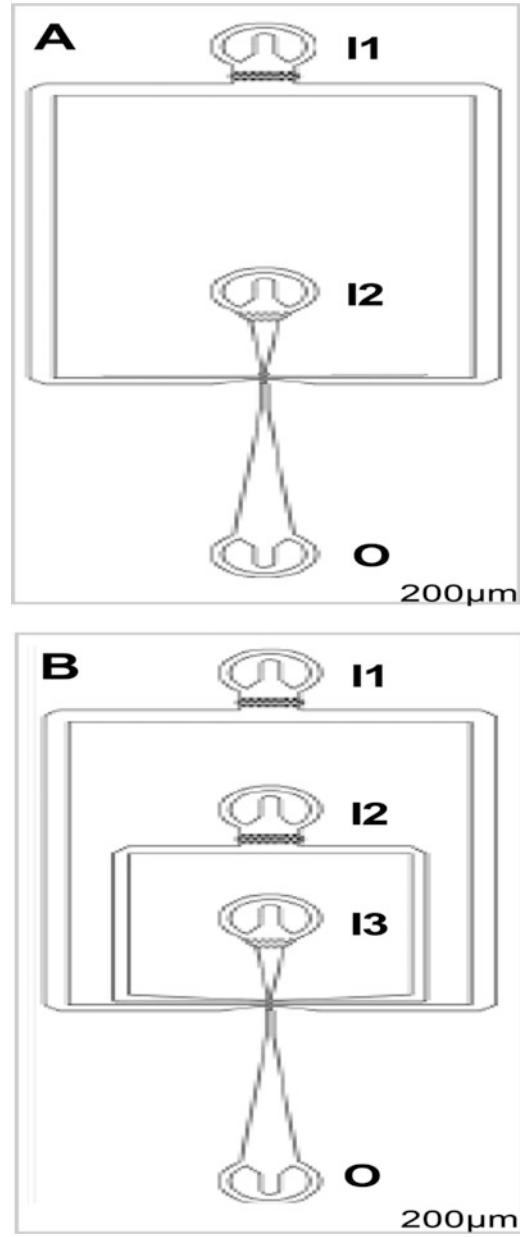

Fig. 2 Different chips assemblies: (**a**) Two inlet points. I1: Inlet point for the continuous phase; I2: Inlet point of the dispersed phase; O: Outlet point; (**b**) Three inlet points. I1: Inlet of the continuous phase; I2: Outlet of the first dispersed phase 2; I3: Inlet of the second dispersed phase 1; O: Outlet point

For generating the water-in-oil emulsions (W/O) both the continuous and dispersed phases should be injected with glass syringes (1 mL and 100 µL, respectively) using Nemesys Apparatus pumps controlled through a Nemesys software (Nemesys Interface, Madrid, Spain). To connect the syringes to the chips' inlet and outlet points, use capillary polyethylene tubes (internal diameter:

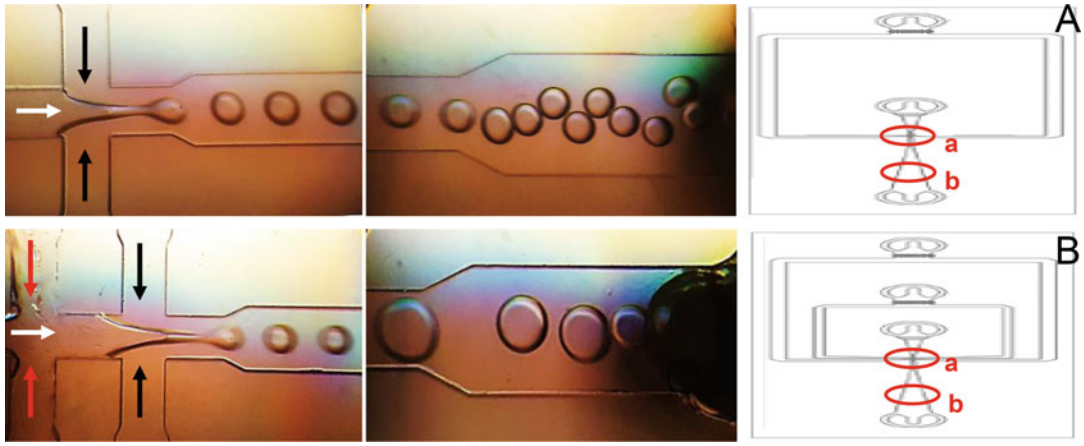

Fig. 3 Microscopic images from W/O emulsions obtained using microfluidic devices. White arrows represent the entrance of the dispersed phase; black arrows, the input of the continuous phase; and red arrows, the input of dispersed phases in emulsions having two dispersed phases. *a* indicates T-junctions of the two phases, and *b*: end point before the outlet. A scheme of the devices is shown on the right part of the figure. (**a**) Chips having two inlet and one outlet points; (**b**) Chips having three inlet and one outlet points

0.38 mm). The formation of the emulsions in the chips can be monitored in real time in an optical microscope connected to a camera, using Vimba software (Vimba v3.1 ARM64, Germany) (Fig. 3).

3.3 Formulation of Emulsions

The continuous and disperse phases used in microfluidic devices can be as follows:

3.3.1 Composition of the Continuous and Dispersed Phases

Continuous phase: soybean oil containing 4% (w/v) of lecithin (emulsifier).

Dispersed phases: sodium caseinate prepared at 6% (w/v) in 0.5 M NaCl or sodium alginate prepared at 1.5% (w/v) in distilled water.

When the goal is to encapsulate microorganisms, possible compositions of disperse and continuous phases are shown in Table 1 (*see* **Note** 3). Note that such compositions are defined for devices containing two inlet and one outlet (Fig. 2a), and three inlet and one outlet points (Fig. 2b).

3.3.2 Microorganisms

- Grow *L. plantarum* CIDCA 83114 in MRS broth at 37 °C in aerobic conditions to the stationary phase (~2 × 10^8 CFU/mL, overnight).
- Harvest cells by centrifugation, wash them twice with phosphate buffer saline (PBS; K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L, pH 7), collect the pellets, and suspend them in the corresponding dispersed phase (sodium caseinate or alginate). Add 6-carboxyfluorescein (CF, 0.5 μM) as marker of the emulsions (Table 1).

Table 1
Examples for the composition of dispersed and continuous phases of emulsions obtained using microfluidic devices

W/O emulsions		
Assay	Phase	Composition
A	Dispersed Continuous	*L. plantarum* CIDCA 83114 in sodium caseinate + CF Soybean oil+lecithin
B	Dispersed Continuous	*L. plantarum* CIDCA 83114 in alginate + CF Soybean oil+lecithin
C	Dispersed 1 Dispersed 2 Continuous	*L. plantarum* CIDCA 83114 in sodium caseinate + CF Alginate Soybean oil+lecithin
D	Dispersed 1 Dispersed 2 Continuous	*L. plantarum* CIDCA 83114 in alginate + CF Sodium caseinate Soy oil+lecithin
Control	Dispersed Continuous	*L. plantarum* CIDCA 83114 in water + CF[a] Soybean oil+lecithin

[a]*CF* carboxyfluorescein

3.4 Fluorescence Microscopy and Droplets' Size

Observe the fresh and freeze-dried emulsions stained with 6-carboxyfluorescein in a fluorescence microscope (Nikon Model Eclipse TS100LED-F MV, Germany) using Vimba software (Vimba v3.1 ARM64, Germany).

Determine the size of the droplets in a fast Read 102^{R} plates composed of 10 counting chambers (Fiji software, Madison, Java8, Germany).

3.5 Freeze-Drying Process

- Freeze the emulsions at −80 °C for 48 h, and then, freeze-dry them at −85 °C for 48 h.
- Store samples at 4 °C in silica gel containing recipients.

3.6 Microbiological and Chemical Characterization of the Emulsions

- Dilute serially the emulsions in PBS.
- Plate them in MRS agar and incubate at 37°C in aerobic conditions for 48 h (*see* **Note 4**).
- Express results as log colony forming units (CFU) per mL.
- Carry out this procedure before and after freeze-drying and along storage.

3.6.1 Bacterial Plate Counts

3.6.2 Scanning Electron Microscopy (SEM)

The morphological features and size of resulting microcapsules should be evaluated by SEM as follows:

- Mount the freeze-dried emulsions on metal stubs with double-sided adhesive tape.

- Coat the stubs with gold using a sputter coater under vacuum and 18 mA at room temperature [10].
- Observe the beads' morphology on a SEM at 14 kV (electron detector for low vacuum conditions).

3.6.3 Particle Size and Zeta (ζ) Potential Measurement

- Place samples in sample holders of a Nano-SZ analyzer, at 37 °C.
- Determine the average hydrodynamic particle size (Z-average) using dynamic light scattering at backward scattering (173°) using the equipment software.
- Determine the ζ potential by measuring at least 10 determinations for each sample.

4 Notes

1. Because of the high viscosity of the elastomer, it should be carefully added either with a spatula or with a pipette. In turn, the curing agent should be dropped to achieve the desired weight.
2. The size of the holes determines the feasibility of formulating emulsions. Holes too small significantly difficult the passage of viscous polymers (e.g., alginate, chitosan). Therefore, it is better to check whether the polymer to be used can flow pass through the defined holes.
3. Although Table 1 shows different compositions of disperse and continuous phases for the encapsulation of *L. plantarum* CIDCA 83114, they can be intended just as examples. Other bioactive compounds (e.g., vitamins, polyphenols) can be also encapsulated following a similar protocol.
4. As lactobacilli are microaerophilic bacteria, incubation in anaerobic conditions is also possible.

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Chapter 8

State-of-the-Art of Encapsulation Based on the Spray-Drying Technique for Carotenoids from Plant Material: Methods and Mechanism

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Abstract

Encapsulation involves the incorporation of food ingredients, enzymes, cells, drugs, or other materials in small capsules. This process consists of surrounding tiny particles with a coating layer conformed of a homogeneous or heterogeneous matrix, to produce small capsules. Therefore, it is a useful tool to fortify foods with bioactive molecules and/or living cells that improve their intact delivery to the target organ, mostly the intestinal tract. Encapsulation goals are to protect, stabilize, and slow down the release of food ingredients. Materials used for designing the protective shell of encapsulates must be food-grade, biodegradable, and able to form a barrier between the internal phase and the external one. Various techniques are employed to form the capsules such as drying, extrusion, emulsification, fluidized bed coating, molecular inclusion, or liposome entrapment. This chapter focuses on reviewing the available spray-drying technique protocols for the specific purpose of encapsulating natural carotenoids.

Key words Microencapsulation, Bioactive molecules, Carotenoid encapsulation, Spray drying, Food application

1 Encapsulating Natural Carotenoids with Spray Drying

Microencapsulation is a technique aimed to enclose tiny particles or droplets of a sensitive substance (core) within a film or coating material in a solid homogeneous or heterogeneous matrix of polymers [1]. Spray drying is a well-known and commonly used method for particle production that comprises the transformation of fluid material into dried particles, taking advantage of a gaseous hot drying medium [2]. This technique has been evolving for over a century, being initially developed for dairy products and volume reduction. Later advances have allowed further applications in

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food, pharmaceutical, chemical, ceramic, and polymer industries [3].

The interest on encapsulating ingredients lies on their chemical instability, thus their bioactivities are protected by encapsulation. This is the case, for example, of carotenoids which are pigments that can be synthesized exclusively by photosynthetic bacteria, fungi, algae, and plants. That is the reason why animals must incorporate them through the diet. For that purpose, this technique is very useful for developing new functional foods based on the incorporation of active compounds into food matrix, providing a protection mechanism for said molecules. However, the stabilization of bioactive molecules is not its only function, as encapsulation also allows controlling the release of the compounds in food, maximizing shelf life, increasing bioavailability mask, preserving flavors and aromas, and turning liquids into easy-to-handle solid ingredients [4].

Therefore, the interest on applying this technique to carotenoids is that it facilitates its intake, which is highly beneficial since this chemical group presents several bioactivities such as photoprotection, antioxidant capacity, enhancement of immunity, precursors of vitamin A, and contribution to reproduction [5]. The main purpose of the use of carotenoids in the food industry is as colorants; however, their low resistance to changes in pH, oxidizing agents, heating, and light exposure may hinder their application. Nevertheless, encapsulation offers protection from oxidation, controlled release, and enables the use of powder format (convenience) and incorporation in aqueous-based food and beverages. Spray drying allows obtaining a product with the desired physical properties by applying variable drying parameters such as temperature, feed flux, and by changing the type of carrier material or pretreatment of the raw material [6].

The addition of encapsulated compounds to food matrixes leads to an increase in total solids content, reducing stickiness and humidity, thus achieving a final product with greater stability and durability. Besides, spray drying is a technique which has been described to preserve antioxidant activity. Vitamins, minerals, colorants, fat and oil flavor, aroma compounds, oleoresins, and enzymes have been encapsulated using this technique due to its effectiveness and low cost [7].

2 Materials for Encapsulation of Carotenoids with Spray Drying

Among the necessary reagents to apply this technique, it is primordial to perform an exhaustive research about the encapsulating wall material. Its selection is critical since it will determine the efficiency and stability of the capsules obtained. To select the most suitable

material, its physicochemical properties must be considered, including solubility, molecular weight, glass/fusion transition, crystallinity, film-forming diffusibility, and emulsifying capacity. The economic cost of the material is another important factor. Concerning carotenoids, the materials of choice to encapsulate them using the spray-drying technique are characterized for being low molecular weight compounds such as polysaccharides (*e.g.*, acacia gum, pectin, cellulose, cellulose derivatives) and/or modified polysaccharides. According to several studies, the best options are modified tapioca starch, maltodextrins, and gum arabic that may be used individually or in combination, arranging them in different layers [6, 8].

3 Methods for Encapsulation of Carotenoids with Spray Drying

Regarding the mechanism of spray-drying encapsulation, it is based on evaporating the humidity of a sample using a heated atmosphere. The process can be divided into three main phases, which are atomization, droplet-to-particle conversion, and particle collection [9]. The correct development of the process requires the previous selection of the dispersant agent to create a homogenize emulsion that will be atomized and dehydrated to obtain a batch of particles that comply the ideal characteristics for encapsulating the target compound. All of these steps, jointly with the conditions in which they are processed, play a crucial impact in the yield of spray-drying mechanism as well as in the final particle properties [2]. A summary of the whole process is shown in Fig. 1. Even though spray drying represents a small step (the ingredients encapsulation) of a complex process, it is the first stage of a chained system and thus its beginning. The final aim is the application of microencapsulated compounds and its use for formulation into food (Fig. 2). Several factors have to be taken into account during the full process design: properties of the active or main ingredient (core), application mode, processing conditions and capabilities, release mechanism, particle size, volume, load requirements, storage stability, legal issues, and cost. Every single step of this mechanism will be described in detail below.

The choice of formulation, processing, and format of the microcapsules depends on the active compound (core) and the final product into which they will be introduced. This chapter is focused on carotenoids as core compounds but choosing the most appropriate methodology also depends on the final application, so covering all the possibilities in detail becomes a difficult duty due to the wide variety of applications associated to these compounds. For this reason, a conceptual scheme will be addressed based on some specific developed examples.

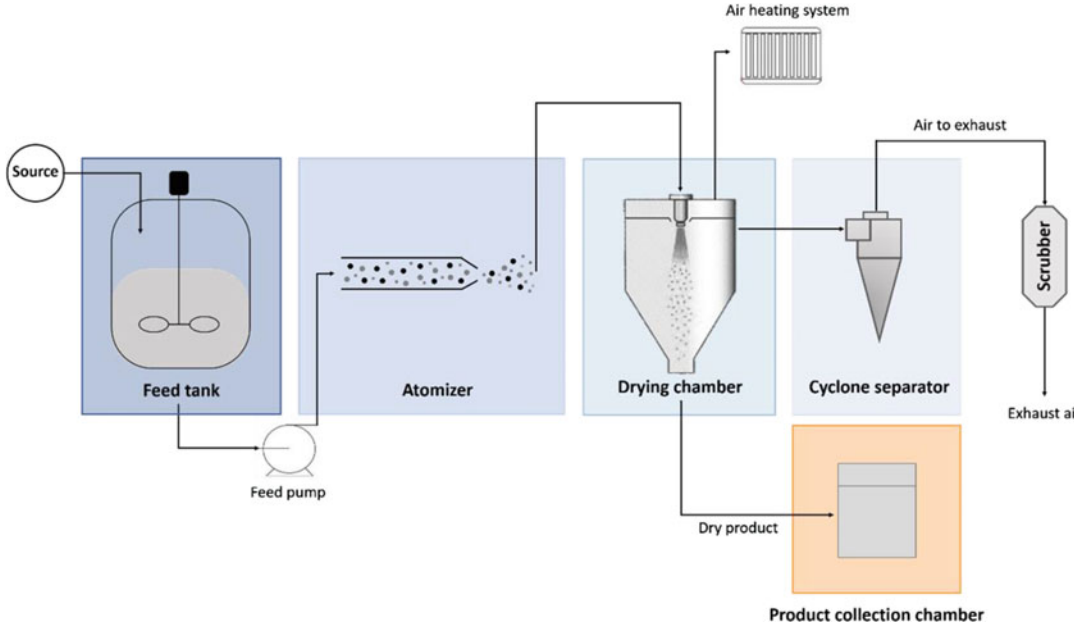

Fig. 1 Schematic representation of spray-drying mechanism. The mixture is pumped from the feed tank to the drying chamber, which has an atomizer inlet that breaks the liquid into a mist of fine droplets that are dried in the chamber forming dry particles. Finally, using an appropriate device, the dried particles are separated from the drying medium and subsequently collected in a tank

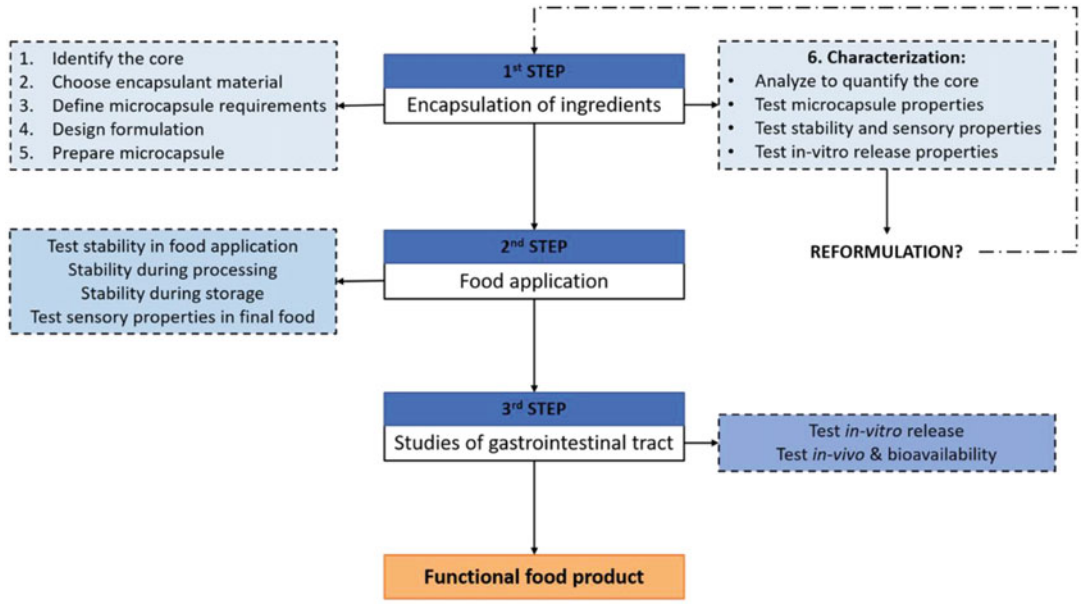

Fig. 2 Design of a functional product by adding capsules generated with the spray-drying method

3.1 Carotenoids as Target Compounds

3.1.1 Pretreatment of the Sample

The natural carotenoid source needs to be subjected to a prior selection to avoid the entry of defective products into the process. Once checked, the sample is submitted to a stage of drying in the darkness. For low-fat raw material, this step can be carried out in stoves at 45°C during the necessary days to dry the plant material. On the other hand, fatty matrices can be damaged if they reach temperatures over 45°C for long periods, so lyophilization is recommended. Once the highest water content has been removed from the sample, grinding and sieving are carried out to increase the contact surface, improving the accessibility and maximizing the carotenoids extraction [10].

3.1.2 Carotenoids Extraction from Plant Material

The selection of a natural source containing a remarkable amount of carotenoids is the most critical step followed by the choice of an adequate [11, 12] extraction method to recover the maximum carotenoid content, even if they are sought. Physical and chemical characteristics of the target compounds and the matrixes in which they are embedded have to be considered to optimize their recovery. Chemical solubility is a relevant feature which will determine the selection of the extractive solvent. Even though solvents may be applied individually, they are frequently used as a mixture in order to maximize the extraction process, chloroform or hydroalcoholic mixtures, consisting in ethanol:methanol (1:1, v/v) or ethanol: water (1:1, v/v) [11, 12], have been reported by different protocols as efficient solutions for extracting internal carotenoids [13]. The variability in the extraction solvents is conditioned to the nature and chemical complexity of the source of pigments. The incubation time required for obtaining a high-throughput extraction usually varies depending on the matrix structure and the particle size, but an immovable condition of the extraction process is that it has to be carried out in the absence of light to minimize degradation losses, due to the photosensitivity of these compounds. Therefore, it is advisable to develop a previous optimization study of the extraction protocol based on an evaluation of the recovery rates which will permit to establish the experimental parameters that will lead to achieve maximum yields.

Additional steps of purification or concentration may be applied in order to obtain high amounts of carotenoids in a smaller volume of samples. The purification may be developed using automatic instruments which permit to track the elution of the target compound or solid phase extraction columns in which the carotenoid-rich fraction has to be further checked. The concentration process consists of the evaporation of the solvent to reduce its final volume or even to completely dry the sample which may be useful for storing purposes [14].

3.1.3 Quantification of Carotenoids

Nowadays, several methodologies have been set up to analyze the carotenoid content of a matrix. Nevertheless, techniques based on spectrometers using absorbance are recommendable due to their speed and simplicity. For this aim, the carotenoid sample is read at 450 nm, later its concentration will be calculated through an interpolation using a calibration curve. This calibration curve is constructed by, at least, five serial dilutions obtained from an initial standard solution of the compound of interest, such as β-carotene. The serial dilutions are made using the same solvent employed for the extraction and dilution of the sample, for example ethanol: methanol (1:1) v/v. The calibration curve has to cover a concentration range wide enough to interpolate the sample concentration, otherwise the sample may be diluted to make it fall among its bottom and top values [15].

3.2 Spray Drying-Based Carotenoids Encapsulation

This step aims to add a substance to the suspension to increase its stability, mainly due to steric hindrance and electrostatic stabilization, which prevent agglomeration. For pigments encapsulation purposes, modified starch, maltodextrin, gum, or others are hydrated to be used as the carrier or wall material [16].

3.2.1 Selection of Dispersant Agent

3.2.2 Emulsion and Homogenization of the Dispersion

To carry out a correct emulsion, it is necessary to homogenize the target compound by the aid of dispersing agents, previously selected, and adding complexing agents later which will stabilize the carotenoids. In this regard, not only the stability will be improved but also the correct release and bioavailability of the compound of interest. The addition of these stabilizers to the emulsion is performed under agitation. In both steps, optimal conditions are achieved at 15,000 rpm (equivalent to 3276 G-force) for 1 min (using an Ultra-Turrax homogenizer), because lower speeds require more stirring time. The active compound (core) is emulsified together with the wall or carrier material usually at a ratio of 1:4 [7]. Once the emulsion is achieved, it is necessary to continue with constant agitation to keep the sample homogeneous [17]. This agitation must be maintained until the entire sample is fed into the atomizer applying a heat source (30°C) that will also facilitate the process and improve performance.

3.2.3 Atomization of the Infeed Emulsion

The main objective of this stage is to create a maximum heat transferring surface between the emulsion [18] and the dry air which indirectly optimizes the heat and mass transfer operations [19]. For this, it is necessary to reduce the particle size of the homogenized sample, which may be accomplished by pressure, centrifugal, electrostatic, or ultrasonic energy, using specific devices called atomizers [9]. Therefore, once the mixture is homogenized in a liquid solution (feed stream), it is fed into the spray dryer,

which atomized the solution, by a nozzle or a spinning wheel, using a carrier gas stream (compressed N_2) [20]. Both sample and hot air flows should be counter current in order to limit thermal degradation.

3.2.4 Dehydration of the Atomized Particles

In this context, dehydration can be defined as the almost complete elimination of water, with a maximum permitted amount of 5%, and it has to be fulfilled in seconds. As room temperature increases it does the droplet temperature and the moisture diffusion takes place, from the core to the cortex, until the droplet fully reaches the air temperature. The resulting particles undergo to an additional process in the drying chamber supported by another nitrogen stream (drying N_2). Rapid evaporation of moisture in a short period (10–15 s) ensures that the core material temperature is below 40°C [2, 21, 22]. The obtained product consists of microcapsules of an approximate spherical shape with size ranges from 5 to 600 μm, coated with a porous cover [23]. Finally, the capsules are collected after they fall to the bottom of the drier [18].

3.3 Evaluation of the Encapsulation: Efficiency, Yield, and Characterization

3.3.1 Efficiency and Yield Determination

The optimal microencapsulation efficiency is variable and dependent on the selected spray-drying conditions; therefore, a previous optimization of the microencapsulation protocol should be performed. The most relevant factors to be set up include feed flow rate, air inlet temperature, and feed temperature. It has been observed that increasing the drying temperature raises the speed of the process and reduces viscosity and droplet size modifying its fluidity and its capacity to be homogeneously sprayed, with a consequent increment in the efficiency rate. The establishment of the optimum speed is crucial to obtain the desired drying level before the droplet gets in contact with the drying chamber surface. Common temperatures used in this process are 160–190°C for air inlet temperature and 80–110°C for air outlet temperature. Other determining parameters affecting the microencapsulation efficacy which are independent of the instrument are the nature of the core and the wall materials as well as the interactions that could take place between them. Different studies have shown that the best wall material for encapsulating carotenoids is modified tapioca starch, maltodextrins, and arabica gum [6, 8].

To measure the yield of the process, it is necessary to determine the carotenoid content in both the inner (C_i) and outer layers (C_s). The more quantity remains in the inner layer, the most efficacy is achieved during the process. Therefore, the yield can be calculated using Eq. 1. On the other hand, when the final aim of the addition of the carotenoid is to exploit its bioactivities, the efficiency of the process also refers to the capacity of the compound to exert its bioactivities when embedded into the microcapsules. To provide this efficiency result, it is needed to measure the bioactivity of the carotenoid added to the initial emulsion and its bioactivity at the

microcapsules. This efficiency data can be calculated using Eq. 2, where A_f represents the carotenoids quantity after encapsulation and A_b, the carotenoids quantity initially added into the emulsion or formulation.

$$\text{Yield } (\%) = \frac{C_i}{C_i + C_s} \times 100 \tag{1}$$

$$\text{Efficiency } (\%) = \frac{A_f}{A_b} \times 100 \tag{2}$$

As previously explained, one of the first requirements that the encapsulation must meet is the controlled release of the compound of interest. When carotenoids are encapsulated and used for fortifying food matrixes, the main release evaluation is performed at intestinal tract level. In this context, the main objectives are to prevent the enzymatic and acidic degradation of the pigment and to control its release by coating it using an adequate capsule. The encapsulation of β-carotene using as external capsule layer a coat consisting of a chitosan-alginate coacervate or consisting on isabgol fiber, obtained from *Psyllium husk*, has been demonstrated to protect the low gastric pH and also allowed to reach the maximum release percentage [8]. Different wall capsule materials provide diverse pigment protection and controlled release which will be also dependent on the nature of the matrix food in which the microcapsules would be embedded. Therefore, a detailed evaluation of the microencapsulated pigments is necessary to demonstrate the compound release and the protection level of the capsule.

3.3.2 Characterization of Microcapsules

Microcapsules characterization represents another important step to determine the encapsulation yield and effectiveness and can be held using different spectral and thermal tools: characterization of powder microcapsules, characterization of microcapsules to monitor, determination of the core stability from degradation, and determination of the release properties of microcapsules [24]. The main parameters to be determined for the characterization are humidity, hygroscopicity, solubility, apparent density, and yield. Thereby, it is possible to know the level of complexation and determine the dairy intake quantities recommended of the obtained final product [1].

4 Notes for Encapsulation of Carotenoids with Spray Drying

1. Optimization of instrumental parameters: feed temperature and air inlet temperature. Due to the high number of variables that influence drying kinetics and final product properties, granular materials with very different characteristics can be obtained depending on the experimental conditions under which the drying process is carried out. Control, quantify,

and model the influence that each variable exerts on the drying kinetics and the final properties of the granule, and thus produce optimal materials for each determined application. Optimization will prevent stickiness problems while reducing the concentration of the encapsulating agent that will minimize costs and its effect in the final organoleptic properties of the product to which it is added [25].

Droplet size is an important factor that is conditioned by pressure: the higher the pressure is applied, the smaller droplet size is achieved. When rotary atomizers are used, both wheel rotation speed and diameter display an inverse relationship to the droplet size [26].

