Development and Characterization of Microencapsulation Systems for Bioactive Ingredients of Interest in the Development of Functional Foods

Desarrollo y Caracterización de Sistemas de Microencapsulación de Ingredientes Bioactivos de Interés en el Desarrollo de Alimentos Funcionales

TESIS DOCTORAL INTERNACIONAL

Laura Gómez Gómez-Mascaraque

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Supervised by: Dra. Amparo López Rubio

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Directora:

Dra. Amparo López Rubio

“Let food be thy medicine and medicine be thy food.”

Hippocrates

Dra. Amparo López Rubio, Científica Titular del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA)

CERTIFICA

Que la presente memoria titulada "Development and Characterization of Nano- and Microencapsulation Systems for Bioactive Ingredients of Interest in the Development of Functional Foods" que, para aspirar al grado de Doctor, presenta Laura Gómez Gómez-Mascaraque, realizada bajo su dirección en el Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), reúne las condiciones adecuadas para su presentación como Tesis Doctoral, por lo que AUTORIZA a la interesada a su presentación. Asimismo, CERTIFICA haber dirigido y supervisado tanto los distintos aspectos del trabajo como la redacción.

Y para que así conste a los efectos oportunos, firmo la presente en Valencia a 18 de Agosto de 2017.

Fdo:

Dra. Amparo López Rubio

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Como decía al principio y, para resumir:

¡MIL GRACIAS A TODOS LOS QUE HABÉIS FORMADO PARTE DE ESTE PROYECTO!

Abstract

The aim of the present work was to design and characterize novel encapsulation structures of interest in the development of functional foods. For this purpose, various biopolymer-based microencapsulation structures were obtained through different processing technologies, with an emphasis on electrospraying as an advantageous alternative to conventional microencapsulation techniques.

Firstly, novel microencapsulation structures were produced from aqueous solutions and in mild conditions by electrospraying, using different biopolymers as encapsulation matrices. For this purpose, the processing conditions were optimized and the relationship between the feed solution properties and the morphology of the electrosprayed materials was studied.

The developed microstructures were then used to microencapsulate model bioactive ingredients with different properties, including hydrophilic molecules, hydrophobic compounds and probiotic microorganisms. For the hydrophobic ingredients, different strategies were evaluated in order to disperse them within the aqueous biopolymer solutions, such as the preparation of emulsions and liposome dispersions prior to electrospraying. An in-line setup for the continuous mixing of liposomes with the biopolymer and their subsequent hybrid capsule formation was also developed by combining microfluidic and electrospraying technologies. For the probiotic microorganisms, the convenience of preparing the feed suspensions from fresh culture or freeze-dried bacteria, as well as the impact of adding a surfactant and a prebiotic carbohydrate to the formulation, were also evaluated.

The performance of the proposed encapsulation systems was evaluated in terms of microencapsulation efficiency, stabilization of the bioactive ingredients against degradation under detrimental conditions and/or their impact on the bioaccessibility of the microencapsulated ingredients after *in-vitro* digestion.

Spray-drying was also used to microencapsulate some of the bioactive ingredients, in order to compare the results obtained through electrospraying with a more conventional encapsulation technique and using different encapsulation matrices.

Additionally, a novel concept of bio-inspired encapsulation was proposed in this thesis: the potential of intact plant cells isolated from potato tubers to bind phenolic compounds was explored, and the impact of starch gelatinization on the loading capacity of these proposed encapsulation vehicles was also assessed.

Finally, the impact of microencapsulation in real food systems was also studied. Yoghurts and biscuits were enriched with a peptide hydrolysate and a green tea extract, respectively, and the stabilization effect of the protective matrices during food manufacturing was assessed. The consumers' acceptability of the enriched biscuits was also studied.

Resumen

El objetivo de este trabajo fue el diseño y caracterización de nuevas estructuras de encapsulación de interés en el desarrollo de alimentos funcionales. Para ello, se obtuvieron distintas microestructuras de encapsulación biopoliméricas utilizando diferentes tecnologías de procesado, con especial hincapié en el *electroesprayado* como alternativa ventajosa a las técnicas de microencapsulación convencionales.

En primer lugar, se desarrollaron nuevas microestructuras de encapsulación a partir de disoluciones acuosas y en condiciones suaves mediante la técnica de *electroesprayado*, utilizando diferentes biopolímeros como matrices de encapsulación. Para ello, se optimizaron las condiciones de procesado y se estudió la relación existente entre las propiedades de las disoluciones de partida y la morfología de los materiales *electroesprayados* obtenidos a partir de ellas.

Posteriormente, se utilizaron las estructuras desarrolladas para microencapsular ingredientes bioactivos modelo con diferentes propiedades, incluyendo moléculas hidrofílicas, compuestos hidrofóbicos y microorganismos probióticos. En el caso de los ingredientes hidrofóbicos, se evaluaron diferentes estrategias para dispersarlos en las disoluciones poliméricas acuosas, como la preparación de emulsiones y de liposomas para su posterior procesado mediante *electroesprayado*. También se desarrolló un proceso en línea para mezclar los liposomas con el biopolímero y *electroesprayarlos* en continuo, combinando las tecnologías de microfluídica y *electroesprayado*. En el caso de los microorganismos probióticos, se evaluó también la conveniencia de preparar las suspensiones bacterianas a partir de cultivo fresco o de liófilos, así como el impacto de añadir un surfactante y un carbohidrato prebiótico a la formulación.

El comportamiento de los sistemas de encapsulación propuestos se evaluó en cuanto a eficiencia de encapsulación, estabilización de los ingredientes bioactivos frente a condiciones de estrés y/o impacto sobre la bioaccesibilidad de dichos ingredientes tras un proceso de digestión *in-vitro*.

También se utilizó la técnica de secado por pulverización para microencapsular alguno de los ingredientes bioactivos, con el fin de comparar los resultados obtenidos mediante *electroesprayado* con los de una técnica de encapsulación convencional, y utilizando diferentes matrices de encapsulación.

Además, en esta tesis se propone un nuevo concepto de encapsulación bio-inspirada, basada en el potencial de células vegetales de patata intactas para unirse a compuestos fenólicos. Asimismo, se evaluó el efecto de la gelatinización previa del almidón presente en las células en la capacidad de carga de estos potenciales vehículos de encapsulación.

Por último, se estudió el impacto de la microencapsulación en alimentos reales. Se enriquecieron yogures y galletas con un hidrolizado de péptidos y un extracto de té, respectivamente, y se estudió el efecto estabilizador de las microcápsulas durante el

procesado de estos alimentos. También se estudió la aceptabilidad por parte de los consumidores de estas galletas enriquecidas.

Resum

L'objectiu d'aquest treball va ser el disseny i caracterització de noves estructures d'encapsulació d'interès en el desenvolupament d'aliments funcionals. Amb aquesta finalitat, es van obtenir diferents microestructures d'encapsulació biopolimèriques utilitzant diferents tecnologies de processat, amb especial èmfasi en el electrosprayat com a alternativa avantatjosa a les tècniques de microencapsulació convencionals.

En primer lloc, es van desenvolupar noves microestructures d'encapsulació a partir de dissolucions aquoses i en condicions suaus mitjançant la tècnica de electrosprayat, utilitzant diferents biopolímers com a matrius d'encapsulació. Per a això, es van optimitzar les condicions de processament i es va estudiar la relació existent entre les propietats de les dissolucions de partida i la morfologia dels materials electrosprayats obtinguts a partir d'elles.

Posteriorment, es van utilitzar les estructures desenvolupades per microencapsular ingredients bioactius model amb diferents propietats, incloent molècules hidròfiles, compostos hidrofòbics i microorganismes probiòtics. En el cas dels ingredients hidrofòbics, es van avaluar diferents estratègies per a dispersar-los en les dissolucions polimèriques aquoses, com la preparació d'emulsions i de liposomes per al seu posterior processat mitjançant electrosprayat. També es va desenvolupar un procés en línia per a mesclar els liposomes amb el biopolímer i electrosprayar-los en continu, combinant les tecnologies de microfluídica i electrosprayat. En el cas dels microorganismes probiòtics, es va avaluar també la conveniència de preparar les suspensions bacterianes a partir de cultiu fresc o de liòfils, així com l'impacte d'afegir un surfactant i un carbohidrat prebiòtic a la formulació.

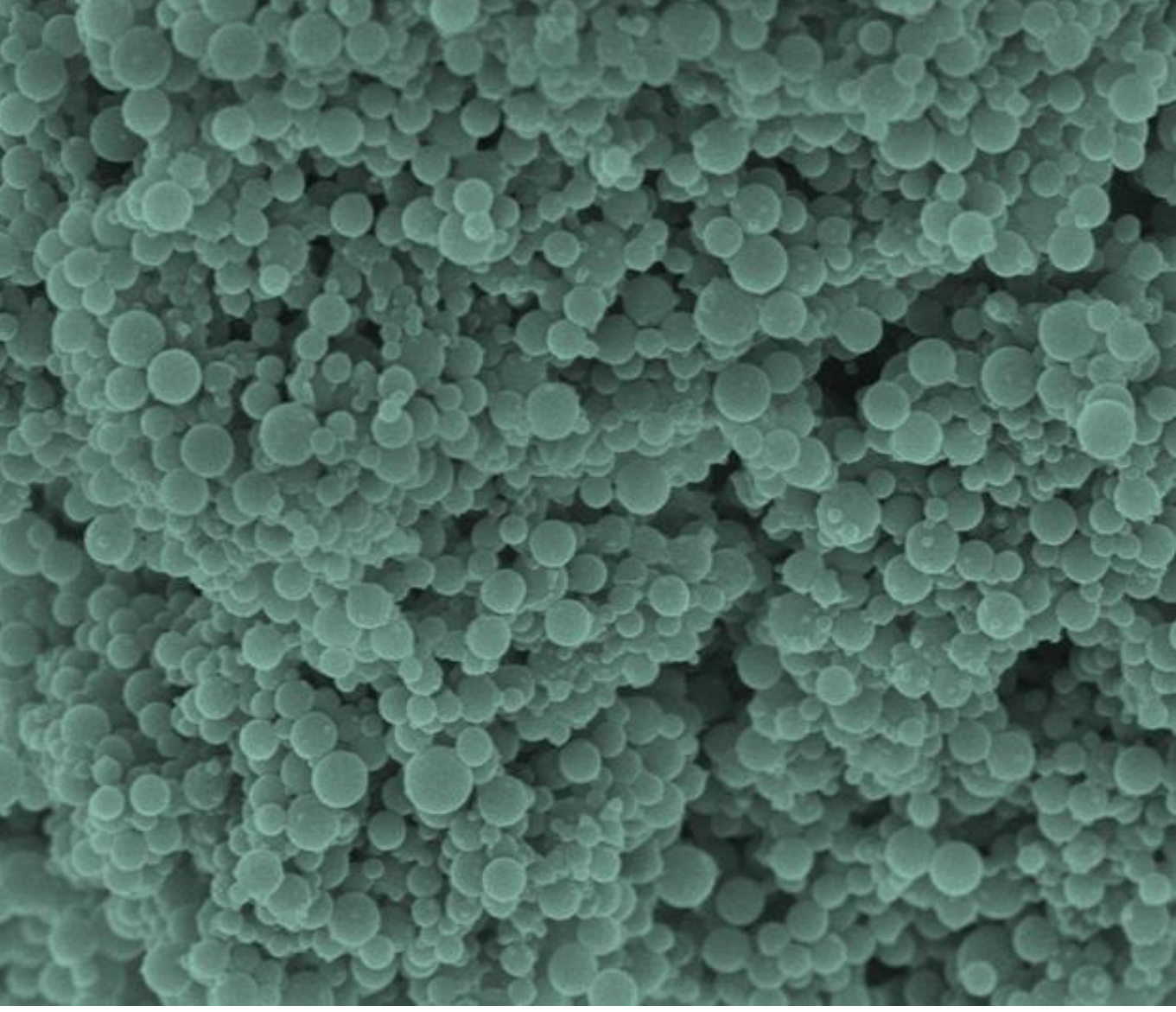
El comportament dels sistemes d'encapsulació proposats es va avaluar respecte a eficiència d'encapsulació, estabilització dels ingredients bioactius front a condicions d'estrès i / o l'impacte sobre la bioaccesibilitat d'aquests ingredients després de la seva digestió *in-vitro*.

També es va utilitzar la tècnica d'assecat per polvorització per microencapsular algun dels ingredients bioactius, per tal de comparar els resultats obtinguts mitjançant electrosprayat amb els d'una tècnica d'encapsulació convencional, i utilitzant diferents matrius d'encapsulació.

A més, en aquesta tesi es proposa un nou concepte d'encapsulació bio-inspirada, basada en el potencial de cèl·lules vegetals de creïlles intactes per unir-se a compostos fenòlics. Així mateix, es va avaluar l'efecte de la gelatinització prèvia del seu midó en la capacitat de càrrega d'aquests potencials vehicles d'encapsulació.

Finalment, es va estudiar l'impacte de la microencapsulació en aliments reals. Es van enriquir iogurts i galetes amb un hidrolitzat de pèptids i un extracte de té, respectivament, i es va estudiar l'efecte estabilitzador de les microcàpsules durant el processat dels aliments. També es va estudiar l'acceptabilitat per part dels consumidors d'aquestes galetes enriquides.

Index



I. INTRODUCTION

1. Functional foods

1.1. Definition

A number of definitions have been proposed for the term 'functional food' since it was first introduced in Japan in the 1980's [1]. Probably, one of the simplest ones was given by the International Food Information Council (IFIC, USA) in 1999 which considers functional foods as 'foods that *may* provide health benefits beyond basic nutrition' [2]. The European Commission Concerted Action Group on Functional Food Science in Europe (FUSOSE) was more specific and considered that a food can be regarded as functional only if it has '*satisfactorily demonstrated* to beneficially affect one or more functions in the body, beyond adequate nutritional effects, in a way which is relevant to either an improved state of health and well-being and/or a reduction of disease risk' [3]. This evidence requirement for the claimed health benefits was later gathered in the Regulation (EC) Nº 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods [4], to protect the consumers against unproven health claims on food products.

Generally, it is considered that functional foods should be similar to conventional foods, which are consumed as part of a normal diet, but should improve some health aspects beyond their primary nutritional function. The FUFOSE states that 'the amount of intake and form of the functional food should be as it is normally expected for dietary purposes' [5]. This concept is somehow different to that of nutraceuticals, which are products produced from foods, also with demonstrated health benefits, but manufactured in medicinal forms such as capsules, tablets or powders [6]. Hence, while nutraceuticals are meant to be consumed as supplements, functional foods should be designed to be ingested as part of our normal diet. This circumstance represents a number of additional challenges related to the incorporation of the functional ingredients to food matrices, as discussed below.

It is worth mentioning that conventional foods containing natural bioactive compounds with positive health effects (e.g. normal tomatoes, which contain naturally occurring carotenoids) are strictly not considered functional foods when consumed as such. However, if the health-promoting compound is isolated and added to another food product, the latter could be considered a functional food [1]. That is, functional foods must be 'designed' to exert the claimed health effect, according to one of the following strategies [5]:

- Increasing the amount of a beneficial component in a conventional product (product fortification).
- Addition of new components not normally found in a particular food product (product enrichment).

- Removing or reduction of deleterious components from a food product, or replacing them with another substance with beneficial effects (product alteration).
- Enhancing one of the components of a product naturally through special growing conditions, new feed compositions, genetic manipulation, etc. (product enhancing).

1.2. Interest in functional foods

The commercial interest in functional foods has grown in the last decades due to the increased consumer awareness of the relationship between nutrition and health and, thus, the promotion of healthy eating and lifestyle [2, 5]. Consequently, the market for functional foods has expanded in the last years in response to the increased demand for foods and beverages with health benefits [7]. Due to the existence of various definitions and unclear borderlines of what is considered as functional foods, the estimation of their exact market size is quite difficult and variable [8]. According to Leatherhead Food Research, the global sales of functional foods and beverages increased 31.5% from 2007 to 2011, and reached 25.3 billion U.S. dollars in 2011. Figure 1 shows the market size of functional foods worldwide in the last decades, according to different sources. Despite the divergence of the data obtained from different sources, it is unquestionable that the market of functional foods is growing, and is projected to further grow in the next few years.

The concept of functional foods is not only appealing for consumers and the food industry, but is also a potential way of reducing the burden on the healthcare system by a continuous preventive mechanism [6]. This generalized interest is naturally shared by the scientific community, whose research efforts support the continuous growth of this sector. The increasing scientific attention paid to functional foods is revealed by the boost of the number of scientific publications registered in this field in the last decades. Figure 2 illustrates the number of documents registered in Scopus per year which contain the words 'functional food*' in their title, abstract and/or keywords, as an indicative. Many of these works are aimed at confronting the challenges faced during the design of new functional foods.

1.3. Challenges in the design of enriched functional foods

Each of the design strategies mentioned in Section 1.1 entail their particular challenges related to the specific technologies used to achieve their purpose. Here, the main challenges faced in the design of enriched functional foods, which are within the scope of the present Doctoral Thesis, are discussed. As previously stated, the design of enriched products involves the addition of ingredients which are not normally found in the food product in which they are to be incorporated. Hence, a number of technical difficulties may be encountered, including the following:

- Incompatibility of some bioactive ingredients with the food matrix in which they are incorporated.
- Alteration of the organoleptic properties of the food product upon addition of the bioactive ingredients.
- Reduction or loss of the activity of the added bioactive ingredients during the storage, commercialization or food consumption.
- Poor bioavailability of the added bioactive ingredients.

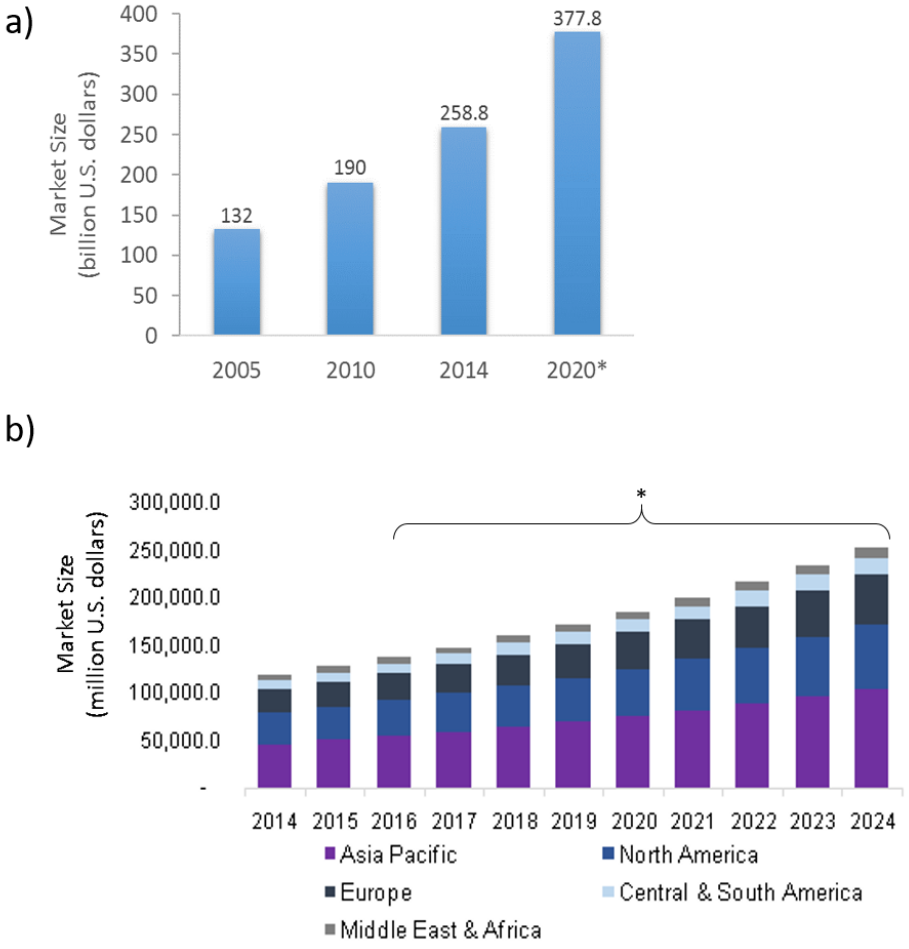


Figure 1. Worldwide market size of functional foods, including projected values (*). Sources: a) www.statista.com; b) www.grandviewresearch.com/industry-analysis/functional-food-market

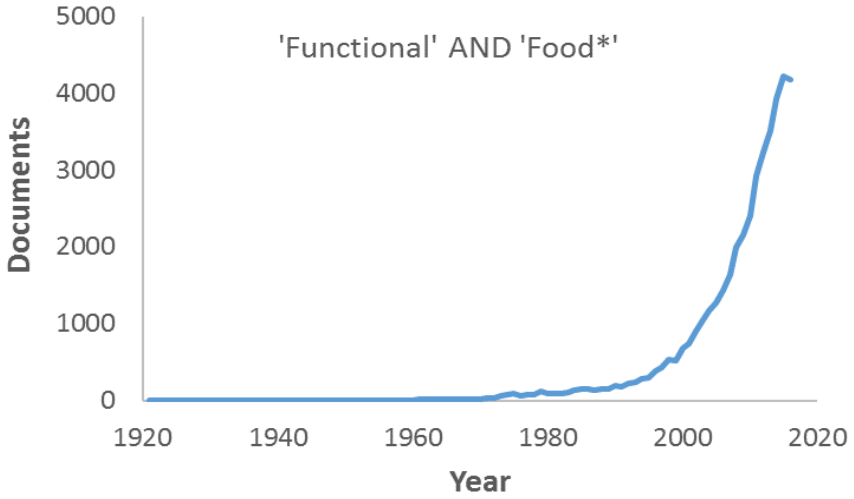


Figure 2. Number of scientific documents per year registered in Scopus containing 'functional food*' in their title, abstract and/or keywords. Data obtained from Scopus (Elsevier) using the *Analyze search results* tool.

The incompatibility of bioactive ingredients with the food matrix may occur when trying to incorporate lipophilic components within hydrophilic foods or beverages, or vice versa. For instance, many bioactive compounds are hydrophobic, such as polyunsaturated fatty acids or carotenoids, and thus insoluble in aqueous media [9]. Different technologies commonly used in the food industry can be employed to overcome this limitation and achieve homogeneous dispersions of the ingredient in the desired matrix, being emulsification the most widely applied strategy [10]. However, oil-in-water emulsions are, in general, thermodynamically unstable [11] and technological efforts must be made to optimize the formulations of poorly water-soluble ingredients [12].

Numerous bioactive ingredients can also affect the organoleptic properties of the food products in which they are incorporated, by modifying their texture, colour, odour and/or flavour. In some cases, these alterations can be regarded as positive, improving the acceptability of the consumers towards the functional food products. This may be the case of some bioactive ingredients which are natural pigments (e.g. anthocyanins), for which the colour of the enriched product is positively associated to its content of health-promoting compounds by the consumers and is of fundamental importance for their acceptance [13]. However, in most cases the sensorial changes caused by the addition of functional ingredients are undesirable. For instance, bioactive peptides usually impart negative sensory attributes such as bitter taste [14, 15]. Similarly, due to their high susceptibility to oxidation, omega-3 fatty acids are important precursors of flavor reversion (i.e. development of off-flavours) [16]. These unpleasant odours and/or off-flavours are unacceptable for the consumers and strategies must be developed to avoid them.

Another intrinsic limitation of most bioactive ingredients is their sensitivity to different environmental stresses such as high temperatures, light, oxygen, certain pH conditions, etc. Consequently, they can lose their expected beneficial bioactivity during food processing, storage and/or consumption [17]. Regarding the health claims made on foods, the Regulation EC/1924/2006 states that 'In order to ensure that the claims made are truthful, it is necessary that the substance that is the subject of the claim is present in the final product in quantities that are sufficient […] to produce the nutritional or physiological effect claimed' [4]. In spite of this, a number of studies have reported low levels of, for instance, viable probiotic bacteria in commercial products which claim to be probiotic [18-20]. Therefore, it is essential to design stabilization strategies to avoid or, at least, minimize the degradation of the bioactive compounds prior to their addition to the food matrix so as their bioactivity is maintained until consumption.

The Regulation EC/1924/2006 also states that 'The substance should also be available to be used by the body' [4]. That is, the functional ingredient must be readily absorbable in the small intestine if entering the blood stream is required to reach the target organs. Consequently, with the exception of those components whose beneficial effects are exerted in the large intestine, the active ingredients should be formulated to be bioaccessible and bioavailable. This represents an additional challenge in the case of some functional ingredients (e.g. carotenoids) whose bioavailability is extremely low [21].

2. Microencapsulation

2.1. Definition and advantages

Microencapsulation has been envisaged as a promising approach to overcome the aforementioned limitations in the development of new enriched functional foods. The microencapsulation concept was initially developed by the pharmaceutical industry, and later expanded to the food and cosmetics sectors [22]. Microencapsulation technologies can be defined as processes in which a compound or ingredient of interest is coated with or embedded within a protective matrix [23] obtaining microstructured or micron-sized materials. The produced microencapsulates can exhibit a variety of structures as illustrated in Figure 3.

Microencapsulation can help solving some of the technological problems previously mentioned, as the encapsulation matrix limits the direct contact of the bioactive ingredients with the detrimental environment [24], working as a physical barrier. Thus, it has been successfully used for the stabilization of biologically active ingredients in food systems [25, 26], preserving their bioactivity and avoiding off-flavours derived from their degradation products [27]. This barrier can also help masking the bitterness of certain bioactive compounds by limiting their contact with

the salivary fluids [28, 29]. Microencapsulation can also enhance the compatibility of hydrophobic compounds with aqueous food matrices, increasing their solubility by dispersing them within hydrophilic encapsulation matrices [30, 31], and even improve the bioavailability of certain bioactive molecules [32, 33].

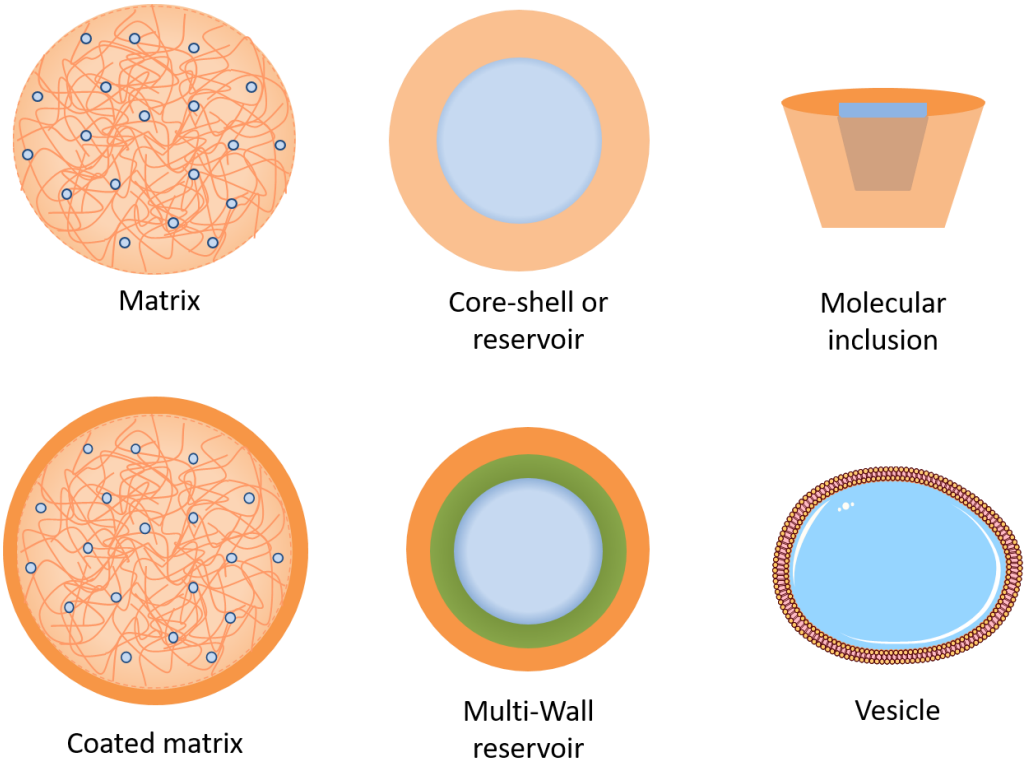


Figure 3. Schematic illustration of different morphologies of encapsulates. Adapted from Đorđević, Balanč, Belščak-Cvitanović, Lević, Trifković, Kalušević, Kostić, Komes, Bugarski and Nedović (2015) [34].

2.2. Microencapsulation methods

A number of microencapsulation methods have been developed, many of which have already been used for food applications. Their classification is complex and variable due to the diversity of existing techniques. A tentative classification is presented in Figure 4, where the main microencapsulation techniques are summarized. An extensive review on these technologies applied in the food industry was recently published by Đorđević et al. (2015) [34].

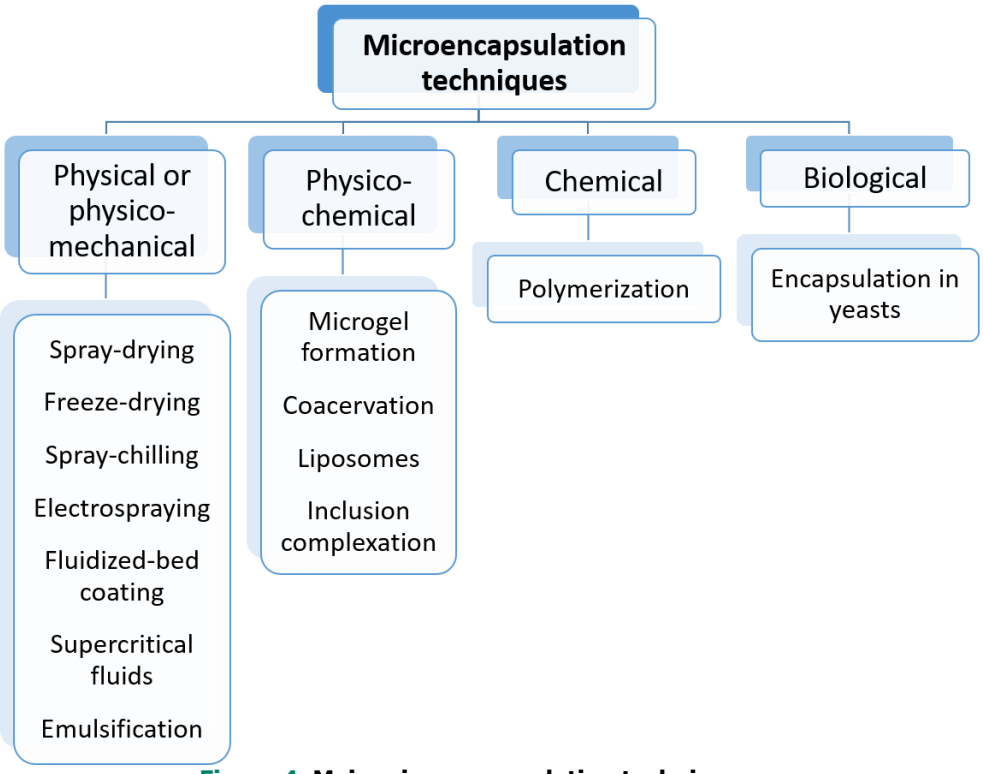


Figure 4. Main microencapsulation techniques.

2.2.1. Physical/physico-mechanical microencapsulation techniques

Spray-drying is the most commonly used microencapsulation technology in the food industry [35]. Nedović et al. (2013) estimated that about 90% of the produced microencapsulation structures in the food industry are prepared by spray-drying [36]. This technology has been extensively employed for the encapsulation of numerous bioactive ingredients, including polyunsaturated fatty acids [37-39], phenolic compounds [40, 41], carotenoids [42, 43] and probiotic microorganisms [44, 45], among others. This technology involves dissolving or dispersing the bioactive ingredient in an aqueous solution or dispersion of the carrier material prior to their atomization or spraying through a nozzle. The generated droplets are then rapidly dried using a hot gas stream, obtaining convenient dry powders [46] with sizes ranging from 10 μm to 3mm depending on the processing conditions [34]. Some of the advantages of spray-drying include the ease of operation, high production rates, low operating costs, reproducibility of the technique and the possibility of using a wide range of encapsulating materials [34]. However, the high temperatures used in the drying step make this technique unsuitable for thermosensitive bioactives [47].

Freeze-drying can be used to entrap bioactive ingredients within a protective matrix by dissolving or dispersing both of them in water, freezing the mixture and

subsequently drying the material by water sublimation under low pressure and reduced temperature [48]. To obtain a fine powder, the obtained material can then be broken into smaller pieces, for instance by grinding. Freeze-drying is a very simple technique to entrap a compound of interest within a protective matrix and together with spray-drying is one of the most commonly used microencapsulation technologies in the food industry [49]. However, it is approximately 30-50 times more expensive than spray-drying [35]. Moreover, the processing time is very long and the materials obtained are highly porous, which generally results in a poor barrier between the active ingredient and the environment [48].

Spray-chilling exploits a similar atomization process to that of spray-drying, but an opposite principle. It consists of solidifying an atomized liquid spray into particles [50]. Thus, the carriers in this case are generally molten oils which solidify upon contact with cold air. Spray-chilling has been already exploited for the encapsulation of probiotics [51, 52] and vitamins [53, 54]. The advantages of this technique include lower operating costs compared to spray-drying and its suitability for heat-sensitive ingredients. However, the solid lipid particles generated through this technique are themselves a constraint for some food applications, being generally restricted to foods containing high levels of fat [46]. Moreover, the active ingredient may be lost by diffusion from the lipid matrix during storage and degradation of the carrier oil may occur during their shelf life [55], limiting their application.

Fluidized-bed coating is generally used to coat preformed powder particles by suspending them in an air stream and spraying them with a coating material [48]. Compared to spray-drying, the heating temperatures used in this technique are significantly lower, and the cost of processing is relatively low. However, its application is limited to preformed spherical particles with a narrow size distribution and good flowability, and uncontrolled agglomeration of the particles usually occurs upon processing [34]. The prominent application of this technique in the food industry is the encapsulation of food supplements such as vitamins and minerals [56, 57].

Supercritical fluids are substances at a temperature and pressure above their critical point at which their liquid and gas phases are indistinguishable [58], being their properties intermediate between those of liquids and gases. Although many compounds can be brought to a supercritical state, supercritical carbon dioxide is arguably the most interesting solvent for encapsulation purposes, because it is environmentally friendly, it minimizes the use of organic solvents and it can be applied at reasonable pressures and temperatures [48]. Different supercritical fluid technologies can be applied for encapsulation purposes, which have been reviewed elsewhere [59]. However, the solubility limitations when using biopolymers and the difficulty to control de process limit their application for edible microcapsules [34]. Hence, in this field, supercritical fluid technologies are mainly used to improve the performance of other encapsulation techniques such as spray-drying, co-precipitation or liposome formation [48].

Emulsions are dispersions of small, spherical droplets of one liquid in another immiscible liquid (typically an oil phase and an aqueous phase) [11]. Emulsions are

present in many conventional food products, such as mayonnaise, ice-creams, milk or dressings [60, 61]. However, they can also be designed to entrap bioactive ingredients in the disperse phase, which can help overcoming the problems related to the low solubility and bioavailability of some compounds, and even delay the degradation of some labile compounds [34]. However, as mentioned in Section 1.3, emulsions are in general thermodynamically unstable, so further processing is generally required to extend their shelf-lives. Powder formation from emulsion by combining emulsification with a drying technique is a plausible technological solution in this regard. Spray-drying is the most commonly used technology to obtain dry encapsulation structures from emulsions in the food industry [35].

2.2.2. Physico-chemical microencapsulation techniques

Microgels, i.e. micron-sized gel structures, are attractive encapsulation vehicles for bioactive ingredients. One of the most commonly used processes to produce microgels for encapsulation purposes is through extrusion. Its basic principle consists of extruding a liquid mixture of the bioactive ingredient with the encapsulating matrix through an orifice and subsequent gelation of the obtained droplets by physical (e.g. temperature changes) or chemical (e.g. crosslinking) means, usually by dripping them onto a bath [62]. Different variations exist depending on the technology used for droplet formation, but in general, the production rate of these extrusion techniques is quite low. Moreover, due to the high viscosity of many biopolymers and the subsequent low concentrations required for processing through extrusion, low density gel networks are generally obtained, which do not provide sufficient barrier effect. Furthermore, the encapsulation efficiencies for small water-soluble compounds is considerable low, as they can easily diffuse to the bath solution. This liquid must additionally be separated from the microgels, and drying would also be necessary to extend their shelf-life, increasing the number of production steps and costs [34].

An easier-to-scale-up alternative to produce microgels is by emulsification, where droplets are formed through dispersion of the polymer solution in an oil phase before promoting gelation by adjusting the environmental conditions [48]. However, the microgel particles must be still separated from the liquid bath and the process is more expensive than the extrusion techniques [34].

Coacervation, the first microencapsulation technique which was developed [63] and one of the simplest [64], consists of forming a liquid phase rich in polymer in equilibrium with another liquid phase. This associative phase separation can be induced in a single polymer system (simple coacervation) or a multi-polymer system (complex coacervation) by altering conditions such as the pH, ionic strength, temperature, etc., or by electrostatic interaction between oppositely charged polymers in complex coacervation [65]. In many cases, microencapsulation through coacervation can be achieved in mild conditions and without toxic solvents, and it yields considerably high encapsulation efficiencies for hydrophobic bioactive ingredients [66]. However, the encapsulation of hydrophilic compounds using this method is not very efficient. Moreover, its high production cost and complexity limit its

application in the food industry [34]. As in the case of the microgels, in order to increase the shelf-stability of the coacervate microcapsules, they are usually dried after production, so this technique is normally combined with others such as spray-drying or freeze-drying [67, 68].

Liposomes are lipid vesicles, i.e. structures consisting of lipid bilayers that confine aqueous compartments [69]. They are formed by self-assembly [70] as a result of the unfavorable hydrophilic-hydrophobic interactions that occur between polar lipid compounds (usually phospholipids) and water molecules [71]. Different methodologies can be used to produce liposome dispersions, which have been reviewed elsewhere [72, 73]. If bioactive compounds are present in the media during the self-assembling process, they can be entrapped either in the lipid or aqueous phases of the vesicles, so liposomes are promising vehicles for the encapsulation of water-soluble, lipid-soluble, and amphiphilic materials [74]. In fact, they have long been utilized as carriers for therapeutic agents [75], and more recently investigated to deliver functional ingredients in the food industry [76], with a positive impact on their stability and bioavailability [77]. However, these vehicles have been described to lose entrapped material during storage and to become instable due to osmotic pressure in contact with certain food components such as sugars or salts [78, 79], or in gastrointestinal conditions, which together with the high cost of scaling-up the production processes [34] limits their practical application in some cases.

Inclusion complexation is based on the unique structure of hollow molecules like cyclodextrins. Cyclodextrins are cyclic oligosaccharides consisting of glucopyranose units linked by α-(1,4) glycoside bonds in a truncated-cone type structure with a hydrophilic exterior and somewhat hydrophobic inner walls [80]. This distintive structure has been exploited to form inclusion complexes in which compounds of interest are stabilized in the interior of these molecules, mainly by co-precipitation techniques. However, the application of cyclodextrins for encapsulation is restricted to low molecular weight compounds which can fit into the cyclodextrins' structures [34]. But the main limitations to their industrial application are the high cost of the cyclodextrins (especially α- and γ-cyclodextrins) and, more importantly, the regulatory issues involving these molecules in some countries. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), for instance, recommended a maximum level of β-cyclodextrins in foods of 5 mg/kg per day [81].

2.2.3. Chemical microencapsulation technologies

Polymerization as a microencapsulation technique exploits the formation of new chemical bonds between reactive monomers to synthetize an encapsulation matrix for a compound of interest *in situ* [82]. Despite being of exceptional interest in diverse fields, including the design of sensors [83], textiles [84], self-healing materials [85] or aerospace coatings [86], this technique entails a critical limitation for its application in food products, as it involves the use of toxic solvents and/or reactants.

2.2.4. Biological microencapsulation technologies

An interesting encapsulation technology is the use of whole **yeast cells** as carriers to stabilize bioactive compounds [87]. Especially, the strain *Saccharomyces cerevisiae* has been extensively investigated for this purpose [88], as it can be easily produced on a large scale and constitutes a food-grade material with high nutritional value [89]. In the food sector, encapsulation in yeasts has been mainly applied for aromas, flavours and antioxidants [90-92]. Being a simple and cost-effective process, the main challenge of this technology is to achieve an effective transfer of the active molecules across the cell wall and plasma membrane of the yeast cells, for which different techniques are being developed [93]. As for many other encapsulation techniques, to achieve the final form of the encapsulates (i.e. a dry powder with long shelf-life), the microencapsulation in yeasts is usually combined with one of the drying techniques previously described, such as spray-drying or freeze-drying [34].

3. Food-grade microencapsulation matrices[1]

The use of microencapsulation matrices for food applications is limited to edible, biodegradable and preferably inexpensive materials, being biopolymers the ideal candidates meeting these requirements. Both proteins and polysaccharides have been proposed as promising vehicles for the protection and/or delivery of bioactive ingredients, having each of them their own advantages and drawbacks. Lipids are also of interest for the microencapsulation of bioactive ingredients, especially in the form of emulsions and liposomes, as described above.

3.1. Carbohydrates

Among carbohydrates, i.e. monosaccharides, oligosaccharides and polysaccharides, the latter are considered the most suitable as encapsulation matrices due to their large molecular structure, which allows effective entrapment of bioactive ingredients [94]. Polysaccharides are very abundant and relatively inexpensive biopolymers [15]. They also possess different reactive groups in their structure which facilitate interaction with a range of bioactive compounds and even allows functionalization of their backbone to achieve tailored functionalities, including the possibility of designing self-assembling structures for encapsulation [95]. It must be noted, however, that the reactivity of their functional groups has also been envisaged as a disadvantage for the encapsulation of certain bioactive ingredients capable of

[1] This section is an adapted version of part of the book chapter: L.G. Gómez-Mascaraque, M. J. Fabra, J. L. Castro-Mayoraga, G. Sánchez, M. Martínez-Sanz and A. López-Rubio (*In preparation*). *Nanostructuring biopolymers for improved food quality and safety*, in Handbook of Food Bioengineering, vol. 20 (Biopolymers for Food Design), Elsevier.

reacting with them, generating toxic products and/or precluding their subsequent release [15]. Polysaccharides are very versatile due to the variety of chemical structures (cf. Figure 5), functional groups and molecular weights available, and the presence of both hydrophilic and hydrophobic motifs along their molecular chains [96].

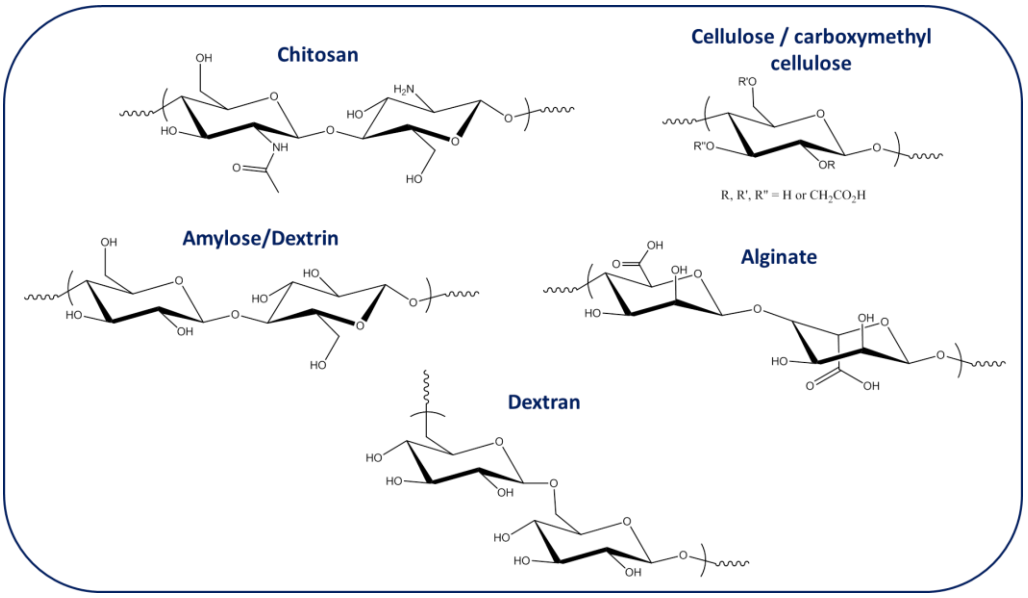


Figure 5. Chemical structure of some polysaccharides used for the encapsulation of bioactive ingredients.

Another interesting feature of polysaccharides is the great stability of their structure, even under harsh temperature conditions, in contrast with proteins, which can be denatured during processing of the materials at high temperatures. Furthermore, a number of polysaccharides are considered dietary fibers, being resistant to enzymatic degradation during oral, gastric and small intestine digestion but vulnerable to microbial fermentation in the colon [94]. Most of the commonly used polysaccharides for encapsulation have a plant origin, like starch, pectin, cellulose, alginate or guar gum, but animal-derived polysaccharides such as chitosan or those from bacterial origin such as dextran are also widespread for this application. An exhaustive review highlighting the advantages and disadvantages of each of them as delivery vehicles in food systems has been recently published [94].

3.2. Proteins

Food proteins are excellent candidates as microencapsulation matrices because, in addition to the protective effects they might exert on the bioactive ingredients, they have a high nutritional value and generally exhibit functional properties themselves [97], being considered the most nutritionally beneficial delivery systems [15]. Moreover, as Livney (2010) points out, some proteins are natural vehicles whose main function is to store, carry and/or deliver essential compounds throughout the organism, so they could also be exploited to deliver other molecules of interest [98]. Some of the characteristics that make proteins natural delivery agents include their capability of binding both hydrophobic and hydrophilic small molecules [99], and having self-assembling properties, surface activity and good gelation characteristics [98]. In general, proteins from animal origin, especially dairy proteins, but also gelatin and egg proteins, are more commonly employed than plant proteins for encapsulation purposes [100]. Some of the advantages of milk proteins over plant proteins include their higher solubility over a broader range of pH, their lower molecular weight and their greater flexibility. On the other hand, plant proteins have a better acceptance from some groups of consumers and are generally less expensive [100]. The most widely used milk proteins are caseins and whey proteins, and their application as encapsulation vehicles has been recently reviewed by Tavares, Croguennec, Carvalho and Bouhallab (2014) [101]. Regarding plant proteins, soy proteins are the predominant choice as wall materials, being those obtained from pulses (such as pea, lentil or chickpea) and cereal grains (for instance zein or barley protein) the most relevant alternatives [100]. Table 1 shows some examples of food ingredients which have been encapsulated using proteins.

3.3. Lipids

Lipids such as glycerides, oils or phospholipids are also used as carrier materials in microencapsulation [102-104]. They can create a good barrier for the protection of sensitive ingredients against moisture, and serve as carriers to disperse lipophilic compounds in aqueous media [105]. Lipid-based carriers are employed as matrices in certain microencapsulation techniques, mainly spray-chilling, emulsification and entrapment within liposomes. However, lipid carriers are only appropriate if the intended food product allows high levels of fat. Moreover, diffusion of the active ingredients from lipid matrices may occur, leading to losses of compound during storage (cf. Section 2). In the case of emulsions and liposomes dispersions, in addition, the liquid nature of the obtained systems complicates their handling and limits their shelf-life. Coating of lipid carriers with biopolymers (proteins or polysaccharides) has been proposed as a plausible strategy to increase their stability and shelf-life [106-108]. Coated lipid carriers can also be dried, for instance by spray-drying or freeze-drying, to produce convenient powdery microencapsulated ingredients [77, 109, 110].

Table 1. Some of the most commonly used proteins for microencapsulation and examples of applications.

Protein	Origin	Examples of encapsulated ingredients
Whey proteins	Animal (milk)	α-linolenic acid [9], lycopene [47], cardamom essential oil [111], folic acid [112], probiotics [113, 114].
Caseins	Animal (milk)	Garden cress seed oil [115], ω-3 fatty acids [26], curcumin [116], indonesian propolis [117].
Gelatin	Animal	Green tea polyphenols [17, 118], holy basil essential oil [119], sunflower oil [120].
Soy proteins	Vegetal	Polyphenols and anthocyanins from pomegranate [121], ω-3 fatty acids [9, 26].
Pea proteins	Vegetal	Conjugated linoleic acid [122], α-tocopherol [123], oils [124].
Chickpea proteins	Vegetal	Flaxseed oil [125, 126], folate [127], probiotics [128].
Lentil proteins	Vegetal	Flaxseed oil [125, 126], probiotics [129].
Zein	Vegetal	Fish oil [130, 131], green tea polyphenols [132].

3.4. Selection of the encapsulation matrix

As inferred from the previous discussion, proteins, polysaccharides and lipids are generally good candidates for the encapsulation of bioactive ingredients. However, there is no consensus in the literature regarding the best choice among them. Indeed, each particular compound to be protected has unique characteristics and distinct physical-chemical behavior and, thus, the choice of an optimal matrix for encapsulation should be done taking into account the properties of both components. Bearing in mind that the choice of wall material will have an impact not only on the encapsulation efficiency and protection of the bioactive ingredient of interest, but also in its release behavior [133], the selection of the most adequate encapsulation matrix is of outmost importance in the development of microencapsulation structures.

4. Electrohydrodynamic processing

As discussed in Section 2.2, drying generally increases the stability of the formulations compared to the original solutions or suspensions, extending the shelf-life of the functional ingredients [49]. This is one of the reasons why, as commented on above, spray-drying and freeze-drying are the most commonly used microencapsulation technologies used in the food industry [35, 49]. This is also the reason why many of the other techniques described above are usually combined with a drying technique in order to obtain a convenient dry powder in the final step. Therefore, the application of drying technologies is, in many cases, unavoidable for the production of microencapsulated functional ingredients. However, both spray-drying and freeze-drying involve important burdens, related with the need of employing high temperatures or with the high cost of processing, respectively, emphasizing the need for alternatives.

Electrospraying, another drying technique based on the electrohydrodynamic processing of polymer melts, solutions or dispersions, has very recently been proposed as an alternative for the microencapsulation of labile bioactive agents [134] with promising applications in the field of functional foods [17, 47, 112] since it can be performed under mild conditions [135]. Due to its novelty, some recent reviews on microencapsulation for food purposes do not yet include this technology in their list of available microencapsulation methods [34, 48]. In this section, this pioneering microencapsulation technique will be discussed.

4.1. Fundamentals of electrohydrodynamic processing

Electrohydrodynamic processing (i.e. processing of electrically charged fluids) allows the production of dry nano- and microstructures by subjecting a polymeric fluid to a high voltage electric field. Usually, the fluid is pumped at certain flow rate through a conductive capillary (e.g. a stainless steel needle) to which the voltage is applied. As a result, electrostatic interactions are generated within the liquid flowing out from the capillary, and when the electrical forces overcome the forces of surface tension, a charged polymer jet is ejected towards the opposite electrode, usually a grounded stainless steel collector. During the flight, the solvent in the jet is evaporated, thus rendering a dry material which is finally obtained in the collector [136-138]. A scheme of a simple setup for electrohydrodynamic processing is depicted in Figure 6.

If the molecular cohesion between the polymeric chains in the charged jet is high enough, this jet is elongated and ultrathin fibres are produced upon drying. This process is known as 'electrospinning' (cf. Figure 6a). Conversely, if the intermolecular cohesion is sufficiently low, the jet breaks into fine droplets. Due to the surface tension, the jet fragments acquire a spherical shape and yield micro- or nanoparticles upon solvent evaporation. In the latter case the process is known as 'electrospraying'

or 'electrohydrodynamic atomization' [139] (cf. Figure 6b). The production of either fibres or particles by electrohydrodynamic processing may be tailored by adjusting the solution properties and the process parameters. For application as food ingredients, particles rather than fibres are preferred, as they are easier to handle and to disperse within the food matrices [17]. Therefore, electrospraying conditions are usually pursued for this purpose.

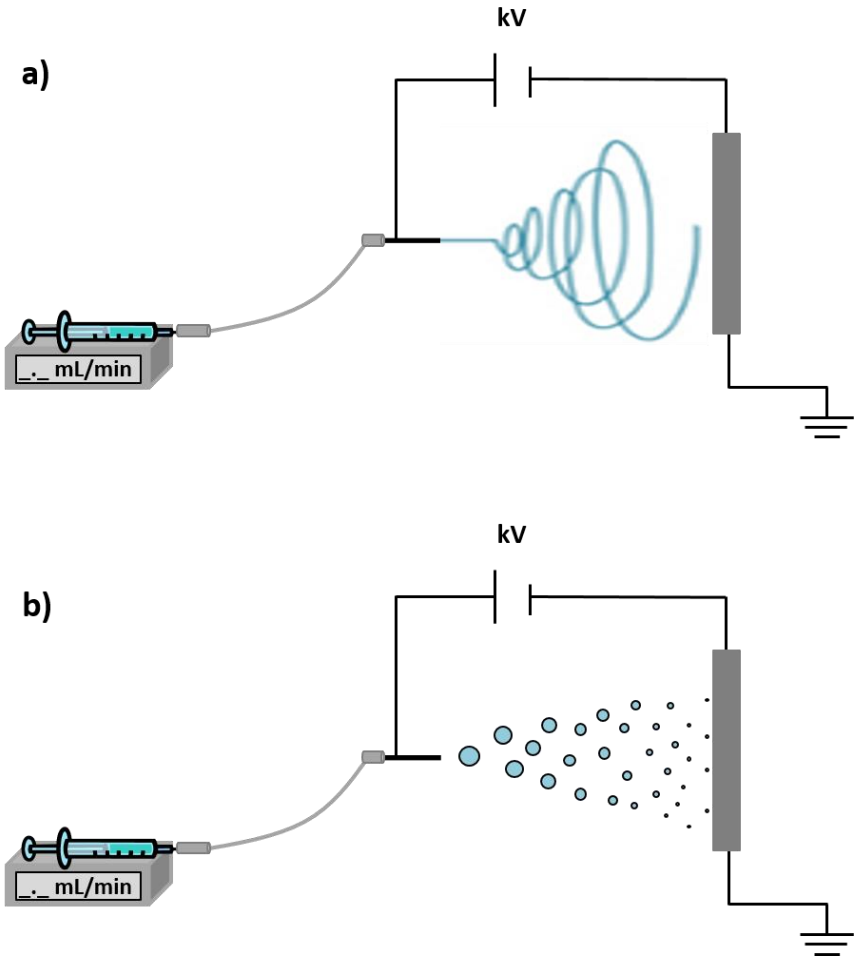


Figure 6. Basic setup for electrohydrodynamic processing working in the electrospinning (a) and electrospaying (b) modes.

Jaworek and Sobczyk (2008) summarized the complex physics involved in the electrospraying process and provided equations for the balance of bulk forces and the balance of stress tensors to which the polymer jet is subjected upon electrospraying, which include electrodynamic, gravitational, inertial and drag contributions [140]. However, they stated that no solutions to the general equations had been provided

yet, and only simplified equations for particular spraying modes and specific considerations had been solved. This gives an idea of the complexity of the physics involved. As a consequence, the process parameters generally need to be optimized for each polymer system, or for each polymer-bioactive combination if microencapsulation is the purpose of the process.

4.1.1. Influence of the solution properties

In order to optimize an electrospraying process for a specific purpose, both the solution properties and the process parameters have to be adjusted. The main solution properties having an impact on the performance of the hydrodynamic processing and the morphology of the resulting materials are the rheological characteristics of the fluid, its surface tension and its conductivity. All these properties in turn depend on the type of polymer used, its molecular weight, its concentration, the solvent used and the addition of other substances (including the bioactive compounds to be encapsulated).

The viscosity and rheological behaviour is a key parameter, as it is related to the extent of chain entanglements within the polymer fluid and, thus, has a decisive impact on the preferential formation of fibres or capsules [141]. At lower viscosities (lower molecular weights and polymer concentrations), the polymer jet is more likely to break up and form droplets [139]. Some authors have tried to generalize the ideal viscosity ranges for the formation of fibres or particles. For instance, Doshi et al. (1995) suggested that the ideal viscosity to achieve electrospinning ranges from 800 to 4000 cP, considering solutions below 800 cP too dilute to undergo chain entanglement [142]. However, nanofibers have been successfully obtained from polymeric solutions with lower viscosities [17]. Indeed, the ideal ranges greatly vary depending on the polymer type and entanglement behaviour in the selected solvent [139]. Moreover, the rheological properties are not the only factor involved. An increase in the surface tension usually favours jet break up, while an increase in the electrical conductivity tends to elongate the jet favouring fibre formation [141], although too high conductivities may lead to jet instability.

Another critical factor is the volatility of the solvent used, which determines whether sufficient solvent evaporation can occur during the flight of the polymeric fluid from the tip of the capillary to the collector [138].

4.1.2. Influence of the process parameters

Electrospraying/electrospinning is affected by a number of process parameters which include the applied voltage, the feed flow-rate, the capillary diameter, the tip-to-collector distance and environmental conditions such as the temperature, pressure and relative humidity.

The applied voltage, which usually varies in the range of 6-20 kV, determines the electrostatic forces applied to the polymer yet (depending on the solution properties).

A sufficient voltage must be applied to overcome the surface tension of the polymer at the tip of the capillary [138]. An increase in the applied voltage increases the charge density causing the jet to accelerate faster and to stretch to a greater extent. This usually results in a reduction of the fibres or particles diameter, although some exceptions have been observed [139] given that the resulting morphology depends on a number of factors. Thus, generalizations hold true only within certain ranges which are often dictated by practical considerations [140].

On the other hand, an increase in the flow rate and/or the needle diameter, or a decrease in the tip-to-collector distance usually leads to an increase in the diameter of the obtained structures. Flow rates must be low enough to allow sufficient time for solvent evaporation in combination with an adequate flying distance, so the optimal ranges strongly depend on the solvent volatility [139].

Environmental factors also play a role in electrospraying/electrospinning, since they have an impact on the solution properties. For instance, higher temperatures increase the molecular mobility and, thus, increase the solution conductivity, while they would decrease the viscosity and surface tension. Moreover, the evaporation of the solvent would be faster. On the contrary, an increase in the relative humidity decreases the evaporation rate of the solvent, which causes an increase in the diameter of the generated structures [139, 140].

4.2. Advantages of electrospraying for microencapsulation

Compared to other drying techniques, the main advantages of electrospraying for obtaining dry microcapsules include the following:

- Microparticles are obtained in a one-step process from the feed solutions [143]
- It does not require high temperatures nor freezing, so it is suitable for thermo-sensitive and cryo-sensitive bioactive ingredients [144].
- Once the process conditions are optimized, its operation is quite easy and cost-effective [138].
- Electrohydrodynamic techniques can achieve uniform dispersion of compounds within the polymeric matrix with high loading capacity and minimal compound loss [138].
- The droplet size is generally smaller than in conventional mechanical atomizers, and with narrower size distributions [140]. Microparticles in the nano- or submicron range are generally obtained, which is preferred to minimize their impact on the textural properties of the food products.
- Due to the mutual repulsion of the charged droplets generated, they are self-dispersing in space, so aggregation is prevented. Moreover the deposition

efficiency of a charged spray is considerably higher than for uncharged droplets [140].

- Although capsule-like microstructures are preferred for their application as food ingredients, electrohydrodynamic processing also allows the design of different nano- and micron-sized morphologies, which in turn allows tailoring their release properties.
- Quality check on the particles can be performed by briefly interrupting the process, which is very convenient [138].

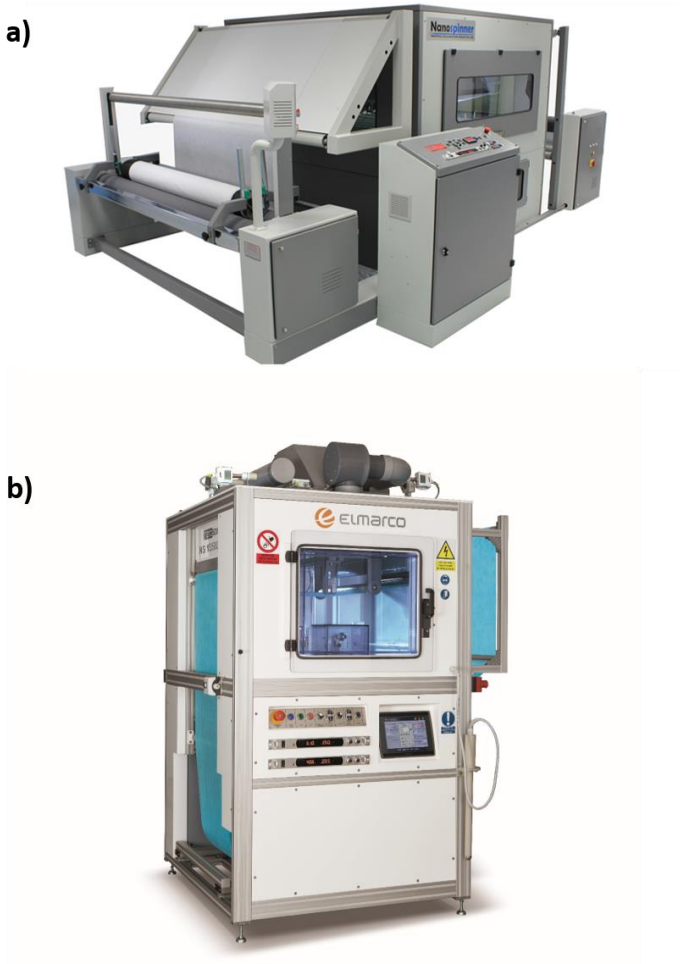


Figure 7. Industrial-scale electrospinning equipment. a) Nanospinner 416, produced by Inovenso Ltd. Reproduced with permission from Inovenso Ltd. b) Nanospider™, produced by Elmarco. Reproduced with permission from Elmarco.

Regarding the scalability, as the application of electrospraying for microencapsulation is still in its exploratory phase [144], most of the research works published or patented in this field have been conducted at lab scale, using one single needle or nozzle. Given the resulting low flow rates which can be processed, the production capacity of these lab-scale equipments is considerably low. However, due to the increased interest in the electrohydrodynamic processing techniques for production of different materials in various sectors, many technological solutions are already being proposed to scale up the production [145]. Some industrial-scale setups are already commercially available for the production of electrospun materials (cf. Figure 7). For instance, Inovenso Ltd. supplies an apparatus with a production rate of about 0.2 kg/h, and the company Elmarco commercializes an electrospinning system with a claimed annual throughput of up to $50x10^6$ m^2 of non-woven nanofibers [146]. Although these industrial solutions have not been applied for microencapsulation purposes in the food sector yet, the industrialization perspectives of the technique are promising.

4.3. Challenges of electrospraying for food applications[2]

Although there is an increased interest in electrospraying as an alternative technique for the microencapsulation of health-promoting compounds, there are still challenges that need to be faced when aiming at applying these microencapsulation structures in food products.

4.3.1. High surface tension and conductivity of aqueous solutions

One of the main challenges in the production of electrosprayed encapsulation matrices for nutraceutical and food applications is the imperative use of aqueous solutions or suspensions to avoid toxicity issues associated with potential residual traces of organic solvents on the materials [147]. Aqueous solutions generally have high surface tension values (the surface tension of water is 72 mN/m [138]), plus they impede the use of high voltages in electrohydrodynamic processes, as ionization of water molecules would occur leading to corona discharges [148]. In practice, the formation of stable jets in electrospraying from aqueous media is complicated and often leads to dripping of the polymeric solution.

The addition of surfactants to the feed solutions has been proposed as a promising strategy for the successful production of electrosprayed materials from aqueous solutions, as they reduce their surface tension and, thus, facilitate their spraying at acceptable voltages. This approach has been previously employed to enhance the spinnability of polymer solutions, obtaining defect-free fibres and

[2] This section is an adapted version of part of the book chapter: L.G. Gómez-Mascaraque, J. Ambrosio-Martín, M. J. Fabra, R. Pérez-Masiá, J. M. Lagaron and A. López-Rubio (2016). *Novel Nanoencapsulation Structures for Functional Foods and Nutraceutical Applications*, in *Nanotechnology in Nutraceuticals*, CRC Press 2016, pp. 373-395.

reducing the fibres diameter [149-151]. Not only surfactants lead to reduced surface tensions, but they can also have an impact on the solution conductivity and rheological properties. Nagarajan et al. (2007) observed an increase in the solution viscosity and conductivity upon addition of sodium dodecyl sulfate, an anionic surfactant, to polyethylene oxide, a non-ionic polymer [152]. Similarly, Talwar et al. (2010) found that non-ionic surfactants considerably altered the viscoelastic properties of associative polymer solutions, specially the relaxation time, hence significantly improving nanofiber morphology [153].

Regarding edible biopolymer structures, food-grade ultrathin fibres from amaranth protein and pullulan blends were obtained through electrohydrodynamic processing, where the addition of a surfactant, Tween80®, proved to be essential for the production of defect-free fibres for the blends with higher protein contents [154]. The addition of the surfactant Tween40® also facilitated the formation of edible blend nanofibers made of cellulose acetate and egg albumen by reducing both the electrical conductivity and the surface tension of the blend solutions [155].

More recently, electrosprayed nanoparticles were successfully obtained from solutions of edible, low molecular weight biopolymers like resistant starch and maltodextrin by reducing their surface tension upon addition of different surfactants. In this work, better results were obtained using non-ionic surfactants like Span20® or Tween20® than employing lecithin, a zwitterionic surfactant [148]. Surfactant-aided electrospraying has also been achieved for other food hydrocolloids, such as soy protein isolate (SPI) or whey protein concentrate (WPC), from aqueous dispersions [156]. Furthermore, some of these electrosprayed submicro- and nanostructures obtained by addition of surfactants have already been used to effectively encapsulate and protect micronutrients such as folic acid [112], with potential application in nutraceuticals.

4.3.2. Incorporation of hydrophobic bioactive ingredients

The compatibility between the bioactive molecule to be incorporated into the nutraceuticals or functional foods and the biopolymeric solution to be electrosprayed may also be an issue. As discussed before, the use of aqueous media for the electrospraying process is almost a requisite when the final application of the materials is an edible product. However, the majority of the bioactive ingredients of interest are not readily soluble in water. In these cases, a plausible strategy would be to prepare an oil-in-water (O/W) emulsion prior to electrospraying, in which the hydrophobic bioactive would be dispersed in a continuous aqueous solution containing the water-soluble biopolymer.

This approach has been extensively used for the entrapment of a number of compounds within polymeric micro- or nanofibers through electrospinning of emulsions [157-159] and has more recently been applied for encapsulation within polymeric particles by electrospraying too [160]. In the field of functional foods, this emulsion-electrospraying approach has very recently been used to encapsulate a

carotenoid ingredient, namely lycopene, in different biopolymeric matrices [47]. In this work, the encapsulation efficiency through emulsion-electrospraying was much higher using a protein matrix (WPC) than employing a polysaccharide (dextran), and this was ascribed to the great differences found in the stability of the emulsions, pointing out that one key factor in the production of encapsulation structures through emulsion-electrospraying is the stability of the emulsions to be processed.

As previously discussed (cf. Section 1.3), O/W emulsions are thermodynamically unstable. The addition of surfactants, which are commonly used to stabilize emulsions, is a plausible option to increase the encapsulation efficiencies in emulsion-electrospraying [134]. Also, proteins are widely used as emulsifiers to facilitate the formation and improve the stability of emulsions [161] because of their amphiphilic nature [10], so these biopolymers are of particular interest for the production of edible capsules through emulsion-electrospraying, as they could both serve as wall materials and aid emulsion stabilization. On the contrary, most polysaccharides do not exhibit interfacial activity due to their hydrophilic nature, not being adsorbed at the fluid interfaces [162]. Nevertheless, some of them are amphiphilic, like gum arabic or modified starch, and others can aid emulsion stabilization by increasing the viscosity of the aqueous phase, thus stopping (or delaying) gravitational separation (e.g., xanthan, pectin or carrageenan) [163, 164].

4.3.3. Electrospraying of biopolymeric matrices

Another limitation imposed for the application of electrosprayed materials to nutraceuticals and functional foods is the mandatory use of edible or food-grade matrices for the encapsulation of the bioactive agents, mainly biopolymers such as proteins or carbohydrates (cf. section 4). However, electrospraying from biopolymer solutions is intricate in most cases as some of them are poorly soluble in water, others have a polycationic or polyanionic nature, and many have too low molecular weights. Moreover, they generally have low chain flexibilities or are organized in globular structures (i.e. globular proteins), which further complicates the electrospraying process because of the absence of enough chain entanglements [152, 165].

One approach to increase chain entanglements between protein molecules is denaturation. It has been long-established that heating globular proteins in aqueous solutions can induce aggregation as a consequence of a denaturation process which is temperature-, pH- and ionic strength-dependent [166]. Denaturation leads to protein unfolding and, hence, to the exposure of its functional groups which can then interact among themselves [156]. Thus, chain entanglements are favored in aqueous dispersions of denatured proteins via increased intermolecular interactions. Accordingly, protein denaturation has been extensively used to produce protein hydrogels, where the amount of entanglements is big enough to induce the formation of a polymeric network [167-169].

Based on the same principles, denaturation of globular proteins could be used as a strategy to improve their processability by electrospinning or electrospraying. Indeed,

Dror et al. (2008) demonstrated the feasibility of obtaining nanofibers from solutions of a model globular protein, namely bovine serum albumin, by denaturation in trifluoroethanol in combination with the use of reducing agents and changes in the pH [170]. Their work showed that a low-viscosity globular protein solution with poor viscoelastic properties could be modified into a spinnable solution by denaturation and manipulation of the protein chain conformation.

Although denaturation of proteins can be conducted by changes in pH, solvents, temperature or by addition of co-solutes, heat is the major means of denaturation used for food purposes, as it avoids the use of toxic compounds [171]. Recently, Pérez-Masiá et al. (2014) found that thermal denaturation of globular proteins such as soy proteins can improve their electrosprayability in aqueous dispersions [156]. Specifically, a soy protein isolate (Mw ≈30,000–350,000 Da) that could not be electrosprayed in its native form gave rise to fine nanoparticles after thermal denaturation. The enhanced rheological properties of soy protein isolates after thermal treatment had previously been studied and exploited for the production of hybrid fibers based on mixtures of soy protein and poly(lactic acid), where bead formation was reduced during electrospinning by means of optimizing the denaturation conditions [172].

The inverse problem, i.e. the presence of too many chain entanglements, can also be encountered when attempting to electrospray biopolymeric solutions. This may be the case of biopolymers with hydrogel-forming capacity, as gelation could occur during processing depending on the selected conditions, hindering the formation of the electrospraying jet [173]. Even if the selected conditions do not trigger gel formation, the process parameters and solution properties must be optimized in order to obtain the desired morphology of the encapsulation structures, which for application as food ingredients should be particles rather than fibres as previously discussed. Since electrohydrodynamic processing of polymeric solutions may yield different morphologies depending on the processing conditions, these must be thoroughly adjusted in order to obtain capsule-like structures. Due to the variety of biopolymers with diverse properties in solution, the optimization of the electrospraying conditions must be carried out individually for each encapsulation matrix of interest. This challenge has been addressed for selected biopolymers in this doctoral thesis.

4.4. Trends in electrospraying for food applications

The application of the electrospraying technique for microencapsulation was first proposed for the protection and controlled release of drugs for biomedical purposes [134], and has only been recently extended to the field of food technology [144]. In this area, electrohydrodynamic processing has already been extensively applied for the development of novel and improved food packaging [174-176].

Electrospun fibres based on edible biopolymers incorporating bioactive compounds begun emerging in the last decade. Zein was the main protein used to incorporate different bioactive compounds, such as antioxidants [132, 177] or omega-3 fatty acids

[130, 131]. The reason why zein attracted so much interest was probably its solubility in ethanol, as this solvent exhibits lower conductivity, surface tension and boiling point than water, and thus it is easier to process by electrohydrodynamic processing (cf. Section 3.3.1). Soon, food-grade electrospun nanofibers started to be developed using other edible biopolymers or combinations of biopolymers. Some examples include nanofibers made from amaranth proteins [149], amaranth proteins and pullulans [154] or cellulose acetate and egg albumen [155]. These were also applied for the encapsulation of bioactive ingredients [178].

Bearing in mind the convenience of formulating encapsulated ingredients as powdery materials instead of fibrilar mats, López-Rubio and Lagaron (2011, 2012) developed, for the first time, electrosprayed capsules from aqueous solutions based on a whey protein concentrate, and loaded them with the antioxidant β-carotene as a proof of concept [147, 179]. Subsequently, Pérez-Masiá, Lagaron and López-Rubio (2014) widened the range of edible electrosprayed microencapsulation structures by developing a battery of microcapsules based on food hydrocolloids, which were proposed as delivery vehicles for incorporation within functional foods. For this purpose, strategies such as the addition of surfactants or the denaturation of proteins were used to improve the sprayability of the hydrocolloids [156]. These structures have been used for the encapsulation of a number of different functional ingredients, including bifidobacteria [114], omega-3 fatty acids [180], vitamins [112] or carotenoids [47].

To continue this ongoing work, the present doctoral thesis aims at contributing to the development of novel food-grade electrosprayed microencapsulation structures capable of protecting and delivering functional ingredients of different nature with their underlying challenges. Moreover, the behaviour of these encapsulation structures during *in-vitro* digestion and when incorporated within real food products has not been explored yet. Given the importance of these aspects for the potential application of these technologies in the food industry, this doctoral thesis also addresses these practical issues.

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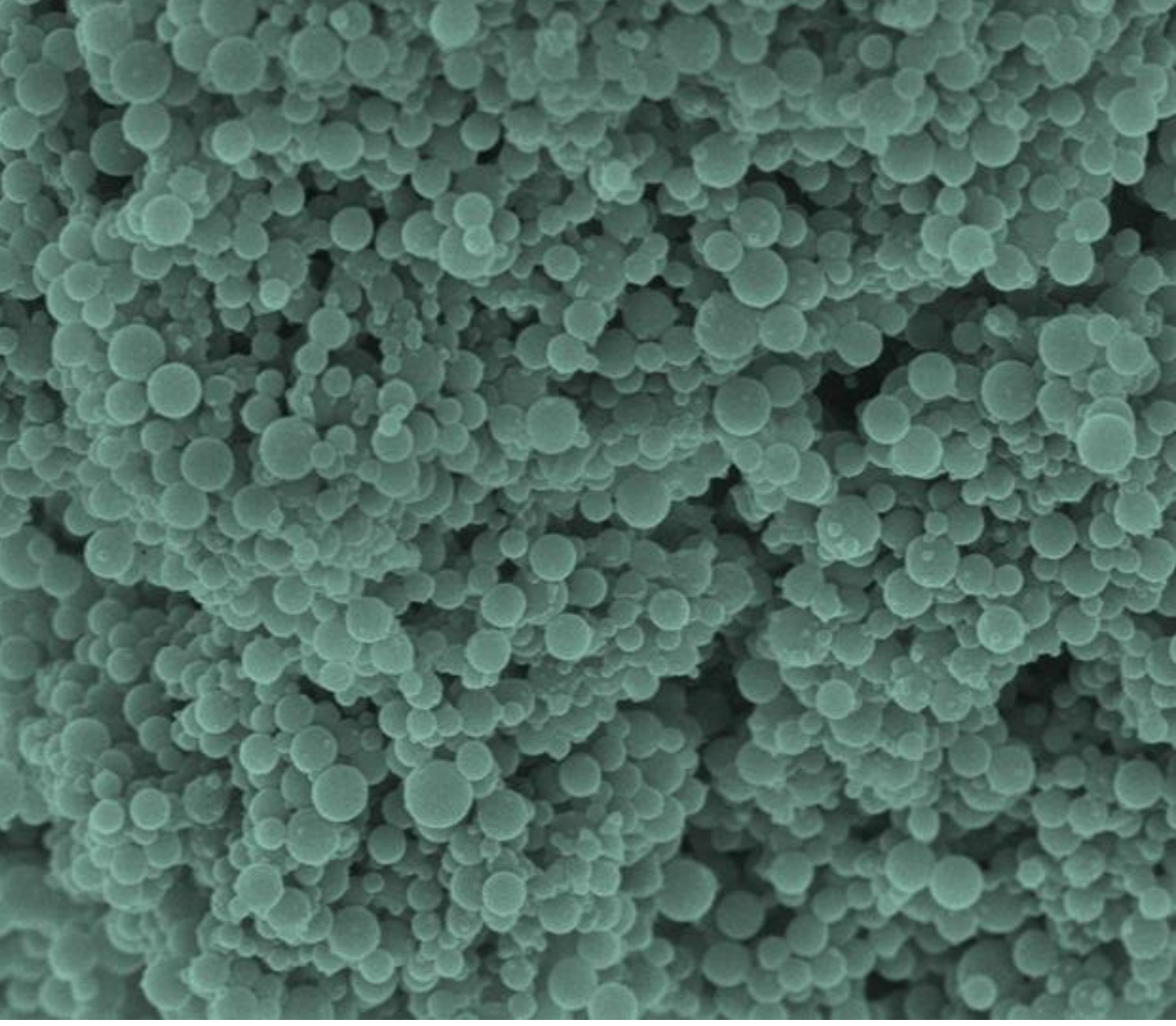


II. Objectives

Objectives

The **general objective** of the present doctoral thesis was to design and characterize novel encapsulation structures of interest in the development of functional foods.