2. Under same pressure conditions, the droplet size increases proportionally to the feed flow rates, since the atomization energy is the same, but the feeding volumes are higher [27].
3. Highly viscous/dense inlet solutions make the process difficult by plugging the needles. This is because much of the atomization energy supplied to the nozzle is used to overcome the high viscous forces of the solution, resulting in larger droplet sizes [28].
4. Controlling evaporation is essential, since it is the phase in which all physical-structural transformations that give the granule the final microstructure and geometry take place, and therefore, it is the stage that determines the microcapsule physical properties [29].
5. Although high inlet temperatures would allow higher evaporation rates due to the greater ability to dry the atomized drops, they should not be excessively high to prevent the degradation of the final product. Therefore, an equilibrated choice of inlet temperature should be made according to the properties of the raw material [29].
6. Outlet temperature should be the highest temperature to which the dried powder can be heated [26].
7. Due to the porous surface of the resulting capsule, it is convenient to not use large concentrations of the ingredient to be encapsulated, since it would prevent its correct protection [30].
8. The sample should not be pumped until the atomizer has reached the optimized temperature. Otherwise, evaporation does not take place correctly [26].
9. It is necessary to ensure that wall and matrix materials do not impede the active compound analysis [31].
10. The major limitation of microencapsulation by spray drying is the limited number of wall materials available that have to additionally be hydrosoluble [16].
11. The resulting products could be sticky and could have a high hygroscopicity if the encapsulating material is not adequately chosen [16].

12. The drying air flow indicates the incoming air in the spray cylinder for drying. The actual flow depends on the pressure loss of the whole system [29].
13. It should be kept in mind that the drying of some products can lead to the formation of dust in dry areas, at the outlet of the dryer or cyclones, which can cause pollution problems.

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Chapter 9

Freeze-Drying Encapsulation as a Mechanism of Choice in Oils: Methods and Mechanism

Maria Carpena, Paula Garcia-Oliveira, Caterina Lourenço-Lopes, Antia Gonzalez Pereira, Maria Fraga-Corral, Miguel Angel Prieto, and Jesus Simal-Gandara

Abstract

Freeze-drying–based encapsulation involves the generation of an emulsion solution formed by the target compound and the encapsulating materials to later convert them into microcapsules applying the freeze-drying technique. Microcapsules are aimed to contain diverse ingredients such as active biomolecules, cells, or other materials. Later, the microcapsules containing the molecules of interest may be incorporated to different food matrixes to fortify them and provide functional or nutritionally improved products. Microcapsules represent a useful tool to perform and control delivery of bioactive molecules and living cells into the target organ, mostly the intestinal tract. Therefore, encapsulation goals are to protect, stabilize, and slow down the release of food ingredients. The wall materials need to comply few features since they have to be food-grade, biodegradable, and able to form a protective barrier to separate the core from the external medium in which it will be embedded and thus prevent the degradation of the core. There are many different techniques to create capsules: freeze-drying, spray-drying, extrusion, emulsification, fluidized bed coating, molecular inclusion, or liposome entrapment. This chapter focuses on reviewing the available freeze-drying technique protocols with the purpose of encapsulating different kinds of oils.

Key words Microencapsulation, Freeze-drying technique, Active biomolecules, Protocol, Oil products application

1 Freeze-Drying–Based Methods for Encapsulating Oil Products

Freeze-drying, also known as lyophilization, consists of a process of dehydration which promotes long-term stability of a product susceptible to decomposing. Likewise, and due to this added stability, this technique facilitates the transport and store of the product. To carry out this process, firstly the material of interest is frozen, and then, high-

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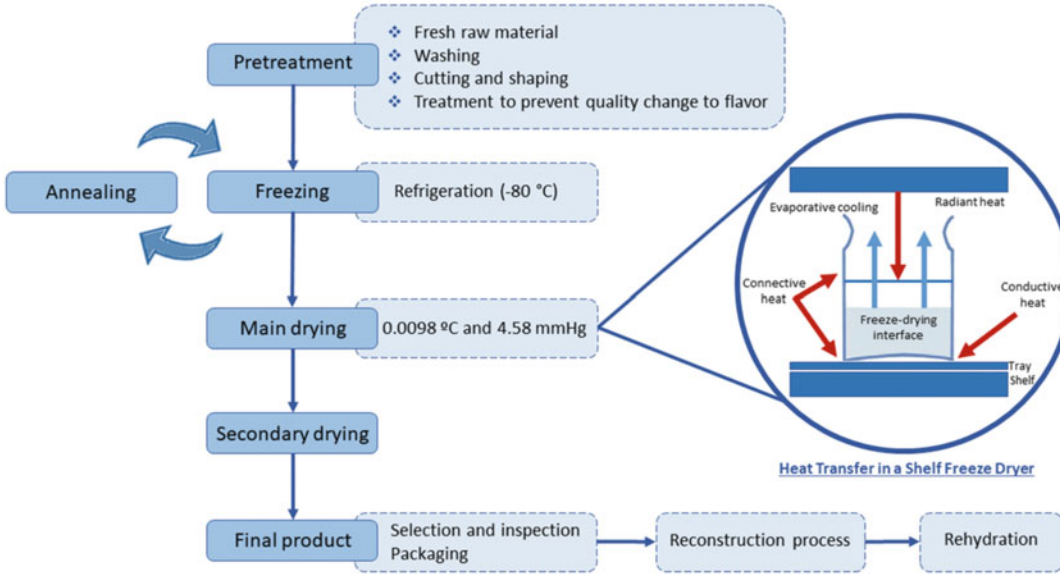

Fig. 1 Design of a functional product by adding capsules generated with the freeze-drying method

pressure vacuum is applied in order to sublimate the water, obtaining a dry final product. A summary of the process is shown in Fig. 1. Thereby, freeze-drying allows converting an unstable or a moisture substance into a dried and stable formulation [1]. Ultra-Turrax (T50, IKA Labortechnik, Staufen, Germany is a technique is of great interest when a substance is unstable in water, as is the case of oils [2].

The equipment necessary to carry out this process consists of a drying chamber with one or more heating shelves, a vacuum pump, a condenser for trapping steam and maintaining vacuum, and an electronic control unit with different sensors to monitor the process [3] as it can be seen in Fig. 2.

Sublimation of water takes place at pressures and temperatures below the triple point (0.0098 °C and 4.58 mmHg) as it is shown in Fig. 3. The products resulting from applying this method have a high quality, because the process takes place at moderate conditions contributing to the formation of highly porous solids able to retain aroma, color, and flavor [4]. The maintenance of these natural properties enhances the self-protection of the molecules and improves their delivery, as in the case of lipophilic compounds with recognized nutritional benefits such as long-chain polyunsaturated fatty acids, essential oils, or carotenoids [5]. Therefore, these characteristics will avoid oil degradation and ensure the conservation of their sensory properties. Freeze-drying also shows other advantages, for instance, the cost is reduced (lightweight, no refrigeration), the physical structure is not altered, reconstitution is quick and easy, and the resultant products have a longer shelf life when packed up properly.

The properties of the microcapsules obtained by this drying technique can be influenced by the sample preparation. The diverse oily nature of the emulsions or suspensions may affect the types of

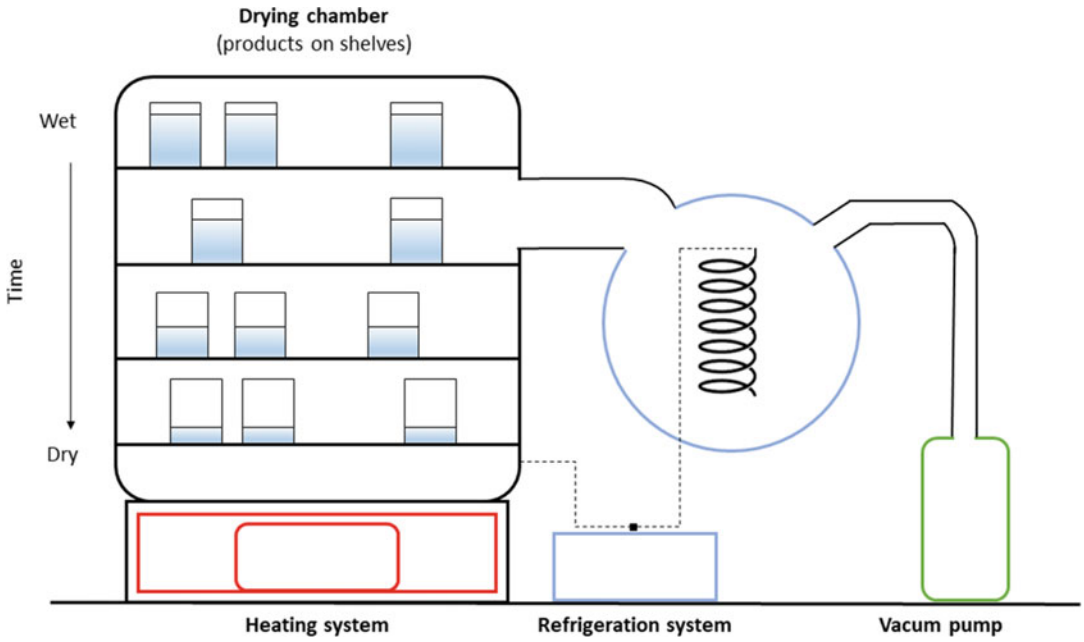

Fig. 2 Main components of freeze-drying equipment

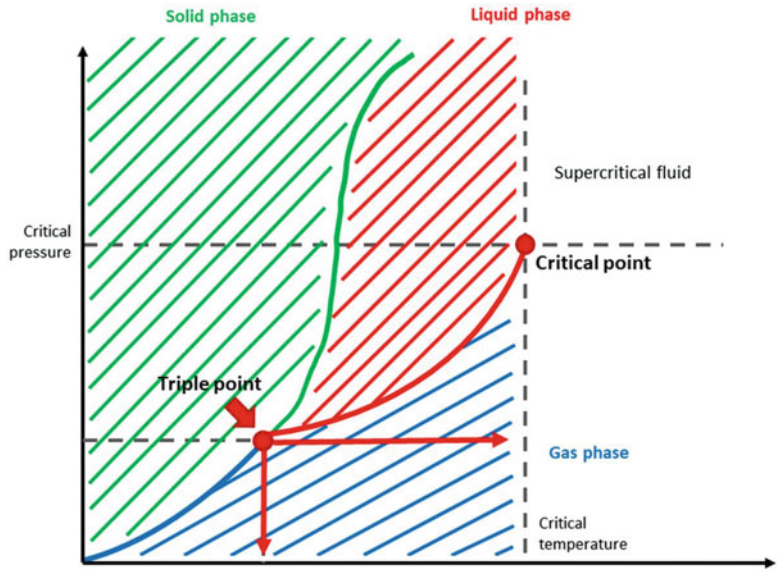

Fig. 3 Phase diagram. The green line corresponds to the freezing point and the red one, the boiling point. The red points show the triple and critical points, respectively. The diagram shows the region on which the freeze-drying process can take place, the gas phase

wall materials required to use, to the ratio wall-core, solid content, viscosity, stability, droplet size, and processing conditions [6].

Generating oil microcapsules has a great interest in the food industry, as they could be added to meals and therefore increase the

consumption of essential fatty acids such as omega 3, which are known for having numerous bioactivities (antithrombin, antimicrobial, or antioxidant, among others) [7]. These fatty acids are unstable when oxidation phenomena caused by light or temperature are produced. Microencapsulation is a useful tool to protect and preserve their bioactivity and allows the addition of other compounds of interest [8]. The main target molecules for freeze-drying encapsulation are those heat sensitive. This technique has shown the highest capacity for retaining volatile compounds compared to others when applied to oils such as fish, walnut, flax, or olive oil. Likewise, microcapsules obtained by this method have been demonstrated to possess high resistance to oxidation. Its main disadvantages are long processing times, high energy requirements, and low encapsulation efficiency [9].

The potential of this technique concerning oils has been reflected through few bibliographic references for its application [8, 10–13]. Co-encapsulation of oils can improve the final properties of a product once the capsules have been incorporated to that matrix. For instance, encapsulation of fish oils with other substances such as limonene can enhance the nutritional value of a food while preventing the modification of its organoleptic features, since microcapsules provide the capacity of masking bad odors [6].

2 Materials

For carrying out the process of freeze-drying, several components are needed to set up a functional system for encapsulation: a drying chamber, a vacuum system, a refrigeration or cooling system, a heating system, and an electronic control unit (Fig. 2).

The drying chamber is formed by one or more shelves where the samples will be placed on bottles or other containers. In this chamber, vacuum is generated through a rotary vane vacuum pump, and heat is usually transferred by conduction from the lower part of the chamber or shelves, although sometimes it can be applied by radiation from the upper plate. The final purpose of the vacuum system is to withdraw the gases of the freeze-drying process, such as the steam, to create a dry and vacuum environment [14]. In the case of the refrigeration system, its task relies on creating a cold environment for the freezing phase. At this point, the cold trap or condenser plays an important role since it allows the condensation of the produced vapors into a solid or liquid, avoiding its mixture with the product. The refrigeration system should guarantee that the cold trap is always at a temperature lower than the sample. The temperatures of the equipment, both heat and cold, are controlled and constant along the surfaces. At last, an electronic control unit is necessary to fix and monitor the different parameters [14].

3 Methods of Freeze-Drying for Encapsulating Oil Products

3.1 Preparation and Homogenization of the Emulsions

The pretreatment of the sample includes the concentration of the target product, formulation revision, decreasing a high-vapor-pressure solvent, or increasing the surface area. Oils can be extracted from diverse matrixes, both animal and vegetal, in different ways, one of the most common is the extraction by dragging with steam. Once the raw material has been subjected to a stream of steam, the dragged volatile fraction is condensed, collected, and separated from the aqueous fraction. This process can be repeated to increase extraction performance. The separation of the oil/water mixture is carried out by decantation, and the extracted oil can be purified by using a saturated solution of sodium sulfate, among others. Once extracted and concentrated the oil, it must be stored in amber bottles for preservation [15]. After obtaining the target oil, a portion may be kept analyzing its chemical profile, as explained later, and the remaining is mixed with different encapsulating materials to create the emulsion. In the case of essential oils of vegetal origin, it was found that a quality encapsulating material is achieved by mixing gum arabic (50%), maltodextrin (40%), and xanthan gum (10%) resulting in high retention of volatile compounds when using slow freezing methods [13]. However, depending on the origin of the oils, these proportions may vary a little, being necessary to adjust them according to the emulsion stability index and its viscosity [16]. The arabic gum, maltodextrin, and xanthan gum must be hydrated one by one with distilled water for 24 h at 8°C before its utilization at concentrations of 30% (w/v) for the arabic gum and maltodextrin and 1% (w/v) for the xanthan gum [17]. The ratio of these encapsulating agents will depend on the oil chosen for encapsulation, so it is necessary to carry out an optimization of these variables.

For example, in the case of fish oils, the optimal working conditions are 10 wt% fish oil, 10 wt% sodium caseinate, 10 wt% carbohydrate, and 70 wt% water. Once the desired concentrations are obtained, ingredients are mixed progressively, only adding carbohydrates after the complete dissolution of sodium caseinate and adding oil in the last place, at an optimal temperature between 5 and 10°C [8]. Additionally, other compounds have been suggested as coating materials alone or in combination, as zein, lecithin, carboxymethyl cellulose, or alginate [9, 12, 16].

Once the solution, containing the lipids of interest and the encapsulating reagents, has been prepared it has to be homogenized to create the emulsion. For this purpose, the mixture is homogenized using an Ultra-Turrax for 1 min at 10,000 rpm [10]. In order to obtain the best possible emulsion, some parameters are usually studied for their characterization, such as emulsion stability, viscosity, zeta potential, or droplet size [16, 18].

3.2 Freezing

This step consists of changing the state of matter to a solid state by freezing with the consequent nucleation phenomena. In this stage, the emulsion is placed into aluminum plates and frozen at −70/−80°C for 24 h [10], an indispensable condition for low-temperature drying. Along this phase, it is important to highlight that not only the solvent (free water) is frozen but all the water content and components of the sample, resulting in the formation of a noncrystalline solid (vitreous solid) [14]. During freezing, the solvent crystallizes under atmospheric conditions and is separated from the residual sample. Consequently, the unfrozen fraction is concentrated [3]. For the achievement of an optimal freezing process, the temperature must be lower than eutectic temperature (in the case of crystalline solids) or glass transition temperature (in the case of amorphous solids) [14, 19]. Here, another parameter must be taken into account: the temperature decreasing rate. Different speeds have distinct effects on the material, in general, a rapid decrease avoids dehydration but too fast cooling could cause alterations as materials' fracture and other detrimental effects [14].

In this phase, there is an optional step called annealing. This procedure consists of submitting the products to repetitive cycles of a predetermined temperature for a specific time and therefore, achieve a complete crystallization of all the formulation components, avoiding heterogeneity [20].

3.3 Drying

Once the sample is frozen, the water is removed employing a drying process that can be divided into two phases. The first consists of the sublimation or primary drying of the frozen water molecules and the second consists of a process of desorption.

3.3.1 Primary or Sublimation Drying (PD)

During the main drying, the sublimation of the ice molecules takes place. Sublimation consists of removing the frozen solvent by transferring it directly from the solid to the gaseous state and concomitant avoidance of the liquid aggregate state [3]. This phenomenon occurs due to the pressure reduction and temperature applied. Controlling these two parameters is of vital importance as they will not only determine the efficiency of the process but also the time limiting stage. This phase may be slow in some cases because if the temperature reaches excessive values, the structure of the material could be altered (collapse). In all cases, temperature and pressure are below the triple point of water (0.0098°C and 4.58 mmHg), and they cannot be higher than eutectic or glass transition temperature (Fig. 3). This process removes up to 98% of moisture content. These conditions are achieved, for example, with −50°C and 112.51 mmHg pressure for 72 h [16]. For this purpose, heat can be applied by radiation or conduction, and saturation vapor pressure is modified by the vacuum system. Moreover, it is also important to maintain equilibrium between the produced

steam and the heat transference to the sample. These variables are submitted to various restrictions, so this process usually takes a long time [14].

3.3.2 Secondary or Desorption Drying (SD)

This phase consists of removing the unfrozen solvent from the sample by desorption, thus reaching the final content in water. For this purpose, it is necessary to apply higher temperatures than in the previous phase to break any physicochemical interaction that has been formed between the solvent molecules and the frozen material. However, as the working drying speed is low, the process stretches on for a whole day but after carrying it out, the bound water is eliminated. Along this stage, the temperature is raised (50–60°C), and the vacuum is lowered to about 50 mmHg. The freeze-dried products are then grounded by using a mortar and a pestle immediately after drying. The microcapsules obtained are directly weighed for each test and stored in specific conditions required by different tests [6].

3.4 Packing and Storage Conditions

Both the desorption of the liquids attached to the solid particles at the previous phase and the packaging of the final product must be carried out in containers able to prevent the reabsorption of water and/or oxygen from the environment [3]. Therefore, packing should be developed in a vacuum chamber or the presence of inert gases as argon or nitrogen [14].

In general terms, the two main problems associated with storage are residual moisture and the temperature. Higher contents of water and elevated temperatures are usually not recommended for storage as they accelerate reactions and thus, cause alterations on the products [14]. A widely used way to store the samples is in properly sealed glass bottles stored at room temperature with relative humidity around 30% and in the absence of light. This way the samples remain uniform for 12 weeks, without alterations [8, 10].

3.5 Characterization of Microcapsules

Several parameters are calculated and studied for the characterization of the obtained microcapsules. The first indicator is the encapsulation efficiency. This value is closely related to oil recovery. To calculate this value, two methods are applied: Röse-Gottlieb method for nonvolatile oil in the capsules and Clevenger distillation for volatile oil during drying [6]. The efficiency of the encapsulation process can be calculated using Eq. 1, where A_f corresponds to the amount of oil after encapsulation and A_b to the amount of oil added in the formulation (before encapsulation) [18].

$$\text{Efficiency } (\%) = \frac{A_f}{A_b} \times 100 \tag{1}$$

Moreover, there is another method for calculating this efficiency which consists of the previous calculation of two parameters theoretical oil content (O_T) and loaded oil content (O_L), according

to Eqs. 2 and 3, respectively. At these equations, Oi and O_f correspond to initial and final content of oil while C_i and C_f to the initial and final mass of the capsule [21].

$$O_T = \frac{O_i}{C_i} \times 100 \tag{2}$$

$$O_L = \frac{O_f}{C_f} \times 100 \tag{3}$$

Then, encapsulation efficiency is calculated following Eq. 4 and corresponds to the value of O_T divided by O_L.

$$\text{Efficiency } (\%) = \frac{O_T}{O_L} \times 100 \tag{4}$$

Although some oils (*i.e.*, rosemary, olive, fish oils) have been encapsulated with high efficiency, the majority of the studies show a slightly lower encapsulation efficiency when compared to other techniques as spray drying [6, 10].

Retention of oil can also be determined by gas chromatography or mass spectrometry when the intention is to characterize and quantify the different oils present in the capsules. To carry out the chromatographic analysis, it is necessary to pretreat the capsules. A common protocol involves the crushing of 100 mg of capsules that will be mixed with 3 mL of distilled water and 5 mL of hexane. This solution is sonicated for 10 min at 80% amplitude (90 W) at 22°C. Once this step is complete, the sample can be injected for its analysis [13].

Another parameter widely employed when characterizing microcapsules is the study of their morphology. The surface of these capsules is studied by using an optical microscope or using a scanning electron microscopy or X-ray diffraction [18, 21, 22].

Since most of the oil quality loss is due to their oxidation, the degree of oxidation present in the samples must also be determined. Considering this issue, it is necessary to determinate peroxide value by iodometric titration, that is, a simple method based on the titration of iodine released from potassium iodide by peroxide using a standardized thiosulfate solution as the titrant and a starch solution as indicator. This method detects all substances that oxidize potassium iodide under acidic conditions [23]. Sometimes, thiobarbituric acid-reactive substances (TBARS) assay is also carried out when studying the oxidative stability of the microcapsules [18]. Another studied parameter is surface oil content or nonencapsulated oil. This process consists of weighing 500 mg of the encapsulated sample and washes it with 1 mL of petroleum ether to make a final volume of 5 mL. After the solvent evaporation, the oil recovered from the microcapsules surface is gravimetrically quantified [24]. Significant higher surface oil and lower volatile retention are achieved on the freeze-drying process when compared to spray-

drying due to dehydration of emulsifiers during the freezing of the water phase, which promotes droplet–droplet interactions in emulsion creating complexes of higher size and less stability [11].

Another characteristic to be determined is the release kinetics of these compounds of interest during storage to assure that it is higher the first 48 h and then, it is stabilized [13], as well as water activity determination [16]. Moisture content can be determined using a Sartorius moisture analyzer [10]. Furthermore, the percentage of bio accessibility can be studied to evaluate its availability at the gastrointestinal tract and its release capacity [18].

4 Notes for Freeze Drying Method for Encapsulating Oil Products

1. The freeze-drying process is expensive, so its application is restricted to high-value products with specific biological and phytochemical properties. However, new technical solutions are applied to modify the freeze-drying process and transform it into a time and cost-effective procedure [4].
2. The solution must be frozen at a temperature below its eutectic temperature [19].
3. The rate of ice crystallization defines the freezing process and efficiency of primary drying [3].
4. Encapsulated materials could be released from the core when ice crystals are removed during the drying stage [20].
5. During the drying phase, the crucial factor is the relation between the steam flow and the heat transference [14].
6. Since the time required for freeze-drying is relatively long, the major loss of the volatile fraction could occur in the emulsion state [11].
7. Since emulsions are highly unstable, approaches are necessary to incorporate oil-based molecules into foods [18].
8. The structure of the capsules is porous, so storage conditions are important to prevent lipid oxidation. Oxygen diffusion or oxygen permeation into matrices becomes a limiting factor in determining the rate of oxidation especially in particles with a large surface area [8, 23].
9. Solid oil formulations showed higher process stability than those with liquid oil [25].
10. Porous materials employed at the freeze-drying process allow a better drug release [4].
11. The major limitation of the freeze-drying process is a high energy requirement, long times, and low encapsulation efficiency [9].

Acknowledgments

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Chapter 10

Development of Novel Inulin-Based Electrosprayed Microparticles for the Stabilization and Delivery of Phlorotannin Extracts

Lucía Cassani and Andrea Gómez-Zavaglia

Abstract

Adding phlorotannins (a group of potent antioxidant and antimicrobial compounds derived from seaweed) to food products is certainly an interesting strategy to diversify the highly demanded functional foods by health-conscious consumers. However, many technological processes (from extraction to food processing and storage) may affect the phlorotannins stability and thus, their biological activity. Therefore, encapsulation of phlorotannins through electrospraying may serve as a solution to overcome the potential problems arising from chemical instability, as this innovative technology does not involve high temperature. In this context, we describe here two important protocols that allow obtaining a high recovery of phlorotannins from *Ascophyllum nodosum* with maximized antioxidant activity (through an optimized microwave-assisted extraction procedure) and also, the stabilization of phlorotannins extract-containing inulin microcapsules (through an encapsulation methodology based on electrospraying) that ensures the maintenance of the phlorotannins' active molecular form up to the consumption time and their safe arrival to the gut. Physicochemical, mechanical, and thermal characterization of the developed electrosprayed microcapsules is also presented.

Key words Electrospraying, Encapsulation, Prebiotic-based microcapsules, Seaweed polyphenols, Characterization

1 Introduction

In the last years, algae have gained attention as rich sources of underexploited bioactive compounds, potentially useful as novel functional ingredients. In particular, phlorotannins, an important group of phytochemicals derived from brown algae, are considered as potent natural antioxidants and antimicrobials compounds with well-demonstrated health beneficial properties by in vitro assays and thus, are valuable for the formulation of functional foods [1, 2].

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The extraction procedure represents an important factor that significantly affects the phlorotannins content and concentration. Considering that phlorotannins are thermosensitive compounds, there is a growing interest in developing novel, efficient, and environmentally friendly extraction techniques focused on improving the extraction yield without affecting the bioactivity of these phytochemicals [3]. For example, microwave-assisted extraction is an alternative green technology which has demonstrated to achieve a higher extraction yield of phlorotannins in comparison to conventional thermal extraction [4].

On the other hand, food manufacturing, long-term storage (light, dissolved oxygen, and high temperature), and digestion conditions (enzymes, interactions with other nutrients, and pH) play important roles in improving or reducing the phlorotannins content which may affect their incorporation into foodstuffs and also their health benefits. These drawbacks expose the need of applying smart strategies to develop a final product providing the necessary protective mechanisms to maintain the active molecular form of phlorotannins up to the consumption time, also controlling their release upon arrival to the gut. In this context, encapsulation appears as a promising approach to overcome the limitations of phlorotannins.

Electrospraying process is a cost-effective and scalable technology that uses a high-voltage electric field to break up a jet of biopolymer solution containing the target compound to fine highly charged droplets from where the solvent is evaporated at room temperature in a very simple one-step process [5, 6]. Thus, this innovative encapsulation technology, which does not involve high temperatures nor organic solvents, is an ideal method for protecting the chemical stability and biological functions of sensitive bioactive compounds such as, phlorotannins, in an environmentally friendly way [5].

The properties of the electrospraying feed solution, as well as the process parameters, should be optimized for each polymer-bioactive combination in order to ensure the successful performance of the electrospraying process leading to encapsulated structures with proper morphology and size [7].

Among the wall materials, inulin, an edible biopolymer composed of fructose units, is not degraded nor absorbed in the stomach or the small intestine, reaching intact the gut (the phlorotannins target), and hence, this characteristic makes inulin a promising natural carrier for these seaweed polyphenols [8].

Regarding solvent, water is preferred over organic acids, due to its nontoxic properties which is beneficial from an environmental point of view [9]. However, the physical properties of water are not the most suitable for electrospraying processing since the surface tension is high (affecting the jet stability), and also when high voltage is applied, ionization of the water molecules could occur

causing corona discharges [7]. Thus, the addition of gums and/or surfactants to aqueous inulin solutions can be a smart strategy to modify the physical properties of feed solutions and thus, enhance their electrosprayability [9, 10].

In light of the above, we described here two important protocols that allow obtaining a high recovery of phlorotannins from *Ascophyllum nodosum* with maximized antioxidant activity (through an optimized microwave-assisted extraction procedure) and also, the stabilization of phlorotannins extract-containing inulin microcapsules (through an encapsulation methodology based on electrospraying). Physicochemical, mechanical, and thermal characterization of the developed electrosprayed microcapsules is also presented.

2 Materials

2.1 Seaweed Material

1. Collect fresh seaweeds (*Ascophyllum nodosum*) on the shore at the season when the phlorotannins concentration is maximum (it will depend on the geographical location).
2. Remove natural residues (e.g., stones, sand, shells, and pieces of wood) attached to the surface of algae, place them in a plastic black box with seawater to protect them from light, and immediately transport to the laboratory.
3. Once at the laboratory, rinse seaweed with fresh water, drain samples, and put them in ziplock bags.
4. Freeze samples at −80 °C for 24 h.
5. Freeze-dry algae for 48 h on an equipment which operates with the condenser at −45 °C at a chamber pressure of 0.04 mbar, for example, Heto FD4 equipment (Heto Lab Equipment, Denmark).
6. Grind dried samples until obtaining a particle size lower than 0.6 mm, and store the resulting powder at −20 °C for further analysis.

2.2 Extraction of Phlorotannins

1. Regarding extraction solvent, use mixtures of ethanol and deionized water since ethanol has low toxicity and GRAS status.
2. Microwave, for example, multiwave-3000 reaction system (Anton-Paar, Germany) (Fig. 1) is mainly composed of 16 vessel extraction chambers equipped with infrared sensor, pressure and temperature (P/T) sensor, vessel mark sensor, and a magnetic stirrer at the base. Also, other microwaves with similar specifications can be used.