For this purpose, several **specific objectives** were proposed:

- The development of novel, edible, biopolymer-based microencapsulation structures using mainly (but not exclusively) the electrospraying technique.
- The morphological and physical-chemical characterization of the developed microencapsulation structures.
- The incorporation of various bioactive ingredients of interest within the selected microencapsulation structures.
- The assessment of the protective effect exerted on the bioactive ingredients upon microencapsulation in the selected structures.
- The evaluation of the release of the bioactive ingredients from the microcapsules in food simulants or during *in-vitro* digestion.
- The incorporation of selected microencapsulation structures to model food matrices and study of their impact on the final food products.

These general objectives were materialized in 11 individual case studies, which have been structured in 5 chapters in this thesis:

Chapter 1: Microencapsulation of hydrophilic bioactive ingredients.

1.1. Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods.

1.2. Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds.

1.3. Stability and bioaccessibility of EGCG within edible micro-hydrogels. Chitosan vs. gelatin, a comparative study.

Chapter 2: Microencapsulation of hydrophobic bioactive ingredients.

2.1. Protein-based emulsion electrosprayed micro- and submicroparticles for the encapsulation and stabilization of thermosensitive hydrophobic bioactives.

2.2. Potential of microencapsulation through emulsion-electrospraying to improve the bioaccesibility of β-carotene.

2.3. Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin.

2.4. A step forward towards the design of a continuous process to produce hybrid liposome/protein microcapsules.

Chapter 3: Microencapsulation of probiotic microorganisms.

3.1. Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and *in-vitro* digestion.

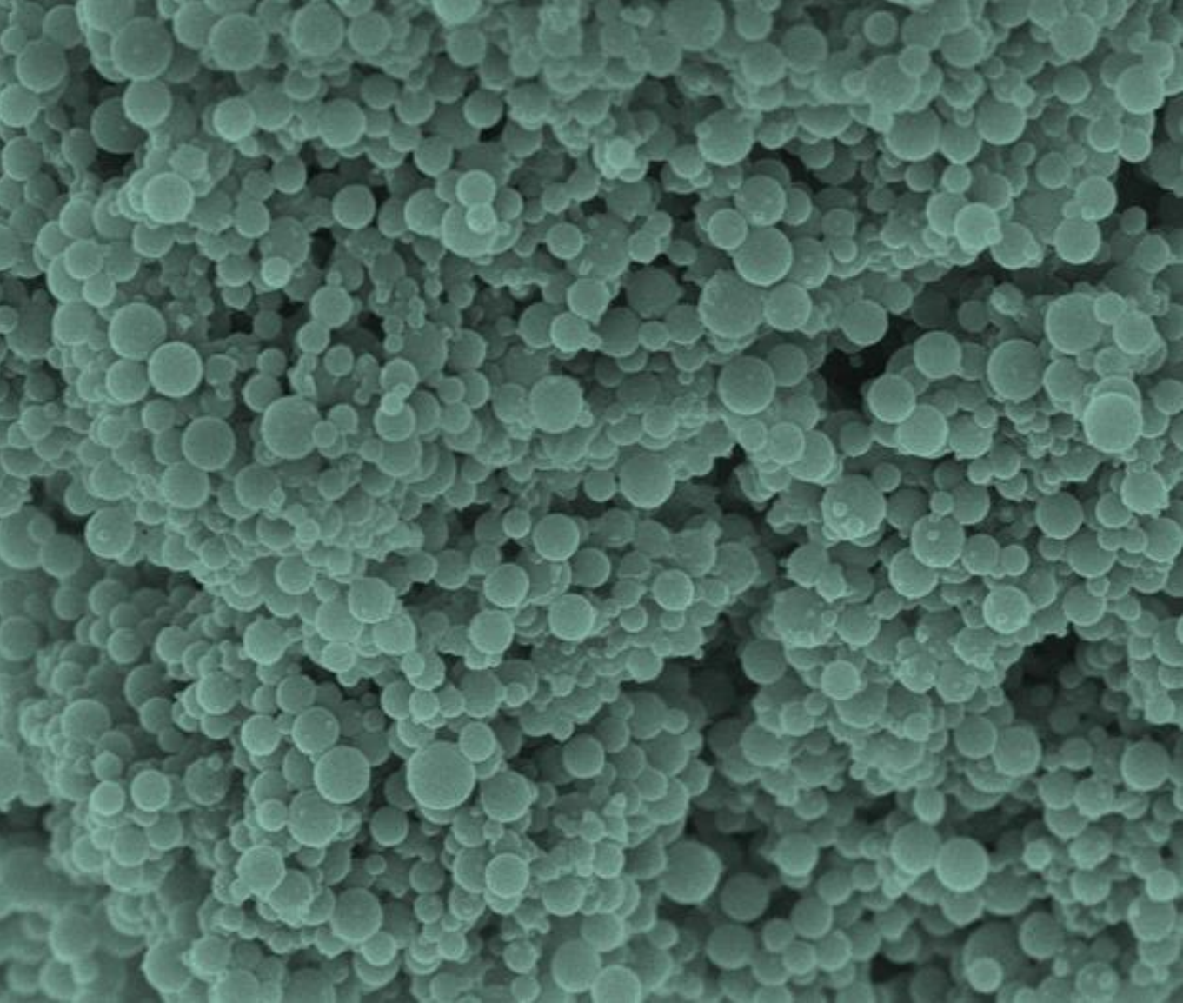
Chapter 4: Potential of plant cells as delivery vehicles for functional ingredients.

4.1. Binding of dietary phenolic compounds to potato cells and individual cell components - nutritional and industrial implications.

Chapter 5: Application of microencapsulation in real food systems.

5.1. Microencapsulation of a whey protein hydrolysate within micro-hydrogels: Impact on gastrointestinal stability and potential for functional yoghurt development.

5.2. Impact of microencapsulation within electrosprayed proteins on the formulation of green tea extract-enriched biscuits.



III. RESULTS

Results

This section includes the results from 11 case studies, structured in 5 chapters:

Chapter 1: Microencapsulation of hydrophilic bioactive ingredients.

1.1. Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods.

1.2. Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds.

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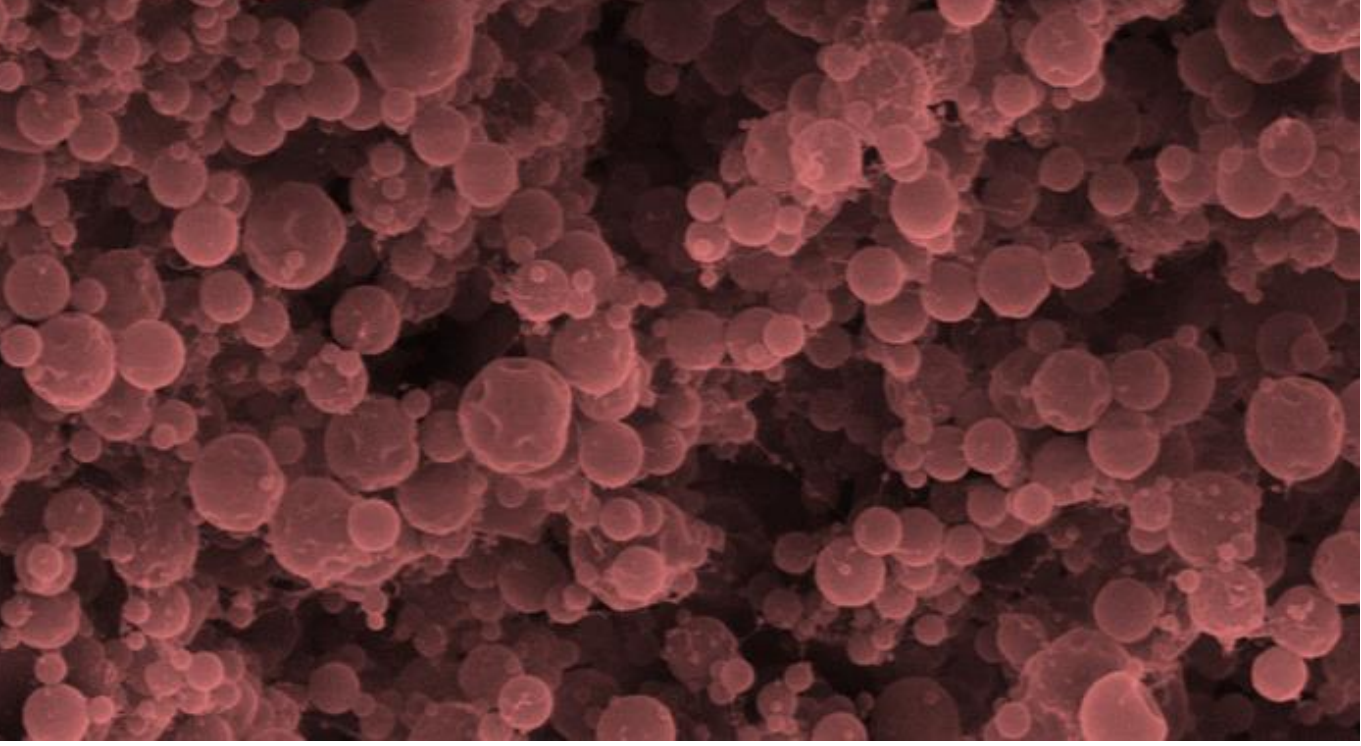


CHAPTER 1

MICROENCAPSULATION OF HYDROPHILIC BIOACTIVE INGREDIENTS

1.1. Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods.

1.2. Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds.

1.3. Stability and bioaccessibility of EGCG within edible micro-hydrogels. Chitosan vs. gelatin, a comparative study.

Introduction to Chapter 1

(–)-Epigallocatechin gallate (EGCG), a powerful antioxidant compound found predominantly in green tea, was selected as a model water-soluble bioactive ingredient in this chapter due to its many attributed health-promoting properties. The interest in its microencapsulation resides in its poor stability in aqueous environments, especially in neutral and alkaline conditions, which limits its direct addition to certain food products. Thus, different microencapsulation structures were designed in an attempt to stabilize this polyphenol against degradation in adverse conditions.

Gelatin and chitosan were selected as encapsulation matrices for EGCG because they are both edible, naturally-derived hydrogel-forming biopolymers, which can be processed in aqueous solutions giving rise to insoluble microcapsules under certain conditions. Moreover, they could be processed in acidic conditions favourable for the stability of EGCG. Two encapsulation techniques, electrospraying and spray-drying, were used to microencapsulate EGCG within these matrices.

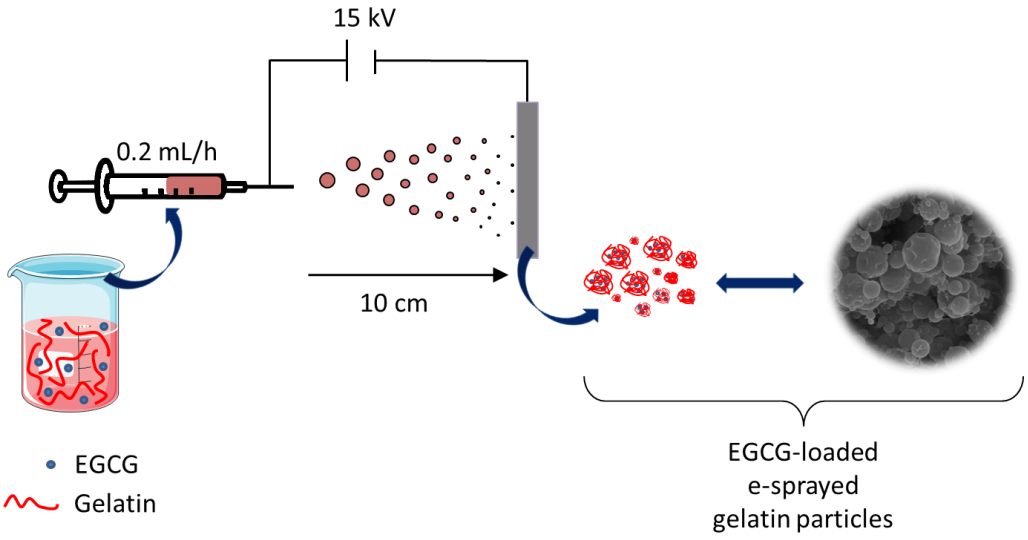
For electrospraying, the processing conditions had to be optimized individually for each biopolymer, given the great impact that solution properties have on the processability of the materials and the different characteristics of each matrix. Therefore, in a first work, the electrospraying process was optimized for the gelatin matrix in order to obtain particulate encapsulation structures as free of fibrils as possible. The obtained capsules were subsequently loaded with EGCG, and their encapsulation efficiency, antioxidant activity, release profiles and stabilization effect in slightly alkaline conditions was assessed.

In a second work, the electrospraying process was similarly optimized for the production of particulate encapsulation structures from chitosan, paying special attention to the impact that the molecular weight of this polysaccharide had on the morphology of the obtained materials. The optimal electrosprayed chitosan capsules were also loaded with EGCG and their antioxidant activity and encapsulation efficiency were assessed. The antiviral activity of the polyphenol was used as a tool to assess the stabilization effect that the capsules conferred to the bioactive molecule.

In parallel, in a third work, both gelatin and chitosan were processed under the same processing conditions through spray-drying. The spray-dried structures were used to encapsulate EGCG and the performance of both encapsulation matrices was compared in terms of their encapsulation efficiency, release properties and bioaccessibility of EGCG after *in-vitro* digestion of the microparticles.

1.1

ELECTROSPRAYED GELATIN SUBMICROPARTICLES AS EDIBLE CARRIERS FOR THE ENCAPSULATION OF POLYPHENOLS OF INTEREST IN FUNCTIONAL FOODS



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Lagarón, J. M., & López-Rubio, A. (2015). *Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods*. Food Hydrocolloids, 49, 42-52. (DOI: http://dx.doi.org/10.1016/j.foodhyd.2015.03.006).

1. Abstract

In this work, the potential of the electrospraying technique to obtain food-grade gelatin capsules in the submicron range for sensitive bioactive protection was explored, studying the influence of the protein concentration on the size and morphology of the obtained particles. Gelatin was selected as encapsulating material because, being commonly used as a food ingredient, it possesses unique gelation properties and is commercially available at a low cost. The electrosprayed matrices were used to encapsulate a model antioxidant molecule, (−)-epigallocatechin gallate (EGCG). Very high encapsulation efficiencies, close to 100%, were achieved, and the antioxidant activity of the bioactive was fully retained upon encapsulation. The EGCG release profiles showed a delayed release of the encapsulated antioxidant in aqueous solutions. Furthermore, while free EGCG in PBS lost a 30% of their antioxidant activity being completely degraded in 100 hrs, encapsulated EGCG retained its whole antioxidant activity within this time period.

2. Introduction

One of the main challenges in the development of functional foods is the preservation of the activity and bioavailability of the bioactive ingredients during food processing, storage and passage through the gastrointestinal tract. The development of edible nano- or microencapsulation matrices has been envisaged as a plausible option to protect these biologically active compounds against adverse conditions [1]. There are a number of encapsulation techniques which can be used to produce nano- or microparticulate systems, being emulsification-evaporation, spray-drying and coacervation the most extensively used [2]. However, some of these production methods involve exposure of the bioactives to high temperatures and/or the use of organic solvents, factors which can affect the stability of sensitive nutrients and preclude their use for food applications due to toxicity concerns associated with the residual traces of solvents [3].

Electrospraying (e-spraying) has recently emerged as an alternative for the generation of polymeric particles incorporating bioactive agents [4] with application in therapeutics, cosmetics and the food industry [5]. E-spraying, together with electrospinning (e-spinning), are versatile electrohydrodynamic fabrication methods which can generate encapsulation structures in a one-step process [6] without the need of employing high temperatures or toxic solvents [7]. A polymer solution flowing out from a nozzle is subjected to an external electrical field in such a way that when the electrical forces overcome the forces of surface tension, a charged jet is ejected towards a grounded collector. During the flight, the jet is elongated and the solvent

evaporates, producing dry continuous fibres in the case of e-spinning [8]. In e-spraying, the jet breaks down into fine droplets which acquire spherical shapes due to the surface tension [6], subsequently producing solid nano- or microparticles upon solvent evaporation. Apart from the feasibility of working at mild ambient conditions and using food-grade solvents, e-spraying has many other advantages as compared to other encapsulation techniques, including high encapsulation efficiencies, uniform bioactive distribution in the matrix, ease of operation and industrial scalability [4, 6]. Moreover, particles aggregation could be prevented due to their own mutual electrical repulsion, and smaller droplet sizes than in conventional mechanical atomisers can be obtained [5].

Among the different food-grade biopolymers which may be used as encapsulating materials, protein hydrogels are of particular interest as they are readily used as food ingredients, for instance to modify food texture or sensorial properties [9]. Specifically, gelatin has been widely employed for enhancing elasticity, stability and consistency of food products [10]. Moreover, it has been traditionally used by the pharmaceutical industry for the manufacture of hard and soft capsules to protect drugs from external agents such as atmospheric oxygen [11]. Gelatin is obtained from partial hydrolysis of collagen which contains repeating sequences of glycine-aa_1-aa_2, where amino acids aa_1 and aa_2 are mainly proline and hydroxyproline (see Figure 1) [12]. This biopolymer has drawn much research attention also in the field of controlled release of drugs due to its biodegradability and electrical properties, and because of its commercial availability and low cost [10, 12]. One of the most interesting properties of gelatin is its ability to form thermoreversible hydrogels in water due to the formation of collagen-like triple helices, interconnected with amorphous regions of randomly coiled segments, and subsequent chains entanglement and network formation below the so-called helix–coil transition temperature [13, 14]. This characteristic of the polypeptide makes it ideal to be processed in aqueous media, while avoiding complete disruption of the obtained capsules when submerged in aqueous foods below its gel-sol transition temperature.

However, gelatin cannot be e-sprayed at room temperature when dissolved in water [15] as gelation would occur [16]. Different solvents, such as fluoroalcohols [15], have been suggested as alternatives to process gelatin by e-spinning/spraying with positive results, but their high toxicity limits their use for food applications. The use of diluted carboxylic acids [17, 18] or ethyl acetate [19] has also been proposed as non-toxic solvents in various works. Particularly, e-spinning of gelatin solutions in acetic acid has been previously reported for type B gelatin from bovine skin [10, 16].

Green tea polyphenols are powerful antioxidants which have attracted great interest in the field of functional foods due to their numerous attributed health benefits. (–)-Epigallocatechin gallate (EGCG), the most abundant and biologically active compound in green tea [20], was selected in this work as a model antioxidant molecule due to its numerous attributed health benefits. It has shown protective effects against infections [21], cardiovascular and neurodegenerative diseases [22], inflammation and arthritis [23] and cancer [24-26], among other therapeutic benefits. However, its poor stability in aqueous solutions [27, 28] limits its direct addition to

food products. Several carrier systems have been developed to protect EGCG from degradation [20, 27-31].

In this work, for the first time, e-sprayed gelatin micro- and submicroparticles are proposed as food-grade encapsulating matrices for EGCG. A type A gelatin from porcine skin was selected in order to confirm whether previous results on the processability of bovine type B gelatin in diluted acetic acid could be applicable to a gelatin from a different origin. Thus, gelatin micro- and submicroparticles were produced in food-grade conditions by electrohydrodynamic treatment, and their ability for the encapsulation and stabilization of bioactives was studied using EGCG as a model water-soluble antioxidant.

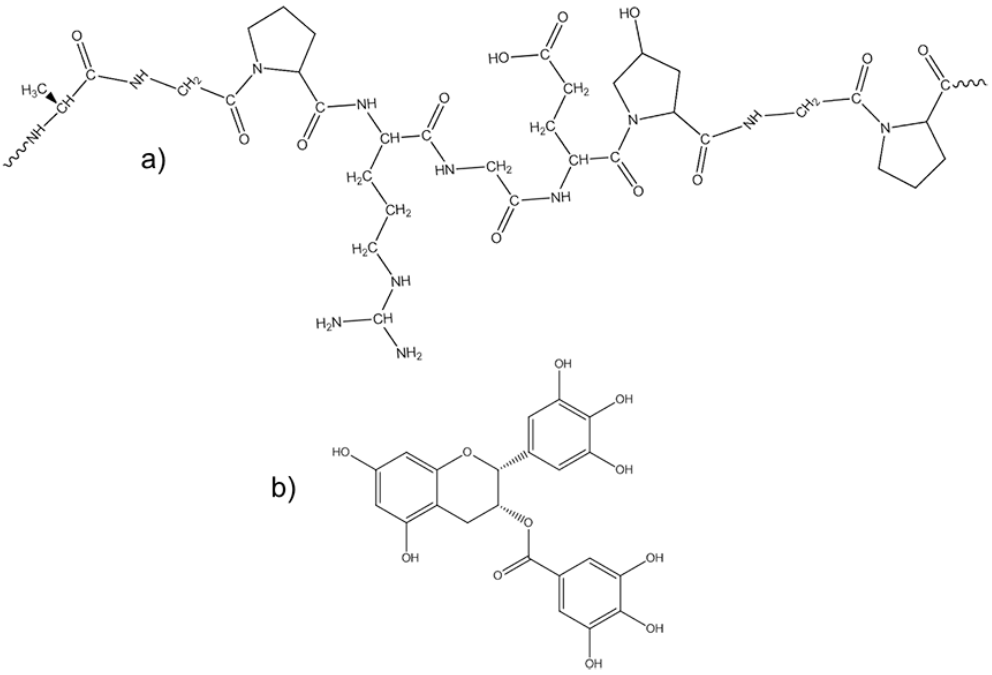


Figure 1. Schematic chemical structures of raw materials: a) gelatin and b) EGCG.

3. Materials and methods

3.1. Materials

Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom was obtained from Sigma-Aldrich. (–)-Epigallocatechin gallate (EGCG), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium

persulfate ($K_2O_8S_2$), buffer solutions of pH 7.4 (phosphate buffered saline system, PBS) and pH 6.1 (2-(N-morpholino)ethanesulfonic acid hemisodium salt, MES), and potassium bromide FTIR grade (KBr) were also obtained from Sigma-Aldrich. Acetic acid (96% v/v, Scharlab) and ethanol (96% v/v, Panreac) were used as received.

3.2. Preparation of gelatin solutions

Gelatin aqueous solutions of different concentrations, i.e. 5, 8, 10 and 20% (w/v), were prepared by dissolving the biopolymer in acetic acid 20% (v/v) at 40ºC under magnetic agitation, and cooled down to room temperature before processing. Gelation of the solutions was not observed for any of the samples.

When EGCG was incorporated for its encapsulation, it was added to the gelatin solutions at room temperature under magnetic stirring, at a concentration of 10 wt.-% of the total solids content.

3.3. Characterization of the solutions

The surface tension of the solutions was measured using the Wilhelmy plate method in an EasyDyne K20 tensiometer (Krüss GmbH, Hamburg, Germany) at room temperature.

The electrical conductivity of the solutions was measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain) at room temperature.

The rheological behaviour of the solutions was studied using a rheometer model AR-G2 (TA Instruments, USA), with a parallel plate geometry. The stainless steel plate diameter was 60 mm and the gap was fixed to 0.5 mm. The tests were performed at a controlled temperature of 25ºC ± 0.1ºC. Continuous shear rate ramps were performed from 0.1 to 200 s^{-1} during 15 min after equilibrating the samples for 5 min, and the shear stress of the samples was registered. All measurements were made at least in triplicate.

3.4. Electrohydrodynamic processing of the solutions

The solutions were processed using a Fluidnatek® LE-10 electrospinning/ electrospraying apparatus, equipped with a variable high voltage 0–30 kV power supply, purchased from BioInicia S.L. (Valencia, Spain). Solutions were introduced in a 5 mL plastic syringe and were pumped at a steady flow-rate through a stainless-steel needle (0.9 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a stainless-steel plate connected to the cathode of the power supply and placed horizontally with respect to the syringe. The processing

parameters were empirically optimized for each gelatin concentration in order to attain stable electrospraying avoiding dripping of the solution. Briefly, the flow rate varied from 0.15 to 0.5 mL/h and the voltage was maintained within the range 15-28 kV. The distance between the tip of the syringe and the collector was 10 cm in all cases.

3.5. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4100) at an accelerating voltage of 10 kV and a working distance of 9-16 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

3.6. Fourier transform infrared (FT-IR) analysis of the particles

Empty and bioactive-containing capsules of ca. 1 mg were grounded and dispersed in 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

3.7. Encapsulation efficiency

The encapsulation efficiency (EE) of the EGCG-loaded capsules was determined based on FT-IR absorbance measurements. A calibration curve (R^2 = 0.995) was obtained using gelatin/EGCG mixtures of known relative concentrations (0, 5, 10 and 15 % w/w of EGCG). The relative maximum absorbances at 1409 cm^{-1} (corresponding to gelatin) and 1039 cm^{-1} (attributed to EGCG) were plotted against the EGCG concentration in the mixtures (cf. Figure S1 in the supplementary material). The EGCG content in the capsules was interpolated from the obtained linear calibration equation. The EE of the EGCG-loaded capsules was then calculated using Eq. (1):

$$EE\ (\%) = \frac{Actual\ EGCG\ content\ in\ the\ capsules}{Theoretical\ EGCG\ content\ in\ the\ capsules} \times 100 \qquad \text{Eq. (1)}$$

3.8. Thermal Properties of the particles

Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q500 TGA. The samples (ca. 8 mg) were heated from room temperature to 600ºC

with a heating rate of 10ºC/min under dynamic air atmosphere. Derivative thermogravimetric (DTG) curves express the weight loss rate as a function of temperature.

3.9. EGCG release from the gelatin particles

10 mg of gelatin/EGCG capsules were suspended in 20 mL of release medium and kept at 20ºC under agitation in a Selecta thermostatic bath model Unitronic Reciprocal C (Barcelona, Spain). Three different release media were used: ethanol (96% v/v), MES aqueous buffer (pH=6.1) and PBS aqueous buffer (pH=7.4). At different time intervals, the suspensions were centrifuged at 3500 rpm and ambient temperature during 10 min using a centrifuge from Labortechnik model Hermle Z 400 K (Wasserburg, Germany), and 1 mL aliquot of the supernatant was removed for sample analysis. The aliquot volume was then replaced with fresh release medium and the particles re-suspended and left back in the thermostatic bath.

The extracted aliquots were analysed by UV-Vis spectroscopy (Shanghai Spectrum model SP-2000UV, Shanghai, China) by measuring the absorbance at 274 nm (maximum of absorbance of EGCG [29]). Calibration curves for EGCG quantification in solution by UV-Vis absorbance were previously obtained for the three different release media ($R^2_{ethanol}$ = 0.998, R^2_{MES} = 0.999, R^2_{PBS} = 0.999). The EGCG release values were obtained from three independent experiments.

3.10. *In-vitro* antioxidant activity

$ABTS^{+\bullet}$ radical scavenging assay was performed in order to quantify the antioxidant activity of both free and encapsulated EGCG, following the decolourization assay protocol described by Re et al. (1999). Briefly, a stock solution of $ABTS^{+\bullet}$ was prepared by reacting ABTS with potassium persulfate (7 and 2.45 mM in distilled water, respectively) and allowing the mixture to stand in the dark at room temperature for 24 hrs. The $ABTS^{+\bullet}$ stock solution was then diluted with acetic acid 20% v/v (same solvent in which the samples were dissolved) to an absorbance of 0.70±0.02 at 734 nm. Stock solutions of free and encapsulated EGCG (5 mM of EGCG in both cases) were prepared in acetic acid 20% v/v to facilitate the dissolution of the gelatin matrix and subsequent complete release of EGCG from the capsules. Then, these stock solutions were diluted 20-fold. 10 µL of diluted sample solution were added to 1 mL of diluted $ABTS^{\bullet+}$, and the absorbance at 734 nm measured at room temperature 1 min after initial mixing. The radical scavenging activity (RSA), expressed as the percentage of reduction of the absorbance at 734 nm after sample addition, was calculated using Eq. (2):

$$RSA\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \qquad \text{Eq. (2)}$$

Where A_0 and A_1 are the absorbances at 734 nm of $ABTS^{+\bullet}$ before and 1 min after addition of the antioxidant samples, respectively.

Experiments were performed on a Shanghai Spectrum spectrophotometer model SP-2000UV (Shanghai, China), at least in triplicate. Solvent blanks were also run in each assay. Unloaded gelatin particles were also evaluated (same particle concentration as in loaded samples) to take into account the potential antioxidant activity of the encapsulation matrix.

3.11. EGCG degradation assays

Solutions/suspensions of 5 mM EGCG and EGCG-loaded gelatin capsules with theoretical EGCG concentrations of 5 mM in PBS were prepared. After selected time intervals, the solutions/suspensions were diluted 20-fold with acetic acid 20% v/v and the $ABTS^{+\bullet}$ radical scavenging assay was conducted as previously explained. The radical scavenging activity (RSA) at the different time intervals was calculated using eq. (2).

3.12. Statistical analysis

A statistical analysis of experimental data was performed through analysis of variance (one-way ANOVA) using OriginPro 8 (OriginLab Corp., Northampton, USA). Homogeneous sample groups were obtained by using Fisher LSD test (95% significance level, $p < 0.05$).

4. Results and discussion

4.1. Optimization of the e-spraying process for obtaining EGCG-loaded gelatin particles

Gelatin solutions were prepared in diluted acetic acid (20% v/v) to enable their processing using e-spraying, as premature gelation of the protein precludes capsule formation using this technique. Moreover, this solvent was considered appropriate for the expected final application as it does not leave toxic residues on the dry materials [33]. Under these food-grade conditions, gelation of the solutions was not observed for any of the samples during the electrohydrodynamic processing at room temperature.

Different material morphologies can be obtained through electrohydrodynamic processing of polymer solutions depending on the process parameters and the solution properties. For food applications, particles rather than fibres are preferred,

since they are easier to handle and to subsequently disperse within the food products. Therefore, various concentrations of gelatin were tested in order to optimize the e-spraying process, with the objective to obtain neat individual particles as free of residual fibrils as possible. Electrohydrodynamic processing of bovine type B gelatin solutions in acetic acid had been previously attempted [10, 16], and the reported results served as a starting point to select a set of protein concentrations to be tested for the optimization of the e-spraying process for type A gelatin from porcine origin. Four different gelatin concentrations (5, 8, 10 and 20% w/v, with sample codes Gel5, Gel8, Gel10 and Gel20, respectively) were finally selected, and the processing parameters (i.e. flow rate and voltage) were also adjusted to maximize the production rate while keeping a stabilized jet, thus, avoiding dripping of the solution. The optimal processing parameters found in this study for the different compositions are summarized in Table 1.

Table 1. Gelatin concentrations tested and their optimal processing parameters.

Sample Code	[Gelatin] (% w/v)	Flow rate (mL/h)	Voltage (kV)
Gel20	20	0.15	28
Gel10	10	0.5	20
Gel8	8	0.2	15
Gel5	5	0.2	17

The size and morphology of materials obtained through electrohydrodynamic processing is strongly dependent on the properties of the polymer solutions [34]. Thus, the selected gelatin solutions were characterized in terms of surface tension, electrical conductivity and rheological behaviour prior to their processing and the results are summarized in Figure 2. On the other hand, Figure 3 shows the morphology of the processed structures obtained from the different solutions together with the particle size distributions for the e-sprayed samples.

Fibres were obtained for the sample with the greatest protein concentration (i.e. Gel20), while pseudo-spherical particles typical of the discontinuous e-spraying process, with more or less residual fibrils, were produced for lower gelatin concentrations. These results are consistent with the electrospinnability domains recently established by Erencia et al. (2014) for gelatin-water-acetic acid systems using a type B gelatin from bovine skin [16]. A certain protein concentration is needed to establish the necessary peptide chain entanglements and chain-chain interactions leading to fibre formation. The particle size distributions of the e-sprayed samples (cf. Figure 3) reflected a decrease in the particle diameter and greater heterogeneity of capsule sizes as the gelatin concentration decreased. In all cases, the majority of the particles had a size in the submicron range, having their maximum in the nanoscale.

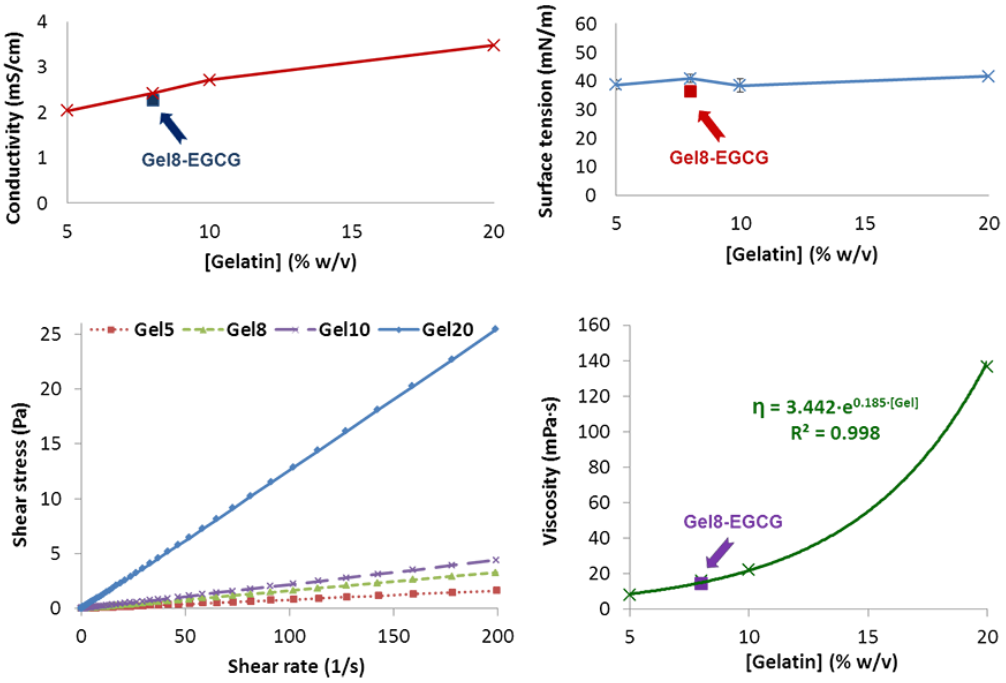
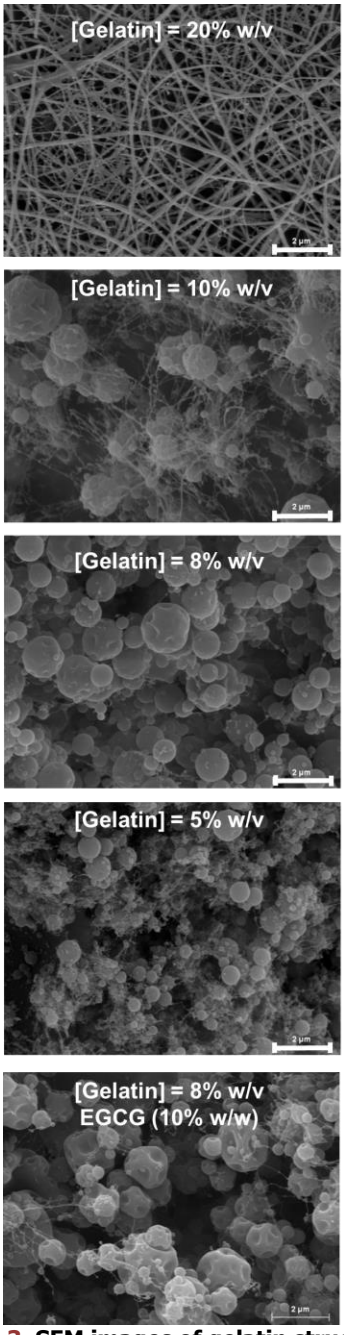


Figure 2. Electrical conductivity, surface tension and rheological behaviour of gelatin solutions in diluted acetic acid (20% v/v). Properties of gelatin solution containing EGCG are also shown (emphasised by arrows).

Regarding the solution properties, in general, a slight increase in the conductivity of the solutions was observed with the polymer concentration, whereas no significant variation was observed for their surface tension. Therefore, differences in the morphology of the processed materials could be mainly attributed to changes in the rheological properties of the solutions. All tested solutions exhibited a Newtonian behaviour, with a linear relationship between the shear stress and the shear rate in the whole range of study (cf. Figure 2) and, thus, the viscosity was calculated from the slope of the shear stress vs. shear rate curves. The Newtonian behaviour of gelatin in aqueous solutions, even at high concentrations, had been previously reported at neutral pH [35]. As expected, the viscosity exponentially increased with the gelatin concentration [16], which resulted in sample Gel20 having a viscosity considerably higher than the rest of the samples and above the so-called critical entanglement concentration, defined as the crossover of concentration from the semidilute unentangled to the semidilute entangled regimes in polymeric solutions [36]. The presence of sufficient chain entanglements for a 20% gelatin concentration explains the production of e-spun fibres instead of e-sprayed particles [37] in sample Gel20, as jet fragmentation during processing was prevented by the strong intermolecular cohesion of this concentrated solution [6].



Sample	Gel5 (*)	Gel8	Gel10	Gel8-EGCG
Mean diameter (µm)	0.23	0.48	0.49	0.47
Standard deviation (µm)	0.17	0.38	0.40	0.37
Minimum diameter (µm)	0.05	0.06	0.07	0.07
Maximum diameter (µm)	0.88	1.84	2.12	2.00

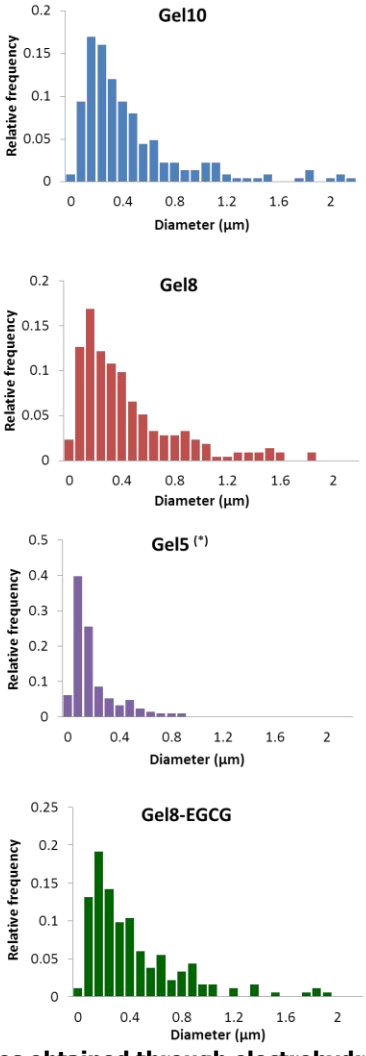


Figure 3. SEM images of gelatin structures obtained through electrohydrodynamic processing from different protein concentrations (left) and particle size distributions for the e-sprayed samples (right). The image and size distribution at the bottom correspond to EGCG-loaded capsules. Scale bars in SEM images correspond to 2 µm. Asterisk (*) depicts significant differences for the particle size distribution ($p<0.05$).

The most spherical morphology, almost free of residual fibrils, was exhibited by Gel8. Higher concentrations led to fibrils formation while lower concentrations resulted in some dripping of the solution. Hence, this gelatin concentration was selected as optimal for further experiments.

Once the conditions for the production of e-sprayed gelatin capsules were optimized, these vehicles were loaded with EGCG as a model water-soluble antioxidant. The gelatin solution (8% w/v) was prepared as in previous experiments, and EGCG was subsequently added at room temperature to achieve a final theoretical EGCG concentration of 10% w/w in the capsules. The morphology and particle size distribution of the resulting encapsulates was similar to those of their unloaded counterparts (cf. Figure 3). A very similar morphology with slightly rougher surface of the capsules was observed for the loaded structures, as solution properties were not considerably affected upon EGCG addition (cf. Figure 2).

4.2. Molecular organization and encapsulation efficiency

The e-sprayed gelatin capsules, both unloaded and loaded with EGCG, were characterized by FTIR spectroscopy along with the commercial untreated gelatin and EGCG.

The spectrum of commercial gelatin showed its four most characteristic bands centred at 3430 cm^{-1} (Amide A, NH stretching), 1642 cm^{-1} (Amide I, C=O and CN stretching), 1543 cm^{-1}, (Amide II, N-H bending) and 1244 cm^{-1} (Amide III, C-N stretching) [38-40]. Also, a band corresponding to the asymmetric stretching vibration of =C–H and $–NH_3^+$ (Amide B) was observed at 3085 cm^{-1} [41]. The bands observed at 2960 and 2928 cm^{-1} correspond to CH_2 asymmetric and symmetric stretching vibrations, respectively, mainly from the glycine backbone and proline side-chains [41, 42].

After the e-spraying treatment, sample Gel8 exhibited the same characteristic bands as the commercial gelatin, but a considerable narrowing and better definition of the bands was apparent, which has been previously observed for other biopolymers after capsule formation (Pérez-Masiá et al., 2014). Moreover, significant displacements were detected for the bands corresponding to the Amides A and I from 3430 and 1642 cm^{-1} to 3402 and 1653 cm^{-1}, respectively, which can be attributed to differences in hydrogen bonding and protein conformation [41] caused by the e-spraying processing. Specifically, shifts of the Amide A band to lower wavenumbers indicate hydrogen bond formation via the N-H groups of the peptides [43], while the shift of the amide I band to 1653 cm^{-1} can be correlated with β-sheet peptide conformation as previously observed for other proteins [44].

The spectrum of commercial EGCG showed an intense band at 3358 cm^{-1} due to the stretching of O-H groups, and other characteristic bands at 1618 cm^{-1}, attributed to the aromatic ring quadrant, at 1544, 1528 and 1518 cm^{-1}, corresponding to the aromatic semicircle stretch, at 1294 cm^{-1}, due to the deformation vibration of O-H

groups of the aromatic alcohol, and at 1097 cm^{-1}, owed to the aromatic rings stretch [45]. The presence of EGCG in the loaded capsules was evidenced by the existence of absorption bands corresponding to this polyphenol in their infrared spectrum, in particular the bands at 1042 cm^{-1} (which shifted to 1038 cm^{-1} in the capsules) and 1148 cm^{-1} (cf. arrows in the inset of Figure 4).

Proteins have been described to strongly interact with polyphenol molecules through hydrogen bonding and hydrophobic interactions [14, 28]. In the e-sprayed loaded gelatin particles developed in this work, apart from the displacement of the 1042 cm^{-1} band of the EGCG, other changes were observed in the infrared spectrum from gelatin upon encapsulation of the bioactive compound. For instance, the maximum of the Amide A band shifted to even lower wavenumbers (3358 cm^{-1}), thus suggesting, as previously explained, that hydrogen bonding between the gelatin matrix and the bioactive took place. The Amide III band also shifted from 1245 cm^{-1} in Gel8 to 1241 cm^{-1} in the EGCG-loaded capsules. These differences suggest the presence of intermolecular interactions between the antioxidant and the biopolymer within the developed capsules, which might contribute to the stabilization of the former.

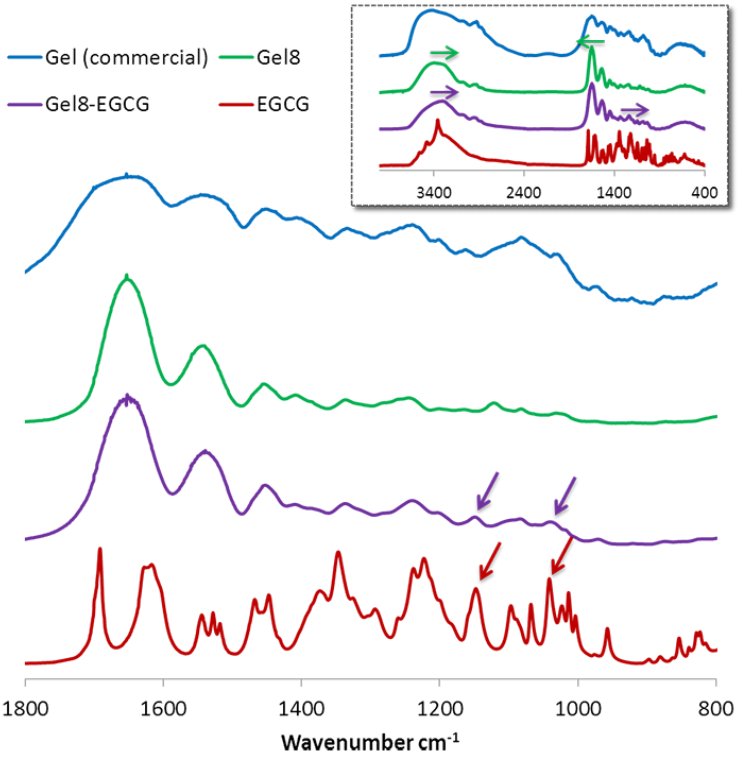


Figure 4. Infrared spectra of commercial and e-sprayed materials in the region from 1800 to 800 cm^{-1}. Arrows indicate bands corresponding to EGCG. The whole spectra are depicted in the inset, where arrows indicate band displacements.

Infrared spectroscopy was also used to estimate the encapsulation efficiency of the samples. Based on the measurements of absorbance intensities from the isolated spectral bands from the protein matrix and the bioactive at 1409 cm^{-1} and 1039 cm^{-1}, respectively, a calibration curve (R^2 = 0.995) was constructed using physical mixtures of gelatin and EGCG of known relative concentrations (cf. Figure S1 in the Supplementary data). The EE of the EGCG-loaded capsules was 96% ± 3%, i.e., almost all the antioxidant added to the solution was effectively incorporated within the capsules. This value was considerably higher than those reported for other encapsulation systems for the protection of cathequins [27, 46-48] and can be explained taking into account the great solubility of EGCG in the polymeric solution and the absence of partitioning effects [27] when using e-spraying as the encapsulation technique.

4.3. TGA. Thermal stability of the particles

Thermogravimetric analysis of raw materials and electrosprayed particles (Gel 8% w/v, both empty and EGCG-loaded), were performed in order to study possible thermostability changes of the ingredients upon electrohydrodynamic treatment. Table 2 and Figure 5 summarize the main results.

Three different stages were observed in the weight loss curve of gelatin. The first stage, observed at temperatures up to 200ºC, is related to the loss of adsorbed and bound water present in the gelatin samples due to its hygroscopic character. The second stage, corresponding to the major weight loss, occurred between 200 and 400°C and has been associated with the protein chain rupture and peptide bonds breakage [49]. The last stage, observed between 400ºC and 600ºC, has been attributed to the thermal decomposition of the gelatin networks [50]. Other authors relate these second and third stages to the elimination of aminoacid fragments in oxidant atmosphere, mainly proline, and the degradation of glycine, respectively [51].

Table 2. Onset temperature, temperatures of maximum degradation rate and corresponding weight losses of the two degradation stages for unprocessed EGCG and gelatin, and for the e-sprayed particles.

Sample	T_{onset} (ºC)	T_{max1}[a] (ºC)	WL_1[b] (%)	T_{max2}[a] (ºC)	WL_2[b]
Gelatin	265.6	301.3	45.2	537.7	36.9
EGCG	228.8	235.4	33.7	483.9	62.3
Gel8	241.3	324.4	53.7	531.8	34.5
Gel8-EGCG	235.8	321.6	50.2	534.4	37.0

[a] Temperature of maximum degradation rate
[b] Weight loss of the corresponding degradation stage

The thermogravimetric curves of the e-sprayed gelatin particles showed similar degradation profiles to that of the original gelatin powder. However, slight changes in the degradation profile of the main stage were observed. The temperature of maximum degradation rate of this stage (T_{max1}) increased upon e-spraying of the protein, both in the presence and in the absence of antioxidant, although degradation was extended over a wider range of temperatures. Specifically, a slight decrease in the onset temperature (T_{onset}) was observed, which cannot be ascribed to EGCG degradation as it was also seen for the unloaded structures.

Regarding the water loss during the first stage, noticeable differences were observed between unprocessed and e-sprayed gelatin samples. Different types of water bound to proteins have been reported [50], including absorbed and structural water. Absorbed water is removed from the samples up to 100ºC, while structural water needs more energy and is eliminated at higher temperatures. Figure 5 shows that, while the first weight loss stage of raw gelatin was extended up to 200ºC due to the presence of structural water, the water loss of e-sprayed gelatin samples, both loaded and unloaded, was only seen up to 100ºC, suggesting that only absorbed water was present in these samples. In fact, the weight loss in this first step was greater for raw gelatin (9.5%) than for both e-sprayed materials (7.2% for Gel8 and 6.0% for Gel8-EGCG). These findings support that the fast drying of the samples during electrohydrodynamic processing of gelatin solutions is capable of removing structural water from the protein, and promoting hydrogen bonding between polypeptide chains and also between the protein and the polyphenol molecules as observed by infrared spectroscopy.

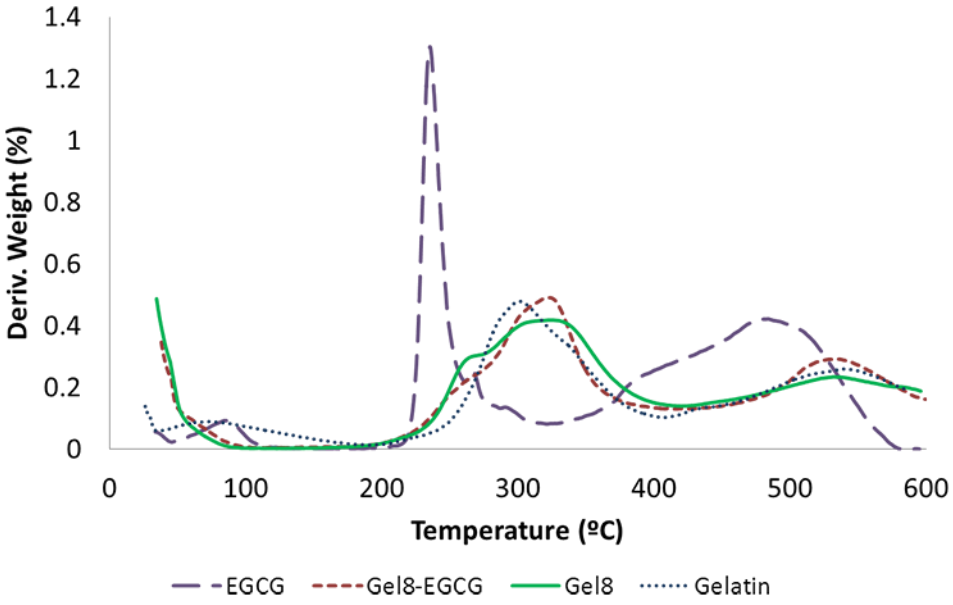


Figure 5. DTG curves of raw EGCG and gelatin, and e-sprayed particles.

No peaks attributed to the degradation of EGCG were detected in the TGA curve of the EGCG-loaded capsules. This could be explained by the good compatibility and intermolecular interactions between the polymer and the bioactive, as previously inferred from the infrared results, which contributed to the stabilization of the latter until the protecting matrix was degraded.

4.4. EGCG release from the electrosprayed gelatin particles

The release of EGCG from the e-sprayed gelatin capsules was studied in three different media. PBS aqueous buffer (pH=7.4) was selected as a simulated biological fluid, as it is one of the most commonly used blood plasma simulant [52]. On the other hand, ethanol is a good simulant for fatty foods and it is easy to work with analytically [53, 54], so it was selected as a fatty food simulant, while MES aqueous buffer (pH=6.1) was selected as a simulant for slightly acidic aqueous foods such as juices [55].

The resulting release profiles are depicted in Figure 6. An initial burst release was observed in all tested media, followed by a slower sustained release which was more clearly observed in the ethanolic suspension. The release was faster in aqueous media as a consequence of the swelling of the gelatin matrix, but still, due to the encapsulation of the antioxidant molecule its dissolution in these media was delayed. This will consequently impact on the degradation kinetics in solution.

A number of semi-empirical mathematical models have been proposed in the literature to describe the release kinetics of bioactive molecules from a carrier or delivery system [56], and some of the most commonly used ones have been applied to the experimental data in Figure 6, including the Higuchi equation [57], the power law model or Ritger-Peppas semiempirical equation [58], and the Peppas-Sahlin model [59]. The last two take into account the combination of Fickian (diffusion) and non-Fickian (polymer relaxation) release mechanisms. These models usually fit experimental data only in the early time points of the release profile [58], and thus only the data corresponding to the so called 'burst release phase' [60] was fitted to the models.

The Peppas-Sahlin model was the one which better fitted our experimental data. Its general equation is shown in Eq. (3), where M_t is the mass of EGCG released at time t, M_0 is the total mass of EGCG loaded in the particles, m is the Fickian diffusional exponent, and k_i are kinetic constants [56]. For an aspect ratio of 1 (i.e. spherical geometry of the carrier), m = 0.425 [59]. Table 3 shows the EGCG release kinetic parameters for the Peppas-Sahlin equation in the different food simulants.

$$\frac{M_t}{M_0} = k_1 \cdot t^m + k_2 \cdot t^{2m} \qquad \text{Eq. (3)}$$

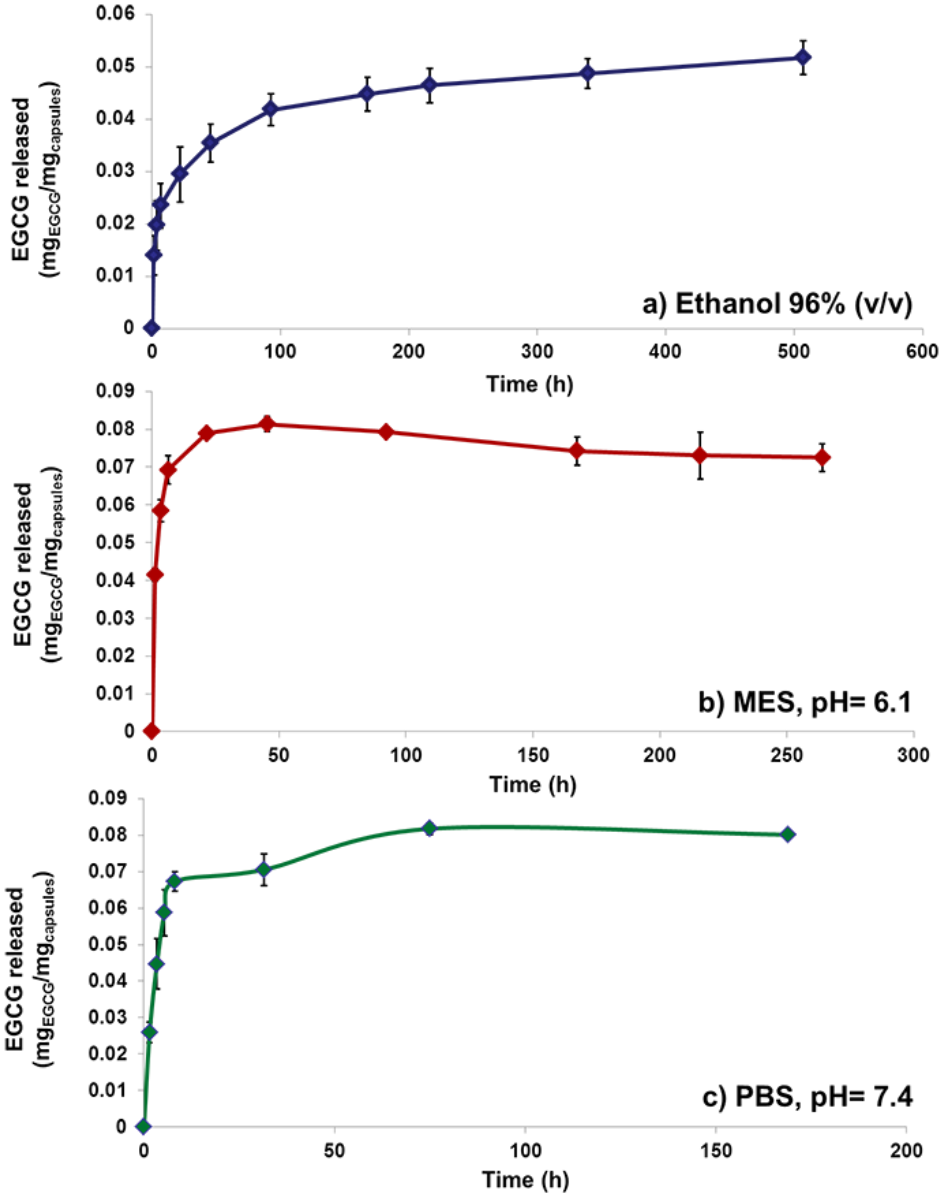


Figure 6. EGCG release profiles from e-sprayed gelatin particles in a) ethanol, b) MES and c) PBS.

Table 3. EGCG release kinetic parameters (k_i) and the linear correlation coefficients (R^2).

Release medium	k_1 ($h^{-0.425}$)	k_2 ($h^{-0.850}$)	R^2
Ethanol 96%	0.10±0.01	-0.006±0.002	0.98
MES	0.42±0.02	-0.053±0.006	0.99
PBS	0.35±0.04	-0.043±0.007	0.95

The Peppas-Sahlin model allows estimation of the relative contribution of the relaxational phenomenon and the diffusional mechanism on the release kinetics. The first term of the equation ($k_1 \cdot t^m$) accounts for the contribution of the diffusion phenomenon to the EGCG release kinetics, while the second term ($k_2 \cdot t^{2m}$) accounts for the case-II transport [59, 61]. For the e-sprayed gelatin particles, k_1 was greater than k_2 in the three food simulants, suggesting that the diffusional mechanism was predominant. Similar behavior has been reported for other spherical carriers based on gelatin microgels [61]. The ratio k_1/k_2 was higher for the release of EGCG in ethanol than in the aqueous media, indicating that the swelling or relaxation of the gelatin matrix had a greater contribution to the release kinetics in the aqueous food simulants, as expected. Moreover, the release was also much faster in these media, as confirmed by the higher values of the kinetic constants in MES and PBS.

4.5. *In-vitro* antioxidant activity and degradation assays

$ABTS^{+\bullet}$ decolourization assay was performed in order to compare the antioxidant activity of encapsulated and free EGCG. For this purpose, diluted acetic acid was used to disrupt the gelatin capsules and dissolve EGCG. The concentration of loaded capsules was calculated to have the same EGCG concentration as in the free EGCG sample (i.e. 0.25 mM), assuming 100% encapsulation efficiency. Unloaded e-sprayed gelatin particles were also evaluated in order to disregard possible contributions of the encapsulating matrix to the total antioxidant activity of the encapsulates, using the same particles concentration as for the loaded capsules. Solvent blanks were run too. The radical scavenging activity (RSA) of each antioxidant solution was calculated using eq. (2).

No statistically significant differences ($p < 0.05$) were observed between the inhibition of the absorbance caused by the e-sprayed gelatin matrix (RSA = 3.2 ± 0.2 %) and the solvent blank (RSA= 3.4 ± 0.2 %), so the whole antioxidant activity of the loaded capsules could be attributed to its EGCG content. The antioxidant activity of encapsulated EGCG (RSA = 26.8 ± 0.7 %) was not significantly different from that of free EGCG (RSA = 27.2 ± 1.5 %) either, thus confirming that the encapsulation efficiency was indeed very close to 100%, because the theoretical loading matched the experimental antioxidant activity of free EGCG. These results corroborated the previous estimations obtained from infrared spectroscopy measurements and verified

that the e-spraying process did not damage the bioactive, as its antioxidant activity was kept intact.

The $ABTS^{+\bullet}$ decolourization assay was also used to study the degradation of free and encapsulated EGCG in aqueous solution/suspension, by measuring the decrease in their RSA value with time as an indicative of the loss of their antioxidant activity due to degradation. PBS was the selected degradation media as EGCG is highly unstable in aqueous solution, especially in neutral or alkaline solutions [20, 28], suffering degradation through oxidative processes [27]. Hence, solutions 5 mM of EGCG and suspensions of EGCG-loaded capsules with the same theoretical EGCG concentration were prepared. Fast degradation of EGCG upon dissolution in PBS media could be clearly observed by its gradual transition from a light pink to an intense yellowish colour. At selected time intervals, the solutions/suspensions were diluted 20-fold with acetic acid 20% v/v with the double objective of disrupting the gelatin capsules and stopping the degradation process by acidification of the medium. The inhibition of the absorbance of $ABTS^{+\bullet}$ caused by the resulting solutions was measured and their RSA values calculated. The results are shown in Figure 7.

The free EGCG samples lost a 30% of their initial RSA in 100 hrs. No further antioxidant activity loss was observed after longer time periods, suggesting that EGCG was fully degraded in PBS after 4 days, even though its degradation products also exhibited some antioxidant activity. In contrast, no significant loss of antioxidant activity was observed for the encapsulated molecule within an observation time of 10 days ($p < 0.05$). This finding proved that the encapsulation system proposed in this work was capable of protecting EGCG from degradation in slightly alkaline solutions.

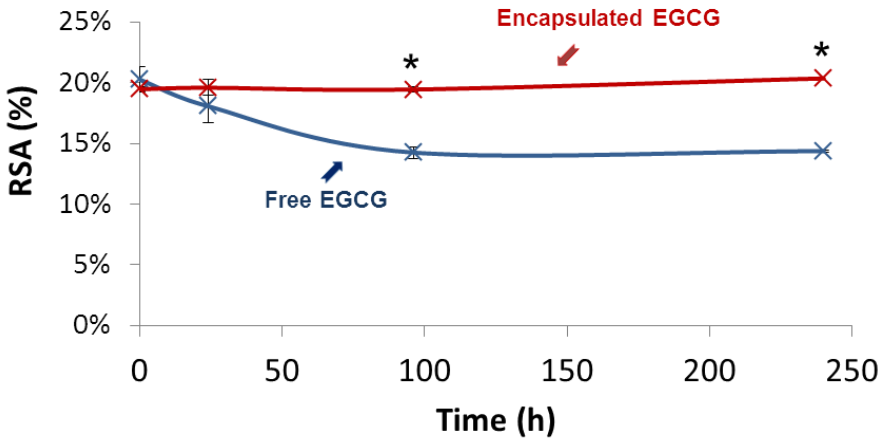


Figure 7. Degradation profiles of free and encapsulated EGCG in PBS. Asterisk (*) depicts significant differences between the two samples at each time point ($p < 0.05$).

5. Conclusions

Gelatin-based encapsulation matrices were produced from food-grade ingredients without the need of employing high temperatures or toxic solvents by electrohydrodynamic treatment of gelatin solutions in diluted acetic acid. The electrospraying process was initially optimized in order to obtain neat particles, almost free of fibrils, to facilitate handling and dispersion into food products. Pseudo-spherical particles with mean sizes in the submicron range were obtained. The potential of these particles to be used as edible carriers for the encapsulation and protection of a model, water-soluble antioxidant, EGCG, was tested by producing electrosprayed gelatin particles with a theoretical antioxidant loading of 10% w/w. Infrared spectroscopy and $ABTS^{+\bullet}$ assays revealed that the encapsulation efficiency of the system was very close to 100%, much higher than that reported for other encapsulation systems for the protection of catechins. Moreover, the radical scavenging assays proved that encapsulation by the e-spraying technique did not damage the bioactive compound, as it retained its antioxidant activity intact. Additionally, this work also proved that the obtained gelatin capsules were capable of stabilizing EGCG against degradation in aqueous solution (pH = 7.4), as its antioxidant activity was better preserved in this media when encapsulated than in its free form. This stabilization can be attributed to both the delay of its dissolution in aqueous media, as observed in the *in-vitro* EGCG release assays, and to the intermolecular interactions which were established between the active molecule and its encapsulating matrix. The overall results presented in this work demonstrate, for the first time, the potential of electrosprayed gelatin particles to be used as encapsulation matrices for polyphenols with application in the development of functional foods.

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8. Supplementary Material

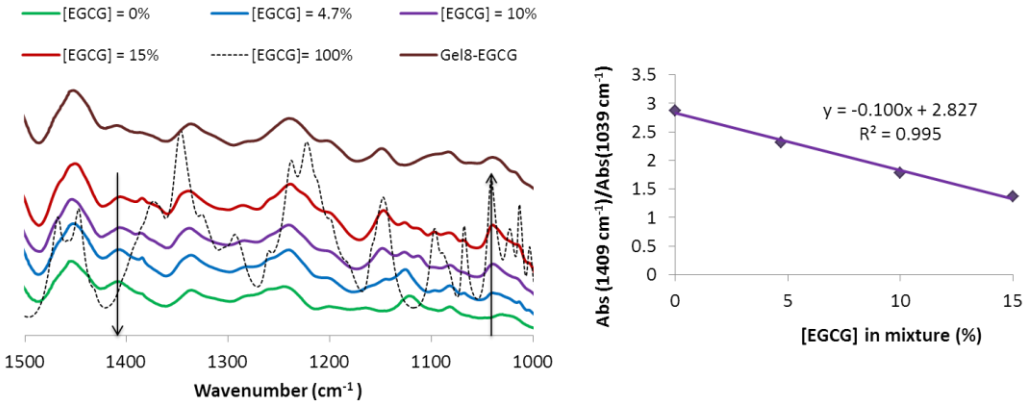
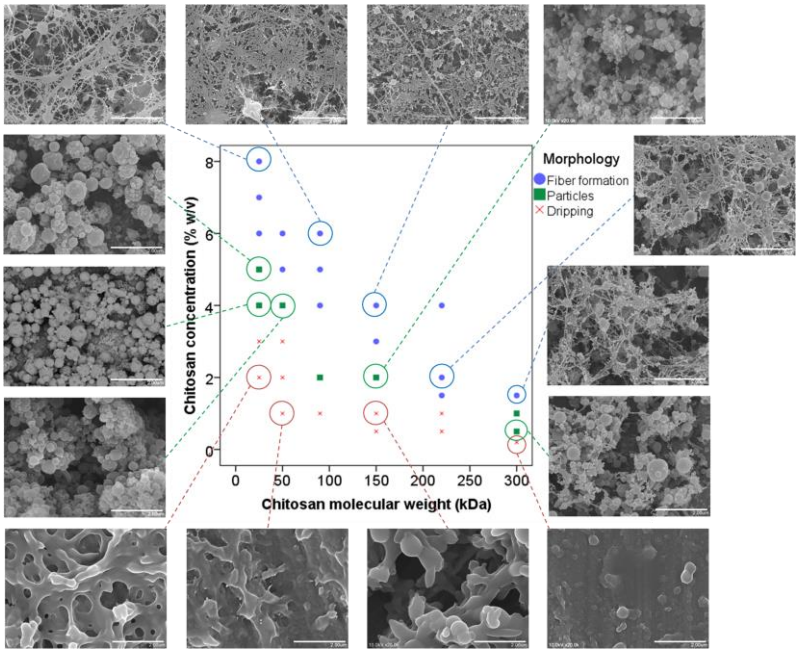


Figure S1. FTIR spectra of KBr pellets used for the calibration curve containing different proportions of gelatin and EGCG (left) and the corresponding calibration curve (right). The spectrum from the EGCG-containing capsules is also included. Arrows point out the intensity changes of the selected bands.

1.2

IMPACT OF MOLECULAR WEIGHT ON THE FORMATION OF ELECTROSPRAYED CHITOSAN MICROCAPSULES AS DELIVERY VEHICLES FOR BIOACTIVE COMPOUNDS



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Sanchez, G., & López-Rubio, A. (2016). *Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds*. Carbohydrate Polymers, 150, 121-130. (DOI: http://dx.doi.org/10.1016/j.carbpol.2016.05.012).

1. Abstract

The molecular weight of chitosan is one of its most determinant characteristics, which affects its processability and its performance as a biomaterial. However, information about the effect of this parameter on the formation of electrosprayed chitosan microcapsules is scarce. In this work, the impact of chitosan molecular weight on its electrosprayability was studied and correlated with its effect on the viscosity, surface tension and electrical conductivity of solutions. A Discriminant Function Analysis revealed that the morphology of the electrosprayed chitosan materials could be correctly predicted using these three parameters for almost 85% of the samples. The suitability of using electrosprayed chitosan capsules as carriers for bioactive agents was also assessed by loading them with a model active compound, (–)-epigallocatechin gallate (EGCG). This encapsulation, with an estimated efficiency of around 80% in terms of preserved antioxidant activity, showed the potential to prolong the antiviral activity of EGCG against murine norovirus via gradual bioactive release combined with its protection against degradation in simulated physiological conditions.

2. Introduction

Micro- and nanoencapsulation, processes in which a compound is embedded within a protective matrix [1] which is organized in the form of micro- or nanosized structures, have attracted increasing research interest for the protection of sensitive bioactive compounds [2] and address current concerns related to their formulation, bioavailability or their delivery to specific sites [3].

Among the different techniques used for microencapsulation, electrohydrodynamic spraying (electrospraying) is rapidly emerging as a promising technology for the production of polymeric microparticles containing bioactive molecules [4], as it overcomes some of the limitations of conventional methods. Electrospraying can generate microencapsulation structures in a one-step process [5] under mild conditions [6, 7] and in the absence of organic/toxic solvents [8], limiting inactivation of the bioactive compounds [9], being adequate for both hydrophilic and hydrophobic drugs or ingredients [10] and generally achieving high loading efficiencies [6, 9]. Therefore, it has found a number of potential applications in various fields, including the pharmaceutical, cosmetic and food industries [11]. It basically consists on subjecting a polymer solution (containing the bioactive to be encapsulated) to a high voltage so that the electric field deforms the interface of the liquid drop and breaks it into fine charged droplets, which are ejected towards a collector while the solvent evaporates, generating dry polymeric microparticles [6, 12].

Biopolymers are preferred as encapsulating matrices for most applications because of their biocompatibility, biodegradability and non-toxicity [13]. Specially, chitosan is a biorenewable, biocompatible and biodegradable polysaccharide considered a GRAS food additive by the FDA [14]. Moreover, it has many attributed functional and bioactive properties, including antioxidant and lipid-lowering capacities, antimicrobial activity, wound healing and antiangiogenic effects, prevention of renal failure, etc. [14-16]. For these reasons chitosan and its derivatives have been widely used in the pharmaceutical [17, 18], biomedical [19, 20], cosmetic [21, 22] and food industries [23, 24], and is considered a good candidate for the encapsulation of bioactive compounds [25, 26]. However, the electrohydrodynamic processing of chitosan is complex due to its particular behavior in solution and its polycationic nature [27], consequence of its structure consisting of β-1,4 linked 2-acetamido-2-deoxy-β-D-glucopyranose units and 2-amino-2-deoxy-b-D-glucopyranose units (cf. Figure S1 of the Supplementary Material) [28].

The electrohydrodynamic spinning (electrospinning) of chitosan for the production of nanofibers from non-toxic solvents has been extensively studied [29], and the impact of different processing parameters, solution properties and/or the molecular weight of the polymer on the morphology of the obtained fibers have been addressed [27, 30]. However, as the focus of these works is the manufacture of fibers, the range of explored conditions does not cover the production of nano/microparticles. The use of electrospraying for the production of dry [31, 32] or gelled [33-35] chitosan micro- and nanospheres has also been reported, however, all these works use only one particular grade of chitosan, with a fixed molecular weight. Given that the molecular weight of chitosan is one of its key characteristics, which can affect not only its processability but also its performance as a delivery vehicle [31], the focus of this work was to study the influence of the molecular weight on the sprayability of chitosan, and to assess the suitability of selected electrosprayed capsules as delivery vehicles for a model bioactive compound: (–)-epigallocatechin gallate (EGCG). EGCG is the most abundant and bioactive compound in green tea [36] and possesses many attributed health benefits [37], including protective effects against infections [38], cardiovascular and neurodegenerative diseases [39], inflammation and arthritis [40] and cancer [41, 42]. In the present work, its antioxidant [39] and antiviral [43, 44] activities were assessed before and after encapsulation within the chitosan electrosprayed capsules.

3. Materials and methods

3.1. Materials

Chitosans with reported degree of deacetylation of 85 ± 2.5 % and different molecular weights, ranging from 25 to 300 kDa, were purchased from Heppe Medical Chitosan GmbH. (–)-Epigallocatechin gallate (EGCG), 2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$) and spectroscopic grade potassium bromide (KBr) were obtained from Sigma-Aldrich. 96% (v/v) acetic acid was supplied by Scharlab.

3.2. Preparation of chitosan solutions

Chitosan solutions of different concentrations, i.e. from 0.5 to 8 % (w/v), were prepared by dissolving the polysaccharide in acetic acid at room temperature under magnetic agitation overnight. Different acetic acid concentrations were used for this purpose, from 20 to 90 % (v/v).

3.3. Characterization of the solutions

The surface tension of the solutions was measured using the Wilhelmy plate method in an EasyDyne K20 tensiometer (Krüss GmbH, Hamburg, Germany) at room temperature.

The electrical conductivity of the solutions was measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain) at room temperature.

The rheological behaviour of the solutions was studied using a rheometer model AR-G2 (TA Instruments, USA), with a parallel plate geometry, and the method described in [45]. Briefly, continuous shear rate ramps were performed from 0.1 to 200 s^{-1} during 15 min at 25 ± 0.1 ºC using a stainless steel plate with a diameter of 60 mm and a gap of 0.5 mm. All measurements were made at least in triplicate.

3.4. Electrohydrodynamic processing of the solutions

The solutions were processed using a homemade electrospinning/electrospraying apparatus, equipped with a variable high-voltage 0-30 kV power supply. Solutions were introduced in a 5 mL syringe and were pumped at a steady flow-rate (0.15 mL/h) through a stainless-steel needle (0.9 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a grounded stainless-steel plate placed at a distance of 10 cm from the tip of the needle in a horizontal configuration. A voltage of 17 kV was applied to the solutions as selected in preliminary trials.

3.5. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of

7-10 mm. As prepared samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination.

3.6. Fourier transform infrared (FT-IR) analysis of the particles

Samples (ca. 1-2 mg) of selected chitosan capsules, both unloaded and EGCG-loaded, were grounded and dispersed in about 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the samples at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

3.7. Antioxidant activity of free and encapsulated EGCG

The $ABTS^{+\bullet}$ radical scavenging assay [46] was performed in order to quantify the antioxidant activity of both free and encapsulated EGCG, following the protocol described in a previous work [45]. Briefly, a stock solution of $ABTS^{+\bullet}$ was prepared by reacting ABTS 7 mM with potassium persulfate 2.45 mM, both in distilled water, and allowing the mixture to stand in the dark at room temperature for 24 h. The stock solution was then diluted with acetic acid 20% v/v to an absorbance at 734 nm of 0.70 ± 0.02. Solutions of free and encapsulated EGCG (0.15 mg/mL of EGCG in both cases) were prepared in acetic acid 20% v/v, and 10 µL aliquots were added to 1 mL of diluted $ABTS^{+\bullet}$, measuring its absorbance at 734 nm after 1 min of mixing. The unloaded chitosan particles were also evaluated, at the same polymer concentration as for the loaded samples. The radical scavenging activity (RSA), expressed as the percentage of reduction of the absorbance at 734 nm after sample addition, was calculated using Eq. (1), where A_0 and A_1 are the absorbances at 734 nm of $ABTS^{+\bullet}$ before and 1 min after addition of the samples, respectively.

$$\text{RSA}\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \qquad \text{Eq. (1)}$$

Experiments were performed on a V-1200 Spectrophotometer from VWR (Pennsylvania, USA), at least in triplicate. Solvent blanks were also run in each assay.

3.8. Microencapsulation efficiency

The microencapsulation efficiency (MEE) of the EGCG-loaded capsules was estimated from the value of their antioxidant activity according to Eq. (2), where both

the free and encapsulated EGCG had the same theoretical concentration in the solutions:

$$MEE\ (\%) = \frac{RSA\ of\ EGCG-loaded\ capsules}{RSA\ of\ free\ EGCG} \times 100 \qquad \text{Eq. (2)}$$

3.9. Virus strain, cell line and infections

The cytopathogenic murine norovirus (MNV-1) strain, a model for human noroviruses, was propagated and assayed in RAW 264.7 (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) as described in Elizaquível, Azizkhani, Aznar and Sánchez (2013) [47]. Semi-purified stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at 660×g for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 µl of inoculum per well.

3.10. Antiviral activity

Different concentrations of free and encapsulated EGCG were added to MNV suspensions (ca. 6 log $TCID_{50}$/mL) and further incubated at 37 °C in a water-bath shaker at 150 rpm for 2 or 16 h (ON). Ten-fold dilutions of treated and untreated virus suspensions were inoculated into confluent RAW monolayers in 96-well plates. Then, infectious viruses were quantified by cell culture assays as described above. Each treatment was done in triplicate. Positive controls were virus suspensions in PBS and virus suspensions added to unloaded chitosan capsules. The decay of MNV was calculated as $\log_{10}(N_t/N_0)$, where N_0 is the infectious MNV titer for untreated sample and N_t is the infectious MNV titer for treated samples.

3.11. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction. This software was also used to carry out a Discriminant Function Analysis (DFA) (cf. Section 4.2.3).

4. Results and discussion

4.1. Impact of the molecular weight on the morphology of electrosprayed chitosan

The use of electrospraying for the production of chitosan micro- and nanospheres for drug delivery applications, and the influence of different processing parameters on the obtained materials has already been reported for specific chitosans [31-34, 48]. However, there is lack of information about the influence that the molecular weight of chitosan itself has on the morphology of the materials obtained through electrospraying. Thus, the formation of microencapsulation structures through electrospinning/electrospraying using different chitosans with varying molecular weights (Mw) and in a wide range of concentrations was evaluated under the same processing conditions (i.e. an applied voltage of 17 kV, a flow rate of 0.15 mL/h and a needle-collector distance of 10 cm), which were selected in preliminary trials so as to be able to process all the solutions. The mean degree of deacetylation (DDA) of all chitosans was 85% (± 2.5 %), and they were all dissolved in 90% acetic acid based on previous works [31, 32, 49]. Figure 1 summarizes the types of morphologies obtained for the different Mw-concentration pairs tested, showing the micrographs obtained by SEM for some representative samples.

The results revealed that there was a small range of Mw-concentration conditions which yielded neat electrosprayed particles under the selected processing parameters. Small deviations from these conditions either caused fiber formation or dripping of the chitosan solutions. As expected, an increase in the molecular weight of chitosan caused a decrease in the concentration at which it could be successfully electrosprayed. Indeed, as the molecular weight of a polymer increases, so does the frequency of chain entanglements and thus the intermolecular cohesion in solution for a fixed concentration. The formation of chain entanglements has been acknowledged as the main factor determining the different morphologies which can be obtained when a polymer solution is processed electrohydrodynamically [50]. Accordingly, when a sufficient entanglement concentration is reached, jet fragmentation during processing is prevented and fibers are formed [45]. In a polymer solution, the number of chain entanglements is affected both by the concentration and the molecular weight of the polymer [50], and is related to the viscosity of the solutions [51]. Thus, in order to better understand the influence of the molecular weight on the morphology of the processed materials, the rheological behaviour of the chitosan solutions was examined, together with other solution properties (surface tension and electrical conductivity) that are known to strongly impact electrohydrodynamic processing [52].

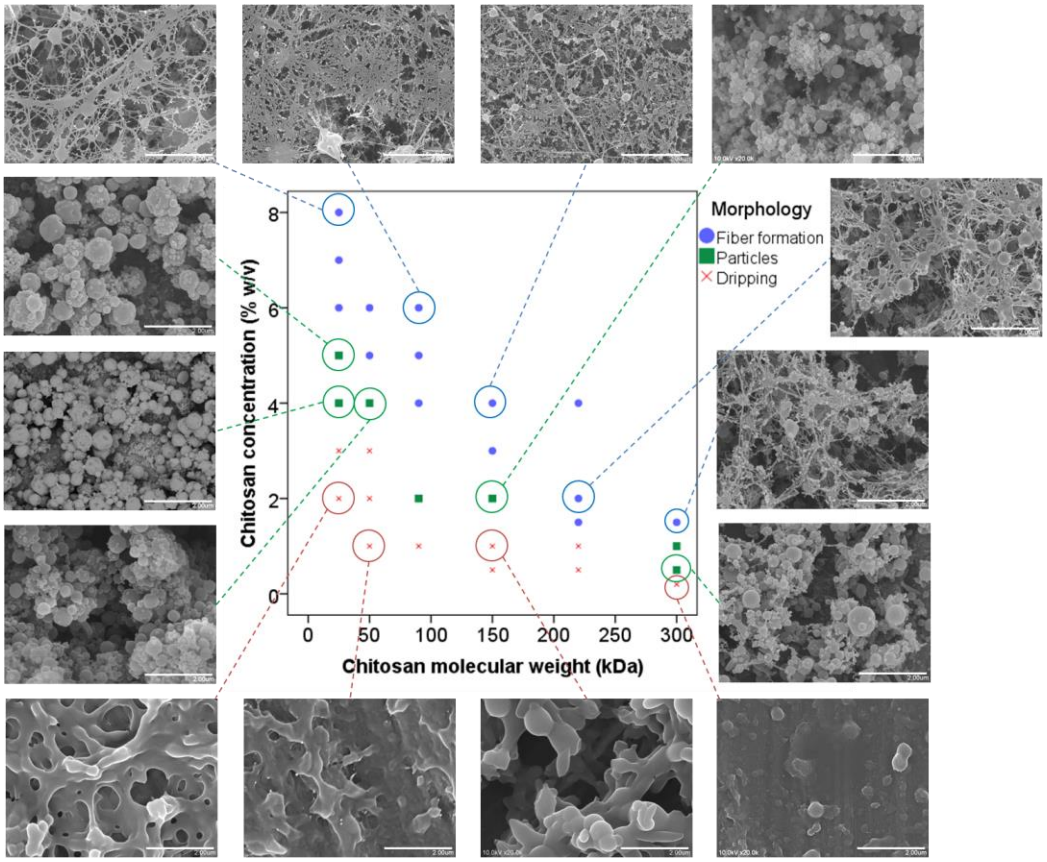


Figure 1. Combined effect of molecular weight and concentration on the morphology of electrosprayed chitosan materials. White scale bars in all the SEM micrographs represent 2 µm.

4.2. Chitosan solution properties

4.2.1. Rheological behaviour

In general, the most diluted chitosan solutions exhibited a Newtonian behavior, with a linear relationship between the shear stress (σ) and the shear rate ($\dot{\gamma}$), regardless of the molecular weight of the polymers. However, as the concentration increased, the chitosan samples showed a shear thinning (pseudoplastic) behavior as previously reported [53], which was manifested at lower concentrations for the higher molecular weight chitosans, and was more evident as both the chitosan concentration and its molecular weight increased. Figure S2 of the Supplementary Material shows the rheological profiles obtained for different concentrations of chitosan with a

molecular weight of 150 kDa, as a representative example. In order to compare the viscosity of the different chitosan solutions, its value at a constant high shear rate (200 s^{-1}) was plotted against the polymer concentration for each molecular weight (cf. Figure 2). As predicted by Al-Fariss and Al-Zahrani (1993) [54] for diluted polymer solutions, the viscosity exponentially increased with the concentration of chitosan at a constant temperature and shear rate, while a potential relationship of the viscosity of chitosan solutions with its molecular weight was observed (cf. Figure S3 of the Supplementary Material) as predicted by the Mark–Houwink equation [55, 56]. Overall, the viscosity could be increased either by increasing the concentration or the mean molecular weight of the polysaccharide, as expected. However, the values of the viscosity alone did not explain the different morphologies obtained, as the ranges of this parameter which yielded neat particles, particles with fibers or just dripping of the solution overlapped (cf. Figure S4 of the Supplementary Material).

4.2.2. Surface tension and electrical conductivity

The surface tension of the chitosan solutions hardly varied with the molecular weight or the concentration of the polysaccharide, increasing only slightly as both increased (cf. Figure 2). This lack of substantial variation with the concentration of biopolymer has also been observed for other systems [45].

The electrical conductivity initially increased with chitosan concentration for the different molecular weights evaluated but eventually showed a maximum for the lower Mw chitosans, i.e. for the solutions where the viscosity allowed evaluation of a wider range of concentrations (cf. Figure 2). This can be explained taking into account that the conductivity depends on both the concentration of charges and their mobility. As the concentration of chitosan, and thus of protonated amino groups increased, the conductivity augmented up to a point when the viscosity was so high that the mobility of the charges was hampered. Indeed, these maximum was found at higher concentrations for lower molecular weights. It was interesting to find that the concentration at which each chitosan grade was successfully electrosprayed in the form of neat particles had an electrical conductivity in the increasing slope of the curves, before the maximum, because the viscosity at higher concentrations was too high and gave rise to fibrils.

Anyhow, as expected, none of the solution properties could explain the differences in the morphology of the obtained materials on its own. In contrast, a combination of them could help predicting the sprayability of chitosan with a specific molecular weight at a particular concentration.

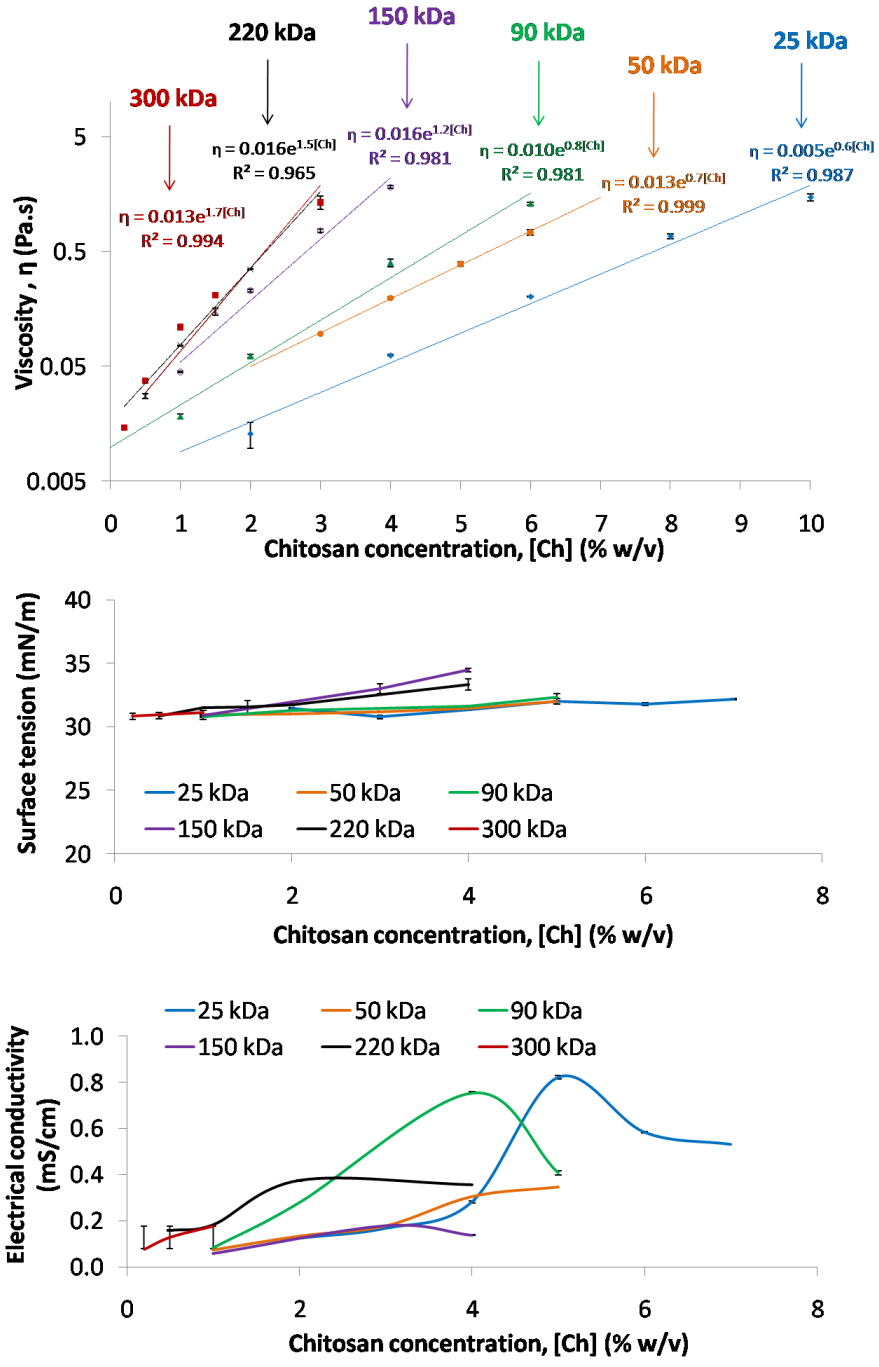


Figure 2. Viscosity, surface tension and electrical conductivity of chitosan solutions as a function of the polymer concentration for different molecular weights.

4.2.3. Combined effect of the solution properties on the morphology

Figure 3 shows the small region where the confluence of certain viscosity, surface tension and electrical conductivity values yielded electrosprayed particles free of residual fibrils. In order to ascertain whether these three properties were effective in predicting the morphology of the electrosprayed materials, a Discriminant Function Analysis (DFA) was conducted using the software IBM SPSS Statistics (v.23). This type of statistical analysis is used to determine if a definite set of variables is successful in predicting a category membership [57]. The DFA revealed that the three variables were relevant to discriminate the different structures. The best rate of discrimination was obtained using the combinations of the variables expressed in Eq. (3) and (4) as standardized canonical discriminant functions, where γ is the surface tension expressed in mN/m, κ is the electrical conductivity expressed in (mS/cm) and η is the viscosity expressed in Pa·s.

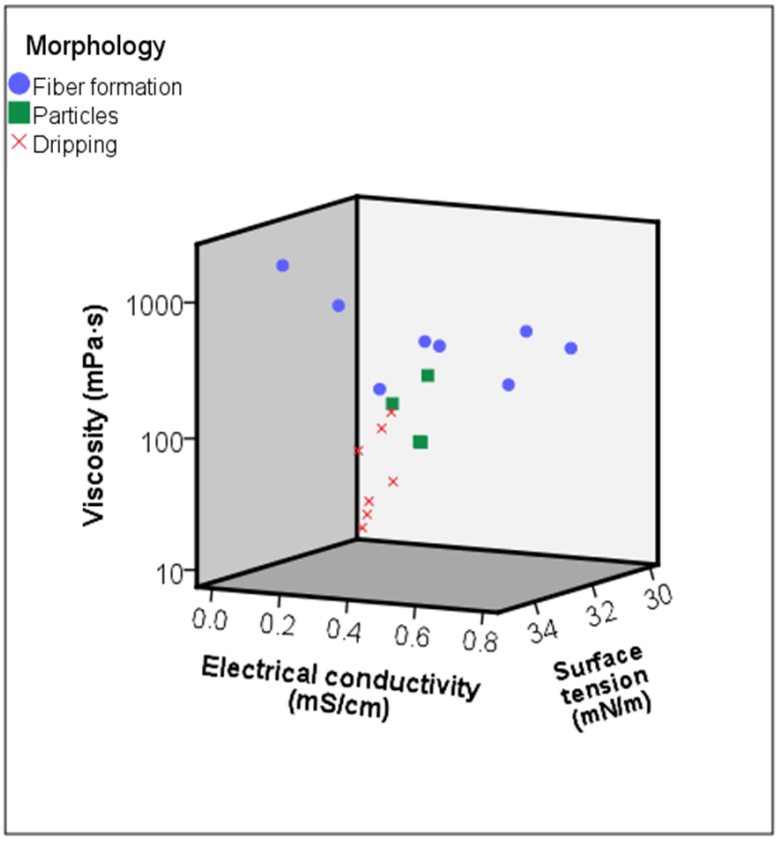
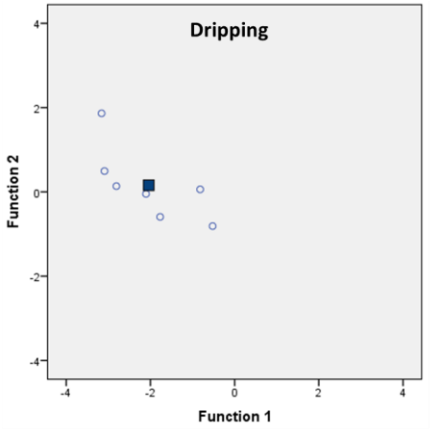


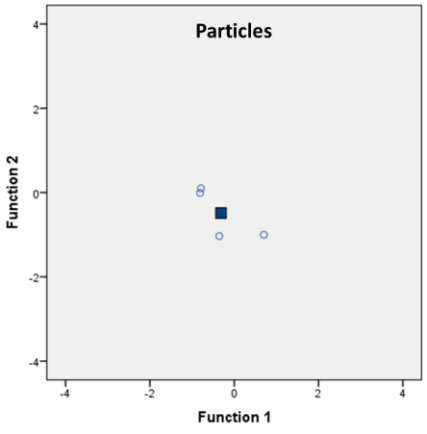
Figure 3. Electrosprayed samples classified by their morphology as a function of the solution properties.

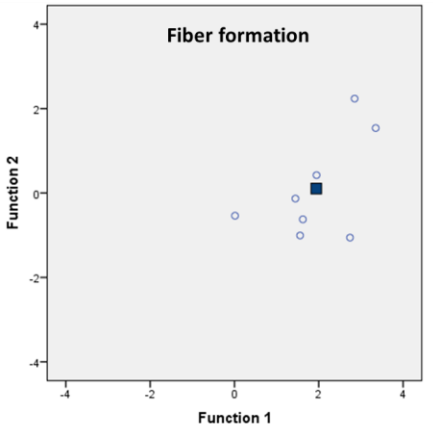
$$Function\ 1 = -0.032 \cdot \gamma + 0.462 \cdot \kappa + 0.928 \cdot \log(\eta) \quad \text{Eq. (3)}$$

$$Function\ 2 = 1.365 \cdot \gamma + 0.0128 \cdot \kappa - 0.811 \cdot \log(\eta) \quad \text{Eq. (4)}$$

With the above discriminant functions, the morphology obtained upon electrospraying of chitosan solutions could be correctly predicted for 84.2% of the assayed solutions (cf. Figure 4). Moreover, all solutions leading to neat particles were correctly classified.







Classification results

Experimental Group Membership	Predicted Group Membership		
	Dripping	Particles	Fiber formation
Dripping	71.4%	28.6%	0%
Particles	0%	100%	0%
Fiber formation	0%	12.5%	87.5%
84.2% of original grouped cases correctly classified			

Figure 4. Group graphs obtained from the discriminant functions (Eq. (3) and (4)) and classification results.

From the different molecular weight – concentration combinations which gave rise to fiber-free electrosprayed particles, the chitosan with the lowest assayed molecular weight was selected, as it allowed the highest electrosprayable concentration and thus

the greatest productivity. Thus, the chitosan of 25 kDa at 5% (w/v) concentration was selected for further analysis.

4.3. Effect of acetic acid concentration

One of the solution parameters that are crucial for the electrohydrodynamic processing of chitosan solutions is the concentration of the acetic acid solution used as solvent. While Homayoni, Ravandi and Valizadeh (2009) [27] stated that the acetic acid concentration should be in the range of 70-90% for optimal electrospinning to obtain nanofibers, Arya, Chakraborty, Dube and Katti (2009) [31] concluded that the optimum concentration for successful electrospraying was 90%, though that might only apply for the particular chitosan they used. This concentration has been selected in other works to produce electrosprayed particles from chitosan [49, 58] without further evaluation. However, Zhang and Kawakami (2010) [32] extended the range of sprayability (again, for a particular chitosan of high molecular weight) down to a 50%, noting that decreasing the acetic acid concentration caused a reduction in the mean particle size. Due to the variability of data found in the literature in this respect, the concentration of acetic acid was optimized for our particular system (chitosan 25 kDa, 5% w/v).

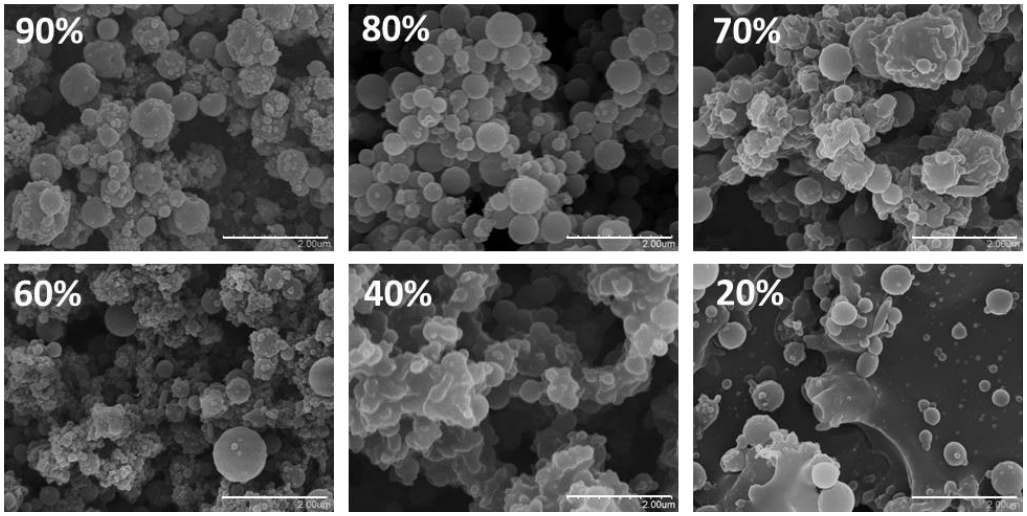


Figure 5. Morphology of electrosprayed chitosan (25 kDa, 5% w/v) obtained from different acetic acid concentrations (90-20% v/v). White scale bars in all the SEM micrographs represent 2 μm.

Figure 5 shows the impact of the acetic acid concentration on the morphology of the electrosprayed chitosan. It could be observed that concentrations below 80% yielded particle aggregates and even dripping of the solution for a 20% acetic acid. As the surface tension of the solutions did not vary significantly with the acetic acid concentration (cf. Figure S5 of the Supplementary Material), this dripping could be attributed to a slight decrease in the viscosity of the solutions in combination with a

sharp increase in their electrical conductivity at low acetic concentrations, which had already been observed by Zhang and Kawakami (2010) [32] and ascribed to the higher degree of dissociation of the acid at low concentrations. However, a concentration of 80% gave rise to more spherical particles with more homogeneous sizes than the 90% used in the previous sections. Having both concentrations the same conductivity, this was attributed to the maximum of viscosity observed at 80% acetic acid (cf. Figure S5 of the Supplementary Material), also patent in the cited work [32] although not commented. Therefore, an optimum acetic acid concentration of 80% was selected to produce our delivery system.

4.4. Morphological and chemical characterization of EGCG-loaded capsules

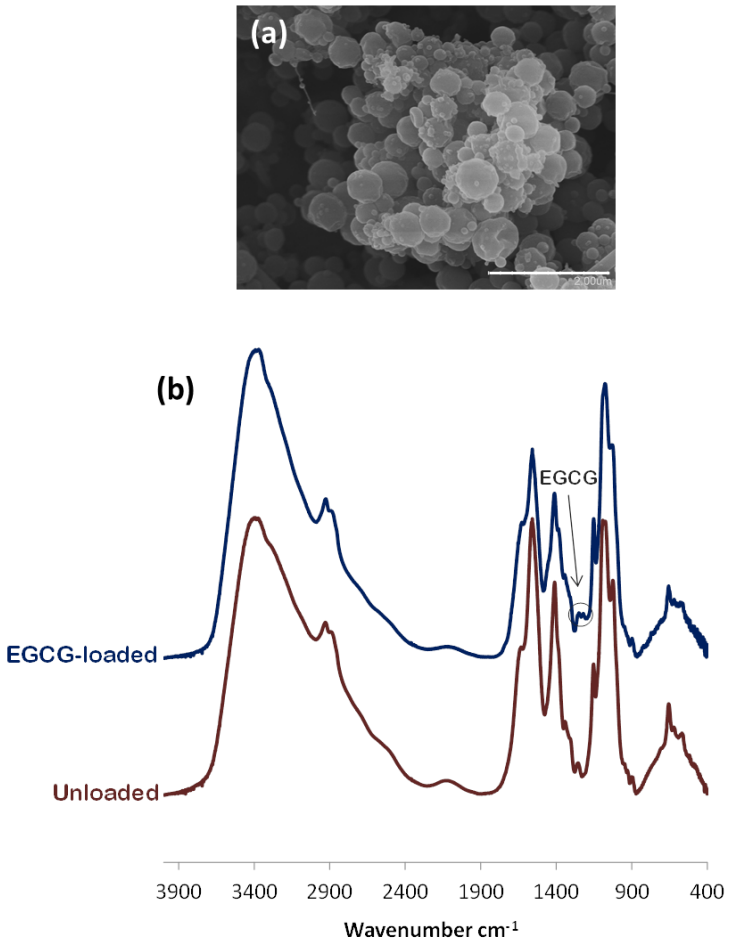


Figure 6. Morphology of EGCG-loaded electrosprayed chitosan particles (a) and infrared spectra of unloaded and EGCG-loaded electrosprayed chitosan particles (b). The white scale bar in the SEM micrograph represents 2 μm.

The conditions selected in the previous section for the production of electrosprayed chitosan delivery vehicles where used to produce EGCG-loaded chitosan particles with a theoretical EGCG content of 10% w/w of the capsules. Figure 6(a) shows a micrograph of the obtained material, which exhibits a similar morphology as that obtained in the absence of the bioactive compound, although slightly less homogeneous in size.

The effective incorporation of EGCG within the chitosan particles was evidenced by the presence of its characteristic infrared absorption band centred at 1221 cm^{-1} (cf. Figure 6(b)), as observed for EGCG-loaded spray-dried chitosan particles obtained in a previous work [59]. This band was slightly displaced in the capsules with respect to free EGCG, being centred at 1223 cm^{-1} in the latter (spectrum showed elsewhere [45]). Other spectral bands of chitosan were also modified. Specifically, the Amide I band shifted from 1634 cm^{-1} in the unloaded particles to 1629 cm^{-1} in the EGCG-loaded capsules. These changes suggested the presence of intermolecular interactions between the bioactive molecule and its biopolymeric vehicle, which might contribute to the stabilization of the former as previously suggested [45].

4.5. Antioxidant activity and encapsulation efficiency

The ABTS radical cation decolourization assay [46] was used to compare the antioxidant activity of free and encapsulated EGCG, and to indirectly estimate the encapsulation efficiency of the system. Therefore, the radical scavenging activities (RSA) of solutions of commercial EGCG (0.15 mg/mL) and encapsulated EGCG (theoretical concentration of 0.15 mg/mL) in acetic acid 20% v/v, as well as solutions of the unloaded chitosan particles (same concentration as for the loaded ones) were calculated using Eq. (1). Solvent blanks were run too.

The results showed no significant differences ($p<0.05$) between the RSA of the unloaded chitosan particles (2.3% ± 0.3%) and that of the solvent blank (2.2% ± 0.4%), suggesting that the polysaccharide exerted no significant antioxidant activity at the assayed concentration. Hence, the potential contribution of the encapsulating matrix to the total antioxidant activity of the EGCG-loaded capsules was neglected. The antioxidant activity of encapsulated EGCG (RSA = 23.0% ± 0.7%) was somewhat lower than that of free EGCG (RSA = 28.9% ± 1.7%) at the same theoretical concentration, implying some bioactivity loss during encapsulation.

From these results, the encapsulation efficiency defined by Eq. (2) was estimated to be 79.6% ± 2.4%. This considerably high value was consistent with similar encapsulation systems based on chitosan used as delivery vehicles for EGCG [59].

4.6. Antiviral activity

Table 1 summarizes the results obtained from the antiviral activity assays performed on murine norovirus. While a concentration of 0.25 mg/mL of EGCG was

insufficient to observe a significant reduction in the recovered titer of the virus after 2 h of incubation, a concentration of 2.5 mg/mL of the polyphenol did cause a significant reduction, which was statistically lower for the encapsulated compound suggesting that 2 h was insufficient for the complete release of the bioactive. On the contrary, when the viruses were exposed to EGCG overnight, the recovered titer was lower than 1.15 $TCID_{50}$/mL for both free and encapsulated EGCG at 2.5 mg/mL and, more interestingly, the reduction was significantly higher for the encapsulated than for the free flavonoid at 0.25 mg/mL.

Table 1. Antiviral activity of free and encapsulated EGCG against murine norovirus

	[Sample] (mg/mL)	Theoretical [EGCG] (mg/mL)	2 h		Overnight	
			Recovered titer ($TCID_{50}$/mL)	Reduction	Recovered titer ($TCID_{50}$/mL)	Reductio
PBS	-		6.45 ± 0.35^{A}	-	5.87 ± 0.38^{A}	-
Free EGCG	2.5	2.5	4.13 ± 0.10^{B}	2.32	$<1.15^{B}$	>4.72
	0.25	0.25	6.03 ± 0.12^{A}	0.42	4.71 ± 0.12^{C}	1.16
EGCG-loaded chitosan	25	2.5	5.20 ± 0.00^{C}	1.25	$<1.15^{B}$	>4.72
	2.5	0.25	6.07 ± 0.21^{A}	0.38	3.48 ± 0.38^{D}	2.39

* Within each column, different letters (A, B, C and D) denote significant differences between treatments ($p < 0.05$).

Given that the unloaded electrosprayed chitosan did not show antiviral activity (data not shown), these results can be explained considering that the bioactive compound might have been gradually released from the microcapsules. In fact, a sustained release of bioactives is often desirable and pursued through their encapsulation [60]. In our system, the lower antiviral activity of encapsulated EGCG at short exposure times and its substantial increase at longer time periods suggested a gradual release of the active compound from the chitosan capsules, mainly ascribed to a Fickian diffusion mechanism with some contribution from polymer relaxation phenomena. This result is consistent with the release profiles obtained for similar systems based on EGCG-loaded chitosan microcapsules [59]. On the other hand, the fact that the reduction in the recovered titer was higher for the encapsulated EGCG after an overnight incubation implied that free EGCG was less active against the MNV than the EGCG released from the capsules. As the assay was carried out in PBS at 37ºC to mimic physiological conditions, this can be easily explained by the great instability of EGCG in slightly alkaline solutions [61-63]. Therefore, the results

suggested that while free EGCG degraded with time in PBS, microencapsulation of the compound within the electrosprayed chitosan particles delayed its degradation, hence protecting its antiviral activity in simulated physiological conditions.

5. Conclusions

There was a small range of molecular weight – concentration combinations which gave rise to electrosprayed particles without leading to solution dripping or fiber formation. Both parameters caused a considerable increase in the viscosity of the solutions, although the values of the viscosity on their own were not enough to predict whether a particular chitosan solution would lead to fiber-free particles. A Discriminant Function Analysis revealed that the surface tension and the electrical conductivity of the solutions were also relevant properties in predicting the obtained morphologies. The analysis allowed the accurate prediction of 84.2% of the electrosprayed chitosan solutions. The chitosan with the lowest assayed molecular weight (25 kDa), which allowed the highest electrosprayable concentration (5% w/v) and thus the highest productivity, was used to produce EGCG-loaded encapsulation structures achieving an encapsulation efficiency close to 80%. Microencapsulated EGCG showed prolonged antiviral activity against murine norovirus as compared to the free compound, suggesting that EGCG was gradually released from the chitosan capsules and that the encapsulation matrix exerted a protective effective on the active compound against degradation in simulated physiological conditions.

6. Acknowledgements

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8. Supplementary Material

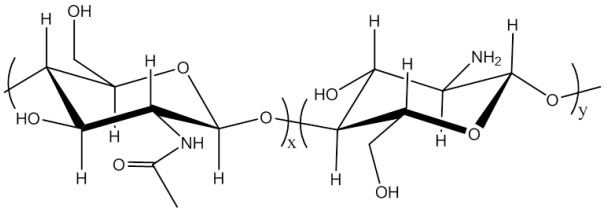


Figure S1. Schematic chemical structure of chitosan.

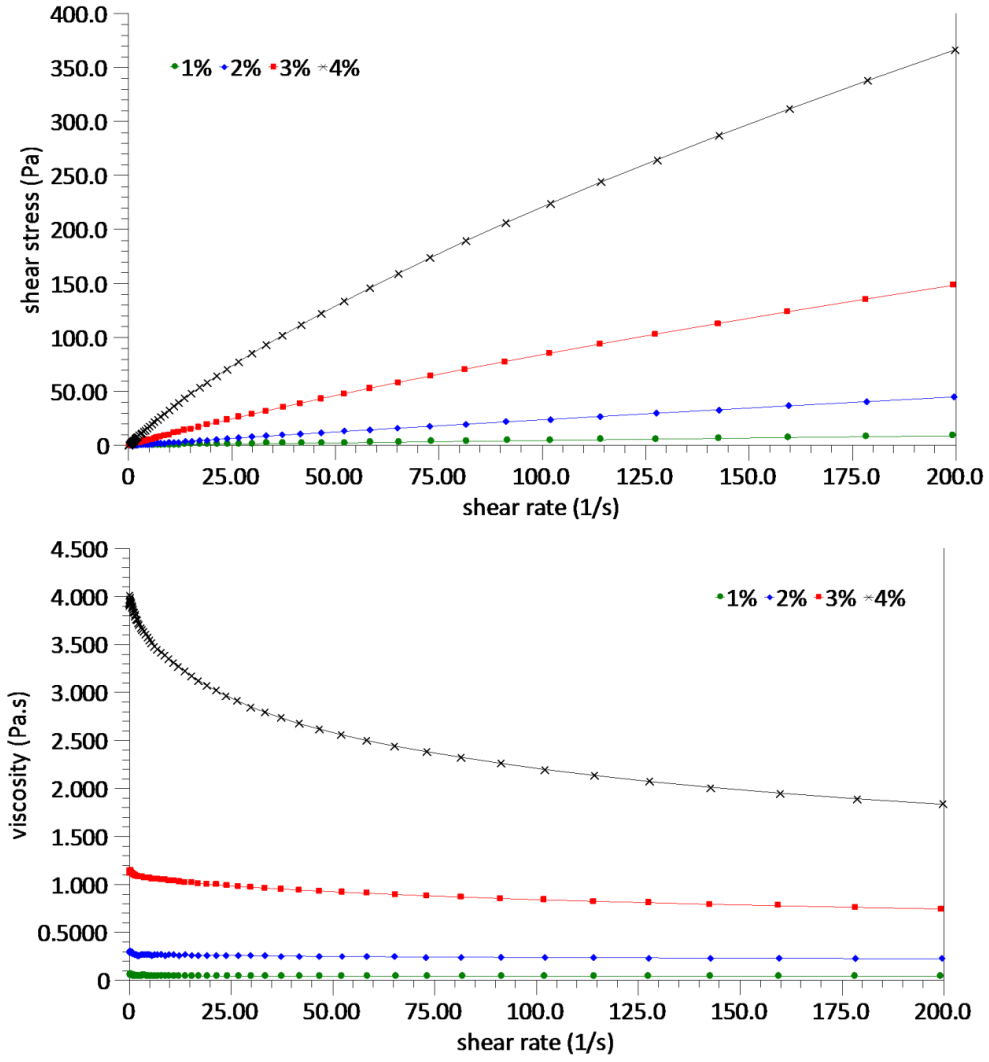


Figure S2. Rheological profiles (shear stress and viscosity *vs.* shear rate) for solutions of the chitosan with a Mw of 150 kDa in 90% acetic acid and different concentrations (1-4% w/v).

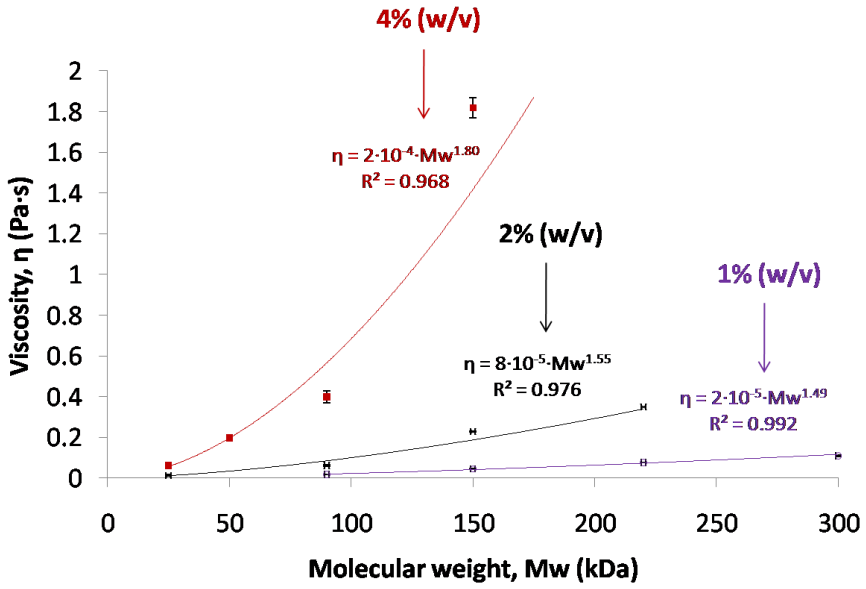


Figure S3. Viscosity of chitosan solutions as a function of the mean molecular weight for different concentrations.

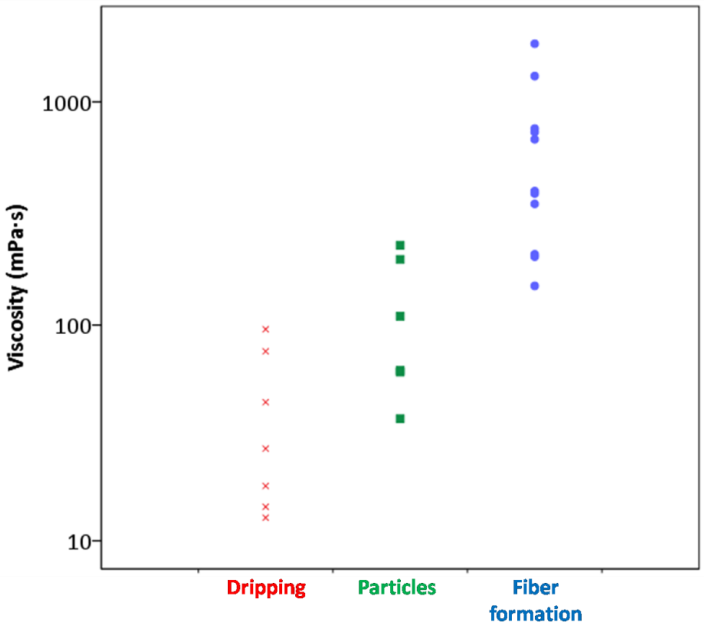


Figure S4. Experimental values of the viscosity (at 200 s^{-1}) of chitosan solutions giving rise to each of the obtained morphologies.

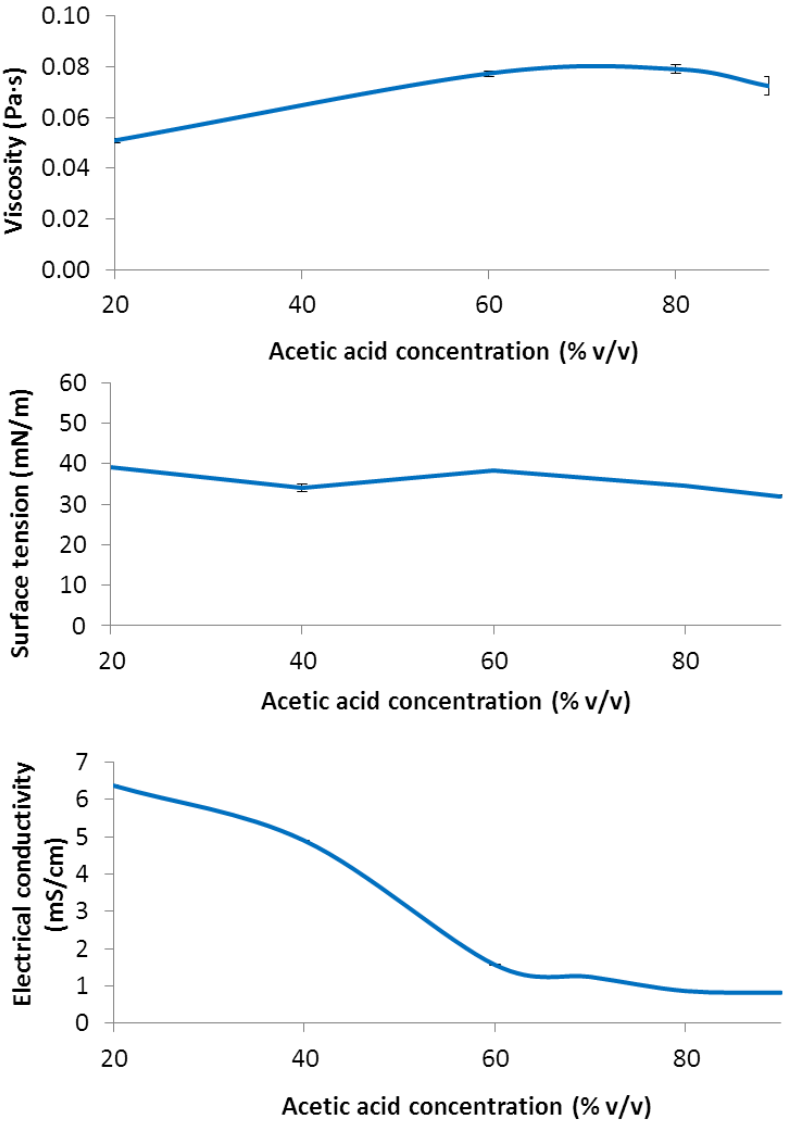
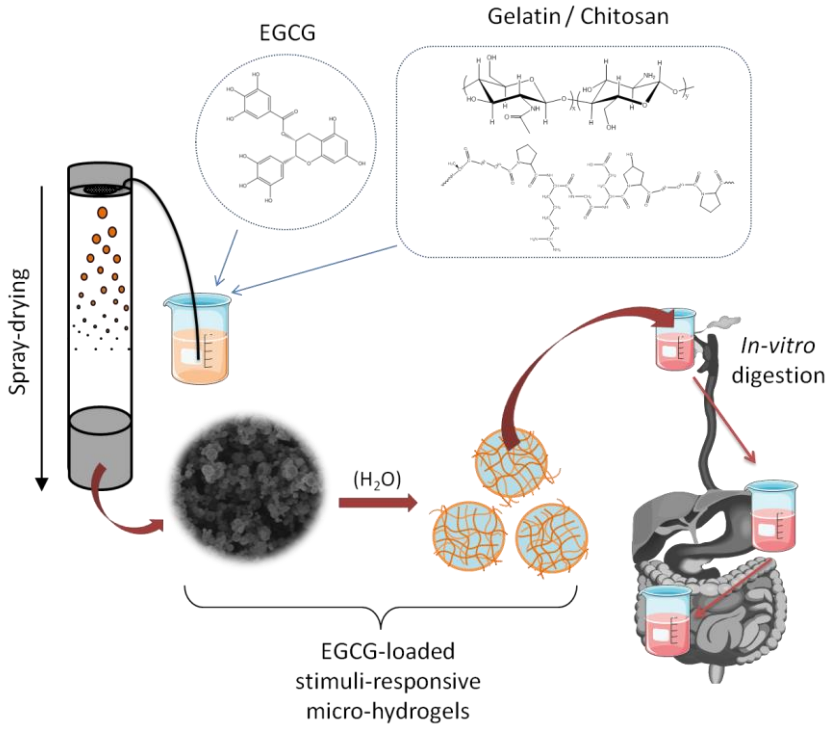


Figure S5. Viscosity, surface tension and electrical conductivity of chitosan solutions (25 kDa, 5% w/v) as a function of the acetic acid concentration in the solvent.

1.3

STABILITY AND BIOACCESSIBILITY OF EGCG WITHIN EDIBLE MICRO-HYDROGELS. CHITOSAN VS. GELATIN, A COMPARATIVE STUDY



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Soler, C., & Lopez-Rubio, A. (2016). *Stability and bioaccessibility of EGCG within edible micro-hydrogels. Chitosan vs. gelatin, a comparative study*. Food Hydrocolloids, 61, 128-138. (DOI: http://dx.doi.org/10.1016/j.foodhyd.2016.05.009).

1. Abstract

Micro-hydrogels are very promising systems for the protection and controlled delivery of sensitive bioactives, but limited knowledge exists regarding the impact of this encapsulation on their bioaccessibility. In this work, two different hydrogel-forming biopolymers (gelatin and chitosan) were compared as wall materials for the microencapsulation of a model flavonoid, (–)-epigallocatechin gallate (EGCG). Results showed that gelatin was more adequate as wall material for the encapsulation of EGCG than chitosan, achieving higher encapsulation efficiencies (95% ± 6%), being more effective in delaying EGCG release and degradation in aqueous solution and exhibiting a 7 times higher bioaccessibility of the bioactive compound (in terms of antioxidant activity) after *in-vitro* gastrointestinal digestion. A very low bioaccessibility of EGCG in chitosan was observed, due to the neutralization of the carbohydrate in the basic simulating salivary conditions, thus precluding subsequent flavonoid release. Moreover, gelatin micro-hydrogels also hindered dimer formation during *in-vitro* digestion, thus suggesting greater bioavailability when compared with free EGCG.

2. Introduction

The development of functional biopolymer nanoparticles or microparticles as encapsulation and delivery systems has enjoyed a great deal of interest in diverse academic fields such as foods, pharmaceutics or cosmetics, highlighting the potential of these structures to protect sensitive bioactives against degradation [1]. Amongst the wide range of bioactive substances studied, green tea flavonoids are powerful antioxidants which have drawn much research attention because of their many attributed therapeutic benefits [2-7], being (–)-epigallocatechin gallate (EGCG) the most abundant polyphenol in green tea possessing the greatest biological activity [8]. However, EGCG is sensitive to heat [9], oxygen [10] and light [11] and, in general, chemically unstable [12], especially in aqueous solutions [13, 14]. Thus, encapsulation of this bioactive compound has been widely explored to improve its stability during food processing and storage [13-18]. Several techniques have been explored for this purpose, being costs and the use of food permitted solvents and matrices the limiting factors for the practical use of encapsulation structures in functional food applications. In this sense, spray-drying is the most widely used encapsulation technique in the food industry [19], as it is a straightforward and cheap procedure which allows the processing of a wide range of food-grade materials with accessible equipment [20]. This technique involves the initial atomization of a formulation containing the wall matrix and the bioactive into fine droplets, followed by their rapid drying using a hot gas stream, which leads to solvent evaporation and rapid formation of the

microparticles. Regarding the encapsulation matrices, food-grade biopolymers capable of forming physical hydrogels are of particular interest. Physical hydrogels are polymer networks characterized by the presence of physical crosslinks, entanglements and/or rearrangements of hydrophobic and hydrophilic domains [21]. Thus, hydrogel-forming biopolymers can be processed in aqueous solutions, while preventing dissolution of the obtained microparticles in aqueous foods under certain conditions. While the protection exerted by the microparticles has obvious benefits during the commercialization stage, an important aspect is the bioaccessibility of the functional compounds after ingestion, as it has been recently observed that encapsulation may decrease it to a certain extent [22]. Another desirable property of potential encapsulating materials for the protection of EGCG is their processability in acidic pHs, as this antioxidant molecule exhibits higher stability in acidic media [12, 23]. Chitosan and gelatin are two edible, naturally-derived biopolymers satisfying both requirements.

Chitosan is a linear polysaccharide obtained by deacetylation of naturally occurring chitin and consists of β-1,4 linked 2-acetamido-2-deoxy-β-D-glucopyranose units and 2-amino-2-deoxy-b-D-glucopyranose units in a proportion which depends on the degree of deacetylation of chitin [24] (cf. Figure 1). Chitosan is soluble in acidic aqueous solutions, where its amino groups are protonated, but gels at neutral or alkaline solutions because of the strong intermolecular interactions, being considered a pH-sensitive hydrogel [25]. Several beneficial properties such as antimicrobial activity [26], lipid-lowering effects [27], and wound healing [28] have been attributed to this polysaccharide, which has been previously used to stabilize EGCG in nanoparticles by the ionic gelation method with tripolyphosphate [12, 13]. Through spray-drying, it has also been used to successfully encapsulate other polyphenolic extracts like olive leaf extract [29] or yerba mate extract [30].

Gelatin is a protein obtained from partial hydrolysis of naturally occurring collagen and contains repeating sequences of glycine-aa_1-aa_2, where amino acids aa_1 and aa_2 are mainly proline and hydroxyproline [31] (cf. Figure 1). Gelatin forms thermoreversible hydrogels in aqueous solutions due to the formation of collagen-like triple helices below the so-called helix–coil transition temperature, leading to chains entanglement and subsequent network formation [32] and, thus, it is considered as a thermo-responsive hydrogel-forming biopolymer. Gelatin has been traditionally used for the production of macro-capsules by the pharmaceutical industry [33], and more recently as a microencapsulation material. Particularly, gelatin has been previously used to protect EGCG in microcapsules produced by layer-by-layer assembly [17, 18], coacervation in combination with different polysaccharides [16] or by electrospraying [15]. Through spray-drying, it has also been used to microencapsulate other flavonoids like naringin [34].

In the present work, spray-dried edible micro-hydrogels based on the polysaccharide chitosan and the protein gelatin were produced and used to microencapsulate EGCG as a model flavonoid antioxidant. The suitability of both

matrices, derived from natural polymers and capable of forming pH-sensitive and thermo-responsive hydrogels, respectively, to encapsulate EGCG was compared for the first time in terms of bioactive release, encapsulation efficiency and bioaccessibility of EGCG using the same processing technique, i.e. spray-drying. The bioaccessibility of EGCG after *in-vitro* gastrointestinal digestion of the capsules was also evaluated. Finally, the ability of the selected encapsulation matrix to protect EGCG against degradation in aqueous solutions was assessed.

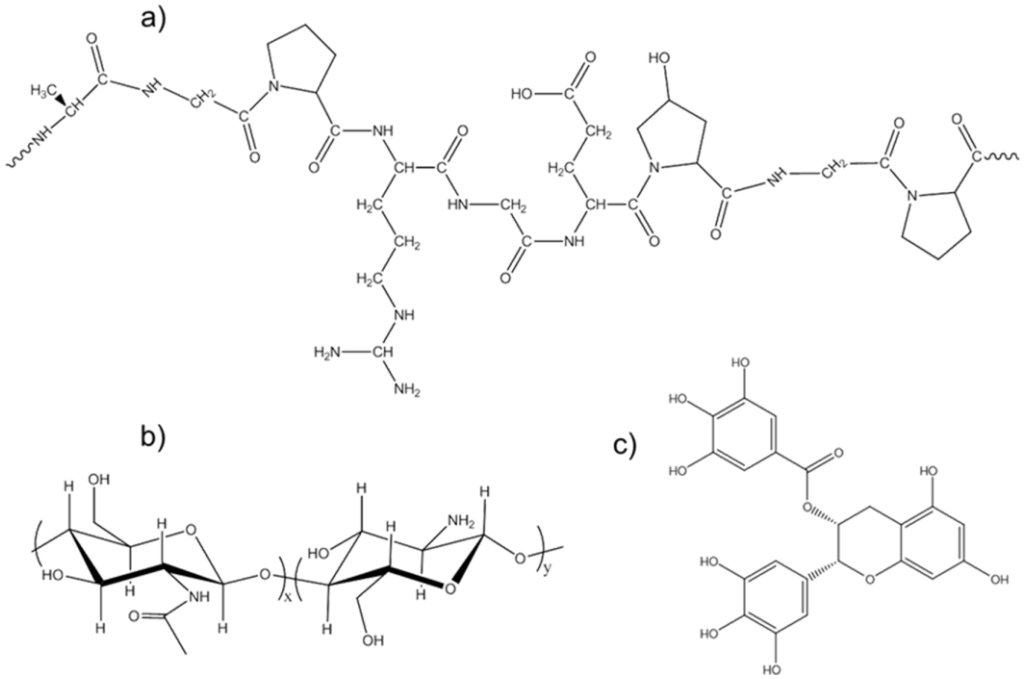


Figure 1. Schematic chemical structures of raw materials: a) gelatin, b) chitosan and c) EGCG.

3. Materials and methods

3.1. Materials

Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, low molecular weight chitosan (Ch), with reported Brookfield viscosity of 20.000 cps, (−)-epigallocatechin gallate (EGCG), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), potassium bromide FTIR grade (KBr), buffer solutions of pH 7.4 (phosphate buffered saline system, PBS)

and pH 6.1 (2-(N-morpholino)ethanesulfonic acid hemisodium salt, MES), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were obtained from Sigma-Aldrich. Acetic acid (96% v/v) was purchased from Scharlab and Pefabloc® from Fluka. All inorganic salts used for the *in-vitro* digestion tests were used as received.

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

3.2. Preparation of biopolymer microparticles

Gelatin stock solutions with a concentration of 10% (w/v) were prepared by dissolving the protein in acetic acid 20% (v/v) at 40 ºC under magnetic agitation. The stock solutions were then cooled down to room temperature and further diluted 50-fold in distilled water before processing.

Chitosan stock solutions with a concentration of 2% (w/v) were prepared by dissolving the polysaccharide in acetic acid 20% (v/v) at room temperature under magnetic agitation. The stock solutions were further diluted 50-fold in distilled water before processing.

When EGCG was incorporated for its encapsulation, it was added to the corresponding biopolymer stock solutions at room temperature under magnetic stirring, at a concentration of 10 (w/w) of the total solids content.

The biopolymer solutions were fed to a Nano Spray Dryer B-90 apparatus (Büchi, Switzerland) equipped with a 7.0 μm pore diameter cap. The inlet air temperature was set at 90 ºC, as it proved to be enough to achieve complete drying of the particles at an inlet air flow of 146 ± 4 L/min and a reduced pressure of 50 ± 3 mbar. Under these conditions, the outlet air temperature varied between 44 and 50 ºC. The spray-dried powders were deposited on the collector electrode by means of an applied voltage of 15 kV.

3.3. Characterization of the microparticles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4100) at an accelerating voltage of 10 kV and a working distance of 9-16 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

The particle size distributions of the spray-dried powders were determined by laser difraction using a Malvern Mastersizer 2000 apparatus equipped with 4 mW He-Ne (632.8 nm) and 0.3 mW LED (470 nm) light sources. The samples were suspended in ethanol to limit swelling of the particles and ultrasonicated to prevent aggregation.

Empty and bioactive-containing capsules of ca. 1 mg were grounded and dispersed in 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution. The microencapsulation efficiency (MEE) of the EGCG-loaded capsules was determined based on FTIR absorbance measurements. Calibration curves (R_{Gel}^2 = 0.995 and R_{Ch}^2 = 0.999) were obtained using Gel/EGCG and Ch/EGCG physical mixtures, respectively, of known relative concentrations (0, 5, 10 and 15 % w/w of EGCG). For the Gel/EGCG calibration curve, the relative maximum absorbances at 1409 cm^{-1} (corresponding to gelatin) and 1039 cm^{-1} (attributed to EGCG) were plotted against the EGCG concentration in the mixtures. For the Ch/EGCG calibration curve, the relative maximum absorbances at 2885 cm^{-1} (corresponding to chitosan) and 1223 cm^{-1} (attributed to EGCG) were used. The EGCG content in the capsules was interpolated from the obtained linear calibration equations, and the MEE of the systems was then calculated using Eq. (1):

$$MEE\ (\%) = \frac{Actual\ EGCG\ content\ in\ the\ capsules}{Theoretical\ EGCG\ content\ in\ the\ capsules} \times 100 \qquad \text{Eq. (1)}$$

Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q500 TGA. The samples (ca. 8 mg) were heated from room temperature to 600 ºC with a heating rate of 10 ºC/min under dynamic air atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

3.4. *In-vitro* EGCG release from the micro-hydrogels

Ten milligrams of EGCG-loaded particles were suspended in 20 mL of release medium (MES aqueous buffer, pH = 6.1) and kept at 20 ºC under agitation at 60 U/min in a Selecta thermostatic bath model Unitronic Reciprocal C (Barcelona, Spain). At different time intervals, the suspensions were centrifuged at 2547 g and room temperature during 10 min using a centrifuge from Labortechnik model Hermle Z 400 K (Wasserburg, Germany), and 1 mL aliquot of the supernatant removed for sample analysis. The aliquot volume was then replaced with fresh release medium and the particles resuspended and left back in the thermostatic bath.

The extracted aliquots were analyzed by UV-vis spectroscopy (Shanghai Spectrum model SP-2000UV, Shanghai, China) by measuring the absorbance at 274 nm

(maximum of absorbance of EGCG). Calibration curves for EGCG quantification in MES solution by UV-vis absorbance were previously obtained (R^2 = 0.999). The EGCG release values were obtained from three independent experiments at the same conditions.

Experimental data were fitted to the Peppas-Sahlin semi-empirical model, whose general equation is Eq. 2, where M_t is the mass of EGCG released at time t, M_0 is the total mass of EGCG loaded in the particles, k_i are kinetic constants, and m is the Fickian diffusional exponent [35].

$$\frac{M_t}{M_0} = k_1 \cdot t^m + k_2 \cdot t^{2m} \qquad \text{Eq. (2)}$$

3.5. Antioxidant activity of EGCG-containing micro-hydrogels

$ABTS^{\bullet+}$ radical scavenging assay was performed in order to quantify the antioxidant activity of both free and encapsulated EGCG, following the decolorization assay protocol described in a previous work [15]. Briefly, a stock solution of $ABTS^{\bullet+}$ was prepared by reacting ABTS with potassium persulfate (7 and 2.45 mM in distilled water, respectively) and allowing the mixture to stand in the dark at room temperature for 24 h. The $ABTS^{\bullet+}$ stock solution was then diluted with acetic acid 20% v/v to an absorbance of 0.70±0.02 at 734 nm. Stock solutions of free and encapsulated EGCG (5 mM of EGCG in all cases) were prepared in acetic acid 20% v/v and subsequently diluted 20-fold. 10 µL of diluted sample solution were added to 1 mL of diluted $ABTS^{\bullet+}$, and the absorbance at 734 nm was measured 1 min after initial mixing. The radical scavenging activity (RSA), expressed as the percentage of reduction of the absorbance at 734 nm after sample addition, was calculated using Eq. (3):

$$\text{RSA}\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \qquad \text{Eq. (3)}$$

where A_0 and A_1 are the absorbances at 734 nm of $ABTS^{\bullet+}$ before and 1 min after addition of the antioxidant samples, respectively.

Experiments were performed on a Shanghai Spectrum spectrophotometer model SP-2000UV (Shanghai, China), at least in triplicate. Solvent blanks were also run in each assay. The unloaded gelatin and chitosan particles were also evaluated (same particle mass concentration as for loaded samples) to take into account the potential antioxidant activity of the encapsulation matrices.

3.6. *In-vitro* gastrointestinal (GI) digestion and bioaccessibility assessment

Suspensions (40 mg/mL) of the EGCG-containing microcapsules and solutions (4 mg/mL) of free EGCG in distilled water were subjected to *in-vitro* GI digestion following to the standardized static *in-vitro* digestion protocol developed within the framework of the Infogest COST Action [36]. Solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized compositions [36]. In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at 37 ºC for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37 ºC for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37 ºC for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively, using 1M HCl or 1M NaOH solutions. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added. Aliquots were collected after the gastric and the duodenal phases and snap-frozen in liquid nitrogen immediately. The antioxidant activity of the digestas was estimated after centrifugation by means of the $ABTS^{\bullet+}$ assay, as an indirect assessment of the bioaccessibility of EGCG after digestion. HPLC-MS was also used to analyze the digestas. The LC system used for this analysis was an Agilent 1290 HPLC system. Separation of EGCG was performed using an Acquity BEH C18 (Waters, 50 mm × 2.1 mm, 1.7 µm of particle size) LC-column. The flow rate was set to 0.4 mL/min and the oven temperature was 30 ºC, being eluent A water slightly acidified with 0.1% of formic acid, and eluent B methanol slightly acidified with 0.1% of formic acid. The elution gradient started with 5% of eluent B during 2 min, increasing to 95% B in 11 min. The injection volume was 10 µl. A Triple TOF™ 5600 system with a DuoSpray™ source operating in the negative ESI mode was used for detection (AB SCIEX, CA, USA). The following parameter settings were used: ion spray voltage, - 4500 ISVF; ion source heater, 400 °C; curtain gas, 25 psi; ion source gas 1.50 - 2.50 psi. For the full MS-IDA (information dependent acquisition) MS/MS analysis, the full MS, the survey scan, and the MS/MS experiments were run in positive mode with a scan range from m/z 100 to m/z 800 and a 250 ms accumulation time for the full MS. The declustering potential was -150 eV and collision energy was -77 eV. The MS was using an IDA acquisition method with two experiments: the survey scan type (TOF-MS) and the dependent scan type (product ion). Data was evaluated using the XIC manager in the PeakView™ software.

3.7. Protection ability of gelatin spray-dried microhydrogels

The $ABTS^{\bullet+}$ assay was also used to evaluate the ability of the selected gelatin micro-hydrogels to protect EGCG from degradation in aqueous media. For this purpose, the antioxidant activity of free and encapsulated EGCG was measured after

dissolution/suspension in PBS. Solutions (5 mM) of EGCG in PBS were prepared. Suspensions of EGCG-loaded capsules in PBS with theoretical EGCG concentrations of 5 mM were also prepared. After specific time intervals, the solutions/suspensions were diluted 20-fold with acetic acid 20% v/v and their RSA was calculated using eq. (2), after conducting the $ABTS^{\bullet+}$ assay.

In order to confirm the results obtained from the degradation assays, HPLC-MS was also used to analyze the samples as described before.

3.8. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

4. Results and discussion

4.1. Characterization of EGCG-loaded spray-dried biopolymeric microparticles

Spray-dried powders were produced from a protein (Gel) and from a polysaccharide (Ch), both derived from naturally occurring biopolymers, and both leading to micro-hydrogels upon hydration in aqueous media. It is important to remark that the solutions were processed at considerable lower temperatures than those described in other works for the production of EGCG-loaded spray-dried biopolymeric particles [37], minimizing the degradation of the bioactive molecule when present.

The morphology of the obtained powders is shown in Figure 2. Pseudo-spherical particles were observed, but different morphologies could be distinguished. Some particles exhibited a corrugated surface, while others revealed a smooth surface. These two types of morphology have been previously described for spray-dried particles obtained from aqueous solutions [2, 38, 39]. Interestingly, a third morphology was also observed only in the gelatin samples, where concave and considerably bigger particles were also generated. This shape is typical of the so-called 'ballooning' effect which occurs at high drying rates when the polymeric matrix is elastic enough to enable this dents formation due to the thermal expansion of air or water vapours inside the drying particles, before solidification of the matrix [37].

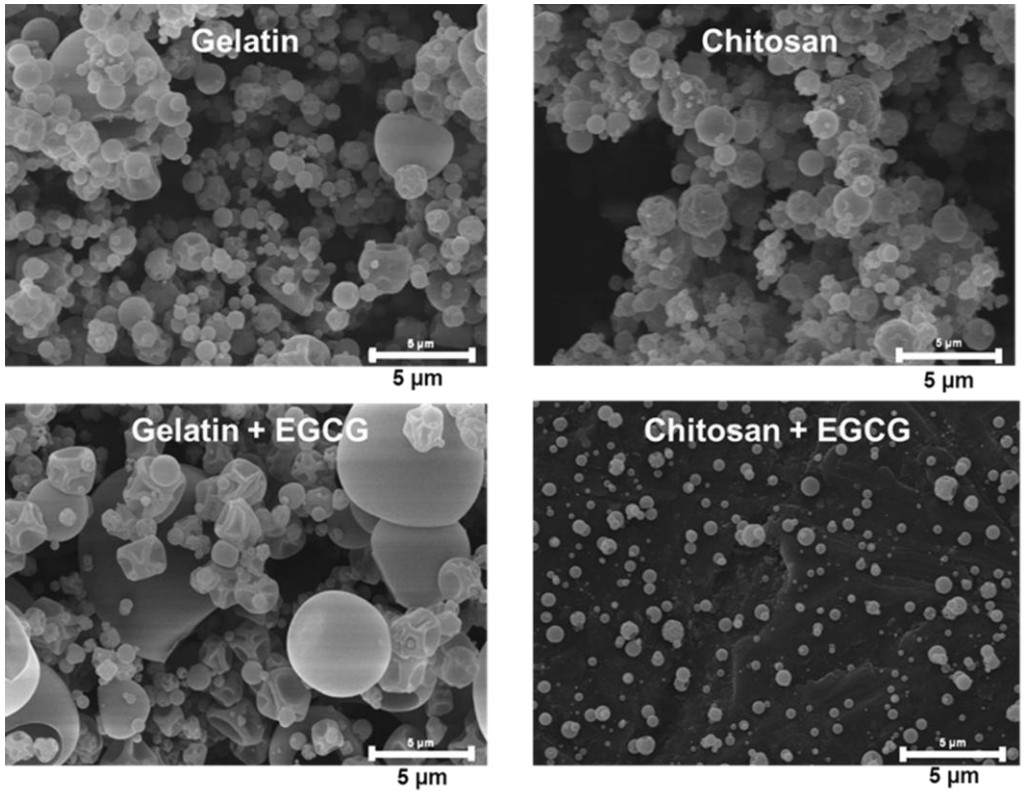


Figure 2. SEM images of spray-dried particles.

The particle size distributions of the spray-dried powders, determined by laser diffraction, are shown in Figure 3. The results were in agreement with the particle sizes observed in Figure 2, and showed that Gel provided significantly bigger microparticles than Ch. This might be due to differences in the packing structure and density of the two polymers, but also to the lower polymer concentration in the Ch solutions, owing to the high viscosity of this polysaccharide. The polydispersity was also lower for microparticles made of Ch. For Gel, the 'ballooning' effect resulted in some particles being substantially bigger than others. Nevertheless, the apparent bimodal distribution observed for this sample was attributed to multi-particle aggregation, as particles having sizes about 100 µm were not observed at all in the SEM micrographs. The EGCG-loaded particles showed similar morphologies and particle size distributions as compared to their unloaded counterparts.

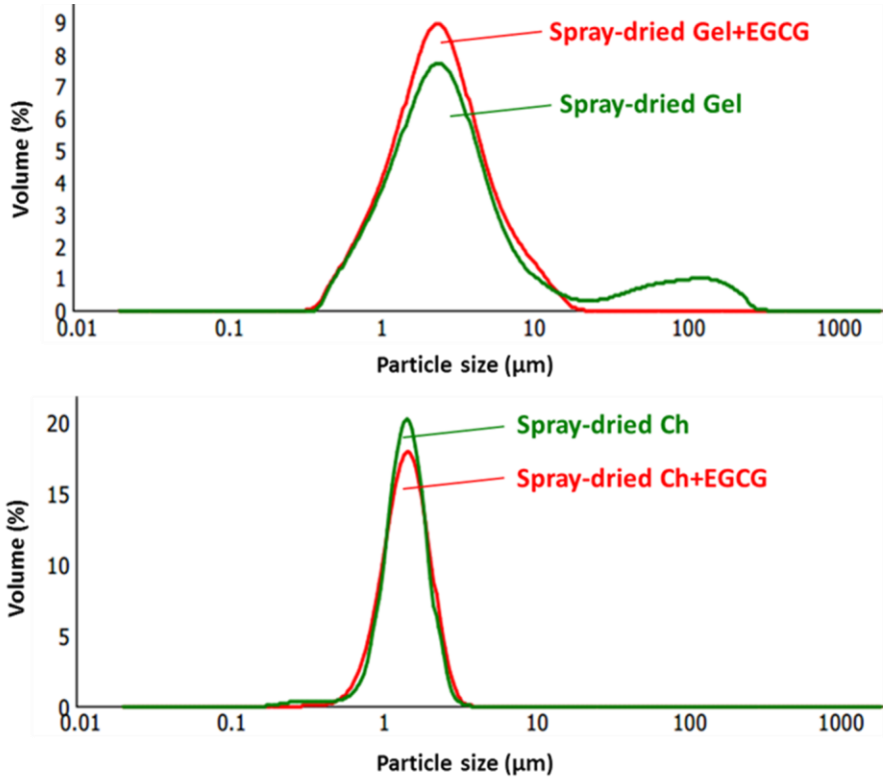


Figure 3. Particle size distributions of spray-dried samples.

The presence of absorption bands attributed to EGCG in the infrared spectra of the loaded particles (cf. Figure 4), specifically the bands at 1042 cm^{-1} (which shifted to 1037 cm^{-1} in the capsules) and 1148 cm^{-1} for gelatin and the spectral band at 1223 cm^{-1} for chitosan, evidenced the presence of the bioactive molecule in the spray-dried capsules and allowed the estimation of the microencapsulation efficiency (MEE) of the system, prior construction of a calibration curve using physical mixtures of spray-dried biopolymers and EGCG of known relative concentrations (R^2 = 0.995 and 0.999 for Gel and Ch, respectively). For this purpose, the relative absorbances of the bands at 1042 or 1223 cm^{-1} (EGCG) and 1409 cm^{-1} (Gel) or 2885 cm^{-1} (Ch) were plotted against the EGCG concentration in the mixtures.

The MEE of the EGCG-loaded capsules was 95% ± 6% and 82% ± 9% for Gel and Ch, respectively. These high encapsulation efficiency values can be explained considering the great solubility of EGCG in the feed solutions and the absence of partitioning effects [13]. Moreover, the EGCG-loaded particles experienced some shifts of their infrared bands with respect to the components alone, such as the amide III band of gelatin which moved to 1240 cm^{-1} or the displacement of the Amide I band of Ch to lower wavenumbers, suggesting intermolecular interactions between the

antioxidant molecule and the protective matrices, fact which is not surprising in the case of Gel as proteins and polyphenols are known to form soluble complexes [40].

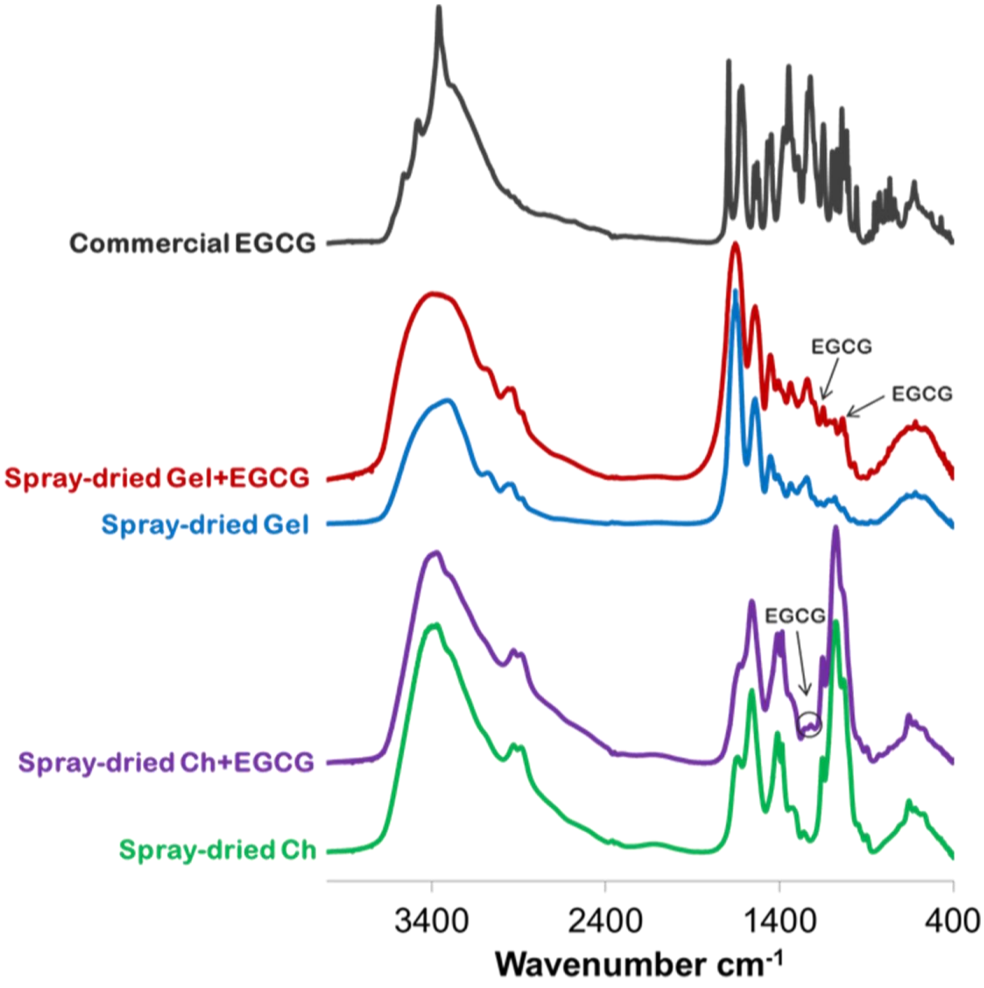


Figure 4. Infrared spectra of commercial EGCG and spray-dried materials.

The thermogravimetric (TG) profiles of pristine materials and their corresponding spray-dried particles, both unloaded and EGCG-loaded, were analyzed to ascertain possible thermostability changes of the ingredients upon processing. The main results are summarized in Table 1 and Supplementary Figure S1.

Table 1. Onset temperature (T_{onset}), temperatures of maximum degradation rate (T_{max1} and T_{max2}) and corresponding weight losses (WL_1 and WL_2) of the two main degradation stages for the raw materials and the spray-dried particles.

Sample	T_{onset} (°C)	T_{max1} (°C)	WL_1 (%)	T_{max2} (°C)	WL_2 (%)
EGCG	228.8	235.4	33.7	483.9	62.3
Gel	265.6	301.3	45.2	537.7	36.9
Ch	268.9	293.5	53.8	542.7	40.4
Spray-dried Gel	244.0	267.0/332.2	62.2	534.9	31.9
Spray-dried Gel+EGCG	238.1	268.7/329.3	65.53	518.4	32.1
Spray-dried Ch	218.1	257.8	62.1	458.4	25.76
Spray-dried Ch+EGCG	222.6	262.3	59.0	471.9	28.5

No peaks attributable to the degradation of EGCG were detected in the DTG curves of the EGCG-loaded particles. This might be due to the good compatibility and molecular interactions between the antioxidant molecule and the biopolymeric matrices (as suggested by FTIR), which delayed the thermal degradation of EGCG until the integrity of the encapsulating materials was lost.

4.2. EGCG release from the micro-hydrogels

The release of EGCG was evaluated upon hydration of the spray-dried particles in MES aqueous buffer (pH = 6.1) as a simulant for slightly acidic aqueous foods such as some juices [41]. Figure 5 shows an initial burst release from both chitosan and gelatin micro-hydrogels, which was more abrupt for the chitosan matrix. The release from the gelatin capsules was noticeably more sustained in the initial hours. While the maximum release from chitosan was observed within the first 10 hours, the maximum EGCG release from gelatin was attained more than 5 times later, suggesting that gelatin micro-hydrogels are more effective in delaying the dissolution of the antioxidant in aqueous media than their chitosan counterparts.

One of the most common semi-empirical models used to describe the release kinetics of bioactive molecules from delivery systems is the Peppas-Sahlin equation [35], which takes into account the combination of Fickian (diffusion) and non-Fickian (polymer relaxation) release mechanisms. Thus, this model was used to fit the first points of the experimental data (up to 10 h), when the sink assumption is valid [42]. Table 2 shows the EGCG release kinetic parameters for both microencapsulation matrices in the MES aqueous food simulant according to the Peppas-Sahlin equation, assuming a spherical morphology (i.e. aspect ratio of 1) for the spray-dried micro-hydrogels and hence a Fickian diffusional exponent (m) of 0.425.

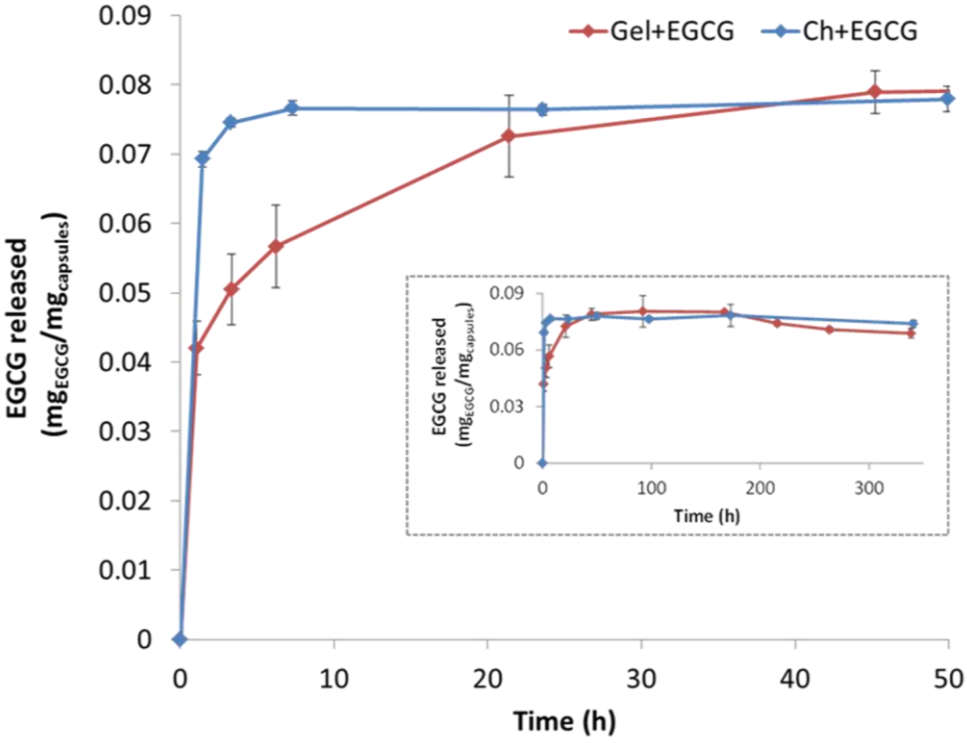


Figure 5. EGCG release profiles from the spray-dried micro-hydrogels. The inset shows an extended release profile up to 350 hours.