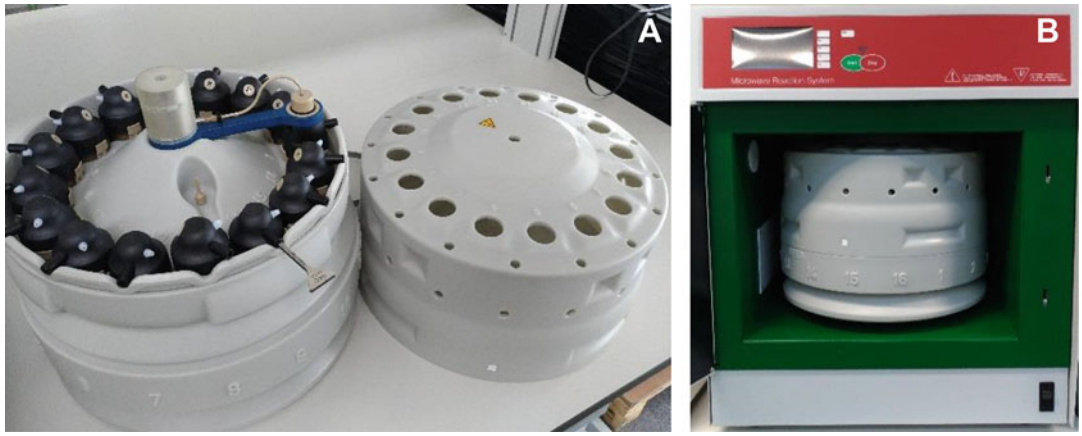

Fig. 1 Microwave extraction unit A 16 vessel chamber with the P/T sensor; B vessel chamber inside the multiwave 3000 reaction system (Anton-Paar, Germany)

2.3 Electrospraying Processing

The typical equipment employed consists of three main elements [6, 7, 11]:

- *High voltage power supply* which usually varies in the range of 6–20 kV and provides high-voltage electricity to generate a jet of feeding fluid.
- *Nozzle or conductive capillary* to which the polymer solution is pumped at a constant rate.
- *Collecting device* to which the charged particles are finally deposited.

3 Methods

3.1 Optimized Extraction of Phlorotannins

1. Weigh 0.6 g of freeze-dried alga material and place it into each of 16 extraction vessels (capacity of 100 mL) which are provided on the pilot-scale multiwave 3000 reaction system (Fig. 1A).
2. Add 20 mL of aqueous ethanol (37.25% v/v) into every extraction vessel to achieve the solid/liquid ratio of 30 g/L.
3. Rapidly add a magnetic stirring bar for agitation to every extraction vessel and then close them with their caps by simply screwing on by hand.
4. Place the closed vessels into the extraction chamber and set the process parameters as following: irradiation time (5 min), pressure (10.5 bar), microwave power (1400 W), and oscillation (ON).
5. Immediately after extraction, cool the extracts in an ice-bath until reaching room temperature.

6. Transfer the slurries to a 50-mL Falcon tube, centrifuge them at 7500 × *g* for 15 min, and discard the pellets.
7. Collect the supernatants and filter them using Whatman filter paper #1.
8. Remove solvent by rotary evaporation and then freeze-dry the concentrated extracts for 48 h.
9. Grind all extracts to fine powders and keep at −20 °C for further analysis.

3.2 Preparation of the Feed Solution

1. Dissolve 20% w/v of inulin in distilled water through gentle stirring at room temperature.
2. Once homogenized, add 1% w/w of guar gum under stirring conditions.
3. Add 5% w/w of sorbitan monolaurate (Span-20) with continuous stirring to ensure complete dissolution (*see* **Note 1**).
4. Next, incorporate 10% w/v of phlorotannins extract (obtained according to Subheading 3.1) into the inulin-based solution, and stir at room temperature until obtaining homogeneous dissolution of all components.

3.3 Characterization of the Feed Solution

The physical properties of the electrospraying solution, in particular viscosity, surface tension, and conductivity, should be thoroughly analyzed to ensure the successful performance of the electrospraying process.

3.3.1 Viscosity

1. Use a controlled-stress rheometer Physica MCR 301 Anton Paar (Ostfildern, Germany) at room temperature and shear rates from 0 to 1000 s^{-1}.
2. Select a coaxial-cylinder geometry (CC27-SN16635).
3. Calculate viscosity from the steady-shear flow curves, as the ratio between shear stress and shear rate.

3.3.2 Surface Tension

1. Prior to running the test, calibrate the tensiometer (Krüss, Germany) by deionized water, and collect data 5 min after setting the plate on the sample's surface.
2. Measure the surface tension of solutions using the Wilhelmy plate method at 25 °C.

3.3.3 Conductivity

1. Measure the electrical conductivity using a portable multiparameter analyzer (DZS-718, Shanghai Precision Science Instrument Co., Ltd., Shanghai, China).

3.4 Preparation of Electrosprayed Microcapsules

Considering that electrospraying conditions have a direct impact on the microstructure morphology and diameter, an optimized protocol that ensures capsule-like structures is mandatory. Therefore, the

efficient microencapsulation of phlorotannins using electrospraying is explained below.

1. Load the prepared solution in a 5-mL plastic syringe connected with a stainless-steel needle of 0.9 mm internal diameter.
2. Place the syringe on a digitally controlled syringe pump and put the needle in horizontal towards the collector.
3. Place a flat sheet of aluminum foil on the negative electrode to collect the electrosprayed microcapsules.
4. Set a distance between the needle and the collector at 10 cm (*see* **Note 2**).
5. Fix the steady flow rate at 0.10 mL/h and apply voltage in a range between 9 and 16 kV.

3.5 Physicochemical Characterization of Microcapsules

The molecular interactions of inulin/guar gum/Span-20/phlorotannins should be analyzed by FTIR spectroscopy according to the following methodology.

3.5.1 Fourier Transform Infrared (FTIR) Spectroscopy

1. Place 5 mg of electrosprayed microcapsules on the sample holder of an ATR-FTIR Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA).
2. Register at least 7 spectra for each sample in the 4000–500 cm^{-1} range by co-adding 100 scans with 4 cm^{-1} spectral resolution.
3. Use OMNIC software (version 8.3, Thermo Scientific, MA, USA) for spectra analysis.

3.5.2 Scanning Electron Microscopy (SEM)

The morphological features and size of resulting microcapsules should be evaluated by SEM.

1. Place the electrosprayed microcapsules on metal stubs with double-sided adhesive tape.
2. Coat the resulting system (metal stub + microparticles) with gold using a sputter coater (Polaron Thermo VGScientific, East Grinstead, Sussex, UK) under vacuum and 18 mA at room temperature.
3. Examine the surface aspect and the inner structure of the capsules using an environmental scanning electron microscope (ESEM) (FEI La B6, Eindhoven, Netherlands) at 14 kV accelerating voltage with an electron detector for low vacuum conditions.
4. For examination of the inner structure, cut microcapsules in half with a steel blade.

3.6 Mechanical Characterization of Microcapsules

Analyzing the tensile properties of the resulting capsules is important to determine the stiffness and flexibility of the microparticles. Tensile tests should be performed according to ASTM D638-94b method (1994).

1. Prior to running the mechanical tests, keep the microparticles membranes at 65 ± 5% relative humidity for 72 h at room temperature.
2. Then, cut the developed microparticles membranes into strips of 5 × 25 mm.
3. Fix strips between the grips with an initial separation of 50 mm using a TA-XT2i Texture Analyser (Stable Microsystems, UK).
4. Set the crosshead speed at 10 mm/min.
5. Calculate the ultimate strength (σ_u), elongation at break (ε_u), and elastic modulus (E).

3.7 Thermal Characterization of Microcapsules

Thermal stability of the resulting microcapsules should be determined by performing thermogravimetric analysis.

3.7.1 Thermogravimetric Analysis (TGA)

1. Weigh 10 mg of microcapsules.
2. Set the heating rate at 10 °C/min in a TGA-40 Shimadzu Thermogravimetric Analyzer.
3. Perform the thermal test by subjecting samples under air atmosphere (35 mL/min) from room temperature to 900 °C.

4 Notes

1. The concentration of added surfactants to solution should be above a critical micelle concentration to ensure that the surface tension of solutions reaches an equilibrium value.
2. Considering that inulin has a high capacity of retaining water, increase the tip-to-collector distance to avoid water drops on the collector, or partial collapse of structures during the production of microcapsules.

Acknowledgments

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Chapter 11

Nanostructures for the Stabilization and Delivery of Lactic Acid Bacteria

Patrícia Alves, Patrícia Coimbra, Florencia Ghibaudo, Andrea Gomez-Zavaglia, and Pedro Nuno Simões

Abstract

Probiotic microorganisms have a striking impact on human life. The advantages associated with these microorganisms are related to therapeutic effects, such as the prevention of gastrointestinal diseases, allergic reactions, and antibacterial, antimutagenic, and anticarcinogenic activity. Within this range of microorganisms, lactic acid bacteria have been used for centuries to ferment various products, which can be consumed daily and which are linked to the gastrointestinal health. The market opportunities and the resulting demands have dictated the development of a long-term protection system for probiotic bacteria. In this context, a protocol that allows the encapsulation of bacteria with and/or without fructo-oligosaccharides as prebiotic ingredient is described. The encapsulation is based on the layer-by-layer method, which consists of depositing several layers of polyelectrolyte with different and alternating surface charges on a substrate, i.e., the bacteria surface.

Key words Probiotics, Lactic acid bacteria (LAB), Encapsulation, Layer-by-layer (LbL), Polyelectrolytes, Fructo-oligosaccharides (FOS)

1 Introduction

The latest definitions state that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1], while prebiotics are "substances selectively used by host microorganisms conferring a health benefit" [2]. The great relevance of both is not only nutritional but also economic. Most probiotics are lactic acid bacteria (LAB), known by their GRAS (Generally Recognized as Safe) attribute, widely used in food and pharmaceutical industries. Fructo-oligosaccharides (FOS) consist of a small number of fructose units linked by glycosidic bonds and are a noticeable example of prebiotics.

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The survival of probiotics in food products and dietetic supplements is affected by a number of factors, including pH, temperature, and relative humidity along storage. Therefore, the viability of probiotic bacteria in the products they integrate is a fundamental feature to guarantee their effectiveness at the time of consumption. This also means that it becomes critical to ensure the survival of the bacterial cultures during processing and storage, as well as to prevent chemical degradation during passage through the gastrointestinal (GI) tract due to the acidic conditions of the food together with the activity of enzymes and bile salts of the intestine [3, 4].

The encapsulation of probiotics aims not only their protection against adverse physical and/or chemical external conditions but also to allow a controlled release in a viable way and in a metabolically active state, in specific parts of the body, mostly in the intestine [5]. Possible release agents are related to pH variations, mechanical stresses, enzymatic activities, osmotic pressure, diffusion of moisture through the layers, presence of some chemical components, and storage time [6]. FOS can be added, not only as prebiotic ingredient but also to further contribute to bacteria preservation during freeze-drying process [7].

The layer-by-layer (LbL) encapsulation technique is one of the simplest, efficient, and low-cost method for encapsulation [8]. It consists in depositing alternating layers of oppositely charged polyelectrolytes, with washing steps in between, to construct nanostructured functional thin films with uniform and controlled properties (Fig. 1).

The final desired properties of each layer (thickness, permeability, ionic strength, and morphology) can be controlled and adapted by changing the pH and ionic strength of the compounds used.

Herein, a protocol for the LbL-based encapsulation of two strains of lactic acid bacteria that allows their stabilization together with the main underlying characterization steps is described.

2 Materials and Methods

All the material (Eppendorf tubes, falcon tubes, pipette tips, and flasks) and all aqueous solutions (polymeric solutions, saline buffer, and culture medium with and without agar) used throughout the LbL procedure must be previously sterilized in an autoclave for 15 min at 121 °C. Furthermore, the entire experimental work involving bacteria manipulation must be carried out under aseptic conditions in a laminar flow chamber in order to prevent contamination. Tables 1 and 2 provide an overview of two LAB strains and natural polymers involved in the LbL approach here described.

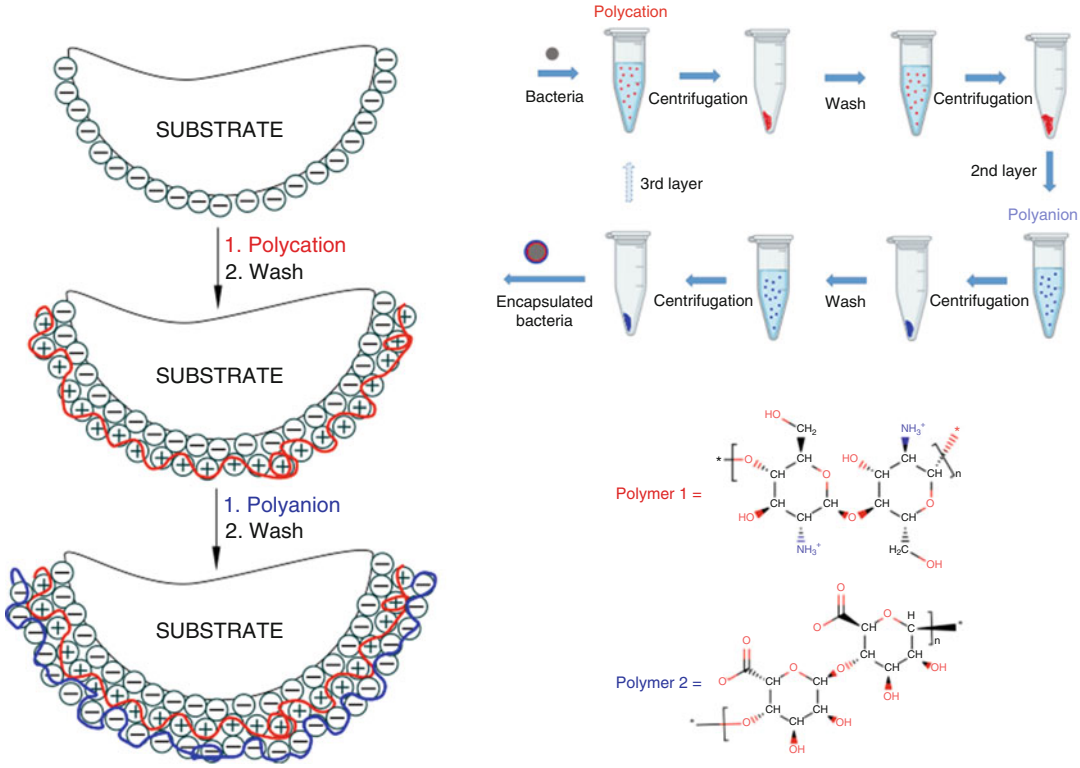

Fig. 1 Schematic representation of LbL assembly on a spherical substrate using oppositely charged polymers (chitosan and alginate as an example)

Table 1
LAB strains selected for the LbL encapsulation tests

Bacteria	Culture medium	T (°C)	Incubation time (hour)
Lactiplantibacillus plantarum CIDCA 83114 (Lp)	MRS	37	14
Lactobacillus delbrueckii ssp. *bulgaricus* CIDCA 331 (Lb)	MRS	42	14

2.1 Preparation of Solutions

1. NaCl 0.15 M solution: Weigh 8.766 g of NaCl into 1 L of milli-Q H_2O.
2. CHI solution:
   (a) Under magnetic stirring, disperse 1.0 g of chitosan powder in about 970 mL of 1% (v/v) acetic acid solution.
   (b) Leave to dissolve overnight.

Table 2
Natural polymers and related solutions selected for the LbL encapsulation tests

Natural polyelectrolytes (Layers)	Preparation solution
Chitosan (+) (CHI)	Acetic acid solution 1% (v/v); NaCl 0.15 M, pH 6
Carboxymethyl Cellulose (−) (CMC)	
Alginate (−) (ALG)	NaCl 0.15 M; pH 6
Pectin (−) (PEC)	

(c) Add 8.766 g of NaCl and adjust pH to 6.0 with 1 M NaOH or 1 M HCl.

(d) Make up solution volume to 1 L with deionized water.

3. PEC, CMC, ALG:
   (a) Under magnetic stirring, disperse 1.0 g of ALG, PEC, or CMC in about 970 mL of deionized water.
   (b) Leave to dissolve overnight.
   (c) Add 8.766 g of NaCl and adjust pH to 6.0 with 1 M NaOH or 1 M HCl.
   (d) Make up solution volume to 1 L with deionized water.
   (e) 0.1% m/v PEC in 0.15 M NaCl.
   (f) 0.1% m/v CMC in 0.15 M NaCl.
4. FOS: 2% m/v FOS in 0.15 M NaCl.

To ensure the self-assembly deposition of the layers, the pH of all solutions should be adjusted to 6 (e.g., by using 1 M solution of either NaOH or HCl). At this pH, the amino groups of CHI are protonated ($pK_a = 6.5$), whereas the carboxyl groups of PEC ($pK_a = 3.5$) or CMC ($pK_a = 4.3$) are deprotonated, thus leading to positive and negative charges on the polyelectrolyte chains, respectively.

2.2 Preparation of Culture Medium with and Without Agar

For each encapsulation assay, it is necessary to prepare MRS broth culture medium (55 g L^{-1}). To evaluate bacteria viability, MRS agar plates are prepared by adding agar (15 g L^{-1}) to MRS medium (55 g L^{-1}). The mixture is autoclaved and the resultant suspension, still hot, plated into Petri dishes in aseptic conditions (about 15 mL for each plate).

2.3 Bacterial Inoculum

1. Inoculate about 0.1 g of lyophilized bacteria in 10 mL of MRS and incubate for 24 h in aerobic conditions in an orbital shaker at 37 °C (Lp) or 42 °C (Lb).

2. Inoculate 10 mL of fresh MRS with 0.1 mL of the previous culture and incubate for another 24 h at the same conditions.
3. Inoculate 250 mL MRS culture medium with 1% (v/v) of the previous culture and incubate in an orbital shaker at 37 °C (Lp) or 42 °C (Lb) for 14–16 h.

2.4 LbL Encapsulation of LAB

The LbL encapsulation of bacteria with CHI and ALG or PEC or CMC is performed as follows:

1. Harvest, by centrifugation at 5630 g for 10 min, bacteria cultures in the stationary phase (~5×10^8 CFU mL^{-1}) and wash twice with 0.15 M NaCl. The pellets will be used for LbL encapsulation.
2. Suspend the bacterial pellets in 20 mL CHI solution to attain 10^7 to 10^8 CFU mL^{-1}. The deposition of the first layer is carried out at 37 °C under orbital shaking at 100 rpm for 1 h. The excess of CHI is removed by centrifugation (5630 × *g*, 5 min, 4 °C), and microorganisms should be washed twice with 0.15 M NaCl.
3. The second layer is deposited by adding 20 mL ALG, PEC, or CMC to the one-layered microorganisms, and then incubated at 37 °C under gentle stirring at 100 rpm for 1 h.
4. Continue this procedure according to the requested number of layers, interspersed between layers of CHI and ALG (or PEC or CMC), respectively (*see* **Note 1**).

2.5 Freeze-Drying

For freeze-drying assays:

1. The encapsulated bacteria should be suspended in a 2% m/v FOS solution.
2. Suspensions should be frozen at −80 °C for 12 h, and freeze-dried (Telstar LyoQuest), operating with the condenser at −80 °C in a chamber pressure of 0.10 mbar for 48 h (*see* **Note 2**).

2.6 Characterization of the Encapsulated Bacteria

The efficiency of the encapsulation method and the use of FOS to ensure the protective effect on the bacteria should be evaluated by different characterization methods, for each formulation, through the determination of the zeta potential, the bacteria growth curve and their viability, both before and after the process of lyophilization as well as noncoated bacteria (as control).

2.6.1 Zeta Potential

1. In a Malvern Instrument Zetasizer Nano-Z (Malvern Instruments, Malvern, UK), use the combination of measurement techniques: electrophoresis and laser doppler velocimetry (Laser Doppler Electrophoresis).

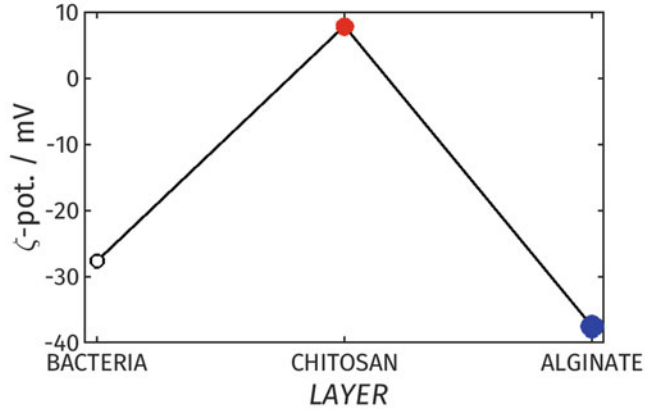

Fig. 2 Representative zeta potential profile in monitoring the LbL procedure

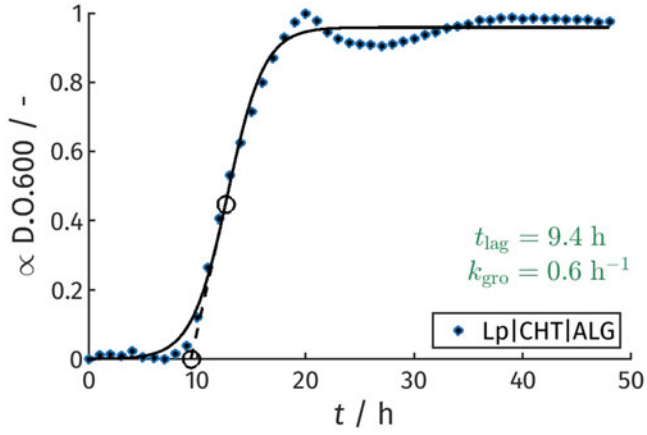

Fig. 3 Representative growth curves for checking the LbL procedure. *Lp L. plantarum*, *CHT* chitosan, *ALG* alginate, *D.O* optical density

2. Suspend bacterial cells in milli-Q water, place 1 mL of the solution in the zeta potential cell, and insert the cell into the equipment.
3. The zeta potential is provided directly by the instrument (Fig. 2).

2.6.2 Bacterial Growth

1. In a microplate reader (Synergy HTX multi-mode microplate reader from BioTek), place a 96-well microplate in which 90 μL of culture medium should be placed in each well and added 10 μL of each bacterial suspension.
2. Growth kinetics should be evaluated for 48 h, registering values hourly, using an optical density (O.D.) of 600 nm and at an optimal growth temperature of the bacterium.
3. Determine the growth curve with the results obtained (Fig. 3).

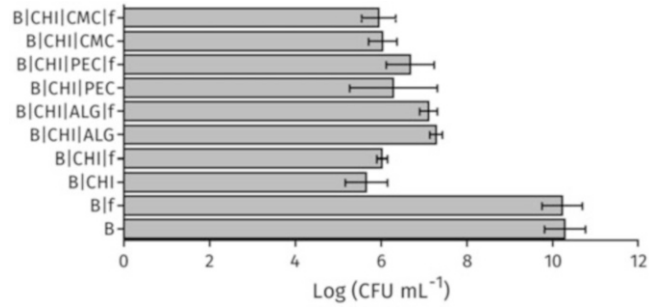

Fig. 4 Representative viability tests for checking the LbL procedure. *B* bacteria, *CHI* chitosan, *CMC* carboxymethyl cellulose, *PEC* pectin, *ALG* alginate, *f* fresh

2.6.3 Bacterial Viability

The counting of viable cells for encapsulated and nonencapsulated bacteria, before and after lyophilization, should be performed using the drop plate counting method.

1. Dilute bacteria suspensions in nine serial tenfold dilutions using 0.15 M NaCl.
2. Divide MRS agar plate into four quadrants, and in each, place several spaced 10 μL drops of the same dilution. Use the last four dilutions of the series.
3. Let the drops be absorbed into the agar for half an hour, and incubate plates in aerobic conditions at the optimal growth temperature of the bacterium for 48 h.
4. Count the colonies of the dilution that gave origin to individualized colonies with a number between 3 and 30 CFU. Average the total count of CFU of at least three drops at the countable dilution and express viability in CFU mL^{-1}.

   Viability should be assessed before and after each coating layer addition and after lyophilization on days 0 and 15 in order to infer the long-term viability of the bacteria (Fig. 4).

2.6.4 Scanning Electron Microscopy (SEM)

The morphological structure and size of encapsulated bacteria should be evaluated by SEM.

1. Place the bacteria onto metal stubs with carbon tape.
2. Place the samples into a gold sputtering system and sputter gold for 30 s at approximately 70 mTorr pressure.
3. Remove sample stub from the gold sputtering system.
4. Place the sample stub into the equipment chamber holder.
5. Examine the shape, size, and surface aspect of the bacteria.

2.6.5 Confocal Laser-Scanning Microscopy Analysis

The integrity, the permeability, and size of resulting encapsulated bacteria should be evaluated using a confocal laser-scanning microscope (Leica TCS SP5 Leica Microsystems, Wetzlar, Germany) [9].

1. Bacteria (encapsulated and nonencapsulated) should be visualized using two dyes: macromolecular fluorescein isothiocyanate (FITC) bound to inulin and To-Pro®-3 iodide, which is a DNA staining dye.
2. Fresh samples of bacteria should be suspended in distilled deionized water at a concentration of 10^8 cells/mL.
3. Add FITC-inulin (final concentration: 0.8 mM) and allow it to interact with the bacteria for 60 min at 37 °C.
4. Samples must be fixed by adding methanol (25% v/v) for 5 min at 20 °C.
5. Bacterial cells should be stained with To-Pro®-3 iodide, with a final concentration of 1 mM, for 5 min at 37 °C.
6. Disperse an aliquot of the suspension on a glass slide and dried under a flow of sterile air.
7. Insert the glass slide on the microscope and observe the results at an excitation wavelength of 488 nm and 633 for FITC-inulin and To-Pro®-3 iodide, respectively.

2.6.6 Simulated Gastric and Intestinal Fluid

Freeze-dried samples should be tested. The assay is divided into two stages: gastric digestion (pepsin, pH 2.5) and intestinal digestion (pancreatin, bile salts, pH 8) (adapted from [9, 10]).

1. Suspend bacteria (encapsulated and nonencapsulated) in simulated gastric solution (3200 units/mL porcine pepsin, 7.2 mM $CaCl_2$, 98 mM NaCl, 13.6 mM KCl, 0.82 mM KH_2PO_4, pH 2.5) and incubate for 90 min at 37 °C under continuous shaking (100 rpm, MaxQ 4000, Thermo Scientific, USA).
2. Centrifuge the bacteria at 5630 × *g* for 5 min and wash once with phosphate buffer saline (PBS) (0.82 mM K_2HPO_4, 98 mM NaCl, 5.5 mM Na_2HPO_4).
3. Suspend the obtained pellet in simulated intestinal fluid (0.1% w/v pancreatin, 0.15% w/v bovine bile salts, 22 mM NaCl, 3.2 mM KCl, 7.6 mM $NaHCO_3$, pH 8).
4. Incubate the resulting for 90 min at 37 °C at 100 rpm under continuous shaking (MaxQ 4000, Thermo Scientific, NJ, USA).
5. After each digestion step, aliquots of 1 mL of uncoated and coated bacteria must be diluted in 0.15 M NaCl, plate counted on MRS agar, and incubated at 37 °C for 48 h in aerobic conditions.
6. The results should be expressed in log CFU mL^{-1}.

2.6.7 Mitochondrial Dehydrogenase Activity

The potential toxicity of polymers on intestinal cells was determined by assessing mitochondrial dehydrogenase activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

1. Caco-2/TC7 cells should be seeded (at least in duplicate) in 24-well plates at 1×10^5 cells per well and incubated for 5 days.
2. Add the bacteria (encapsulated and nonencapsulated) at a concentration of 1×10^8 CFU mL^{-1} and incubate the cell for 24 h.
3. Wash the cells twice with PBS (pH 7.0), and replace the medium by Dulbecco's Modified Eagle's Medium (DMEM) (without phenol red dye) containing 0.5 mg/mL MTT.
4. Incubate for 3 h.
5. Add 0.2 mL dimethyl sulfoxide to remove formazan from cells.
6. Read the absorbance at 560 nm in a microplate reader (Synergy HT microplate reader, Bio-Tek Instruments, Vermont, USA).
7. Determine the cell viability according to Eq. (1):

$$\text{Cell viability } (\%) = \frac{OD_t}{OD_c} \times 100\% \quad (1)$$

where OD_t is the optical density of the treated cells (with uncoated and coated microorganisms) and OD_c is the optical density of the control cells (nontreated cells).

3 Notes

1. Noncoated bacteria must be used as control.
2. Noncoated microorganisms suspended in 2% m/v FOS were used as controls.

Acknowledgments

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Chapter 12

Fish Oil Encapsulation Using Soy Proteins as Wall Material: Protocols to Ensure PUFA Protection

Luciana Di Giorgio, Pablo Rodrigo Salgado, and Adriana Noemí Mauri

Abstract

Currently the incorporation of fish oil in diet is highly recommended due its high content of poly-unsaturated fatty acids, and their proved benefits on health. However, their incorporation into functional food is a significant challenge due to their low solubility in aqueous solvents, undesirable aroma, and high susceptibility to oxidative deterioration. Oxidative reactions could produce free radicals and hydroxides that can be toxic and volatile compounds such as aldehydes and ketones that negatively affect the sensory properties of food. Encapsulation technique could be used to protect fish oils from lipid oxidation, and soy proteins appeared to be suitable as wall material due to their acceptable water solubility, good adsorption at the water–oil interface, and their advantageous gelling and film-forming properties.

The objective of this chapter is to describe and discuss the protocols used to encapsulate fish oil in soy protein microparticles by emulsification and spray drying, focusing the process on the oxidative protection of oil, determining the encapsulation efficiency and the oxidative stability of the fish oil through the peroxide value, thiobarbituric acid reactive substances, and the Rancimat test.

Key words Encapsulation, Fish oil, Encapsulation efficiency, Lipid oxidation, TBA, Peroxide value, Rancimat, Soy proteins, Emulsification, Spray drying

1 Introduction

Omega-3 poly-unsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), provide important health benefits [1, 2]. Their beneficial effect has been proved against cancer, cardiovascular and inflammatory diseases, and immune and neurological disorders [3–9]. They are considered essential, since the human body itself cannot synthesize them [10]; therefore, their incorporation through diet is recommended [2, 11], and healthy foods supplemented with these acids are gaining importance in the food market [12].

Fish oils contain large amounts of essential PUFAs, including ω-3 EPA and DHA, ω-6 PUFAs linoleic (C18:2), and arachidonic (C20:4) acids [13]. However, their incorporation into food is a

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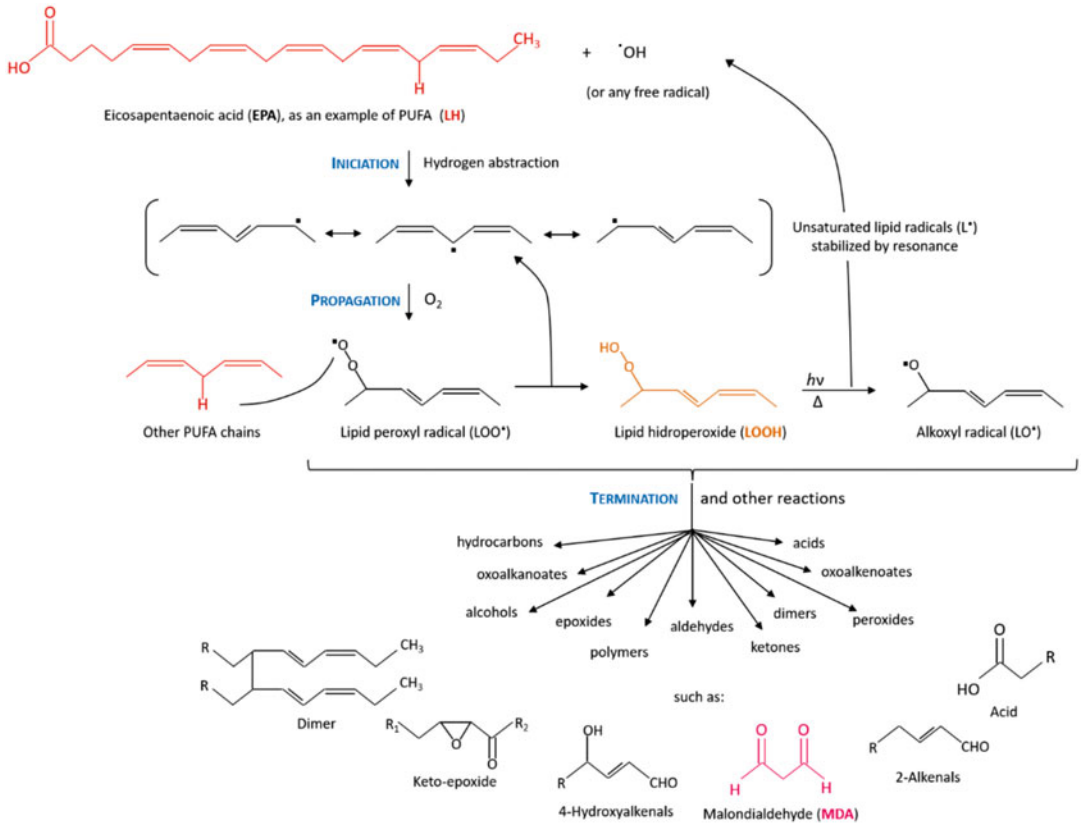

Fig. 1 Simplified scheme of EPA oxidation reactions, as an example of fish oil PUFA, and some of the oxidized products

significant challenge due to their low solubility in aqueous solvents, undesirable aroma, and high susceptibility to oxidative deterioration [14, 15]. EPA and DHA can react rapidly with singlet oxygen in a free radical reaction process known as autoxidation in which hydroperoxides are formed. These compounds can produce toxic effects in the body [16], causing the onset of diseases such as cancer or atherosclerosis [17]. Later, these hydroperoxides degrade to some volatile compounds such as aldehydes and ketones that can be detected by human taste and smell even at low levels of oxidation [18]. Figure 1 shows a simplified scheme of the lipid oxidation reactions (taking eicosapentaenoic acid as an example) and their resulting products.

Therefore, these oils must be stabilized against oxidation before being added to food to avoid affecting the taste, odor, or shelf life of the product [19]. This is primarily achieved by preserving them in the presence of antioxidant compounds, encapsulating them within a proper material, or combining both techniques. Encapsulation technique deals with coating or trapping an active compound to protect it, transport it, and/or control its release

[20, 21]. It has been found to be an excellent method for stabilization [22] and preservation of omega-3 fatty acid against oxidation by restricting undesirable influences from the environment [23–26]. Furthermore, encapsulation also allows masking the unpleasant flavor of fish oil [1, 10, 27].

Most processes used for oil encapsulation include an emulsification previous step. An ideal material for oil encapsulation should have good emulsifying and film-forming properties. In addition, it should have low viscosity in high solid contents, low hygroscopicity, and low cost [26]. Many proteins fulfill these characteristics [28–30]. Those derived from bovine milk are the most used as emulsifiers by the food industry. But currently there is a special interest in its replacement by plant proteins [28, 31]. Soy proteins appear to be suitable for the encapsulation of fish oil, as they have acceptable water solubility, good adsorption at the water–oil interface, and advantageous gelling and film-forming properties [32–34].

As far as we know, only Di Giorgio et al. (2019) [34] studied the encapsulation of fish oil into soybean microparticles by emulsification and spray drying, analyzing the effect of the formulation and the emulsification technique on the physicochemical properties of the resulting emulsions and encapsulates. Other authors had previously studied the encapsulation of fish oil using soy proteins together with banana starch or maltodextrin by emulsifying and lyophilization [35, 36].

The objective of this chapter was to describe and discuss the protocols used to encapsulate fish oil in soy protein microparticles by emulsification and spray drying, focusing the process on the oxidative protection of oil, determining the encapsulation efficiency and the oxidative stability of the fish oil through the peroxide value, thiobarbituric acid reactive substances, and the Rancimat test.

2 Materials

Fish oil (C22:6 docosahexaenoic acid, DHA, >22%, C20:5 eicosapentaenoic acid, EPA, >7% with vitamin E, rosemary extract and BHA as antioxidant) was kindly supplied by OmegaSur (Argentina) and used as oil phase. Soy protein isolate (SPI) SUPRO 500E, kindly supplied by DuPont N&H (Brazil) (protein content: 85 ± 2% w/w on dry basis; N × 5.71, measured by Kjeldahl method), was used as emulsifying agent. Ethyl ether (Merck, Argentina), isooctane (Dorwill, Argentina), isopropanol (Cicarelli, Argentina), methanol (Merck, Argentina), butanol (Cicarelli, Argentina), NH_4SCN (Biopack, Argentina), $FeSO_4 \cdot 7H_2O$ (Cicarelli, Argentina), $BaCl_2 \cdot 2H_2O$ (Cicarelli, Argentina), HCl (Biopack, Argentina), cumene hydroperoxide (Sigma Aldrich, USA), TBA (Sigma-Aldrich, USA), and TCA (Cicarelli, Argentina).

3 Methods

3.1 Fish Oil Encapsulation by Emulsification and Spray Drying

1. An aqueous dispersion of soy protein isolate (5% w/v) was prepared by magnetically stirring with a Dragonlab OS20-Pro equipment (Dragonlab, China) at 250–300 rpm during 45–50 min at room temperature (*see* **Note 1**). This dispersion (pH ≅ 7) was used as aqueous phase to prepare O/W emulsions.
2. Fish oil was added as the oily phase in a protein:oil ratio of 1:1, 2:1, 3:1, and 4:1 w/w (*see* **Note 2**).
3. These systems were pre-emulsified with an Ultra-Turrax T-25 high shear probe mixer (IKA®, Werke GmbH & Co. KG, Germany) using a dispersion tool S25N-18G (rotor diameter 13.4 mm) at 13,500 rpm for 90 s (*see* **Note 3**).
4. Then, these systems were emulsified with an ultrasonic homogenizer (VCX 750 Vibra-Cell, Sonics & Materials Inc., USA) at 50% power (350 W) for 5 min, with 30 s pulses (*see* **Note 4**).
5. The spray drying process was performed in a laboratory-scale Mini Spray Dryer Büchi B-290 (Büchi Labortechnik AG, Switzerland) equipped with an atomizer nozzle of 700μm diameter. The emulsions were fed into the main chamber (70 cm diameter) through a peristaltic pump, and the feed flow rate was controlled by the pump rotation speed (3.1 mL/min). Drying air flow rate was 357 L/h, and compressor air pressure was 6–8 bars. Inlet and outlet air temperature were 180 ± 2 °C and 96 ± 8 °C, respectively (*see* **Note 5**).
6. The spray-dried microcapsules were collected and stored in caramel-colored glass containers wrapped with aluminum foil, at room temperature in the dark for further analysis. Figure 2 shows the visual appearance of soy proteins' microcapsules containing fish oil (**A**) and their morphology observed by scanning electron microscopy (SEM) (**B**) (*see* **Note 6**).

3.2 Encapsulation Efficiency (EE)

1. Total oil content (TO) of microcapsules was determined by Soxhlet method [37] using ethyl ether as extraction solvent. The TO extracted was expressed as weight percent on dry basis of the microcapsules (*see* **Note 7**).
2. The free oil content (FO) (nonencapsulated oil fraction) of microcapsules was determined according to Di Giorgio et al. (2019) [34]. Briefly, ethyl ether (7.5 mL) was added to 0.5 g powder and the mixture stirred in a capped glass jar for 2 min at room temperature (20 °C) (*see* **Note 8**).
3. The mixture was filtered through a Whatman filter paper no. 1, and the powder retained on the filter was rinsed three times with 10 mL of ethyl ether to ensure complete removal of

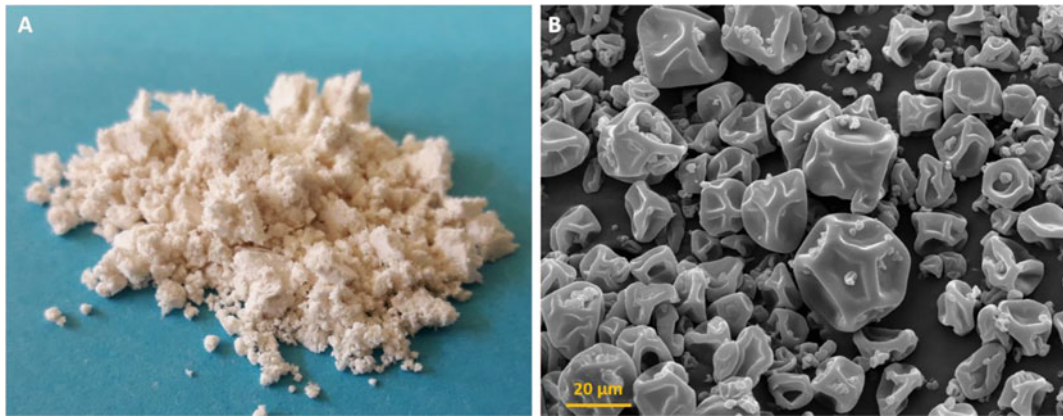

Fig. 2 Soy proteins microcapsules containing fish oil: (**a**) visual appearance and (**b**) morphology observed by scanning electron microscopy (SEM) at 500×

free oil. The filtrate (free oil and solvent) was collected in a clean and tared container.

4. Then, the solvent was left to evaporate at room temperature until constant weight (*see* **Note 9**).
5. The FO extracted was weighed and expressed as a percentage respect to the weight (dry basis) of the microcapsules.
6. Encapsulation efficiency (EE) was determined according to Eq. (1) (*see* **Note 10**):

$$\text{EE}\ (\%) = \frac{(TO - FO)}{TO}\ 100\% \qquad (1)$$

where EE is the encapsulation efficiency (%), T_O is the total oil content of microcapsules (% in dry basis), and F_O is the free oil content of microcapsules (% in dry basis).

3.3 Oxidative Stability

3.3.1 Peroxide Value (PV)

1. The oil was extracted according to the method described by Partanen et al. (2005) [38]. The sample (0.5 g) was weighed into a test tube and suspended in 5 mL of distilled water. The tube was shaken until complete powder dispersion (*see* **Note 11**). Dispersion (300μL) was vortexed three times for 10 s with 1.5 mL of an isooctane/isopropanol mixture (3:1 v:v) (*see* **Note 12**). Then, it was centrifuged at maximum speed in a centrifuge (HERMLE Z 326 K, Germany) for 2 min at 20 °C, and the upper phase (oil phase) was taken for analysis (*see* **Note 13**). Fish oil (300μL) was used as control to determine their initial peroxide content.
2. Peroxide value was determined spectrophotometrically, according to the method described by Mancuso et al. (2000) [39] (*see* **Note 14**). A portion of the oil phase /fish oil (20–200μL) (*see* **Note 15**) was added to 2.8 mL of a

methanol/butanol (2:1 v:v), followed by 15μL of thiocyanate aqueous solution (3.94 mol/L), and 15μL ferrous iron (0.072 mol/L acidic solution) (*see* **Note 16**).

3. The sample was briefly vortexed and allowed to react in the dark for 20 min at room temperature. Finally, the developed absorbance was measured at 510 nm with a Beckman DU650 spectrophotometer (Beckman, Germany) (*see* **Note 17**).
4. Lipid hydroperoxide concentrations were determined using a cumene hydroperoxide (Sigma-Aldrich Inc., St. Louis, USA) standard curve (*see* **Note 18**).

3.3.2 Thiobarbituric Acid Reactive Substances (TBARS)

The number of 2-thiobarbituric acid (TBA) was determined according to the method proposed by Tironi et al. 2007 [40] with some modifications.

1. Powder sample (0.22 g) was homogenized with 1.78 mL of trichloroacetic acid solution (TCA; 5% w/v) during 30 min, and then centrifuged at 10,000 × *g* for 10 min at 20 °C (HERMLE Z 326 K, Germany) (*see* **Note 19**).
2. Supernatant (0.5 mL) was mixed with 0.5 mL of TBA 0.5% w/v aqueous solution in capped tubes (*see* **Note 20**).
3. The mixture was incubated 30 min at 70 °C with gentle agitation (*see* **Note 21**), and then absorbance was determined at 532 nm using a Beckman DU650 spectrophotometer (Fullerton, CA, USA) (*see* **Note 22**).
4. TBA values were expressed as mg of malondialdehyde/kg of oil according to Eq. (2):

$$\mathrm{TBA} = \frac{\mathrm{Abs}\ M\ V\mathrm{s}\ V\mathrm{e}\ 1000}{\varepsilon\ l\ m} \tag{2}$$

where TBA is the TBA number (mg of malondialdehyde/kg of oil), Abs is the absorbance at 532 nm, M is the malondialdehyde molar mass (72 g/mol), V_s is the sample volume (0.5 mL), V_e is the extract volume (1.78 mL), ε is the molar extinction coefficient of the colored complex ($1.56 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$), l is the optical path (1 cm), and m is the sample mass (g).

3.3.3 Accelerated Oxidation Test: The Rancimat Method

1. An accelerated oxidation test of the microcapsules was performed in a Rancimat (Metrohm 743, Switzerland) equipment (*see* **Note 23**). Nonencapsulated fish oil was used as a control.
2. The sample (1 g of microcapsules or fish oil) was exposed to an air flow (20 L/h) at a constant temperature (90 °C) inside the equipment (*see* **Note 24**).
3. The electrical conductivity was continuously registered in the measuring vessel.

4. The oxidative stability index (OSI) was considered as the induction time till conductivity increased sharply [41] (*see* **Note 25**).

4 Notes

1. Water should be added slowly to thoroughly moisten the soy protein isolate. In this way, proteins could absorb water, swell, and disperse or solubilize homogeneously, avoiding the formation of agglomerates that later became difficult to disintegrate. Stirring conditions should also be set to obtain homogeneous protein solutions or dispersions.
2. Protein:oil ratios should be determined considering proteins' emulsifying and encapsulating capacity as well as the used techniques. Protein amount should be sufficient to form and stabilize the emulsion and to physically retain the oil to avoid its loss in the drying chamber or the cyclone during spray drying.
3. Pre-emulsification step was carried out in a 70-mm-diameter beaker (250 mL volume) at room temperature. During this process, the beaker must be moved manually to ensure the homogenization of all content, avoiding air incorporation. Therefore, the hole on the dispersion tool should be submerged in the sample or covered with film. The speed and time of pre-emulsification step as well as the composition of the aqueous phase and the protein:oil ratio would determine the properties of the resulting emulsion [42]. O/W emulsions with particles diameters of ≈20–30μm were obtained under the conditions described in Subheading 3.1.
4. In this second emulsification stage, a standard tip (13 mm diameter) immersed two-thirds in a 70 mm diameter beaker (250 mL volume) was used. The beaker should be kept in agitation with a magnetic stirrer (at low or moderate agitation) to ensure that treatment was performed on the entire sample volume. As the ultrasound process could heat the sample, it should be carried out in an ice bath. O/W emulsions with particles diameters of ≈1–1.6μm were obtained under the described conditions.
5. Spray drying operational variables that can be modified are: the temperature, flow and pressure of the inlet air as well as the feed rate and temperature of the sample [43]. Their selection would depend on sample formulation such as the encapsulating agent or the active compound. Working with soy proteins and fish oil, O/W emulsions were kept without stirring at room temperature during its feeding to the dryer (in the case of samples that precipitate, continuous agitation is necessary). The air inlet

temperature was set at 180 °C, and the combination of air flow rate and pressure and sample feed flow was selected in preliminary tests in order to obtain the highest solid recovery but keeping the outlet air temperature close to 95 °C as a higher temperature generally implies a greater lipid oxidation.

6. Soy protein microcapsules containing fish oil presented a slightly yellowish coloration (Fig. 2a) quite like SPI's one. These powders did not turn out to be oily to the touch nor exhibited the characteristic odor of fish oil [34]. These particles showed a spherical and smooth shape, characterized by the presence of dents on the surface (indentations) (Fig. 2b) and had mean apparent diameters of 15–20μm.
7. Different aspects should be considered when selecting the most suitable solvent for lipid extraction: polarity, boiling temperature, price, equipment limitations, and safety conditions, among others. Ethyl ether has an intermediate polarity and a relatively low boiling temperature (34.6 °C). These characteristics ensured a fast and almost complete (≈99.9%) lipids extraction from the microcapsules even using low temperatures (35–40 °C) by performing eight semicontinuous extraction cycles in Soxhlet equipment. But ethyl ether is a highly volatile and flammable liquid that can form explosive peroxides, so its handling must be done carefully, in a well-ventilated area or in extraction hood, using the appropriate safety elements to avoid inhalation and prolonged contact with the skin.
8. There is no standardized technique for determining free oil content in encapsulates. The method described in this chapter is based on those developed by other authors [44–46]. In this case, the same solvent as the one employed to determine total oil was used, but under milder conditions: batch extraction, room temperature, and shorter contact time between the sample and the solvent, in order to extract only the free oil, not the encapsulated onto.
9. Solvent evaporation could be accelerated by placing the container on a hot plate and/or under an extraction hood. The selected temperature should favor solvent evaporation rate without affecting the oxidative stability of the extracted lipids. In this case, the selected temperature was ≈35 °C. As the volume of solvent was low, evaporation was fast, ensuring that lipids did not degrade during the process.
10. Equation (1) determines the encapsulation efficiency of different oils in different matrices [47]. Experimental techniques used to determine the percentages of total and free oil may vary in terms of the solvent used (as sometimes solvent mixtures are preferred in order to achieve a certain polarity), or the equipment used depending on availability [44, 48–54].

11. Oil extraction from the microencapsulates was the first step in the peroxide value determination. As the microcapsules obtained had hydrophilic nature, they should be initially resuspended in distilled water. Microencapsulate:water ratio was defined taking as reference the publications of Partanen et al. (2005) and Ixtaina et al. (2015) [38, 55] and allowed the microcapsules to be resuspended efficiently. Alternatively, ethanol could be added to improve sample resuspension.
12. The relatively low polarity of isooctane/isopropanol solution made possible the complete extraction of lipids from the microcapsules. Therefore this mixture of solvents is widely used [55–59].
13. Both organic and aqueous phases were clearly distinguished in the test tube during lipid extraction. After centrifugation, the oily phase in the upper part of the tube was separated. Fish oil was used as a control in order to determine the peroxides initially present, and those produced as a consequence of encapsulation processing.
14. Although there are several techniques to evaluate PV, one of the most widely used is based on the ability of hydroperoxides (LOOH) to oxidize iodide ion (I^-) to iodine (I_2) in acidic medium. The iodine formed is titrated with sodium thiosulfate using a starch solution as indicator [60] that turned from a blue/violet color (attributed to amylose-iodine complex) to colorless. Eqs. (3) and (4) [61] show the chemical reactions involved in the test.

$$LOOH + 2H^+ + 2KI \rightarrow LOH + H_2O + 2\,K^+ + I_2 \quad (3)$$

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_2O_6 + 2NaI \quad (4)$$

It should be noted that starch addition should be done near the titration end point (when yellowish iodine color was faded) because at high iodine concentration starch is decomposed to products whose indicator properties are not entirely reversible. Although this technique is widely used, it has certain limitations. The identification of the titration end point could become difficult, for example, with samples with a certain coloration, such as the ones described in this work. The yellowish color of microcapsules may make it difficult to see the indicator turn. Therefore, in these cases, it is convenient to use other techniques, such as spectrophotometric ones.

Ferric thiocyanate spectrophotometric method is described in this chapter due to its simplicity and reproducibility. It also has greater sensitivity (0.05 mEq peroxide/kg) than the standard iodometric titration method, due to the lower susceptibility of ferrous ions (Fe^{2+}) to spontaneous oxidation by oxygen in air, compared to that of iodide solutions.

15. The volume variation in this protocol was established to achieve a sample absorbance between 0.2 and 1. High volumes (200μL) were used with samples with incipient oxidation, while smaller volumes (20μL) were advisable to use with more oxidized samples.
16. This method is based on the ability of hydroperoxides (LOOH) to oxidize Fe^{2+} to ferric ions (Fe^{3+}) in an acid medium [62]. Fe^{3+} formed chromophores when they complexed with thiocyanate that can be measured by spectrophotometry [63]. The reactions are described in Eqs. (5)–(7) [64]:

$$\mathrm{LOOH} + \mathrm{Fe}^{2+} \rightarrow \mathrm{LO}^{\tilde{n}} + \mathrm{Fe}^{3+} \tag{5}$$

$$\mathrm{LO}^{\tilde{n}} + \mathrm{Fe}^{2+} + \mathrm{H}^{+} \rightarrow \mathrm{LOH} + \mathrm{Fe}^{3+} \tag{6}$$

$$\mathrm{Fe}^{3+} + 5\mathrm{SCN}^{-} \rightarrow \mathrm{Fe(SCN)_5}^{2-} \tag{7}$$

17. The mixture is left in contact for such a time as to ensure that the reaction was completed (20 min). Light and oxygen could interfere on PV determination. Dissolved oxygen could also oxidize Fe^{2+} in the presence of peroxides, so nitrogen could be previously bubbled into the solvents that would be used in the determination. The $Fe(SCN)_5{}^{2-}$ complex formation in the solvent mixture developed a pink-violet coloration with absorption maximum at 500–510 nm [63].
18. Different peroxides (hydrogen peroxide, succinyl peroxide, cumene hydroperoxide, nonlipidic tert-butyl hydroperoxide, cumyl hydroperoxide) or standard solutions of Fe^{3+} ($FeCl_3$) can be used to perform the calibration curve. Cumene hydroperoxide is one of the most used for this technique. It is a highly reactive compound, very oxidizable, so it should be prepared at the time the calibration curve would be done. Peroxide concentrations from 0 to 180–200μM were used to perform the calibration curve in the linear range.

    Several experiments have been done to evaluate the correlation between PV results obtained by the ferric thiocyanate method and by iodometric titration. PV values obtained by ferric thiocyanate method doubled those of iodometric one [60, 65]; therefore, results obtained by the ferric thiocyanate method should be adjusted by a 0.5 correction factor [66].
19. TBARS determination could be carried out on samples treated differently:
    (a) by direct heating the sample with TBA [67, 68];
    (b) by sample distillation followed by reaction of the distillate with TBA [69–71];
    (c) by extraction of the lipid portion of the sample with solvents and reaction of the extract with TBA [72, 73]; and

(d) by MDA extraction using aqueous trichloroacetic acid [74–77] or perchloric acid [73, 78] and reaction with TBA.

The most preferred method is the aqueous acid extraction one [40, 79–81] because it is simple and gives highly correlated results with the distillation [73] and sensory evaluation [78] methods.

The sample weight:TCA extraction volume ratio should be at least 1:8–1:10 to ensure effective extraction.

20. It is advisable to slightly heat the 0.5% w/v TBA aqueous solution while preparing it, keeping it magnetically stirred, to facilitate the reagent dissolution.
21. It was observed that the reaction yield increased while the temperature decreased from 100 °C to 70 °C. Likewise, erroneous values could be obtained if turbidity was generated (by decomposition of the TBA reagent) when the heating time was greater than 30 min. Botsoglou et al. (1994) [81] described as optimal the reaction conditions adopted in this chapter (30 min, 70 °C).
22. TBARS method is based on the formation of a pink complex with absorbance at 532–535 nm when TBA reacts with the different secondary oxidation products of unsaturated fatty acids. Figure 3 shows the reaction of malondialdehyde (MDA) with two molecules of TBA to give the colored complex [82].
23. Figure 4 shows an experimental scheme for determining the oxidative stability index (OSI) using a Rancimat equipment. The fish oil sample was exposed to air flow at a constant temperature, and the highly volatile secondary oxidation products generated were transferred into the measuring vessel with the air flow, where they were absorbed in distilled water. The electrical conductivity was continuously registered, and the volatile compounds could thus be detected by increased conductivity. The induction time until these secondary reaction products appeared is referred to as the oxidative stability index (OSI) [41]. Therefore, higher OSI values implied higher oxidative stability.

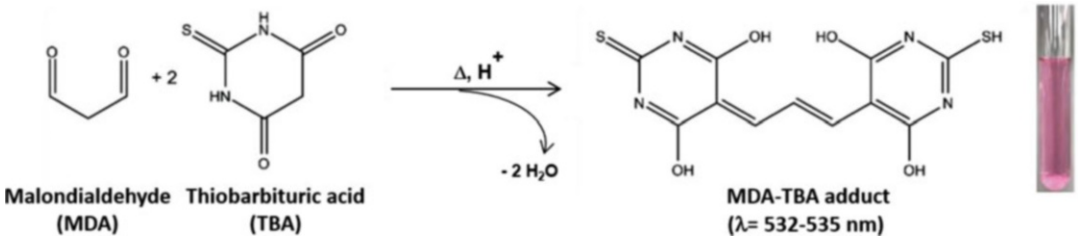

Fig. 3 Reaction of malondialdehyde (MDA) with TBA

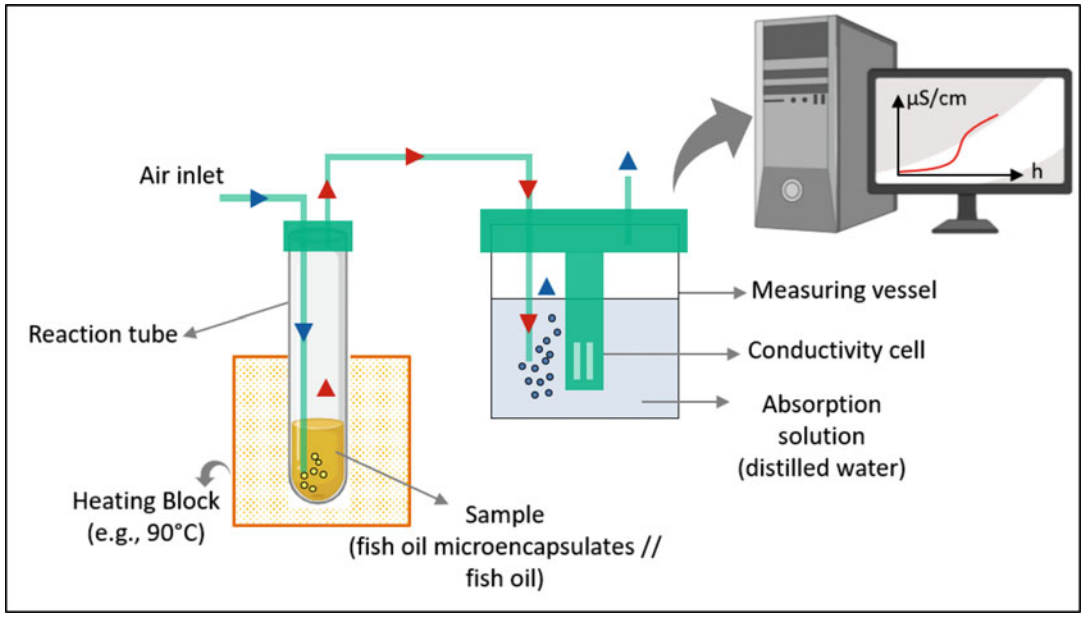

Fig. 4 Experimental scheme for determining the oxidative stability index (OSI) using a Rancimat equipment

24. Sample amount, heating temperature, and air flow are the parameters that could be easily varied in the equipment and could affect OSI value [83, 84]. No significant differences in OSI values were observed working with different amounts of microencapsulates (1, 2, and 3 g). Therefore, the minimum amount of sample was used in the analysis.