Table 2. EGCG release kinetic parameters (k_i) and the linear correlation coefficients (R^2).

	k_1 ($h^{-0.425}$)	k_2 ($h^{-0.850}$)	R^2
Spray-dried Gel+EGCG	0.51±0.06	-0.12±0.02	0.996
Spray-dried Ch+EGCG	0.82±0.01	-0.21±0.01	0.996

Both spray-dried matrices exhibited higher absolute values for k_1 than for k_2. Given that the first term of the Peppas-Sahlin equation (k_1) is related to the contribution of the diffusion phenomenon to the overall release kinetics, and the second term (k_2) accounts for the case-II transport or relaxational phenomenon [35], the values in Table 2 suggest that the predominant release mechanism for these micro-hydrogels was a diffusion phenomenon. These results are in good agreement with those previously reported for EGCG-loaded gelatin microparticles obtained by electrospraying [15]. The negative values obtained for k_2 in both cases indicated that the swelling (or relaxation) of the polymeric matrices impeded the EGCG release in the

initial burst release phase, due to the fast solvent uptake. The Peppas-Sahlin model also confirmed the faster EGCG release kinetics from the Ch hydrogels than from the Gel matrix, which could in part be attributed to their smaller particle size and consequent higher specific area, besides the intrinsic differences in the release mechanisms from both matrices.

Even though the release was slower from the gelatin matrix, it was still relatively fast for these capsules to be directly applied to beverage foods, as the antioxidant molecule would be released during their storage. Rather, they would be more appropriate for the formulation of dry food products, which may require processing as a liquid or humid paste for a limited time but dried before storage, such as pastry or bakery products.

4.3. Antioxidant activity of EGCG-loaded micro-hydrogels

The radical scavenging activity of both encapsulated and free EGCG was assessed by means of the $ABTS^{\bullet+}$ decolourization assay to ascertain whether the micro-encapsulation process had an impact on the antioxidant activity of the bioactive. There were no significant differences between the inhibition of the absorbance caused by the solvent blank and the two unloaded hydrogels (cf. Table 3), so the antioxidant activity of the matrices was indeed neglected. The RSA of the encapsulates was thus attributed only to the contribution of their EGCG content. While no significant differences were found between the RSA of the gelatin encapsulates and the free EGCG, suggesting that the antioxidant activity of the bioactive was fully retained during the encapsulation process, the chitosan encapsulates showed a lower RSA than EGCG in its free form, supporting the lower encapsulation observed when chitosan was used as the encapsulating matrix. In fact, the antioxidant activities measured are in close agreement with encapsulation efficiencies estimated from the infrared spectra of the materials, as the gelatin micro-hydrogels retained 97% of the antioxidant activity of free EGCG, whereas chitosan encapsulates showed only 84% of its RSA.

Table 3. Antioxidant activity of free and encapsulated EGCG (theoretical EGCG concentration: 0.25 mM), together with solvent and matrices blanks.

Sample	RSA (%)	Standard deviation (%)
Solvent	3.2 [a]	0.3
Spray-dried Gel	2.8 [a]	0.3
Spray-dried Ch	2.4 [a]	1.1
EGCG	26.9 [b]	0.9
Spray-dried Gel+EGCG	26.0 [b]	2.4
Spray-dried Ch+EGCG	22.7 [c]	1.0

Different letters (a-c) within the same column indicate significant differences among the samples.

4.4. *In-vitro* GI digestion and bioaccessibility assessment

Although microencapsulation has proven to be efficient in preventing degradation of bioactive substances, it can also have an impact on their bioaccessibility [22]. Thus, the assessment of the bioaccessibility of the encapsulated functional ingredients is of outmost importance in the design of novel functional foods, given that the Regulation (EC) 1924/2006 on nutrition and health claims made on foods declares (in Section 15) that "In order to ensure that the claims made are truthful […] The substance should also be available to be used by the body" [43]. One of the most simple definitions of bioaccessibility states that it is "the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption" [44], that is, "the fraction that is soluble in the gastrointestinal environment" [45].

Thus, knowledge about the bioaccessibility of EGCG encapsulated in gelatin or chitosan microcapsules is crucial to assess the suitability of these matrices as carriers for the bioactive compound in functional foods, but this information is scarce in the literature. Therefore, a bioaccessibility assessment was carried out in this work for the prepared microparticles. For this purpose, free and encapsulated EGCG were subjected to static *in-vitro* GI digestion and the soluble fraction of the digestas (i.e. the supernatant obtained after centrifugation) was analyzed by means of the $ABTS^{\bullet+}$ assay, which provided an indirect estimation of the EGCG content released from the matrix during digestion. The unloaded spray-dried micro-hydrogels, as well as blank samples (water) were also digested in order to disregard possible contributions of the encapsulation matrices and/or the enzymes added during digestion to the total antioxidant activity of the digestas. The value of RSA obtained for the digestas of the unloaded hydrogels and the blanks were then substracted from the RSA of the corresponding EGCG-containing digestas in order to take into account only the contribution of their EGCG content to their total antioxidant activity. The results are summarized in Table 4.

Table 4. Antioxidant activity of supernatants from gastric and duodenal digestas.

Sample	RSA (%) of gastric digesta(*)	RSA (%) of duodenal digesta
EGCG	84 ± 8^{a}	52 ± 5^{a}
Spray-dried Gel+EGCG	23 ± 2^{b}	36 ± 3^{b}
Spray-dried Ch+EGCG	15 ± 3^{c}	5 ± 2^{c}

(*) The supernatant from the gastric digesta was diluted 2-fold before analysis, as the as-prepared samples provided a complete inhibition of the absorbance of $ABTS^{\bullet+}$ at 734nm. Different letters (a-c) within the same column indicate significant differences among the samples.

The RSA of the digestas of free EGCG and, consequently, its bioaccessibility, was significantly higher than the value obtained for encapsulated EGCG, suggesting that only part of the EGCG content was released from the microcapsules during *in-vitro* GI digestion, while free EGCG was already completely dissolved in water before digestion. This resulted in the partial degradation of free EGCG, as observed from the decrease in the antioxidant activity after the duodenal phase, while an increase in this antioxidant activity was observed for the soluble fraction of digested EGCG-loaded Gel microcapsules during digestion. Therefore, even though this fraction only represented the 27% of the value obtained for free EGCG after the gastric phase, it increased up to a 68% of the activity of free EGCG after the duodenal phase. These values are consistent with the EGCG release profile obtained for the gelatin matrix in an aqueous buffer (cf. Figure 5). From these results it was seen that around 50% of the EGCG content was released in MES after 3.3 hours. Given that digestion lasted 4 hours and that digestive enzymes were present in the simulated fluids, plus differences in the pH and ionic strength, and a slightly higher temperature, a release of a 68% of the EGCG content during digestion was in good agreement with the release results obtained.

To further confirm these results, the supernatant of the digestas of EGCG-loaded Gel micro-hydrogels were analyzed by HPLC-MS. The mass spectrum of EGCG is shown in Supplementary Figure S2. The HPLC-MS experimental results obtained from the encapsulates followed the same trend as in the antioxidant studies, i.e. a greater difference in EGCG content was observed after the gastric phase, while only slight differences were observed in the digestas from the free and Gel encapsulated EGCG after the duodenal phase (cf. Supplementary Figure S3).

Interestingly, the mass spectra shown in Figure 6 suggested that after the gastric phase, the amount of dimer EGCG (m/z 915) in the digesta obtained from free EGCG was greater than in the Gel-encapsulated counterpart, where the monomeric compound (m/z 457) was most abundant. It is important to emphasize that while catechin monomers are readily absorbed in human subjects and animals, there are controversies about the bioavailability of oligomeric forms [46] thus suggesting that encapsulation could improve bioavailability by hindering the formation of oligomeric species.

At the end of the intestinal digestion process, there was a marked decrease in the amount of EGCG which, in agreement with previous studies, showed that the major degradation of EGCG occurs in intestinal fluids [46].

Regarding the bioaccessibility of EGCG microencapsulated in Ch, it was considerably lower than for the protein matrix. These results were unexpected considering the faster release of EGCG in aqueous media from the polysaccharide than from gelatin. However, they could be explained in light of the pH-responsive behavior of Ch hydrogels [25]. In fact, the short oral phase of the digestion, although frequently disregarded in many studies [36], seemed to be crucial for assessing the bioaccessibility of EGCG encapsulated in Ch. The neutral pH of the simulated salivary fluid would have neutralized the previously protonated amino groups of spray-dried Ch (as a consequence of its processing in acetic acid), favoring the formation of strong intra- and intermolecular interactions through hydrogen bonding (possibly involving the bioactive molecule) which are characteristic of Ch and are the main reason for the poor solubility of this polysaccharide in aqueous media [47]. This

neutralization of the Ch molecules would have hindered swelling of the particles and release of their EGCG contents. Previous studies had observed that neutralization of chitosan membranes prepared from acetic acid solutions triggered a molecular rearrangement of its polymer chains, modifying its physico-chemical properties and swelling behavior [48]. In order to confirm this neutralization during the oral phase, spray-dried Ch was subjected to the neutral pH found in the salivary solution and dried under vacuum. The bands in the 1800 – 1500 cm^{-1} region of the infrared spectrum of the resulting sample shifted back to the profile exhibited by the raw chitosan (cf. Figures S4 and S5 in the Supplementary material), thus supporting our hypothesis.

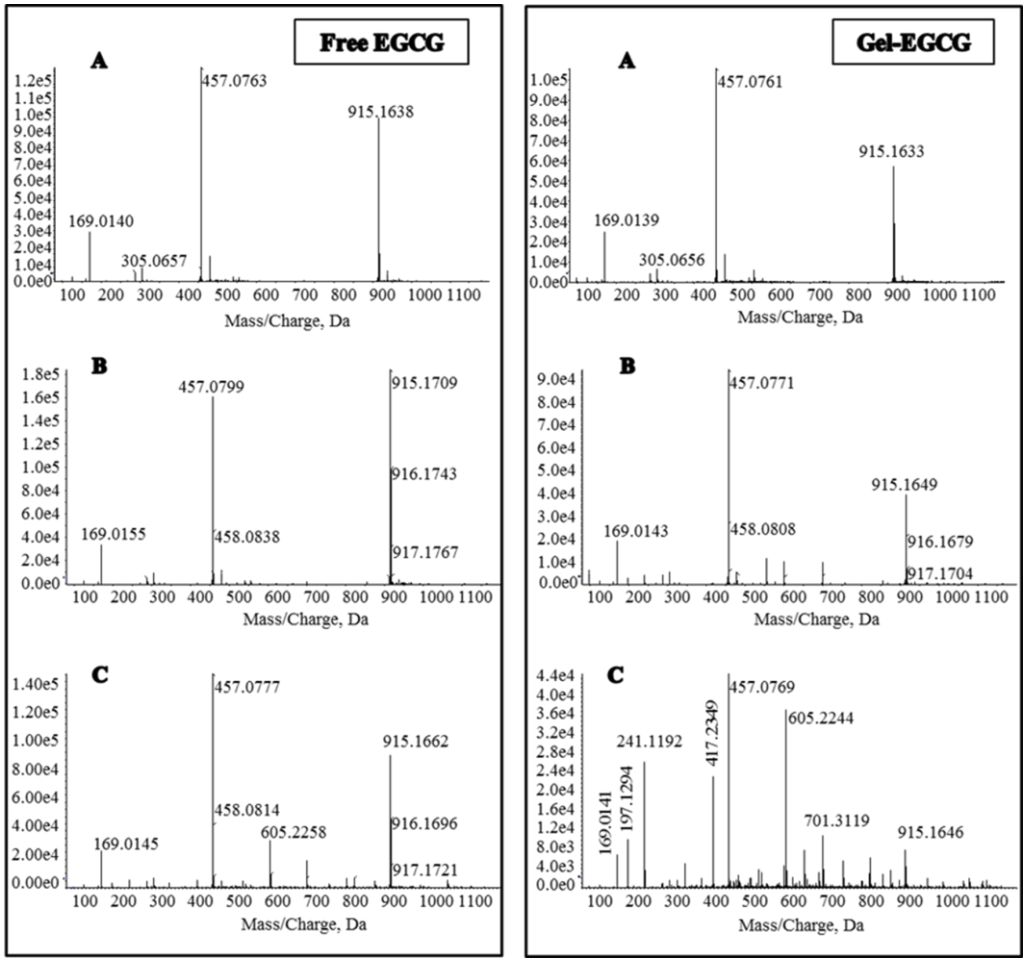


Figure 6. Mass spectra of free (left) and Gel-encapsulated EGCG (right) before digestion (A) and after *in-vitro* gastric (B) and intestinal digestion (C).

With the aim of confirming that the differences observed in Table 4 were attributable to a lack of EGCG release from the capsules during digestion, the duodenal digestas (including the insoluble fraction) were subjected to treatment with acetic acid (20% v/v) and vigorous agitation during 4 hours, with the aim of dissolving the encapsulation structures. The treated digestas were then subjected to the $ABTS^{\bullet+}$ assay, observing no significant differences between the RSA corresponding to free EGCG and both encapsulated EGCG samples (cf. Table S1 in the Supplementary material). Hence, the presence of the antioxidant compound in the insoluble fraction of the duodenal digestas of the encapsulates was corroborated.

Comparing both matrices, although the bioaccessibility of EGCG decreased upon microencapsulation in both cases, it was significantly higher for Gel than for Ch, so the protein was considered to be a more suitable encapsulation matrix than the polysaccharide. Moreover, as commented on above, although a greater bio-accessibility was observed, the bioavailability of free EGCG during digestion might be compromised by the formation of oligomeric species, as deduced from the mass spectra in Figure 6, which was much more limited when EGCG was encapsulated within the developed Gel micro-hydrogels.

4.5. Protection ability of spray-dried gelatin micro-hydrogels

As gelatin proved to be a more adequate matrix for the encapsulation of EGCG than chitosan, not only exhibiting higher encapsulation efficiencies and, thus, higher retention of the antioxidant activity, but also a more delayed EGCG release in aqueous solution and higher bioaccessibility of the bioactive after digestion, this matrix was selected to carry out an *in-vitro* degradation test. This test was also based on the $ABTS^{\bullet+}$ decolorization assay and was used to compare the degradation profiles of free and gelatin-encapsulated EGCG in PBS, by monitoring the decrease in their RSA value with time after dissolution or suspension in this medium.

Hence, solutions of EGCG (5 mM) and suspensions of EGCG-loaded gelatin micro-hydrogels (theoretical EGCG concentration also 5 mM) in PBS were prepared. The fast degradation of EGCG upon dissolution in this buffer could be visually observed by a change in color from a light pink to an intense yellowish color, as seen in previous work [15]. After different degradation periods, the samples in PBS were diluted 20-fold with acetic acid 20% v/v to stop the degradation process by lowering the pH of the medium, and to facilitate the complete dissolution of the Gel micro-hydrogels. The RSA of the resulting solutions was then calculated by means of the $ABTS^{\bullet+}$ assay and the results are shown in Figure 7.

Free EGCG experienced a rapid loss of antioxidant activity. After 4 days, it had lost almost one third of its initial activity, while no significant loss was observed in the RSA of the EGCG-loaded gelatin capsules within that time period. The RSA only slightly decreased after 10 days in PBS. These results suggest that the encapsulation of EGCG in gelatin micro-hydrogels by the spray-drying technique could effectively protect

EGCG from degradation in slightly alkaline solutions, in which free EGCG is highly unstable [8, 14].

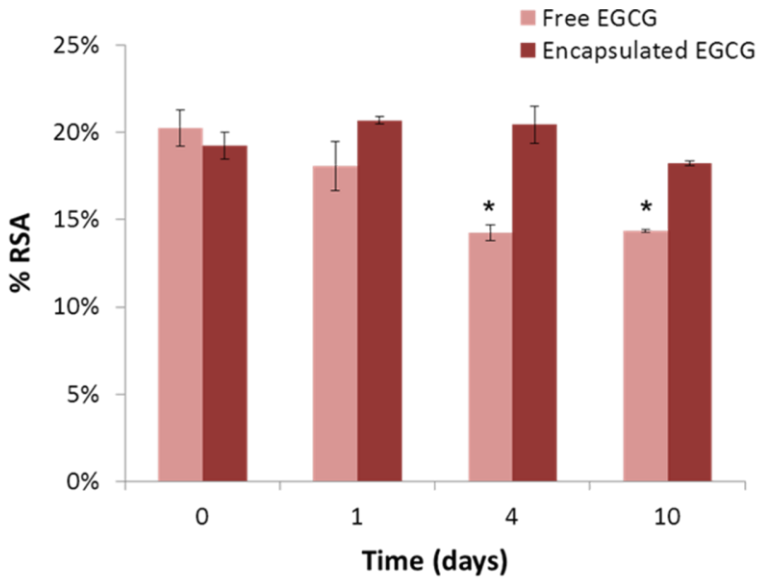


Figure 7. Radical scavenging activity of free EGCG and EGCG-loaded gelatin micro-hydrogels after different degradation periods in PBS. Asterisk (*) depicts a significant difference between results corresponding to free EGCG with respect to encapsulated EGCG ($p<0.05$).

An HPLC-MS/MS analysis was carried out to confirm these results. As shown in Fig. 8A, after 24 hours in PBS, the percentage of EGCG considerably decreased for the free (non-encapsulated) bioactive. In contrast, the stability was significantly increased when EGCG was incorporated in the gelatin micro-hydrogels since just slight changes were observed in the recovery of EGCG after 24 hours (cf. Fig. 8B and Supplementary Figure S6 for more details).

Moreover, as observed from the chromatograms of non-encapsulated EGCG in Figure 8, the decrease in the EGCG peak was accompanied by the appearance of a peak at 2.1 minutes which increased with incubation time and that corresponded to EGCG degradation products as shown in Supplementary Figure S7 (cf. supporting information for a more in-depth analysis). Since EGCG is a potent anti-oxidant, it tends to be oxidized within biological environment, leading to lower bioavailability and short half-life limiting its therapeutic efficiency [49]. Interestingly, this peak was absent in the chromatograms from the EGCG encapsulated within the gelatin micro-hydrogels, thus confirming the protection ability of the structures developed.

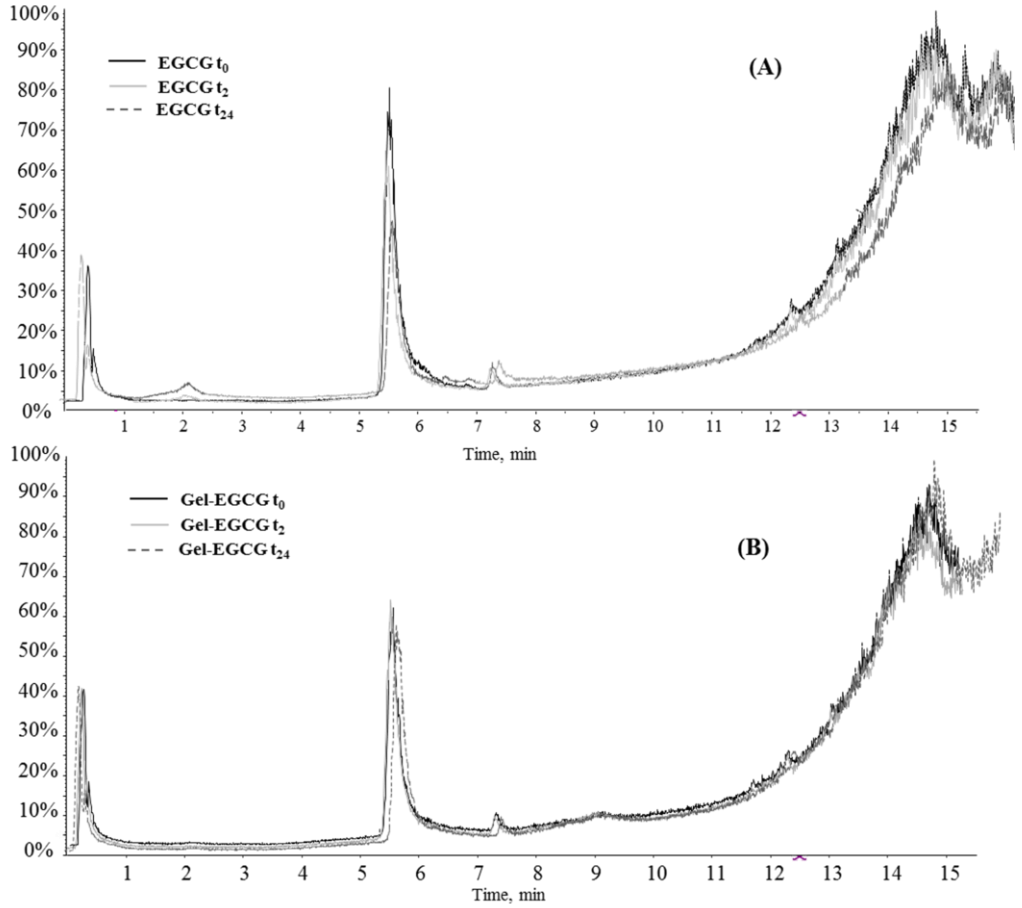


Figure 8. Total ion chromatogram of (A) free-EGCG and (B) Gel-EGCG in PBS at different times of incubation (0 h, 2 h and 24 h).

5. Conclusions

Spray-dried micro-hydrogels based on gelatin and chitosan were proposed as encapsulating matrices for the model flavonoid EGCG. Although the polysaccharide gave rise to smaller microparticles with narrower size polydispersity than the protein, the latter achieved higher microencapsulation efficiencies (95% ± 6%) than the former (82% ± 9%), as estimated by infrared spectroscopy. These results were confirmed by $ABTS^{\bullet+}$ assays, which corroborated that the gelatin micro-hydrogels retained 97% of the antioxidant activity of free EGCG, while chitosan showed only 84% of its radical scavenging activity. The TGA profiles of the samples suggested that microencapsulation was successful in stabilizing the bioactive thermally, as only the

degradation steps attributed to the matrices were detected. The release of EGCG from the spray-dried particles when suspended in slightly acidic aqueous solution was faster from the chitosan matrix, so gelatin was more effective in delaying the solubility of the flavonoid in this medium. Furthermore, the bioaccessibility of EGCG after digestion was higher when microencapsulated in gelatin than in chitosan, as the latter pH-sensitive micro-hydrogel precluded the release of the bioactive after being neutralized in the basic mouth conditions. Moreover, encapsulation in gelatin hindered EGCG dimer formation, which may also have an impact from the bioavailability viewpoint. The overall results suggested that gelatin-based micro-hydrogels are more adequate as encapsulation matrices for flavonoids than chitosan for application in the development of functional foods. The capability of gelatin to stabilize EGCG against degradation in slightly alkaline aqueous solution was also demonstrated, thus broadening the potential for food incorporation.

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8. Supplementary Material

8.1. Thermal stability of the microparticles

Figure S1 shows that three main stages were observed in all the TG curves. Due to the hygroscopic character of the biopolymers, an initial stage corresponding to the loss of water was observed. The second stage occurred at temperatures of maximum degradation rate close to 300ºC for both the polysaccharide and the protein. This thermo-oxidative degradation has been associated to deacetylation and depolymerisation processes in Ch [1], and to protein chain rupture and peptide bonds breakage in Gel [2]. The last stage, observed between 400ºC and 600ºC, has been related to the thermal destruction of the pyranose ring and the oxidative decomposition of residual carbon in Ch [3], and to thermal decomposition of the polymer networks in Gel [4].

The TG curves of the spray-dried Ch particles showed a noticeable decrease in the thermal stability of the polysaccharide after processing, both in the presence and in the absence of antioxidant. This could be mainly attributed to the presence of residual solvent molecules which modified the thermal stability of the biopolymer [5].

Thermostability changes are also observed for processed Gel. In this case, the main degradation stage exhibits two different maxima which might be attributed to two different structures or morphologies. This observation is consistent with the existence of a few apparently hollow, thin-walled structures together with more compact rounded particles in these samples, as observed by SEM. Our group had observed similar changes towards the appearance of two maxima in the main stage of the TG curves of Gel when the protein was subjected to electrospraying, where two different morphologies could be differentiated too (particles and residual fibrils) [6].

It is worth noting that the water loss during the first stage also changed after spray-drying the biopolymers. While the raw materials experienced a weight loss which extended well above 100ºC, especially in the case of gelatin, all spray-dried samples lost the water they contained before reaching 100ºC. This suggests that only absorbed water was present in the spray-dried samples, and that structural water was removed during processing due to the rapid drying of the materials.

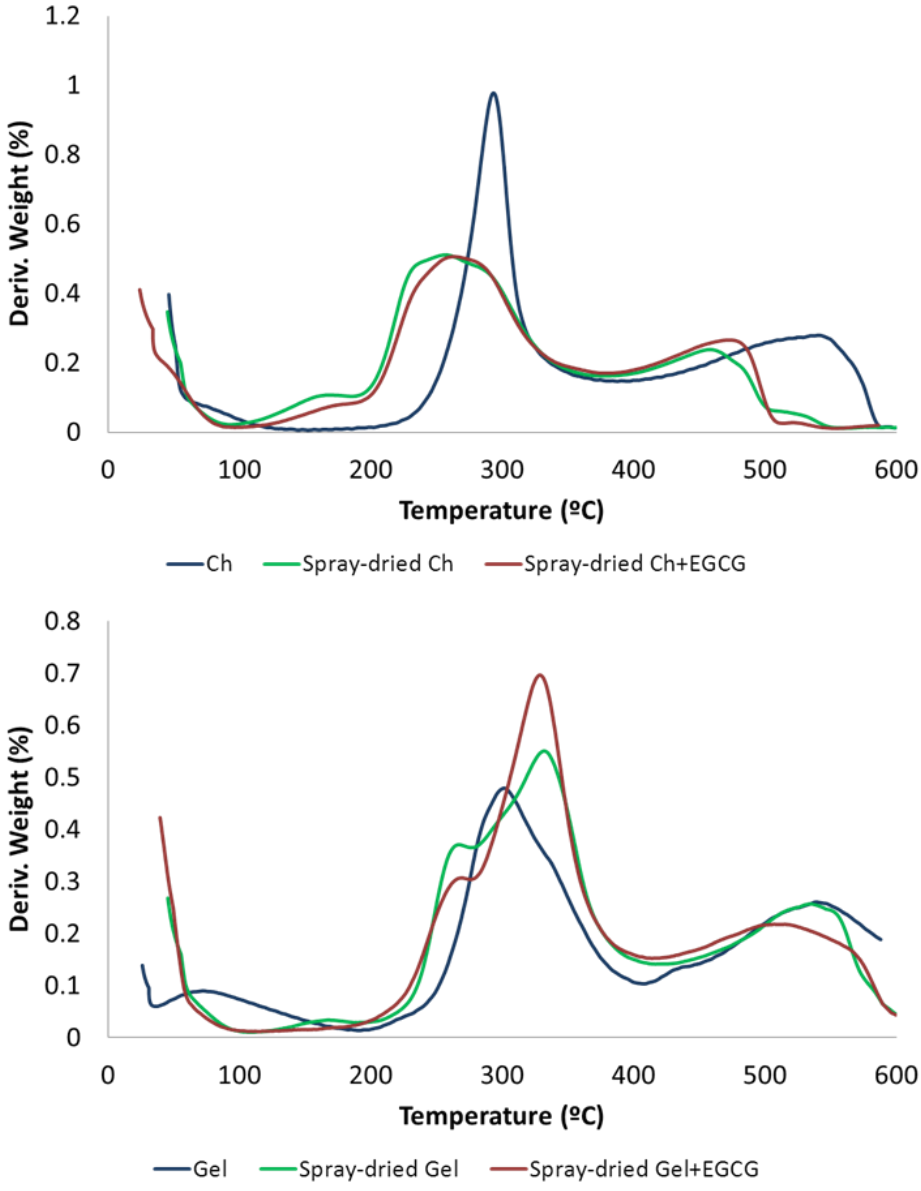

Figure S1. DTG curves of raw matrices and spray-dried particles of Ch (top) and Gel (bottom).

8.2. *In-vitro* GI digestion and bioaccessibility assessment

Supplementary Figure 2 showed that, according to previous studies [7] EGCG gave a high intensity of the deprotonated molecular ion, [M-H]-, at m/z 457 in negative ESI analyses. Besides the deprotonated molecular ion, two adduct ions at m/z 915 [2M-H]-, m/z 537 [M-H+80]- and m/z 493 M-H+36]- were obtained in the mass spectrum. The adduct ion [2M-H]- was formed because the effect of the hydrogen bond. The adduct [M-H+80]- was formed from EGCG with the mobile phase modifier, formic acid. Finally, the adduct ion at m/z 493, showed the chlorine adduct ion.

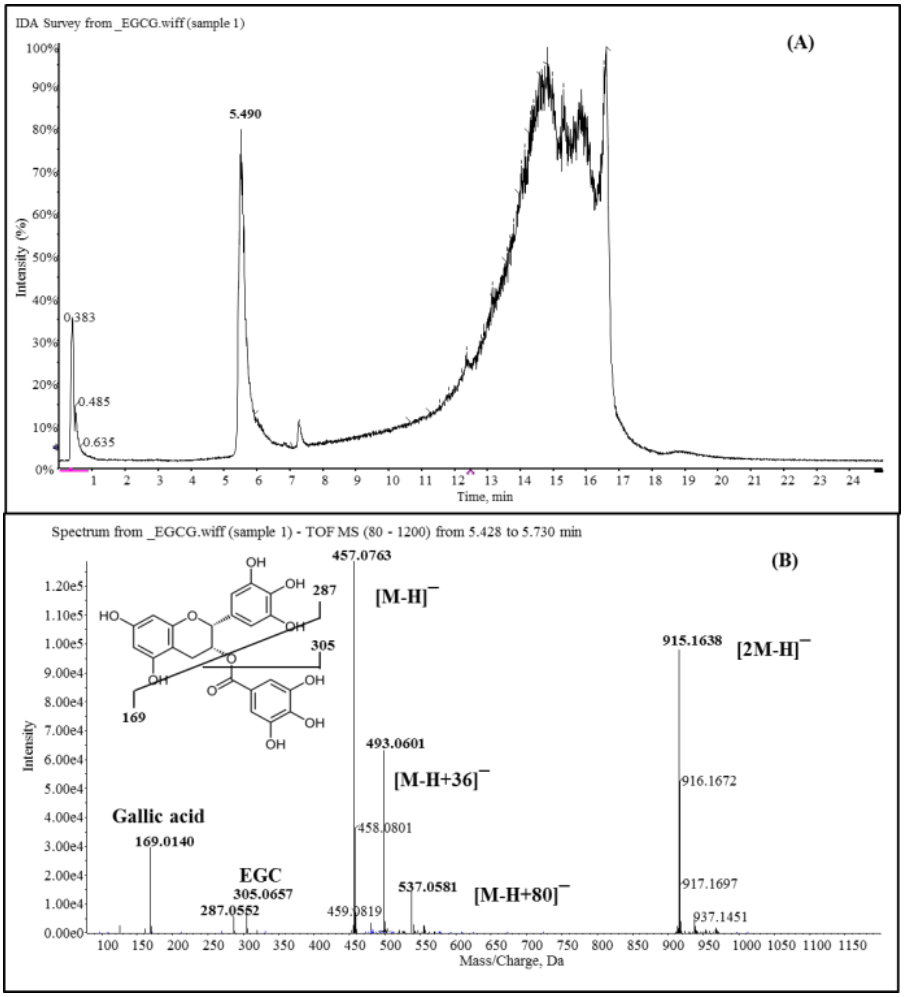

Figure S2. Information dependant acquisition (IDA) experiment of EGCG in PBS sample (t=0h) (A) Total Ion Chromatogram (B) EGCG spectrum and compound identification.

Figure S3 shows the chromatograms of free and Gel-encapsulated EGCG after *in-vitro* gastric and intestinal digestion. As observed from this Figure, there was an excellent match between the HPLC-MS experimental results and the antioxidant studies after the gastric phase, being the percentage of decrease in antioxidant activity and total ion count similar (around 60%) between the free and encapsulated EGCG. It should be stressed that the low signal observed after the intestinal phase was ascribed to the low concentration of EGCG in the extract at the end of the simulated digestion process, but the mass spectra confirmed the presence of the bioactive as shown in Figure 6 of the manuscript.

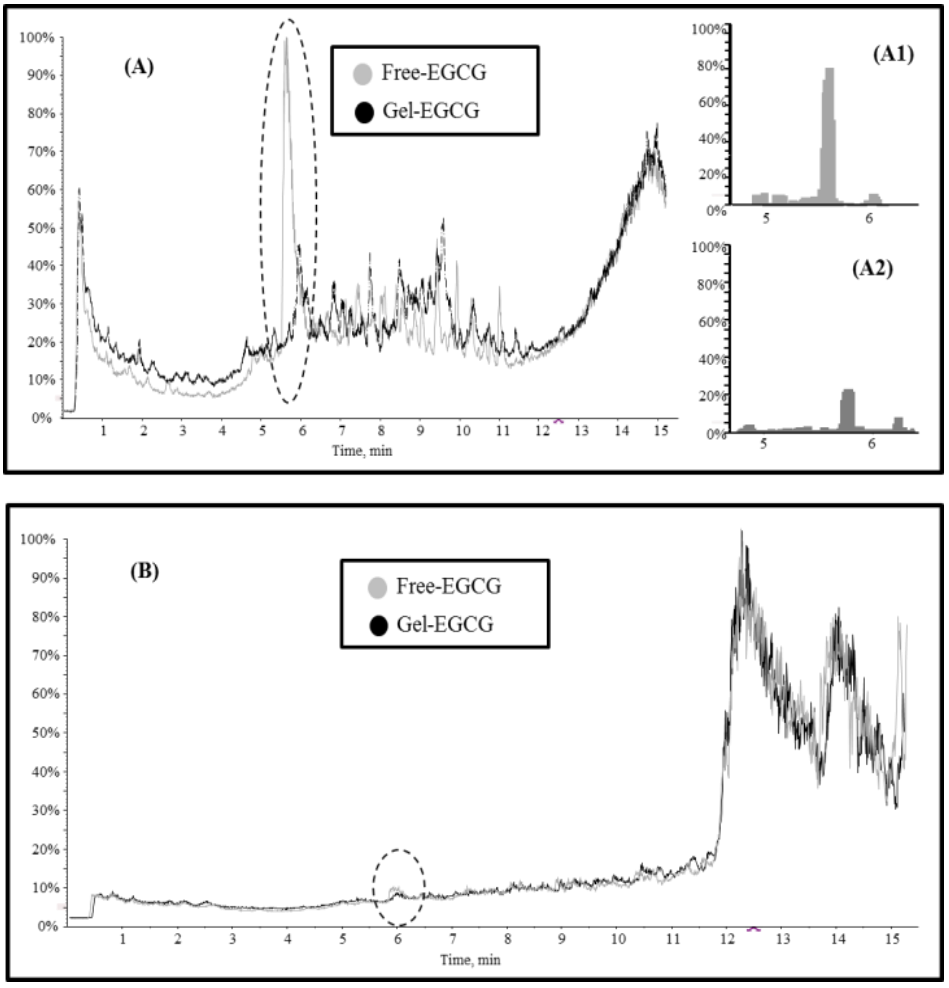

Figure S3. Information dependant acquisition (IDA) experiment of EGCG (A) TIC of gastric digesta extract (A1 corresponding to free-EGCG and A2 to Gel-encapsulated EGCG extracted chromatogram) and (B) TIC of intestinal digesta extract.

In this work it was also observed that the bioaccessibility of EGCG in the chitosan micro-hydrogels was very low, fact which was ascribed to the neutralization of the encapsulating matrix in the simulated salivary fluid. To confirm this hypothesis, spray-dried Ch was subjected to the neutral pH found in the salivary solution and dried under vacuum. Figure S4 shows that the bands in the 1800 – 1500 cm^{-1} region of the infrared spectrum of the resulting sample shifted back to the profile exhibited by the raw chitosan.

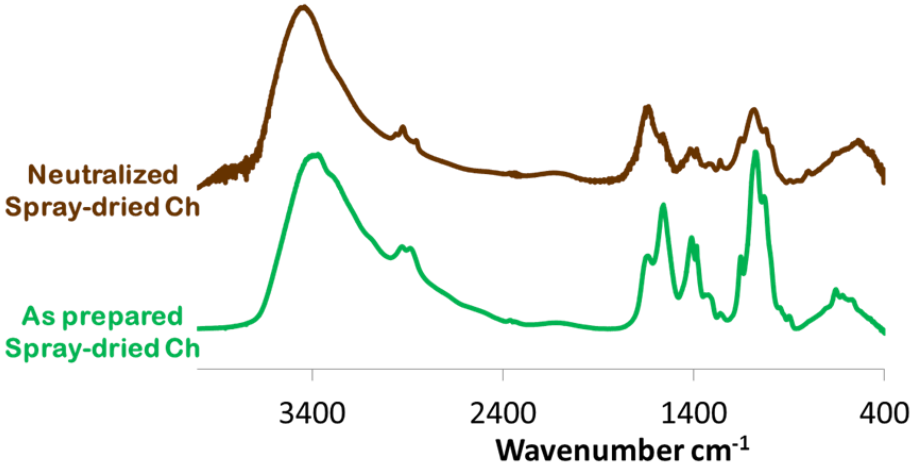

Figure S4. Spray-dried chitosan before (as prepared) and after contacting the simulated salivary fluid (neutralized).

8.3. Total EGCG content in duodenal digestas

In order to confirm that the differences in the antioxidant activity found between the supernatant of the duodenal digestas of free EGCG and encapsulated EGCG (cf. Table 4) were due to a lack of EGCG release from the capsules during digestion, the whole duodenal digestas (including the insoluble fraction) were subjected to treatment with acetic acid (20% v/v) and vigorous agitation during 4 hours, with the aim of dissolving the encapsulation structures. The treated digestas were then subjected to the $ABTS^{\bullet+}$ assay, observing no significant differences between the RSA corresponding to free EGCG and both encapsulated EGCG samples, as seen in Table S1. Hence, the presence of the antioxidant compound in the insoluble fraction of the duodenal digestas of the encapsulates was corroborated.

Table S1. Antioxidant activity of duodenal digestas treated with acetic acid (20% v/v).

Sample	RSA (%) of treated duodenal digesta
EGCG	14.7 ± 1.4 [a]
Spray-dried Gel+EGCG	15.9 ± 1.5 [a]
Spray-dried Ch+EGCG	13.6 ± 1.2 [a]

(*) The same letter (a) within the results indicate there were no significant differences among the samples.

8.4. Protection ability of spray-dried gelatin micro-hydrogels

Basic pH is known to affect EGCG stability and, thus, the degradation of free EGCG and gelatin encapsulated EGCG was studied after incubating them in PBS solution during 2 and 24 hours. To determine the recovery, solutions (5 mM) of EGCG in PBS and suspensions of EGCG-loaded gelatin micro-hydrogels in PBS with theoretical EGCG concentrations of 5 mM were and the samples were incubated during 2 and 24 hours and analyzed through HPLC-MS/MS. The recovery of EGCG was determined considering 100% recovery of free EGCG at the initial time. Figure S5 shows that while a marked recovery decrease was observed for the non-encapsulated compound after 24 hours, EGCG concentration only marginally decreased when encapsulated within gelatin, thus highlighting the protection ability in this medium of the developed micro-hydrogels.

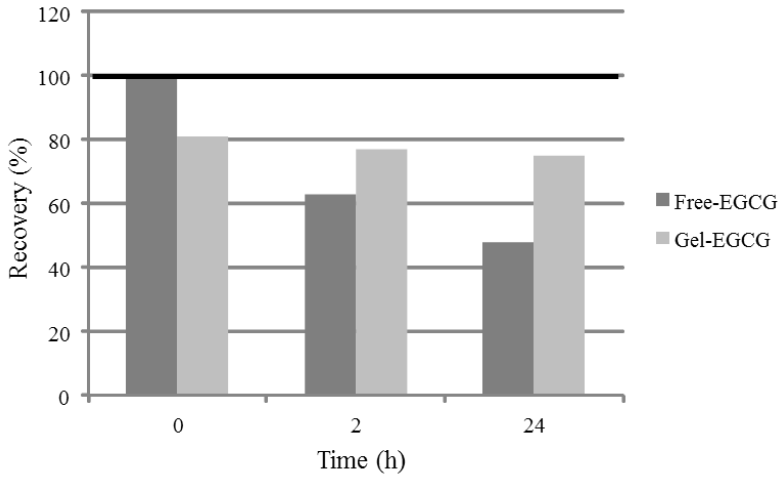

Figure S5. Stability studies of EGCG in PBS medium after 2 and 24 hours incubation.

Although the main goal of HPLC-MS analysis was not to identify the degradation products of EGCG, particular attention was given to the degradation of free EGCG in PBS to explain its antioxidant capacity since despite the significant decrease in its content after 24 hours of incubation in PBS, a high antioxidant activity remained. Therefore, the peak arising at 2.1 minutes in the chromatograms of the non-encapsulated bioactive was characterized and the results are shown in Figure S6. Since the QqTOF system used in this study has the capacity to be used for screening non-target and/or unknown compounds, the identification of molecules responsible of this signal was carried out.

As it can be observed in Figure S6, the mass spectrum of this peak exhibits the ions m/z 455 and m/z 473, which increased along incubation time. After an in-depth and exhaustive review of the published literature about EGCG degradation, these ions were identified as O-methyl derivatives [8]. Since EGCG is a potent antioxidant, it tends to be oxidized within biological environments, thus leading to lower bioavailability and short half-life limiting its therapeutic efficiency [9]. Unfortunately, and as far as we know, no previous studies have been published regarding the antioxidant activity of EGCG O-methyl derivatives. However, Dueñas et al. (2010) evaluated the antioxidant capacity of O-methylated metabolites of catechin, epicatechin and quecertin, concluding that despite the relative decrease of antioxidant capacity of these metabolites, they still presented relatively high antioxidant activity [10].

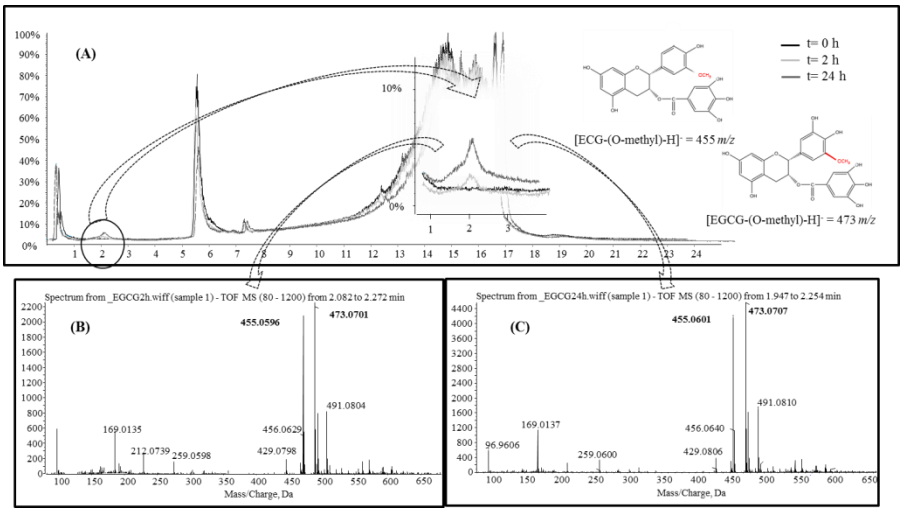

Figure S6. Information dependant acquisition (IDA) experiment of EGCG degradation after one day in PBS (A) Total Ion Chromatogram at different times (0 hour, 2 hours and 24 hours); (B) EGCG Spectrum at 2 hours and (C) EGCG Spectrum at 24 hours corresponding to 2.1 minutes peak.

8.5. References

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CHAPTER 2

MICROENCAPSULATION OF HYDROPHOBIC BIOACTIVE INGREDIENTS

2.1. Protein-based emulsion electrosprayed micro- and submicroparticles for the encapsulation and stabilization of thermosensitive hydrophobic bioactives.

2.2. Potential of microencapsulation through emulsion-electrospraying to improve the bioaccesibility of β-carotene.

2.3. Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin.

2.4. A step forward towards the design of a continuous process to produce hybrid liposome/protein microcapsules.

Introduction to Chapter 2

Compared to hydrophilic ingredients, hydrophobic compounds entail an additional challenge for their microencapsulation, since the use of aqueous media for the dissolution or suspension of the biopolymeric matrices is almost imperative for the production of edible products. Hence, in this second chapter, different strategies were used to disperse lipophilic bioactive ingredients within the polymer solutions prior to their microencapsulation by drying techniques such as electrospraying or spray-drying. These approaches involved the preparation of oil-in-water emulsions or liposome dispersions.

Different proteins were selected in this chapter as encapsulation matrices, including gelatin, a whey protein concentrate, a soy protein isolate and zein. Proteins were preferred over polysaccharides in this case due to their amphiphilic nature and subsequent superior performance as emulsifiers. On the other hand, three different lipophilic ingredients with recognized health-promoting properties were selected to develop microencapsulated functional ingredients, namely α-linolenic acid, β-carotene and curcumin, each of them presenting different problematics.

α-Linolenic acid, as an ω-3 fatty acid, is extremely thermosensitive and highly susceptible to oxidative degradation. Hence, its stabilization is of outmost importance for the development of functional foods fortified with this compound in order to extend their shelf-life. Moreover, high temperatures should be avoided during the processing of the encapsulation structures in order to prevent bioactive degradation. Hence, electrospraying of protein-stabilized α-linolenic acid emulsions was proposed in the first work, and the results were compared with those obtained using spray-drying. Also, two different emulsification procedures were used to prepare the feed emulsions, one consisting of a single high-speed homogenization step and the other including a second ultrasonication step. The impact of the emulsification procedure, the microencapsulation technique used and the protein matrix on the encapsulation efficiency and stabilization of α-linolenic acid at high temperatures was studied.

One of the main limitations for the incorporation of β-carotene as a functional ingredient is its low bioavailability. One approach to enhance its bioaccessibility is its incorporation within digestible lipids in oil-in-water emulsions. However, the shelf-life of emulsions is limited. Hence, in the second work, the emulsion-electrospraying technique previously proposed for α-linolenic acid was employed to microencapsulate β-carotene, using soy bean oil as a carrier oil to prepare the protein-stabilized feed emulsions. The effect of the emulsification procedure and the protein matrix on the encapsulation efficiency was studied, and the potential of this technique to increase the bioaccessibility after *in-vitro* digestion was assessed.

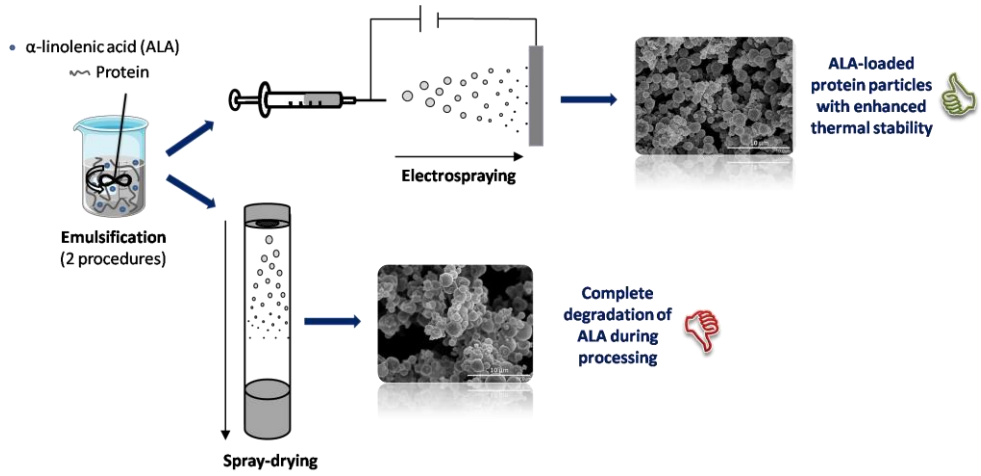
An alternative to emulsions as vehicles for lipophilic bioactive ingredients is their incorporation within liposome dispersions. This approach was explored in the third

work of this chapter, in which curcumin was microencapsulated with the aim of increasing both its stability in solution and its bioaccessibility. For this purpose, phosphatidylcholine liposomes were produced through the ethanol injection method. The effect of the lipid concentration on the liposomes size distribution, and the impact of this size on the subsequent electrospraying process was studied using whey protein concentrate as encapsulation matrix. The developed microencapsulation structures were then loaded with curcumin and the microencapsulation efficiency was evaluated. The stabilization of curcumin upon microencapsulation was assessed by comparing its degradation rate in its free and microencapsulated form when dispersed in phosphate buffered saline solution. Its bioaccessibility after *in-vitro* digestion was also compared.

In the last work of this chapter, a continuous manufacturing process was proposed for the production of the aforementioned liposome/protein micro-encapsulation structures, and part of the process was implemented. The concept involved the combination of microfluidic and electrospraying technologies. Microfluidic techniques, based on the manipulation of tiny volumes of fluids inside micron-sized channels, allow the continuous production of liposomes. Then, an intermediate mixing step would be necessary in order to blend the liposomes with the protein dispersion prior to electrospraying the mixture. Due to the low flow rates used in both techniques, this mixing step should be also accomplished by means of microfluidics, in a so-called 'micromixer'. In this preliminary work, the mixing performance of three different designs of microfluidic devices was evaluated for mixing a stream of liposomes with a dispersion of whey protein concentrate. An assembled micromixer-electrospraying setup was then built using the selected micromixer, and the arrangement was used to produce the hybrid liposome/protein microcapsules in a semi-continuous process.

2.1

PROTEIN-BASED EMULSION ELECTROSPRAYED MICRO- AND SUBMICROPARTICLES FOR THE ENCAPSULATION AND STABILIZATION OF THERMOSENSITIVE HYDROPHOBIC BIOACTIVES



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., & López-Rubio, A. (2016). *Protein-based emulsion electrosprayed micro- and submicroparticles for the encapsulation and stabilization of thermosensitive hydrophobic bioactives*. Journal of Colloid and Interface Science, 465, 259-270. (DOI: http://dx.doi.org/10.1016/j.jcis.2015.11.061).

1. Abstract

This work shows the potential of emulsion electrospraying of proteins using food-grade emulsions for the microencapsulation and enhanced protection of a model thermosensitive hydrophobic bioactive. Specifically, gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) were compared as emulsion stabilizers and wall matrices for encapsulation of α-linolenic acid. In a preliminary stage, soy bean oil was used as the hydrophobic component for the implementation of the emulsion electrospraying process, investigating the effect of protein type and emulsion protocol used (i.e. with or without ultrasound treatment) on colloidal stability. This oil was then substituted by the ω-3 fatty acid and the emulsions were processed by electrospraying and spray-drying, comparing both techniques. While the latter resulted in massive bioactive degradation, electrospraying proved to be a suitable alternative, achieving microencapsulation efficiencies (MEE) of up to ~70%. Although gelatin yielded low MEEs due to the need of employing acetic acid for its processing by electrospraying, SPI and WPC achieved MEEs over 60% for the non-sonicated emulsions. Moreover, the degradation of α-linolenic acid at 80ºC was significantly delayed when encapsulated within both matrices. Whilst less than an 8% of its alkene groups were detected after 27 hours of thermal treatment for free α-linolenic acid, up to 43% and 67% still remained intact within the electrosprayed SPI and WPC capsules, respectively.

2. Introduction

One of the most promising approaches to preserve hydrophobic bioactive ingredients in food systems is their nano- or microencapsulation within protective matrices [1], as they act as barriers, thus limiting direct contact of the bioactives with the detrimental agents of the environment [2]. Moreover, microencapsulation can also help overcoming the incompatibility between the hydrophobic compounds and the aqueous matrix of many food products, potentially increasing their bioavailability [3]. However, it also represents an additional challenge, given that the use of aqueous media for the dissolution or suspension of the polymers to be used as encapsulating matrices is almost imperative for the production of edible products, in order to avoid toxicity issues [4]. A plausible strategy to disperse the lipophilic bioactive into the aqueous polymer solution is to prepare oil-in-water (O/W) emulsions prior to microencapsulation. Although O/W emulsions are, in general, thermodynamically unstable [5] there are several strategies which can be used to increase their stability and the subsequent encapsulation efficiency [6], such as reducing the size of the oil droplets or addition of tensioactive compounds [7]. Spray-drying is the most commonly used technology in the food industry to obtain dry encapsulation structures

from emulsions [8]. However, spray-drying involves the use of a hot gas stream to rapidly dry the fine droplets produced in its initial atomization step, which results detrimental for hydrophobic thermosensitive bioactives such as ω-3 fatty acids. In contrast, electrospraying (i.e. a technique based on the electrohydrodynamic processing of polymer melts, solutions or dispersions) can be performed under mild conditions [4], so it has recently been proposed as an alternative for the microencapsulation of labile bioactive agents [6] with promising applications in the field of functional foods [9, 10]. This technology allows the production of nano- and microencapsulation structures by subjecting the polymeric fluid, which is pumped through a conductive capillary, to a high voltage electric field. As a result, a charged polymer jet is ejected towards the opposite electrode, which is broken down into fine droplets during the flight, generating dry polymeric particles upon solvent evaporation before being deposited on the collector [11-13]. Emulsion electrospraying has been recently proposed for drug encapsulation [14] and for the development of cytocompatible microcapsules [15] using carbohydrate matrices, but to the best of our knowledge only the electrospraying of whey protein concentrate (WPC)-stabilized emulsions has been reported for the microencapsulation and protection of bioactive compounds of interest in functional foods [10] to date.

Proteins are particularly interesting molecules for emulsion electrospraying, as their amphiphilic structures allow their use as effective emulsifiers [16] in addition to their primary function as wall materials. Indeed, proteins are often used as ingredients in food emulsions, providing both electrostatic and steric stabilization, in addition to their own nutritional properties [7].

In this work, three different protein types, specifically gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) were used as encapsulation matrices of α-linolenic acid (ALA) as a model hydrophobic bioactive by the emulsion electrospraying technique, with the aim of comparing their protection ability against oxidation. ALA was chosen for this purpose as, apart from being one of the most relevant ω-3 fatty acids playing an important role in the regulation of cellular functionality [17] and the preservation of the cardiovascular, neurovascular and mental health [18], it is highly susceptible to oxidative degradation when exposed to oxygen, light and/or heat [19]. In fact, ALA is considered to be the most important precursor of flavor reversion (i.e. development of off-flavors) [20] and, thus, its high instability can compromise not only the nutritional value of ALA-enriched food products but also their sensorial properties, reducing their shelf-life [21]. Furthermore, the well-established spray-drying technique was used to dry the emulsions for comparison purposes. Two emulsification protocols were carried out prior to microencapsulation using both techniques, and the influence of emulsion properties, drying technique and type of protein on the microencapsulation efficiency and on the stabilization of ALA against degradation at high temperatures were studied.

3. Materials and methods

3.1. Materials

Whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090 and with a w/w composition of ~80% protein, ~9% lactose and ~8% lipids, was kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark). Soy protein isolate (SPI) was kindly donated by The Solae Company (Switzerland). Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, soy bean oil (SBO), α-linolenic acid (≥99%) (ALA), Tween® 20 and potassium bromide FTIR grade (KBr) were obtained from Sigma-Aldrich. 96% (v/v) acetic acid (Scharlab) was used as received.

3.2. Preparation of oil in water (O/W) emulsions

The aqueous phase of each emulsion consisted of a protein solution/dispersion. Three different proteins were used to prepare the O/W emulsions: gelatin (Gel), soy protein isolate (SPI) and whey protein concentrate (WPC). In a preliminary stage, soy bean oil (SBO) was used as the oily phase in order to optimize the production of the encapsulating structures containing lipophilic compounds, using an inexpensive oil. Afterwards, SBO was substituted by a ω-3 fatty acid, linolenic acid (ALA), as a model functional oil. In all cases, the oil was added in a proportion of 10% (w/w) with respect to the total mass of non-volatile compounds in the capsules. The use of a surfactant, Tween20®, was also considered for the stabilization of some of the emulsions, as described below. The preparation of the O/W emulsions using each of the three different proteins was slightly different, as illustrated in Figure 1.

The emulsification step itself was conducted using two different procedures. The first one consisted of a one-step high-speed homogenization process conducted using an IKA T-25 Digital ULTRA-TURRAX® equipped with a S 25N - 25F dispersing element whose stator diameter was 25 mm (Germany) at 6000 rpm during 2 min. The other approach included a second step consisting of an ultrasonication treatment, which was aimed at reducing the drop size of the oil phase. For this purpose, an ultrasonic probe (Bandelin electronic, Germany) was used at an amplitude of 10% and a frequency of 20 kHz for 2 min, in intervals of 30 s to avoid excessive heating. An ice bath was also used to prevent overheating of the samples.

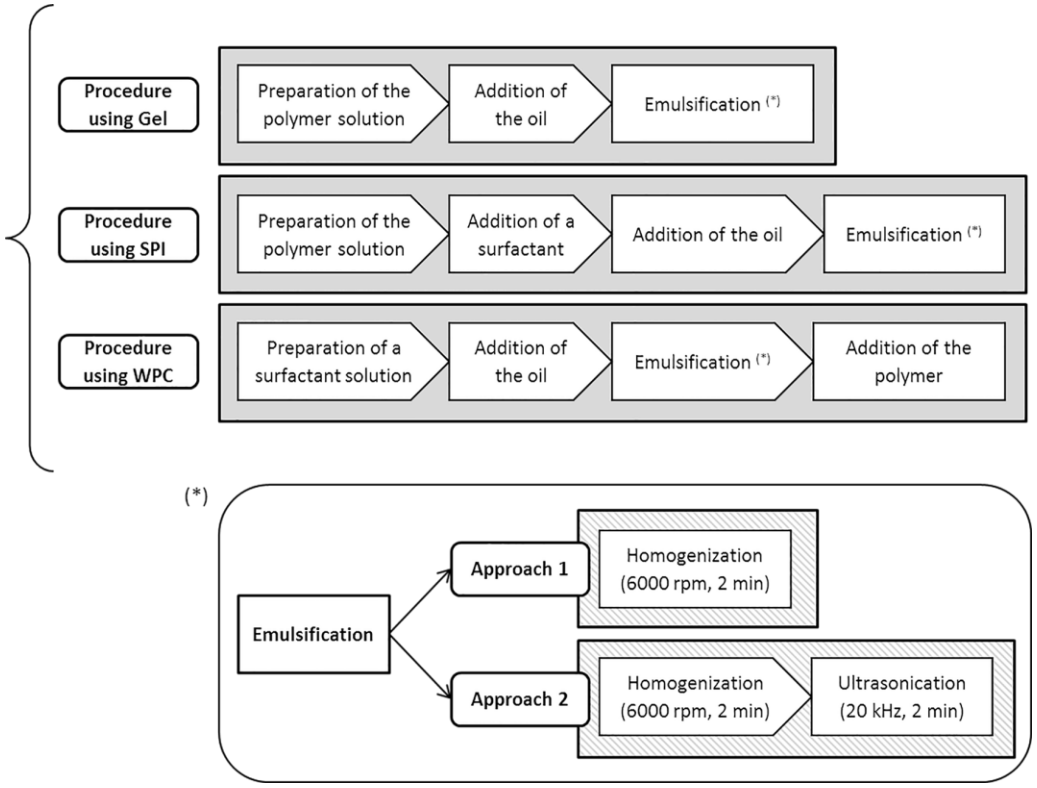


Figure 1. Scheme of the preparation of O/W emulsions.

3.2.1. Preparation of O/W emulsions using gelatin

Gelatin aqueous solutions (8% w/v) were prepared as described in [9] and cooled down to room temperature before preparation of the emulsions. The use of a surfactant for the formation of stable emulsions was not necessary in this case. In fact, preliminary optimization tests showed that the addition of either Tween20® or soy lecithin as surfactants resulted in the coalescence of the oil droplets, due to the aggregation of the protein and the surfactant molecules at acidic pH. Conversely, the emulsions were stable for weeks when gelatin was used alone, both as emulsifier and as wall matrix for the capsules. Consequently, the oil phase was directly incorporated to the premix and the emulsions were prepared following both approaches described in Figure 1.

3.2.2. Preparation of O/W emulsions using SPI

SPI (10% w/v) was dissolved in distilled water and denaturation of the protein was carried out to improve its electrosprayability [22], by heating the solution to 90ºC

for 30 min. Then, the solution was cooled down to room temperature in an ice bath before preparation of the emulsions. The addition of the surfactant Tween20® (5% w/v) was necessary to obtain stable emulsions in this case. Lastly, the oil phase was added to the premix and the emulsions were prepared following both approaches described in Figure 1.

3.2.3. Preparation of O/W emulsions using WPC

The preparation of SBO/WPC emulsions has already been reported for the encapsulation of lipophilic bioactive ingredients [10]. Based on this work, an aqueous surfactant solution was first prepared by dissolving 5 % (w/v) of Tween20® in distilled water. Afterwards, the oil phase was added, and pre-emulsions were prepared following both approaches described in Figure 1. Lastly, the required mass of WPC to achieve a protein concentration of 20% (w/v) in the aqueous phase was added to the preformed emulsions and magnetically stirred until a homogeneous emulsion was obtained.

3.3. Characterization of the emulsions

The rheological behaviour of the emulsions at 20ºC ± 0.1ºC was studied using a rheometer model AR-G2 (TA Instruments, USA) with a parallel plate geometry, using the methodology described in Gómez-Mascaraque, Lagarón and López-Rubio (2015) [9] after equilibrating the samples for 2 min. All measurements were made at least in triplicate.

In addition, optical microscopy images were taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital camera head (Nikon DS-5Mc). Nis Elements software was used for image capturing.

3.4. Stability of the emulsions

The stability of the emulsions was assessed following the creaming index method proposed in [23]. Briefly, each emulsion was transferred into a sealed tube and stored for 5, 24 and 48 h at room temperature. When the emulsions separated into two different phases, the height of the top opaque ('cream') layer was measured (H_c), and the creaming index (CI) was calculated following Eq. (1), where H_E is the total height of each emulsion in the tube.

$$CI = 100\ (H_c/H_E) \qquad \text{Eq. (1)}$$

3.5. Production of microencapsulation matrices by spray-drying

The emulsions were diluted 20-fold in distilled water prior to their processing by spray-drying, to avoid too high viscosities which would block the spraying head. The emulsions were subsequently fed to a Nano Spray Dryer B-90 apparatus (Büchi, Switzerland) equipped with a 7.0 μm pore diameter cap. The inlet air temperature was set at 90ºC, as it proved to be enough to achieve complete drying of the particles at an inlet air flow of 146 ± 4 L/min and a reduced pressure of 50 ± 3 mbar. Under these conditions, the outlet air temperature varied between 50 and 65ºC. The spray-dried powders were deposited on the collector electrode by means of an applied voltage of 15 kV.

3.6. Production of microencapsulation matrices by emulsion electrospraying

The emulsions were processed without further dilution using a homemade electrospinning/electrospraying apparatus, equipped with a variable high-voltage 0-30 kV power supply. The emulsions were introduced in a 5 mL plastic syringe and were pumped at a flow-rate of 0.15 mL/h through a stainless-steel needle (0.9 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a stainless-steel plate connected to the cathode of the power supply and placed facing the syringe in a horizontal configuration, at a distance of 10 cm. The applied voltage was 15 kV for the gelatin emulsions and 17 kV for SPI and WPC emulsions. The above processing parameters were selected from preliminary tests in order to attain stable electrospraying, avoiding dripping of the solution.

3.7. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-9 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

3.8. Fourier transform infrared (FT-IR) analysis of the samples

FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment following the methodology described in Gómez-Mascaraque, Lagarón and López-Rubio (2015) [9].

3.9. Microencapsulation efficiency

The microencapsulation efficiency (MEE) of the ALA-loaded capsules was determined based on FT-IR absorbance measurements. A calibration curve was obtained for each encapsulation matrix (R_{Gel}^2 = 0.999, R_{SPI}^2 = 0.993, R_{WPC}^2 = 0.986) from the spectra of protein/ALA mixtures of known relative concentrations (0, 5, 10 and 15 % w/w of ALA). The relative absorbance intensities of the peaks at 3012-3013 cm^{-1} (attributed to ALA) and at 1541-1543 cm^{-1} (corresponding to the Amide II band of the proteins) were plotted against the ALA concentration in the mixtures. The intact ALA content in the capsules was interpolated from the obtained linear calibration equations. The MEE of the ALA-loaded particles was then calculated using Eq. (2):

$$MEE\ (\%) = \frac{Content\ of\ ALA\ in\ the\ capsules}{Content\ of\ ALA\ initially\ added\ to\ the\ emulsions} \times 100 \qquad \text{Eq. (2)}$$

3.10. Thermal Properties of the materials

Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q500 TGA. The samples (ca. 8 mg) were heated from 25ºC to 600ºC with a heating rate of 10ºC/min under dynamic air atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

3.11. Accelerated oxidation assays for free and microencapsulated ALA

Non-encapsulated and microencapsulated ALA was subjected to thermal treatment at 80°C in order to evaluate the protective effect of each wall material. After selected time intervals, FT-IR spectra were recorded for each sample, and the absorbance intensity of the band at 3012-3013 cm^{-1}, corresponding to ALA, was measured. The decrease in the relative intensity of the aforementioned band was related to the extent of degradation of ALA within the capsules or in its native form, as previously reported [24].

3.12. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

4. Results and discussion

4.1. Characterization of O/W emulsions

Two different procedures were used for the preparation of the emulsions, as illustrated in Figure 1. The first approach consisted of a simple high-speed homogenization treatment, while the second one included a second ultrasonication step aimed at reducing the droplet size of the emulsions [25]. In general, smaller droplets lead to increased stability of emulsions and improved bioavailability of the active ingredients [5, 26], also facilitating their inclusion and dispersion within the fine microencapsulation structures to be produced. However, ultrasonic treatments may heat the emulsions, potentially leading to partial degradation of thermosensitive bioactives. Therefore, both approaches were used for the preparation of the emulsions and the impact of the ultrasonication treatment was studied. Figure 2 shows the images obtained by optical microscopy for the different emulsions produced using SBO as a model oily phase.

The appearance of the emulsions produced using the first approach (single high-speed homogenization step) was dramatically different for each protein. Gel and WPC led to well dispersed droplets, which were significantly smaller for Gel, while flocculated droplets were observed for SPI emulsions. These differences were also manifested in their rheological behaviour, i.e. while the emulsions prepared using Gel and WPC exhibited quite a Newtonian behaviour in the range of study, the emulsion prepared in the single high-speed homogenization step using SPI showed a manifest shear thinning behaviour (cf. Figure S1 of the Supplementary Material), usually associated to a high degree of droplet flocculation [23, 27]. This strong shear thinning behaviour, together with the high viscosity of the emulsion suggested that the mechanism of flocs formation was by bridging flocculation, which occurs when the protein chains or aggregates are shared between two droplets [7]. A plausible explanation for the occurrence of bridging flocculation when using SPI involves the previous denaturation step which is carried out for this protein as a requirement for the subsequent electrospraying process [22]. Denaturation leads to protein unfolding and thus to increased exposure of its non-polar amino acids. This may promote droplet flocculation in oil-in-water emulsions through increased hydrophobic attraction between protein chains adsorbed onto different droplets [16].

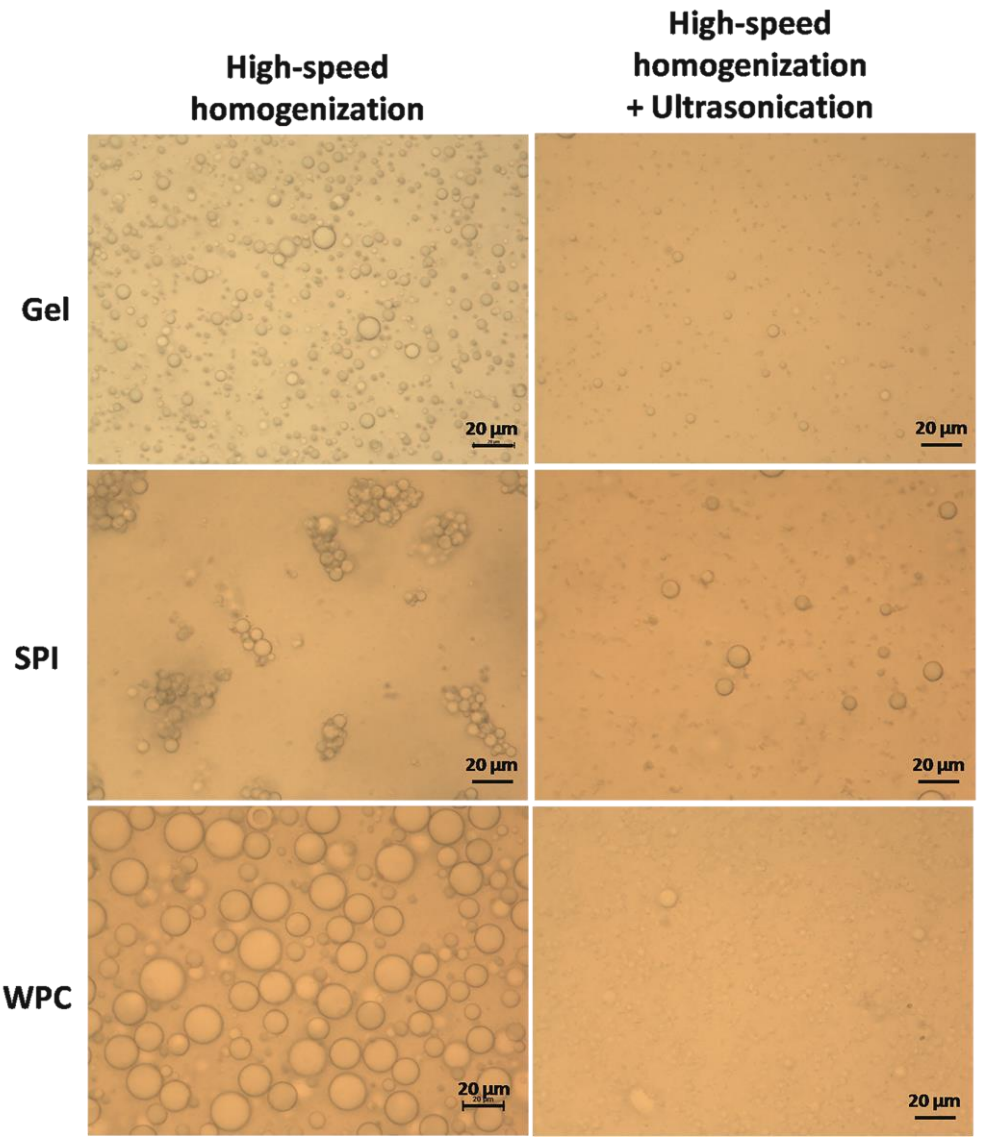


Figure 2. Optical micrographs for the different emulsions produced using SBO. Scale bars represent 20 µm.

When the second emulsification approach was applied (i.e. including an ultrasonication treatment) the droplet size was indeed greatly reduced for the three protein formulations tested. Moreover, the SPI-stabilized emulsion turned Newtonian, with a substantial decrease in its viscosity, suggesting that the flocs were disrupted. Previous studies had shown a decrease in the extent of droplet aggregation and apparent viscosity upon ultrasound treatments, in addition to a reduction of the mean particle size [23].

4.2. Creaming stability of the emulsions

The creaming index of an emulsion after a particular time lapse is an indicative of its stability to gravitational separation. As the density of the oil droplets in an O/W emulsion is lower than that of its aqueous environment, they tend to move upwards unless efficiently stabilized [27]. The creaming index (CI) of the emulsions prepared in this work after 5, 24 and 48 h are summarized in Table 1, and the appearance of the emulsions with or without a cream layer is shown in Figure S2 of the Supplementary Material.

Table 1. Creaming index (CI) of the emulsions, calculated according to Eq. (1).

Emulsion		5 h	24 h	48 h
Protein	Emulsion procedure			
Gel	HSH	0%	0%	1%
Gel	HSH+US	0%	0%	0%
SPI	HSH	4%	4%	4%
SPI	HSH+US	0%	0%	0%
WPC	HSH	0%	24%	15%
WPC	HSH+US	0%	13%	9%

Five hours after preparation the SPI emulsion prepared through high-speed homogenization (without ultrasound treatment) already experienced creaming, which was not surprising taking into account the presence of big flocs in this sample (cf. Figure 2). The rest of the emulsions did not show signs of gravitational separation during these first 5 hours, meaning that they were stable to creaming during at least the time required to electrospray them. Gelatin was the most efficient protein system evaluated for the stabilization against gravitational separation of the emulsions, only experiencing subtle creaming after 48 h when no ultrasound treatment was applied, due to the bigger droplet sizes. On the other hand, sonication had a positive effect on the stability of SPI-based emulsions, as the disruption of the flocs together with the droplet size reduction avoided the creaming phenomenon during at least 48 h. Regarding WPC, a thick cream layer appeared after 24 h, which further compacted during the following day reaching smaller CIs at 48 h. This cream layer was obviously thicker for the non-sonicated emulsion, due to its considerably bigger droplet size. Thick cream layers are usually caused by bridging flocculation, as strong attractive forces yield less packed flocs [27]. However, unlike the SPI emulsion produced through procedure 1, WPC emulsions did not show signs of flocculation during the

first hours after preparation. The reason for this late flocculation might be related to the conformational changes that globular proteins may suffer resulting from adsorption to an interface [16], consequently exposing their non-polar and cysteine residues to the aqueous phase. This phenomenon has been described for whey proteins, where disulphide cross-linking can occur at the interface [7]. In SPI, as denaturation was forced through the previous thermal treatment, a much earlier flocculation was observed until the emulsion was ultrasonicated. Conversely, the ultrasound treatment in the WPC-stabilized emulsion was applied before the addition of the protein, so it could not affect its reassembling.

4.3. Morphology of electrosprayed capsules from O/W emulsions

The production of microcapsules from protein-stabilized emulsions by spray-drying has been studied in a number of works. However, the emulsion electrospraying approach has only recently been proposed for the microencapsulation of functional ingredients [10]. Thus, in order to ascertain the feasibility of producing electrosprayed microencapsulation structures from the prepared emulsions (i.e. using SBO as a model oil), these were subjected to hydrodynamic processing (cf. Section 3.6. for process parameters) and the obtained structures are shown in Figure 3.

From the micrographs it was concluded that gelatin was the only protein which yielded proper microparticulate structures when the first emulsification procedure was used. The structure obtained using SPI showed signs of dripping and wetted particles while WPC yielded a mixed structure exhibiting a continuous polymeric surface below some spherical microcapsules. This can be explained in the light of the properties and droplet size distribution of each emulsion. While gelatin exhibited the smallest droplet sizes using Procedure 1 (cf. Figure 2), SPI emulsion formed large floccules and the size the majority of the droplets in the WPC emulsion was too big to be encapsulated within the generated smaller microcapsules. Although the effective volume of the droplet is significantly increased by the absorbed proteins on their surface [7, 28], which means that the actual oil volume is smaller than the apparent droplet size, some of the droplets in WPC and the floccules in SPI were still too big for microcapsule formation.

Upon ultrasound treatment, the droplet size was dramatically reduced for the three protein systems, so that all of them could yield neat microcapsules when electrosprayed, even though the particle size also decreased (except for WPC). The size and morphology of these particles varied from one protein to another, and this can be attributed not only to the characteristics of the emulsion droplets but also to the properties of the proteins themselves. WPC dispersions in the absence of added oils or bioactive compounds usually give rise to bigger particles than gelatin or SPI [9, 22] for similar concentrations and electrospraying conditions as the ones used in this work.

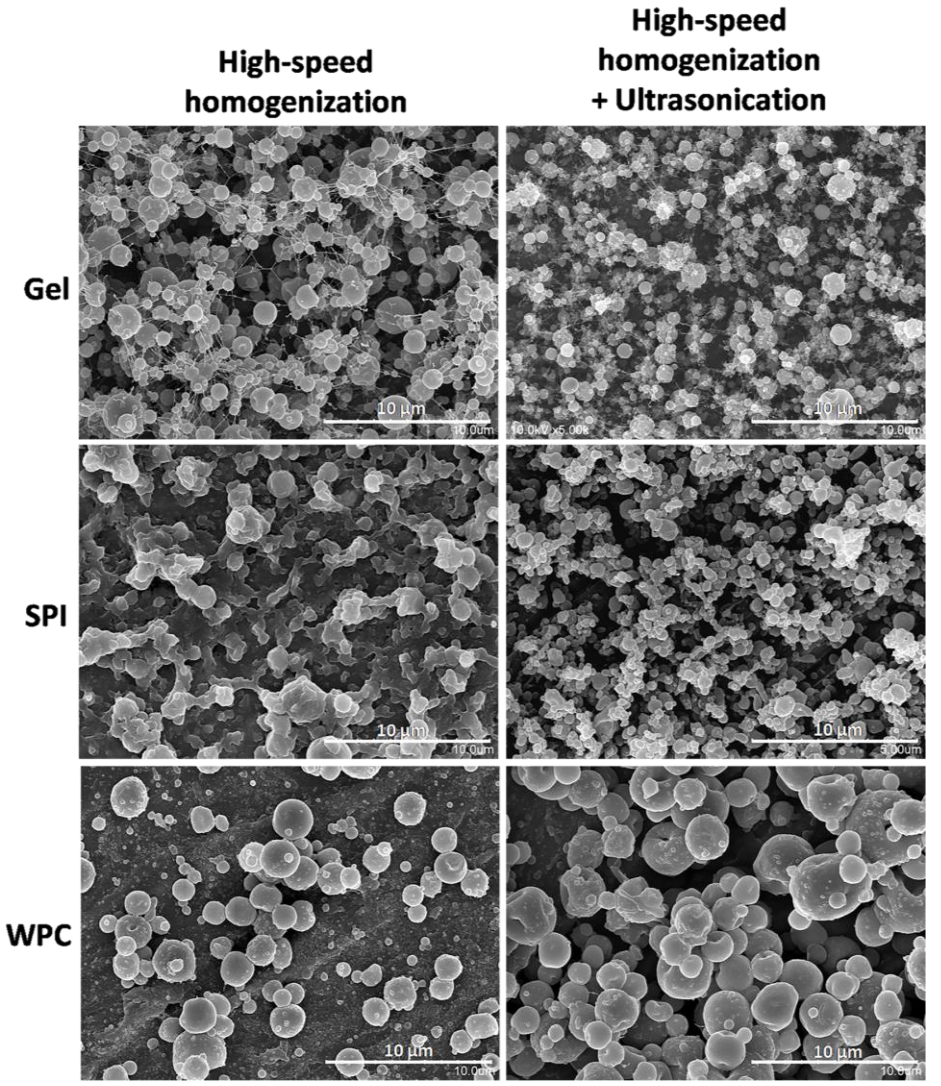


Figure 3. SEM images of electrosprayed structures obtained from the protein-stabilized SBO/W emulsions prepared using Procedure 1 (left) and Procedure 2 (right). Scale bars correspond to 10 µm.