    On the other hand, the heating temperature and the air flow could be varied between 50 and 220 °C and 7 and 25 L/h, respectively. The Rancimat test for vegetable oil samples is normally carried out at 100–140 °C. While in the case of fish oil, which is highly susceptible to lipid oxidation, temperatures below 100 °C are preferred in order to have reproducible OSI values [85, 86]. Taking into account these antecedents and some preliminary results, it was decided to perform the Rancimat test at 90 °C and air flow of 20 L/h.

25. Previous studies have shown the correlation between OSI values obtained by the Rancimat test and those determined by other sensory and/or analytical methods [87–89]. Méndez et al. (1996) [85] reported that peroxides decomposition in fish oil followed first-order kinetics and could satisfactorily relate it to their Rancimat test results. OSI values determined by Rancimat method at elevated temperatures for a particular oil could be extrapolated to storage studies at low temperature. Extrapolation methods included reaction kinetics, and some based on the Arrhenius equation [90] could be used to estimate the shelf life of the product.

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Chapter 13

Obtention and Characterization of Cyclodextrins Complexes for the Development of Food Ingredients

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Abstract

Encapsulation is an interesting and feasible strategy to increase natural compounds solubility and stability, improving their application as food ingredients. In this sense, encapsulation in cyclodextrins emerges as a promising tool for the food industry. This chapter describes practical methods and protocols we have optimized for the obtention and characterization of inclusion complexes of different compounds such as α-terpineol, myristic acid, and cholesterol in β-cyclodextrin (BCD) or in a modified BCD as 2-hydroxypropyl-β-cyclodextrin (HPBCD). We show with these examples of relevance in foods, how the spatial configuration, size, and polarity of the ligand and of the cyclodextrin could significantly affect the degree of encapsulation, water interactions, thermodynamics parameters, and complex structure. We explain how the optimal encapsulation conditions (ligand:cyclodextrin molar ratio, temperature, and stirring time), water solubility, and complexes stability could be evaluated. Protocols about how to verify and study the encapsulation through spectrophotometry, water sorption, phase solubility, and differential scanning calorimetry (DSC) techniques are presented. The chapter provides information about methods of encapsulation of natural compounds in cyclodextrins and its analysis to develop ingredients for functional food formulations or nutraceuticals.

Key words Encapsulation, Cyclodextrins, Ultrasound, Food ingredients, DSC, Phase solubility studies

1 Introduction

Food processing has a particular interest in technologies of preservation, transformation, and extraction [1]. Nowadays, there is a growing consumer demand for foods formulated with natural compounds to replace synthetic additives or provide nutraceutical and/or nutritional value [2]. These compounds are frequently susceptible to unfavorable environmental, processing and/or gastrointestinal conditions. In this sense, encapsulation is an interest-

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ing way to preserve them during processing and storage [3]. Among the methods, molecular encapsulation in cyclodextrins is an innovative and attractive option for the food industry [4].

1.1 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of D-glucopyranose linked by α-1,4 glycosidic bonds, synthesized by enzymatic (cyclodextrin-glycosyltransferase) degradation of starch. α-CDs, β-CDs, and γ-CDs are natural cyclodextrins with six, seven, and eight glucose units, respectively [5]. They present a truncated cone spatial shape with hydrogen bonds between hydroxyl groups (HO) of glucose molecules. Cyclodextrin outer surface is hydrophilic, while the inner cavity is hydrophobic [6]. This spatial geometry gives them the possibility of interacting with a wide variety of compounds through noncovalent interactions and form host–guest complexes including molecules within their cavity [7]. The inclusion of these molecules, commonly called "ligands," changes their physicochemical properties. In this way, CDs have been used to increase compounds' solubility in aqueous medium and to improve the stability of bioactive compounds against degradation agents [3]. Studies for their oral administration have shown that natural CDs are not toxic due to their low or no absorption in the human intestine [8]. They are accepted as food additives in the United States and Japan and are listed by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) [7]. CDs can be used in many food products as carriers of polyunsaturated fatty acids (PUFA), vitamins, polyphenols, among others [9]. Currently, β-cyclodextrin (BCD) is the most commonly used in food and pharmaceutical formulations due to its low price, good availability, and proper cavity size allowing the inclusion of a wide range of compounds [10]. However, compared to other CDs, it has the lowest water solubility (*see* Table 1) due to its higher intramolecular interactions [11]. Some modifications are usually made on the natural BCD molecules by substituting hydroxyl groups of the

Table 1
Characteristics of α-, β-, γ-, and 2-hydroxypropyl-β cyclodextrins

CD type	Glucose units	Molecular weight (g/mol)	Cavity diameter (Å)	Water solubility at 25 °C (g/100 mL)
α-CD	6	972	4.7–5.3	14.5
β-CD	7	1135	6.0–6.5	1.85
γ-CD	8	1297	7.5–8.3	23.2
2-hydroxypropyl- βCD (HPBCD)	7	1400	6	>60[a]

Source: [14–16]
[a]The water solubility depends on the degree of substitution and location of the substituents

glucoses with hydroxypropyl or another residue to increase its water solubility. The different substituent groups and the degree of substitution conduct to notable changes in BCD properties and on its complexes [12]. The most used and low-cost modified cyclodextrin (CD) is the 2-hydroxypropyl-β-cyclodextrin (HPBCD). The number of hydroxypropyl groups (DS) in the HPBCD molecule is an important parameter, and the manufacturer usually provides this data. HPBCD (DS ≈ 6–7) has been approved by the European Medicine Agency (EMA) and the FDA as safe for humans due to its lower toxicity, higher water solubility nearly tenfold more soluble, and for certain types of ligands stronger inclusion ability than BCD [13].

Cyclodextrins have also the advantage of being neutral in terms of odor and taste which extend their possible applications [17]. For an efficient encapsulation, it is essential to select the appropriate type and concentration of CD and the right solvent. A dynamic equilibrium is established in the solution inclusion process, through noncovalent interactions and with a defined ligand:CD stoichiometry (Fig. 1). Different factors can promote the displacement of the equilibrium towards complex formation [18]. To reach that goal, it is necessary to define process variables such as time, temperature, and ligand:CD ratio. Complexes in solid state are commonly obtained through dehydration by freeze-drying or spray drying of the ligand:CD aqueous solutions, however these processes can affect the encapsulation. Therefore, complexes must be characterized/verified not only in solution but also in solid state.

When the solid CD complexes are hydrated under stirring, the equilibrium is reestablished since it is a reversible process [7]. This particular aspect of CD complexes allows obtaining systems where CDs could act as delivery agents, controlling the release, the stability, and the concentration of the ligand in the solution.

For the encapsulation process, ligands could be added to the CD solution as a liquid, solid, or dissolved in aqueous solution or in organic solvent [19, 21]. The selection of the type of CD, the solvent, and the encapsulation method depends on the properties of the ligand and the CD, the available equipment, and the costs. If the CD is dissolved in an aqueous-based solution, the solubility of

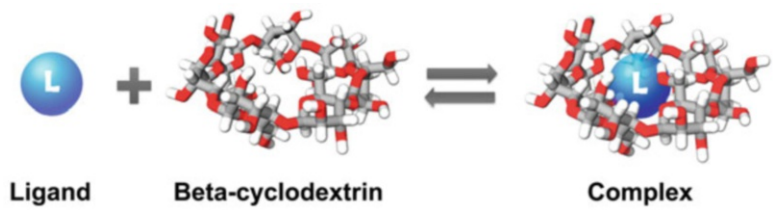

Fig. 1 Schematic representation of the dynamic equilibrium established in the inclusion process of a ligand in BCD with a stoichiometric 1:1

the ligand in this medium must be analyzed [22, 23]. There is no ideal technique to obtain solid-state complexes with CDs, but a method that maximizes the encapsulation must be sought. The most frequently used are [17]:

1. *Coprecipitation*. In this technique, the ligand is added to a CD aqueous solution, stirring at constant temperature long enough to favor the inclusion process and establish an equilibrium between the free and associated species. Stirring speed and time as well as temperature and ligand:CD molar ratio are essential factors in this process. Then, the precipitation of the complexes is promoted by keeping the solution under refrigeration temperatures. Solvent removal can be performed by different methods as freeze-drying, oven drying, or adding a matrix and proceeding with spray drying to obtain dehydrated systems.
2. *Freeze-drying or spray drying of solutions or suspensions*. It is a modification of the coprecipitation method. The step of cooling to favor the complexes precipitation is avoided, and the complete system is freeze-dried, or spray dried.
3. *Kneading or grinding methods*. In the kneading method, solid CDs and ligands are mixed with a small amount of solvent. In the grinding, the solvent is avoided. In both techniques, the preparation of the complex is by mechanochemical activation through manual kneading using a mortar or mechanical grinding using a ball or vibrating mills. This mechanical mixing provides the energy to favor the encapsulation. They are fast and eco-friendly methods to obtain inclusion complexes directly in the solid state but are not recommended for sensitive ligands that could be degraded or evaporated during the process [14].

1.2 Ultrasound Assistance in the Encapsulation Process

Ultrasonication (US) is an environmentally friendly technique as it is relatively safe and nontoxic, with low energy consumption and high efficiency [24]. High-intensity ultrasound is an efficient physical approach for preparing complexes faster, with high yield and reduced purification steps. When cavitation and collapse of bubbles occurs during the ultrasonic irradiation, energy is released and converted to high pressure and temperature. This process could accelerate dissolution of compounds breaking their intermolecular interactions, favoring the inclusion in the CD [25]. The US is currently being studied to reduce BCD encapsulation times of compounds like fatty acids or essential oils [26]. On the other hand, the application of ultrasound is also an ecological alternative to increase the extraction of bioactive compounds from agro-industrial by-products. Particularly, when aqueous solutions of CD are used as a solvent to increase the solubility and extraction

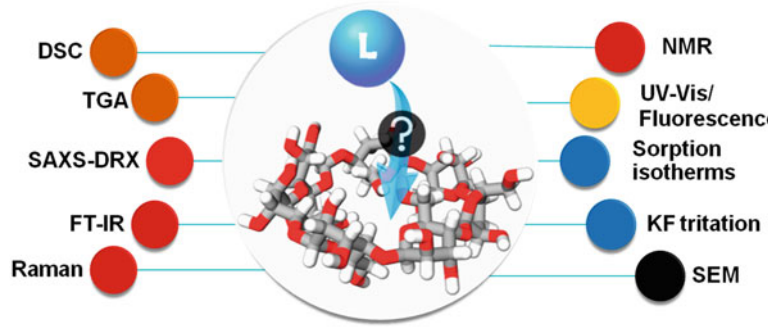

Fig. 2 Schematic representation of different techniques used to verify encapsulation, characterize inclusion complexes, or study stability and water sorption of the complexes. Differential scanning calorimetry (DSC), thermogravimetry analysis (TGA), x-rays (SAXS and DRX), infrared, Raman, and nuclear magnetic resonance spectroscopies (FTIR, Raman, and NMR), spectrophotometry (UV-Vis, fluorescence), water sorption properties (isotherms, Karl Fischer titration), and scanning electron microscopy (SEM)

of hydrophobic compounds, the application of US favors the inclusion of such compounds in the cyclodextrin [19]. The optimal parameters such as power, frequency, sample volume, and sonication time must be studied to obtain the desired effect without affecting the compounds of interest [27].

1.3 Verification and Characterization of the Encapsulation

Molecular encapsulation produces modifications in the physicochemical properties of both the cyclodextrin and the ligand. The study of these changes allows us to determine the stoichiometry and stability of the complexes, the thermodynamic of the inclusion process, and the type and intensity of interactions between the compounds. The characterization of the ligand:CD complexes in solution or in solid state is essential to select the optimal formulation that suits the desired purposes [28]. Figure 2 shows different techniques commonly used to verify and characterize CD complexes. The selection of the adequate method depends on the type of ligand, solvent and CD, the costs, the available equipment, and the future application of the complexes. Also, an essential aspect is whether the complexes are in solution or solid state since there are suitable techniques for each case.

This chapter describes the fundamentals of some of the most used techniques shown in Fig. 2 and the results we obtained of their application for studying BCD and HPBCD complexes with ligands of food interest (cholesterol, myristic acid, and α-terpineol).

1.3.1 Spectrophotometric Methods

They are widely used to determine the concentration of a ligand that absorbs in UV or visible or presents fluorescence. They are generally based on determining the change in the absorption or emission of the compound when it is encapsulated. These

techniques are simple, versatile, fast, and accurate, providing very reliable data. However, if the aqueous solubility of the ligands is low, the classical quantification techniques must sometimes be modified to improve the sensitivity [29].

1.3.2 Phase Solubility Study

The phase solubility methods developed by Higuchi and Connors [30] are commonly used to study the ligand-CD equilibrium in solution. They are also employed to calculate the thermodynamics parameters of the inclusion process and the stoichiometry of the complexes. They consist of studying the effect of the variation of CD concentration on the solubility of the ligand, whose initial concentration remains constant, forming a supersaturated solution (over its solubility). As ligand is added, a shift in equilibrium occurs towards the formation of the inclusion complex. The concentrations of the complex or of the ligand in equilibrium are determined by evaluating a suitable physical or chemical property as a spectrophotometric one. Then, the ligand molar concentration in solution is plotted as a function of the CD concentration at the equilibrium. The graphs obtained are called phase solubility diagrams (Fig. 3).

Higuchi and Connors [30] classified the systems according to the type of empirical phase diagram.

1. Type A_L: indicates the formation of soluble complexes. In this system, the increase in the solubility of the ligand is linear with the increase in CD concentration. The stoichiometric ratio in these cases is 1:1.

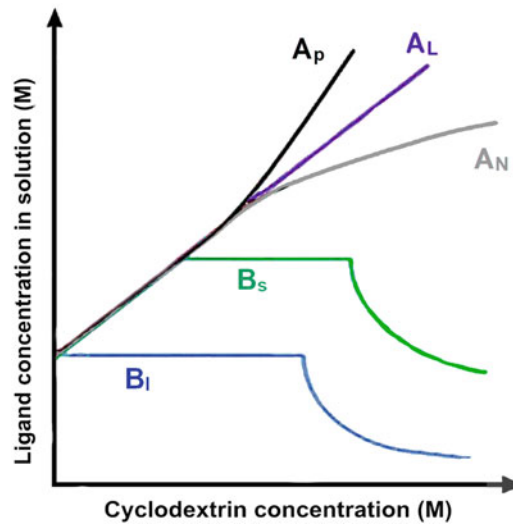

Fig. 3 Phase equilibrium solubility diagrams adapted from Higuchi and Connors (1965)

2. Type A_P: the positive curvature of the diagram indicates that the stoichiometric ratio is not 1:1, but rather that the order of the CD is greater than that of the ligands.
3. Type A_N: is difficult to interpret since the negative deviations can be associated with many reasons as guest molecular associations.
4. Type B: suggests the formation of poorly soluble (B_S) or insoluble (B_I) in aqueous medium complexes.

Most of the ligand:CD complexes present 1:1 stoichiometry. In this case, stability constants (K_s) of complexes in solution at a specific temperature can be obtained from the linearization of the A_L type phase diagrams. The following equation can be used to fit the phase diagram data:

$$K_s = K_{1:1} = \frac{[L - CD]}{[L][CD]} = \frac{\text{Slope}}{1 - \text{Slope}} \quad (1)$$

where [L], [CD], and [L−CD] are the equilibrium concentrations of the ligand, the cyclodextrin, and the complex formed, respectively. The intercept (S_0) can be estimated as the solubility of the ligand in water [15].

The thermodynamic parameters associated with the molecular encapsulation of the ligand, such as the variation of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG), can be obtained by studying the dependence of the stability constants of the complex with temperature [31]. This method determines K_s at different temperatures using the phase solubility method, as previously described. Then, the integrated equation of van't Hoff (Equation 2) could be applied:

$$\ln \; K_s^T = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad (2)$$

where K_s^T is the stability constant calculated at temperature T (in kelvin, K). With the data of enthalpy and entropy changes, the variation of free energy can be evaluated by applying the following equation (Equation 3):

$$\Delta G^0 = \Delta H - T\Delta S \quad (3)$$

where ΔG^0 is the free energy variation at 298 K. The analysis of the entropy, free energy, and enthalpy variations allows us to conclude whether a particular molecular encapsulation process is exothermic or endothermic, spontaneous, and whether it gives rise to more or less ordered systems.

1.3.3 Differential Scanning Calorimetry (DSC)

DSC is a frequently used method to verify and study solid-state complex formation [29, 32]. The total or partial disappearance of a characteristic transition of the guest molecule (e.g., endothermic

melting) is a strong evidence of complete or partial inclusion in the CD [23, 33], and encapsulation efficiency can be calculated. DSC also allows determining the glass transition temperature of the system, which occurs in a temperature range that depends on the rate of heating, the thermal history of the product, molar mass, and water content. BCD has a glass transition temperature difficult to determine as it is mostly a hydrate, while HPBCD, being an amorphous derivative, present a clear glass transition [22]. DSC can also be used to analyze oxidative stability of ligands and complexes by determining the oxidation initiation temperature.

1.3.4 Water Sorption Studies

Water sorption depends on solid–solid and solid–water interactions in the system and is affected by the physical state, the chemical composition, and the physical structure of the sample. Considering the strong influence of water on complex formation and stability, the analysis of water content in ligand:CD systems not only can give evidence of the complexation but also becomes of fundamental importance to define the appropriate storage conditions of the powder formulations [20]. If the equilibrium water content of the samples is plotted as a function of a_w, water sorption isotherms could be obtained.

2 Materials and Methods

2.1 Preparation of CD Solutions

To regularly prepare a saturated solution of BCD (15 mM), we weigh 1.85 g of the solid CD (Table 1) and add 100 ml of distilled water. Due to its difficulty to dissolve, BCD must be incorporated under stirring and heating at a temperature between 45 and 50 °C for 30 min. With this procedure, we obtain transparent solutions, stable at room temperature (20–25 °C). To prepare solutions with different concentrations of CD, it is convenient to make dilutions from the saturated solution (*see* **Note 1**).

2.2 Encapsulation with CD by Coprecipitation

2.2.1 Regular Protocol

In the coprecipitation method, the ligand is dispersed or dissolved in the CD aqueous solution in a suitable ligand:CD molar ratio (*see* **Note 2**). Depending on the properties of the ligand, it could be added directly in solid state or dissolved in water or aqueous ethanol. Controls of CD and ligand solutions must be prepared in the same conditions as complexes for comparison and for verifying complexation. Characteristics of the ligands, such as solubility, size, structure, spatial geometry, and polarity, are important to properly design the encapsulation experiment. To promote ligand–CD interactions, the system is stirred under heating at a constant rate for a certain time and then for a period of time at room temperature (*see* **Note 3**). For thermolabile ligands,

complexes can be prepared at room temperature optimizing stirring conditions. For example, to maximize the encapsulation of α-terpineol, we obtained an optimized time of 3 h at 50 °C and then 3 h at room temperature [20] and for cholesterol, 1 h at 50 °C followed by 23 h at 25 °C [21]. After stirring, the next step is to store the obtained suspensions overnight at 3 °C to promote the precipitation of the complexes (*see* **Note 4**). Then, the suspensions are filtered (PTFE filters of 0.45 μm average pore diameter), and the filtrates are dehydrated in a vacuum oven (50 °C) until constant weight, or frozen at −26 °C for 24 h and freeze-dried (operating at a condenser plate temperature of −111 °C, chamber pressure of 30 Pa, and shelf temperature of 25 °C).

2.2.2 Modifications to Regular Protocol

This protocol is recommended to study complexes of high aqueous solubility or that are part of a mixture, such as essential oils or vegetable extracts, and when a powder with all compounds present in the solution is required. As in the regular coprecipitation technique, CD solution is mixed with the ligand at the selected molar ratio (*see* **Note 5**). The combined solution is stirred during the selected time and temperature as previously described (*see* **Note 3**). The difference with the regular protocol is that, in this case, precipitation and filtering are not carried out, and the complete solution/suspension is frozen at −26 °C for 24 h and then freeze-dried in the same conditions explained for the coprecipitation method (Subheading 2.2.1).

Freeze-Drying of the Complete Solution/Suspension

Ultrasound Assistance

Protocols for ligand:CD complexation could be improved using ultrasound assistance. In this case, the mixture is ultrasonicated (US) (in an ice bath to avoid changes in temperature due to the process) for a time in the order of minutes (*see* **Note 6**) and then is stirred (*see* **Note 3**). To obtain complexes in solid state from aqueous solution, the dehydration of the systems is carried out by freeze-drying as explained in Subheading 2.2.1.

For achieving higher yields of extraction and encapsulation, we optimized a protocol using both modifications mentioned above to extract and encapsulate bioactive compounds from olive pomace, a by-product of the olive oil industry. In this experiment, the optimum sonication time was 10 min (100 W power, 30 Hz frequency, continuous pulse, titanium probe M2), and stirring during 21 h at 60 °C [19].

Hence, before selecting a protocol for encapsulation, it is essential to define and be clear about the optimization's goal and, also, how to follow the encapsulation of the ligand.

2.3 Study of Inclusion Complexes in Liquid Systems

2.3.1 Spectrophotometric Determination

Spectrophotometric techniques are commonly used for studying complexes in solution. The steps to quantify the ligand are listed below:

1. Obtention of the spectra (UV-Visible or fluorescence) of the ligand in the required solvent.
2. Analysis of the spectral data to obtain the maximum absorption UV-vis wavelength, absorptivity coefficient, fluorescence absorbance, etc.
3. A calibration curve must be done with different ligand concentrations, determining the absorbance at the maximum UV-Visible absorption wavelength. The range of ligand concentrations selected for the calibration curve should include the concentration of ligand used to prepare the combined ligand: CD solutions. In this way, the free ligand could be determined in the system.
4. In fluorescence studies, it is common to use standard reference solutions of compounds with high quantum yield, such as quinine sulfate (QS) (*see* **Note 7**). Solutions of different concentrations of the ligand of interest are then referenced to the calibration curve of QS. The results are commonly expressed in QS counts. Fluorescence is more sensitive than UV or visible techniques but is limited only to fluorescent ligands.

Figure 4 shows the absorption spectrum we obtained for an aqueous solution of α-terpineol (TER) 5 mM at 27 ± 2 °C. TER absorbs between 260 and 240 nm without a defined maximum. This type of spectra is typical of terpenoid compounds. The insert

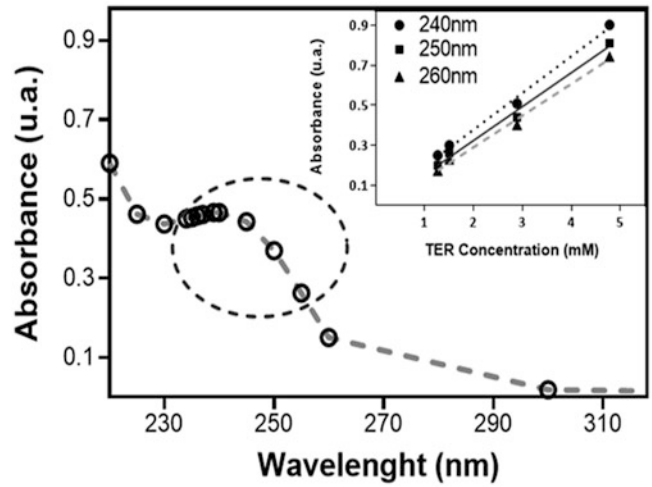

Fig. 4 UV absorption spectrum obtained for a 5 mM α-terpineol (TER) aqueous solution at 27 ± 2 °C. Insert graph shows the calibration curves made considering the absorbance at different wavelengths (240, 250, and 260 ± 4 nm) for 1.2 to 5 mM TER solutions

Table 2
Data of linearization of the TER calibration curves in the range 1.2–5 mM at 240, 250, and 260 nm. Limits of detection (LD = 3StDv$_0$) and quantification (LQ = 10StDv$_0$) values of TER were evaluated in saturated aqueous solutions of BCD (15 mM). (StDv$_0$ = the standard deviation)

(nm)	R^2	LD (mM)	LQ (mM)
240	0.994	0.34	2.89
250	0.991	0.21	1.27
260	0.993	0.38	2.36

in Fig. 4 shows the calibration curves performed considering the absorbance of standard solutions at different wavelengths within the TER absorption range (240, 250, and 260 nm).

The concentration range of TER standard solutions was 1.2 to 5 mM. All the linearizations presented a similar slope and a good regression coefficient. We performed a statistical analysis of the linearization at each wavelength, using the limits of quantification (LQ) and detection limit (LD) as selection criteria (*see* **Note 8**). In our experiments, we used a saturated solution of BCD (15 mM) without ligand as blank. Definitions: LQ = 10 StDv$_0$ and LD = 3 StDv$_0$. In Table 2, we show the results of this analysis.

Considering the results, we decided to perform the TER determinations at 250 nm as it maximized the sensitivity of the technique (lower LQ and LD).

In the introduction, we explain that one of the principal disadvantages of spectrophotometric determinations is that, in general, the solubility of the ligands in water is low, and the classical quantification techniques must be modified to improve its sensitivity. We determined a ligand as cholesterol with a very poor water solubility by modifying a commercial enzymatic kit test (COLESTAT, Wiener Laboratory, Rosario, Argentina) [21]. The determination of the chromophore product was made at 505 nm. By comparing the spectrophotometric absorption data of the standard ligand solutions with those of ligand:CD solutions, it is possible to know if the complex was formed and to quantify the ligand in solution during inclusion without modifying the equilibrium process. When the ligand is included in the inner cavity of the cyclodextrin, its absorption or emission signals could be modified in the spectra [34] due to electronic interactions. These spectrophotometric techniques allow not only to quantify but also to study the noncovalent ligand–CD interactions.

2.3.2 *Phase Solubility*

To determine stability constants (K_s) of the complexes, a phase solubility study could be carried out according to Higuchi and Connors method [30], as described in the introduction. Figure 5 shows an example of phase solubility diagrams we obtained for

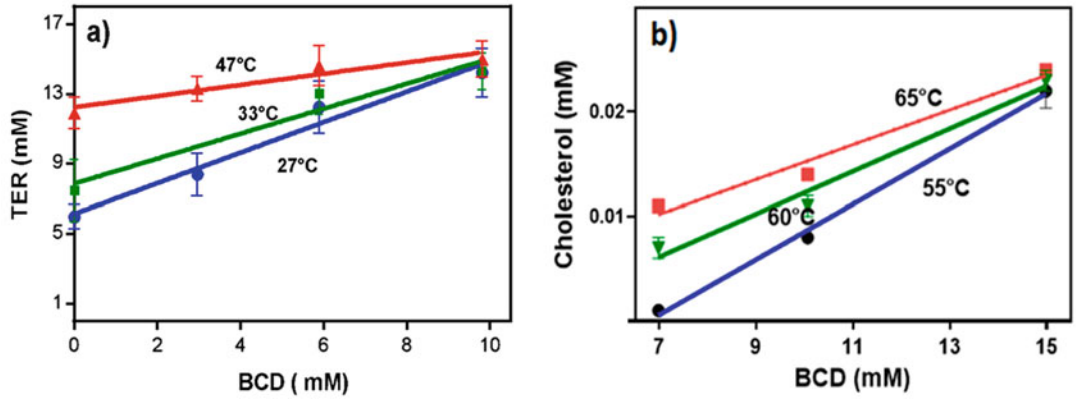

Fig. 5 Phase solubility diagrams for α-terpineol:BCD (**a**) and for cholesterol:BCD (**b**) at 27, 33, 47 °C and 55, 60, 65 °C, respectively

complexes of α-terpineol and cholesterol (CHO) with BCD (TER:BCD and CHO:BCD, respectively). These complexes were prepared by mixing an excess amount of ligand (TER 5 mM or CHO 5 mg/L) in an aqueous solution containing increasing amounts of the CD (0 to 15 mM for BCD) [20, 21]. After equilibration for 24 h, we determined the concentration of the ligands in the solutions spectrophotometrically (*see* **Notes 9** and **10**). We recommend to carry out the phase solubility experiments at least in triplicate. If the idea is to calculate thermodynamic parameters such as enthalpy or entropy changes, the K_s value must be obtained at three or more temperatures.

To characterize the complexes, we calculated K_s for each temperature from the fitted curve over the linear portion of the phase solubility diagrams using Eq. (1). Figure 5a shows that TER solubility increases linearly along with the assayed concentrations of BCD showing typical A_L-type diagrams that suggest the formation of 1:1 complexes. On the other hand, CHO solubility does not show typical linear phase solubility diagrams with all BCD concentrations. CHO water solubility changed very slightly with BCD concentrations less than 7 mM, regardless of temperature. Then, the solubility increased linearly with BCD concentrations higher than 7 mM (Fig. 5b). So, we consider CHO:BCD phase diagrams as type A_L from 7 mM CD concentration. The small change in cholesterol solubility at low BCD concentrations could be explained by the self-association of its molecules in aqueous solution. This particular characteristic of CHO led us to add an extra step of centrifugation at low temperature in the coprecipitation method (Subheading 2.2.1) [21]. This example clearly shows the need to adapt the methods to each ligand:CD system considering its particular and characteristic physicochemical properties.