4.4. Morphology of ALA-loaded electrosprayed and spray-dried capsules

Once the feasibility of the emulsion electrospraying technique had been confirmed for the three proteins, the model SBO was substituted by the bioactive ω-3 fatty acid, ALA. The emulsions were prepared using the second procedure, including the

ultrasound treatment, as it proved to be more adequate for the encapsulation of oil droplets in the previous section, and they were processed both by electrospraying and spray-drying. Emulsions prepared using Procedure 1 were also electrosprayed for comparison purposes. Figure 4 shows the micrographs of the obtained structures and Figure S3 of the Supplementary Material summarizes the particle size distribution for each sample.

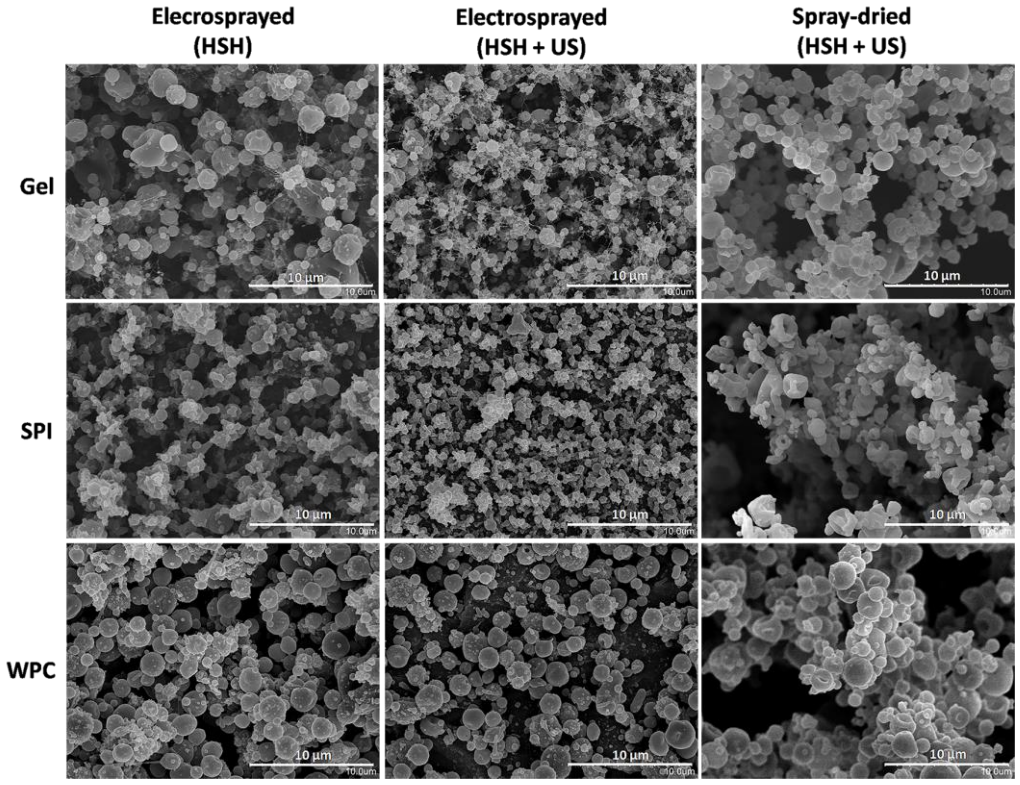


Figure 4. SEM images of ALA-loaded electrosprayed and spray-dried structures obtained from the protein-stabilized emulsions. Scale bars correspond to 10 µm.

Surprisingly, both the emulsions prepared through Procedures 1 and 2 allowed the production of micro- or submicroparticles through electrospraying. These results suggest smaller droplet sizes of the emulsions prepared with ALA in comparison with the ones prepared using the model oil, SBO. In fact, although ALA is water insoluble, it has a polar head in its structure which provides enhanced compatibility through reorganization of the lipid molecules to expose their carboxyl groups to the water interface, fact which can contribute to increased stability of the emulsions and decreased droplet size. Also, the extent of protein unfolding at the interface is usually larger for more non-polar oils [16], so the flocculation of the emulsions prepared with

the globular proteins might have been prevented. As a result, the six emulsions yielded ALA-loaded microencapsulation structures through electrospraying.

In general, the ultrasonicated emulsions led to smaller particle size distributions, with the exception of WPC, where little differences in the particle diameters were observed. This can be attributed to the increase in surface tension and electrical conductivity [29] found after the ultrasound treatment, and also to their expected smaller droplet sizes. The structures obtained through spray-drying showed bigger mean diameters than the ones obtained through electrospraying, as observed in previous works [10].

4.5. Molecular organization

FTIR spectroscopy was used to characterize the molecular organization of the microencapsulation structures. For this purpose, the spectra of emulsion electrosprayed and spray-dried ALA-loaded particles were compared to those of the raw proteins and the free fatty acid. The spectra of the unloaded particles were also obtained.

The IR spectrum of commercial ALA showed its most representative bands centered at 3013 cm^{-1} (stretching of *cis*-alkene groups -HC=CH- in PUFAs) and 1711 cm^{-1} (C=O stretching in fatty acids) [30]. Other relevant bands of the bioactive compound were found at 2965, 2932 and 2856 cm^{-1} due to the methyl asymmetrical stretching, the methylene asymmetrical stretching and the methylene symmetrical stretching vibrations, respectively [31]. On the other hand, the spectra of the three as-received proteins showed the characteristic bands ascribed to the vibration of the bonds in their amide groups, referred as the Amide A (N-H stretching), Amide B (asymmetric stretching vibration of =C-H and $-NH_3^+$), Amide I (C=O stretching), Amide II (N-H bending and stretching) and Amide III (C-N stretching) bands [32, 33]. Also noticeable are the bands observed around 2960 cm^{-1} and 2930 cm^{-1}, corresponding to the $-CH_2$ asymmetric and symmetric stretching vibrations, respectively [34].

Regarding the microencapsulation structures, differences in their characteristic bands were observed in comparison with the as-received proteins. Table 2 shows a summary of the wavenumbers at which each of these characteristic bands were found, and the complete spectra are provided as supplementary data (cf. Figure S4). Interestingly, ALA-loaded particles produced using different emulsification protocols (i.e. with or without the ultrasonication step) yielded similar infrared spectra, only differing in the intensity of the peak at 3013 cm^{-1}, due to the presence of ALA. Thus, for simplification purposes only the results for the materials obtained following the first emulsification approach are displayed. The spectral data of unloaded electrosprayed particles are also provided.

Table 2. Characteristic FTIR absorption bands (wavelengths in cm^{-1}) of as-received proteins and microencapsulation structures thereof.

	Gel (raw)	Gel ES (unloaded)	ALA-loaded Gel ES	ALA-loaded Gel SD
Amide A	3430	3312	3313	3313
Amide B	3085	3080	3080	3080
Amide I	1642	1653	1653	1653
Amide II	1543	1541	1541	1541
Amide III	1244	1244	1244	1244
-CH2	2960, 2928	2961, 2939	2961, 2931	2961, 2938
ALA	-	-	3013	-
	SPI (raw)	**SPI ES (unloaded)**	**ALA-loaded SPI ES**	**ALA-loaded SPI SD**
Amide A	3294	3289	3289	3289
Amide B	3075	3075	3075	3075
Amide I	1653	1653	1653	1655
Amide II	1534	1542	1543	1543
Amide III	1240	1251	1250	1250
-CH2	2961, 2928	2956, 2925	2957, 2927	2957, 2927
ALA	-	-	3012	-
	WPC (raw)	**WPC ES (unloaded)**	**ALA-loaded WPC ES**	**ALA-loaded WPC SD**
Amide A	3297	3293	3293	3293
Amide B	3078	3078	3077	3079
Amide I	1651	1654	1654	1654
Amide II	1540	1540	1543	1543
Amide III	1262	1249	1248	1248
-CH2	2963, 2924	2959, 2927	2959, 2927	2958, 2926
ALA	-	-	3013	-

ES = Electrosprayed; SD = Spray-dried

After processing the proteins, both by emulsion electrospraying and spray-drying, a general narrowing of the peaks was observed, which has already been described in previous works [4, 9]. Moreover, the Amide A band shifted to significantly lower wavenumbers for the three proteins, indicating changes in the hydrogen bonding structure of the proteins [35]. These changes have been previously attributed to the removal of the structural water during the rapid drying process in the formation of electrosprayed gelatin particles [9], and this hypothesis could be extended to the other proteins as inferred from the TGA analysis (see below). The Amide B band only experienced a displacement towards lower wavenumbers for gelatin, not being

significantly affected upon processing of SPI or WPC. This is attributed to the processing conditions of gelatin in diluted acetic acid and subsequent protonation of its amino groups, being this band partially due to the vibration of $-NH_3^+$ groups. Gelatin also showed the most significant changes in the displacement of the Amide I band upon processing, which is attributed to changes in the secondary structure of proteins [36]. Although the denaturation of SPI prior to processing did not lead to displacements in the Amide I band, which only narrowed, it did have an impact on the Amide II band, which is also conformationally sensitive [37, 38]), producing a considerable shift towards higher wavenumbers, as previously observed [22]. The Amide III band also experienced changes in its shape for both WPC and SPI, but not for gelatin, although the complex mixture of globular proteins present in these samples limits the interpretation of these results. Finally, the relative intensities of the bands corresponding to the asymmetric and symmetric stretching vibrations of methylene groups and their peak maxima changed upon processing of the proteins, especially for SPI and WPC (which contained the surfactant). It is worth noting that these general comments are valid for both electrosprayed and spray-dried capsules, which exhibited little differences between them in terms of the bands ascribed to the proteins.

The presence of the ω-3 fatty acid in the electrosprayed encapsulation microstructures was evidenced by the existence of its characteristic absorption band at 3013 cm^{-1} in these samples. However, this peak was not detected for any of the spray-dried materials, suggesting that the bioactive compound was completely degraded during processing through the latter technique, due to the high temperatures required for the production of the encapsulation structures in this case. As the aforementioned band is related to the presence of *cis*-alkene groups in the samples, its disappearance implies that these double bonds were no longer present in the samples, i.e. they had been oxidized. Therefore, regardless of the protein used as wall material, electrospraying proved to be an effective technique for the microencapsulation of the thermosensitive bioactive while spray-drying resulted in complete ALA degradation. Other works had reported the successful encapsulation of ALA-rich oils, such as linseed oil [39] or chia oil [40], within proteins and polyssacharides through spray-drying. However, pure ALA (>99%) showed extreme sensitivity to heat and thus it could not be detected in any of the spray-dried samples. Therefore, only the electrosprayed capsules will be considered in the following sections.

4.6. Microencapsulation efficiency

The band at 3013 cm^{-1} was also used to estimate the microencapsulation efficiency (MEE) for the electrosprayed materials, as it did not overlap with any of the bands of the proteins and it was a good indicator for the integrity of the bioactive compound. Hence, the MEE estimated in this work is based on the presence of intact double bonds in the bioactive fatty acid rather than the mere content of oil (oxidized or not) which is measured in other works by gravimetric techniques [40-42].

Calibration curves were constructed for each ALA-protein system (R^2_{Gel} = 0.999, R^2_{SPI} = 0.993 and R^2_{WPC} = 0.986) using physical mixtures of the unloaded electrosprayed proteins with known relative concentrations of the fatty acid, and using the Amide II band of each protein as a reference. The MEE of the ALA-loaded particles was then calculated using Eq. (2) and the results are summarized in Table 3.

Table 3. Microencapsulation efficiencies for the electrosprayed materials. Different letters (a-e) within the same column indicate significant differences at $p < 0.05$ among the samples.

Electrosprayed sample		
Protein	**Emulsion procedure**	**MEE (%)**
Gel	HSH	$40 \pm 4^{a,c}$
Gel	HSH+US	23 ± 12^{a}
SPI	HSH	61 ± 4^{b}
SPI	HSH+US	30 ± 3^{a}
WPC	HSH	67 ± 5^{b}
WPC	HSH+US	$50 \pm 3^{b,c}$

HSH = High-speed homogeneization; US = Ultrasonication

In general, the estimated MEE was considerably higher when no ultrasound was used for emulsion preparation. This was directly related to the heating of the emulsions during the ultrasonication treatment, which must have partially degraded the thermosensitive fatty acid. In fact, despite the short duration (i.e. 30 s) of the treatment intervals and the use of an ice bath to cool down the samples, the temperature of the emulsions after de ultrasonication treatment raised up to 45 ºC. Hence, although the second emulsification approach proved to be useful to decrease de droplet size of the emulsions and increase their stability, it caused partial oxidation of the bioactive oil before encapsulation.

Concerning the MEE of the different proteins, gelatin yielded the lowest values regardless of the emulsification protocol employed. This was attributed to the low pH of the gelatin solution prepared in diluted acetic acid, which might have contributed to the greater degradation of the fatty acid before and during processing. Recent studies also found greater extents of lipid oxidation in ALA-containing SBO at acidic pH than at neutral pH [43], supporting this hypothesis.

The best results were achieved when either SPI or WPC were used as wall matrices and the first and simplest emulsification approach was employed, both samples belonging to the same statistical group (cf. Table 3). Remarkably, those two emulsions were the most unstable when SBO was used as a model oil, even leading to bridging flocculation. However, the differences in the model oil and the bioactive oil structures might have led to improved stability of the latter, as commented above.

4.7. Thermogravimetric analysis of the materials

Thermogravimetric analysis of the raw proteins and the free ALA, as well as the unloaded and ALA-loaded electrosprayed particles, was conducted in oxidative conditions at 10ºC/min in order to assess potential thermostability changes of the bioactive ingredient upon microencapsulation. A physical mixture of the unloaded electrosprayed materials and ALA (10% w/w) was also analysed. Figure 5 shows the obtained DTG curves.

The main degradation stage (major weight loss) for free ALA had its temperature of maximum degradation rate (T_{max}) at 228 ºC. In contrast, the main degradation stage for the three proteins took place in the range of 250-400ºC for all the materials. Thus, the degradation of both components could be distinguished in the physical mixtures. For instance, a small peak at T_{max}=219 ºC was observed for SPI and a small shoulder at T_{max}=215 ºC for WPC, which were both attributed to the presence of ALA, as they were not present in the unloaded electrosprayed proteins alone. The decrease in the thermal stability of ALA in these mixtures could be attributed to its increased exposed surface when physically absorbed on the proteins. For gelatin, its physical mixture with the fatty acid exhibited a small weight loss well below the T_{max} of the free bioactive, which was absent in the neat electrosprayed gelatin, and hence was similarly attributed to the degradation of free ALA absorbed on the gelatin surface. The exceptional decrease in stability in this case could be motivated by the presence of residual acetic acid in the capsules.

The DTG curves of the ALA-loaded encapsulation structures showed similar degradation profiles to those of the unloaded particles. Both exhibited slight changes in the degradation profile of the main stage when compared to the as-received proteins. As previously reported for gelatin [9], the T_{max} of the main degradation stage of the three protein systems increased upon electrospraying, although the onset temperature decreased (i.e. the degradation started at lower temperatures). These changes have been attributed to the structural changes caused by the electrospraying process and the reduction of the particle size, which results in an increase of the specific area and, consequently, of their susceptibility to thermal degradation [9]. Regardless, the weight losses attributed to ALA in the mixtures were not found in the ALA-containing encapsulation structures, suggesting an increase in the thermal stability of the bioactive fatty acid upon encapsulation, as its degradation was delayed until its protective matrices themselves degraded.

Another interesting feature, also observed in previous works dealing with electrospraying of proteins [9], is that their fast drying triggered the removal of their structural water, so that only losses of absorbed water were afterwards detected in the processed materials (up to 100ºC), while the as-received proteins prolonged the weight loss attributed to solvent (water) evaporation up to around 125ºC in SPI and WPC and even close to 200ºC for gelatin. This would have an impact on the structural changes detected by infrared spectroscopy.

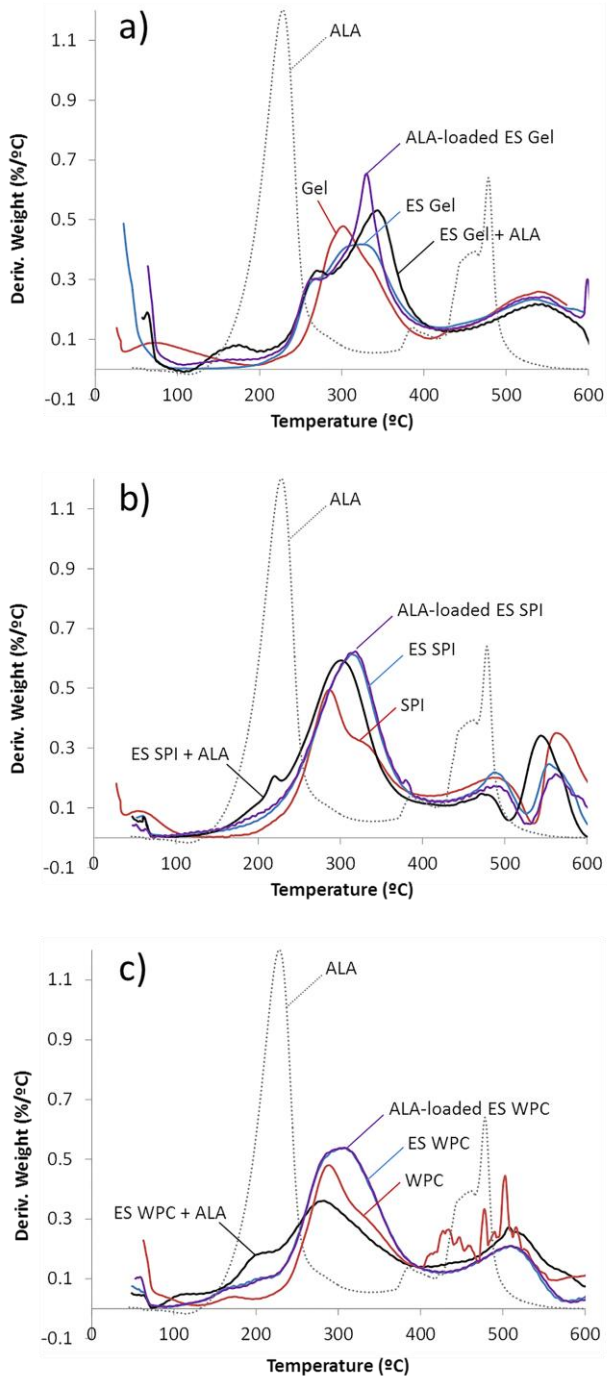


Figure 5. DTG curves of raw ALA, as-received proteins, e-sprayed particles and their mixtures for gelatin (a), SPI (b) and WPC (c).

4.8. Accelerated oxidation/degradation assays

The protective effect of the different emulsion electrosprayed encapsulation structures on the oxidative stability of ALA was assessed through an accelerated degradation assay at 80ºC. For this purpose, the relative intensity of the infrared band attributed to the presence of alkene groups (3012-3013 cm^{-1}) was measured after different time periods at this temperature. The decrease in the relative absorbance of this band was related to the extent of ALA degradation [24]. Results were normalized to the initial ALA content in the capsules for a better comparison of the different matrices, and they are shown in Figure 6.

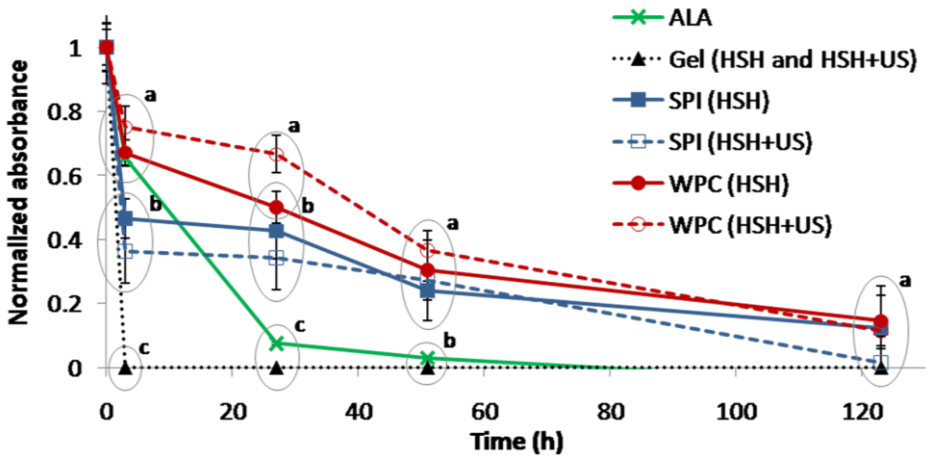


Figure 6. Degradation profiles at 80ºC for free and encapsulated ALA. Different letters (a-c) within the same time period indicate different statistical groups with significant differences among them at $p < 0.05$.

The peak of interest was not detected in any of the gelatin samples after only 3 hours of thermal treatment, emphasizing that not only this matrix did not protect ALA from thermal oxidation, but it also accelerated its degradation, most probably due to the presence of residual acetic acid in the capsules.

Regarding the globular proteins, the degradation of ALA within the WPC matrices did not show significant differences with that of free ALA during the first 3 h, although the stability of the encapsulated fatty acid was significantly improved during the following days. This fast, initial degradation could be attributed to the fraction of the bioactive allocated on the surface or very close to the surface of the particles.

A similar degradation profile was found for ALA-loaded SPI capsules. However, in this case during the first hours of high temperature exposure, a greater extent of degradation was observed in comparison with that of free ALA. This might be attributed to the increased specific area of the ALA domains located on the surface of the particles. Compared to free ALA in bulk, whose specific area exposed to air was

low, the encapsulated fatty acid was fragmented into very small domains (droplets) by emulsification prior to electrospraying, greatly increasing its specific area. Thus, if a fraction of this oil remained on the surface of the particles, it would be more exposed to the environment. This statement would also be applicable to WPC, meaning that the fraction of oil on the surface of the protein particles would be lower for the WPC than for SPI. This difference could be attributed, among other factors, to the bigger particle sizes of the WPC capsules, which thus had smaller specific surface area. After the first three hours, the degradation of encapsulated ALA, both within SPI and WPC particles, was significantly delayed with respect to free ALA, highlighting the effective protection of these electrosprayed matrices against oxidation at high temperatures.

While the protection exerted by the WPC capsules was enhanced compared to the SPI particles during the first hours of treatment, no significant differences were observed among the samples after 2 days. Furthermore, the procedure used for the preparation of the emulsions had little effect on the degradation profiles, despite its impact on the encapsulation efficiency. Only WPC showed a significant difference between both methodologies after the first 27 hours of treatment, most probably due to the bigger droplet sizes of the non-ultrasonicated emulsion, which led to a bigger fraction of non-encapsulated or superficial oil, taking into account that the particle size of the capsules was very similar for both approaches.

5. Conclusions

A novel emulsion electrospraying technique has been used to develop protein-based microencapsulation structures for the protection of ALA (used as a model thermosensitive hydrophobic bioactive compound) and compared with a well-established technology used in the food industry such as spray-drying. Being ALA a thermosensitive compound, spray-drying was inappropriate for this purpose, completely degrading the ω-3 fatty acid. As hypothesised, the electrospraying technique proved to be a satisfactory alternative, achieving microencapsulation efficiencies of up to 67% ± 5%. It was also found that the low pH required for processing gelatin through electropraying resulted in quick degradation of the encapsulated bioactive, while the ultrasound treatment for emulsion preparation also decreased the MEE due to heating. Thus, the best results were achieved using the globular proteins (WPC and SPI) and the simple homogenization procedure for the preparation of the emulsions, significantly delaying ALA oxidation during accelerated degradation assays at 80ºC. The overall results of the present work demonstrate the potential of electrospraying of protein-stabilized emulsions for the microencapsulation and enhanced protection of thermosensitive and hydrophobic bioactive ingredients, specifically ω-3 fatty acids, offering an improved alternative to traditional technologies used in the food industry such as spray-drying, which gives rise to oxidative degradation and does not significantly protect ω-3 fatty acids [44]. Further research

will be needed to extend the applicability of these results to a wider range of hydrophobic bioactive ingredients and wall materials.

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8. Supplementary Material

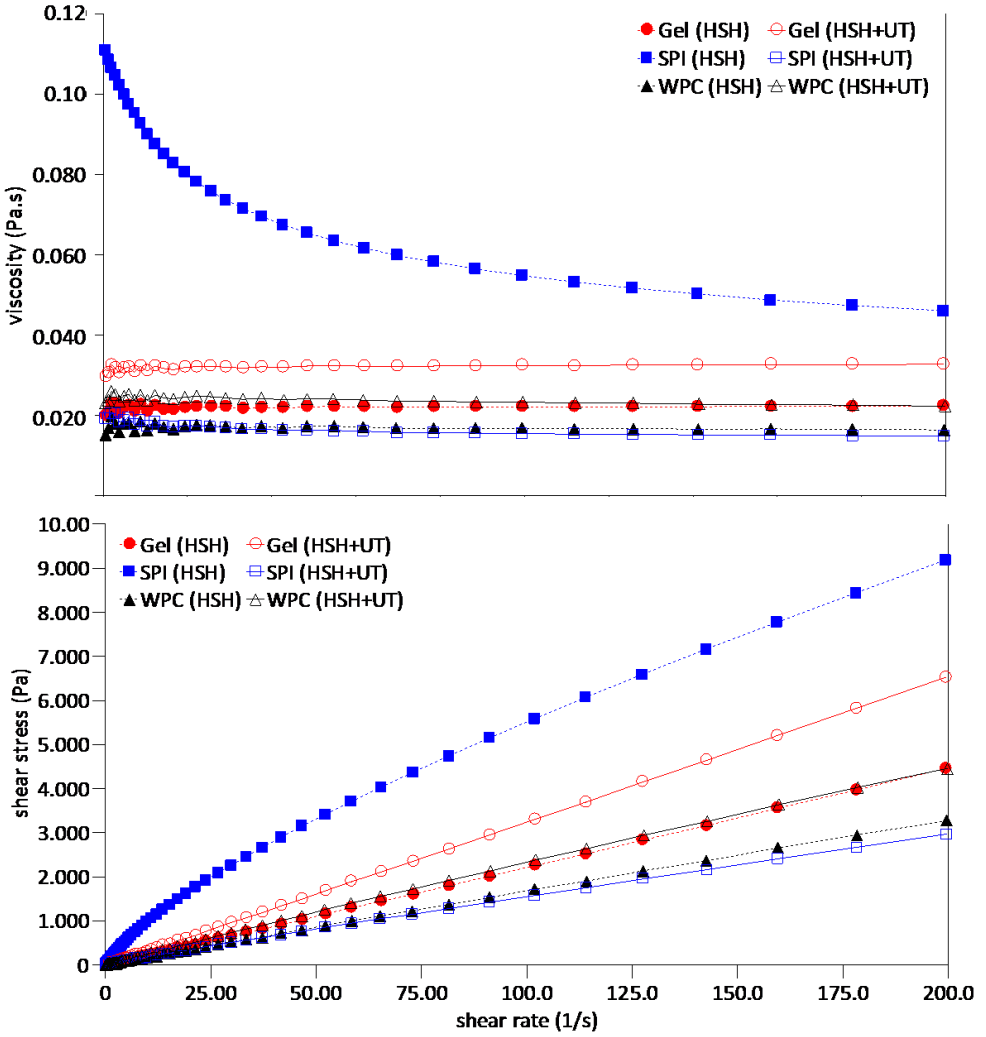


Figure S1. Rheological behaviour of the protein-stabilized emulsions: variation of the viscosity (up) and the shear stress (bottom) as a function of the shear rate.

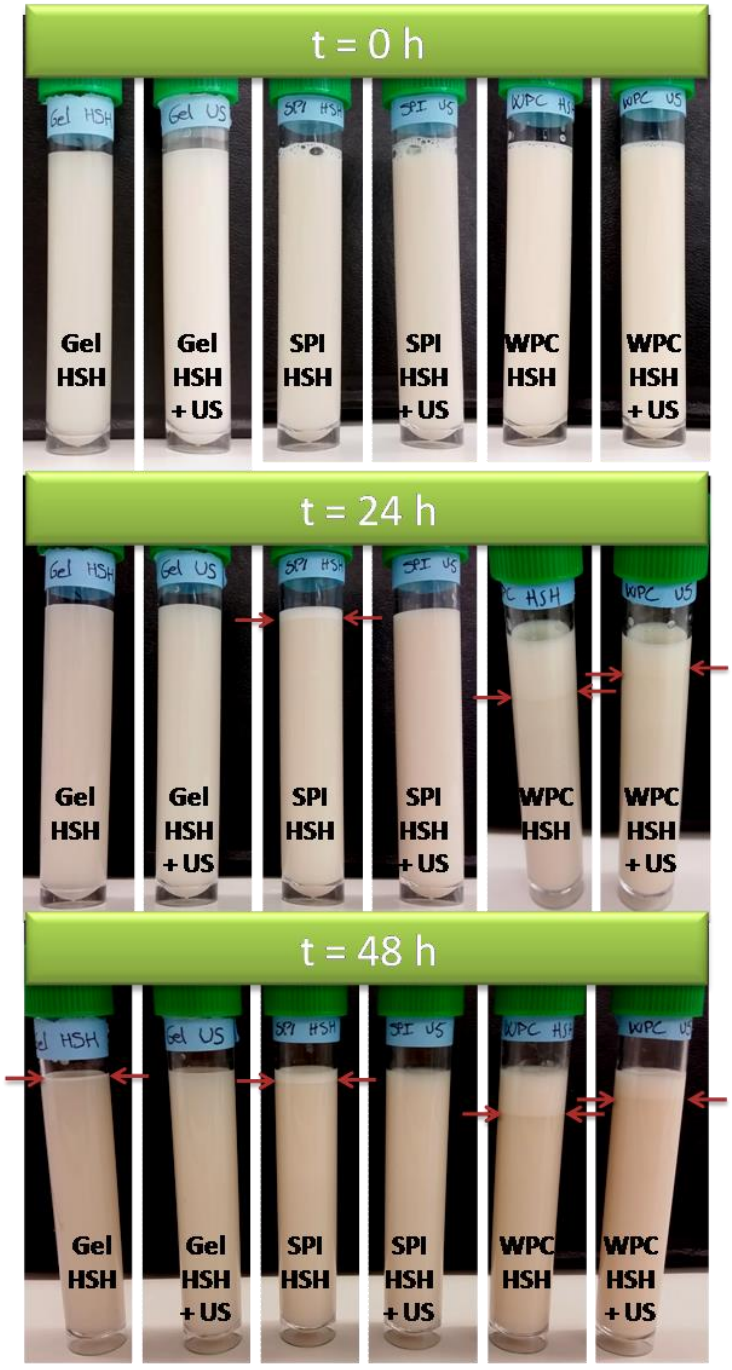


Figure S2. Appearance of the protein-stabilized emulsions during the creaming index assay. Arrows indicate the presence of a cream layer.

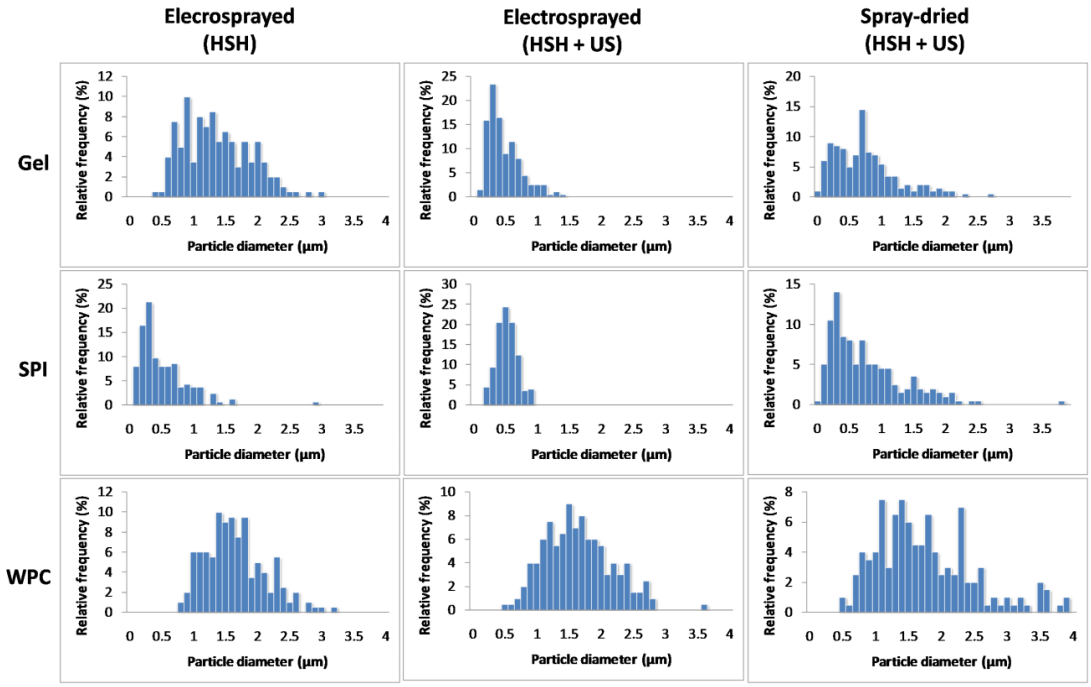


Figure S3. Particle size distributions for ALA-loaded electrosprayed and spray-dried encapsulation structures.

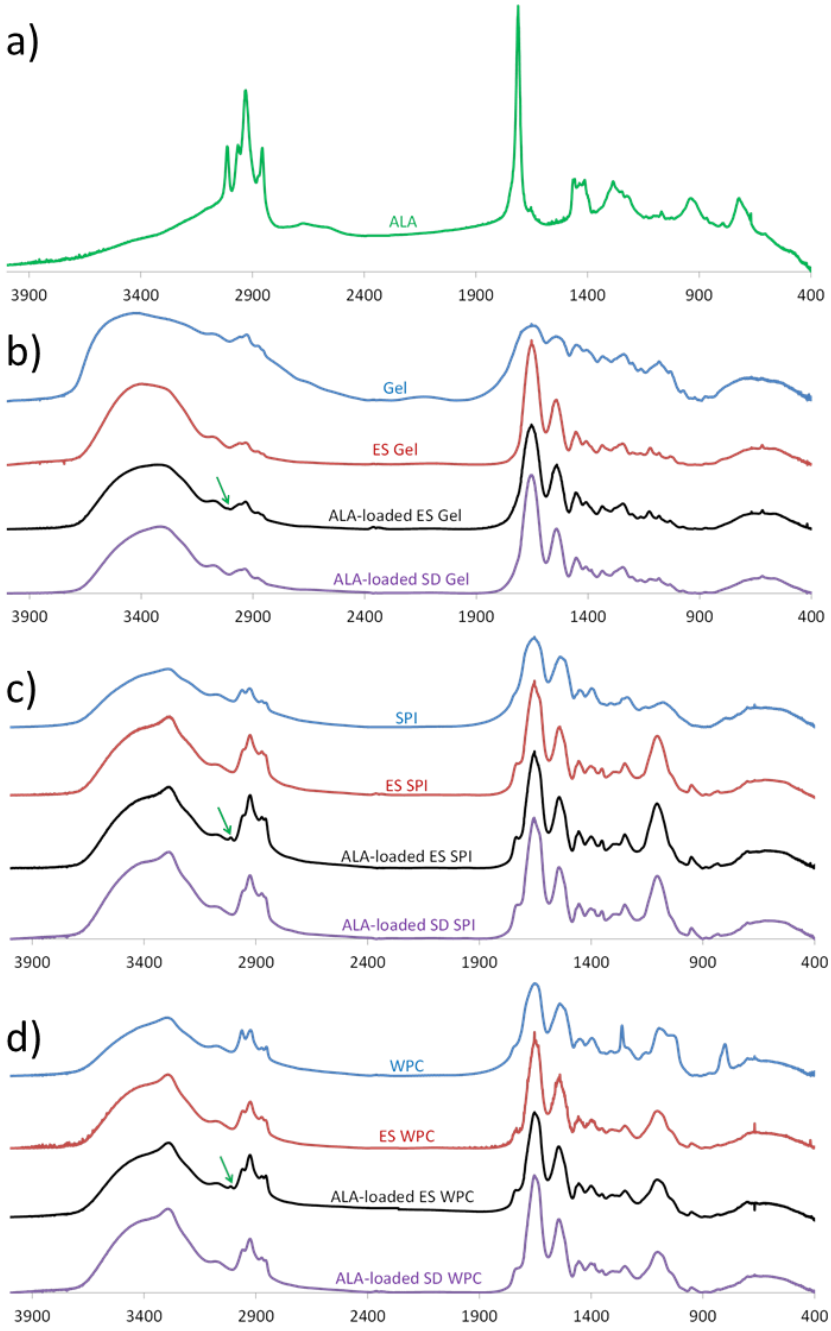
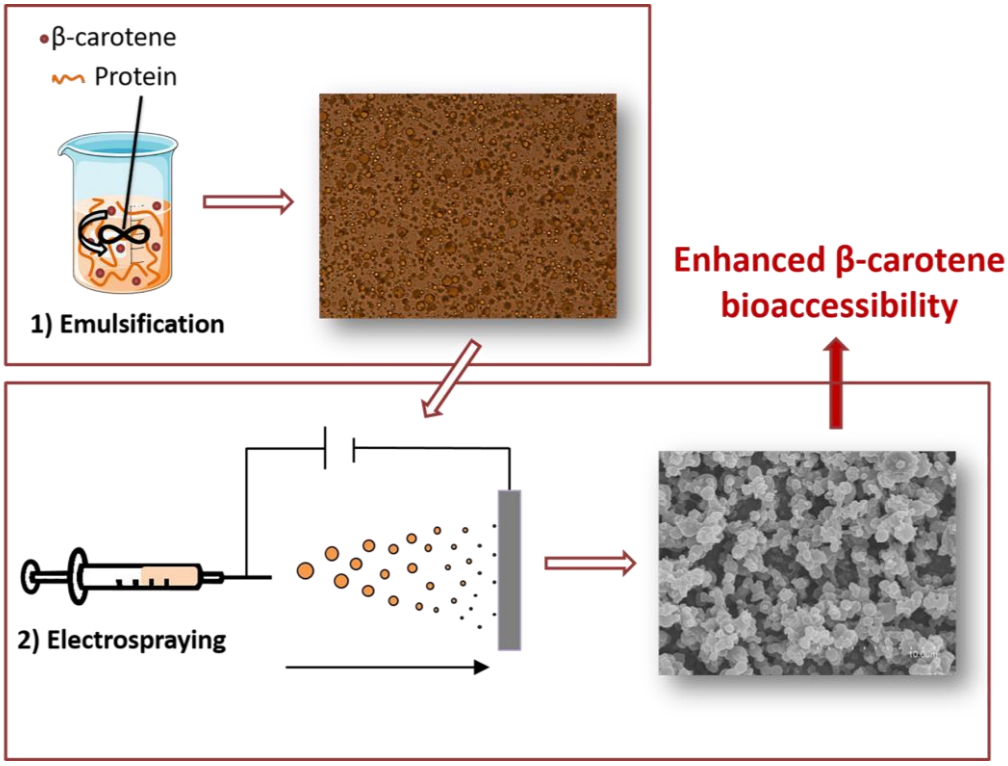


Figure S4. FTIR spectra of raw ALA (a) and as-received proteins, unloaded electrosprayed (ES) particles, and ALA-loaded electrosprayed (ES) and spray-dried (SD) encapsulation structures for gelatin (b), SPI (c) and WPC (d).

2.2

POTENTIAL OF MICROENCAPSULATION THROUGH EMULSION-ELECTROSPRAYING TO IMPROVE THE BIOACCESIBILITY OF β-CAROTENE



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Pérez-Masiá, R., González-Barrio, R., Periago, M. J., & López-Rubio, A. (2017). *Potential of microencapsulation through emulsion-electrospraying to improve the bioaccesibility of β-carotene*. Food Hydrocolloids, 73, 1-12. (DOI: https://doi.org/10.1016/j.foodhyd.2017.06.019).

1. Abstract

The development of carotenoid-enriched functional foods is limited by the low bioaccessibility of these bioactive compounds. The aim of this work was to improve the bioaccessibility of β-carotene after *in-vitro* digestion through its encapsulation within electrosprayed protein microparticles. Two different protein matrices (zein and a whey protein concentrate, WPC) and two emulsification procedures (high-speed homogenization and ultrasonication) were used to prepare the microcapsules through emulsion-electrospraying, using a soy bean oil as lipid carrier, and the impact of the emulsion properties on the microencapsulation efficiency (MEE) and the bioaccessibility of β-carotene was studied. Results showed that the stability of the prepared emulsions was the main factor affecting the microencapsulation efficiency. The application of an ultrasonic treatment was necessary to stabilize the WPC emulsions and increase the MEE of the WPC microcapsules, but had a slight negative impact on the total β-carotene content of the zein particles, due to thermal degradation of β-carotene, without significantly affecting their MEE. The highest MEE was achieved for the capsules obtained from zein emulsions (34±7%). All the encapsulation structures, except those obtained from WPC emulsions prepared by high-speed homogenization, increased the bioaccessibility of β-carotene after *in-vitro* digestion, which was negligible in its free form.

2. Introduction

Carotenoids are a group of natural pigments with many attributed health benefits when consumed in sufficient levels [1, 2]. Especially, β-carotene has been described to exert protection against a number of severe health disorders, including cancer, cardiovascular diseases or macular degeneration [3, 4]. Hence, there is increasing interest in the incorporation of β-carotene as a functional ingredient in food formulations. However, the poor solubility of this compound in aqueous media complicates its application in the food industry and causes its bioavailability to be extremely low [5].

In order for β-carotene to be uptaken by the enterocytes in the small intestine, it has to be previously incorporated into mixed micelles during digestion [6]. Its simultaneous consumption with digestible lipids has shown to enhance its bioavailability [7-9], since they can solubilize β-carotene and transport it to the mixed micelles via the free fatty acids which are released upon lipid digestion, thanks to the biliary salts and pancreatic lipase [10]. Hence, emulsion-based systems have been proposed as vehicles for the incorporation of β-carotene into aqueous food products in

order to increase its bioaccessibility [1]. However, the microbiological stability of oil-in-water emulsions is generally poor, especially when proteins are used as emulsifiers, since they favour the intensive growth of microorganisms [11]. In order to improve their storage stability, these emulsions can then be dried to obtain microencapsulation structures [5] obtaining easy-to-handle powdery ingredients.

Spray-drying is the most frequently employed technique in the food industry to produce dry microencapsulation structures from emulsions [12]. It has been previously employed for the microencapsulation of carotenoids, including β-carotene, within a number of biopolymeric matrices, being the most commonly used arabic gum, maltodextrins and modified starches [13-16]. However, this technology involves the use of high temperatures, which may have a negative impact on thermosensitive bioactives like carotenoids. Alternatively, electrospraying is emerging as a promising technology for the microencapsulation of labile bioactive compounds, as it allows processing at room temperature [17]. It is a technique based on the electrohydrodynamic processing of polymeric fluids, which consists of subjecting the feed solution or emulsion to a high voltage electric field in such a way that a charged polymer jet is ejected from a conductive capillary through which it is pumped, to a grounded collector. Due to the electrostatic forces generated within the fluid, the jet breaks into small droplets and the solvent evaporates before the material is deposited on the collector in the form of dry micro- or nanoparticles [18].

Recently, Pérez-Masiá et al. (2015) used the emulsion-electrospraying technique to microencapsulate lycopene, another prominent carotenoid, and compared the performance of the obtained structures with those obtained by spray-drying. Their results showed that much higher encapsulation efficiencies were obtained through electrospraying, due to the thermal degradation of lycopene upon spray-drying [19]. Similar results were also obtained for other thermosensitive bioactive compounds, such as α-linolenic acid [17]. Furthermore, the encapsulation of these and other labile bioactive ingredients (e.g. polyphenols, vitamins and probiotics) within electrosprayed microparticles has proven to successfully increase their stability in detrimental pH conditions [18], high relative humidity [20] or when subjected to a thermal treatment [21]. β-Carotene has been previously incorporated within protein fibers by electrospinning, increasing its light stability [22]. However, the potential of electrosprayed microencapsulation structures, which are easier to disperse into food products than fiber matts [18], to increase the bioaccessibility of carotenoids has not been explored yet.

In the present work, β-carotene was microencapsulated through emulsion-electrospraying for the first time, and the potential of the proposed systems to increase its bioaccessibility after *in-vitro* digestion was assessed. For this purpose, two different proteins were used as encapsulation matrices, i.e. zein and a whey protein concentrate (WPC). Proteins are particularly interesting matrices for emulsion electrospraying, since their amphiphilic structures allow their use as effective emulsifiers [23], and both electrospun/sprayed zein and WPC had previously shown to enhance the stability of β-carotene [22, 24]. A soy bean oil (SBO) was used as a carrier oil to dissolve β-carotene and oil-in-water emulsions were prepared through

two different procedures, the first one consisting of a single high-speed homogenization step and the second one including a subsequent ultrasonication treatment. The emulsions and the encapsulation structures obtained thereof were characterized and the microencapsulation efficiency of the different systems was estimated. Moreover, the recovery of β-carotene from the mixed micelles fraction of the digestas was assessed after an *in-vitro* digestion simulation.

3. MATERIALS AND METHODS

3.1. Materials

Whey protein concentrate (WPC) was kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark). Under the commercial name of Lacprodan® DI-8090, the composition per 100 g of product consisted of ~80 g of protein, ~9 g of lactose, and ~8 g of lipids, being the rest water and minerals like sodium and potassium. Zein from corn (grade Z3625, 22–24 kDa), β-carotene, soybean oil (SBO), Tween20®, pepsin from porcine gastric mucosa, bile extract porcine, pancreatin from porcine pancreas and dimethylformamide (DMF) were supplied by Sigma-Aldrich (Spain). 96% (v/v) ethanol was purchased from Panreac (Spain). All inorganic salts used for the *in-vitro* digestion assays were used as received. Methanol and tert-butyl methyl ether (HPLC grade quality) were provided by Sigma-Aldrich (Spain).

3.2. Preparation of the emulsions

The emulsions were prepared based on the procedures described in Gómez-Mascaraque and López-Rubio (2016) [17], with some modifications. The aqueous phase of each emulsion consisted of a protein solution/dispersion in water or in a water-ethanol mixture. Two different proteins were used to prepare the emulsions: whey protein concentrate (WPC) and zein. Soy bean oil (SBO) was used as the oily phase. When β-carotene was incorporated into the emulsions, it was previously dissolved in SBO at a concentration of 5% (w/w). For this purpose, brief heating (4 to 5 min on a plate at 90 ºC) was necessary [1]. Tween20® was used as a surfactant to help stabilizing the emulsions and decreasing the surface tension to improve the subsequent electrospraying process.

3.2.1. Preparation of the protein solutions/dispersions

Each protein was dissolved/dispersed in an adequate solvent at the optimal concentration determined in previous works to allow subsequent processing by electrospraying. Thus, WPC (20% w/v) was dispersed in distilled water [17], and zein (12% w/v) was dissolved in a water-ethanol mixture (20:80 v/v) [25].

3.2.2. Preparation of the premix

Tween20® (5% w/v) was added to the protein dispersions and the mixtures were stirred until homogenization. Then, SBO (or β-carotene-containing SBO) was added in a proportion of 50% (w/w) with respect to the mass of protein, to achieve a final content of β-carotene of 10% (w/w) with respect to the mass of protein in the final bioactive-containing capsules.

3.2.3. Emulsification

The premixes were emulsified using two different procedures, based on the protocols described in [17]. The first one consisted of a one-step high-speed homogenization process conducted using an IKA T-25 Digital ULTRA-TURRAX® (Germany) equipped with a S 25N-25F dispersing element (stator diameter of 25 mm) at 6000 rpm during 5 min. The other approach included a second step of ultrasonication aimed at reducing the droplet size [26], which was performed using a ultrasonic probe (Bandelin electronic, Germany) at an amplitude of 10% and a frequency of 20 kHz for 2 min in pulse mode (50% active cycle), using an ice bath to avoid excessive heating of the samples.

3.3. Characterization of the emulsions

The surface tension of the emulsions was measured using the Wilhelmy plate method in an EasyDyne K20 tensiometer (Krüss GmbH, Hamburg, Germany) at room temperature.

The electrical conductivity of the emulsions was measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain) at room temperature.

The rheological behaviour of the emulsions was studied using a rheometer model AR-G2 (TA Instruments, USA), with a parallel plate geometry, and the method described in [27]. Briefly, continuous shear rate ramps were performed from 1 to 200 s^{-1} during 20 min at 25ºC ± 0.1ºC using a stainless steel plate with a diameter of 60 mm and a gap of 1 mm. All measurements were made at least in triplicate.

Optical and fluorescence microscopy images were also taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. The autofluorescence of β-carotene in the emulsions was captured using a 480/15 excitation filter and 535/20 emission filter. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

3.4. Stability of the emulsions

The creaming index method [28] was used to assess the stability of the emulsions. The emulsions were introduced in sealed tubes and stored for different time periods (i.e. 5, 24 and 48 h) at room temperature. When phase separation occurred, the height of the cream layer (H_c, phase rich in oil) and the total height of each emulsion in the tube (H_E) where measured, and the creaming index (CI) was calculated according to Eq. (1):

$$CI = 100\ [(H_E - H_c)/H_E] \qquad \text{Eq. (1)}$$

3.5. Encapsulation of β-carotene through electrospraying

The emulsions were processed using an electrospinning/electrospraying apparatus assembled in house, equipped with a variable high-voltage 0-30 kV power supply and a Fluidnatek® L-10 syringe pump. Emulsions were introduced in a 5 mL plastic syringe and pumped at a steady flow-rate of 0.15 mL/h through a stainless-steel needle, which was connected to the syringe through a PTFE tube and placed perpendicularly to the stainless-steel plate used as collector, at a distance of 10 cm. The applied voltage was 13kV for zein emulsions and 15-16 kV for WPC emulsions, based on preliminary trials. Samples were processed through electrospraying during 5 hours after preparation of the emulsions.

3.6. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of ~10 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the FIJI software [29]. Size distributions were obtained from a minimum of 200 measurements.

3.7. Fourier transform infrared (FT-IR) analysis of the particles

Free and microencapsulated β-carotene (ca. 1-2 mg) was grounded and dispersed in about 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the samples at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

3.8. Microencapsulation efficiency

The total amount of β-carotene incorporated within the electrosprayed materials was calculated as the percentage of the initial β-carotene content added to the emulsions that was present in the electrosprayed materials, according to Eq. (2).

$$\text{Total } \beta - \text{carotene } (\%) = \frac{\text{total } \beta-\text{carotene content in electrosprayed materials}}{\text{theoretical } \beta-\text{carotene content in feed emulsions}} \times 100 \quad \text{Eq. (2)}$$

The microencapsulation efficiency (MEE) of the β-carotene loaded capsules was calculated as the percentage of the initial β-carotene content added to the emulsions that was effectively incorporated within the cores of the electrosprayed microparticles, according to Eq. (3).

$$\text{MEE } (\%) = \frac{\text{total } \beta-\text{carotene content in electrosprayed materials} - \beta-\text{carotene content on the surface}}{\text{theoretical } \beta-\text{carotene content in feed emulsions}} \times 100 \quad \text{Eq. (3)}$$

The total content of the carotenoid present within the electrosprayed materials was estimated by UV-vis measurements after extraction of the bioactive compound from the electrosprayed structures. For this purpose, WPC particles (ca. 10 mg) were disrupted in distilled water at 2 mg/mL, releasing their contents. The reconstituted emulsions were subsequently diluted 2-fold with DMF to dissolve the β-carotene content and the WPC was removed by centrifugation. On the other hand, zein particles (ca. 10 mg) were directly dissolved in DMF at 2 mg/mL, as the protein is soluble in this solvent. In both cases, the absorbance of the resulting solutions was measured at 451 nm using a V-1200 spectrophotometer (VWR, Barcelona, Spain). Calibration curves for β-carotene quantification were previously obtained for the carotenoid in DMF/H_2O (50:50) and in DMF in the presence of 1 mg/mL of zein ($R^2_{DMF/H2O}$ = 0.9998, $R^2_{DMF/zein}$ = 0.9991).

The content of β-carotene on the surface of the capsules was estimated according to a method adapted from Umesha, Monahar and Naidu (2013) [30]. Briefly, the capsules (ca. 10 mg) were re-suspended in hexane (1 mg/mL), vortexed for 5 s and immediately filtered using Whatman™ filter devices with 0.2 μm PTFE membranes (GE Healthcare Life Sciences, UK). The absorbance of the filtrate at 451 nm was also measured as explained above, previous calibration (R^2_{hexane} = 0.9994).

3.9. *In-vitro* digestion

Suspensions of pure (ca. 2.5 mg) and microencapsulated β-carotene (ca. 200 mg) in 2.5 mL distilled water, i.e. a theoretical β-carotene concentration of 1 mg/mL in all cases, were subjected to *in-vitro* digestion following the protocol described in [31]. Solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the compositions described in [32]. In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at

37 ºC for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (3.8 mg/mL), and incubated at 37 ºC for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (37.8 mg/mL) and porcine pancreatin (16.25 mg/mL), and incubated at 37 ºC for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively, using 1M HCl or 1M NaOH solutions. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added.

3.10. HPLC analysis of bioaccessible β-carotene after *in-vitro* digestion

The samples obtained from the *in-vitro* digestion assays after the duodenal phase (the 'digestas') were centrifuged at 1795 g and 4ºC for 20 min using an Eppendorf 5804R Centrifuge (Eppendorf, Germany), and aliquots of the clear micelle phase, i.e. disregarding the sediment phase at the bottom and the oily phase at the top when present [1], were collected. One aliquot of each sample was then filtered using 0.45 µm, nylon, Whatman® Syringe filters in order to remove any residual large particles or droplets, as described in [1]. The micelle phase of the digestas was then extracted as follows: 5 mL of methanol/tetrahydrofuran (1/1, v/v) was added to 2 mL of sample, mixed by vortexing during 1 min and evaporated using a speed-vacuum concentrator (Savant™ SPD131DDA SpeedVac™ Concentrator, Thermo Scientific, UK). The residue was resuspended in 0.5 mL of tert-butyl methyl ether and MeOH (50:50, v/v), centrifuged at 20.817*g* during 10 min at room temperature, and analyzed by HPLC-DAD. Chromatography separation was performed using a C30 column 250 x 4.6 mm, 5 µm i.d. (Trentec, Gerlingen, Germany) maintained at 17 ºC, and using tert-butyl methyl ether (A) and MeOH (B) as mobile phases at a flow of 1 mL/min. The gradient started with 2% A in B, to reach 35% A at 35 min, 60% A at 45 min, 60% at 55 min, followed by washing and then a return to the initial conditions. β-carotene (all-*trans*-β-carotene) was identified in the chromatograms according to their UV spectra and retention times, by comparisons with the standard, and the chromatographic peak area, recorded at 450 nm, was quantified as β-carotene.

3.11. Statistical analysis

A statistical analysis of experimental data was performed using IBM SPSS Statistics software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups were obtained through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

4. RESULTS AND DISCUSSION

4.1. Characterization of the emulsions SBO-protein emulsions

The properties of emulsions, which depend both on their composition and on the size and aggregation state of the dispersed phase, determine factors such as their stability or the bioavailability of the active ingredients they may contain [33, 34]. Moreover, the successful production of microcapsules through electrospraying is strongly dependent on the feed solution/emulsion properties. For instance, the viscosity should be enough to lead to the necessary polymer entanglements to form the encapsulation structures, the surface tension should be low enough for the Taylor cone to form and yield a stable electrospraying process, and the electrical conductivity should not be too high as to not destabilize the electrospraying jet [35-37]. Therefore, the SBO/protein emulsions prepared through both emulsification procedures were thoroughly characterized before incorporation of β-carotene to produce the encapsulation structures. Table 1 shows the viscosity (at 200 s^{-1}), surface tension and electrical conductivity of all the different formulations assayed. The complete rheological profiles of the emulsions are also shown in Figure 1.

The surface tension of all the emulsions was low enough to allow the success of the electrospraying process, as they did not surpass the limit of 50 mN/m above which, according to previous studies, a liquid cannot be electrosprayed [38]. The lower values achieved for the zein emulsions can be related to the presence of ethanol in the continuous phase, whose surface tension is lower than that of water. Moreover, no significant differences were observed before and after the application of the ultrasound treatment for a given composition. Neither did the electrical conductivity significantly change upon ultrasonication which, as expected, was considerably higher for the WPC emulsions, due to the greater water content of their continuous phase and higher protein concentration. The rheological properties of the emulsions, however, did change upon ultrasonication for the WPC-stabilized emulsions. These exhibited a shear-thinning behaviour, which is generally indicative of a certain degree of flocculation [28]. In this case, the viscosity significantly increased upon ultrasonication, which generally suggests a decrease in the droplet size since the average distance of separation between the droplets decreases [39]. Moreover, ultrasound treatments have been previously reported to alter the structural characteristics of whey proteins [40, 41]. Unfolding of globular proteins and subsequent increased exposure of their non-polar amino acids, which would facilitate the interaction among different protein chains, could also contribute to the observed change in the rheological properties. Conversely, zein emulsions exhibited a Newtonian behaviour, indicative of well dispersed and non-flocculated droplets [28],

and their viscosity was not altered after the ultrasound treatment. Apparently, the non-globular structure of this protein, with a dominant α-helical conformation in ethanolic solution [42], was less affected by the ultrasound treatment.

Table 1. Properties of the SBO/protein emulsions. Different letters (a-b) within the same column indicate significant differences at $p < 0.05$ among the samples.

Emulsion		Surface tension (mN/m)	Electrical conductivity (μS/cm)	Viscosity (mPa·s)
Protein	Emulsion procedure			
Zein	HSH	25.9 ± 0.1 [a]	386 ± 1 [a]	19.7 ± 0.9 [a]
Zein	HSH+US	26.1 ± 0.1 [a]	373 ± 5 [a]	19.6 ± 0.9 [a]
WPC	HSH	36.5 ± 0.2 [b]	1102 ± 51 [b]	21.3 ± 0.1 [a]
WPC	HSH+US	36.5 ± 0.2 [b]	1149 ± 9 [b]	25.7 ± 0.3 [b]

HSH = High-speed homogenization; US = Ultrasonication

4.2. Morphology and creaming stability of the β-carotene loaded emulsions

Once the emulsions had been characterized, β-carotene was incorporated in their oil phase by dissolving it in the SBO before emulsification. Figure 2 shows the appearance of the obtained emulsions. Due to the extremely high turbidity of the WPC emulsions, their accurate observation was not possible. However, it can be inferred from the pictures that some big-sized droplets were present in the WPC emulsion prepared using only high-speed homogenization (Figure 2a), while their size was reduced upon ultrasonication (Figure 2b), as previously observed for similar emulsions [17]. In contrast, due to the good solubility of zein in ethanol and the subsequent transparency of the continuous phase of its emulsions, the droplets in that case could be clearly observed. Again, some bigger droplets were observed in the emulsions prepared using only high-speed homogenization (Figures 2c, e), and the size was slightly reduced upon ultrasonication (Figures 2d, f). It is also noticeable that, although β-carotene has a low solubility in ethanol, this solubility is so low that the carotenoid is preferentially located in the disperse phase of the zein emulsions (Figures 2e, f, were fluorescence from β-carotene can be observed).

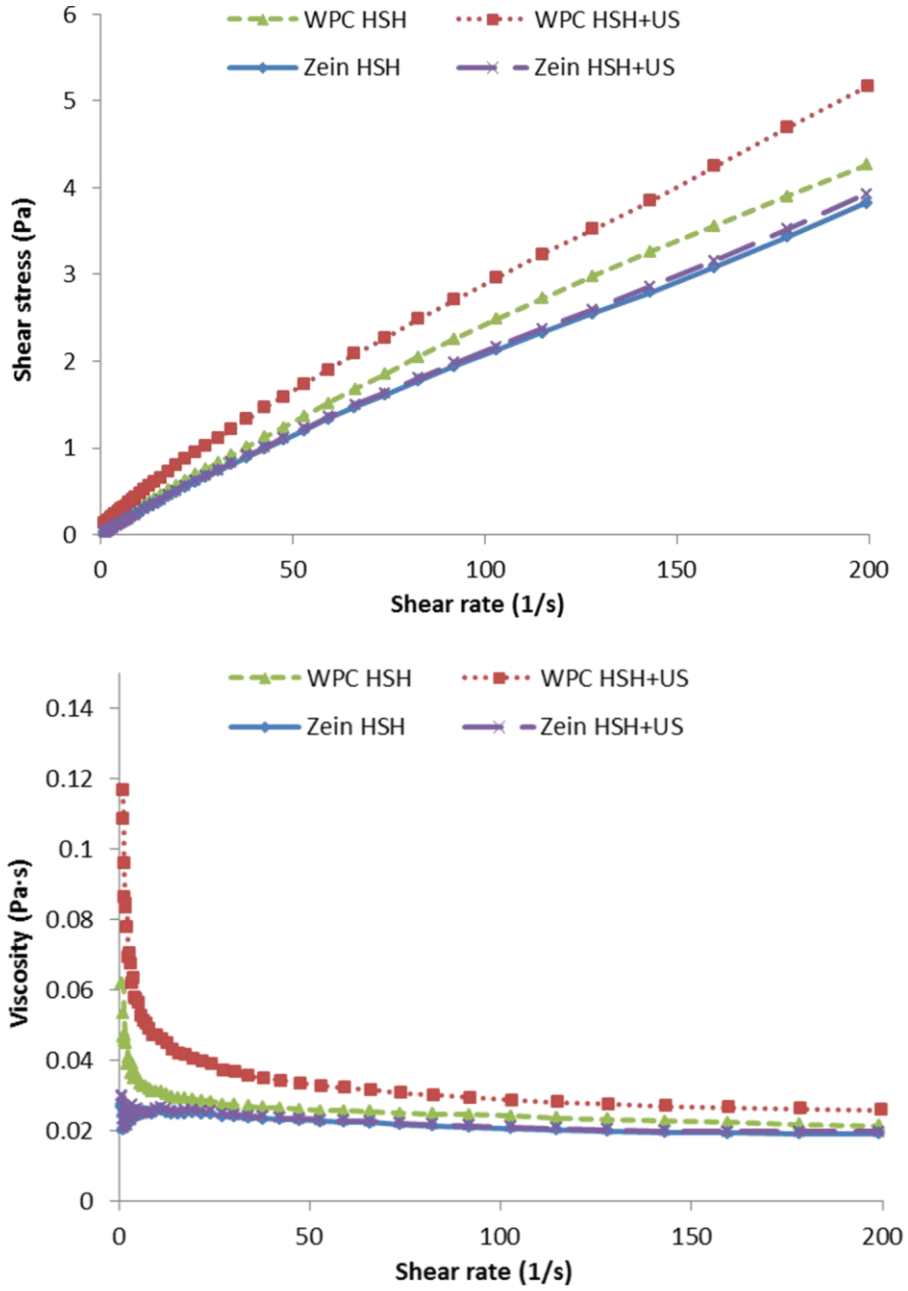


Figure 1. Rheological behaviour of the emulsions: variation of the shear stress (up) and viscosity (bottom) as a function of the shear rate.

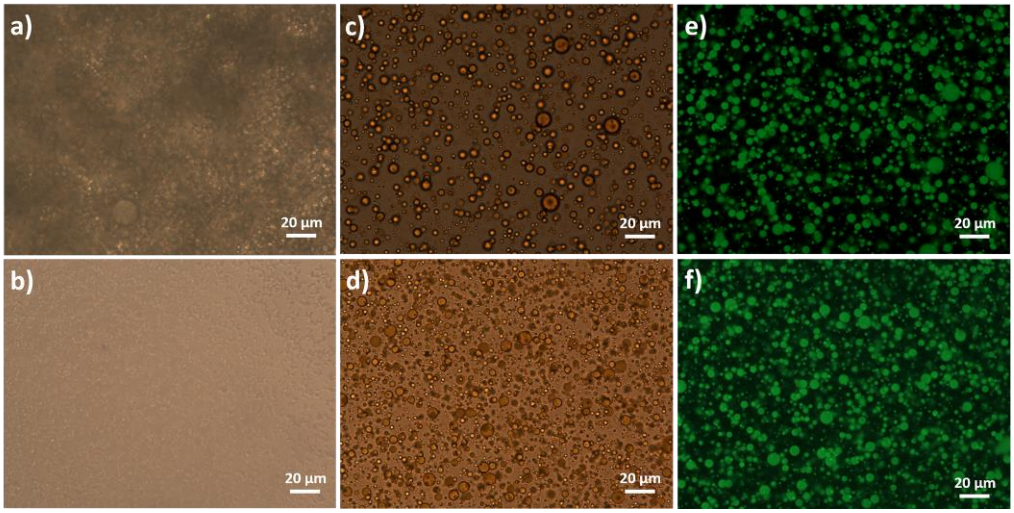


Figure 2. Optical micrographs of the β-carotene loaded emulsions prepared using WPC (a, b) and zein (c, d), and fluorescence micrographs of zein emulsions containing β-carotene (e, f). Emulsions a, c and e were prepared by high-speed homogenization. Emulsions b, d and f were prepared applying an additional ultrasonication step. Scale bars represent 20 µm.

The creaming index of the different emulsions was also calculated after different storage periods as a measure of their stability to gravitational separation. It is worth mentioning that the cream layer was placed at the top in the WPC emulsions, as the density of the oil droplets is lower than the aqueous WPC dispersions, but was placed at the bottom in the zein emulsions, due to the lower density of the continuous phase consisting of an ethanolic zein solution. Results are shown in Figure 3.

From the analysis of the creaming index of the different emulsions it could be concluded that the most unstable formulation was the WPC emulsion prepared with a single high-speed homogenization step, as it experienced creaming even during the first 5 hours of storage. Consequently, phase separation of this emulsion occurred during the electrospraying process. Conversely, when the WPC emulsion was subjected to an additional ultrasonic treatment, it became very stable, not experiencing phase separation even after 48 hours of storage. This was due to both the reduction in the droplet size of the emulsions, as inferred from Figure 2, and the unfolding/reassembly of the proteins upon ultrasonication, which might have enhanced the emulsifying properties of WPC through an increase in the exposure of their non-polar amino acids and thus faster adsorption of the protein to the oil-water interface [43]. Regarding the zein emulsions, little differences in their creaming stability were observed depending on the emulsification procedure used. The droplet deposition at the bottom of the tubes after long storage periods was only slightly

lower when the ultrasonication treatment was applied, presumably due to the presence of slightly smaller droplets (cf. Figure 2). Nevertheless, both zein emulsions were stable during the whole processing time through electrospraying (i.e. 5 h).

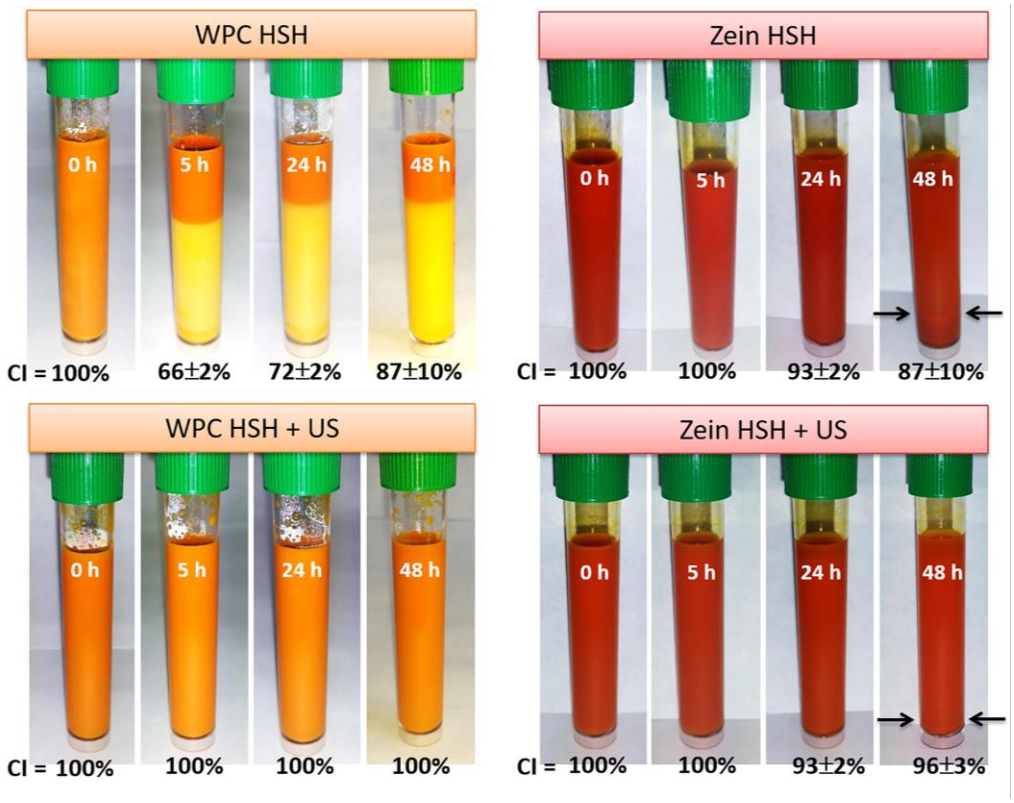


Figure 3. Images of the emulsions after storage for 0, 5, 24 and 48 h, together with the values of their creaming index (CI).

4.3. Morphology of the β-carotene-containing microcapsules

Figure 4 shows the SEM images of the β-carotene-containing encapsulation structures prepared with the different feed emulsions, and their particle size distributions are shown in Figure 5. The WPC particles obtained from the emulsion prepared in a single high-speed homogenization step were quasi-spherical and very similar in size to those obtained in a previous work for SBO/WPC emulsions [17]. However, hollow holes were observed in some of the particles, which suggest that some oil droplets were not successfully trapped within the encapsulation structures, probably due to their big size. Therefore, a low encapsulation efficiency was anticipated for this sample. These holes were not observed when the ultrasound

treatment had been applied. Also, a significant reduction in the particle size was observed for the WPC samples upon ultrasonication. This was attributed to the reduction of the droplet size of the emulsion and the conformational changes of the protein, which had an impact on the rheological properties of the emulsions, as discussed above. On the other hand, zein yielded smaller particles than WPC when the emulsion was prepared by high-speed homogenization, and exhibited a rougher surface with a greater content of nanofibrils. This morphology, which is frequently observed in electrosprayed zein microstructures [25], was very similar for both emulsification methods, as the ultrasound did not cause significant changes in the emulsion properties in this case (cf. Sections 4.1 and 4.2).

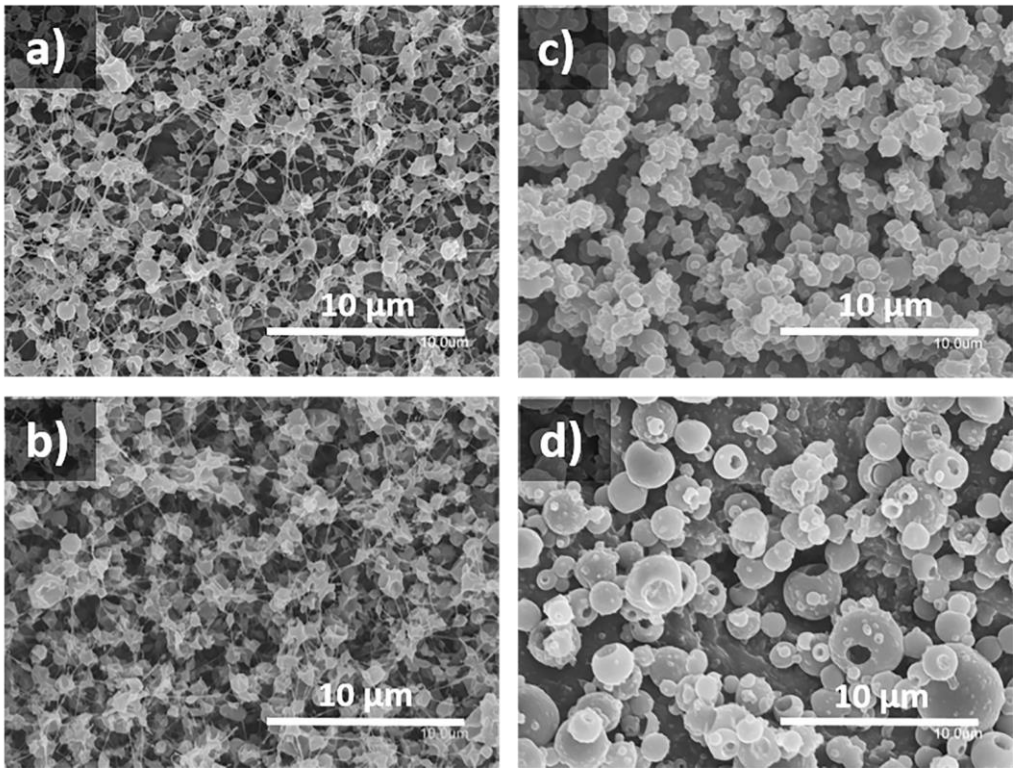


Figure 4. SEM images of the β-carotene loaded electrosprayed capsules prepared using zein (a, b) and WPC (c, d). Feed emulsions for samples in b and d were prepared by high-speed homogenization. Feed emulsions for samples in a and c were prepared applying an additional ultrasonication step. Scale bars represent 10 µm.

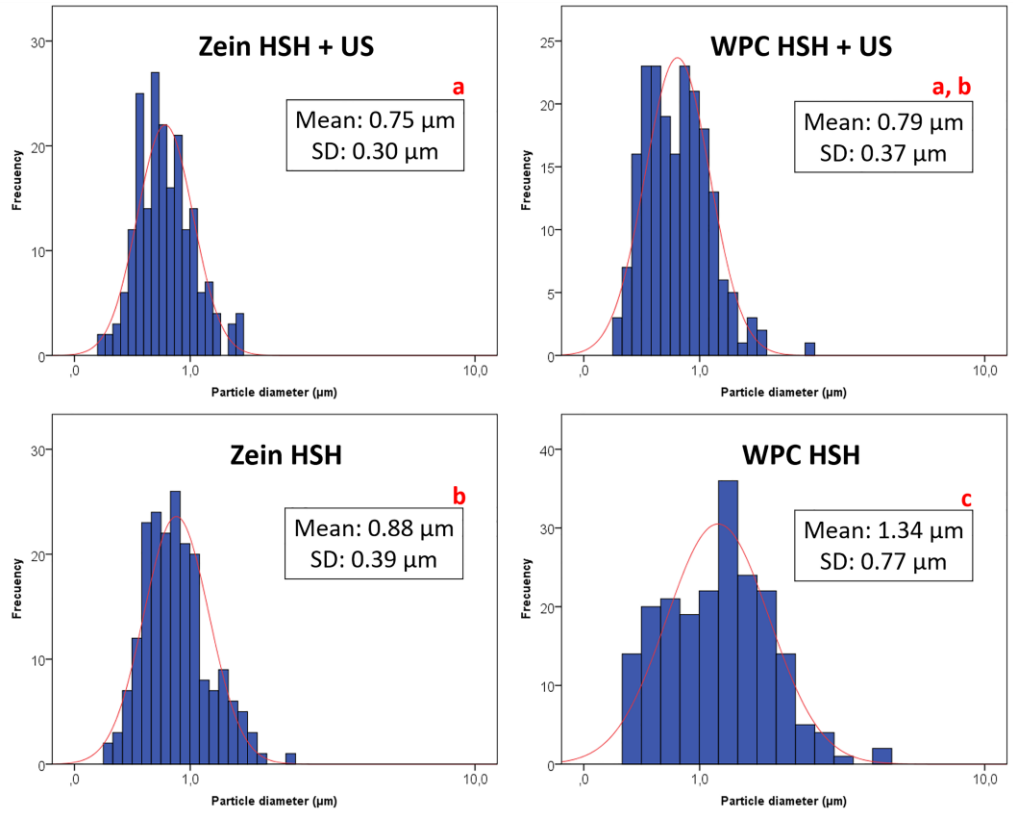


Figure 5. Particle size distributions of the β-carotene loaded electrosprayed capsules. Different letters in red (a-c) placed above the legends indicate significant differences at $p < 0.05$ among the samples.