Through the van't Hoff equations (Eqs 2 and 3), thermodynamic parameters (ΔH, ΔS, and ΔG^0) involved in complex formation can be calculated using the K_s values obtained at the studied

Table 3
Thermodynamic parameters of TER:BCD and CHO:BCD complex formation considering 1:1 molar ratio

System	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG_{25} (kJ mol^{-1})
TER:BCD	−124 ± 5	−355 ± 17	−18 ± 1
CHO:BCD	−43 ± 3	−65 ± 9	−23 ± 1

temperatures. Table 3 shows the thermodynamic parameters we obtained for the systems presented in Fig. 5 (TER:BCD and CHO:BCD). The negative values for ΔH indicate that both ligand inclusion processes are exothermic.

The greater the negative enthalphy change value, the more stable will be the ligand:CD system and more efficient is the inclusion. The relatively small and negative values of the entropy changes (ΔS) confirm that the main interactions that stabilize these complexes are of noncovalent type. The negative ΔS value is related to ligand:CD interactions that energetically favors the exit of water molecules from the inner cavity of the CD [21]. The rotational and translational degrees of freedom in the complex decrease with respect to the free molecules, giving rise to more ordered systems [21, 35]. The negatives values of ΔG indicate that the inclusion process of both ligands was spontaneous.

These thermodynamics studies provide useful information on suitable conditions for encapsulation and storage of inclusion complexes in cyclodextrins with potential application to the development of functional or nutraceutical foods.

2.4 Study of Inclusion Complexes in Solid State

We refer to complexes in solid state as those obtained in solution and then dehydrated by freeze-drying, as described in Subheading 2.2. These studies involve the verification of complexes formation and their characterization. The analyzes require comparing the results of the freeze-dried complexes with those obtained for systems prepared by physical mixture (PM). To prepare the physical mixture, accurately weighed quantities of the CD and the ligand (in the desired molar ratio) should be mixed in a mortar for a certain time (usually 5 to 10 min), enough to achieve a homogeneous powder. The PM is considered as control since no interactions are established between the ligand and the CD during the mixing process, and therefore the ligand remains free.

2.4.1 Differential Scanning Calorimetry (DSC)

In order to analyze solid complexes by DSC, powdered samples (5–20 mg) are weighed in 40 μL aluminum pans, which are then hermetically sealed. An empty pan is usually employed as reference. Dynamic scans over a wide temperature range (e.g., −80 to 180 °C) are performed to identify the characteristic transition temperature (melting, crystallization, evaporation) of the ligands

we want to encapsulate. Once that temperature has been identified, the start temperature of the run must always be approximate 20 °C below than the ligand transition temperature. It must be taken into account that at temperatures higher than 200–250 °C, most of the CDs and therefore their complexes break down. The scans are conducted under nitrogen gas flow. Measurements should be made at least in duplicate.

Determination of Encapsulation Efficiency

From dynamic thermograms, the melting temperature and the enthalpy of fusion of the pure ligand and of the ligand in the complexes could be obtained. Figure 6 shows the thermograms obtained for pure ligands (TER and CHO) and for their complexes with CDs (TER:HPBCD and CHO:BCD) prepared in a 1:1 molar ratio, compared to those obtained for ligand-CD physical mixtures. The thermograms of the CDs and the pure ligands were also included. Each sample was heated at 10 °C/min from −20 to 110 °C. In all systems, the enthalpy of fusion in the physical mixture was similar to the pure ligand, indicating that no CD complexes were formed by mechanical mixing.

The thermograms obtained for TER:HPBCD did not show any endothermic signal in the melting temperature range of TER, which indicates that the ligand was not free and was completely encapsulated in the CD at the studied molar ratio. However, in the CHO:BCD thermograms, the endothermic signal of the ligand was smaller than that of the pure ligand, indicating that CHO was partially encapsulated in BCD. Therefore, these results suggest that inclusion complexes were formed using the coprecipitation/freeze-drying method.

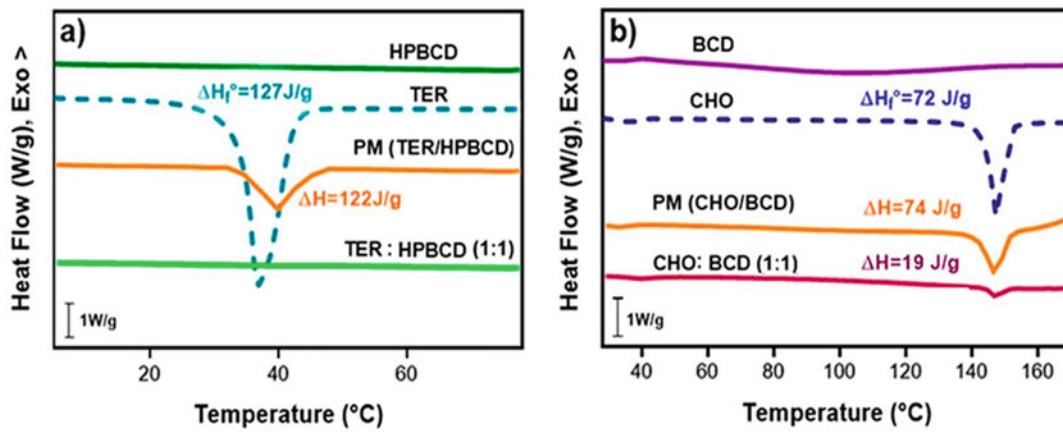

Fig. 6 Thermograms obtained by DSC for (**a**) α-terpineol (TER) and (**b**) cholesterol (CHO) both compared with the thermograms obtained for the ligands combined with 2-hydroxypropyl-beta-cyclodextrin (HPBCD) or beta-cyclodextrin (BCD), respectively, in ligand:CD molar ratios 1:1. The corresponding physical mixture thermogram of each system is also included

Table 4
Optimum stirring time and percentage of encapsulation efficiency (% EE) of each ligand, prepared in ligand:CD molar ratios 1:1 and 1:3, using β-cyclodextrin (BCD) or 2-hydroxypropyl-β-cyclodextrin (HPBCD). Systems were stirred at 25 °C and then freeze-dried as described in Subheading 2.2.1. The standard deviation of the determinations is indicated for $N = 3$. Ligands: cholesterol (CHO), α-terpineol (TER), and myristic acid (MYR)

System	Molar ratio	Optimum stirring time (h)	% EE
CHO:BCD	1:1	24	73 ± 2
CHO:BCD	1:3	24	65 ± 1
TER:BCD	1:1	6	100
TER:BCD	1:3	6	100
TER:HPBCD	1:1	6	100
TER:HPBCD	1:3	6	100
MYR:BCD	1:1	12	35 ± 2
MYR:BCD	1:3	12	45 ± 5
MYR:HPBCD	1:1	12	13 ± 3
MYR:HPBCD	1:3	12	42 ± 5

The encapsulation efficiency (EE%) could be determined as the ratio between the enthalpy of fusion of free ligands in the combined system (corrected considering the mass of ligand in the sample) and the enthalpy of fusion of the pure ligand, measured under identical conditions; according to Equation 4:

$$\text{EE}\ (\%) = \frac{m_0 - m_{\text{L}}}{m_0} \times 100 = \left(1 - \frac{\Delta H_m}{\Delta H_{m_0}}\right) \times 100 \qquad (4)$$

where m_0 is the total mass of the ligand, calculated based on the mass weighed in each DSC pan of the combined systems, m_{L} the nonencapsulated ligand mass, ΔH_{m_0} (J/g ligand) is the enthalpy of fusion of the pure ligand, and ΔH_m (J/g ligand) is the enthalpy of fusion of the ligand in the systems combined with CDs, normalized to the mass ligand ratio in the systems.

Table 4 summarizes the EE% values and the corresponding optimal stirring times we obtained for complexes of CHO, TER, and myristic acid (MYR), prepared in 1:1 and 1:3 ligand:CD molar ratios using the coprecipitation method. The optimal encapsulation conditions for each system were obtained analyzing EE% (Eq. 4) for different stirring times and ligand:CD molar ratios.

From this data, it is observed that the optimal molar ratio for CHO was 1:1. Compared with the other ligands, a longer stirring time was necessary to achieve CHO encapsulation. On the other hand, TER encapsulation seems to be independent of molar ratio and of the type of CD used, and its inclusion was total even with a

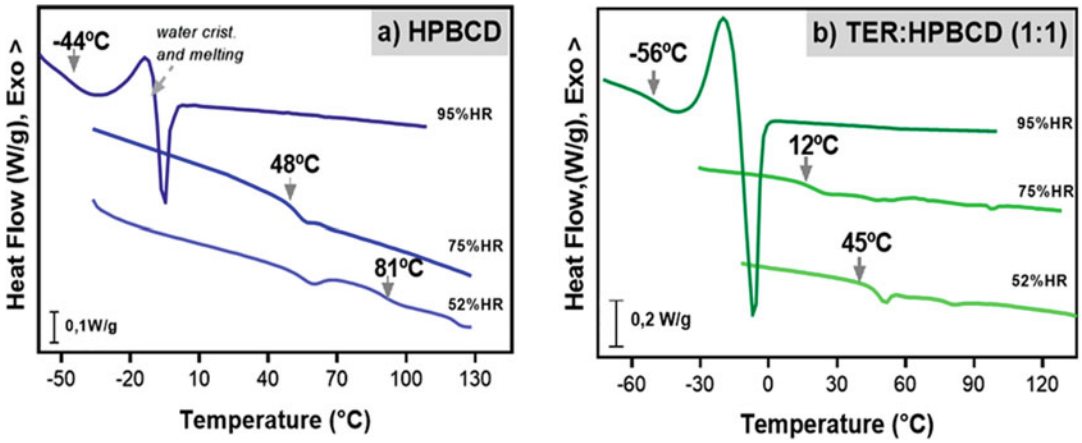

Fig. 7 Thermograms of 2-hydroxypropyl--cyclodextrin (**a**) and α-terpineol:HPBCD complexes (**b**) equilibrated at three relative humidity. Arrows show the onset temperature of the glass transition

shorter stirring time. For MYR, higher EE% was obtained with a 1:3 molar ratio, but encapsulation was partial, showing a lower affinity for HPBCD. This analysis allows us to study host–guest interactions and conclude about how encapsulation is affected by the structure, polarity, and geometry of the compounds.

Determination of Glass Transition Temperature (T_g)

Although many physicochemical properties of different cyclodextrins have been studied, there are not many studies on the influence of the formation of complexes on the glass transition temperature of cyclodextrins. The glass transition temperature is detected by DSC as an endothermic shift in the baseline of dynamic thermograms (obtained as described in Subheading 2.4.1) that occurs due to the change in the specific heat (ΔC_p) of the system at T_g.

Figure 7 shows the thermograms with the step corresponding to the glass transition for HPBCD (a) and for its complexes with TER (b), equilibrated at different relative humidities (% RH). In both systems, the plasticizing effect of water is observed.

For a given water content, the T_g value was lower in the complexes than in the HPBCD, which evidence a certain plasticizing effect of TER. Samples equilibrated at 95% RH (with the higher water content), exhibited exothermic and endothermic peaks corresponding to crystallization and melting of free water in the system. The obtained results show that the encapsulation of the ligand in the CDs involves interactions that promote supramolecular changes in the matrix.

2.4.2 Water Sorption Studies

The water sorption studies are performed by the isopiestic static-gravimetric method. Powdered samples (BCD and CHO:BCD) are distributed into glass vials of 5 mL (around 100 mg/vial) and placed into vacuum desiccators at different relative humidities

provided by different saturated salt solutions: $KCOOCH_3 \cdot 5H_2O$, $MgCl_2 \cdot 6H_2O$, $NaBr \cdot 2H_2O$, NaCl for achieving water activities (a_w) values of 0.22, 0.33, 0.57, 0.75, and 0.84 at 25 °C ± 1 °C, respectively [36]. The water activity of the saturated salt solutions must be confirmed using an a_w meter (AquaLab Series 3, Decagon Devices Inc., Pullman, USA). After reaching the equilibrium condition (difference in weight < 0.0005 g), samples are oven-dried under vacuum (735 mmHg) at 70 °C until constant weight to determine the water content at each relative humidity. The measurements are performed in triplicate, and the average value is informed.

Figure 8a shows the water content of BCD and CHO:BCD complexes at different molar ratios equilibrated at a_w 0.22, 0.33, 0.57, and 0.75 (25 °C).

The complexes show a lower water content than the pure BCD. One of the driving forces behind encapsulation in CDs is the replacement of the water molecules in the cavity by the ligand, so it can be used to verify the encapsulation (Fig. 8b). The presence of cholesterol in the system considerably modified the CD water sorption. The inclusion of the ligand modifies the CD–water interactions, and the water adsorbed by the complexes is less. This study allows us to confirm the encapsulation of the ligand and to analyze some properties of the complexes.

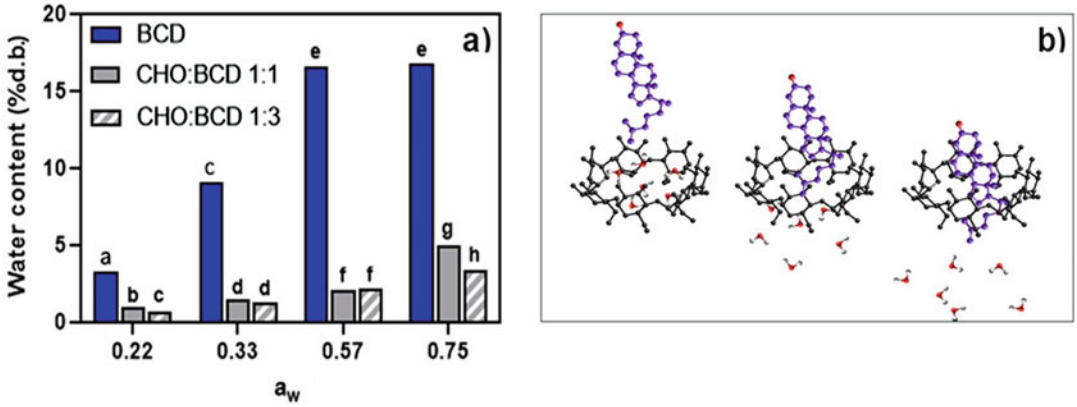

Fig. 8 (**a**) Water content of β-cyclodextrin (BCD) and of the cholesterol complexes (CHO:BCD) with 1:1 and 1:3 ligand:CD molar ratios, as a function of water activity, at 25 °C. Means with the same letter are not significantly different ($p > 0.05$). (**b**) Schematic representation of the displacement of water molecules by cholesterol inclusion in BCD cavity in aqueous solution. Structures of CD (black) and cholesterol (violet) were simulated with the HyperChem Professional V7.5 program. Only water molecules involved in the encapsulation process were represented to simplify the scheme

3 Conclusions

Encapsulation of different compounds in cyclodextrins can be carried out following the methods described in this chapter. Several techniques are described to confirm the formation of these complexes, both in solution and in solid state, and to characterize them for their application as food ingredients.

Through theoretical foundations and practical examples, it is intended to provide simple and clear protocols to encapsulate different types of compounds in cyclodextrins (BCD, HPBCD). We also pretend to contribute to a better understanding of the critical variables of the confinement process and of the interaction mechanisms involved.

4 Notes

1. If solutions are stored at temperatures lower than 4 °C, the CDs precipitate and must be reheated for dissolution. Hence, we suggest preparing only the CD solution volume required for the experiment to avoid solubility and contamination problems.
2. Ligands, such as terpenes and flavonoids that are completely encapsulated in the cavity of the CDs, form complexes at molar ratio 1:1. Other ligands, such as fatty acids, are not entirely encapsulated, and the stable inclusion complex with CD would be obtained with 1:2 or 1:3 ligand:CD molar ratios [22]. We recommend using different molar ratios to verify which is the most suitable to obtain the complexes (Subheading 2.4.1).
3. Stirring time and temperature must be selected according to ligand properties and CD solubility and have to be optimized to achieve a high encapsulation efficiency (Subheading 2.4.1).
4. Some poor water soluble ligands can form emulsions, so a centrifugation step with cooling could be necessary to favor the precipitation of the complexes. For example, in our experiments, a centrifugation of 20 min at 4 °C and 8000 × *g* was necessary to favor the CD:cholesterol complexes precipitation [21].
5. The selection of ligand:CD molar ratio must be based on preliminary calculations of the molecular weight of the ligand and CD characteristics. If ligands are part of a mixture, we recommend selecting the majority compound to calculate this molar ratio. For mixtures of different compounds, we recommend considering the one found in the highest proportion to calculate the ligand:CD molar ratio.

6. As several variables have to be considered, some statistics designs such as multiple response surface to optimize the parameters are recommended.
7. Quinine sulfate could be dissolved in 0.5 M H_2SO_4. Then the standard quinine solutions are measured at an excitation wavelength of 310 nm, to maximize the quantum yield of this molecule. These solutions are very stable at refrigeration temperature.
8. Commonly, statistical parameters "LQ" and "LD" are determined by measuring the standard deviation (StDv0) of the blank system (solvent without the interest compound).
9. It is assumed that the equilibrium between both species is reached after shaking or sonicating the system for a certain time at constant temperature and pressure.
10. The ligand or complex concentration could also be determined by different techniques such as high-performance liquid chromatography or NMR.

Acknowledgments

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Chapter 14

Stability of Antioxidants Encapsulated in Freeze-Dried Prebiotic Matrices

Juan Mauel Faroux, Maria Micaela Ureta, Andrea Gomez-Zavaglia, and Emma Elizabeth Tymczyszyn

Abstract

In this chapter, a method for evaluating the ability of freeze-dried prebiotic matrices (e.g., fructo and galacto-oligosaccharides, FOS and GOS, respectively) to stabilize vitamin C is described. Sucrose, a well-known protective compound, can be used as a control. Storage conditions can also be investigated by equilibrating samples in atmospheres of saturated salts within 11% and 85% relative humidities.

Key words Prebiotic matrices, Ascorbic acid, Sucrose, Relative humidity

1 Introduction

Preservation of bioactive compounds present in food has been the subject of various studies in the last decade. Freeze-drying microencapsulation is a methodology used to preclude degradation of such sensitive compounds [1].

The ability of some prebiotic compounds, such as galacto- and fructo-oligosaccharides (GOS and FOS, respectively), to form amorphous solids (glass matrix) underlines their importance as food components potentially useful to encapsulate functional ingredients. However, these amorphous solids can lose this glassy state under certain storage conditions (e.g., high temperature and humidity). This phenomenon, known as glass transition, can negatively impact the integrity of the bioactive components by collapsing the matrix structure [2]. FOS and GOS are complex mixtures of oligosaccharides with different degrees of polymerization (DP), well known for their prebiotic properties. Additionally, as they have a sweet flavor and are not hydrolyzed or absorbed in the upper part of the gastrointestinal tract, they arrive unaltered to

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the gut, thus contributing to the body weight control as low caloric sweeteners, and also giving the feeling of satiety [3].

Vitamin C, also known as ascorbic acid, is involved in several body functions (e.g., formation of collagen, iron absorption, immunomodulation, wound healing, maintenance of cartilage, bones, and teeth). However, it is very unstable to heat, oxygen, technological, and physiological processes. Therefore, retention of vitamin C is of great importance to ensure its biological functions. Furthermore, as its retention correlates well with that of other nutrients in food, it can be used as a model to study the stability of other bioactive components in processed, dried, and stored food products [2].

In order to evaluate the ability of different freeze-dried prebiotic matrices to stabilize labile nutrients (e.g., vitamin C), solutions of GOS, FOS, and sucrose (control sample) were used as protectant of freeze-dried ascorbic acid samples stored in different conditions.

2 Materials

2.1 Reagents

1. Ascorbic acid, sucrose, LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, KI, NaCl, KCl (pro analysis grade), and silica gel were purchased from Research Ag SA (Buenos Aires, Argentina).
2. Commercial GOS syrup (Cup Oligo H-70 Kowa Company, Tokyo, Japan)®, containing 75% of total GOS of different DP: 4% of high-molecular-weight oligosaccharides (DP $\geq$ 5), 21% of tetrasaccharides (DP4), 47% of trisaccharides (DP3), 23% of disaccharides (DP2) and lactose, and 5% of monosaccharides, including glucose and galactose, all percentages expressed in dry basis (w/w) [4].
3. Commercial FOS from Orafti Beneo p95 (Mannheim, Germany), containing 92% of total FOS of different DP: 6% of DP $\geq$ 7, 15% of DP 6, 16% of DP5, 29% of tetrasaccharides (DP4), 25% of trisaccharides (DP3); 5% of sucrose (DP2), and 3% of monosaccharides (e.g., glucose and fructose), all percentages expressed in dry basis (w/w) [5].
4. Folin–Ciocalteu reagent (BIOPAKC), purchased from Cientifica Nacional (La Plata, Argentina). This reagent can be prepared by dissolving 100 g sodium tungstate(VI) dihydrate and 25 g sodium molybdate(VI) dihydrate in 700 mL distilled water, 100 mL concentrated hydrochloric acid, and 50 mL of 85% w/w phosphoric acid. Then, 150 g of lithium sulphate hydrate is added to the mixture. This reagent is very stable if protected from reducing agents and from light [6].

2.2 Equipment

1. Spectrophotometer: Shimadzu Biospec 1601 UV-vis Spectrophotometer (Kyoto, Japan)

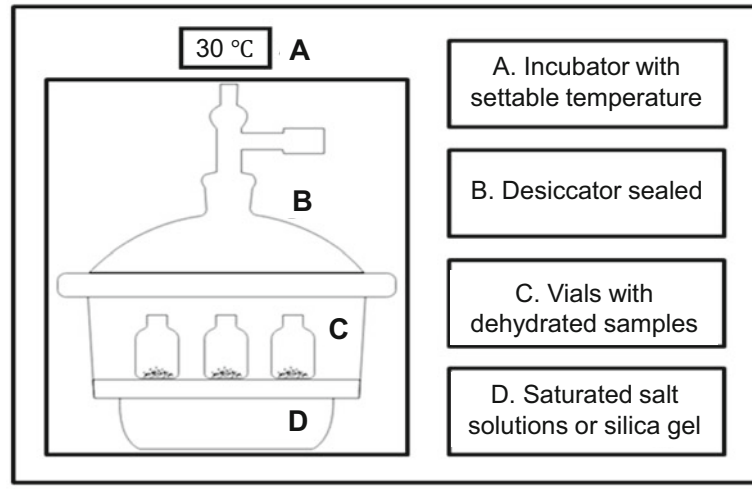

Fig. 1 Storage system with controlled relative humidity and temperature

2. Freeze-dryer equipment: Freeze-dryer (Labconco freeze-dryer system/Freezone 4.4, KansasCity, MO, USA). Condenser temperature: −50°C; chamber pressure: 0.06 mbar.
3. Drying chambers: A set of sealed recipients, with atmospheres of relative humidities (RH) of 11, 22, 33, 40, 70, 75, and 85%, achieved with the following salts: LiCl, CH_3CO_2K, $MgCl_2$, K_2CO_3, IK, NaCl, and KCl, respectively (Fig. 1).
4. Incubator: Laboratory-scale incubator with control temperature with or without cold-heat temperature, according to the temperature selected to storage and room temperature. The range of temperature varied between 4 and 50°C.
5. Freezer: Freezing temperature (e.g., −20, −40, or −80°C) for storage studies.

2.3 Solutions

1. 20 g of each sugar (e.g., GOS, FOS, and sucrose) was dissolved in distilled water to obtain 20% w/w solutions.
2. 1 g of ascorbic acid was dissolved in the sugars' solutions to achieve a concentration of 1% w/v.
3. The solutions were fractionated into 15-mL sterile tubes (1 mL per tube).

3 Methods

3.1 Encapsulation

Samples were frozen at −40°C for 24 h. Tubes were frozen on a tilted plane in order to enlarge the exposure surface. Then, samples were freeze-dried at <0.1 mbar ($T < -40$°C) for 48 h.

3.2 Storage

Freeze-dried samples were stored in sealed desiccators for 120 days at different temperatures (from 4 to 50 °C) at different RH (from 0 to 75% RH) (Subheading 2.2). The dry atmosphere (RH 0%) was made with silica gel previously desiccated at 100–105°C in a stove, until it reached a blue color (*see* **Note 1**) [7].

3.3 Folin–Ciocalteu Reaction

The Folin–Ciocalteu reaction is commonly used for quantification of polyphenols. These compounds react with the redox reagent to form a blue complex that can be quantified [8]. The molybdenum and the tungsten present in the Folin–Ciocalteu reagent are oxidized (VI). When reacting with a reducing agent, the molybdenum blue and the tungsten blue are formed, and the average oxidation state of the metals is between 5 and 6 [6]. The resultant blue complex has a maximal absorption at 760 nm and is proportional to the concentration of phenolic compounds originally present in the sample [9]. In this chapter, this reaction is used to quantify ascorbic acid and evaluate its degradation in the storage conditions.

3.4 Absorbance Measurement

The oxidation of ascorbic acid was evaluated every 30 days (Folin–Ciocalteu reaction).

1. A standard calibration curve was constructed using ascorbic acid (0–50 μg/mL), and the absorbance of the reaction products was determined at 760 nn (*see* **Note 2**).
2. The freeze-dried samples were dissolved in 500 μL of distilled water. Agitation for 15 min at 20 °C is recommended to facilitate the complete dissolution.
3. All samples were diluted to obtain final concentration within the limits of those used in the calibration curve. Aliquots of 15 μL were taken from the 500 μL dissolution, and 985 μL of distilled water were added to obtain 1 mL solution with a concentration of 30 μg/mL.
4. The diluted samples were vigorously mixed with 250 μL of Folin–Ciocalteu reagent (1/10) and left at 20 °C for 30 min to let the color stabilize [2].
5. The absorbance of all samples was read at 760 nm [2].

4 Notes

1. *Storage at different RH.* Samples were stored in hermetic sealed recipients with a saturated salt solution to generate the needed atmosphere. Therefore, each of them had a specific water activity (a_w). Table 2 shows the list of the salts that can be used and corresponding a_w.

   At least three different a_w must be used for all experiments reported in this chapter. In order to ensure their oversaturation, the solutions were prepared with solute in excess. Thus,

Table 1
Volumes of concentrated ascorbic acid solution (500 μg/mL) and water used to prepare the calibration curve

Series number	Ascorbic acid solution (μL)	Distilled water (μL)	Final concentration (μg/mL)
0	0	1000	0
1	20	980	10
2	40	960	20
3	60	940	30
4	80	920	40
5	100	900	50

Table 2
List of saturated salt solutions to induce different RH

Salt	Water activity (20°C)	RH (%)	Water solubility (20°C) (g/100 mL)
LiCl	0.1131	11	77.00
CH_3COOK	0.2311	22	253.00
$MgCl_2$	0.3307	33	54.20
K_2CO_3	0.4316	40	112.00
KI	0.6990	70	0.12
NaCl	0.7547	75	35.90
KCl	0.8511	85	23.80

the oscillations in the a_w values resulting from temperature fluctuations do not affect the RH of the hermetic sealed recipients. The solutions were incubated for 24 h at 20°C.

2. *Calibration curve.* The standard curve was prepared using solutions of ascorbic acid (1 mL) at different concentrations (0 μg/mL, 10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, and 50 μg/mL). One mL of distilled water was used as blank (0 μg/mL). For the 500 μg/mL solution, 0.01 g of ascorbic acid was dissolved in 20 mL of distilled water. Vigorous shaking was used to facilitate ascorbic acid dissolution (Table 1).

   All the solutions were mixed with 250 μL of Folin–Ciocalteu reagent (dilution 1/10), incubated at 20°C for 30 min [2] and read at 760 nm. Absorbance values from calibration curve were plotted as a function of concentration, and fitted using a linear regression. The obtained equation was used to determine the concentrations of ascorbic acid remaining in the stored samples.

Two issues need to be pointed out. The first one is related to the need of making a new standard curve for each measurement. Using an old standard curve can lead to calculation mistakes. Although the experimental procedure to make a standard curve is the same, differences in environmental conditions (e.g., temperature and humidity) can alter results.

The second issue is related to the use of distilled water as blank. To be strict, a standard curve should be carried out for each investigated sugar. When carrying out a standard curve for each solution, no significant differences between the sugar and the water curves were observed. Hence, it can be concluded that the Folin–Ciocalteu reagent just reacts with ascorbic acid, not having relevant interactions with sugars. For this reason, all standard curves were determined using ascorbic acid dissolved only in distilled water.

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Chapter 15

Immobilization of β-Galactosidase in Calcium Alginate Beads

Gonçalo Nuno Martins, Onofre Figueira, and Paula Cristina Castilho

Abstract

Enzyme immobilization comprises several methodologies that take advantage of the properties of different support materials for improving enzymatic processes. Among many techniques, the entrapment of enzymes in calcium alginate beads is one of the simplest and most used. β-Galactosidase is an enzyme relevant in the food industry for its ability to catalyze the hydrolysis of lactose into its constituent monomers, glucose and galactose, and to produce lactose-free consumer goods. The activity of this enzyme can be evaluated before and after its immobilization in calcium alginate beads, by its reaction with *O*-nitrophenyl β-D-galactopyranoside or with lactose.

Key words β-Galactosidase, Calcium alginate, Immobilization, *O*-Nitrophenyl β-D-galactopyranoside, Lactose

1 Introduction

Enzyme immobilization comprises several methodologies, widely known and studied for over 100 years, that take advantage of the properties of different support materials for improving enzymatic processes. The possibility of immobilizing enzymes widened their industrial application by allowing a better handling and easier separation and the possibility of their reusage. The catalysts become more stable under harsh chemical and physical conditions and can be introduced in all types of reactors. Overall, the entire enzymatic process becomes more economically viable and appealing for the adaptation in all industrial processes [1]. Among the different methodologies used over the years (entrapment, adsorption, encapsulation, covalent and ionic binding, and cross-linking), the entrapment, specifically, in calcium alginate beads, is one of the simplest and most used. This technique implies the occlusion of

Gonçalo Nuno Martins and Onofre Figueira contributed equally for the development of this work.

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the enzyme in the polymeric network of the alginate allowing the substrates and the products to be permeated by the polymer, while the enzyme is retained inside. Calcium alginate beads' immobilization usually translates in an increment in mechanical strength and enzyme stability, resulting in higher resistance to pH and temperature variations [2].

β-D-galactosidase (β-Gal, E.C. 3.2.1.23), also known as β-galactosidase and lactase, is an enzyme that can catalyze the hydrolysis of lactose to its constituent monosaccharides, glucose and galactose. However, given the appropriate conditions, such as high lactose concentrations, galacto-oligosaccharides (GOS) can be synthetized through the transference of a galactose moiety from a β-galactoside to a hydroxyl group of an acceptor. Industrially, this enzyme is widely used mainly as a hydrolase, namely in the food industry, for the generation of lactose-free products, important in cases of lactose intolerance, and for tackling lactose crystallization issues, obtaining more soluble and sweeter sugars. Its transferase activity is also commonly studied and used, aiming at the production of GOS for food and pharmaceutical applications, in virtue of their prebiotic and nutritional properties [2–4].

1.1 Objectives

1. Immobilize the enzyme β-galactosidase in calcium alginate beads.
2. Compare the enzymatic activity before and after the immobilization procedures using standard protocols.
3. Use the immobilized β-galactosidase for the hydrolysis of lactose.

2 Materials

Solutions and Reagents

All reagents used should be of analytical grade or higher, and all solutions, unless stated otherwise, should be prepared in ultrapure water. These can be prepared as such:

A. Alginate at 3% (w/v): Weigh 0.3 g of alginic acid and slowly transfer it into 9.6 mL of Tris–HCl buffer at 100 mM, pH = 7.3. Heat the beaker under continuous stirring (≈300 rpm) for 30–40 min with increasing temperature up to 50 °C. Keep under constant stirring until complete dissolution of the alginate. Let it cool to room temperature before use with enzyme (*see* **Notes 1** and **2**).

B. Calcium chloride at 2% (w/v): Weigh 2 g of anhydrous calcium chloride and add 100 mL of Tris–HCl buffer at 100 mM, pH = 7.3 into a beaker. Keep under constant stirring until complete dissolution at room temperature (*see* **Notes 1** and **2**).

C. Enzyme stock solution: 10 mL solution containing 10 mg of β-galactosidase from *Escherichia coli* (G-4155, Sigma-Aldrich) suspended in 50% glycerol, 5 mM Tris buffer salts, 5 mM magnesium chloride, 0.5 mM DTT, and 0.5 mM mercaptoethanol at pH 7.4—in a total of 5000 Units [5]. This solution has an enzyme concentration of 500 Units/mL, 500 Units/mg, and 1 mg/mL.

D. Sodium phosphate buffer at 100 mM, pH = 7.3 [6]: To prepare a 500 mL buffer solution, in a 500 mL beaker, weigh 7.0980 g of Na_2HPO_4 and dissolve in 300 mL of ultrapure water. Adjust the pH of the Na_2HPO_4 solution to 7.3 with a solution of 100 mM NaH_2PO_4 previously prepared by dissolving 1.3798 g of the monohydrated salt in 100 mL of ultrapure water. Make up the buffer solution's volume to 500 mL with ultrapure water. Store at 4 °C.

E. Tris–HCl buffer at 100 mM, pH = 7.3: Prepare a 1 M stock solution, weighing 150.53 g of Tris–HCl into a beaker and dissolving in 800 mL of ultrapure water using magnetic stirrer. Adjust the pH to 7.3 using HCl and/or NaOH. Make up the volume to 1 L with ultrapure water. Afterwards, dilute 20 mL of this solution with 150 mL of ultrapure water, adjust the pH to 7.3 if necessary and make up to 200 mL using ultrapure water. Store at 4 °C (*see* **Note 2**).

F. Magnesium chloride at 30 mM [6]: Weigh 0.0610 g of $MgCl_2 \cdot 6H_2O$ and dissolve in 10 mL of ultrapure water. Store at 4 °C.

G. 2-mercaptoethanol (2-ME) at 3.36 M [6]: Prepare by diluting 470 μL of the commercial product (14.3 M) with 1530 μL of ultrapure water (final volume = 2 mL). Store at 4 °C.

H. *O*-Nitrophenyl β-D-galactopyranoside (ONPG) at 68 mM [6]. Prepare by dissolving 0.1024 g in 5 mL of phosphate buffer (100 mM and pH = 7.3) in a water bath at ≈40–45 °C. Keep the solution at this temperature throughout the assay to prevent precipitation, which usually occurs at temperatures <40 °C.

I. Lactose at 10 g/L: Weigh 2.5 g of lactose. Add it to a 250 mL volumetric flask and add tris–HCl buffer at 100 mM, (*see* **Note 2**) pH = 7.3 to prepare a 250 mL solution.

3 Methods

The experimental procedures are described using a commercially obtained β-galactosidase from *E. coli* as an example. Enzymes from different sources present different properties and optimum working conditions, so caution is needed when replicating these

procedures as they may not be ideal to every β-galactosidase available, particularly when choosing the composition and pH of the buffer and the working temperature (*see* **Note 3**).

3.1 Immobilization Procedure

1. In an Eppendorf, add 200 μL of a 30 mM magnesium chloride (**Solution F**) solution and gently resuspend 200 μL of stock enzyme solution (**Solution C**) in the magnesium solution. Store this mixture at 4 °C until further use.
2. Gently add the 400 μL of enzyme–magnesium mixture into the 9.6 mL of the 3% (w/v) alginate solution (**Solution A**) and keep under stirring conditions for at least 1 min.
3. Transfer the alginate–enzyme mix to a 10 mL syringe with a 2 mm diameter exit.
4. Place a beaker containing the 100 mL calcium chloride solution at 2% (w/v) (**Solution B**) at 10 cm underneath the syringe and keep under constant magnetic stirring (≈300 rpm).
5. Manually push the plunger of the syringe in order to extrude, droplet by droplet, the alginate–enzyme mixture into the calcium chloride solution below.
6. Let the newly formed beads harden in the solution for at least 30 min at room temperature.
7. Collect the beads and wash once with distilled water and twice with Tris–HCl buffer 100 mM, pH 7.3.
8. Store the beads in Tris–HCl buffer, in the fridge, until further use (Fig. 1).

Fig. 1 Formed calcium alginate beads with entrapped β-galactosidase

3.2 Enzymatic Activity Determination

3.2.1 Activity of the Free β-Galactosidase

The enzymatic activity of β-galactosidase is determined spectrophotometrically by its reaction with *o*-nitrophenyl β-D-galactopyranoside (ONPG) and monitoring the release of the yellow-colored *o*-nitrophenol (ONP). The experimental conditions described in this protocol, except stated otherwise, follow those suggested by the supplier in their recommended protocol for the determination of the enzymatic activity of this specific enzyme [6].

1. Analyze the label, the product sheet, or the product's webpage, and determine the enzyme's concentration and Units based on the information available (*see* **Solution C**).
2. Prepare, by dilution, a 1 Unit/mL enzyme solution to be used in the ONPG assay. Use 100 mM phosphate buffer (pH 7.3) as solvent and keep the solution at 4 °C (*see* **Note 4**). First, prepare a 50 Units/mL solution and, from this one, prepare the 1 Units/mL solution.
3. Program the spectrophotometer for reading at 405 nm (*see* **Note 5**) and at 37 °C.
4. Perform the ONPG assay directly inside a cuvette (quartz or polystyrene). Add 2.6 mL of buffer, followed by 0.1 mL of both $MgCl_2$ and 2-ME solutions (*see* **Notes 6** and **7**). Finally, add 0.1 mL of enzyme at 1 Unit/mL or buffer (blank). Mix by inversion. Perform different experiments of the ONPG assay with phosphate and Tris–HCl buffers, both at 100 mM and pH = 7.3.
5. Place the cuvette inside the spectrophotometer. Wait a few moments until the temperature and absorbance stabilize.
6. Add 0.1 mL of ONPG solution and mix the final 3 mL solution. Record the change in absorbance over 10 min (*see* **Note 8**) and confirm a linear increase of absorbance over time as depicted in Fig. 2.
7. Perform this experiment in triplicates.
8. Calculate the $\Delta A_{405\text{nm}}$/min. It can be calculated simply by subtracting the absorbance at 10 min ($A_{10\text{min}}$) by the absorbance at 0 min ($A_{0\text{min}}$) and dividing this number by 10 min (t, duration of the assay), assuming a linear variation is observed. Perform this calculation for the experiments with the enzyme and the buffer.

$$\Delta A_{405\text{nm}}/\text{min} = \frac{A_{10\,\text{min}} - A_{0\,\text{min}}}{t} \quad (1)$$

9. Determine the enzyme's activity using the following formulas:

   First, calculate the number of Units/mL in the mixture of the ONPG assay ($Units/mL_{ONPG\ assay}$). Subtract the $\Delta A_{405\text{nm}}$/min obtained for the enzyme (*Test*) by that obtained for the buffer (*Blank*) and divide by the millimolar extinction coefficient of ONP at 410 nm—$\varepsilon_{\text{ONP}} = 3.5$ (*see* **Note 9**) [6].

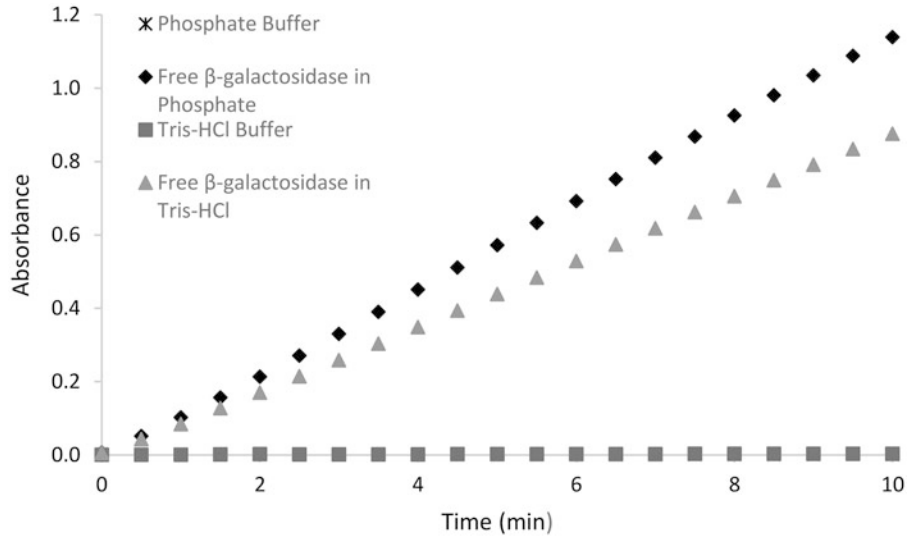

Fig. 2 Change in absorbance at 405 nm during the ONPG assay with free β-galactosidase using phosphate and Tris–HCl buffers as solvents. The plot for the phosphate buffer is superimposed with that for the Tris–HCl buffer

$$\text{Units/mL}_{\text{ONPG assay}} = \frac{(\Delta A_{405\text{nm}}/\min \textit{Test} - \Delta A_{405\text{nm}}/\min \textit{Blank})}{\varepsilon_{\text{ONP}}} \tag{2}$$

Multiply the number of $\text{Units/mL}_{\text{ONPG assay}}$ by 3 mL, the final volume in the assay—V_{assay}; obtain the overall number of *Units* in the cuvette.

$$\text{Units} = \text{Units/mL}_{\text{ONPG assay}} \times V_{\text{assay}} \tag{3}$$

1 Unit is defined as the amount of enzyme capable of hydrolyzing 1.0 μmol of ONPG per minute at 37 °C and pH 7.3.

By dividing the number of *Units* by the volume of enzyme solution used in the assay—in this case, 0.1 mL, the $\text{Units/mL}_{\text{enzyme}}$ are obtained. Multiply this value by the dilution factor (*Df*), if appropriate, and determine the $\textit{Units/mL}_{\textit{enzyme}}$ of the main stock enzyme solution.

$$\textit{Units/mL}_{\textit{enzyme}} = \frac{\text{Units}}{V_{\text{enzyme}}} \times \textit{Df} \tag{4}$$

The number of *Units/mL* obtained for β-galactosidase in phosphate and Tris–HCl buffers is in Table 1.

3.2.2 Characterization of the Immobilized β-Galactosidase

The activity of β-galactosidase after the immobilization in the calcium alginate beads (Subheading 3.1) as well as the amount of immobilized enzyme is determined by the ONPG assay. The latter is determined from a calibration curve, of the Units/mL versus the concentration, prepared with the free enzyme.

Table 1
Activity of free β-galactosidase determined by the ONPG assay using phosphate or Tris–HCl buffers

Sample	Free β-galactosidase	
Buffer	Phosphate	Tris-HCl
U/mL enzyme	1.0 ± 0.0	0.7 ± 0.0

Calibration Curve

1. Prepare the necessary solutions for the ONPG assay as described in Subheading 3.2.1, namely the 1 Unit/mL enzyme, the $MgCl_2$, the 2-ME, and the ONPG solutions. Replace the phosphate buffer by the Tris–HCl buffer (*see* **Note 2**).
2. With the 1 Unit/mL β-galactosidase solution, perform the ONPG assay as described in Subheading 3.2.1 but with decreasing volumes of enzyme. Adjust the volume of buffer used accordingly (Table 2).
3. Calculate the enzyme's concentration (ng/mL) in the various assays (Table 2), knowing that the supplied enzyme solution at 500 Units/mL—1 mg/mL (Subheading 2, **solution C**)—was diluted to 1 Unit/mL—2 μg/mL (Subheading 3.2.1, **step 2**).
4. Calculate the $mUnits/mL_{ONPG\ assay}$ obtained in the 3 mL solution of the ONPG assay for each experiment (Table 2) using the formulas discussed in Subheading 3.2.1, **steps 8** and **9**.
5. Plot the $mUnits/mL_{ONPG\ assay}$ values obtained in **step 4** of this section versus the β-galactosidase concentration in each assay, calculated in **step 3**, and determine the linear plot of this correlation (Table 2).

Activity of the Immobilized β-Galactosidase

For the determination of the activity of the β-galactosidase immobilized in calcium alginate beads, the ONPG assay requires some modifications. Specifically, this form of the assay is performed in a water bath at 37 °C, and using magnetic stirrer for mixing the solution with the immobilized enzyme.

1. Immobilize the enzyme using the procedure described in Subheading 3.1.
2. Prepare a water bath at 37 °C.
3. In a cuvette, add 2.7 mL of Tris–HCl buffer at 100 mM and pH = 7.3, followed by 0.1 mL of the $MgCl_2$ solution and 0.1 mL of the 2-ME solution. Finally, place an appropriate and representative amount of calcium alginate beads with immobilized enzyme (test) inside the cuvette—in this case,

Table 2
Experimental procedure for the determination of the activity of free β-galactosidase at various mass concentrations. The *mUnits/mL*$_{ONPG\ assay}$ refers to the *mUnits/mL* obtained in the final 3 mL solution of the ONPG assay

Experimental preparation				Results
β-gal initial concentration (μg/mL)	$V_{β\text{-gal}}$ (μL)	V_{Buffer} (μL)	β-gal final concentration (ng/mL)	$mUnits/mL_{ONPG\ assay}$
2.0	100	2600	66.7	24.8
	75	2625	50.0	19.4
	50	2650	33.3	12.9
	25	2675	16.7	6.6
	5	2695	3.3	1.4
	1	2699	0.7	0.4
	0, Blank	2700	0.0	0.0
			Equation	m = 37.546 b = 0.213
			R^2	0.991

five beads are used (*see* **Note 10**). Place the cuvette in the water bath and mix the solution and the loaded calcium alginate beads at 300 rpm using a magnetic stirrer, small enough to fit inside the cuvette.

4. Prepare the spectrophotometer for readings at 405 nm and 37 °C.
5. With the cuvette inside the water bath, add 0.1 mL of the ONPG solution, and after mixing, place the cuvette in the spectrophotometer and record the absorbance. Return the cuvette to the water bath and incubate the mixture.

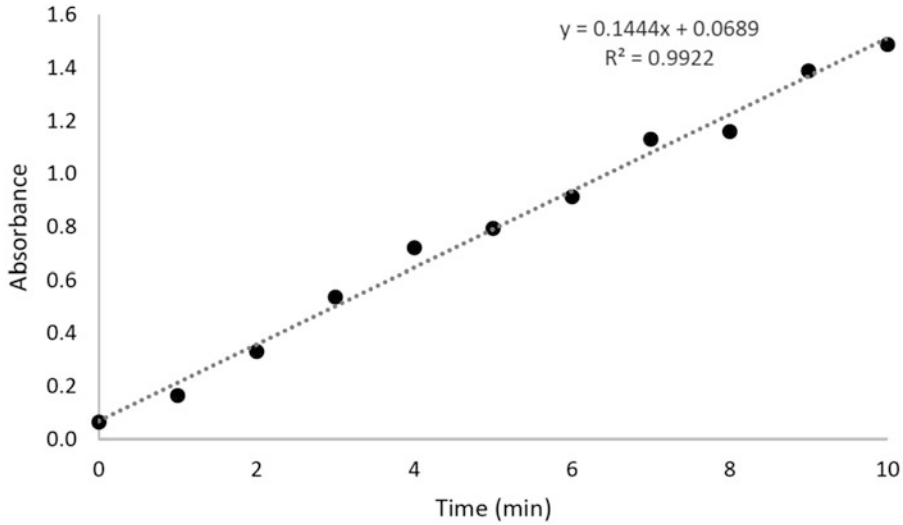

Fig. 3 Change in absorbance at 405 nm during the ONPG assay for immobilized β-galactosidase on calcium alginate beads. The assays were done with Tris–HCl buffer

6. Record the absorbance at least once every minute over 10 min (Fig. 3).
7. Calculate the $\Delta A_{405\text{nm}}$/min and the $mUnits/mL_{ONPG\ assay}$ using the formulas described previously in Subheading 3.2.1, **step 8** and **9**. The $\Delta A_{405\text{nm}}$/min for the buffer is calculated by performing this experiment without the immobilized enzyme.
8. With the value of $mUnits/mL_{ONPG\ assay}$, determine the enzyme concentration in the ONPG assay ($ng\ \beta\text{-}Gal/mL_{ONPG\ assay}$), using the calibration curve (Subheading "Calibration Curve", **step 5**, Table 2).
9. Divide the value of $mUnits/mL_{ONPG\ \text{assay}}$ by the enzyme concentration ($ng\ \beta\text{-}Gal/mL_{ONPG\ assay}$) and obtain the $mUnits/ng$ of enzyme (Table 3). Perform this calculation for both the free and immobilized enzyme, for comparison of the enzyme's activity before and after the immobilization process.

$$mUnits/ng\ \beta - Gal = \frac{mUnits/mL_{ONPG\ assay}}{ng\ Gal/mL_{ONPG\ assay}} \tag{5}$$

10. Multiply the value of the $mUnits/mL_{ONPG\ \text{assay}}$ by 3 mL (final volume in the ONPG assay, V_{assay}), and obtain the Units of enzyme present in the cuvette during the assay. Divide this number by n (number of beads used in the assay, $n = 5$) to determine the *Units/bead* (Table 4).

$$mUnits/bead = \frac{mUnits/mL_{ONPG\ assay} \times V_{assay}}{n} \tag{6}$$

Table 3
Activity of β-galactosidase before and after the immobilization in calcium alginate beads as determined by the ONPG assay using Tris–HCl buffer as solvent

	Free β-galactosidase	Immobilized β-galactosidase
Buffer	Tris-HCl	Tris-HCl
mUnits/ng β-galactosidase	0.4 ± 0.0	0.4 ± 0.0

Table 4
Activity of the immobilized β-galactosidase, as determined by the ONPG assay, expressed as mUnits per number of loaded bead (*mUnits/bead*) and mUnits per g of loaded bead (*mUnits/g bead*) and the average mass of loaded bead (m_{bead})

	Immobilized β-galactosidase
mUnits/bead	24.6 ± 2.3
mUnits/g bead	606.9 ± 55.6
m_{bead} (g)	0.041 ± 0.001

11. several loaded beads and calculate their average mass (m_{bead}) in grams. Divide the $mUnits/bead$ by the average bead's mass and obtain the $mUnits/g_{bead}$ (Table 4).

$$mUnits/g_{bead} = \frac{mU/bead}{m_{bead}} \quad (7)$$

Determination of the Amount of Immobilized β-Galactosidase

The amount of immobilized enzyme is determined from the enzyme concentration in the ONPG assay ($ng\ \beta\text{-}Gal/mL_{ONPG\ assay}$), which is calculated using the calibration curve discussed in Subheading "Calibration Curve", **step 5**.

1. Multiply the value of $ng\beta\text{-}Gal/mL_{ONPG\,assay}$ by the final volume in the ONPG assay ($V_{assay} = 3$ mL), and divide by the number of beads used ($n = 5$) to determine the mass of β-galactosidase (ng) per bead (Table 5).

Table 5
Amount of enzyme immobilized per bead and per g of beads

	Immobilized β-galactosidase
ng β-Gal/bead	65.1 ± 6.0
ng β-Gal/g bead	1607.9 ± 148.2

$$ng\ \beta - Gal/bead = \frac{ng\ \beta - Gal/mL_{ONPG\ assay} \times V_{assay}}{n} \quad (8)$$

2. Divide the mass of enzyme/bead by the average mass of loaded bead (Table 4), and obtain the mass of β-galactosidase per gram of bead (ng β-Gal/g) (Table 5).

$$ng\ \beta - Gal/g_{bead} = \frac{ng\ \beta - Gal/bead}{m_{bead}} \quad (9)$$

3.3 β-Galactosidase Activity over Lactose

The hydrolytic activity of β-galactosidase can be assessed by monitoring its reaction with lactose. The experimental conditions are similar to those used in the ONPG assay except lactose is now the substrate [7].

3.3.1 Reaction of the Free β-Galactosidase

1. Prepare a water bath at 37 °C.
2. Add, to a 25 mL beaker, 15 mL of the previously described lactose solution at 10 g/L in 100 mM Tris–HCl buffer at pH = 7.3 (**Solution I**). Keep under magnetic stirring at 500 rpm.
3. Add 0.5 mL of a 30 mM $MgCl_2$ solution (**Solution F**), maintaining the same final concentration as in the ONPG assay.
4. Start the reaction by adding the enzyme solution of an appropriate concentration. Add 0.03 mL of an enzyme solution with 500 U/mL (**Solution C**)—final concentration of 1 U/mL.
5. Monitor the reaction for 24 h (Fig. 4), and take several aliquots throughout the experiment (0, 1, 2.5, 5, and 24 h).
6. Stop the reaction through enzyme inactivation in a water bath at 100 °C for 10 min.
7. Filter the samples in 0.45 μm cellulose-acetate filters for HPLC and analyze them by HPLC-RI.
8. Determine the amount of lactose in each sample using a calibration curve, prepared with the standard compound. Calculate the percentage of lactose hydrolysis (Table 6) in a sample by comparing the concentration of lactose in that sample ($C_{Lactose}$) with its initial concentration ($Ci_{Lactose}$):

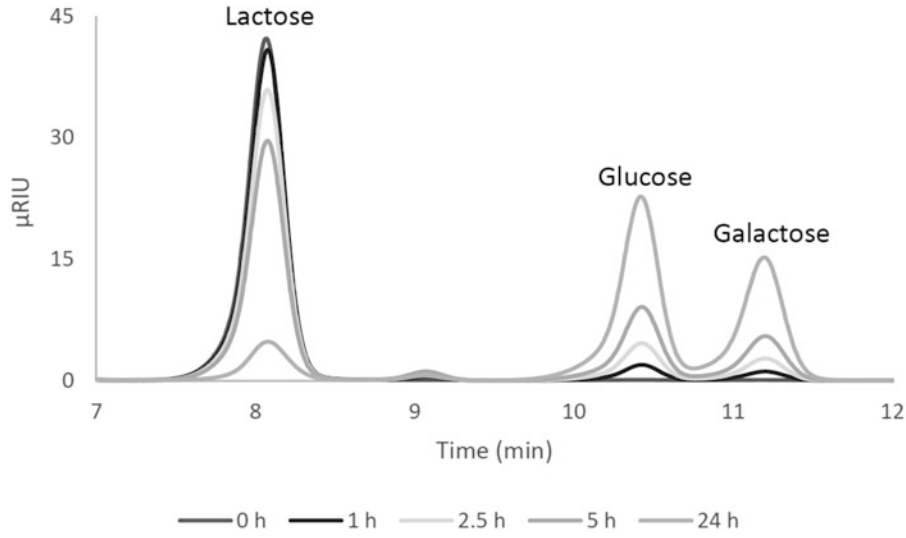

Fig. 4 Chromatograms obtained by HPLC-RI after the reaction of free β-galactosidase at 1 U/mL and lactose at 10 g/L for 5 h in 100 mM Tris–HCl buffer pH = 7.3 at 37 °C

Table 6
Percentage of lactose hydrolysis by the free β-galactosidase at different times

Time	0 h	1 h	2.5 h	5 h	24 h
Lactose Hydrolysis (%)	0.0 ± 0.7	4.7 ± 1.6	17.6 ± 1.3	33.1 ± 1.4	90.8 ± 0.5

$$Lactose\ hydrolysis\ (\%) = 100 - \left(\frac{C_{Lactose}}{Ci_{Lactose}} \times 100\right) \qquad (10)$$

3.3.2 Reaction of the Immobilized β-Galactosidase

1. Prepare a water bath at 37 °C.
2. Add, to a 25 mL beaker, 15 mL of the previously described lactose solution at 10 g/L in 100 mM Tris–HCl buffer at pH = 7.3 (**Solution I**). Keep under magnetic stirring at 500 rpm.
3. Add 0.5 mL of a 30 mM $MgCl_2$ solution (**Solution F**), maintaining the same final concentration as in the ONPG assay.
4. Weigh 700 mg of calcium alginate beads with immobilized β-galactosidase (≈18 to 20 beads).

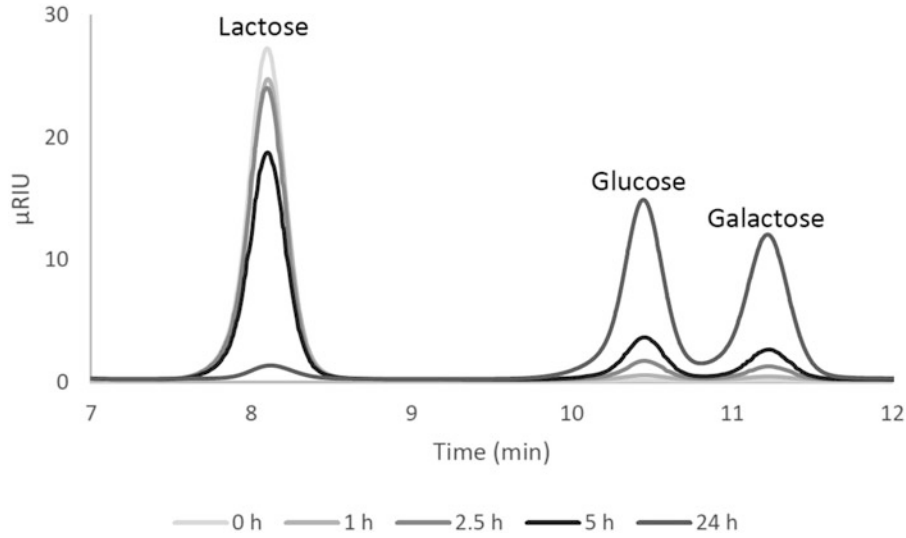

Fig. 5 Chromatograms obtained by HPLC-RI after the reaction of β-galactosidase, immobilized in calcium alginate beads, at 1 U/mL and lactose at 10 g/L for 5 h in 100 mM Tris–HCl buffer pH = 7.3 at 37 °C

5. Start the reaction by adding 700 mg of calcium alginate beads—approximate to 1 U/mL of immobilized enzyme—to the reaction mixture.
6. Monitor the reaction for 24 h, and take several aliquots throughout the experiment (0, 1, 2.5, 5, and 24 h).
7. Stop the reaction by decantation of the beads. Filter the aliquots in 0.45 μm cellulose-acetate filters for HPLC. Analyze the aliquots by HPLC-RI (Fig. 5).
8. Determine the amount of lactose in each sample using a calibration curve prepared using the standard compound and calculate the percentage of lactose hydrolysis (Table 7) using the formula discussed in the previous section (Subheading 3.3.1, **step 8**).

4 Notes

1. Dissolutions of alginic acid (alginate) and calcium chloride are performed with the buffer to maintain the appropriate conditions for the microenvironment of the enzyme when entrapped in the calcium alginate beads. The dissolution of the alginic acid, for example, reduces the pH of the medium, reducing the activity of neutral enzymes.
2. Buffers with different compositions and pH can be used, depending on the properties and nature (acid or neutral) of the enzyme. However, the use of phosphate and citrate buffers is not advisable since they cause the disintegration of the alginate polymer. Since phosphate buffer is incompatible with the

Table 7
Percentage of lactose hydrolysis by β-galactosidase immobilized in calcium alginate beads at different times

Time	0 h	1 h	2.5 h	5 h	24 h
Lactose Hydrolysis (%)	0.0 ± 3.3	4.7 ± 9.8	4.1 ± 10.3	27.0 ± 9.6	98.6 ± 0.3

calcium alginate beads, Tris–HCl buffer is used for consistency between different experiments [8].