4.4. Analysis of the particles through infrared spectroscopy

FTIR spectroscopy was used to chemically analyze the obtained capsules. Also, the spectra of the unloaded electrosprayed proteins (in the absence of β-carotene and its carrier oil) was obtained for comparison purposes. Figure 6 shows the obtained spectra.

The IR spectrum of the β-carotene-loaded capsules showed contributions from both the protein wall material and the carrier oil containing the carotenoid. The characteristic bands of proteins, i.e. amide A or N-H stretching (3294 cm^{-1} for WPC and 3299 cm^{-1} for zein), amide B or asymmetric stretching vibration of =C-H and $-NH_3^+$ (3072 cm^{-1} for WPC and 3065 cm^{-1} for zein), amide I or C=O stretching (1648 cm^{-1} for WPC and 1660 cm^{-1} for zein), amide II or N-H bending and stretching (1546 cm^{-1} for WPC and 1541 cm^{-1} for zein), and amide III or C-N stretching (1241 cm^{-1} for WPC and 1245 cm^{-1} for zein) [44, 45] were present in all the encapsulation structures.

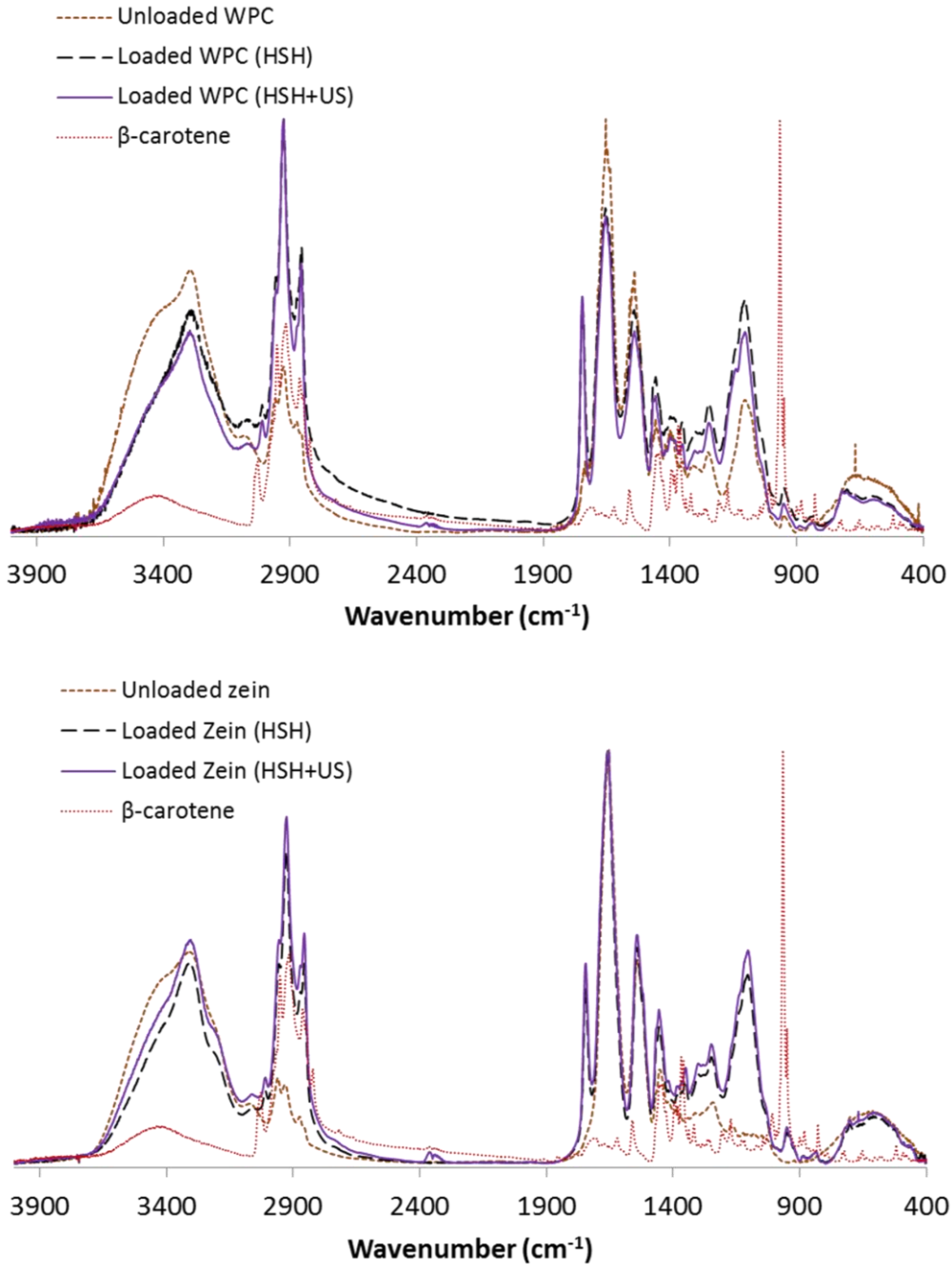


Figure 6. FT-IR spectra of the electrosprayed capsules.

The β-carotene-loaded capsules showed a considerable increase in the absorbance of the bands between 3000 cm^{-1} and 2800 cm^{-1} with respect to their unloaded counterparts, due to the contribution of the SBO [19]. In addition, a band centred at 1748 cm^{-1} corresponding to the C=O groups of the triglycerides from the SBO [46] appeared in the loaded structures. A band at 950 cm^{-1} was also found in the loaded structures. This band might be ascribed to the contribution of C=C bonds found in carotenoids [47], as reported previously for lycopene-loaded capsules [19]. However, the spectra of the electrosprayed capsules produced from the SBO emulsions in the absence of β-carotene also showed this band (results not shown), hindering its sole attribution to the presence of β-carotene. This, together with the low mass ratio of β-carotene in the capsules, hampered the unequivocal identification of β-carotene in the FTIR spectra of the electrosprayed materials, as well as the evaluation of its possible interactions with the proteins. Nevertheless, the presence of β-carotene in the capsules can be indirectly inferred from the presence of the carrier oil in which it was dissolved.

4.5. Microencapsulation efficiency

Table 2 summarizes the total and surface β-carotene contents, and the microencapsulation efficiencies of the different materials. The total β-carotene contents in the electrosprayed materials were reasonably high except for the samples obtained from the WPC emulsions prepared by high-speed homogenization. In that case, the great instability of the emulsions and subsequent phase separation during the electrospraying process explained the great loss of β-carotene via dripping, as the creaming of the emulsions caused the gravitational separation of the oil droplets from the aqueous polymeric dispersion in the syringe. In contrast, the rest of the emulsions were stable throughout their processing by electrospraying (5 h, cf. Figure 3), and consequently the incorporation of β-carotene within the resulting materials was significantly higher. Amongst them, the highest β-carotene content was obtained for the zein emulsions prepared in a single high-speed homogenization step. Ultra-sonication of emulsions containing thermosensitive bioactives has been previously reported to decrease the amount of ingredient incorporated within electrosprayed capsules despite increasing emulsion stability [17], which has been ascribed to an increase in the temperature of the emulsions caused by the ultrasonication treatment. This seemed to be also applicable to the systems developed in this work.

On the other hand, results showed that a considerable amount of the β-carotene present in the electrosprayed materials was located at the surface of the capsules, especially for the WPC emulsions prepared by high-speed homogenization, for which most of their β-carotene content was located on the surface. Consequently, the amount of β-carotene located in the core of these capsules and, thus, their MEE, was

almost negligible. The ultrasonication treatment, however, managed to significantly increase the MEE of the samples prepared from WPC emulsions, due to an increase in their gravitational stability and a reduction of their droplet size distribution (cf. Section 4.2). Zein showed higher MEE values than WPC, although a considerable amount of superficial β-carotene was also found in these samples. The amount of superficial β-carotene significantly decreased upon ultrasonication of the zein emulsions, presumably due to the reduction in their droplet size. Hence, although ultrasonication reduced the total amount of β-carotene in the zein capsules, the remaining β-carotene was more efficiently incorporated within the cores of the capsules, resulting in similar MEE values for the electrosprayed zein microstructures obtained from both emulsification procedures.

Table 2. Total and surface β-carotene contents, and microencapsulation efficiencies of the electrosprayed microcapsules (MEE). Different letters (a-c) within the same column indicate significant differences at $p < 0.05$ among the samples.

Sample		Total β-carotene (%)	Surface β-carotene (%)	MEE (%)
Protein	Emulsion procedure			
Zein	HSH	74 ± 3 [a]	41 ± 6 [a]	33 ± 9 [a]
Zein	HSH+US	61 ± 3 [b]	27 ± 4 [b]	34 ± 7 [a]
WPC	HSH	26 ± 4 [c]	21 ± 4 [b]	6 ± 8 [b]
WPC	HSH+US	57 ± 1 [b]	35 ± 2 [a,b]	22 ± 3 [a,b]

4.6. Bioaccessibility of β-carotene

Free and microencapsulated β-carotene was subjected to *in-vitro* digestion in order to study the impact of microencapsulation on the bioaccessibility of the carotenoid. The clear micelle phase (i.e. disregarding the sediment phase at the bottom and the oily phase at the top when present) of each digesta was then filtered as suggested by Quian et al. (2012) to remove large particles (> 450 nm) that would not be expected to be directly absorbed by epithelium cells [1]. Figure 7 shows the HPLC chromatograms of the filtered clear micelle phase of the obtained digestas.

No peaks attributable to β-carotene were detected in the chromatograms of the digesta obtained from free β-carotene, so its bioaccessibility was considered negligible. Certainly, in the absence of a carrier oil to dissolve it, the crystalline, insoluble carotenoid in its free form could not be incorporated into bioaccessible micelles. Huo, Ferruzzi, Schwartz and Failla (2007) [48] reported that relatively low amounts of triolein and canola oil (0.5-1%) were needed to achieve maximum

micellization of carotenoids, while higher amounts were required (~ 2.5%) when the triglycerides of the oil were mainly constituted by medium-chain saturated fatty acids. For this reason, crystals were collected in the digestas from the pure β-carotene, both in the sediment phase after centrifugation and retained on the filter, which both showed the presence of red precipitates.

Conversely, the presence of β-carotene was detected in the digestas of the microencapsulated carotenoid, except for the sample obtained from the WPC emulsion prepared in a single high-speed homogenization step, probably due to the negligible MEE of this system. In fact, only 26% of the initial β-carotene added was present in these capsules (cf. Table 2), so the content of β-carotene before digestion was considerably low. In the rest of the chromatograms, the peak at a retention time of 39 min was identified as all-trans β-carotene. Other peaks appeared at 27, 31 and 41 min, which showed similar spectra but different retention times as all-trans β-carotene. These were ascribed to β-carotene cis isomers. According to the elution order, the isomers were tentatively identified as 15-cis, 13-cis and 9-cis β-carotene [49]. Although all-trans-β-carotene was used in the encapsulation process, during *in-vitro* digestion the low pH of the gastric phase could have increased the content of *cis*-isomers β-carotene, previously described for other carotenoids [50]. The pH driven isomerization is considered responsible, at least partially, for the high *cis*-isomers proportion of carotenoids found in the human body after their consumption [51, 52].

These results showed that the microencapsulation of β-carotene within protein matrices by emulsion-electrospraying was a successful approach to improve the bioaccessibility of β-carotene. Emulsification had already been described as an effective approach to increase the bioaccessibility of β-carotene [1], but the combined emulsion-electrospraying encapsulation technique adds the advantage of obtaining a dry, easy-to-handle powdery ingredient. However, the obtained bioaccessibilities were still quite low. Only 0.01-0.02 % of the encapsulated all-*trans* β-carotene was detected in the bioaccessible fraction of the digestas (0.03-0.05 % of total carotenoids, taking into account the *cis*-isomers). Previous works demonstrated that factors such as the chain length and saturation degree of the fatty acids have an impact on the incorporation of carotenoids into mixed micelles, and consequently on their bioaccesibility and bioavailability [1]. For instance, carotenoids have shown higher micellarization extent in the presence of triolein and canola oil than in trioctanoin and coconut oil [53]. The main fatty acid in canola oil and triolein is oleic acid, which only represents a 25-30% of total fatty acids in soya oils [54] like the one used in this work. Given the decisive impact that the lipid profile of the carrier oil has on the bioaccessibility of carotenoids, future works should study the impact of different carrier oils on the microencapsulation of β-carotene through emulsion-electrospraying and its subsequent bioaccessibility in order to improve the potential of this technique.

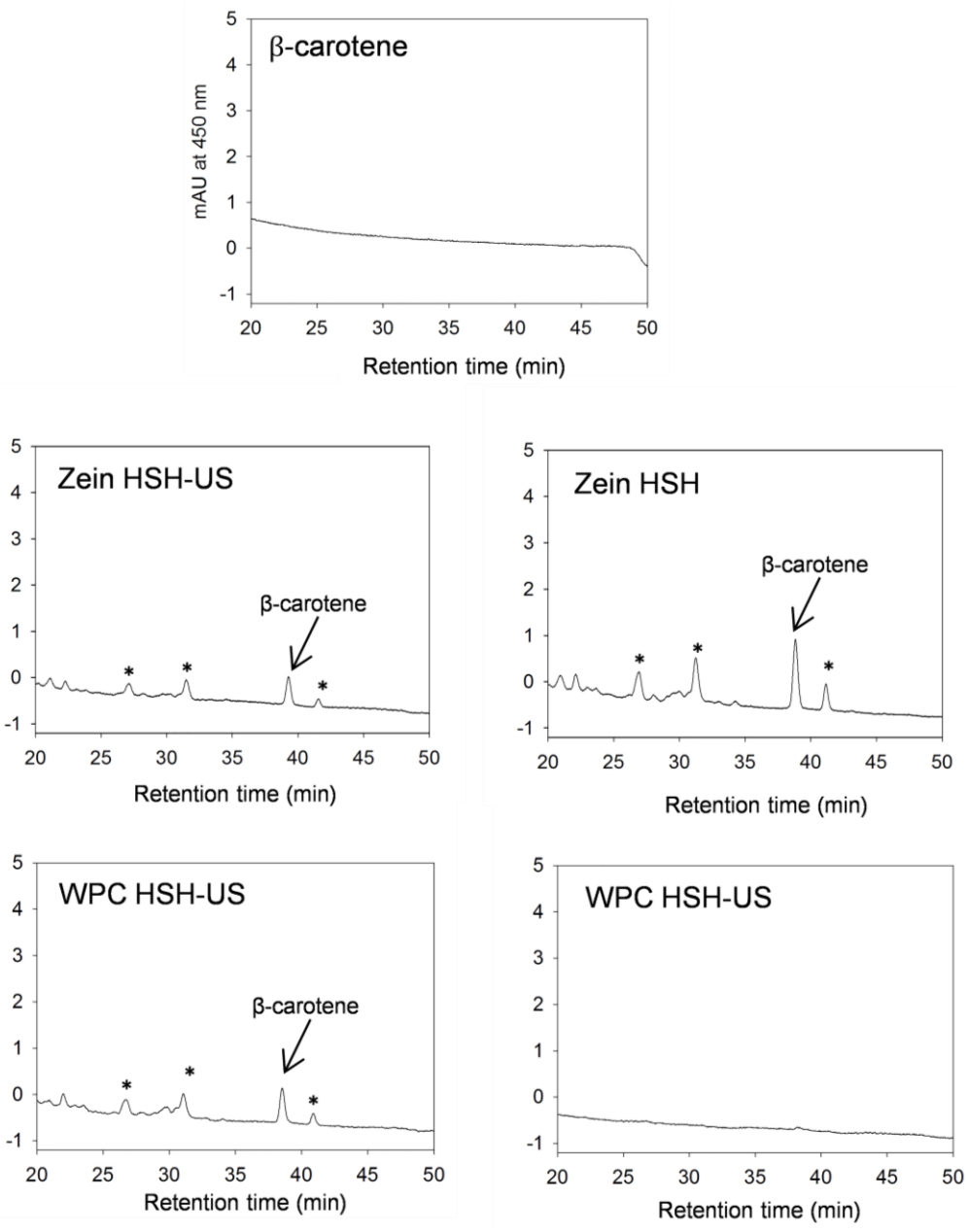


Figure 7. Chromatograms of clear micelle phase of the digestas from free and microencapsulated β-carotene.

5. Conclusions

β-Carotene was encapsulated within zein and WPC microstructures by emulsion-electrospraying, using SBO as carrier oil and two different emulsification procedures, one consisting of a single high-speed homogenization step and the other including a subsequent ultrasonication treatment. The results showed that the properties of the zein emulsions did not significantly change upon ultrasonication, while the rheological profile, the droplet size and the stability of the WPC emulsions were noticeably affected by the emulsification procedure, due to the unfolding and reassembling of the globular proteins caused by the ultrasonication treatment. Although all the formulations could be electrosprayed, the WPC emulsions prepared by high-speed homogenization were unstable to gravitational separation, resulting in a negligible encapsulation efficiency. The rest of the systems exhibited reasonably high β-carotene contents, up to 74 ± 3% for the zein particles obtained from the emulsions prepared by high-speed homogenization. However, only around half of the total β-carotene content in the microcapsules was incorporated in their cores, the rest being located on their surface. Although the ultrasonication treatment was necessary for the stabilization of the WPC emulsions and increase in the microencapsulation efficiency of electrosprayed WPC capsules, it reduced the amount of total β-carotene incorporated within the zein microstructures due to thermal degradation of β-carotene, but did not affect their overall MEE. Nevertheless, all the microencapsulation structures except the WPC particles obtained from unstable emulsions increased the bioaccessibility of β-carotene after *in-vitro* digestion, which was negligible in its free form. Although the bioaccessibility was still considerably low, the present study shows the potential of the emulsion-electrospraying technique to enhance the bioaccessibility of carotenoids, prior optimization of the feed formulations.

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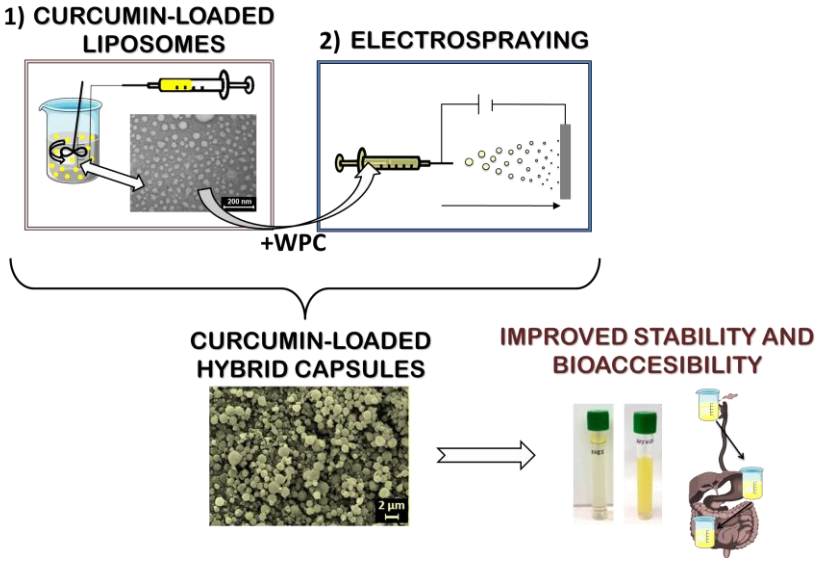
2.3

MICROENCAPSULATION STRUCTURES BASED ON PROTEIN-COATED LIPOSOMES OBTAINED THROUGH ELECTROSPRAYING FOR THE STABILIZATION AND IMPROVED BIOACCESSIBILITY OF CURCUMIN



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Casagrande Sipoli, C., de La Torre, L. G., & López-Rubio, A. (2017). *Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin*. Food Chemistry, 233, 343-350. (DOI: https://doi.org/10.1016/j.foodchem.2017.04.133).

1. Abstract

Novel food-grade hybrid encapsulation structures based on the entrapment of phosphatidylcholine liposomes within a WPC matrix through electrospraying were developed and used as delivery vehicles for curcumin. The loading capacity and encapsulation efficiency of the proposed system was studied, and the suitability of the approach to stabilize curcumin and increase its bioaccessibility was assessed. Results showed that the maximum loading capacity of the liposomes was around 1.5% of curcumin, although the loading capacity of the hybrid microencapsulation structures increased with the curcumin content by incorporation of curcumin microcrystals upon electrospraying. Microencapsulation of curcumin within the proposed hybrid structures significantly increased its bioaccessibility (~1.7-fold) compared to the free compound, and could successfully stabilize it against degradation in PBS (pH = 7.4). The proposed approach thus proved to be a promising alternative to produce powder-like functional ingredients.

2. Introduction

Curcumin, whose chemical structure is depicted in Figure S1 of the Supplementary Material, is a multivalent compound with attributed antioxidant, antiinflamatory and antimutagenic activities [1] and, thus, an attractive bioactive ingredient for the development of functional foods. However, its poor solubility in water and its great chemical instability [2] result in very low bioavailability rates upon oral consumption [3]. These drawbacks limit the direct application of curcumin not only in the food industry but also in the medical field [4, 5]. Hence, a number of strategies have been proposed to design appropriate delivery vehicles for this compound [6, 7].

Liposome dispersions can facilitate the incorporation of lipophilic molecules into food products with a positive impact on their stability and bioavailability [8]. Specifically, degradation of curcumin in alkaline conditions can be considerably reduced within liposome environments [9]. However, these vehicles have been described to lose entrapped material during storage and to become instable due to osmotic pressure in contact with certain food components such as sugars or salts [10, 11]. Coating of liposomes with biopolymers has been proposed in a number of works as a plausible strategy to increase their stability and shelf-life [12-14].

On the other hand, the commercialization of powdery food ingredients is substantially more convenient than handling liquid ingredients such as liposome dispersions, as dried powders are easier to handle and to preserve from contamination during storage and, moreover, they occupy reduced storage volumes [15]. Therefore, biopolymer-coated liposome dry delivery vehicles for food ingredients

have been obtained through spray-drying or freeze-drying the corresponding dispersions [8, 16, 17]. However, spray-drying involves the use of high temperatures which can cause degradation of sensitive bioactives [18], and freeze-drying is a considerably expensive technique [19].

In this context, electrohydrodynamic processing (i.e. electrospinning and electrospraying) has emerged as an alternative drying technique for the production of encapsulation structures under mild conditions by applying a high-voltage electric field to a polymer solution, dispersion or melt [20]. In previous works we have explored this technology to microencapsulate a wide range of bioactive ingredients [21-23] and it has been recently explored for the encapsulation of β-carotene-loaded liposomes within biodegradable electrospun fibres [24].

In this work, the design of novel food-grade hybrid encapsulation vehicles for curcumin, based on the entrapment of curcumin-loaded liposomes within a protein matrix was proposed, and its impact on the stability and bioaccessibility of curcumin was assessed. The maximum loading capacity of these carriers was also investigated. A whey protein concentrate was selected as the encapsulation matrix to produce the hybrid capsules. Whey proteins are cheap by-products from the cheese industry with functional characteristics [25] which are electrosprayable in aqueous media [20, 25]. They have been already used for liposome coating and have previously shown protective effects when used as encapsulation matrix [20, 26].

3. Materials and Methods

3.1. Materials

Whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090, was kindly donated by ARLA (ARLA Food Ingredients, Denmark). Pure phosphatidylcholine (98% ± 4%) stabilized with 0.1% ascorbyl palmitate, under the commercial name of Phospholipon® 90G, was obtained from Phospholipid GmbH (Germany). Curcumin (>99.5%), phosphate buffered saline system (PBS, pH = 7.4), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were obtained from Sigma-Aldrich (Spain). Pefabloc® was supplied by Fluka. All inorganic salts used for the *in-vitro* digestion tests were used as received. Absolute ethanol (>99.5%) was purchased from Synth (Brasil), and acetonitrile from Merck (Germany).

3.2. Liposomes production

Liposomes were prepared using the ethanol injection method based on the protocol described in de Freitas Zômpero, López-Rubio, de Pinho, Lagaron and de la

Torre (2015) [24], with modifications. Briefly, phosphatidylcholine ('the lipids') was dispersed in absolute ethanol at room temperature and added dropwise to milliQ water in a volumetric ratio of 10% under continuous stirring at 1336 rpm using a Cowles type impeller. The agitation was maintained for 5 min after the addition of the lipids. Different lipid concentrations were tested, ranging from 20-80 g/L in the final liposome dispersion.

In order to reduce the size of the liposomes the samples were subjected to ultrasonic treatments using a 2 mm ultrasound probe model SXB30 (Sonomax Srl, Italy) in pulse mode (30% active cycle) for 1 min 30 s in intervals of 30 s at increasing power (i.e. 60, 90 and 120 W, respectively). Curcumin-loaded liposomes were produced by adding different amounts of curcumin to the lipids solutions in ethanol, in mass ratios ranging from 1-8% with respect to the lipids.

3.3. Size distribution of the liposomes

The size distribution of the liposomes was determined via dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments Corp., WORCS, UK), according to the method described in Sipoli, Santana, Shimojo, Azzoni and de la Torre (2015) [27] prior dilution to a lipid concentration of 20 µg/mL with milliQ water, and intensity-weighted results are reported.

3.4. Morphology of the liposomes

Transmission electron microscopy (TEM) was conducted on a LEO 906E microscope (Zeiss, Germany) at 60 kV. Liposome dispersions were diluted to 1 mg/mL lipids and 5 µL of the samples were deposited on 400-mesh copper grids coated with Formvar carbon film. The excess water was removed with blotting paper after 30 s. The samples were subjected to negative staining with 1% (w/v) uranyl acetate prior to examination (5 µL of staining solution was deposited and dried after 10 s).

3.5. Preparation of liposome/protein formulations

WPC (25% w/v) was dispersed in milliQ water at room temperature under vigorous magnetic stirring. The required volume of liposome dispersion was then added to achieve final lipids/WPC mass ratios of 2.5, 5, 7.5 and 10%, under continuous agitation. The final WPC concentration in the formulation was 20% (w/w), as required for the subsequent electrohydrodynamic processing step [18].

3.6. Production of hybrid microencapsulation structures by electrospraying

The previous formulations were processed using a homemade electrospinning/ electrospraying apparatus assembled in-house. The dispersions were pumped with a digitally controlled syringe pump model KDS-100 (KDScientific, USA) at a flow-rate of 0.15 mL/h through a needle with an inner diameter of 0.84 mm. Processed samples were collected on a grounded copper plate covered with aluminium foil which was placed at a distance of 10 cm from the tip of the needle in a horizontal configuration. The applied voltage was 10 kV as selected from preliminary tests in order to attain stable electrospraying.

3.7. Morphological characterization of the particles

Scanning electron microscopy (SEM) was conducted on a Leo 440i microscope (LEO Electron Microscopy/Oxford, Cambridge, United Kingdom) at an accelerating voltage of 10 kV and a working distance of 15 mm after sputter-coating the samples with gold. Particle diameters were measured from the SEM micrographs using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

3.8. Fourier transform infrared (FT-IR) analysis of the samples

FT-IR spectra were collected using a Nicolet 6700 Thermo Scientific FT-IR equipment (USA). The powdery electrosprayed materials were dispersed in spectroscopic grade potassium bromide and analysed in transmission mode. The lipids' spectrum was obtained without further processing in ATR mode. All spectra were obtained by averaging 32-64 scans at 4 cm^{-1} resolution.

3.9. Entrapment efficiency within the liposomes

The liposome dispersions were centrifuged at 100×g and 4 °C for 5 min using a Heal Force Neofuge 23R centrifuge (Thanes Science, Thailand). These conditions were optimized in preliminary trials to precipitate the non-encapsulated crystals of curcumin and not the liposomes. The supernatant was diluted 4-fold with ethanol, dissolving the liposomes and curcumin. The concentration of curcumin was then assessed by UV-vis spectroscopy at 425 nm using a Thermo spectrophotometer model Genesys 6 (New York, USA), prior preparation of a calibration curve (R^2 = 0.9995). The entrapment efficiency (EE) and the loading capacity (LC) of the liposomes were calculated according to Eq. (1) and (2), respectively.

$$EE_{lip}\ (\%) = \frac{Mass\ of\ entraped\ curcumin\ (experimental)}{Total\ mass\ of\ curcumin\ added\ (theoretical)} \cdot 100 \quad \text{Eq. (1)}$$

$$LC_{lip}\ (\%) = \frac{Mass\ of\ entraped\ curcumin\ (experimental)}{Total\ mass\ of\ lipids\ in\ the\ liposomes\ (theoretical)} \cdot 100 \quad \text{Eq. (2)}$$

3.10. Polarized light microscopy

Polarized light microscopy images were taken using a digital microscopy system (Nikon, Multizoom AZ100, Japan) equipped with a polarized light source (Nikon, C-FI115 Fiber Illuminator, Japan) and a digital camera head (Nikon, DS-Ri1, Japan).

3.11. Differential scanning calorimetry (DSC)

DSC was performed using a differential scanning calorimeter model DSC1 from Mettler Toledo (Switzerland) after freeze-drying the liposome suspensions. The samples (ca. 25 mg) were placed in perforated standard aluminium pans (40 µl) and subjected to a heating ramp from -50 to 200 ºC at a scanning rate of 10 ºC/min under a dynamic nitrogen atmosphere (45 mL/min).

3.12. Encapsulation efficiency within the hybrid encapsulation structures

The electrosprayed materials were dispersed in water (16 mg/mL) and vortex-agitated to disrupt the protein capsules. The dispersions were then slowly diluted 4-fold in ethanol to precipitate WPC while dissolving the lipids and curcumin. After centrifugation at 5000 g and 4 ºC during 20 min, the curcumin concentration in the supernatant was analysed by UV-vis spectroscopy as described above. The encapsulation efficiency (EE) and loading capacity (LC) of the electrosprayed hybrid structures were calculated according to Eq. (3) and (4), respectively.

$$EE_{hyb}\ (\%) = \frac{Mass\ of\ entraped\ curcumin\ (experimental)}{Total\ mass\ of\ curcumin\ added\ (theoretical)} \cdot 100 \quad \text{Eq. (3)}$$

$$LC_{hyb}\ (\%) = \frac{Mass\ of\ entraped\ curcumin\ (experimental)}{Total\ mass\ of\ the\ hybrid\ structures\ (experimental)} \cdot 100 \quad \text{Eq. (4)}$$

3.13. Curcumin degradation assays

Free curcumin, curcumin-loaded liposomes and curcumin-loaded hybrid capsules were dissolved/dispersed in PBS (pH=7.4) to achieve theoretical curcumin concentrations of 0.01 mg/mL in all cases. For free curcumin, a 1 mg/mL stock solution was first prepared in absolute ethanol and subsequently diluted 100-fold in

PBS. After selected time intervals, aliquots of the aforementioned solutions/dispersions were diluted 3-fold with absolute ethanol, centrifuged for 30 s at 12000 rpm using an Eppendorf MiniSpin microcentrifuge from Fisher Scientifics and their absorbance at 425 nm was measured as described in section 3.9.

3.14. *In-vitro* gastrointestinal digestion

Suspensions of the curcumin-containing hybrid microcapsules (40 mg/mL) and the equivalent concentration of free curcumin (0.04 mg/mL) in distilled water were subjected to *in-vitro* digestion following the method described by Gómez-Mascaraque, Soler and Lopez-Rubio (2016) [28]. Aliquots collected after the duodenal phases were snap-frozen in liquid nitrogen until further use.

3.15. Bioaccessibility assessment

The amount curcumin released during digestion was estimated after centrifugation of the digestas. The supernatants were freeze-dried, re-suspended in water and extracted with 70 % acetonitrile before HPLC-MS analysis. Eluent A was water slightly acidified with 0.005% acetic acid, and eluent B acetonitrile with 0.005% acetic acid, working in isocratic mode at 70% of eluent B. The LC system used for this analysis was an Acquity® TQD system from Waters. Separation of curcumin was performed using an Acquity UPLC C18 Kinetex (Phenomenex, 100 mm x 2.1 mm, 1.7 µm particle size) LC-column. The flow rate was set to 0.4 mL/min. The injection volume was 5 µl. The mass spectrometer was equipped with a Z-spray electrospray ionization source and spectra were acquired in positive ionization multiple reaction monitoring (MRM) mode with interchannel delay of 0.16 s. The bioaccessibility was estimated according to Eq. (5), where EE_{hyb} refers to the encapsulation efficiency obtained for the hybrid structures (cf. Eq. (3)).

$$Bioaccessibility\ (\%) = \frac{[curcumin]in\ digesta}{[curcumin]_{theoretical} \times EE_{hyb}} \cdot 100 \qquad \text{Eq. (5)}$$

3.16. Statistical analysis

Significant differences between homogeneous sample groups were obtained through two-sided t-tests at $p < 0.05$ using IBM SPSS Statistics software (v.23) (IBM Corp., USA). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

4. Results and discussion

4.1. Phosphatidylcholine liposomes

Suspensions of liposomes with different lipid (phosphatidylcholine) concentrations, ranging from 20-80 g/L, were prepared using the ethanol injection method. The size distribution of the obtained liposomes could not be accurately determined by DLS due to their big sizes and great polydispersity index (results not shown). Jaafar-Maalej and co-workers had also observed an increase in the size of liposomes produced using egg-yolk lecithin by ethanol injection when the lipids concentration was increased from 10 g/L to 60 g/L, and the formation of large aggregates when the lipids concentration was higher than 60 g/L [29].

Subsequent attempts to electrospray the protein dispersions containing the prepared liposomes failed, as the disturbance of the Taylor cone caused by the big-sized liposomes led to dripping of the dispersions. Therefore, the liposomes were subjected to a ultrasonic treatment (cf. Section 3.2) in order to reduce their size [30] while maintaining high lipids concentrations in the final hybrid structures.

Table 1 summarizes the average size and polydispersity index (intensity weighted) of the obtained liposomes after the ultrasonic treatment. The results showed that both the average size of the liposomes and their polydispersity increased with the lipid concentration. In order to confirm the results from DLS, the liposomes were observed by transmission electron microscopy (TEM). Figure S2 of the Supplementary Material shows a TEM image obtained for liposomes prepared with a final lipid concentration of 80 g/L, whose sizes are in the range of those obtained by DLS.

4.2. Hybrid electrosprayed capsules

Electrosprayed capsules were obtained from WPC dispersions containing sonicated phosphatidylcholine liposomes produced at different lipid concentrations in order to ascertain whether the concentration and size of the liposomes had an impact on the sprayability of the suspensions and the morphology and size of the obtained encapsulation structures. All evaluated liposome concentrations (i.e. up to a 10% w/w of lipids with respect to the protein) could be successfully electrosprayed, and the images obtained by scanning electron microscopy (SEM) of the capsules are shown in Figure 1, together with their particle size distributions. From these images it was concluded that all the dispersions containing liposomes at different concentrations and with different size distributions yielded spherical capsules upon electrospraying, finding no significant differences in their size distributions. Therefore, the maximum concentration tested, i.e. 10% w/w of lipids with respect to the protein, was selected for further experiments, in order to maximize the amount of bioactive to be incorporated in the hybrid encapsulation structures.

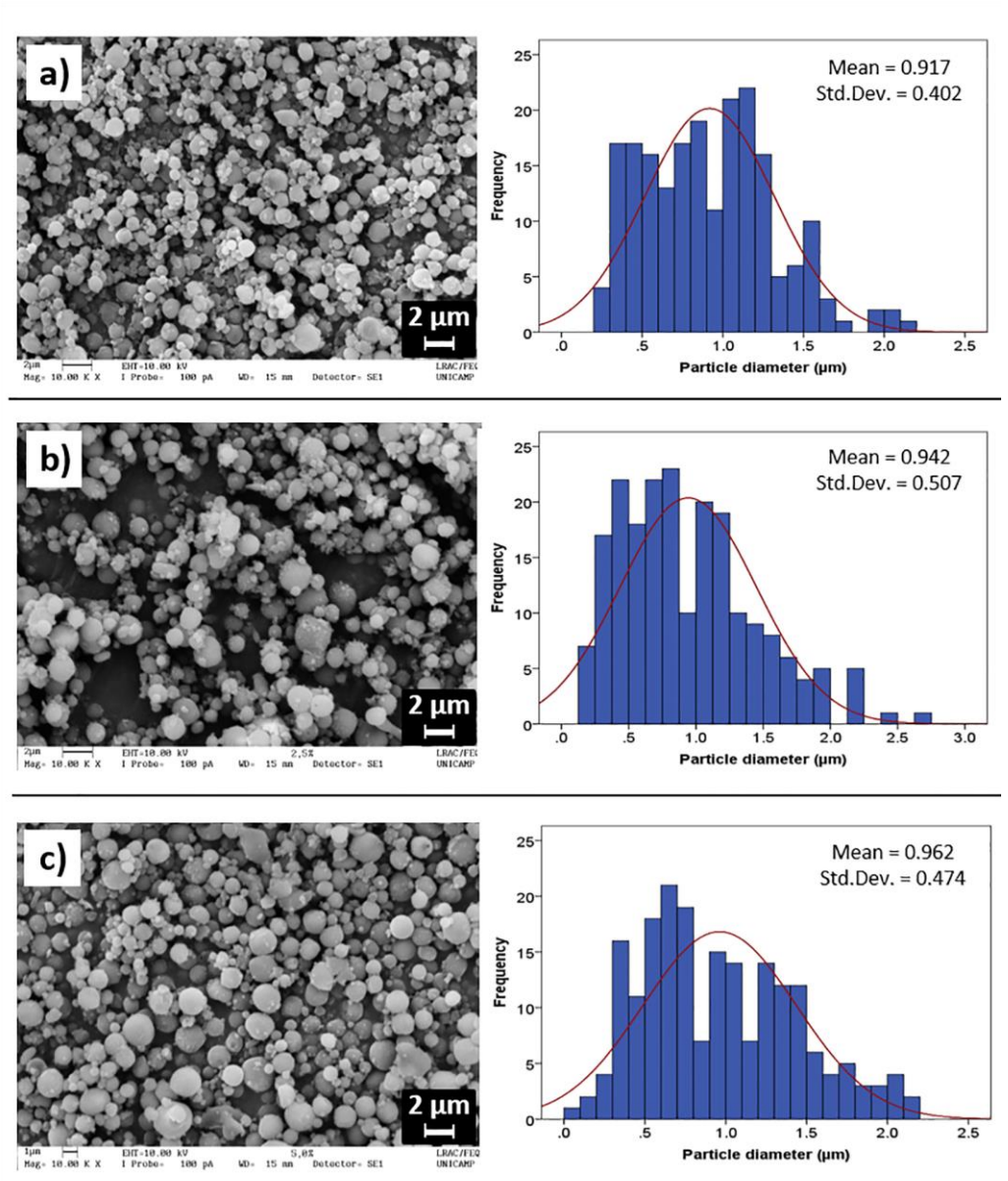


Figure 1. SEM images and particle size distributions of unloaded hybrid encapsulation structures obtained with different liposome concentrations: a) 0% lipid, b) 2.5% lipid, c) 5% lipid.

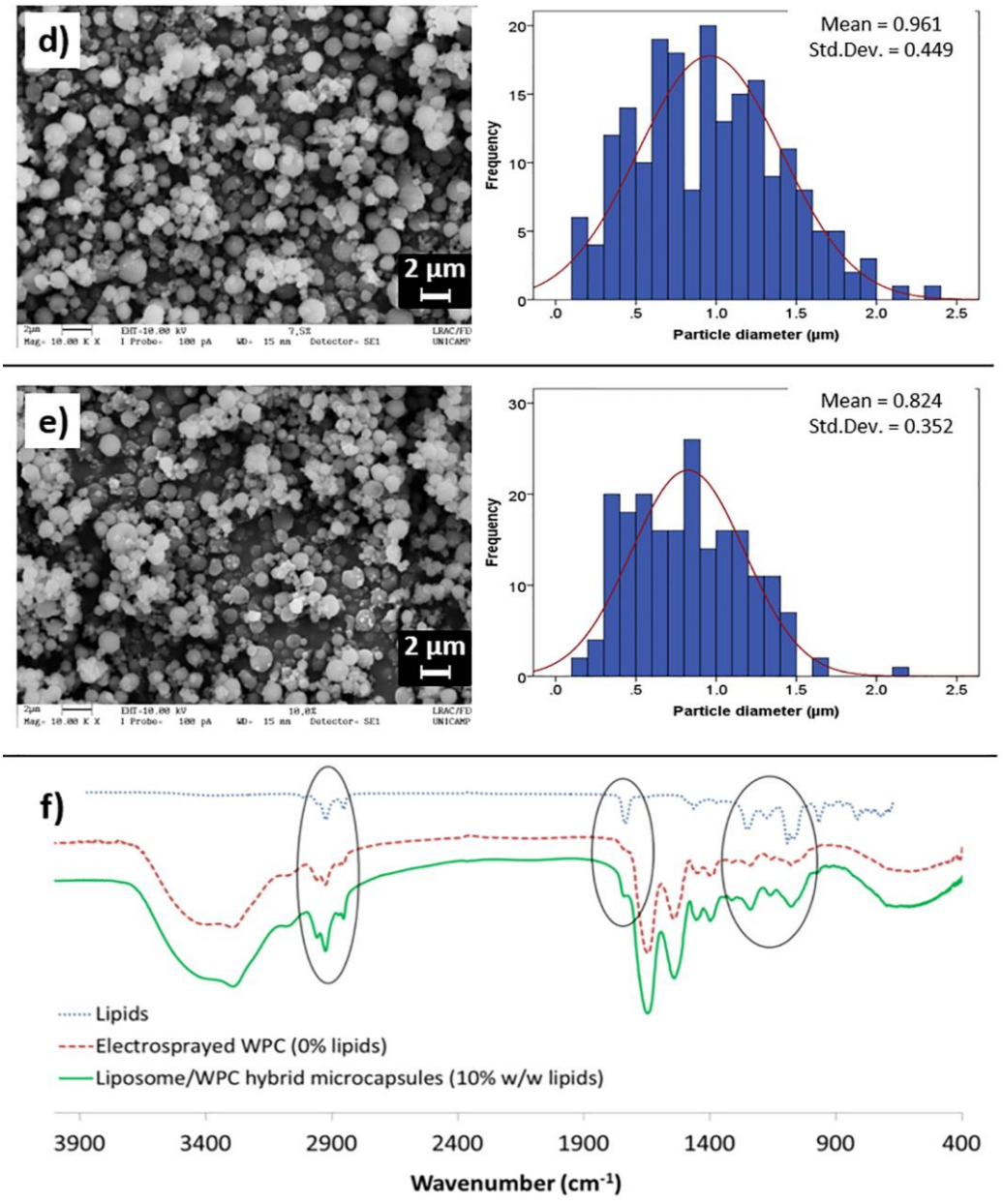


Figure 1 continued. SEM images and particle size distributions of unloaded hybrid encapsulation structures obtained with different liposome concentrations: d) 7.5% lipid and e) 10% lipid; f) FT-IR spectra of commercial lipids, electrosprayed WPC and the liposome-WPC hybrid carriers.

Figure 1 also shows the FT-IR spectra of the capsules containing 10% w/w liposomes, together with the spectrum of WPC capsules containing no lipids and that of the commercial lipids. The spectrum of electrosprayed WPC in the absence of lipids exhibited the characteristic bands of proteins centered at 3411 cm^{-1} (Amide A, N-H stretching), 3075 cm^{-1} (Amide B, asymmetric stretching of =C-H and NH_3^+), 1650 cm^{-1} (Amide I, C=O and C-N stretching), 1547 cm^{-1} (Amide II, N-H bending) and 1242 cm^{-1} (Amide III, C-N stretching) [18]. These bands were also observed in the spectrum of the hybrid capsules, where the presence of lipids within the electrosprayed structures was evidenced by the increase in the intensity of the peaks at 2854 and 2925 cm^{-1} (ascribed to the symmetric and asymmetric vibrational modes of CH_2 groups), the bands at 1242, 1156 and 1079 cm^{-1} (associated to contributions of phosphate groups in the lipids), and the appearance of a shoulder at 1739 cm^{-1} corresponding to the band at 1736 cm^{-1} (vibration of C=O groups) in the pure lipids [31].

Spectral band shifts were observed in the hybrid capsules with respect to its individual components, such as the band ascribed to the Amide B, which shifted to 3072 cm^{-1} in the hybrid structures, or the mentioned band at 1739 cm^{-1}, which was originally centered at 1736 cm^{-1} in the commercial lipids. The spectral changes corresponding to lipid vibrational bands, have been attributed to the reorganization of their supramolecular structure [31] upon liposome production. On the other hand, potential intermolecular interactions between the lipids and the protein might be also taking place, as suggested by the shifts observed in the bands ascribed to the protein. Indeed, globular proteins and, in particular, whey proteins, have been described to suffer conformational changes resulting from adsorption to oil droplets in oil-in-water emulsions [32, 33], consequently exposing their non-polar residues. A similar phenomenon could take place in the prepared liposome dispersions, where the liposomes would act as self-organized oil droplets, which would interact with the exposed non-polar residues of the protein. Previous works have already suggested that phospholipids can modify the secondary structure of whey proteins due to hydrophobic interactions [34], allowing even the insertion of whey proteins into the liposomal membrane [12].

4.3. Curcumin-loaded liposomes and microcapsules

The selected hybrid carrier, i.e. with a lipid content of 10% w/w, was loaded with different concentrations of curcumin. For this purpose, curcumin-loaded liposomes (lipid concentration of 80 g/L) were first produced by adding different amounts of curcumin (1-8% w/w with respect to the lipids) to the lipid solutions before injection. After mixing with the protein dispersion, the mixture was electrosprayed.

Table 1 summarizes the average size and polydispersity index (intensity weighted) of the obtained curcumin-loaded liposomes, which increased with increasing curcumin contents. Being curcumin a lipophilic compound, it was expected to be located in the hydrophobic region of the liposomal bilayer, having an impact on the size and stability of the liposomes. In fact, Karewicz, Bielska, Gzyl-Malcher, Kepczynski, Lach and

Nowakowska (2011) reported that liposomes based on egg yolk phosphatidylcholine and dihexadecyl phosphate were destabilized upon loading with curcumin [35]. Similarly, curcumin affected the phase transition of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes [9].

Table 1. Average size and polydispersity index (PDI) of unloaded liposomes prepared with different lipid concentrations (a), and curcumin-loaded liposomes prepared with 80 g/L lipids (b).

a) Unloaded liposomes			b) Curcumin-loaded liposomes		
[lipid] (g/L)	Average hydrodynamic diameter (nm)	PDI	[curcumin] (% w/w)	Average hydrodynamic diameter (nm)	PDI
20	206 ± 3	0.27 ± 0.01	**0**	407 ± 58	0.66 ± 0.06
40	244 ± 3	0.43 ± 0.02	**1**	508 ± 46	0.75 ± 0.02
60	342 ± 38	0.59 ± 0.06	**2**	656 ± 188	0.73 ± 0.04
80	407 ± 58	0.66 ± 0.06	**8**	771 ± 54	0.87 ± 0.07

Despite this increase in the size and polydispersity of the liposomes, all the above curcumin-loaded liposomes could be successfully electrosprayed when incorporated in the WPC dispersions, causing no substantial changes in the size and morphology of the hybrid capsules (c.f. Section 4.5).

4.4. Entrapment of curcumin within the liposomes

In order to ascertain the amount of curcumin effectively incorporated within the liposomes as a function of the curcumin concentration added to the lipids solution, the entrapment efficiency (EE) and loading capacity (LC) of the systems were calculated according to Eq. (1) and (2), respectively. Results are summarized in Table 2.

The results showed that the encapsulation efficiency decreased with the amount of curcumin added to the lipids solutions. However, no significant differences were observed in the loading capacity of the samples, regardless of the initial curcumin concentration. Therefore, it was concluded that a maximum curcumin loading of about 1.5 % (w/w) with respect to the mass of lipids could be effectively incorporated within the liposomal bilayer. The excess curcumin would then be excluded from the liposomes structure, crystallizing upon contact with water and hence decreasing the entrapment efficiency above the mentioned loading capacity.

To confirm this hypothesis, the curcumin-loaded liposomes suspensions were centrifuged and the obtained pellet was observed under a polarized light microscope to corroborate the presence of curcumin microcrystals. The obtained images are shown in Figure S3 of the Supplementary Material. While very few microcrystals were

observed when a proportion of 1% w/w curcumin was used, the concentration and size of the microcrystals tended to increase as more curcumin was incorporated into the lipids solution.

The above qualitative assessment was complemented with DSC measurements. As inferred in Section 4.3, liposomal curcumin is expected to be located in the phospholipid bilayer and impact its stability. Hence, the liposome phase transition associated with the disruption of the phospholipid bilayer was expected to occur at lower temperatures when curcumin was incorporated in the liposomes [9, 36]. In fact, a reduction of the transition temperature has been already observed in phosphatidylcholine-based liposomes upon curcumin loading [37]. It was hypothesized that the decrease in the phase transition temperature (i.e. 'the destabilization') would be proportional to the amount of curcumin incorporated within the liposomal bilayer. Figure 2 shows the DSC thermograms of unloaded and curcumin-loaded freeze-dried liposomes.

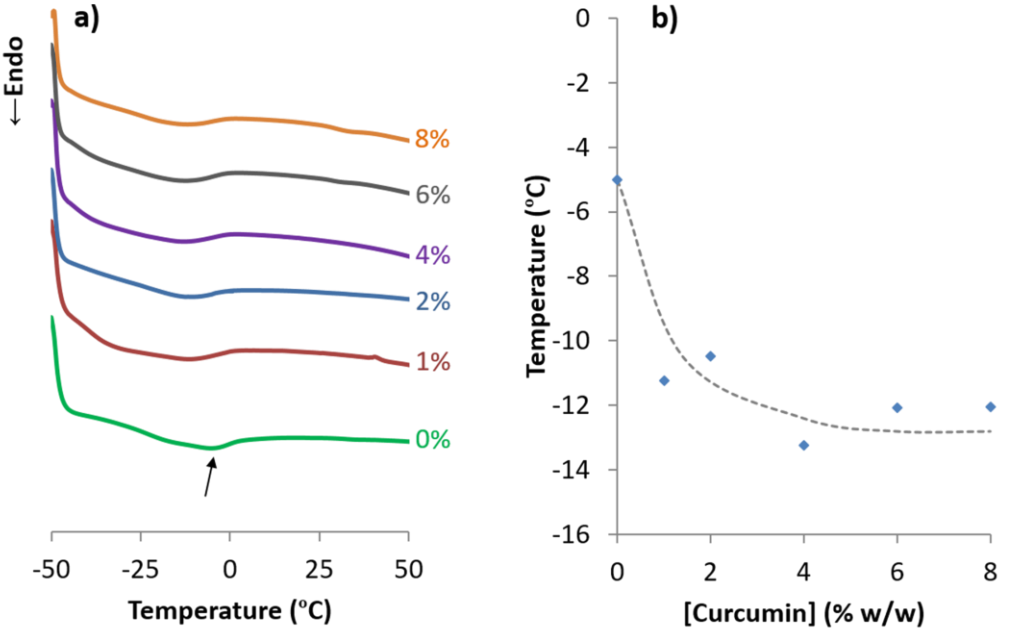


Figure 2. Thermograms of unloaded and curcumin-loaded liposomes with different curcumin w/w concentrations (a) and variation of their transition temperature (pointed by the arrow) with the theoretical concentration of curcumin (b). The dotted line is only for visual guidance purposes.

Indeed, the obtained thermograms showed that curcumin-loading lowered the transition temperature of the liposomes more than 5 ºC, and small differences were observed amongst the transition temperature of the curcumin-loaded liposomes with different curcumin concentrations, suggesting that the amount of curcumin effectively

incorporated in the liposomal bilayer did not differ substantially regardless of the curcumin concentration in the initial lipid solution.

4.5. Encapsulation efficiency within the hybrid microcapsules

The double encapsulation of curcumin within the hybrid structures was accomplished by incorporating the curcumin-loaded liposome suspensions into WPC dispersions (in a lipid:WPC ratio of 10% w/w and different curcumin:lipid ratios) and electrospraying the mixture as previously done with the unloaded liposomes. Again, spherical capsules were obtained with similar size distributions as those observed in Figure 1. A representative SEM image is shown in Figure S4 of the Supplementary Material for a theoretical curcumin concentration of 0.2% w/w with respect to the protein mass. The curcumin encapsulation efficiency (EE) and the loading capacity (LC) of the hybrid microcapsules were calculated according to Eq. (3) and (4), respectively, for different theoretical curcumin concentrations. The results are summarized in Table 2.

Table 2. Entrapment or encapsulation efficiency and loading capacity of curcumin-loaded liposomes (a) and curcumin-loaded hybrid microcapsules (b).

a) Liposomes			b) Hybrid microcapsules		
[curcumin] (% w/w)	EE (%)	LC (%)	[curcumin] (% w/w)	EE (%)	LC (%)
1	79 ± 1^{a}	0.79 ± 0.01^{a}	**0**	-	-
2	72 ± 3^{a}	1.44 ± 0.07^{a}	**0.1**	104 ± 5^{a}	0.104 ± 0.005^{a}
4	32 ± 10^{b}	1.27 ± 0.43^{a}	**0.2**	75 ± 1^{b}	0.150 ± 0.003^{b}
8	11 ± 1^{b}	0.87 ± 0.05^{a}	**0.4**	69 ± 4^{b}	0.275 ± 0.016^{c}

Different letters (a–c) within the same column indicate significant differences at $p<0.05$ among the samples

As previously observed for the simple liposomal encapsulation, the efficiency of the hybrid encapsulation process decreased as the theoretical curcumin concentration increased. However, the loading capacity of the capsules increased as the curcumin concentration did, and was higher than that expected considering the results in the previous section. Considering the maximum curcumin loading capacity of the liposomes previously calculated of around 1.5%, loading capacities of the hybrid capsules greater than 0.15% would imply that part of the curcumin present in those hybrid structures was not entrapped within the lipids and should then be in crystalline form. Accordingly, the smallest curcumin crystals present in the liposomal dispersion might have remained in suspension during the electrospraying process, aided by the

relatively high viscosity of the WPC dispersion. On the other hand, bigger crystals would have precipitated in the syringe, decreasing the encapsulation efficiency as observed when increasing curcumin concentrations. However, although an increase in the loading capacity of the hybrid capsules was observed upon increasing the curcumin concentration in the solutions, the physical form of the curcumin (either crystalline or liposomal) was expected to have implications in its bioaccessibility and, consequently, in its bioavailability (cf. Section 4.4).

4.6. Bioaccessibility of curcumin after *in-vitro* digestion

The bioaccessibility can be defined as the fraction of a compound 'that is soluble in the gastrointestinal (GI) environment and is available for absorption' or 'the fraction of external dose released from its matrix in the GI tract' [38]. In particular, the bioaccessibility of lipophilic ingredients has been related to the fraction of the compounds which is incorporated into the mixed micellar phase formed during digestion in the small intestine [39]. Therefore, in this work the bioaccessibility was calculated as the fraction of the original curcumin content which was recovered from the micellar phase after *in-vitro* digestion (i.e. the clear phase observed after centrifugation of the digestas), as described in previous works [39, 40]. The original curcumin content was calculated taking into account the theoretical loading of the hybrid microcapsules and the encapsulation efficiency, as expressed in Eq. (5). Results are shown in Table 3.

Table 3. Bioaccessibility of pure and microencapsulated curcumin.

Sample – $[curcumin]_{theoretical}$	Bioaccessibility (%)
Free curcumin - pure	3.2 ± 0.5 [a]
Microcapsules - 0.1% w/w	5.4 ± 0.5 [b]
Microcapsules - 0.2% w/w	5.3 ± 0.5 [b]
Microcapsules - 0.4% w/w	4.8 ± 0.3 [b]

Different letters (a–b) within the same column indicate significant differences at $p<0.05$ among the samples

Results showed that the bioaccessibility of pure curcumin was very low, as expected. Microencapsulation of the compound within the proposed hybrid structures, however, significantly increased its bioaccessibility (~1.7-fold), regardless of the curcumin content in the capsules. It is worth noting that, although the loading capacity of the hybrid microcapsules was different for each sample, the loading capacity of the precursor liposomes was not statistically different (cf. Table 2) and thus the amount of 'liposomal curcumin' is expected to be similar in all capsules

subjected to digestion. Accordingly, no statistically significant differences were observed among the microencapsulated samples. In this sense, incorporation of theoretical curcumin contents greater than 1% in the precursor liposomes was considered impractical, as it did not further improve the bioaccessibility of curcumin while it led to lower encapsulation efficiencies.

4.7. Protective effect of microencapsulation

In order to assess whether the microencapsulation of curcumin within the proposed system effectively stabilize the bioactive ingredient, the free and encapsulated compound were dissolved/dispersed in PBS (pH=7.4) medium in which curcumin has been described to be very unstable undergoing rapid hydrolytic degradation [41]. The optimal curcumin load determined from the previous sections (i.e. 1% in the liposomes and 0.1% in the hybrid capsules) was selected for this assay. Figure 3 shows the decay that its absorbance at 425 nm experienced in time due to degradation.

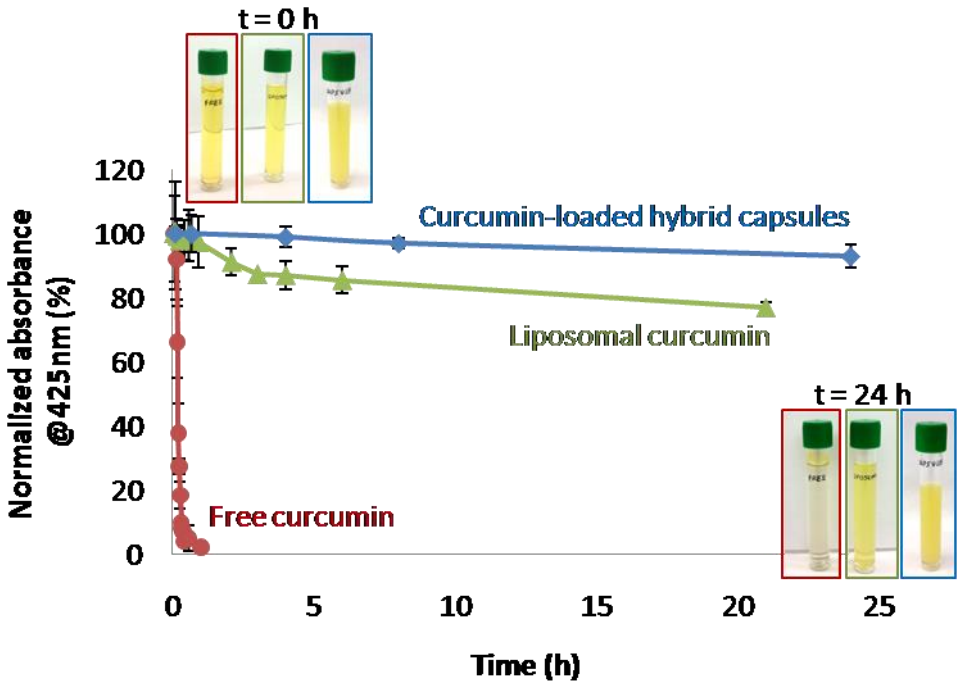


Figure 3. Degradation profiles for free and encapsulated curcumin in PBS (pH=7.4).

As expected, free curcumin rapidly degraded in PBS, being completely degraded in only 1 h. In contrast, microencapsulated curcumin remained stable throughout the experiment, showing minimal absorbance decay. The results also showed that

entrapment of curcumin within the liposomes was enough to greatly improve its stability. However, a small absorbance drop was observed for liposomal curcumin during the first hours, suggesting that the dual encapsulation approach provided enhanced protection against degradation in the buffer. Although the protein concentrate used in this work is water-dispersible, implying that the contents of the capsules would be released upon dispersion of the powder in aqueous solution, whey proteins adsorb at oil/water interfaces where they suffer conformational changes [32, 33], forming a film around the oil droplets which acts as a physical barrier at the interface [42, 43]. As suggested above (cf. Section 4.2), this phenomenon could also take place when the oil phase is organized into liposomes [34], explaining the additional protective effect provided by the protein matrix, despite being water-dispersible. These results are in agreement with those recently reported by Frenzel and co-workers [8], who showed that whey protein coatings increased the physical stability of soy phospholipids-based liposomes and reduced their bilayer semi-permeability.

5. Conclusions

Novel food-grade hybrid encapsulation structures based on the entrapment of phosphatidylcholine liposomes within a WPC matrix through electrospraying were developed and used as delivery vehicles for curcumin. Liposomes produced by the ethanol injection method were ultrasonicated to improve their electrosprayability by reducing their size. Although relatively big (~400 nm) and polydisperse (PI~0.6), liposomes produced at a lipid concentration as high as 80 g/L were successfully incorporated within the WPC capsules. Entrapment of curcumin (1-8% w/w) within the liposomes increased their size and reduced their stability, and the entrapment efficiency of the liposomes decreased with increasing curcumin concentrations, suggesting that their maximum curcumin loading capacity was 1-1.5 % (w/w). The excess curcumin precipitated in the form of microcrystals, a fraction of which could be incorporated together with the liposomal curcumin within the hybrid encapsulation structures upon electrospraying of the WPC-liposome dispersions, increasing the loading capacity of these vehicles despite the decrease in the encapsulation efficiency. Microencapsulation of curcumin within the proposed hybrid structures significantly increased its bioaccessibility (~1.7-fold) compared to the free compound, regardless of the curcumin content in the capsules. Hence, incorporation of curcumin contents greater than 1% in the liposomes was considered impractical, as the lower encapsulation efficiencies did not compensate by an improvement in the bioaccessibility. Finally, the microcapsules could successfully stabilize curcumin against degradation in PBS (pH = 7.4), demonstrating that the whey protein coating of the liposomes obtained by the dual encapsulation strategy provided enhanced protection as compared to the simple entrapment within uncoated liposomes.

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8. Supplementary Material

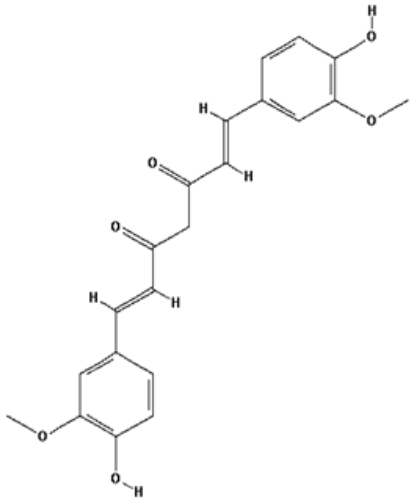


Figure S1. Molecular structure of curcumin. Source: National Center for Biotechnology Information. PubChem Compound Database; CID=969516, https://pubchem.ncbi.nlm.nih.gov/compound/969516.

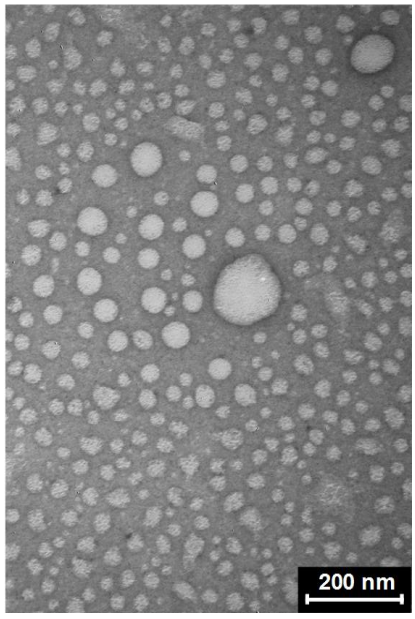


Figure S2. TEM image of liposomes prepared through the ethanol injection method followed by ultrasound treatment, with a final lipid concentration of 80 g/L.

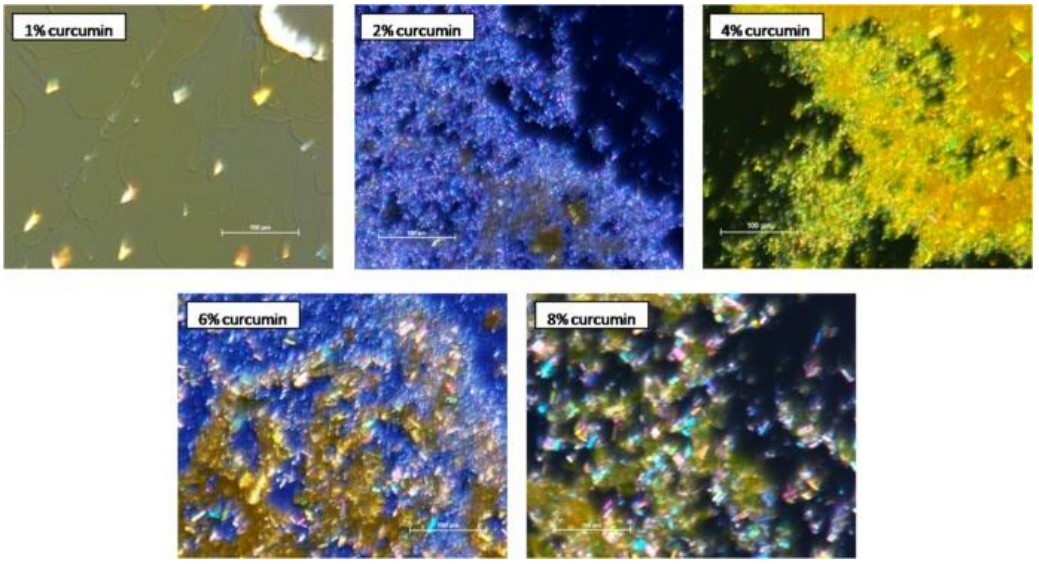


Figure S3. Particle size distributions for ALA-loaded electrosprayed and spray-dried encapsulation structures.

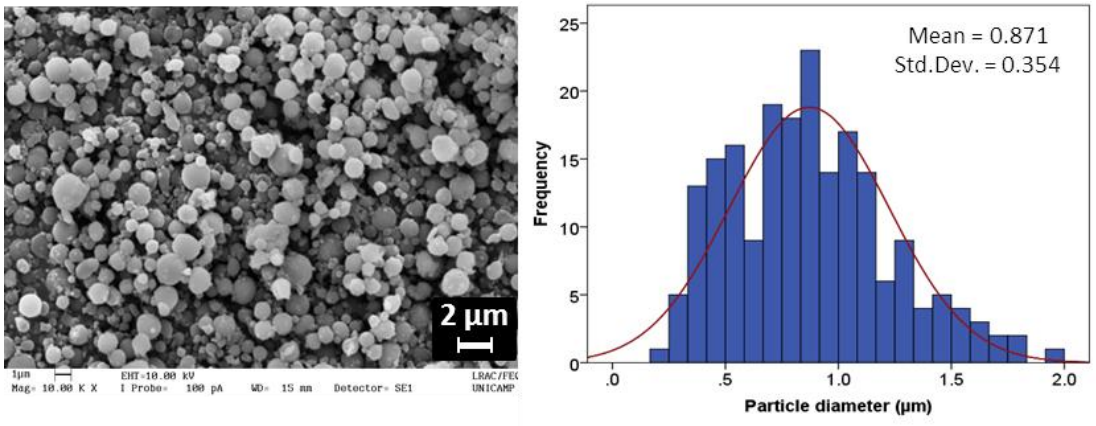
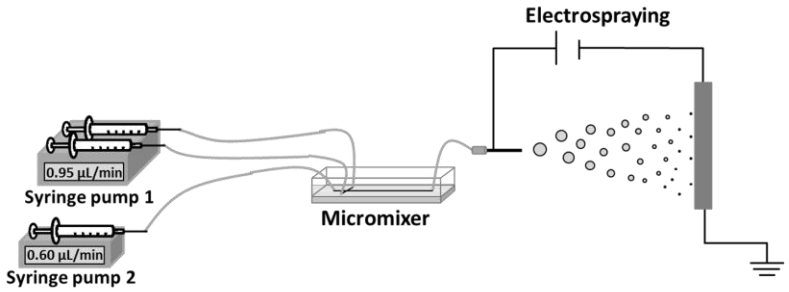
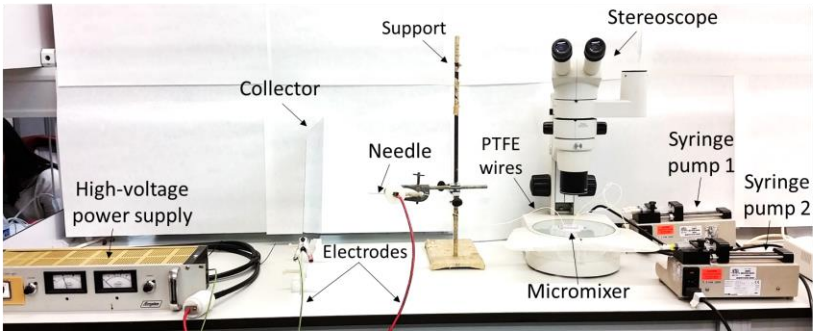


Figure S4. SEM image of curcumin-loaded hybrid encapsulation structures with theoretical curcumin concentration of 0.2% w/w (left) and their particle size distribution (right).

2.4

A STEP FORWARD TOWARDS THE DESIGN OF A CONTINUOUS PROCESS TO PRODUCE HYBRID LIPOSOME/PROTEIN MICROCAPSULES





This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Casagrande Sipoli, C., de La Torre, L. G., & López-Rubio, A. (2017). *A step forward towards the design of a continuous process to produce hybrid liposome/protein microcapsules*. Journal of Food Engineering, 214, 175-181. (DOI: https://doi.org/10.1016/j.jfoodeng.2017.07.003).

1. Abstract

Microfluidics and electrospraying, two revolutionary technologies with industrial potential for the microencapsulation of lipophilic bioactive ingredients, have been combined to produce hybrid liposome/protein microencapsulation structures in a semi-continuous process, reducing the number of steps required for their manufacture. Three different microfluidic mixing devices, one of them consisting of a simple straight microchannel (cross junction design) and the other two exhibiting patterned microchannels with different geometries (Tesla and 'splitting and recombination' designs), were used to mix a liposome suspension with a whey protein concentrate dispersion. The Tesla design showed the best mixing performance, as observed by fluorescence microscopy, so it was selected to be assembled to an electrospraying apparatus. The proposed in-line setup was successfully used to produce the micron-sized encapsulation structures, as observed by scanning electron microscopy.

2. Introduction

Liposomes can be used as delivery vehicles for the incorporation of lipophilic bioactive compounds into food products, increasing their stability and bioavailability [1]. However, the high semi-permeability of their membranes and low physical stability [2] limit their practical application. Microencapsulation of the liposomes within dry biopolymeric matrices, obtaining convenient powdery ingredients, has been proposed as a strategy to overcome these limitations [3-6].

Specifically, the electrospraying technique, a drying technology based on the electrohydrodynamic processing or atomization of polymeric fluids, has been very recently used to produce dry liposome/protein microencapsulation structures, which proved to successfully stabilize and improve the bioaccessibility of curcumin [7]. The main advantage of electrospraying over other more commonly used drying techniques such as spray-drying is its operation under mild temperature conditions avoiding the thermal degradation of thermosensitive bioactive ingredients [8].

On the other hand, conventional technologies used for the manufacturing of liposomes usually require numerous pre- and post-processing steps [9]. Microfluidics, a technology which allows manipulation of tiny volumes of fluids inside micron-sized channels, has been proposed as an alternative for the continuous production of liposomes by flow focusing [10].

The combination of microfluidics and electrospraying would allow the design of an industrially attractive continuous process for the production of liposome/protein microencapsulation structures similar to those previously developed in batch [7]. For this purpose, an intermediate mixing step would be necessary in order to blend the liposomes with the polymer prior to electrospraying the mixture. Due to the low flow

rates used in both techniques, this mixing step should be also accomplished by means of microfluidics.

Several designs for microfluidic mixing devices ('micromixers') have been proposed. The most simple design is based on straight channels [11]. In these devices, mixing occurs through a diffusion mechanism due to the low Reynolds numbers (Re) obtained for the fluids, which flow in the laminar regime as a consequence of the micro scale [12]. However, diffusion-driven mixing is time-consuming and inefficient, and thus passive micromixers based on the modification of the flow channel geometry by adding obstacles in the flow path have been also designed in order to enhance fluid mixing by increasing contact between the two fluids. Several patterned microchannels with different geometries have already been developed [13-16].

In this work, we report on the study of three different micromixer designs based on the cross flow junction, Tesla and SAR ('splitting and recombination') designs [11, 13, 14], to achieve an effective mixing of a liposome suspension with a whey protein concentrate (WPC) dispersion. The selected micromixer was then assembled to an electrospraying apparatus to produce liposome/protein microencapsulation structures in a one-step process.

3. Materials and Methods

3.1. Materials

A whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090 was kindly donated by ARLA (ARLA Food Ingredients, Denmark). Pure phosphatidylcholine (98% ± 4%) stabilized with 0.1% ascorbyl palmitate, was obtained from Lipoid GmbH (Germany). Rhodamine B was obtained from Sigma-Aldrich (Germany). Absolute ethanol (>99.5%) was purchased from Synth (Brasil). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (USA). Sylgard 184 Silicone Elastomer Kit (Dow Corning, Midland, MI, USA) was used as material precursor of PDMS layers for the microfluidic devices, and NANO™ SU-8 photoresist formulation (MicroChem Corp., USA) was used to prepare the masks.

3.2. Production of the microfluidic mixing devices

Three different designs for the micromixers were projected using AutoCAD (Autodesk). The geometry of their channels is depicted in Figure 1. The polydimethylsiloxane (PDMS)/glass microfluidic devices were then produced following the procedure described in [17], which is based on the conventional UV photolithographic and soft-lithography methods. The cross-section of the microchannels was rectangular in all cases, with a depth of 100 µm.

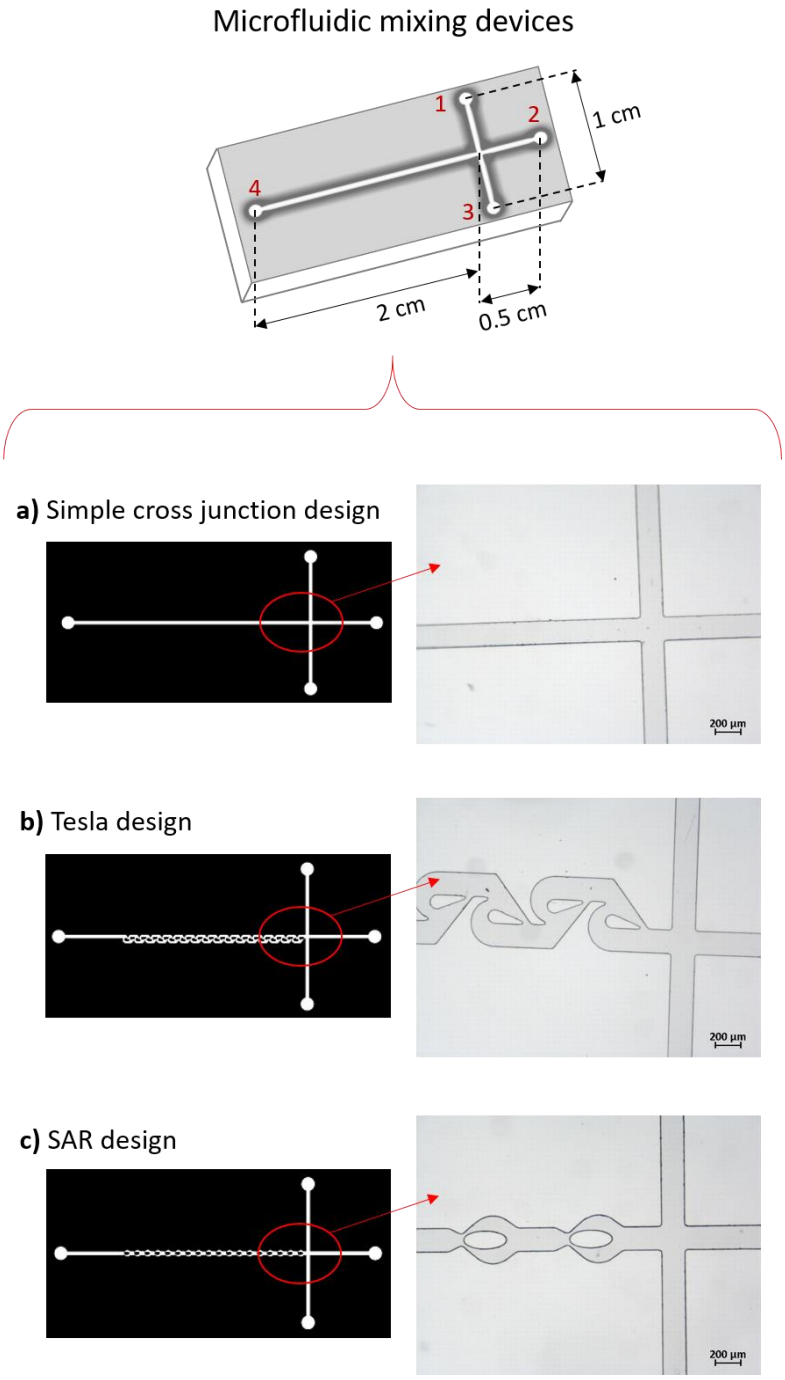


Figure 1. Schemes and corresponding micrographs of the three different designs of microfluidic mixing devices used in this work. The microchannels are 200 µm wide and 50 µm deep.

3.3. Preparation of protein and liposome dispersions

WPC (25% w/v) was dispersed in milliQ water at room temperature under vigorous magnetic stirring. Liposome dispersions (80 g/L) were prepared using the ethanol injection method followed by ultrasonication as described previously [7]. Both dispersions were filtered prior to their pumping into the micromixers, to avoid clotting. The liposome dispersions were filtered using 0.45 μm, nylon, Whatman® syringe filters, while the WPC dispersions were filtered using filter paper under vacuum due to its high viscosity.

3.4. Mixing protein and liposome dispersions in micromixers

The dispersions were introduced in glass syringes and were pumped through the selected microfluidic mixer devices with digitally controlled syringe pumps model KDS-100 (KDScientific, Massachusetts, USA). The protein dispersion was infused through the lateral inlets of the micromixers, each stream flowing at 0.95 μL/min. The liposomes were introduced through the central inlet at 0.6 μL/min. These rates were selected by carrying out a mass balance taking into account the prerequisites of the subsequent electrospraying process, which was optimized in a previous work [7] and required a total flow rate of 2.5 μL/min and a final WPC concentration in the mixture of 20% (w/w).

3.5. Assessment of the quality of mixing

To assess whether the different microfluidic devices were effective in mixing both suspensions, 0.1mM rhodamine B was added to the liposome stream as a fluorescent probe and the flow of the fluids through the microchannels was observed under an inverted research microscope Eclipse Ti-U, (Nikon, Tokyo, Japan) equipped with a digital imaging head which integrates an epifluorescence illuminator. A digital camera head (Nikon 1.5MP DS-Qi1Mc, Japan) was attached to the microscope. Nis Elements software (Nikon, Japan) was used for image capturing, and FIJI [18] was used to process the images. The mixing index in the outlet of each micromixer was calculated according to the method and equations described in [19].

3.6. In-line production of hybrid microencapsulation structures

The outlet of the micromixer was connected to an electrospraying apparatus, equipped with a variable high-voltage (0-30 kV) power supply and assembled in-house. Specifically, the blend dispersion of protein and liposomes coming out from the micromixer was pumped through a polytetrafluoroethylene (PTFE) thin tube connected to a stainless steel needle (0.84 mm inner diameter). Figure 2 shows

shows a schematic representation of the set-up. The dispersions were introduced in 5 mL plastic syringes and were pumped through the selected micromixer with digitally controlled syringe pumps model KDS-100 (KDScientific, Massachusetts, USA) at the same flow-rates used in Section 3.4. A grounded collector was placed facing the needle in a horizontal configuration, at a distance of 10 cm from its tip, and a voltage of 10 kV was applied.

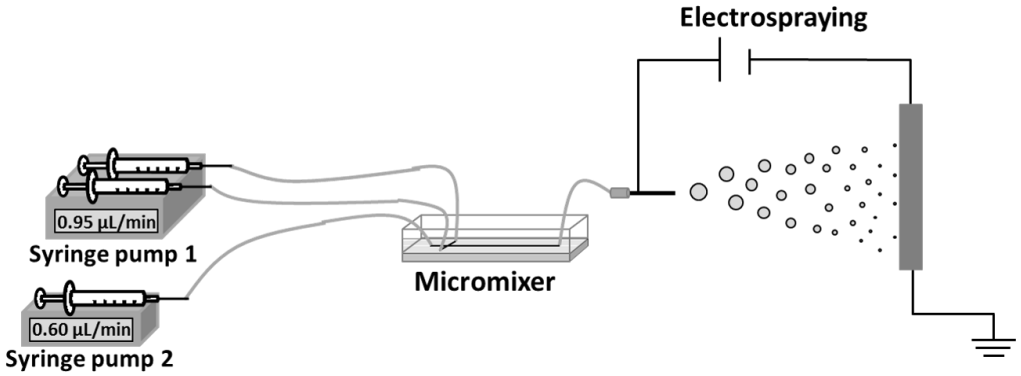


Figure 2. Basic scheme of the proposed micromixer-electrospraying setup.

3.7. Morphological characterization of the hybrid microencapsulation structures

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of ~10 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination.

4. Results and discussion

4.1. Microfluidic mixing devices

In order to achieve an effective mixing of the protein and liposome dispersions prior to their electrospraying, the performance of three different designs of microfluidic devices as micromixers was studied. Figure 1a) shows the simplest microfluidic device with a cross junction design consisting of 3 inlets and a single straight channel [11]. Due to the relatively high viscosity of the polymer solutions or suspensions commonly used to produce electrosprayed microencapsulation structures [8, 20], their mixing with a second fluid in a simple cross junction design may require

long microchannels, as vortex formation was not expected given the low flow rates, and thus low Reynolds numbers, required for subsequent electrospraying [21]. Therefore, two other geometries were also explored. Figures 1b) and 1c) show different geometries based on the Tesla [13] and SAR (splitting and recombination) [14] designs, respectively. These geometries were aimed at creating swirling sections to accelerate mixing. Figure 1 also shows micrographs of the produced micromixers, where the detailed geometry can be observed.

4.2. Mixing performance of the micromixers

The protein and liposome dispersions were prepared in batch as described in a previous work, where their characterization was reported [7]. 0.1mM rhodamine B was added to the liposome dispersion as a fluorescent probe. The mixture of both fluids when infused into the microfluidic device was observed under the microscope. Figure 3 shows fluorescence micrographs of different sections of the microfluidic devices filled with the flowing dispersions, together with a reconstruction of the whole central channel obtained from different pictures and assembled using the software ImageJ.

From the images in Figure 3, the software Fiji was used to calculate the fluorescence profile (i.e. intensity of the pixels) along the main channel of the micromixers. Figure 4 shows, for each micromixer design, the fluorescence profile of different sections of the microchannel, where the baseline corresponded to black pixels. The profiles after the confluence of the three inlets, which are highlighted in black in Figure 4, showed a narrow peak corresponding to the rhodamine-containing liposomes stream. At that point, the protein and liposomes dispersions could be clearly distinguished, both visually in Figure 3 and quantitatively in Figure 4. The following sections along the channels, in all cases, exhibited a gradual widening of the fluorescence signal, due to the progressive mixing of both streams throughout the micromixer, up to the outlet (section 19000 μm), which is highlighted in red in Figure 4. However, the shape of these profiles was different for each micromixer. In the SAR design, a preferential pathway was taken by the liposomes dispersion (cf. arrow in Figure 3), so the 'splitting and recombination' concept did not apply. Consequently, the fluorescence increased preferentially in one of the ends of the microchannel. Conversely, the fluorescence distribution in the cross junction and Tesla designs was more symmetric. In the outlet, the fluorescence profile was flatter for the cross junction and Tesla designs than for the SAR design, due to the aforementioned preferential pathway in the latter, indicating that the former achieved a better mixing of both fluids.

To assess the quality of mixing quantitatively, the mixing index at section 19000 μm, (outlet) was calculated for each micromixer according to the method described in Shiroma, Oliveira, Lobo-Júnior, Coltro, Gobbi, Lucimara and Lima (2017) [19]. The mixing indexes were 0.77, 0.74 and 0.58 for the cross junction, Tesla and SAR designs, respectively. Surprisingly, the simple diffusion in the cross junction design was more effective for mixing both streams than the SAR design, and its performance was similar to that of the Tesla design.

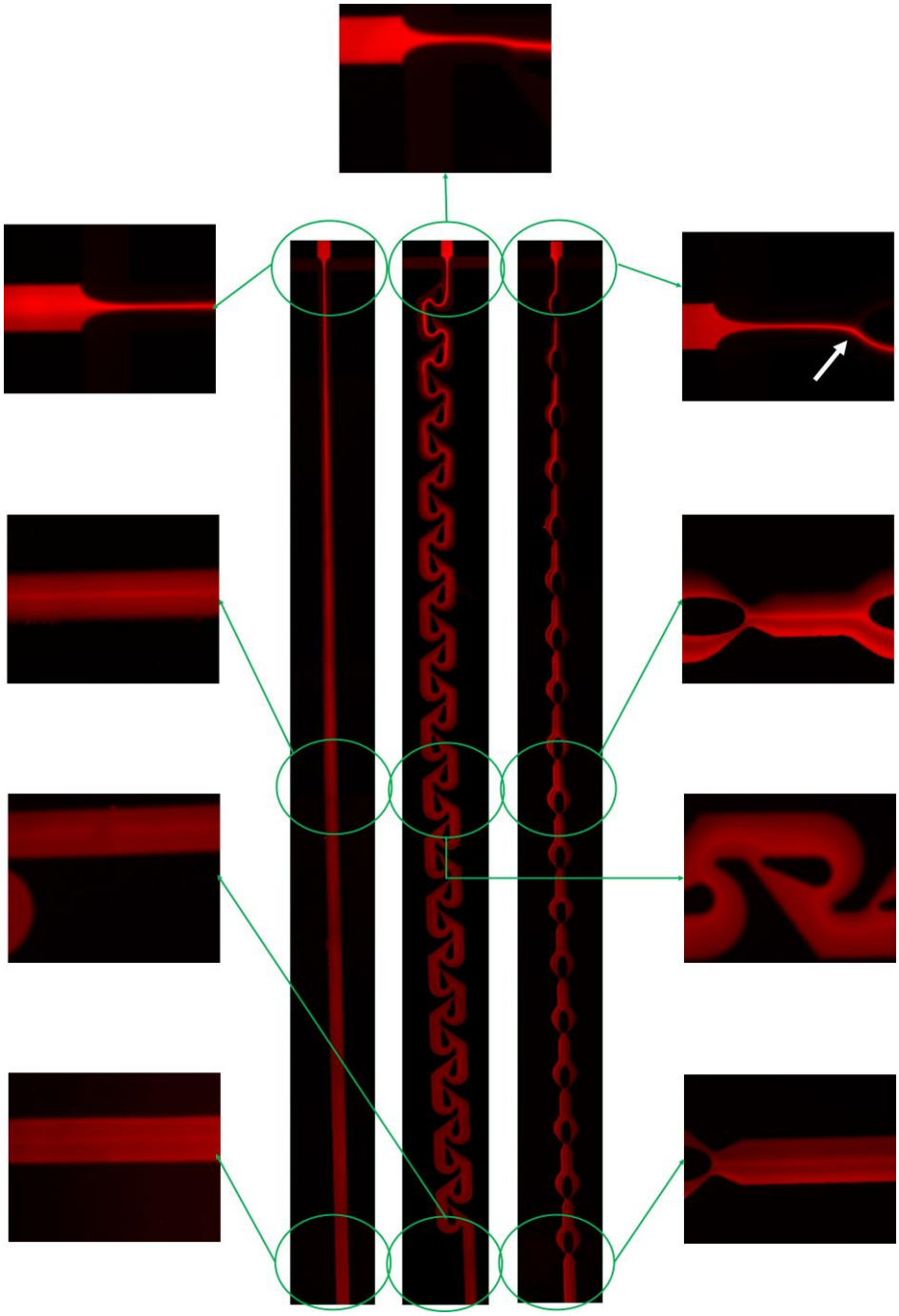


Figure 3. Fluid mixing using microfluidic devices with T (left), Tesla (central), and SAR (right) designs. Relevant sections are shown in detail. The arrow indicates the preferential pathway taken by the liposomes in the SAR design.

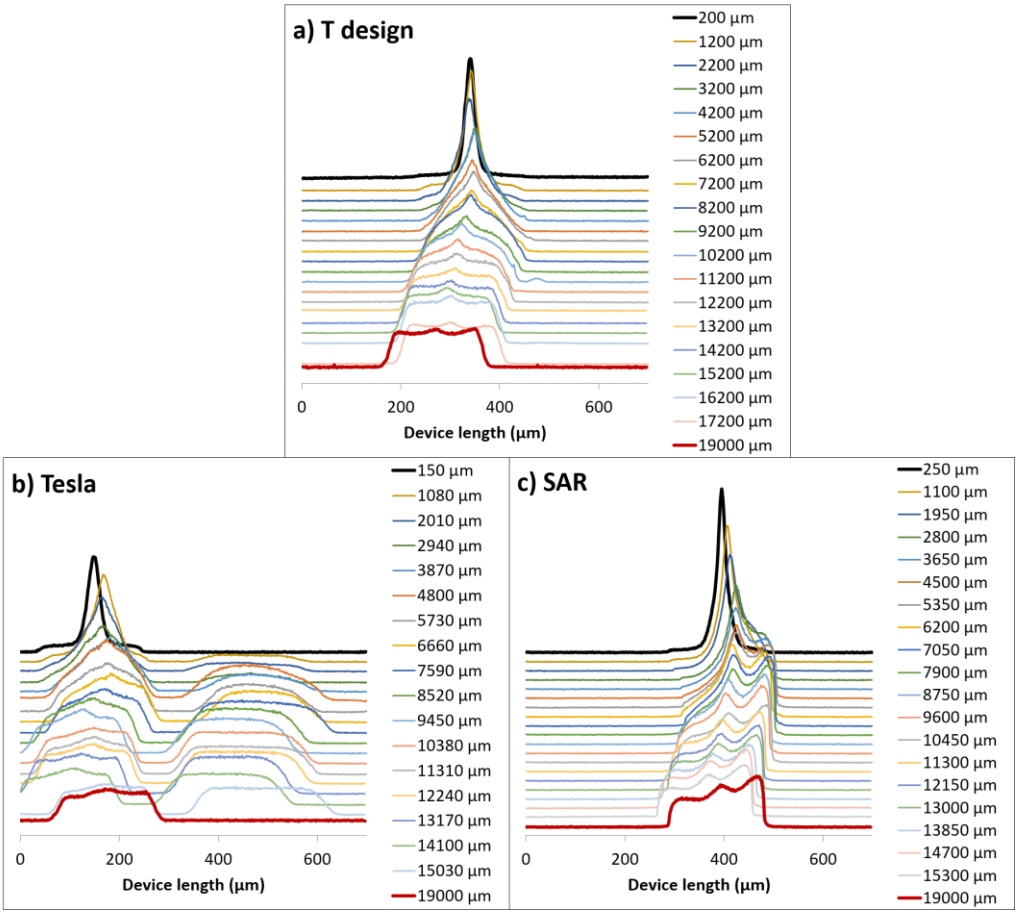


Figure 4. Fluorescence profiles along the length of the main channel of the microfluidic devices.

Although the ellipse-like geometry of the SAR design had proved to be efficient in mixing human blood [14], the flow rates used in that work were considerably higher than the ones required for electrospraying, and the viscosity substantially lower, so the Reynolds number was expected to be significantly higher. The Reynolds number (Re) in our microfluidic devices was calculated according to Eq. (1-3) (assuming channels with rectangular shape), where ρ is the density of the fluids, μ is their viscosity, W is the the width of the microchannels, L is their depth, Dh is their hydraulic diameter, A is the area of their section, Q is the flow rate and V is the flow velocity. And, indeed, it was estimated to be 0.03 when both dispersions were mixed (cf. Table 1). Increasing the flow rate 10-fold in the SAR design avoided the aforementioned preferential pathway taken by the liposomes stream (cf. Figure S1 of

the Supplementary Material). However, the flow rates in this work were fixed as a requirement for the subsequent electrospraying process.

$$Re = \frac{\rho \cdot V \cdot Dh}{\mu} \quad \text{Eq. (1)}$$

$$Dh = 2 \cdot \frac{W \cdot L}{W + L} \quad \text{Eq. (2)}$$

$$V = \frac{Q}{A} = \frac{Q}{W \cdot L} \quad \text{Eq. (3)}$$

Table 1. Calculation of Reynolds number (Re) in the inlets and outlet of the micromixers, according to Eq. (1-3). For the outlet, the viscosity and density of a mixture of the polymer and the liposomes was assumed.

		Inlets 1,3	**Inlet 2**	**Outlet**
Fluid	Density (ρ, kg/m^3)	1023	980	1013
	Viscosity (μ, Pa·s)	0.023	0.00163	0.011
Geometry of the channel	Depth (L, m)	$5.0 \cdot 10^{-5}$	$5.0 \cdot 10^{-5}$	$5.0 \cdot 10^{-5}$
	Width (W, m)	$2.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$
	Hydraulic diameter (Dh, m)	$8.0 \cdot 10^{-5}$	$8.0 \cdot 10^{-5}$	$8.0 \cdot 10^{-5}$
	Area of section (A, m^2)	$1.0 \cdot 10^{-8}$	$1.0 \cdot 10^{-8}$	$1.0 \cdot 10^{-8}$
Flow	Flow rate (Q, m^3/s)	$1.6 \cdot 10^{-11}$	$1.0 \cdot 10^{-11}$	$4.2 \cdot 10^{-11}$
	Flow velocity (V, m/s)	$1.6 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$4.2 \cdot 10^{-3}$
	Re	**0.006**	**0.05**	**0.03**

Low flow rates involve low Peclet numbers, which favour mixing by diffusion [22]. This may be the reason why, for this particular system, the cross junction design provided a better mixing. However, the Tesla micromixer achieved an equally good mixing index. Previous studies showed similar performances for Tesla and cross junction designs at low flow rates (low Peclet and Reynolds numbers, since diffusion is favoured), although the performance of the Tesla design was considerably better at higher flow rates [13]. Since the flow rates used in this work were extremely low, and mixing by diffusion was then favoured, both micromixer designs exhibited similar mixing indexes. However, given the greater potential of the Tesla geometry in terms of flow-rate flexibility, the latter was selected to assemble the in-line mixing and electrospraying set-up.

4.3. In-line production of hybrid microencapsulation structures

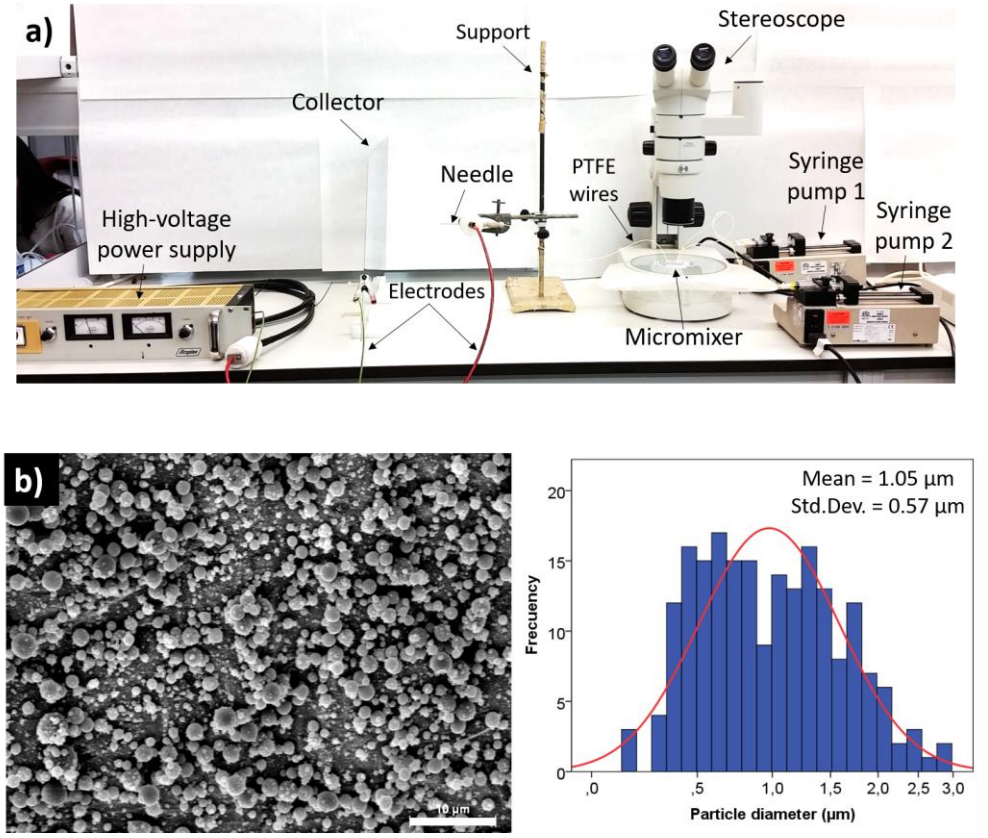


Figure 5. a) In-line micromixer-electrospraying assembled setup. b) SEM micrograph of the obtained hybrid microencapsulation structures together with their particle size distribution. Scale bar indicates 10 µm.

An in-line mixing and electrospraying set-up was assembled using the Tesla micromixer. For this purpose, a PTFE tube was used to connect the outlet of the microfluidic device to the stainless steel needle, combining the microfluidic and electrohydrodynamic technologies in a quasi-continuous process. A stereoscope was used to ensure the correct performance of the micromixer and the absence of clogging. Figure 5 a) illustrates the aforementioned set-up, which was used to produce the novel hybrid encapsulation structures recently proposed in a previous work [7]. Figure 5 b) shows the morphology of the obtained microcapsules, where populations in the micron and sub-micron range can be observed. This morphology was very similar to those previously obtained [7], thus confirming the potential of the developed methodology for semi-continuous production of hybrid encapsulation structures.

5. Conclusions

In this work, the microfluidics and electrospraying technologies were combined to produce hybrid liposome/protein microencapsulation structures in a semi-continuous manufacturing process. For this purpose, three different designs for microfluidic devices were used to mix a stream of liposomes with a dispersion of a whey protein concentrate. These designs were based on the T, Tesla and SAR ('splitting and recombination') designs previously proposed in the literature. Due to the high viscosity of the protein dispersion and the low flow rates required for the subsequent electrospraying process, the SAR design was not effective in mixing both fluids, as a preferential pathway was taken by the liposomes instead of splitting and recombining. Conversely, Tesla and cross junction designs exhibited adequate mixing performances. An assembled micromixer-electrospraying setup was built using the Tesla design, and this arrangement was successfully used to produce the dry liposome/protein microcapsules, which exhibited a similar morphology as those produced in batch in a previous work. The next step towards the design of a fully continuous manufacturing process would involve the incorporation of additional microchannels in the microfluidic devices to continuously produce the liposomes by flow focusing before their mixture with the protein.

6. Acknowledgements

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8. Supplementary Material

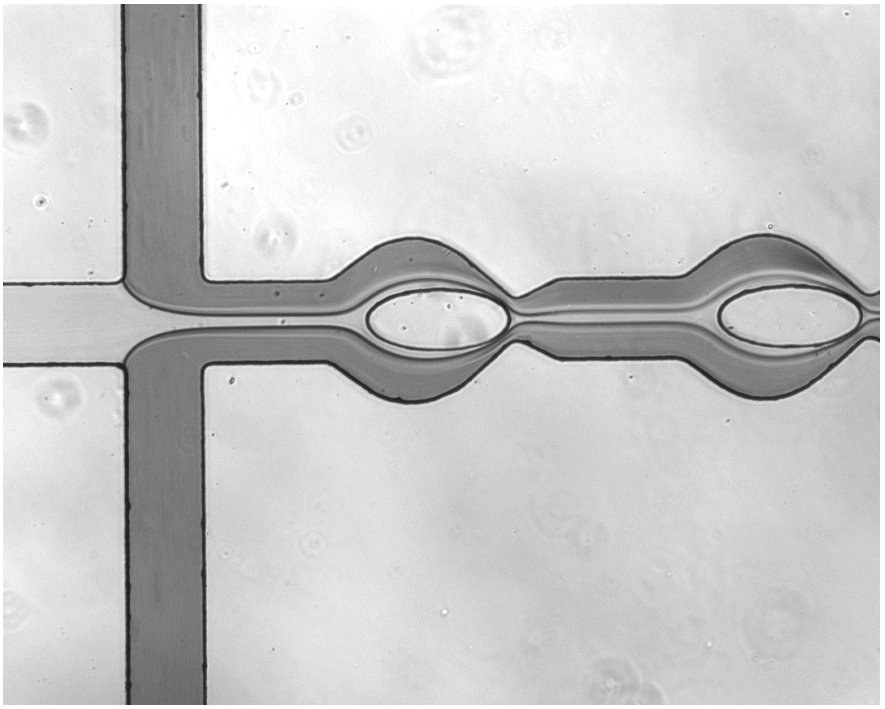
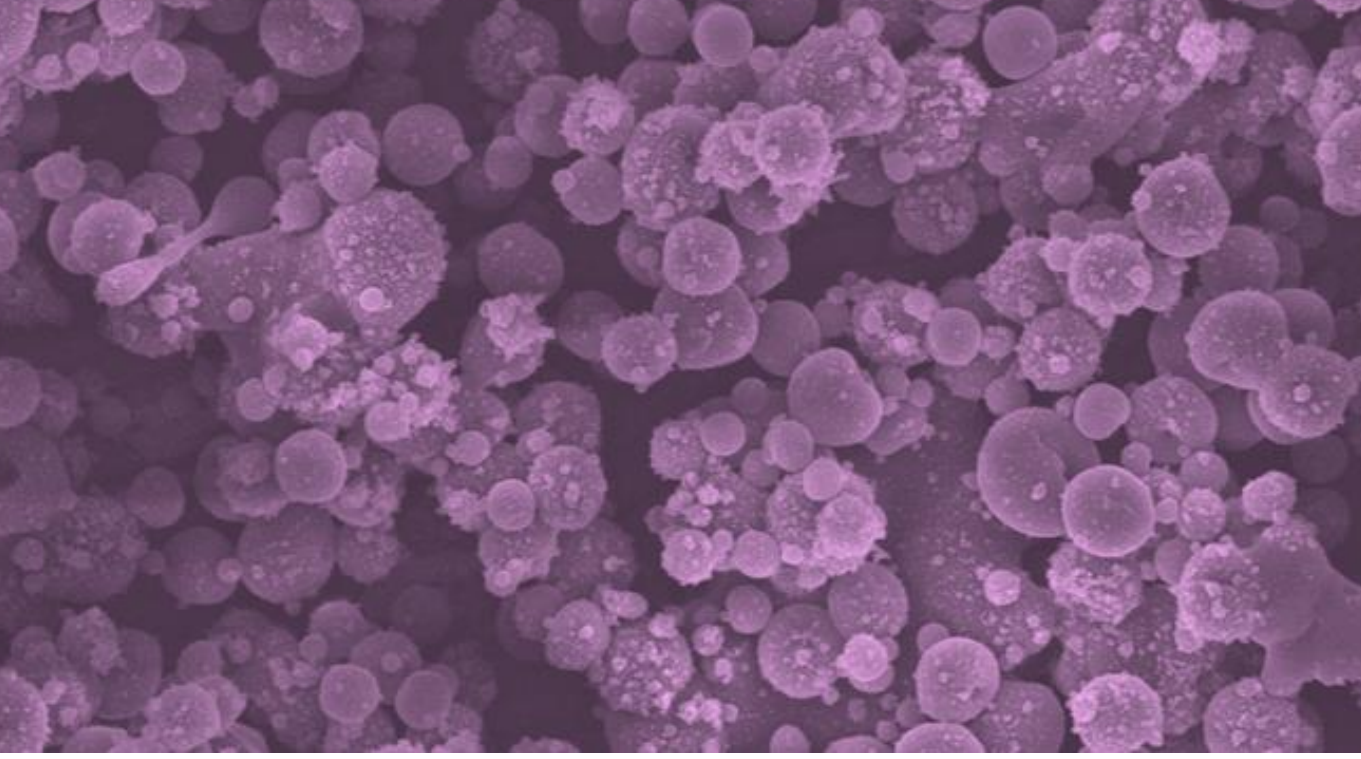


Figure S1. Mixture of the liposomes and WPC dispersions in the SAR micromixer at a flow rate 10-fold higher than the one required for electrospraying. No preferential pathway is taken by the liposome dispersion when the flow rate is increased 10-fold.



CHAPTER 3

MICROENCAPSULATION OF PROBIOTIC MICROORGANISMS

3.1. Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and in-vitro digestion.

Introduction to Chapter 3

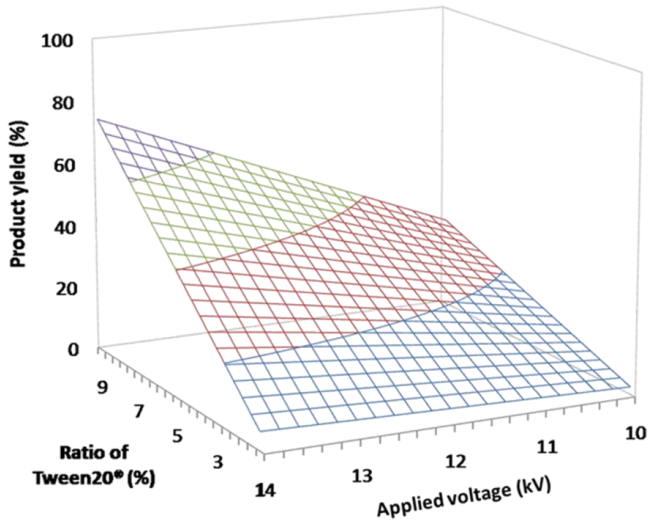
A widely accepted definition of probiotics, recognized by the FAO/WHO, states that they are 'live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host'. These benefits may include prevention of gastrointestinal infections, reduction of serum cholesterol levels, improvement of lactose metabolism and/or enhancement of the immune system. In order to exert these health benefits, the probiotic bacteria must be alive, metabolically active, and their concentration at the time of consumption should be high enough to exert their claimed health benefits. However, a number of studies report low levels of viable probiotic cells in commercial products, possibly due to viability losses during food processing and/or storage. This points out the need for developing efficient microencapsulation technologies capable of protecting probiotic bacteria against detrimental conditions within the food matrices in order to preserve their viability during food fabrication, shelf life and, ideally, during consumption.

Compared to the bioactive ingredients used in the previous chapters, which were chemical compounds, probiotic microorganisms involve additional challenges derived from the fact that they are living organisms. They are complex arrangements of biomolecules whose viability must be preserved during microencapsulation in order for them to survive throughout the process.

In this chapter, the electrospraying technique was used to encapsulate a model probiotic strain, *Lactobacillus plantarum*, within WPC-based microcapsules. For this purpose, the electrospraying conditions were optimized in order to minimize the viability loss during processing while maximizing productivity. Factors such as the convenience of microencapsulating fresh cultures or freeze-dried bacteria, as well as the impact of the applied voltage, surfactant concentration and addition of a prebiotic carbohydrate to the formulation were evaluated. The protection ability of the selected microencapsulation structures towards the probiotic strain was assessed during storage under stress conditions (medium and high relative humidity conditions) and after *in-vitro* digestion.

3.1

OPTIMIZATION OF ELECTROSPRAYING CONDITIONS FOR THE MICROENCAPSULATION OF PROBIOTICS AND EVALUATION OF THEIR RESISTANCE DURING STORAGE AND *IN-VITRO* DIGESTION



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Morfin, R. C., Pérez-Masiá, R., Sanchez, G., & Lopez-Rubio, A. (2016). *Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and in-vitro digestion*. LWT - Food Science and Technology, 69, 438-446. (DOI: http://dx.doi.org/10.1016/j.lwt.2016.01.071).

1. Abstract

Electrospraying has recently emerged as a novel microencapsulation technique with potential for the protection of probiotics. However, research efforts are still needed to minimize the viability loss observed during the processing of sensitive strains, and to maximize productivity. The aim of the present work was the optimization of the electrospraying conditions for the microencapsulation of a model probiotic microorganism, *Lactobacillus plantarum*, within a whey protein concentrate matrix. In a pre-optimization step, the convenience of encapsulating fresh culture instead of freeze-dried bacteria was established. Additionally, a surface response methodology was used to study the effect of the applied voltage, surfactant concentration, and addition of a prebiotic to the formulation on cell viability and productivity. Viability losses lower than 1 $\log_{10}$ CFU were achieved and the bacterial counts of the final products exceeded 8.5 $\log_{10}$ CFU/g. The protection ability of the developed structures during storage and *in-vitro* digestion was also evaluated.

2. Introduction

Microencapsulation technologies constitute a plausible approach for the preservation of biologically active ingredients in food systems including probiotic bacteria [1-3], not only during processing or storage, but also during gastrointestinal transit, improving the delivery of probiotic strains to the large intestine [4]. Several methods have been reported to microencapsulate probiotic microorganisms, including spray-coating [5], emulsion and/or spray-drying [6], extrusion [7], and gel-particle technologies [3]. Being a well-established process that can produce large amounts of material, spray-drying is the most commonly used microencapsulation technique in the food industry [8]. However, this technique involves the use of high temperatures, which results in significant cell mortality [9]. On the other hand, spray-coating and entrapment in gel particles generate relatively big particles (90-250 μm and >200 μm, respectively) that may affect food sensory qualities [10], and the latter is considerably expensive to be scaled up in the food industry [1, 5]. Recently, electrospraying has emerged as an alternative microencapsulation technique [11] which can generate very fine particulate structures in a one-step process [12] under mild conditions [13]. It basically involves the application of a high voltage electrical field to a polymer solution, dispersion or melt which is sprayed towards a charged collector, where the dry nano- or microparticles are deposited [12, 14, 15] (cf. Supplementary material). Some of the advantages of electrospraying include the possibility of working under mild ambient conditions and using food-grade solvents, achieving high encapsulation efficiencies and obtaining smaller particle sizes than in conventional mechanical atomisers [11, 12, 16]. Recently, electrohydrodynamic processes were proposed for

the entrapment of living bifidobacteria within ultrathin polymeric fibers [17], which significantly reduced their viability loss during storage at different temperatures. Furthermore, the electrospraying technique proved to be useful for the encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 within edible hydrocolloids for functional foods applications, effectively prolonging bacterial survival at different relative humidities [18]. Although the viability of the aforementioned commercial strain was not significantly affected by the electrohydrodynamic process, it was found that a certain viability loss occurred when trying to microencapsulate non-commercial probiotic cultures during the electrospraying processing. In addition, the different process parameters and the properties of the probiotic feed suspension have an impact on the productivity of the electrospraying technique, as the formation of stable jets from aqueous media is complicated [19] and often leads to dripping of the polymeric solution if conditions are not optimized. Thus, a study of the impact of different electrospraying variables on the bacterial viability loss and the process yield is needed.

Lactobacillus plantarum, a prominent species among lactic acid bacteria which has been found to colonize healthy human gastrointestinal tracts [20] and to whom a number of health benefits have been attributed, such as reduction of serum cholesterol levels [21] or downregulation of proinflammatory genes [22], has been used as a model probiotic microorganism for the present study. Regarding the encapsulation matrix, a whey protein concentrate (WPC) was selected as the main wall component of the capsules, as its capability for preserving the viability of probiotic microorganisms had already been demonstrated in a previous work using *B. animalis* [18]. Moreover, whey proteins are cheap by-products from the cheese industry and possess functional characteristics [13].

The aim of the present work was to study the impact of the electrospraying conditions on the viability of *L. plantarum* within the obtained WPC microstructures, as well as on the productivity of the process. This mathematical modelling has been attempted following a Design of Experiments (DoE) methodology [23], applying a second-order Box-Behnken design to maximize the bacterial viability and the process yield. The capability of the microencapsulation structures obtained by applying the optimal electrospraying conditions to prolong the survival of *L. plantarum* during storage at different relative humidities, as well as during static *in-vitro* digestion, was also evaluated.

3. Materials and methods

3.1. Materials

Whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090 and with a w/w composition of ~80% protein, ~9% lactose and ~8% lipids, was

kindly donated by ARLA (ARLA Food Ingredients, Viby, Denmark), and was used without further purification. *Lactobacillus plantarum* strain CECT 748 T was obtained from the Spanish Cell Culture Collection (CECT) and routinely grown in Man, Rogosa and Sharpe (MRS) broth (Scharlau, Barcelona, Spain). Serial dilutions were made in 1 g/L meat peptone solution and plate counting was performed on MRS agar, both provided by Conda Pronadisa (Spain). Surfactant Tween20®, maltodextrin (with dextrose equivalent 16.5-19.5), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract porcine and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Spain). The commercial resistant starch Fibersol® was manufactured by ADM/Matsutani (Iowa, USA). The LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Invitrogen (California, USA). All inorganic salts used for the *in-vitro* digestion tests were used as received.

3.2. Preparation of WPC dispersions

WPC dispersions were prepared by mixing the protein concentrate with distilled water or skimmed milk under magnetic stirring at room temperature to achieve a concentration of 0.3 g/mL. This concentration had been previously optimized based on previous works [13, 18] in order to minimize dripping of the suspensions during electrospraying. Fibersol® and/or Tween20® were also added to some of the formulations in variable amounts.

3.3. Preparation of probiotic cells suspensions

Two different strategies were used to incorporate the probiotic cells within the WPC dispersions. The first one involved the use of a fresh culture of *L. plantarum*. Bacteria were grown in MRS broth for 24 h at 37ºC, reaching the growth stationary state as observed from the growth curves (results not shown) constructed using a POLARstar Omega Microplate Reader from BMG LABTECH (Ortenberg, Germany) (final cell density of 9-10 $\log_{10}$ CFU/mL). The lactobacilli were then collected by centrifugation in 50 mL tubes at 4000 rpm for 5 min using an Eppendorf Centrifuge 5804R equipped with an Eppendorf Rotor S-4-72, obtaining a pellet that was subsequently washed twice with PBS and re-suspended in the WPC dispersions. The second approach consisted in freeze-drying the cell culture after re-suspension of the twice-washed pellet in a PBS solution containing 0.1 g/mL of maltodextrin, and subsequent incorporation of the freeze-dried cells into the WPC dispersions.

3.4. Preparation of probiotic-containing capsules through electrospraying

The suspensions were processed using a Fluidnatek® LE-10 electrospinning/ electrospraying apparatus, equipped with a variable high voltage 0–30 kV power

supply, purchased from BioInicia S.L. (Valencia, Spain). Probiotic-containing WPC suspensions were introduced into a sterile 5 mL plastic syringe and pumped at a steady flow-rate of 0.15 mL/h through a stainless-steel needle (2.41 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump (KD Scientific Inc., Holliston, U.S.A.). The obtained encapsulation structures were collected on a stainless-steel plate connected to the ground electrode of the power supply and placed at a distance of 10 cm with respect to the tip of the needle. The suspensions were processed during a fixed time of 4h. Applied voltage varied within the range of 10-14 kV. A schematic representation of the setup used for microencapsulation can be found in the supplementary material.

3.5. Viability of encapsulated and non-encapsulated *L. plantarum*

The viability of *L. plantarum* was evaluated by plate counting. Samples were subjected to 10-fold serial dilutions in 1 g/L meat peptone solution and plated on MRS agar. After 24-48 h incubation at 37ºC, the number of colony-forming units (CFU) was determined. Cell viability was evaluated for the probiotics-containing WPC suspensions before processing (N_0) and in the dry electrosprayed products (N_{ES}) by resuspension of a precise amount of the powder in 1 mL of peptone solution. Tests were made in triplicate.

3.6. Morphological characterization of the capsules

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 10-12 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination.

In addition, optical microscopy images were taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

In order to confirm the presence of the probiotic bacteria within the WPC capsules, the cells were stained using a LIVE/DEAD kit (BacLight® viability kit, Invitrogen) prior to the electrospraying process. Because the dye used to observe live cells in green (SYTO 9®) was found to also stain WPC, it was not possible to discern live bacteria from the encapsulating matrix after the electrospraying process, and only sporadic death cells could be distinguished in red due to the propidium iodide dye. Consequently, bacteria were intentionally killed before staining by resuspension of the twice-washed bacterial pellet in 20 mL of 96% (v/v) ethanol and subsequent

incubation at room temperature for 40 minutes. The ethanolic suspension was then washed and the dead pellet was resuspended in 10 mL of buffered peptone water. Propidium iodide was used as a red dye according to the manufacturer protocol, before incorporating the dead bacteria to the feed WPC dispersions for electrospraying.

3.7. Optimization of electrospraying process parameters through Box-Behnken experimental design

Three key variables were selected for the optimization of the electrospraying process in terms of bacterial viability loss (y_1) and product yield (y_2): the applied voltage (x_1), the concentration of a prebiotic additive (Fibersol®) incorporated into the formulation (x_2), and the concentration of a surfactant (Tween20®) added to the feed suspensions (x_3). A Box-Behnken fractional-factorial experimental design was developed with these three variables at three levels (3^3) in order to reduce the number of experimental runs. This model was used to correlate the response variables, y_1 and y_2, to the independent variables, x_1, x_2 and x_3, by fitting them to a polynomial second order model, whose general equation is Eq. (1), where y_i are each of the predicted responses, x_i and x_j are the input variables affecting the response variables, β_0 is the offset term, β_i are the linear coefficients, β_{ii} are the quadratic coefficients and β_{ij} are the interaction coefficients [24]. Table 1 summarizes the independent variables (factors) used and their assayed levels (coded as +1, 0 and -1).

$$y_i = \beta_0 + \beta_i \sum x_i + \beta_{ii} \sum x_i^2 + \beta_{ij} \sum_i \sum_j x_i x_j \qquad \text{Eq. (1)}$$

Table 1. Factors for the Box-Behnken design and their levels. Mass fractions are expressed with respect to the amount of WPC.

Factors		Levels		
		-1	0	+1
x_1	Voltage (kV)	10	12	14
x_2	Fibersol® (wt.%)	0	10	20
x_3	Tween20® (wt.%)	1	5	9

A small number of experimental runs (i.e. 15 runs, each made in triplicate) was necessary for the optimization process (cf. Table 3). The design included replicated central points. The bacterial viability loss (ΔN) was calculated as the difference in viability per unit mass of dry solids in the suspensions before processing and in the dry electrosprayed products, according to Eq. (2). The product yield was determined according to Eq. (3).

$$\Delta N = N_0 - N_{ES} \quad \text{Eq. (2)}$$

$$Product\ yield\ (\%) = \frac{Mass\ of\ electrosprayed\ product\ recovered\ from\ collector}{Mass\ of\ solids\ in\ the\ processed\ suspension} \times 100 \quad \text{Eq. (3)}$$

3.8. Survival of encapsulated *L. plantarum* under stress conditions

Selected electrosprayed capsules containing *L. plantarum* were stored at medium and high relative humidities (RH), i.e. 53 and 75%, as described in [18], and their bacterial viability was tested after different time intervals. For this purpose, the powders were introduced in desiccators containing $Mg(NO_3)_2$ and NaCl saturated solutions, respectively. Similarly, freeze-dried samples of *L. plantarum* obtained from the same formulations used for the electrospraying process (by lyophilization of the feed WPC-based suspensions) were also stored at the same conditions, in order to compare the proposed microencapsulation technique with a well-established preservation method.

3.9. Survival of encapsulated *L. plantarum* during digestion

Suspensions (0.03 g/mL) of the electrosprayed capsules or their freeze-dried counterparts, respectively, in distilled water were subjected to *in-vitro* gastrointestinal digestion in order to evaluate the survival of protected *L. plantarum* during simulated consumption. Digestion was simulated according to the standardized static *in vitro* digestion protocol developed within the framework of the Infogest COST Action [25]. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized compositions [25]. In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at 37ºC for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37ºC for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37ºC for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively. Aliquots were collected after the gastric and the duodenal phases and the viability of *L. plantarum* in the digestas was assessed by plate counting.

3.10. Statistical analysis

The response surface modelling was conducted using the software Unscrambler X (version 10.1, CAMO software AS, Oslo, Norway, 2010). The statistical analysis of the Box-Behnken model was performed through analysis of variance

(ANOVA). A $p < 0.05$ was considered significant. The determination coefficient (R^2), which measures the goodness of fit of the regression model, was used as an indicator of the quality of the model to predict the experimental data.

A statistical analysis of the rest of the experimental data was performed through analysis of variance (one-way ANOVA) using OriginPro 8 (OriginLab Corp., Northampton, USA). Homogeneous sample groups were obtained by using Fisher LSD test (95% significance level, $p < 0.05$).

4. Results and discussion

4.1. Pre-optimization of the electrospraying conditions

The first (preliminary) step in this process optimization was to determine whether the initial state of the cells had an effect on cell viability after encapsulation and, also, to determine the maximum cell load which could be incorporated within the protein matrix. For this purpose, freeze-dried bacteria were added to the WPC dispersions in different amounts, and compared with addition of bacterial pellets obtained from different volumes of fresh culture in 5 mL of WPC dispersion. The use of a non-ionic surfactant, Tween20® (a polysorbate), to improve the electrospraying process [26] and limit the dripping of the aqueous solutions was also considered, and its influence on the bacterial viability was thus studied. Table 2 summarizes the test conditions used is this first stage. The applied voltage was fixed at 10 kV in all cases, keeping the rest of the processing parameters as stated in Section 3.4.

Table 2. Different conditions evaluated during the pre-optimization stage.

State of bacteria	Amount of bacteria	Proportion of Tween20®
Freeze-dried	5 $mg/mL_{WPC\ susp.}$	-
Freeze-dried	10 $mg/mL_{WPC\ susp.}$	-
Freeze-dried	15 $mg/mL_{WPC\ susp.}$	-
Freeze-dried	5 $mg/mL_{WPC\ susp.}$	1 $g/100g_{\ WPC}$
Freeze-dried	5 $mg/mL_{WPC\ susp.}$	5 $g/100g_{\ WPC}$
Fresh culture	Pellet from 5 mL broth	-
Fresh culture	Pellet from 5 mL broth	5 $g/100g_{\ WPC}$
Fresh culture	Pellet from 10 mL broth	5 $g/100g_{\ WPC}$
Fresh culture	Pellet from 100 mL broth	5 $g/100g_{\ WPC}$

First, the initial viability (N_0) of *L. plantarum* in the WPC suspensions was compared using the different formulations. It was observed that adding higher

amounts of freeze-dried bacteria (i.e. $5 \cdot 10^{-3}$, 10^{-2} and $1.5 \cdot 10^{-2}$ g/mL) did not yield significant differences ($p < 0.05$) in the initial cell counts. All the formulations prepared using freeze-dried bacteria exhibited an initial bacterial viability in the range of 7.23 ± 0.21 $\log_{10}$ CFU/g. Similarly, no significant differences ($p < 0.05$) were found in the initial cell counts when using pellets obtained from 5 mL or 10 mL of fresh culture to prepare the suspensions. In these cases, the average initial viability was 9.38 ± 0.42 $\log_{10}$ CFU/g. Lastly, the suspension prepared using a pellet obtained from 100 mL of fresh culture had an average N_0 of 10.28 ± 0.35 $\log_{10}$ CFU/g. These results showed that significantly higher initial cell counts were achieved when using fresh culture for the preparation of the suspensions. It is worth mentioning that higher amounts of freeze-dried bacteria could not successfully be incorporated into the WPC dispersions because the resultant feed suspensions aggregated and precipitated in the syringe during the electropraying process.

Figure 1 summarizes the results from viability loss (ΔN) and product yields of *L. plantarum* after the electrospraying process of the different formulations containing freeze-dried bacteria (a, c) and fresh culture (b, d). In general, greater viability losses and lower productivities were observed when using freeze-dried microorganisms than for fresh cultures, mainly explained by the poor bacterial dispersion within the WPC suspension when using the freeze-dried form of the probiotic strain. However, the suspension prepared using 100 mL of fresh culture also exhibited a high viability drop and lower productivities, which could be attributed to an excess of biomass in the formulation which apart from hindering proper microencapsulation of the bacteria, led to extensive dripping of the solution. In general, the product yield was considerably improved by the addition of Tween20® which, apart from favouring bacterial dispersion, led to lower surface tensions of the suspensions [19] and it did not significantly affect the viability loss of *L. plantarum* at the low concentrations used (1-5% w/w).

In view of the results, the formulation containing a bacterial pellet obtained from 10 mL of fresh broth and 5 wt.% of Tween20® provided the greatest product recovery percentage (16.1 ± 2.1%) while experiencing one of the lowest viability losses (0.6 ± 0.1 $\log_{10}$ CFU/g). This specific formulation resulted in capsules containing over 10^9 CFU/g (cf. Figure 2), a final bacterial count in the dry product which was similar to that obtained from a 100 mL-pellet, although with much higher productivity. Thus, this formulation was chosen as the starting point for further optimization of the electrospraying process through a Box-Behnken experimental design, in order to increase the product yield while maintaining high bacterial viabilities in the final product.

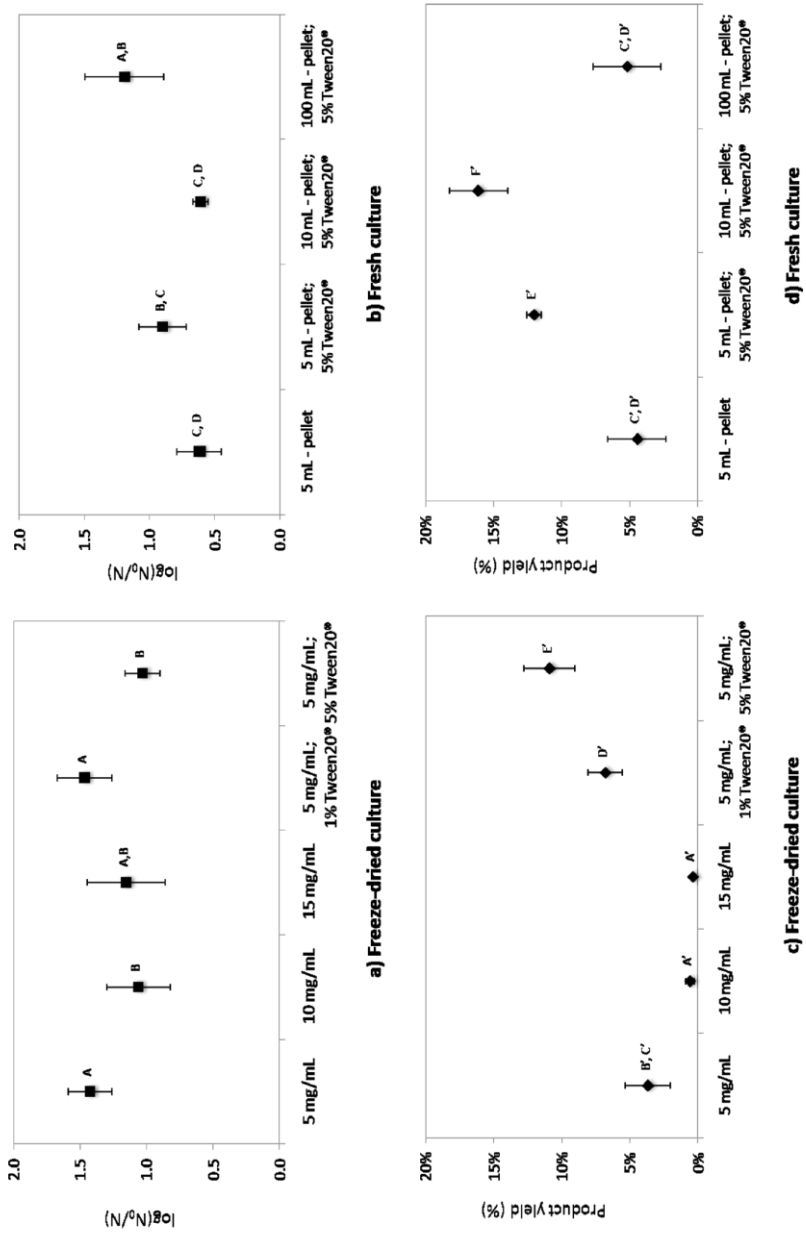


Figure 1. Viability losses obtained using a) freeze-dried bacteria and b) fresh culture, and product yields using c) freeze-dried bacteria and d) fresh culture. Different letters in each pair of graphs denote statistically significant differences between results ($p<0.05$).

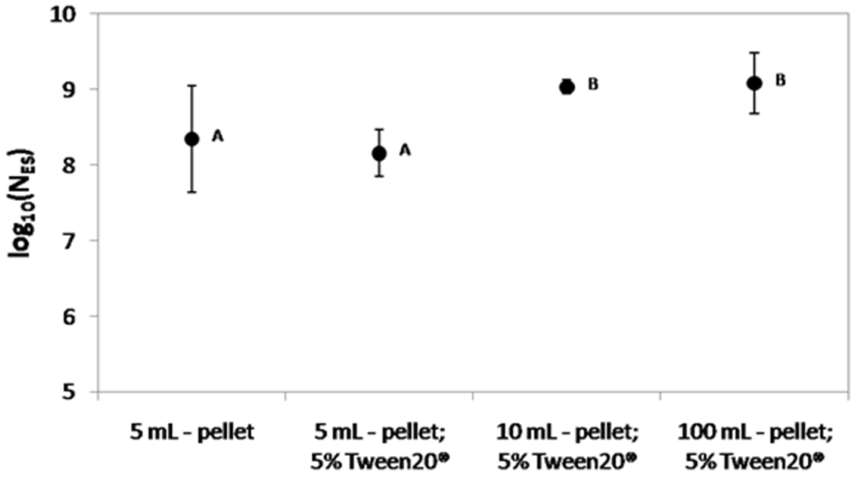


Figure 2. Cell viability of the final electrosprayed products obtained from pellets of fresh culture of L. plantarum. Different letters denote statistically significant differences between results ($p<0.05$).

4.2. Mathematical modelling of the electrospraying process

Three key factors potentially influencing the bacterial viability and product yield in the microencapsulation of probiotics through electrospraying processes were selected for the Box-Behnken modelling: the applied voltage (x_1), the concentration of Fibersol® in the formulations (x_2), and the ratio of Tween20® in the feed suspensions (x_3). The applied voltage is known to exert an effect on the properties of electrosprayed materials [11]. On one hand it must be sufficiently high to overcome the surface tension of the suspensions, in order to efficiently produce the microcapsules. On the other hand, it was hypothesized that too strong electric fields might impose a source of stress on the probiotic strain, having an impact on its viability. Fibersol® is a commercial resistant starch recognized as GRAS by the FDA, so it was used as a prebiotic additive [27] to ascertain whether the addition of the carbohydrate, apart from giving rise to a symbiotic product, could enhance bacterial viability within the capsules. Other prebiotics have been previously reported to increase the viability of probiotic bacteria upon microencapsulation through spray-drying [28]. Lastly, the positive effect of Tween20® on the product yield was evidenced in the previous section and a more exhaustive study of its impact on bacterial viability loss should be carried out in order to optimize the feed formulation.

Hence, a Box-Behnken design was developed with these three factors at three levels in order to assess their impact on the response variables and find the optimum combination of these parameters able to yield the best results. The lower and upper levels of each factor (cf. Table 1) were fixed based on preliminary experiments carried out to determine the limits which allowed a stable electrospraying process (results not shown). A total of 15 experimental runs, each made in triplicate, were necessary to

construct the design models. Table 3 summarizes the full design and the experimental values obtained for the response variables in each run.

Table 3. Complete full design and results for the response variables.

Run	x_1 (kV)	x_2 (wt.%)	x_3 (wt.%)	y_1 (log_{10} CFU/g)	y_2 (%)
1(*)	10	0	5	0.92 ± 0.16	16.8 ± 1.6
2	14	0	5	0.96 ± 0.13	54.5 ± 8.4
3	10	20	5	0.76 ± 0.15	24.6 ± 1.7
4	14	20	5	0.74 ± 0.03	56.4 ± 4.1
5	10	10	1	0.64 ± 0.09	9.3 ± 7.9
6	14	10	1	0.50 ± 0.12	3.2 ± 3.0
7	10	10	9	0.66 ± 0.12	29.6 ± 9.4
8	14	10	9	0.83 ± 0.05	65.7 ± 9.2
9	12	0	1	0.72 ± 0.15	5.0 ± 1.6
10	12	20	1	0.84 ± 0.20	7.2 ± 2.5
11	12	0	9	0.99 ± 0.09	50.6 ± 10.1
12	12	20	9	0.89 ± 0.18	68.5 ± 3.5
13-15	12	10	5	0.54 ± 0.04	31.0 ± 7.8

(*) From previous section

The results in Table 3 were used to construct two polynomial second order models, according to Eq. (1), with the aid of the software Unscrambler X, each corresponding to one of the response variables y_1 and y_2. Both models were statistically analyzed using analysis of variance (ANOVA) in order to check the significance of their linear, quadratic and interaction terms, as well as the significance of the models themselves. The quality of the models was also checked by comparing the experimental results in Table 3 with the values predicted by the models. As observed in Figure 3, the model obtained for y_1 did not accurately describe the experimental values. This was attributed to the intrinsic variability of the results and consequent high deviations that are obtained when studying microbiological systems. Due to its great lack of fit, the use of this model for the prediction and optimization of the viability loss during electrospraying was considered risky and thus the model was disregarded. Conversely, an acceptable value was obtained for the lack of fit of the model for y_2 and, thus, this model was considered adequate for the prediction and optimization of the product yield for the proposed electrospraying process.

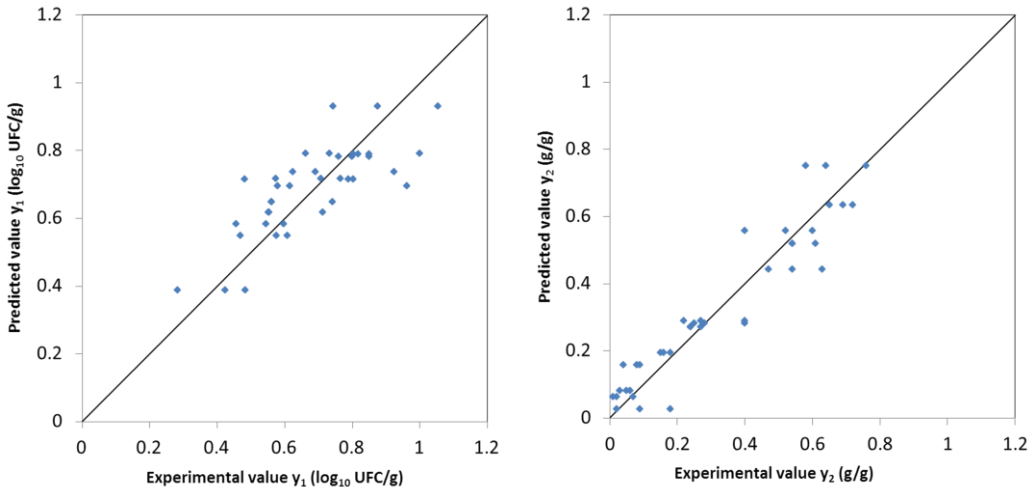


Figure 3. Predicted versus experimental values for a) viability loss and b) product yield.

The final model equation which correlates the product yield with the three factors, after disregarding non significant terms, is expressed in Eq. (4), where x_i are the coded values of the factors (from -1 to +1, cf. Table 1). Table 4 shows the results of the ANOVA analysis for this model, from where it could be concluded that the linear terms corresponding to the applied voltage and the ratio of Tween20®, as well as their interaction, are highly significant ($p<0.01$). The linear and quadratic terms involving the ratio of Fibersol® in the formulation were also statistically significant ($p<0.05$). The offset term (β_0 = 27.9) corresponds to the predicted value of the product yield at the central point ($x_1=0$; $x_2=0$; $x_3=0$), and its value is not significantly different from the experimental result (31.0 ± 7.8 %). This further confirms the goodness of fitting of this model.

$$y_2(\%) = (27.9 + 12.4x_1 + 3.8x_2 + 23.8x_3 + 10.7x_3x_1 + 7.5x_2^2) \quad \text{Eq. (4)}$$

In practice, the mathematical model in Eq. (4) can be interpreted by comparing the magnitude of its coefficients. Firstly, all of them are positive, which means that an increase in any of the three factors within the limits of the model had a favourable effect on product yield. The factors which had the greatest impact were the concentration of surfactant (β_3 = 23.8) and the applied voltage (β_1 = 12.4). Indeed, the addition of surfactants has been proposed as a useful strategy for the successful production of electrosprayed materials from biopolymeric aqueous solutions or dispersions, as they reduce their high surface tension and thus facilitate their spraying at acceptable voltages [19]. On the other hand, increasing the applied voltage helps overcoming the surface tension of the fluid and facilitates the electrospraying process [11]. In fact, both factors had a synergistic effect, as evidenced from their high interaction coefficient (β_{13} = 10.7). Figure 5 shows the interaction effect of varying

these two factors on the product yield according to the model, for a constant level of x_2. The addition of the prebiotic carbohydrate also improved the product yield, although to a lesser extent (β_2 = 3.8; β_2^2 = 7.5). It is worth noting that product yields over 65% were achieved for the best combinations, that is, more than three times greater than the best result obtained from the pre-optimization stage, showing a considerable improvement.

Table 4. ANOVA analysis for the response surface quadratic model in Eq. (4).

	Sum of Squares	DF(*)	Mean Square	F ratio	p-value
Model	1.955	5	0.3909	55.54	4.1×10^{-15}
x_1	0.373	1	0.3725	52.92	2.4×10^{-8}
x_2	0.035	1	0.0353	5.01	3.2×10^{-2}
x_3	1.359	1	1.3585	193.01	2.4×10^{-15}
x_1x_3	0.137	1	0.1365	19.40	1.0×10^{-4}
x_2^2	0.052	1	0.0517	7.34	1.0×10^{-2}
Lack of Fit	0.129	7	0.0184	4.63	0.0018
$R^2 = 0.894$					

(*) DF = Degrees of freedom

Regarding the viability loss during electrospraying, although a successful mathematical modelling was not achieved in terms of the three proposed factors, the results allowed the extraction of some qualitative conclusions about the effects of these variables on the process. While a clear tendency was not observed for variations in the applied voltage or the concentration of Fibersol® in the formulation, a slight increase in the viability loss during electrospraying was found in average for increasing Tween20® contents. In any case, the viability loss always remained below 1 $\log_{10}$ CFU/g, thus yielding average bacterial counts in the final electrosprayed materials in the range of 8.7-9.3 $\log_{10}$ CFU/g.

4.3. Morphology of selected encapsulation structures

Two of the electrosprayed materials obtained from section 4.2. were selected for further evaluation. The first one was the powder produced in Run 8, as the conditions used for these tests (x_1=1; x_2=0; x_3=1) resulted in one of the highest product yields. No significant differences were found between the product yields obtained for Run 8 and 12 (cf. Table 3), but the former showed a slightly lower viability loss and, thus, this sample was chosen. The second one was selected in order to obtain minimal viability losses. However, the runs which resulted in the minimum losses had too low product yields. Thus, a minimum product yield of 50% was fixed as an acceptable limit considering that lower yields would not be industrially attractive for production. The conditions used in Run 4 (x_1=1; x_2=1; x_3=0) resulted in the electrosprayed

capsules which, meeting this requirement, experienced the lowest viability loss, so the product obtained from this specific composition was chosen for further testing.

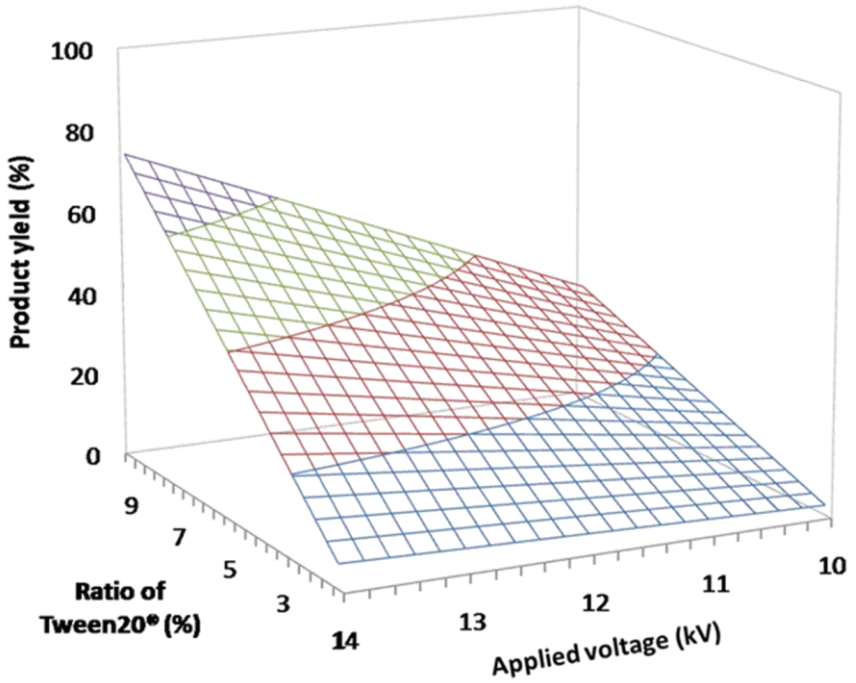


Figure 4. Response surface for the product yield, showing the interaction of the applied voltage and the ratio of Tween20® at a constant level of prebiotic concentration (level 0).

Figure 5 shows the morphology of the selected samples. The electrosprayed product obtained from Run 8 exhibits a more homogeneous structure, where individual and spherical particles could be distinguished. In contrast, the powder obtained in Run 4 presents a rather amorphous shape, with some individual capsules but also some fused structures. These differences might be attributed to the greater ratio of Tween20® used in Run 8, which facilitated the electrospraying process by reducing the surface tension of the suspensions. The location of the lactobacilli inside the electrosprayed capsules was confirmed by the optical and fluorescence microscopy images of the materials (cf. Figure 5 C and D). As stated in Section 3.6, bacteria had to be killed before staining to avoid the use of the dye SYTO 9®, which also stained the WPC matrix and precluded the identification of live cells. Although many of the particles did not contain microorganisms, the presence of dead bacteria within some of the WPC-based capsules was confirmed.

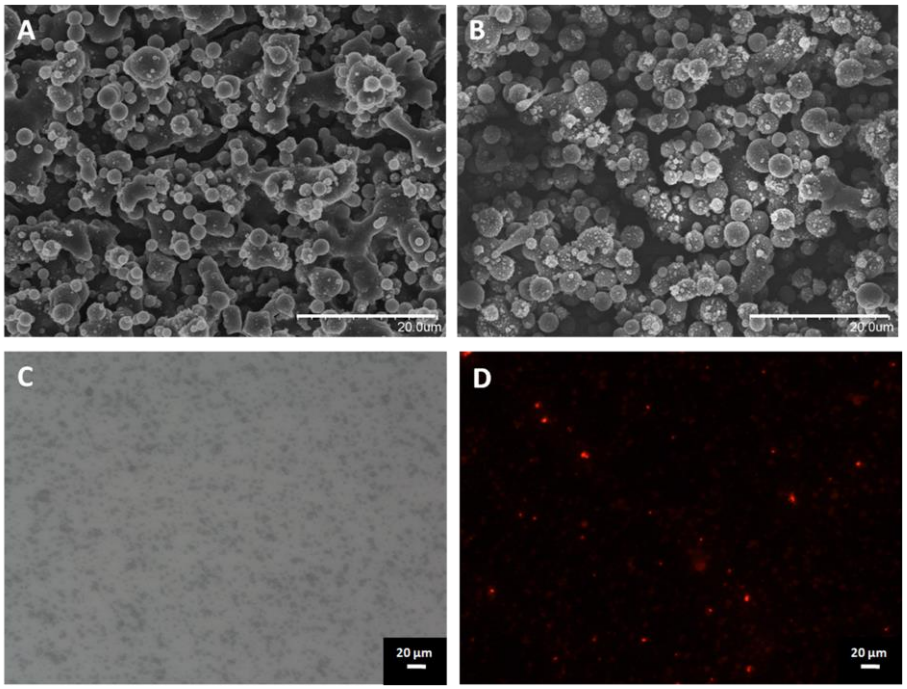


Figure 5. SEM images of WPC-based electrosprayed microcapsules containing *L. plantarum*, obtained under conditions in Run 4 (A) and Run 8 (B), and optical micrographs of the latter under normal light (C) and using a fluorescence source (D). Scale bars in all images correspond to 20 µm.

4.4. Survival of encapsulated *L. plantarum* under stress conditions

The ability of the selected capsules to protect *L. plantarum* when subjected to stress conditions was assessed by measuring the viability of the probiotic within the materials after storage during certain time periods at different relative humidity conditions (i.e. 53% and 75%). The survival of the lactobacilli within the electrosprayed capsules was compared to that of freeze-dried samples containing the same formulations as in Runs 4 and 8, respectively. All materials exhibited similar initial cell counts regardless of the method used for their preservation, confirming that the viability losses observed upon electrospraying are similar to those produced during freeze-drying. Figure 6 shows that electrosprayed microcapsules provided enhanced protection to the bacteria compared to freeze-drying for the same formulations at both storage conditions. Indeed, for the freeze-dried materials, a reduction of 1 $\log_{10}$ CFU/g was observed after 1 day of storage at 75% RH, and there were no cell counts after 1 week. In contrast, no viability losses were found for microencapsulated bacteria after 24h and their survival was prolonged for 10 days at the same conditions. Similarly, less than 1 $\log_{10}$ CFU/g reduction was observed for

microencapsulated bacteria after 3 weeks of storage at 53% RH while the viability of freeze-dried bacteria decreased almost 3 log_{10} CFU/g in the same period.

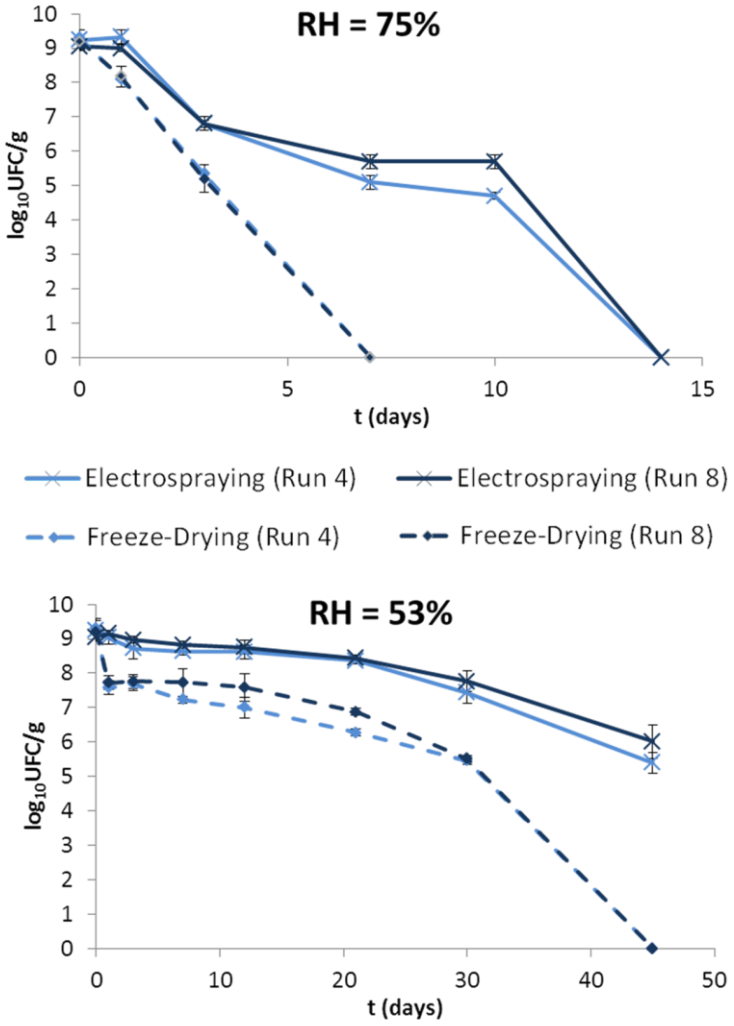


Figure 6. Survival of electrosprayed microcapsules (continuous lines) vs. freeze-dried materials (dotted lines) after storage at different relative humidities.

Furthermore, while no cell counts were found for the freeze-dried samples after 45 days, the microcapsules only experienced about 3 log_{10} CFU/g viability loss in the same time period. These results are attributed to a better material organization in the compact, capsular assemblies than in the porous, random structure of the freeze-dried material, which resulted in the prolonged viability of the encapsulated bacteria.

4.5. Survival of encapsulated *L. plantarum* during digestion

Viability of *L. plantarum* microcapsules and freeze-dried material was also evaluated after an *in-vitro* digestion process. Table 5 shows the cell counts obtained initially and after the gastric and duodenal phases of the digestion. Again, all the materials presented similar cell counts prior to the digestion process. It was observed that the main viability loss occurred after the gastric phase, due to the acidic conditions of this stage (pH=3). However, the microencapsulation achieved a slightly better protection for the bacteria than freeze-drying, probably because the arrangement of the wall material into capsular structures delayed its dissolution, thus slightly delaying the exposure of the probiotic bacteria to the simulated gastric fluid and enhancing the protective effect of the matrix in comparison with the freeze-dried samples. However, very small differences were observed after the intestinal phase, probably because in this step the capsules were completely disrupted and could not protect the bacteria. Nevertheless, the slight differences observed between both processing techniques could also be ascribed to the high resistance of this specific strain to acidic conditions, as observed in preliminary trials which showed that *L. plantarum* viability was hardly affected by acid conditions (pH = 3.8) after 1h of exposure (data not shown).

Table 5. Viability of *L. plantarum* before digestion and after the gastric and duodenal phases. Different letters (a-c) within the same column indicate significant differences among the samples.

Sample	Initial (UFC/g)	After Gastric Phase (UFC/g)	After Intestinal Phase (UFC/g)
Freeze-Drying (Run 4)	$(2.3 \pm 1.4) \times 10^{9\,a}$	$(1.5 \pm 0.1) \times 10^{7\,a}$	$(4.7 \pm 1.1) \times 10^{6\,a}$
Electrospraying (Run 4)	$(2.0 \pm 1.4) \times 10^{9\,a}$	$(2.7 \pm 0.5) \times 10^{7\,b}$	$(8.7 \pm 3.3) \times 10^{6\,a}$
Freeze-Dring (Run 8)	$(1.7 \pm 0.8) \times 10^{9\,a}$	$(1.4 \pm 0.4) \times 10^{7\,a}$	$(6.7 \pm 1.6) \times 10^{6\,a}$
Electrospraying (Run 8)	$(2.3 \pm 0.6) \times 10^{9\,a}$	$(7.3 \pm 0.7) \times 10^{7\,c}$	$(1.6 \pm 0.2) \times 10^{7\,b}$

5. Conclusions

The present work shows the convenience of using fresh culture of *L. plantarum* over freeze-dried bacteria for the preparation of the feed suspensions, as this approach led to higher initial cell counts in the WPC suspensions, lower viability losses during electrospraying and greater process productivities. Also, the addition of a surfactant, Tween20®, to the feed suspensions considerably increased the product yield. Although the model obtained for the viability loss could not explain the experimental results with statistical significance, a model for the product yield was

successfully developed through a Box-Behnken experimental design. According to this model, an increase in any of the three selected factors selected (applied voltage, surfactant concentration and addition of a prebiotic) had a favourable effect on the product yield, being the concentration of surfactant and the applied voltage the factors which had the greatest impact on the product yield, exhibiting a synergistic effect. Regarding the bacterial viability loss during electrospraying, it remained below 1 $\log_{10}$ CFU/g in all tests, so that the final electrosprayed materials had average bacterial counts in the range of 8.7-9.3 $\log_{10}$ CFU/g. Finally, while the electrosprayed microcapsules conferred *L. plantarum* similar protection against digestion as compared to a more widely-used preservation method such as freeze-drying, they proved to offer enhanced protection during storage of the food ingredient at high relative humidity. Since the ingredients used to prepare the encapsulating matrices in this study are edible and/or recognized by the FDA as GRAS, incorporation of the proposed structures into food products would be a feasible approach for the development of enhanced functional food products containing probiotics.