3. β-Galactosidases can be of two natures: acid or neutral. Neutral β-galactosidases have an optimal pH ranging between 6.0 and 7.0 and therefore neutral buffers, such as sodium phosphate buffer or Tris–HCl, are more suitable. Acid β-galactosidases have an optimal pH ranging between 3.0 and 5.0 being advisable the use of more acidic buffers, such as sodium acetate buffer [9].
4. The usage of fresh solutions is recommended but it was observed that enzyme solutions can be kept at 4 °C for days and still maintain their activity.
5. Different protocols for this assay use different wavelengths for ONP detection—in the range of 400 nm–420 nm [3]. In this experiment, the analyses are performed at 405 nm because this method is to be adapted into a microplate reader format, and this is the only wavelength available in the equipment. Additionally, this wavelength has also been used by the manufacturer before in the determination of the activity of this enzyme [10].
6. The buffer and other reagents can be kept at 37 °C prior to being used.
7. The inclusion of the Mg^{2+} metal ion is important for enzyme stabilization [3]. The purpose of 2-ME is to act as a reducing agent that can increase the activity of enzymes that contain sulfhydryl groups in the enzyme's active site [3, 11].
8. The absorbance at 405 nm was initially monitored for only 5 min as described in the supplier's recommended protocol [6]; however, it was latter increased for 10 min when determining the activity of the immobilized enzyme.
9. Although the wavelength used in the assay is 405 nm, instead of the 410 nm suggested by the supplier, the value of the

millimolar extinction coefficient of ONP at 410 nm was used for the calculations.

10. The number of beads used was chosen to be five in order to have a representative amount of immobilized enzyme, accounting for differences in enzyme loading onto the different calcium alginate beads produced, and also to not cause a significant change in the final volume of the mixture.

Acknowledgments

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Chapter 16

Bacterial S-Layer Proteins for Stabilization of Food Ingredients Encapsulated in Liposomes

Maria de los Ángeles Serradell and E. Elizabeth Tymczyszyn

Abstract

To protect food ingredients from degradation that can occur in the harsh environmental conditions of the gastrointestinal tract, many different oral delivery systems such as liposomes have been developed. However, liposomes are dynamic entities which tend to aggregate and/or fuse, and thus some strategies can be used to improve their features. In this sense, bacterial S-layer proteins (SLPs) can reassemble as a rigid nanostructured lattice covering different surfaces completely, and they have been used to stabilize liposomes.

In this chapter, we will describe the experimental protocols to prepare and characterize SLP-coated multilamellar lipid vesicles which can be applied to the encapsulation of food ingredients.

Key words S-layer proteins, Lipid membranes, Degradation, Gastrointestinal tract

1 Introduction

The harsh environment that reigns over the gastrointestinal tract such as low pH and presence of bile salts and enzymes forces the need for the development of oral delivery systems that protect the substances or structures (e.g., vitamins, antioxidants, fatty acids, essential oils, hormones, antimicrobial peptides, drugs, antigens, enzymes, and probiotic microorganisms) that must reach the intestine without being degraded, so that those systems must tolerate the adverse conditions themselves. Moreover, these ingredients may become hazardous due to oxidation reactions or can also react with other components present in the food, which might limit bioavailability, or change the color or taste of a product, thus favoring spoilage and deterioration. In many cases, microencapsulation can be used to overcome these challenges [1].

Regarding many different systems that have been developed, liposomes are among the safest ones because of their biocompatibility and biodegradability [2]. They are nano- to micro-sized

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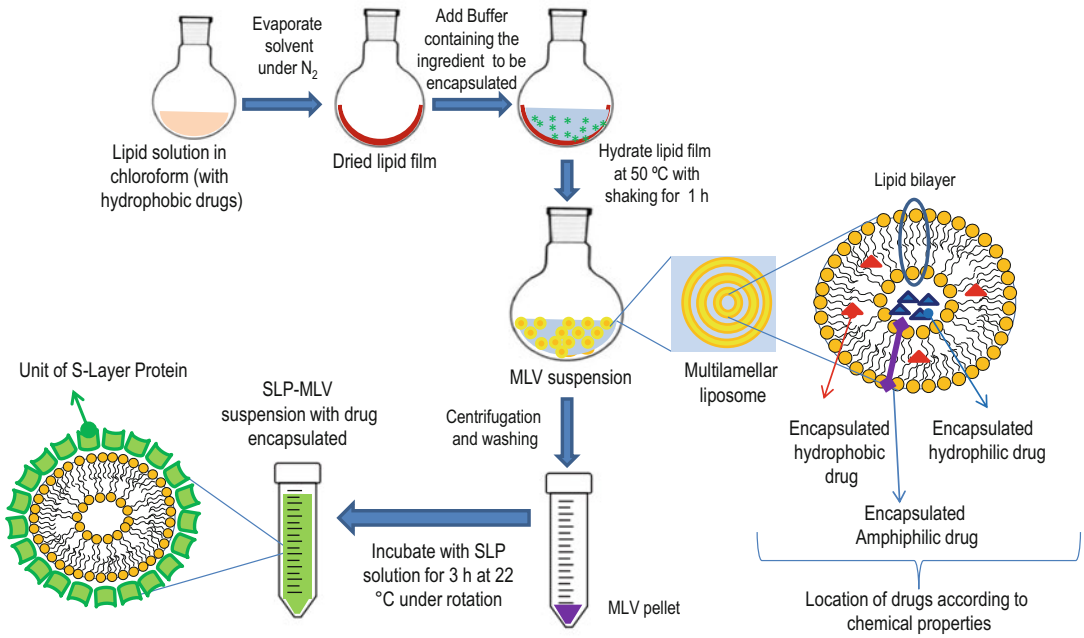

Fig. 1 Scheme for the preparation of liposomes coated with SLPs

spherical lipid vesicles having a cell-like morphology consisting of an internal aqueous core surrounded by an external phospholipid bilayer envelope, able to encapsulate and deliver both lipophilic and hydrophilic substances as it is shown in Fig. 1 [3]. Different types of food ingredients can be encapsulated in liposomes and nanoliposomes such as flavoring agents, essential oils, amino acids, vitamins, minerals, enzymes, microorganisms, antioxidants, antimicrobials, preservatives, among others [1]. However, due to liposomes are dynamic entities which tend to aggregate and/or fuse, the poor stability of conventional liposomes could limit their applications; therefore, increasing attention has been paid to the surface engineering to improve their features [4].

The S-layer is a rigid nanostructured envelope formed by protein subunits that covers the surface of different prokaryotes, and it has been maintained throughout the evolution both in *Bacteria* and *Archaea* [5].

Bacterial S-layer proteins (SLPs) account for approximately 10–15% of total cellular proteins thus, regarding that the biomass of prokaryotic microorganisms surpasses the biomass of the eukaryotic organisms, these surface proteins can be considered as one of the most abundant biopolymers on our planet [6]. S-layers are composed of numerous identical proteins or glycoprotein subunits, 40–200 kDa in molecular weight, which form a two-dimensional, regular, and highly porous array (unit cell sizes in the range of 3 to 30 nm, and thicknesses of roughly 10 nm) with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry [7].

Many different functional properties have been proposed for bacterial S-layers. Thus far, they include protection from adverse environmental conditions or antimicrobial peptides or radiation or bacteriolytic enzymes, functioning as molecular sieves, as binding sites for ions, phages, or toxins, mediation of bacterial adhesion to different substrates, and also immunomodulation [8, 9].

The proteinaceous subunits are held together and attached to the underlying cell surface by noncovalent interactions. Thus, some detergents or chaotropic agents are usually employed for the isolation and purification of the SLPs [10].

The isolated SLPs are able to reassemble into mono- and double layers at solid supports, at the air–water interface, at lipid films, and to cover nanocapsules, nanoparticles, emulsomes, and liposomes completely [4, 10–12]. This unique capability can be exploited in different areas such as medicine, pharmacology, bioengineering, and food industry, among others.

For coating liposomes, the SLPs from different Gram-positive bacterial species such as *Lactobacillus kefiri*, *Lactobacillus brevis*, *Lactobacillus helveticus*, and *Bacillus stearothermophilus* [13–16] have been used. There is evidence that coating with bacterial SLPs improve membrane stability [13–16] and gastrointestinal adhesion of liposomes [16]. Moreover, delivering of calcein to human colon adenocarcinoma cells has been achieved in a safe and efficient way using SLP-coated liposomes [17]. Considering all these findings, SLP-coated liposomes constitute a promising stable carrier system for sensible biomolecules and nutraceutics in food industry.

This chapter will be focused on the experimental aspects for preparation, characterization, and stability assay of SLP-coated liposomes.

2 Materials

2.1 Bacterial Strains

As a source of SLPs, *Lactobacillus kefiri* strains will be used in this chapter (*see* **Note 1**).

2.2 Chemical Reagents

Prepare all solutions using deionized water and analytical grade reagents. For bacterial cultures, de Man-Rogosa-Sharpe (MRS) commercial broth must be prepared by dissolving 55.5 g of powder per liter of water (*see* **Note 2**), and then sterilizing at 121 °C and 2 atm for 15 min. For S-layer protein isolation, prepare phosphate-buffered saline (PBS; KH_2PO_4 0.144 g/l, NaCl 9 g/l, Na_2HPO_4 0.795 g/l, pH 7.2) and 5 M LiCl. For preparation of liposomes, the following substances will be needed: soybean lecithin, cholesterol, stearylamine, chloroform, and calcein. To evaluate the stability of SLP-coated liposomes under gastrointestinal conditions, simulated gastric solution (in w/v: 0.73% NaCl, 0.05% KCl, 0.4% $NaHCO_3$, and 0.3% pepsin U/ml) at pH 2.5 (adjusted with 18.5%

w/v HCl) and simulated intestinal fluid (in w/v: 0.1% pancreatin and 0.15% bovine bile salts) at pH 8.0 (adjusted with 20% w/v NaOH) have to be prepared.

2.3 Equipment

1. Laboratory centrifuge: Maximum speed 20,000 × *g*. Rotor interchangeable for different types and size of tubes.
2. Rotavap: Rotative equipment with vacuum for evaporation of solvent.
3. Fluorescence spectrometer; minimal requirements: fluorescence emission detection at 520 nm, excitation wavelength of 492 nm.
4. Test tube rotator; rotation speed 6–60 rpm.

3 Methods

3.1 Bacterial Growth

Lactobacilli are usually grown to stationary phase in 250 ml of MRS broth at 37 °C in anaerobic or aerobic condition [18].

3.2 S-Layer Isolation

Harvest bacterial cells by centrifugation (5000 × *g*, 15 min, 4 °C), and wash twice with PBS. Resuspend the pellet in a tenfold volume of 5 M LiCl and agitate for 20 min–1 h at 20–25 °C (*see* **Note 3**). After centrifugation (5000 × *g*, 15 min, 4 °C), dialyze the extract against distilled water at 20–25 °C for 24 h under agitation, changing the water every 2 h.

To assure the absence of large aggregates, a centrifugation at 25,000 × *g* for 20 min at 4 °C should be carried out. The quality of the clear supernatant can be performed by SDS-PAGE [19], and the SLP concentration can be determined by a protein detection assay (bicinchoninic acid or Bradford's method [20]).

3.3 Liposome Preparation

To obtain positively charged liposomes, prepare a mixture composed of 625 nmol of soybean lecithin, 312 nmol of cholesterol, and 63 nmol of stearylamine in 100μl of chloroform (*see* **Note 4**). Then, put necessary volume of chloroform solution in a glass tube or balloon and evaporate the solvent under nitrogen flow to obtain lipid film according to Fig. 1 (*see* **Note 5**). Storage the lipid film under vacuum overnight, to remove the solvent completely. To encapsulate hydrophobic compounds (such as fatty acids, tocopherol, DHA), they can be dissolved together with the mixture of lipids (*see* **Note 6**). Rehydrate the dry lipid film by addition of PBS pH 7.0 to obtain a final concentration of 1 mM of total lipids (for example, for 100μl of chloroform solution evaporated, add 1 ml of buffer). Do it under vigorous and continuous agitation above the transition temperature (~50 °C, *see* **Note 7**) for 1 h (or until lipid film was completely loos from glass wall) to obtain multilamellar lipid vesicles (MLV) (Fig. 1). To encapsulate hydrophilic

compounds, rehydrate the lipid film with a solution of interest prepared in PBS buffer pH 7.0.

To remove non-entrapped compounds, centrifuge 5 min at 5600 × *g* and wash twice with PBS pH 7.0 (*see* Fig. 1).

3.4 Recrystallization of the SLPs on Liposomes

Mix 5 ml of the SLP solution adjusted to a concentration of 10μg/ml with 1.0 ml of liposome suspension containing 1μM of lipids (*see* **Note 8**). Incubate for 3 h at 22 °C in a test tube rotator with a rotation speed of 10 min^{-1}.

3.5 Stability of SLP-Coated Liposomes

To evaluate the SLP-coated liposomes stability, prepare calcein-loaded liposomes according to Fig. 1, but rehydrate the lipid film in a 65 mM calcein solution in 10 mM HEPES pH 8.5 (*see* **Note 9**). Non-entrapped calcein can be removed by centrifugation and washing as it was described in Subheading 3.3.

3.5.1 Retention of Entrapped Calcein Method

Stability of SLP-coated liposomes can be estimated by % of calcein retention since entrapped calcein is quenched by concentration and its dilution after release produce a dequenching and a dramatic increase of fluorescence at 520 nm. Stability of SLP-coated liposomes submitted to different treatments (freezing, freeze-drying, heating, storage, addition to a harsh environmental food matrix, tolerance to gastrointestinal conditions, etc.) can be monitored by increase of fluorescence emission at 520 nm, using an excitation wavelength of 492 nm. The percentage of entrapped calcein can be calculated as follows:

$$100 - \frac{(F - FI)}{(FT - FI)} \quad (1)$$

where *F* is the fluorescence of the sample after each treatment, *FI* is the fluorescence of the sample immediately after washing liposomes and before treatment, and *FT* is the fluorescence of the sample the addition of Triton X-100 solution (20% v/v in deionized water) and represents the 100% of entrapped calcein.

3.5.2 Tolerance to Gastrointestinal Conditions

Dilute SLP-coated liposomes with entrapped calcein in simulated gastric fluid and incubate at 37 °C with stirring at 200 rpm for 90 min. After incubation, wash the SLP-coated liposomes twice with PBS pH 7.2. Then, resuspend them in simulated intestinal fluid, and incubate at 37 °C with stirring at 200 rpm for 3 h. Calculate the percentage of calcein retention using Eq. (1).

4 Notes

1. The SLPs from other bacterial species can be used. The experimental protocols for SLP isolation should be optimized accordingly (*see* **Note 3**).

2. The amount of MRS powder to be added per liter of water can vary slightly depending on the brand.
3. Alternatively, harvest bacterial cells by centrifugation (5000 × *g*, 15 min, 4 °C) and wash twice with NaCl 9 g/l. Resuspend the pellet in a tenfold volume of guanidine hydrochloride solution (GnCl 5 M in 50 mM Tris–HCl buffer, pH 7.2) and incubate at 20–25 °C with constant agitation for 20 min^{-1} h. After incubation, centrifuge again (5000 × *g*, 15 min, 4 °C) and dialyze the GnCl-extracted SLPs against distilled water at 20–25 °C for at least 2 h.
4. If the mixture of lipids is not completely soluble in chloroform, add few quantities of methanol until complete dissolution. Additionally, for pharmacological applications, soybean lecithin can be replaced with dipalmitoylphosphatidylcholine (DPPC) in the same ratio.
5. For evaporation of a higher volume of solvent, chloroform lipid solution could be evaporated to a dry film at 60 °C under vacuum in a rotavapor equipment. If applicable, the solvent can be removed by lyophilization.
6. The addition of a hydrophobic compound into the lipid bilayer could affect liposome properties. For this reason, the ratio of the ingredient to total lipids must be checked previously according to the physicochemical properties of the ingredient to be encapsulated.
7. The transition temperature can change slightly depending on the lipid composition.
8. The protein/lipid ratio needed to obtain completely covered liposomes depends on the SLP nature.
9. Calcein-quenched solution must be checked until complete dissolution by adding NaOH to obtain an intense red-brown solution without precipitate.

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Chapter 17

Fructosyltransferase Immobilization Via Entrapment

Maria Micaela Ureta and Andrea Gómez-Zavaglia

Abstract

Immobilization has emerged as a suitable approach to ensure the stability of enzymes, simplifying their removal once the reaction has been completed and enabling their reutilization. Entrapment is an immobilization method consisting in the occlusion of a given enzyme in synthetic or natural polymeric networks that act as permeable membranes for substrates and products, retaining the enzyme inside (encapsulated enzyme). In this chapter, a protocol for the entrapment of frutctosyltransferase in calcium alginate beads both in batch and in continuous processes is described. The physicochemical characterization of the enzyme–alginate beads (scanning electron microscopy, infrared spectroscopy, determination of water content) and the determination of the enzymatic activity of the entrapped enzyme are also described.

Key words Fructosyltransferase, Immobilization, Alginate, Ionic gelation, Fructo-oligosaccharides

1 Introduction

Many industrial chemical reactions employing enzymes as biocatalysts occur under extreme conditions, in terms of temperature, pH and presence of salts, surfactants, and organic solvents. During the last decades, enzymes' immobilization has emerged as a suitable methodology to guarantee enzymes' stability during industrial processes. This strategy also allows reusability of the catalyst, simplifying its removal from the reaction medium [1]. There are several immobilization techniques, and there are no standardized concepts about the most appropriate one for the different biocatalysts in the industry. The selection is via trial and error, by comparing the activity, stability, and reusability of free vs immobilized enzymes [2].

Among all of the immobilization methods, entrapment consists in the occlusion of a given enzyme in synthetic or natural polymeric networks (gel, fiber, or microcapsule) that act as permeable membranes to substrates and products, retaining the enzyme inside [3, 4]. It is a fast, cost-effective, and feasible strategy of immobili-

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zation. As a drawback, immobilization often presents mass transfer limitations due to diffusional problems between substrates, products, and carriers. In this sense, the support should overcome this issue.

Alginate is one of the most employed material for enzymes' immobilization via entrapment. It can be chemically modified enhancing biocompatibility and affinity to biomolecules [5, 6], also providing a good activity recovery and easy diffusion of substrates and products through the matrix pores [7]. Additionally, it has low associated costs, since alginate is generally obtained from by-products of different industries [1, 5]. The main drawback of alginate entrapment method is associated to enzyme outflows from the gel beads; nevertheless, it can be minimized by regulating the alginate concentration, as well as by decreasing the pH [8].

Fructo-oligosaccharides (FOS) are nondigestible oligosaccharides with prebiotic properties that can be incorporated in the formulation of many food products, beverages, and infant formula, to stimulate the development of intestinal microbiota [1]. FOS can be obtained by industrial enzymatic synthesis using sucrose as substrate and fructosyltransferases as biocatalysts, promoting the obtaining of short-chain FOS [degree of polymerization (DP) ranging from 3 to 7] [9]. Particularly, fructosyltransferase have both transferase and hydrolase activities, meaning that it is capable of catalyzing the transfer of functional groups (glycosylic) and, at the same time, the hydrolysis of organic molecules (sucrose). This double function considerably decreases the enzymes' costs, but the nonspecificity represents a technological challenge because the products' yield is lower than that obtained using more specific enzymes. For this reason, the industry must implement production processes considering both economic and technological aspects [9, 10]. The scale-up production of FOS by enzymatic synthesis can be performed through batch or continuous processes. In the former, after the time needed for the reaction to be completed, the enzyme is separated from the products and unreacted substrate, often leading to its inhibition or inactivation. In continuous processes, there is constant and simultaneous renewal of reactants and removal of products. In this case, immobilized enzymes are often used until a significant loss of activity is observed. Therefore, optimization is needed to determine the number of cycles the enzymes can perform [10].

In this chapter, immobilization of fructosyltransferases via entrapment is described using dried sodium alginate as support. Additionally, we present the methodology to test the enzyme–alginate gel beads in both batch and continuous FOS production process.

2 Materials

2.1 Immobilization

1. Enzyme: Viscozyme L (Novozyme, Denmark) (56 FU/mL; FU: fructosyltransferase units).
2. Support: Sodium alginate from brown algae from Sigma-Aldrich (St. Louis, MI, USA).
3. $CaCl_2$ and acetate buffer from Sigma-Aldrich (St. Louis, MI, USA).

2.2 FOS Synthesis

1. Standards: 1-Kestose (DP3), nystose (DP4), and 1F-fructofuranosylnystose (DP5) standards from Wako Chemicals (Richmond, VA, USA).
2. Reactant: Sucrose and citric acid from Sigma-Aldrich (St. Louis, MI, USA).

2.3 Equipment

1. Peristaltic pump (Gilson Miniplus 3, USA).
2. Freeze-dryer Heto FD4 (Heto Lab Equipment, Denmark).
3. Scanning Electron Microscope (SEM) FEI Quanta 3D 200 dual beam Focused Ion Beam (FEI, Hillsboro, Oregon, USA).
4. ATR-FTIR Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA).
5. Volumetric Karl–Fisher titrator (Mettler Toledo, model DL31, Waltham, MA, USA).
6. HPLC Perkin-Elmer Series 200 equipment (Waltham, MA, USA) with refractive index detector and autosampler. Chromatographic column: Sugar Pak I column (10μm, 6.5 × 300 mm) with Guard Pak LC pre-column inserts (10μm) (Waters, Milford, MA, USA).

3 Methods

3.1 Fructosyltransferase Immobilization in Dried Alginate

1. Prepare a 4% (w/v) sodium alginate solution in distilled water and stir to homogenize. Let the liquid rest for 2 h.
2. To obtain the enzyme solution, dilute 3.5% (v/v) of Viscozyme L in sodium acetate buffer, pH 4.5.
3. Mix the alginate solution with the enzyme solution in a 1:1 (w:w) ratio. Using a peristaltic pump, drop the resulting mixture onto a 0.2 M $CaCl_2$ solution in sodium acetate buffer (pH 4.5). Maintain constant magnetic stirring at 100 rpm for 20 min.
4. Separate the formed gel beads (ionotropic gelation) from the solution and wash them with sodium acetate buffer (pH 4.5) for 30 min with continuous replacement of the washing solution.

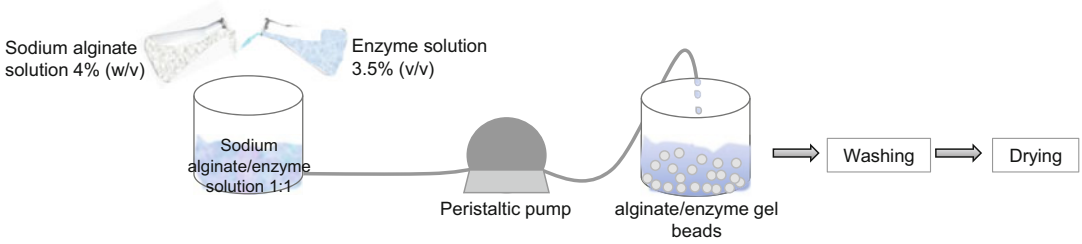

Fig. 1 Schematic representation of enzyme immobilization in alginate gel beads

5. Freeze-dry the beads (pressure = 0.021 mbar, temperature = −55 °C), for 48 h.

Figure 1 shows a schematic representation of this process.

3.2 Characterization of Dried Enzyme–Alginate Beads

1. Obtain SEM images of the dried beads to determine their pore size and morphology.
2. Characterize the immobilized enzyme complex in terms of morphology and intermolecular bonds by Fourier Transform Infrared (FTIR-ATR) spectroscopy in the 4000–550 cm^{-1} region, at a spectral resolution of 4 cm^{-1}.
3. Measure water content of the dried alginate–enzyme complex using a volumetric Karl–Fisher titrator employing Hydranal composite 5 as reagent.

3.3 Enzymatic Activity

1. Prepare a sucrose solution 500 g/L in sodium acetate buffer (pH 4.5).
2. Incubate 100 mg of alginate beads in 1 mL of sucrose solution at 45 °C under constant stirring (150 rpm) for 15 min (*see* **Note 1**).
3. After 15 min, determine product's composition by HPLC (refraction index detector). Use standards of fructose, glucose, sucrose, 1-kestose (DP3), nystose (DP4), and 1F-fructofuranosyl nystose (DP5) to determine their retention times and check the linear range of the measurements. One unit of transfructosylation activity (TU) is defined as the amount of catalyst that produces 1μmol of kestose or nystose from sucrose per minute under the above-described conditions.

3.4 Batch FOS Production with Immobilized Enzyme

Figure 2 presents a schematic representation of the two methods described to produce FOS with the immobilized enzyme: batch (stirred tank) and continuous (packed bed column).

1. Prepare reaction medium: a 400 g/L sucrose solution and stabilize pH at 4.5 using citric acid solution 2 M and medium temperature at 40 ± 5 °C.

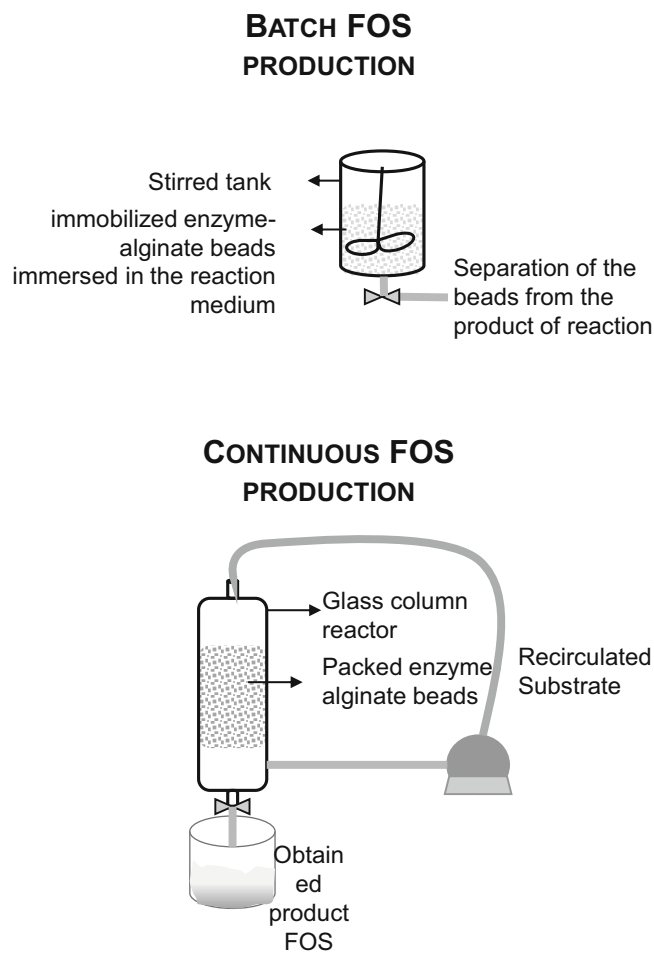

Fig. 2 Batch and continuous production of FOS

2. Add the catalyst (dried alginate beads) approximately 20 mg per 500 mL of reaction medium. Maintain continuous stirring for 4.5 h.
3. To follow reaction progress, take samples at regular intervals (every 15 min the first hour and every 30 min until the end of reaction).
4. Separate the alginate beads from the reaction medium with the aid of a filter or a spoon.
5. Wash the catalyst with sodium acetate buffer (pH 4.5) for 30 min with continuous replacement of the washing solution and repeat the drying step to preserve it in the freezer (−15 °C) (*see* **Note 2**).
6. In case there is any lixiviated enzyme, heat the reaction medium at 85 °C for 5 min.
7. Determine the composition of products along the reaction progress by HPLC (refraction index detector).

3.5 Continuous FOS Production with Immobilized Enzyme

1. In a glass cylinder (3.5 × 60 cm), pack 150 g of enzyme–alginate beads.
2. Recirculate (6 cm^3/min) 600 cm^3 of 400 g/L sucrose solution at 40 °C.
3. Renovate the substrate solution every 24 h (*see* **Note 3**).
4. After 24 h of recycling, determine product composition, by HPLC (refraction index detector).

4 Notes

1. To evaluate the thermal stability of the immobilized enzyme, the same procedure to measure enzyme activity should be repeated at different temperatures.
2. Ideally the catalyst should not be preserved unused for long periods. We strongly recommend determining the enzyme activity assay before reusing it.
3. After 30 days of continuous operation, we recommend the measurement of the enzyme–alginate complex activity and the renewal of the reactor catalyst if necessary.

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Correction to: Basic Protocols in Encapsulation of Food Ingredients

Andrea Gomez-Zavaglia

Correction to:
FM and Chapter 9 in: Andrea Gomez-Zavaglia (ed.), ***Basic Protocols in Encapsulation of Food Ingredients, Methods and Protocols in Food Science,***
https://doi.org/10.1007/978-1-0716-1649-9

This book was inadvertently published without including the series preface in the frontmatter. This was corrected by including the series preface in the frontmatter.

In Chapter 9, the first name of a co-author was incorrectly published as Cecilia Lourenço-Lopes, and it was corrected as Caterina Lourenço-Lopes.

The updated online version of the frontmatter and Chapter 9 can be found at https://doi.org/10.1007/978-1-0716-1649-9_9 and https://doi.org/10.1007/978-1-0716-1649-9

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