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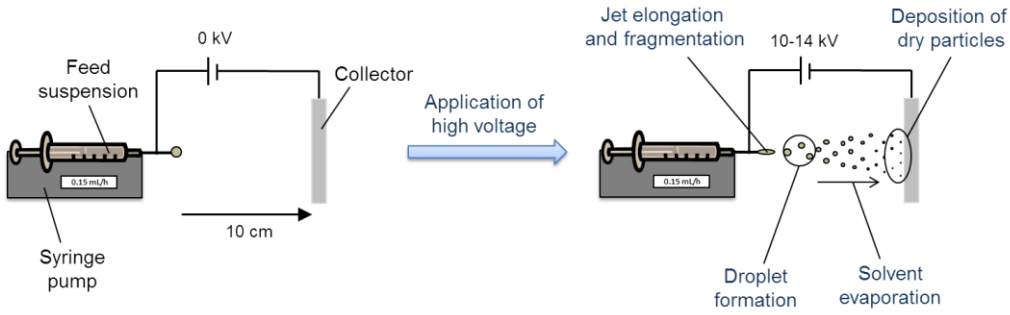
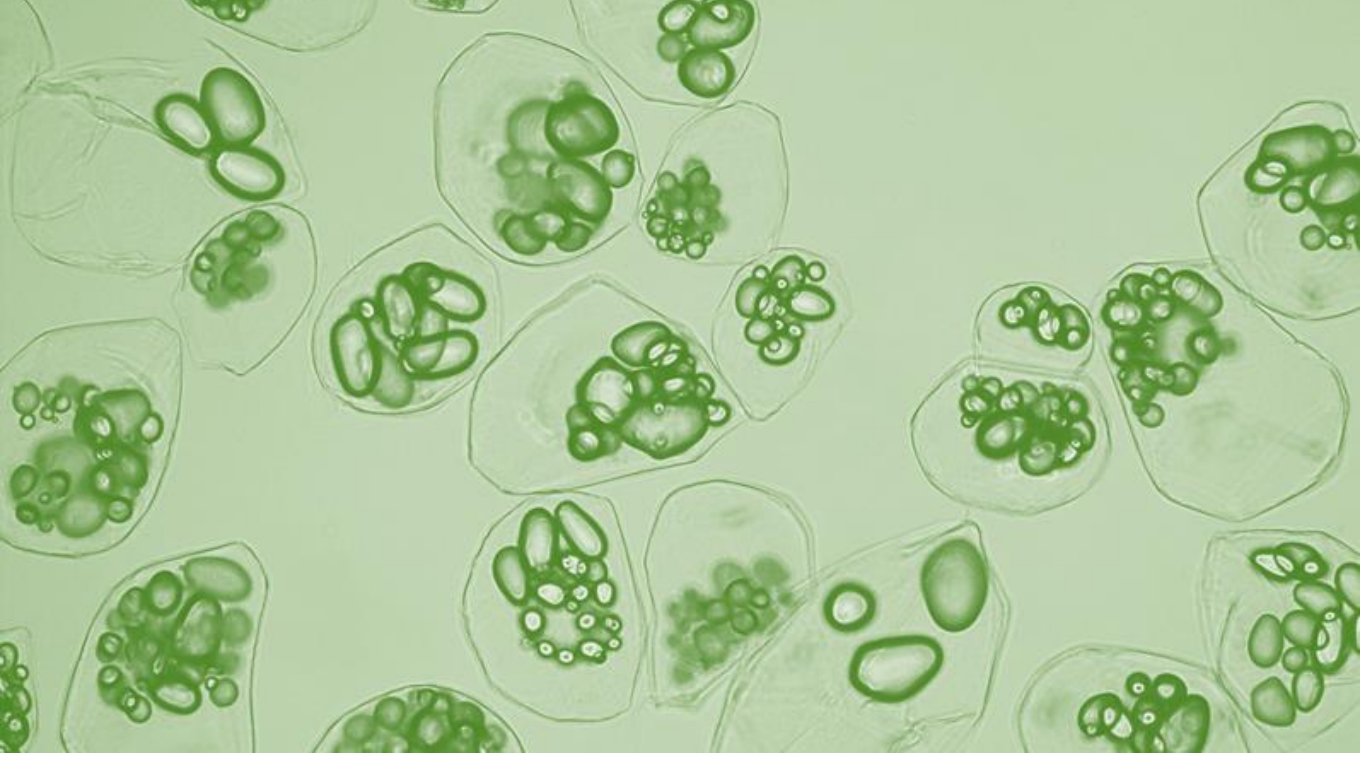
8. Supplementary Material

Scheme S1. Simplified representation of the electrospraying setup used for the microencapsulation of *L. plantarum*.





CHAPTER 4

POTENTIAL OF PLANT CELLS AS DELIVERY VEHICLES FOR FUNCTIONAL INGREDIENTS

4.1. Binding of dietary phenolic compounds to potato cells and individual cell components - nutritional and industrial implications.

Introduction to Chapter 4

An alternative microencapsulation approach is the use of biological systems as vehicles for bioactive ingredients. Cells can be regarded as natural carriers since they consist of a number of biomolecules enclosed within a semi-permeable membrane. Indeed, microorganisms such as yeasts have been already proposed as stabilizing vehicles for bioactive compounds such as certain antioxidants.

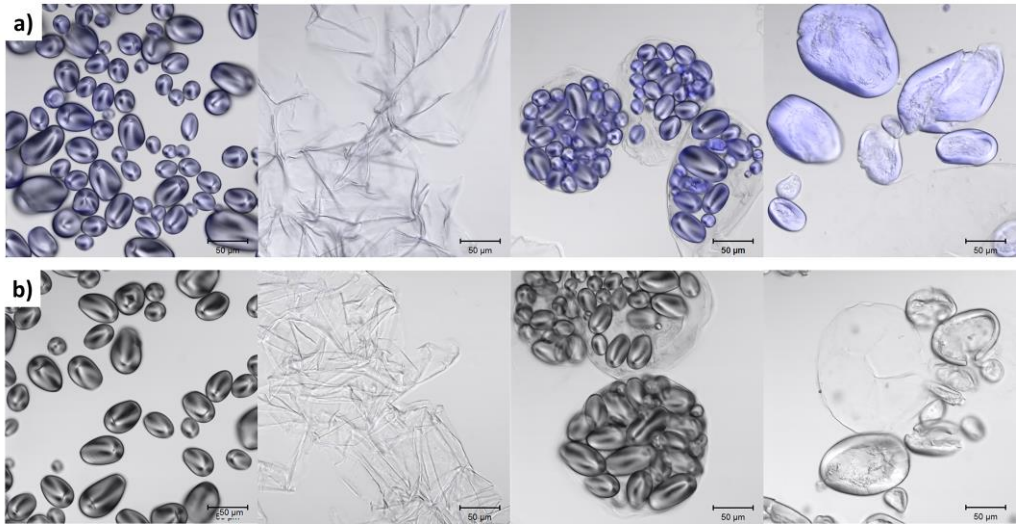
In this chapter, the potential of plant cells for the encapsulation of functional ingredients was explored. Specifically, cells from potato tubers were selected in this exploratory work because their structure is simpler than that of most plant cells, mainly consisting of starch granules and cell walls. Moreover, potatoes are an essential part of the human diet, being amongst the three most important food crops and the most widely consumed plant root.

Phenolic compounds were selected as model antioxidant ingredients, due to their ability to bind to different macromolecules and, particularly, to dietary polysaccharides such as starch and plant cell wall components. Representative phenolic compounds from diverse origins and with different molecular structures were used for this purpose, namely (+)-catechin, phloridzin and vanillic acid.

The ability of different potato cell components, i.e. isolated starch and isolated cell walls, to bind the selected phenolic compounds was first assessed and compared to the binding capacity of intact potato cells and disrupted potato cells, varying the concentration of polyphenols, pH and incubation times. Additionally, the binding capacity of intact potato cells was compared to that of cooked potato cells, in order to assess the impact of starch gelatinization on the loading capacity of the proposed encapsulation vehicles.

4.1

BINDING OF DIETARY PHENOLIC COMPOUNDS TO POTATO CELLS AND INDIVIDUAL CELL COMPONENTS - NUTRITIONAL AND INDUSTRIAL IMPLICATIONS.



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Dhital, S., López-Rubio, A., & Gidley, M. J. (2017). *Dietary polyphenols bind to potato cells and cellular components*. Journal of Functional Foods, 37, 283-292. (DOI: https://doi.org/10.1016/j.jff.2017.07.062).

1. Abstract

The ability of phenolic compounds to bind to dietary polysaccharides such as starch and plant cell wall components impacts their nutritional value. Here, we report interactions between potato cells and three different phenolic compounds (+)-catechin, phloridzin and vanillic acid. The binding interactions of the phenolic compounds with intact potato cells, as well as disrupted cells, cooked cells, isolated cell walls and starch granules was explored varying polyphenol concentration, pH and incubation time. Results showed that binding capacity depends on the type of phenolic compound ((+)-catechin > phloridzin > vanillic acid) as well as the type of substrate, to a maximum of ~50 μmol/g (dry weight). The observed differences ($p < 0.05$) were ascribed to the amount and accessibility of potential binding sites in both the phenolic compounds and the polysaccharides. Remarkably, polyphenols could penetrate intact cells and bind the starch within them, suggesting their potential as delivery vehicles, whose loading capacity more than doubled after cooking.

2. Introduction

The application of phenolic compounds as functional ingredients is widely studied [1-5]. In a complex food system, interactions between these bioactive compounds and the food matrix may affect the quality of polyphenol-rich food products, as these interactions impact both the bioavailability and bioactivity of the phenolic compounds [6, 7].

Recent studies using *in-vitro* systems suggest that nutritionally-significant amounts of phenolic compounds can bind to plant cell walls and cell wall analogues [8-10]. These bound phenolics are, therefore, delivered to the large intestine where both dietary fiber and polyphenols have been shown to exert positive effects on microbial populations, thereby promoting health benefits [11-15]. This and other practical implications have led to an increase in the research attention paid to the binding of phenolic compounds to plant cell walls [16-18]. Apart from cell walls, the interactions between phenolic compounds and starch, another major plant-derived carbohydrate in human diet [19, 20], may affect not only the nutritional properties of the phenolic compounds but also the processability as well as enzymic susceptibility of starches [21].

It is generally agreed that the binding of polyphenols to macromolecules, and specifically polysaccharides, is mainly mediated by hydrogen bonds and hydrophobic interactions [6, 16, 17], although the exact mechanisms are not fully understood yet. Covalent bonding between phenolic compounds and polysaccharides has been reported too [22]. The occurrence of ionic interactions has also been proposed for

charged molecules, such as the binding of anthocyanins to pectins [8]. Most works report that the binding capacity increases with the molecular weight of the phenolic compounds [16, 17, 23-25], although other factors such as the stereochemistry or the degree of galloylation of polyphenols have been suggested to have an effect on the absorption capacity [17].

Regarding the associations with plant cell walls, i.e. complex polysaccharide networks, some studies highlight the role of specific polysaccharide composition on their binding capacity towards polyphenols. For instance, procyanidins and anthocyanins were found to preferentially bind to pectins as compared to other cell wall components [8, 25, 26], while phenolic acids bound faster to cellulose than to cellulose-pectin composites [9]. The complex porous structure and distribution of hydrophilic/hydrophobic domains within the cell walls has also been observed to play an important role in their absorption capacity [16, 24]. On the other hand, starch, compared to pectin and cellulose, has shown an intermediate affinity for procyanidins [25].

Both starch and dietary fiber are major components of plant cells in e.g. cereals, legumes, bananas and tubers, and food processing frequently involves tissue disruption and cellular breakage, making both components readily available for interaction with polyphenols. However, many plant-derived foods are also consumed without major tissue disruption and cell breakage, and not all cells are ruptured during mastication, as the size of the average plant cell (of the order of 100 μm) is smaller than that of e.g. chewed vegetables pieces [27]. Hence, the interactions between polyphenols and intact plant cells are relevant to the nutritional functionality of foods, but little research attention has been paid to it yet.

In this manuscript, we report the binding of polyphenols to different potato cell components (i.e. cell walls and starch granules), as well as intact and broken cells, with the aim of elucidating the interactions taking place in this model food system. The cells from potato tubers were selected in this work as a model due to the ease of isolation of starch, cell wall and cells from both raw and cooked tissues. Three molecules featuring diverse origins and different molecular structures and sizes (i.e. (+)-catechin, phloridzin and vanillic acid) were used as representative food phenolic compounds. In view of the obtained results, the potential for intact cells, with or without cooking, to act as encapsulation vehicles for phenolic compounds was also discussed.

3. Materials and Methods

3.1. Materials

Sebago cultivar potato tubers were purchased from a local supermarket and used as such for isolation of potato cells and cell components as described in Sections 3.2

and 3.3. Vanillic acid, phloridzin dihydrate, (+)-catechin hydrate, sodium azide, hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), ethanol and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Australia). Reagents for total starch determination were supplied by Megazyme International (Wicklow, Ireland).

3.2. Isolation of potato cells

Potato cells were isolated under mild conditions to minimise cellular damage. Cell detachment was achieved by successive treatment with mild acid and alkali solutions in order to remove pectin from middle lamella between cells [28, 29]. In brief, approximately 5 g of peeled potatoes were cut into cubes (~5 mm)^3, introduced into 50 mL tubes and immersed in 40 mL of 0.05M hydrochloric acid. The tubes were incubated at 100 rpm for 6 hours at room temperature on a rocking shaker (MS-NRK, Major Science, Taiwan). The potato cubes were then washed 3 times with distilled water and incubated in 40 mL of 0.025M sodium hydroxide overnight. The resulting detached cells were thoroughly washed with running tap water on a stack of 250, 175 and 90 µm sieves. The isolated and predominantly intact cells (confirmed by microscopy, see Section 3.4.1) retained on the 90 µm sieve were collected and stored in PBS buffer with 0.02% sodium azide under refrigeration until further use.

In order to assess the impact of the intactness of the cells on their binding affinity for the phenolic compounds, 'broken cells' samples were also generated by an overnight intense magnetic agitation of the intact cells, consequently disrupting them (confirmed by microscopy, see Section 3.4.1) as reported by [30] for legumes cells.

3.3. Isolation of cell walls and starch granules

Potato cell walls and starch granules were isolated by mechanical disruption of cells followed by selective sieving. For this purpose, potato tubers were peeled, cut into pieces and gently blended with excess water using a kitchen blender. The obtained blend was sieved and the filtrate containing the < 90 µm fraction (starch granules) was collected and decanted. After several washing (with distilled water) and decanting steps, the starch granules were vacuum dried at room temperature. The > 300 µm fraction retained on the biggest sieve was introduced again in the blender and homogenized with excess water, this time at the maximum intensity. The cell walls fraction was collected on a 150 µm sieve after thoroughly washing with distilled water. Microscopic observations showed that some intact cells were still present in the collected fraction at that stage, so complete breakage of cells was accomplished by intense agitation for 2 days with magnetic stirrer bars in the presence of 0.02% sodium azide [30]. Complete removal of starch granules from the

cell walls fraction was confirmed by microscopy after sieving. Cell walls were then thoroughly washed with distilled water and vacuum dried at room temperature.

3.4. Characterisation of potato cells and cell components

3.4.1. Confocal laser scanning microscopy (CLSM)

Potato cells and cell components were observed by CLSM using a LSM 700 microscope (Zeiss, Jena, Germany), both before and after conducting the binding assays. For fluorescence images capture, a 405 nm laser was used for excitation and emissions between 400 and 487 nm were collected. Objectives of 10x and 20x were used.

3.4.2. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were collected in attenuated total reflectance (ATR) mode using a Perkin Elmer Spectrum 100 spectrometer coupled to a Universal ATR sampling accessory. The spectra were obtained in the range 4000-650 cm^{-1} by averaging 32 scans at 2 cm^{-1} resolution.

3.4.3. Total starch analysis

The starch content of the potato cells was determined using the total starch Megazyme assay kit (K-TSTA 08/16, Megazyme International, Wicklow, Ireland), based on the AOAC Method 996.11 and AACC Method 76-13.01, and following the instructions given by the supplier. The cells were broken by intense agitation with magnetic stirrer bars overnight prior to analysis, to ensure complete access of the enzymes to starch [30].

3.5. Standard phenolic solutions

0.25-3.0 mM solutions of the phenolic compounds were freshly prepared prior to use by dissolving them in phosphate buffered saline solution (PBS, pH=7.4) in the presence of 0.02% sodium azide. Brief mild heating was applied to dissolve phloridzin in the aqueous buffer.

3.6. Binding assays

Binding assays were conducted following a method adapted from [8] and [10]. Briefly, ca. 20 mg (expressed as dry weight) of substrate (i.e. potato cells, starch or cell walls) were incubated in 5 mL of phenolic solutions in a 15 mL Falcon tube completely wrapped with aluminium foil with shaking at 100 rpm for 2 h on an orbital shaker at room temperature. Control samples in the absence of substrate, and blanks

in the absence of phenolic compounds, were simultaneously incubated under the same conditions. Samples were then centrifuged at 4000 g (or 200 g in the case of intact cells to prevent breakage) and their absorbance at their λ_{max} (278 nm for (+)-catechin, 285 nm for vanillic acid and 246 nm for phloridzin) was measured using a UV-1700 Pharma Spec UV–Vis spectrophotometer (Shimadzu, Japan), after dilution as required. The concentration of free (unbound) phenolic compounds in each sample after the assay was determined based on external standard curves ($R^2 \geq 0.9998$), and the amount of bound phenolic compounds was calculated according to Eq. (1). Results were obtained from independent triplicates.

$$Bound\ (\%) = \frac{Amount\ of\ phenolics\ in\ control\ -\ Amount\ of\ phenolics\ in\ sample}{Dry\ mass\ of\ cells\ or\ cell\ component} \cdot 100 \qquad \text{Eq. (1)}$$

3.7. Binding kinetics

Binding assays were also conducted after different incubation periods using the method described in Section 3.6, to ascertain the relative binding rates to the different potato cell derived substrates. For this purpose, 250 µL aliquots were sampled at different times of incubation (up to 6 hours) and the amount of bound phenolic compounds at each time period was calculated according to Eq. (1).

3.8. Effect of pH on the binding extent

Binding assays were also conducted at pH=3 (representing gastric pH [31]) to ascertain the effect of pH on the binding of phenolic compounds to potato cells and cell components. For this purpose, the PBS buffer was replaced by a 0.001N HCl solution and the assays were conducted following the procedure described in Section 3.6 for a concentration of phenolic compounds of 2mM. The absorbance values in this medium were determined at 278 nm for (+)-catechin, 292 nm for vanillic acid and 283 nm for phloridzin (standard curves with $R^2 \geq 0.9999$).

3.9. Effect of cooking on the binding extent

In order to assess the impact of cooking on the affinity of potato cells for the phenolic compounds, 'cooked potato cells' were isolated by boiling potato cubes for 15 min, and selectively sieving the boiled potato cells as described in Section 2.2. by gently pressing the cooked potato through the sieves in excess water. The cells were resuspended in PBS buffer in the presence of 0.02% sodium azide. Their binding to the phenolic compounds (2mM) was studied following the procedure in Section 2.6.

3.10. Statistical analysis

IBM SPSS Statistics software (v.23) (IBM Corp., USA) was used to perform the statistical analysis of the data. The significance of the differences observed between samples was assessed through two-sided t-tests at $p < 0.05$. For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

4. Results and discussion

4.1. Isolation of potato cells, cell walls and starch

Mature potato tuber cells mainly consist of starch granules, which generally account for more than a 60% of their dry weight, and dietary fiber (cell walls) [32, 33]. These cell walls comprise a network of cellulose, cross-linking glycans, and pectins [34]. Figure 1 shows CLSM images of the intact cells and individual cell components isolated from potato tubers (without any autofluorescence from native polyphenols) and used in subsequent sections, together with their FT-IR spectra.

As observed in the micrographs (Figure 1a), the methodology employed for the isolation of intact cells and cell components achieved effective separation of the different constituents. Their FT-IR spectra exhibited characteristic bands of polysaccharides. Specifically, the spectrum of the cell walls showed characteristic bands of pectins and cellulose, such as those ascribed to the esterified (1732 cm^{-1}) and non-esterified carboxyl groups (1603 cm^{-1}) of pectins, methyl esters of pectins (1416 cm^{-1}), CH_2 groups in cellulose (1317 cm^{-1}) or ring stretching indicative of β-glycosidic linkages in cellulose (893 cm^{-1}) [35]. On the other hand, starch (Figure 1b) exhibited its characteristic C–O and C–C stretching vibrations modes in the range 1200-1000 cm^{-1}, showing bands centered at 991, 1075, and 1148 cm^{-1} [36]. The intact cells showed the contribution of both cell walls and starch in their spectra (Figure 1b).

The results from the total starch content assay indicated that the cells isolated from the potato tubers used in this work had a starch content of 74 ± 2 % of their dry weight. This value is in agreement with starch contents previously reported for Sebago potato tubers, which vary in the range of 57.2 – 74.7 % depending on the collection period [33].

4.2. Binding capacity

Figure 2 shows the binding isotherms for the different phenolic compounds and potato cell components studied in this work. Complementary Figure S1 of the Supplementary Material shows the statistical analysis of the data. The amount of bound phenolics after 2 hours of incubation generally increased with their concentration in solution, and was both polyphenol- and substrate-dependent.

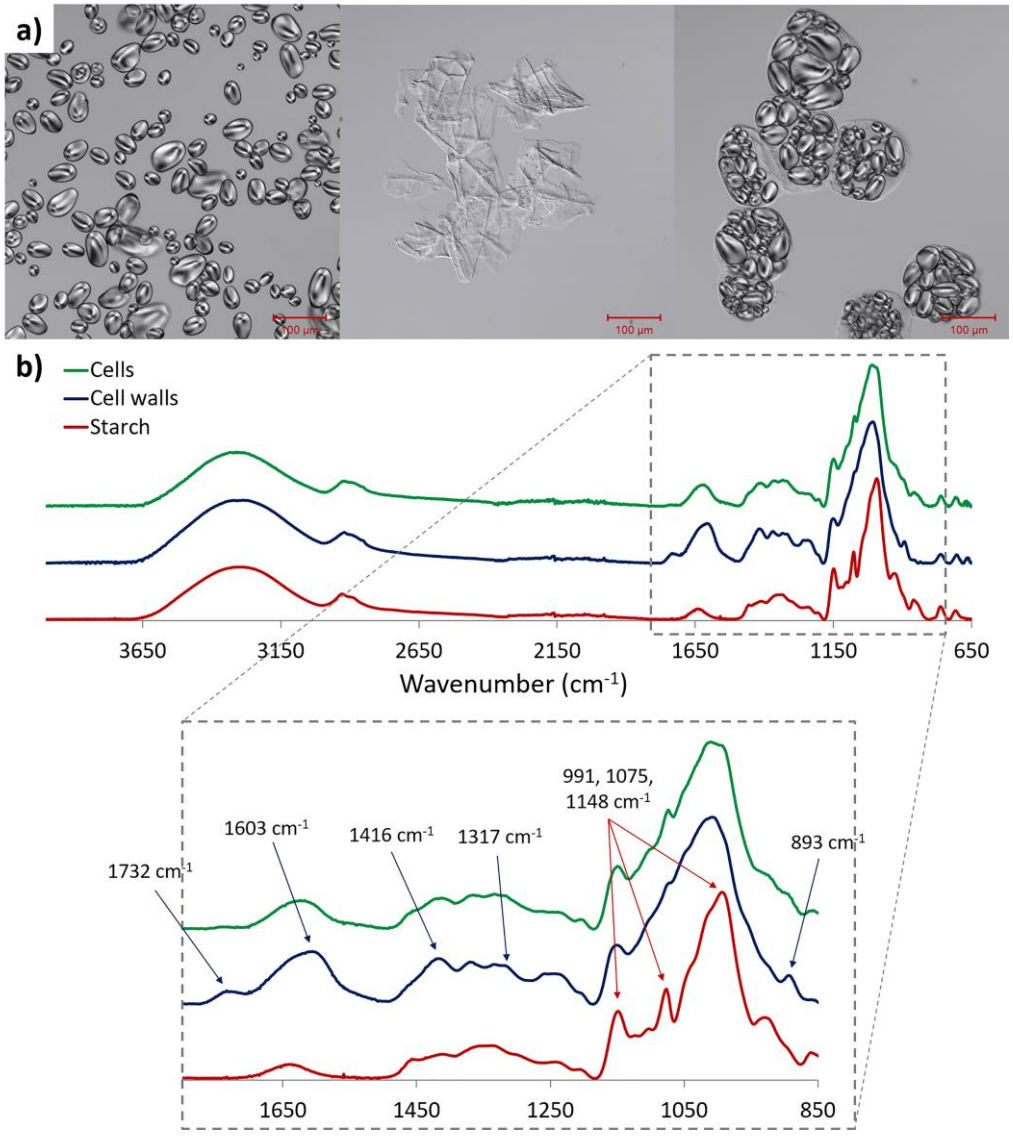


Figure 1. a) CLSM images from suspensions of isolated potato cells and cell components (from left to right: starch, cell walls and intact cells). All scale bars correspond to 100 µm. b) FT-IR spectra of dried cells and cell components (from top to bottom: intact cells, cell walls and starch).

In general, (+)-catechin exhibited the highest binding for all substrates (up to ~50 µmol/g) amongst the studied phenolic compounds, although binding capacity to the cell walls was similar for phloridzin. On the other hand, the binding capacity of vanillic acid was found to be consistently low. Various studies have reported increased

binding affinity of phenolic compounds for polysaccharides with increasing molecular weight [16, 17, 23-25]. Although these studies refer to olimeric phenolic compounds (condensed tannins) and may not be directly comparable with our results, in general, a greater number of hydroxyl groups in larger molecules would imply additional potential interaction sites [7]. Vanillic acid is both a smaller molecule and has only a single hydroxyl group per molecule and, thus, its lower binding capacity was not surprising. However, having more hydroxyl groups in its structure, phloridzin did not exhibit the greatest binding affinities for potato cells or isolated starch, only showing comparable capacity to that of (+)-catechin with isolated cell walls. Factors such as the stereochemistry of polyphenols have also been suggested to have an effect on their absorption capacity [17], and the steric hindrance of phloridzin due to its attached glucose molecule might have limited its absorption onto starch granules and the cells, whose specific surface areas readily available for interaction may be smaller than that of the broken cell walls. In a previous work, Le Bourvellec and Renard (2005) showed that the physical state of the cell walls (dried under different conditions and, thus, having different physical characteristics but the same polysaccharide composition) affected their adsorption capacity, due to their different surface area. Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert and Gidley (2017) [37] also showed that the structural modification induced on cellulose by different processing techniques had an impact on the adsorption of polyphenols. Compared to cell walls, the starch is organized as a compact semi-crystalline structure and has lower swelling ability than cell walls. Thus a lower binding affinity of phloridzin on starch may be expected due to steric hindrance. Differences in polarity amongst the phenolic compounds and the polysaccharides constituting the various cell components may also play a role in the observed differences, as adsorption from solution is actually the result of a balance between the affinity of the phenolic compounds for the substrate versus their affinity for the solvent.

In the case of (+)-catechin, no significant differences were observed between the amount of polyphenol bound to starch, cell walls and intact cells. Interestingly, (+)-catechin was able to penetrate the cell wall barrier and interact with the starch granules inside the cells. Otherwise, the 74% of the cells' dry weight which is accounted by starch granules would not participate in the absorption of (+)-catechin and the bound amount per weight of dry cells would have been lower. CLSM observations were conducted in order to confirm the presence of (+)-catechin inside the potato cells (see Section 4.4, Figure 5).

Interestingly, the amount of (+)-catechin bound to broken cells was significantly greater than that bound to individual cell components (i.e. starch and cell walls) or intact cells (cf. Figure S1 of the Supplementary Material). A plausible explanation for these results might be related to the physical state of the substrates and the resulting specific surface available for interaction. The intense magnetic agitation treatment carried out to break the cells also damaged the starch granules increasing their surface area for binding as shown in Figure 3. In order to ascertain whether this

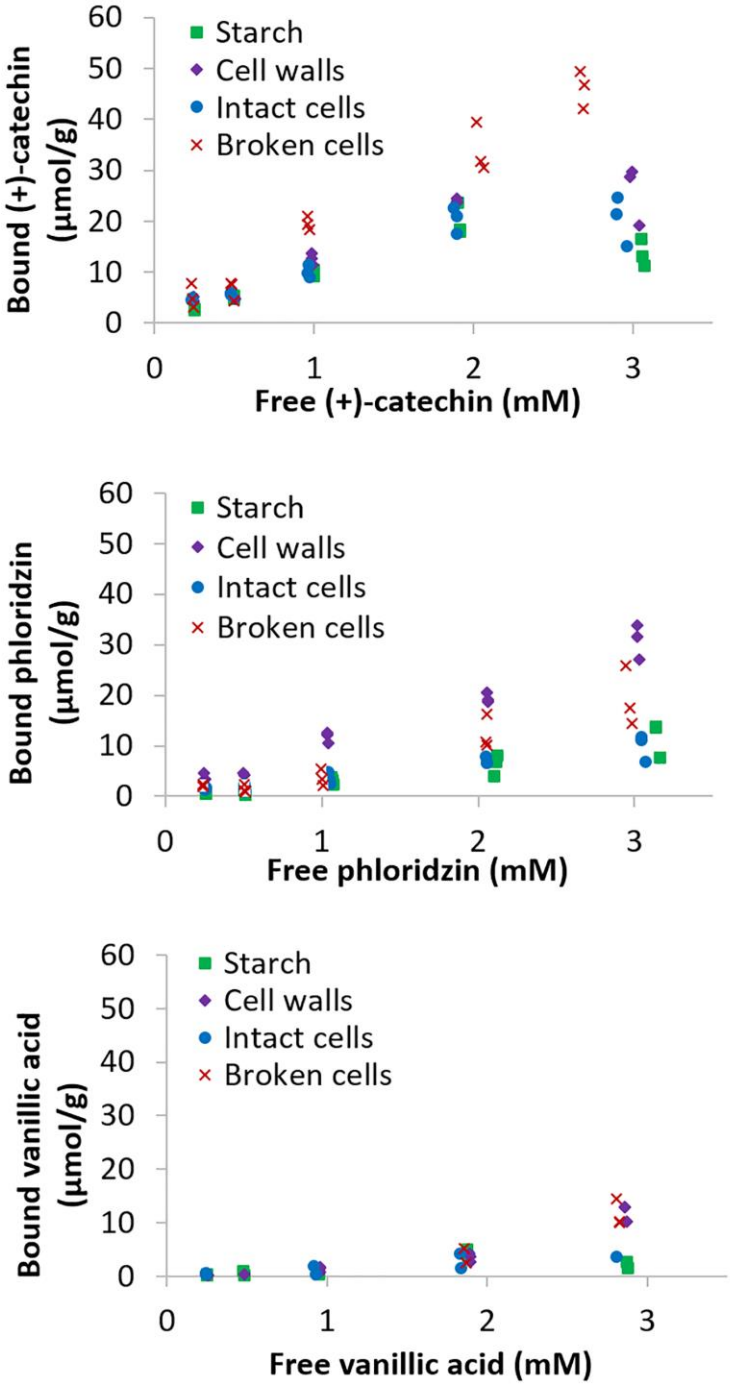


Figure 2. Binding isotherms for the different phenolic compounds - potato cells systems after 2 h incubation.

damage was the cause of the significant increase in the binding extent observed for (+)-catechin towards the broken cells, isolated starch granules were also subjected to overnight intense magnetic agitation following the same procedure used to break the cells, before evaluating their binding to (+)-catechin at 2 mM concentration. As shown in Figure 3a (left column) the damaged starch granules in the 'broken cells' samples have distinctive granule morphology compared to intact starch granules (right column). A 40% increase in the amount of bound (+)-catechin was indeed observed, providing a plausible explanation of the increased binding reported for the broken cells.

Regarding phloridzin, a different trend was observed. In this case, the binding affinity of the polyphenol for the cell walls was significantly higher than for starch (cf. Figure S1 of the Supplementary Material). As mentioned above, the high number of hydroxyl groups in its structure involves a number of potential interaction sites with the polysaccharides via hydrogen bonds. However, steric hindrance might represent a limitation for its access and, thus, binding to compact structures such as that of starch granules. Han et al. (2015) recently showed that while catechin could successfully diffuse into the pores of starch granules, rutin, which has a similar phenolic structure but is conjugated with a disaccharide, did not diffuse at all [20]. A similar phenomenon might occur in the case of phloridzin, significantly reducing its absorption onto the starch granules. Again, no significant differences were found between the amount of phloridzin bound to intact cells and starch. Being starch the majoritarian component of the cells, these results suggest that phloridzin was also capable of penetrating the cell walls and bind to intracellular starch.

Finally, vanillic acid showed little affinity for potato cells and isolated cell components, presumably because it is a small molecule with only one hydroxyl group per molecule. A higher concentration of the phenolic compound was needed to observe significant solution depletion in the presence of the substrates, and only cell walls and broken cells (the components with higher available surface areas) showed convincing evidence for binding through a reduction in vanillic acid concentration in the solution. Unlike (+)-catechin and phloridzin, vanillic acid is negatively charged in neutral solution, which might also have impacted its binding to the potato cells and cell components due to electrostatic repulsions. However, the effect of this negative charge was found to be negligible as binding was similar at pH values where the negative charge was neutralised (see Section 4.6).

It is worth mentioning that the data obtained for binding of phenolic compounds to polysaccharides at different concentrations and at equilibrium can be generally fitted to type I (Langmuir) isotherms [10, 17, 25]. However, in the binding isotherms presented in this work, a plateau value was not reached for some of the systems (cf. Figure 2), due to the impossibility of increasing the polyphenol concentration because of solubility limitations.

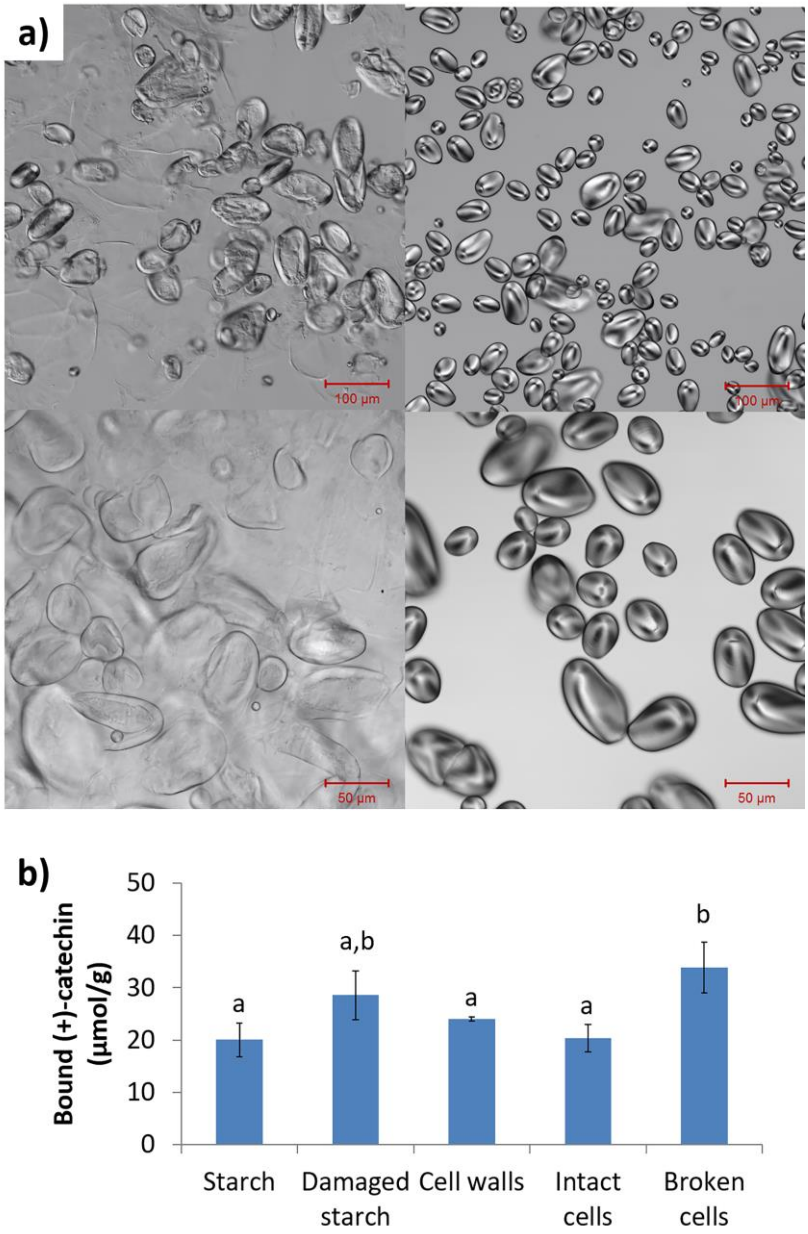


Figure 3. a) CLSM images of broken cells (left) in comparison with intact starch (right) at two different magnifications. Scale bars correspond to 100 µm in upper images and 50 µm in lower images. b) Binding extent of 2 mM (+)-catechin to potato cells and cell components including 'damaged starch' after 2 h incubation. Experiments were performed at least in triplicate. Error bars represent standard deviations. Different letters (a–b) indicate significant differences at $p < 0.05$ among the samples.

4.3. Binding kinetics

Figure 4 shows the binding kinetic profiles of (+)-catechin and phloridzin (2 mM) to the different plant-based substrates assayed in this work. Vanillic acid was excluded from this assay due to its low binding capacities. All the curves show an initial increase in the amount of bound polyphenol with time until a plateau was reached. In all cases, a 2h incubation period was enough to reach the plateau.

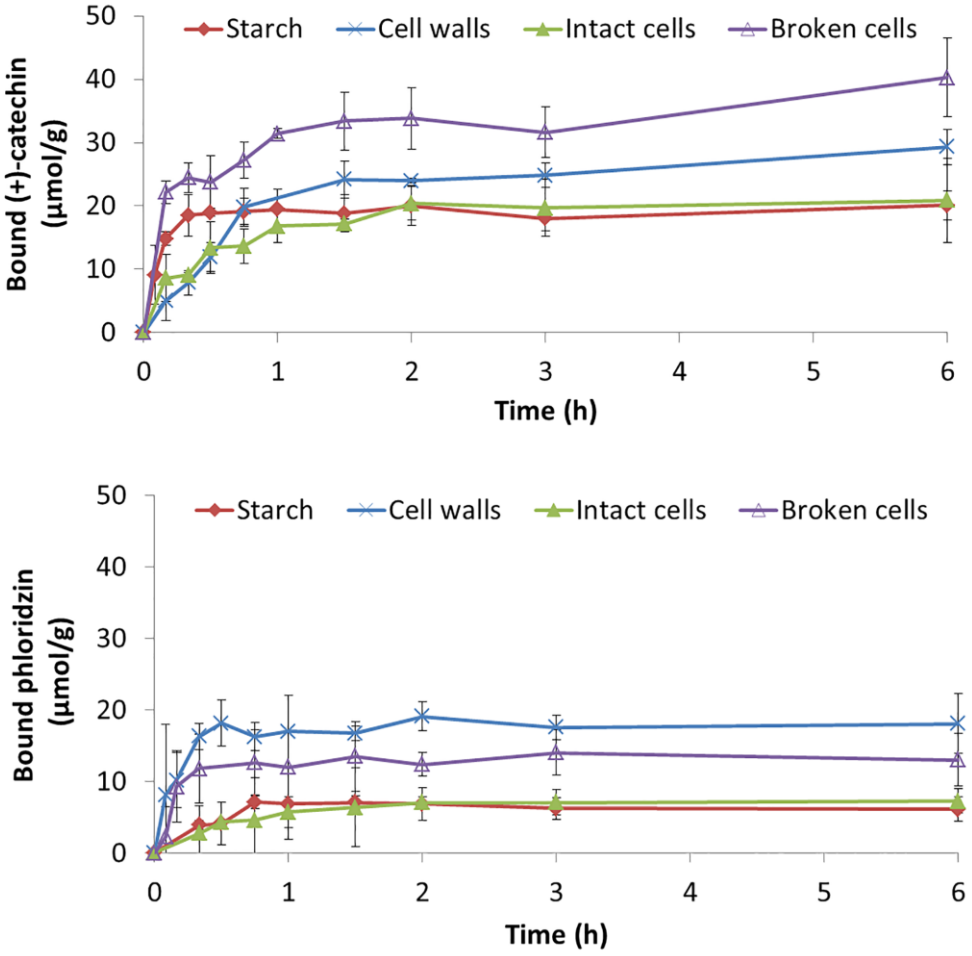


Figure 4. Binding of (+)-catechin and phloridzin (2mM) to potato cells and cell components after different incubation times. Experiments were performed at least in triplicate. Error bars represent standard deviations.

The polyphenols bound the different cell components at different rates in the first 2h of incubation. For (+)-catechin, binding to starch was found to be the fastest to

reach the plateau (after only 20 min), while the cell walls absorbed polyphenol molecules in a slower manner. Again, the observed differences can be attributed to the structure and physical organisation of the polysaccharide-based substrates. Starch is organised in the form of compact granules, whose available surface will be only marginally altered upon hydration in the polyphenol solution. In contrast, cell walls are organised in the form of interpenetrating hydrogels with great water uptake capacity, whose fibrillar structure swells in aqueous solution. As the fibre swell, more binding sites become available and the polyphenol diffuses inside its porous structure, and thus the binding progresses over time, eventually surpassing the binding capacity of starch after ≈1h incubation. These results are in agreement with previous reports which suggested that the conformational organisation of polysaccharides play an important role on their binding to polyphenols [25]. Regarding the broken cells, a very fast binding was observed in the first 10 minutes, since most of the available binding sites were readily available for interaction due to the disruption of the cell walls and the hydrated state of these samples.

In the case of phloridzin, again, a different trend was observed. Not only did it bind to cell walls to a greater extent than to starch, but it also bound them at a higher rate. Thus, both the binding capacity and affinity of phloridzin were higher for cell walls than for starch. These results agree with the previous suggestion that the steric hindrance of this larger molecule might represent a limitation for its binding to compact structures, while its affinity for swollen polysaccharides is higher.

The slowest binding profiles in both cases were observed for the binding of the polyphenols with the intact cells. As the polyphenols would have to penetrate the cell wall barrier to interact with the starch granules, a slow solution depletion could be expected, as the binding rate in this case would be limited by the diffusion of the polyphenols through the intact cell walls. This slow binding rate together with the binding extent to intact cells being comparable to that of starch are indicative of the ability of the polyphenols to penetrate into the potato cells.

In all cases, the binding of polyphenols to potato cells and cell components took place within the first 2 h of incubation, indicating that the duration of a typical human digestion process would be enough for these interactions to take place upon simultaneous consumption of polyphenol ingredients and plant-based foods (refer to Section 4.6. for results at gastric pH), not to mention the long-term storage of polyphenol-rich vegetable and other products.

4.4. Location of bound polyphenols within the cells and cell components

Figure 5 shows representative images of starch, cell walls, intact cells and broken cells after 2h incubation in the presence of 2mM solutions of (+)-catechin, and respective controls in the absence of phenolic compounds. The intrinsic fluorescence of (+)-catechin allowed confirmation of its location of binding within the cells and cell components. However, no significant autofluorescence (as compared with the

background signal) could be detected for phloridzin or vanillic acid, so the comparison could not be made (results not shown).

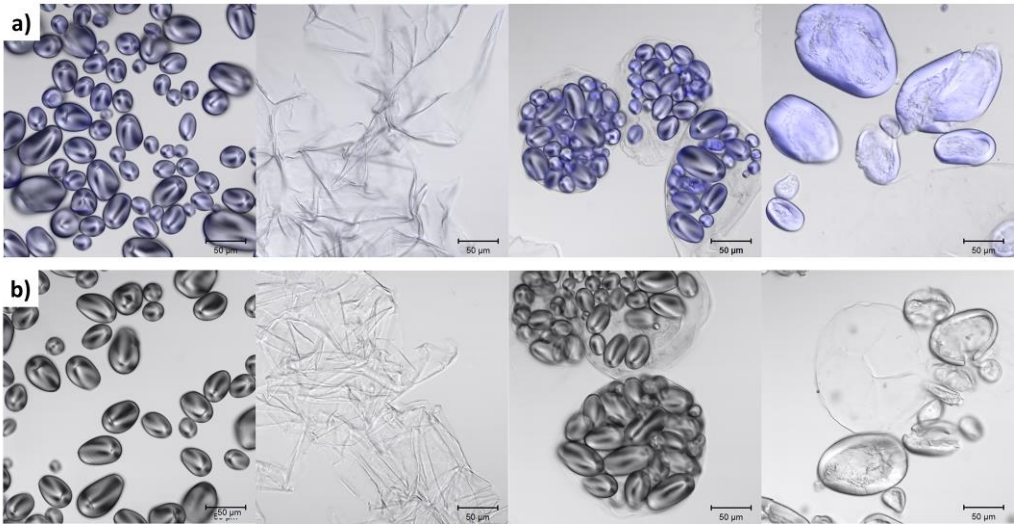


Figure 5. CLSM images of potato cells and cell components (starch, cell walls, intact cells and broken cells, from left to right) after 2h contact with (+)-catechin (a) and controls in the absence of phenolic compound (b). All scale bars correspond to 50 µm.

Although the fluorescence of the cell walls in the presence of (+)-catechin was not so marked, presumably due to their high swelling degree and thus low density, the binding of this polyphenol to the starch granules was evidenced by the intense and localized fluorescence shown in Figure 5a. Moreover, not only did (+)-catechin bind to the isolated starch granules, but it could also bind to them inside the intact cells, which confirmed the capability of polyphenols, specifically (+)-catechin, to cross the cell walls as hypothesized in Section 4.2.

These interesting results suggest, for the first time, the potential of plant cells and, in particular, potato cells as delivery vehicles for bioactive ingredients, specifically polyphenols. As cell walls (dietary fiber) are not digestible, the contents of the intact cells are expected to be delivered to the large intestine where the fermentation by bacteria would allow their release, provided the cells remain intact upon passage through the gastrointestinal tract [30]. Intracellular starch is probably acting as a sink, with binding of (+)-catechin promoting uptake of additional (+)-catechin across the cell wall.

4.5. Effect of pH on the binding extent

In order to assess the effect of the pH on the binding of the phenolic compounds to the different substrates, binding assays were also conducted at pH=3, for a fixed

phenolic compound concentration (2mM) and incubation period (2 h). This pH was selected because it is close to the human gastric pH [31], and it is below the pK_a of ca. 4.5 for vanillic acid [38], so its dissociation and thus negative charge could be repressed. Results are shown in Figure 6, together with the data obtained at neutral pH for comparison purposes.

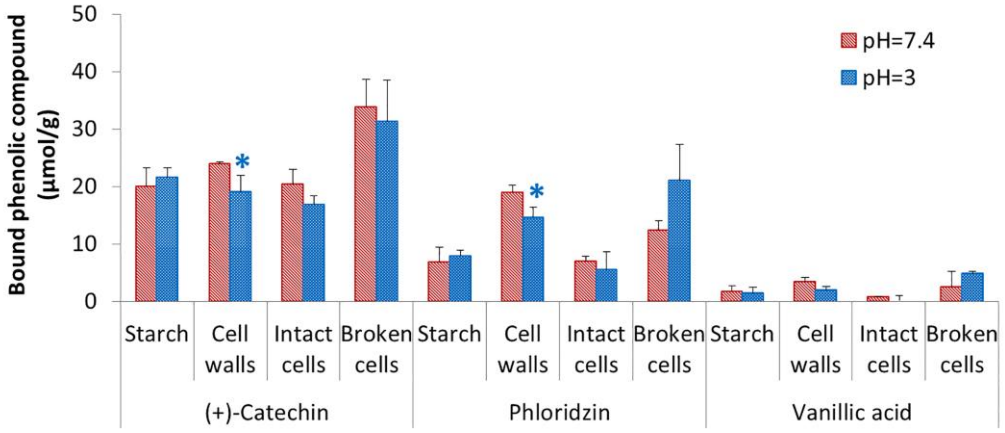


Figure 6. Binding capacity of the phenolic compounds (2mM) to potato cells and cell components at pH 3.0 and 7.4 after 2 h incubation. Experiments were performed at least in triplicate. Error bars represent standard deviations. Asterisks (*) indicate significant differences between the results at different pH values for a given phenolic compound and substrate.

The binding extent for the three phenolic compounds was similar at both pH values. No significant differences were observed except for the binding of (+)-catechin and phloridzin to cell walls, which was significantly lower at pH 3.

The fact that the binding of vanillic acid to potato cells and cell components did not increase at pH 3 compared to neutral pH suggests that the potential electrostatic repulsion between the negatively charged phenolic acid (present at pH 7 but essentially not at pH 3) and the polysaccharides did not significantly affect the affinity of vanillic acid for the assayed substrates. These results are in agreement with previous work which concluded that the native charge of polyphenols is only a secondary factor in the interactions between polyphenols and neutral polysaccharides such as cellulose [10]. Regarding the presence of negatively charged polysaccharides (i.e. pectins) in the potato cell walls, our results are also in agreement with Padayachee et al. (2012) who reported similar binding affinities for phenolic acids towards cellulose and cellulose-pectin composites at equilibrium, despite the negative charge of the latter, further suggesting that the impact of electrostatic repulsions was a minor factor [9]. These findings support the hypothesis that the binding of the phenolic compounds to the polysaccharides in potato cells is mainly mediated by hydrogen bonds and hydrophobic interactions with the aromatic ring.

4.6. Effect of cooking on binding

Plant-derived ingredients, especially potatoes, are usually cooked before consumption, which generally involves subjecting them to thermal treatments. The hydrothermal processing of plant cells has a substantial impact on their structure, with major changes caused by the gelatinization of the starch granules [39]. In order to assess the effect of cooking on the affinity of potato cells for the phenolic compounds, these were incubated in the presence of 'cooked cells', i.e. cells isolated from boiled potato tubers, and the amount of bound phenolic compounds from 2mM solutions was determined after 2h. As starch becomes soluble in aqueous solution after gelatinization, and therefore it cannot be separated from the binding media by centrifugation, the assay was only carried out for the intact cells in this case. Results are shown in Figure 7a, together with those obtained for the raw cells from Section 4.2.

The amount of all the phenolic compounds bound to the potato cells significantly increased when the latter had been previously cooked. As observed in Figure 7b, the integrity of the starch granules within the cells was lost, and a swollen mass of gelatinized starch filled the cooked cells. Rather than being organized in the form of compact, semi-crystalline granules as in the raw cells, the starch in the cooked cells exhibited an amorphous swollen structure which would considerably increase the accessibility of its potential binding sites, consistent with the observed increase in the binding capacity of the boiled cells. Again, these results suggest that the conformational organization of the polysaccharides within plant cells plays a key role in their binding to phenolic compounds.

Finally, the potential of potato cells as delivery vehicles for the encapsulation of phenolic compounds seemed to be enhanced upon cooking, as their loading capacity significantly increased. The ability of the cooked cells to accumulate phenolic compounds was further confirmed by the localised fluorescence of (+)-catechin confined within the limits of their cell walls in Figure 7b. Future studies should explore the impact of the gelatinization of starch on the release of the phenolic compounds throughout the gastrointestinal tract.

Overall, the present work provides new information about the binding of phenolic compounds to potato cells and cell components, as a model polysaccharide-based food system. The obtained results suggest, for the first time, the potential of potato cells as encapsulation vehicles for phenolic compounds. Future, complementary studies should explore the reversibility of these polyphenol-polysaccharide binding and its effect on the release of the phenolic compounds throughout the gastrointestinal tract, with an emphasis on the impact of gelatinization of starch on the performance of potato cells as potential delivery vehicles for these bioactive compounds.

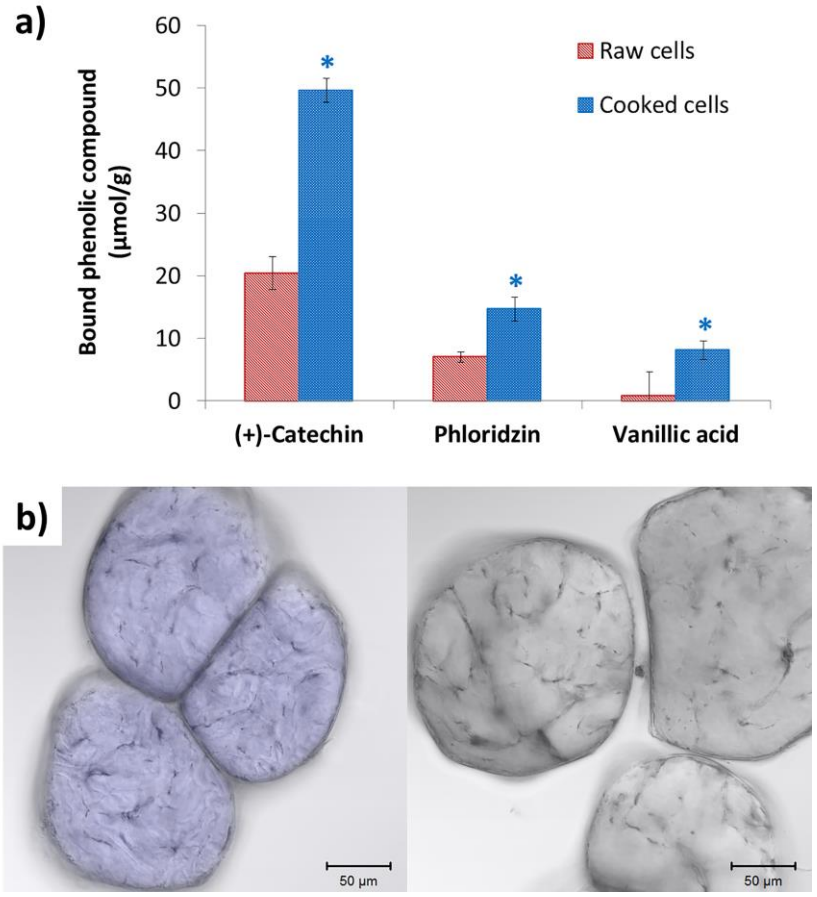


Figure 7. a) Amount of bound phenolic compounds after incubation with raw and cooked potato cells. Experiments were performed at least in triplicate. Error bars represent standard deviations. Asterisks (*) indicate significant differences within the same phenolic compound. b) CLSM images of cooked potato cells after 2h contact with (+)-catechin (left) and a control in the absence of (+)-catechin (right). Scale bars correspond to 50 µm.

5. Conclusions

The binding of three phenolic compounds (vanillic acid, phloridzin and (+)-catechin) to potato cells and individual cell components, i.e. cell walls and starch granules, was studied in this work. The cells and cell components were successfully isolated from potato tubers and mainly consisted of polysaccharides, as inferred from their FT-IR spectra. Significant amounts of phenolic compounds (up to ~50 µmol/g) bound to the different substrates. In general, the extent of binding to the different substrates

followed the order (+)-catechin > phloridzin > vanillic acid, which could be mainly attributed to the amount of potential binding sites (hydroxyl groups) in their structure as well as the greater steric hindrance of phloridzin. The electrostatic interactions did not significantly impact the binding capacities, which were similar at pH 3.0 and 7.4. The physical organization of the polysaccharides in each cell component was found to affect both the binding extent of the phenolic compounds and their binding rate. Phloridzin, possibly due to its large steric volume, exhibited a similar binding capacity as (+)-catechin only towards the isolated cell walls, whose swollen structure facilitated the exposure of their binding sites, while its binding capacity towards the starch granules (compact structures) and the intact cells (consisting mainly of starch granules) was significantly lower. After 2 h of incubation, the binding profiles approached a plateau, suggesting that the duration of a digestion process upon simultaneous consumption of phenolic compounds and plant-based food products would be enough for these interactions to take place. Of particular interest, the phenolic compounds (especially (+)-catechin) were able to penetrate the intact cells and bind to the starch granules within them. Consequently, the potential of intact potato cells to be exploited as natural delivery vehicles for the encapsulation of polyphenols is suggested, which could have industrial applications besides nutritional implications. This potential could be enhanced by previously cooking (boiling) the potato cells, as their loading capacity dramatically increased due to the gelatinization of the starch and consequent increased accessibility of its binding sites. If these cellular capsules survive the stomach and small intestine, they would also deliver significant payloads of resistant starch to the microbiota in the large intestine.

6. Acknowledgements

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8. Supplementary material

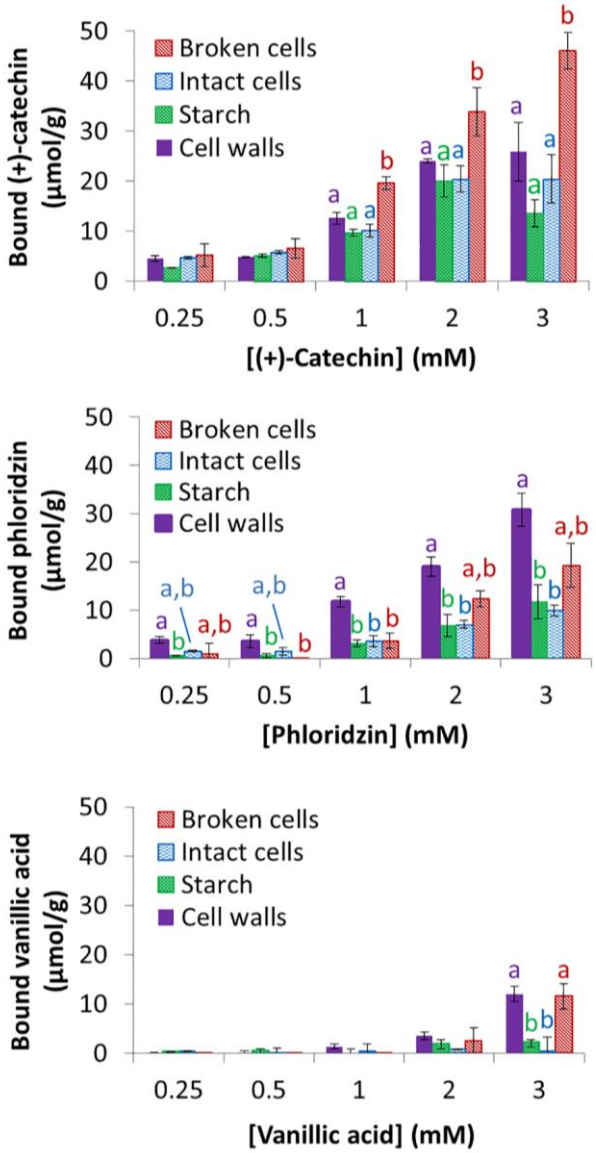


Figure S1. Comparison between the binding capacity of polyphenols to potato cells and cell components at different concentrations after 2 h incubation. Experiments were performed at least in triplicate. Error bars represent standard deviations. Different letters (a–c) within the same series indicate significant differences at $p < 0.05$ among the samples. No letters indicate no significant differences within the series.

CHAPTER 5

APPLICATION OF MICROENCAPSULATION IN REAL FOOD SYSTEMS

5.1. Microencapsulation of a whey protein hydrolysate within micro-hydrogels: Impact on gastrointestinal stability and potential for functional yoghurt development.

5.2. Impact of microencapsulation within electrosprayed proteins on the formulation of green tea extract-enriched biscuits.

Introduction to Chapter 5

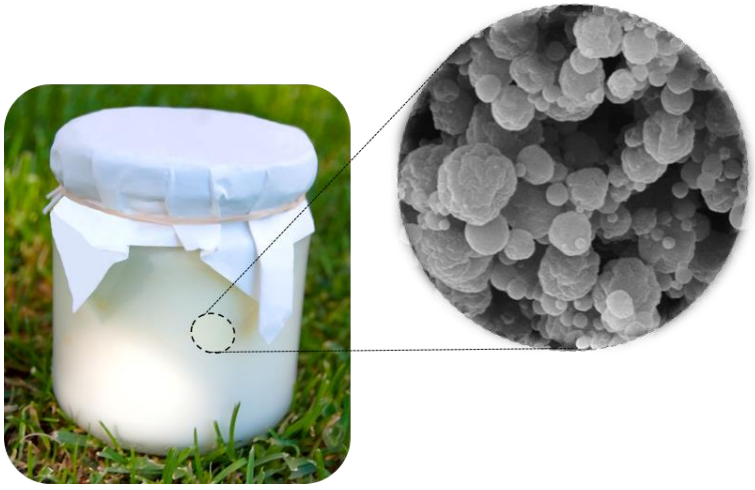
Most of the research works dealing with microencapsulation of bioactive ingredients for the development of functional foods, including those presented in the previous chapters, assess the behaviour of the microencapsulates *in-vitro*, using simple food simulants instead of complex food matrices. However, evaluating the impact of microencapsulation in real food systems is the necessary last step, in order to confirm the suitability of the proposed delivery systems to achieve the desired goals. In this chapter, two different case studies are presented, each of them addressing a particular challenge in the design of real functional food products.

In the first work, the aim was the enrichment of yogurts with a potentially bioactive peptide hydrolysate. One of the main challenges of adding bioactive peptides to fermented dairy products is precisely their susceptibility to degradation by the lactic acid bacteria which uptake them as a source of nitrogen during fermentation. Hence, microencapsulation was proposed to minimize the loss of peptides during the production of natural yogurts. For this purpose, a whey protein hydrolysate was produced and microencapsulated by spray-drying using gelatin and chitosan matrices previously explored in chapter 1. Both the free and microencapsulated hydrolysate were added to UHT low fat milk, which was then fermented to produce peptide-enriched yogurts. The impact of microencapsulation on the protection of the peptides during lactic acid fermentation was assessed.

In the second work, the aim was the enrichment of biscuits with a green tea extract. Catechins, the active compounds in green tea, are thermosensitive and very unstable in alkaline conditions. Given that biscuit doughs generally have high pH values, and high temperatures are used to bake them, the bioactivity of the green tea extract might be compromised during biscuit manufacturing. Again, micro-encapsulation was proposed to minimize the degradation of tea catechins during the biscuit preparation process. In this case, the electrospraying technique was used to microencapsulate the green tea extract within gelatin and zein matrices. The free and microencapsulated extract was then added to biscuit doughs, and the protective effect of microencapsulation on the tea catechins upon baking was evaluated. Additionally, a sensorial analysis was conducted to assess whether the incorporation of the GTE in its free or microencapsulated form had an impact on the consumers' acceptability.

5.1

MICROENCAPSULATION OF A WHEY PROTEIN HYDROLYSATE WITHIN MICRO-HYDROGELS: IMPACT ON GASTROINTESTINAL STABILITY AND POTENTIAL FOR FUNCTIONAL YOGHURT DEVELOPMENT



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Miralles, B., Recio, I., & López-Rubio, A. (2016). *Microencapsulation of a whey protein hydrolysate within micro-hydrogels: Impact on gastrointestinal stability and potential for functional yoghurt development*. Journal of Functional Foods, 26, 290-300. (DOI: http://dx.doi.org/10.1016/j.jff.2016.08.006).

1. Abstract

Gelatin and chitosan micro-hydrogels containing a potentially bioactive whey protein hydrolysate were developed through spray drying and the impact of microencapsulation on protection during digestion and peptide stability against lactic acid fermentation during yoghurt manufacturing was assessed. The results showed that the protection exerted by the encapsulation structures during milk fermentation was sequence- and matrix-dependent, being chitosan more effective than gelatin in stabilizing the peptides. However, only 5 out of the 21 fermentation-susceptible peptides identified could be protected through encapsulation within chitosan (1 of which was also protected by gelatin). Moreover, the encapsulation within chitosan microparticles did not substantially affect the peptide profile of the digested hydrolysate, and therefore, the peptide bioaccessibility was not expected to be compromised.

2. Introduction

Biologically active peptides are specific fragments of proteins with 2 to 20 amino acids that have desirable biological activities [1]. Specifically, bioactive peptides derived from milk proteins have attracted great interest in the field of functional foods [2, 3] because of their potential ability to promote human health by reducing the risk of chronic diseases or enhancing our natural immune system [4, 5]. These peptides are inactive within the sequence of the precursor proteins and need to be released (by proteolysis) to exert their physiological functions [6]. Although normal gastrointestinal digestion of milk leads to some release of active peptides, a number of techniques based on fermentation and/or enzymatic hydrolysis have been investigated to produce bioactive peptide-enriched protein fractions [1], while adding value to by-products from the food industry [7].

A number of bioactive peptides have already been studied and recent reviews suggest that new research should focus on the application of these functional ingredients to commercial food products [8]. Functional foods have become popular and commercially successful in some sectors of the food industry, especially in fermented dairy products, partly due to their general acceptance among consumers [9]. However, fortification of these food products with protein hydrolysates is challenging, not only because of their low bioavailability, bitter taste, hygroscopicity and their likelihood of interacting with the food matrix thus altering food texture and colour [8, 10], but also because of their susceptibility to degradation by lactic acid bacteria during fermentation [11, 12].

Microencapsulation technologies, i.e. processes in which the ingredients of interest are coated with or embedded within a protective matrix [13] obtaining micron-sized materials, are regarded as an effective approach to overcome the aforementioned limitations [14], and have been successfully used for the preservation of biologically active ingredients in food systems [15, 16], including protein hydrolysates and peptides [8]. Among the numerous encapsulation techniques, spray-drying is the most commonly used one in the food industry [17]. It consists of an initial atomization of a formulation containing the protective matrix and the bioactive, and subsequent rapid drying of the obtained droplets using a hot gas stream to produce dry microparticles. Although spray-drying has been extensively applied for the protection of peptides and hydrolysates [18-21], there is still lack of information about the impact that encapsulation may have on the functionality and stability of the peptides [8].

Both proteins and polysaccharides can be used as protective matrices for the encapsulation of protein hydrolysates by spray-drying [8]. However, there is no consensus in the literature regarding the best choice among them, an aspect which should also be explored. In general, hydrogel-forming biopolymers are particularly interesting, as they can be processed in aqueous solutions while preventing disruption of the produced microparticles in aqueous environments under certain conditions [22]. In this sense, chitosan, a linear polysaccharide obtained by deacetylation of chitin and consisting of β-1,4 linked 2-acetamido-2-deoxy-β-D-glucopyranose units and 2-amino-2-deoxy-b-D-glucopyranose units in a proportion which depends on its degree of deacetylation [23], is considered a pH-sensitive hydrogel-forming biopolymer [24]. On the other hand, gelatin, a protein obtained from partial hydrolysis of collagen and containing repeating sequences of glycine-aa_1-aa_2, where amino acids aa_1 and aa_2 are mainly proline and hydroxyproline [25], is considered a thermo-responsive hydrogel-forming biopolymer. Thus, both chitosan and gelatin are edible, naturally-derived and hydrogel-forming biopolymers with potential application in the microencapsulation of protein hydrolysates.

In this work, a whey protein hydrolysate was produced and used as a model peptide-enriched protein fraction to study its microencapsulation by spray-drying within two different biopolymers, a polysaccharide (chitosan) and a protein matrix (gelatin). The implications of its microencapsulation, in terms of protection of the peptides during gastrointestinal digestion and lactic acid fermentation, were studied and the results were compared for both encapsulation matrices. For this purpose, the free and encapsulated hydrolysate were subjected to *in-vitro* gastrointestinal digestion and the peptide profiles were obtained by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). In addition, commercial UHT low fat milk was supplemented with the microencapsulated and non-encapsulated hydrolysate and fermented to produce yoghurts. The protective ability at peptide level of chitosan and gelatin during the assays was compared.

3. Materials and methods

3.1. Materials

A bovine whey protein concentrate (WPC) was purchased from Friesland Campina Ingredients (Zwolle, The Netherlands). Type A gelatin from porcine skin, with reported gel strength of 175 g Bloom, low molecular weight chitosan, with reported Brookfield viscosity of 20.000 cps, potassium bromide FT-IR grade (KBr), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile extract porcine were all obtained from Sigma-Aldrich (Madrid, Spain). 96% (v/v) Acetic acid was purchased from Scharlab (Barcelona, Spain) and Pefabloc® from Fluka-Sigma-Aldrich. All inorganic salts used for the *in-vitro* digestion tests were used as received. Freeze-dried concentrated lactic cultures sachets, under the commercial name of YO-MIX™, were obtained from Danisco (Sassenage, France). Commercial UHT low fat milk was bought from a local supermarket (Hacendado, Valencia, Spain).

3.2. Preparation of the hydrolysate

The WPC was dissolved in water 5% (w/v) and heated at 90 ºC for 10 min. Hydrolysis was carried out in triplicate at 37 ºC and pH 8.0 by addition of 1M NaOH for 3 h with constant agitation. Food grade trypsin (Biocatalyst, Nantgarw, UK) was used at an enzyme-to-substrate ratio of 1:20 (w/w). Reactions were stopped by heating at 95 ºC for 15 min, to ensure the complete inactivation of the enzyme. The hydrolysate was then spray-dried. The inlet temperature of spray drying was maintained at 140 ºC and the outlet temperature was between 75 and 100 ºC, following the method described in [26].

3.3. Microencapsulation of the hydrolysate

The hydrolysate was microencapsulated within gelatin and chitosan particles by spray-drying. The hydrolysate (30% w/w with respect to the total solids mass) was dispersed in gelatin (10% w/v) or chitosan (2% w/v) stock solutions in acetic acid 20% (v/v). After a 50-fold dilution, the dispersions were fed to a Nano Spray Dryer B-90 apparatus (Büchi, Switzerland) equipped with a 7.0 µm pore diameter cap. The inlet air temperature was set at 90 ºC, the inlet air flow rate was 150 L/min and the pressure 50 mbar. The outlet air temperature was 50 ± 5 ºC. The spray-dried powders were deposited on the collector electrode by means of an applied voltage of 15 kV.

3.4. Morphological characterization of the particles

Samples were sputter-coated with a gold-palladium mixture under vacuum and observed by scanning electron microscopy (SEM) using a Hitachi microscope (Hitachi S-4100) at an accelerating voltage of 10 kV and a working distance of 15-16 mm. Particle diameters were measured from the SEM micrographs using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

3.5. Fourier transform infrared (FT-IR) analysis of the samples

The hydrolysate, both in its free form and microencapsulated within the biopolymers, was dispersed in spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa and FT-IR spectra were collected in transmission mode using a Bruker FT-IR Tensor 37 equipment (Rheinstetten, Germany). The spectra were obtained by averaging 10 scans at 1 cm^{-1} resolution.

3.6. Static *in-vitro* digestion

Dispersions of the free hydrolysate (12 mg/mL) or suspensions of the hydrolysate-loaded microcapsules (40 mg/mL, i.e. the equivalent of 12 mg/mL of hydrolysate) in distilled water were subjected to *in-vitro* gastrointestinal digestion according to the standardized static *in vitro* digestion protocol [27]. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the reported compositions [27]. In the oral phase, the dispersions were mixed with SSF (50:50 v/v) and incubated at 37 ºC for 2 min in a shaking incubator at 150 rpm. In the gastric phase, the oral digest was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37 ºC for 2 h in a shaking incubator at 150 rpm). In the duodenal phase, the gastric digest was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37 ºC for 2 h as described above. The pH was initially adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added and the digests were snap-frozen in liquid nitrogen for subsequent lyophilisation.

Freeze-dried samples were re-suspended in 10 mL of milliQ water and centrifuged for 20 min at 1795 g and 4 ºC. The supernatant was then ultracentrifuged using Centriprep® Ultracel® YM-3 centrifugal filter units (Millipore, Cork, Ireland) with a molecular weight cut-off of 3 kDa. The ultracentrifugation was carried out at 3000 g and 4 ºC in three steps of 95 min, 35 min and 10 min, respectively, according to the supplier's instructions. The ultrafiltrates were freeze-dried for storage and re-dissolved in milliQ water prior to HPLC-MS/MS analysis.

Samples of the hydrolysate-loaded microcapsules (non-digested) were suspended in acetic acid (20% v/v) under vigorous agitation to dissolve the encapsulation matrices, and subsequently ultrafiltered following the same procedure described above for the digests in order to assess the effective release of the peptides from their encapsulation matrices.

3.7. Lactic fermentation of hydrolysate-containing milk

Commercial UHT low fat milk was supplemented with the hydrolysate and fermented to produce peptide-enriched yoghurts. For this purpose, 1 sachet of freeze-dried concentrated lactic cultures (YO-MIX ™) was dispersed in 1 L of milk, and 1 mL of this mixture was further diluted in 1 L of milk. Both the free hydrolysate (200 mg) and the hydrolysate-loaded capsules (667 mg) were dispersed into 15 mL aliquots of the inoculated milk and incubated overnight at 42 ºC (until pH 5 was reached). 'Blank' yoghurts (i.e. without hydrolysate) were also produced.

The obtained yoghurts were then freeze-dried and re-suspended in 10 mL of acetic acid (20% v/v) under vigorous agitation for 5 h in order to dissolve the encapsulation matrices and release the peptides for analysis, as described above. The resulting suspensions were centrifuged to remove solids and the supernatant was subsequently ultracentrifuged using Centriprep® Ultracel® YM-3 centrifugal filter units as described above for the digests. The ultrafiltrates were freeze-dried for storage and re-dissolved in milliQ water prior to HPLC-MS/MS analysis.

3.8. HPLC-MS/MS

Samples were analysed on a 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) coupled to an Esquire 3000 ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was carried out using a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The injection volume was 50 µL and the flow rate 0.2 mL/min. A linear gradient from 0 to 45% of solvent B (acetonitrile/formic acid 0.1%) and 55% of solvent A (water/ formic acid 0.1%) in 120 min was used. In these analyses, the target mass was set at 750 *m/z.* Spectra were recorded over the mass/charge (*m/z*) range of 200-1500.

Data processing was done with Data Analysis™ (version 4.0; Bruker Daltoniks, GmbH Germany). Peptide sequencing was assisted by MASCOT, using a homemade database that includes the most abundant cow's whey proteins. The matched MS/MS spectra were interpreted with BioTools version 3.2, both from Bruker Daltoniks GmbH (Germany). Comparison of peptide profiles were performed with Venny®. (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

4. Results and discussion

4.1. Peptide profile of the hydrolysate

A tryptic whey protein concentrate hydrolysate was produced in order to obtain a range of peptides which would allow the study of the impact of microencapsulation on their resistance to gastrointestinal conditions and their stability against lactic acid fermentation. A total of 47 β-lactoglobulin (β-Lg) peptide sequences were identified in the protein hydrolysate, 27 of which had been previously identified in a tryptic hydrolysate of a β-lactoglobulin enriched whey protein concentrate prepared in a similar way [28]. In addition, 11 α-lactalbumin (α-La) peptide sequences were also found. Cleavages corresponded mostly to specific trypsin sites after Arg and Lys residues, but also peptides with Leu, Phe, Glu and Tyr at the C-terminal position were found. A food-grade trypsin was used in this study, which explains the broad specificity, since this enzyme preparation has chymotrypsin activity. Table 1 summarizes the 58 identified peptides in the hydrolysate. These sequences covered almost the whole protein sequence except those regions containing disulphide bridges, where the identification was impaired. Some of the identified sequences have been reported to exert different bioactivities, such as antimicrobial (β-Lg fragments 15-20, VAGTWY, 78-83, IPAVFK, 92-100, VLVLDTDYK and α-La 1-5, EQLTK) [29, 30], ACE-inhibitory(β-Lg fragment 9-14, GLDIQK) [31], hypocholesterolemic (β-Lg fragment 70-74, IIAEK) [32] and DPP-IV–inhibitory activity (β-Lg fragments 15-20, VAGTWY and 78-82, IPAVF) [33, 34].

4.2. Morphological characterization of the microcapsules

The whey protein hydrolysate was microencapsulated using a protein (gelatin) and a polysaccharide (chitosan) as wall materials by spray-drying in order to compare the suitability of both biopolymers to protect the different peptides during gastrointestinal digestion and against lactic acid fermentation. Fig. 1 shows the SEM micrographs of the hydrolysate-containing spray-dried powders, which exhibited a pseudo-spherical morphology with varying roughness of their surfaces. These heterogeneous shapes are typically observed for spray-dried particles obtained from aqueous solutions [35-37] due to the fast evaporation of the solvent. In general, larger microparticles were obtained when gelatin was used as encapsulating matrix, partially due to the lower concentration of the feed suspensions containing chitosan as the wall material, which was a processing requirement due to the high viscosity of the polysaccharide solution.

Table 1. Peptides identified in the protein hydrolysate and microcapsules with chitosan and gelatin.

Protein	Fragment	Experimental mass	Theoretical mass	Sequence	Detected within the microcapsules	
					Chitosan	Gelatin
β–Lg	1 – 5	572.2	572.4	LIVTQ	✓	
	1 – 8	932.5	932.5	LIVTQTMK	✓	✓
	2 – 8	819.4	819.5	IVTQTMK	✓	✓
	8 – 14	800.5	800.5	KGLDIQK	✓	✓
	9 – 14	672.4	672.4	GLDIQK		
	15 – 20	695.2	695.3	VAGTWY	✓	✓
	21 – 26	561.2	561.3	SLAMAA	✓	✓
	21 – 32	1190.6	1190.6	SLAMAASDISLL		
	25 – 32	788.4	788.4	AASDISLL	✓	✓
	21 – 26	562.2	562.3	SLAMAA	✓	✓
	27 – 43	1846.2	1846.0	SDISLLDAQSAPLRVYV		
	23 – 32	990.5	990.5	AMAASDISLL		
	33 – 40	856.4	856.4	DAQSAPLR	✓	✓
	36 – 40	542.3	542.3	SAPLR		
	41 – 46	750.3	750.4	VYVEEL		
	41 – 57	1943.8	1943.0	VYVEELKPTPEGDLEIL		✓
	41 – 58	2057.0	2056.1	VYVEELKPTPEGDLEILL		
	43 – 57	1680.8	1681.0	VEELKPTPEGDLEIL		✓
	43 – 60	2051.0	2050.1	VEELKPTPEGDLEILLQK		✓
	70 – 75	700.4	700.5	KIIAEK	✓	✓
	71 – 75	572.3	572.4	IIAEK	✓	✓
	76 – 82	774.4	774.5	TKIPAVF	✓	✓
	77 – 82	673.4	673.4	KIPAVF	✓	✓
	78 – 82	545.3	545.3	IPAVF	✓	✓
	78 – 83	673.4	673.4	IPAVFK	✓	✓
	83 –87	558.3	558.3	KIDAL	✓	✓
	83 – 91	1043.6	1043.6	KIDALNENK	✓	✓
	84 – 91	915.5	915.5	IDALNENK	✓	✓
	91 – 100	1192.6	1192.7	KVLVLDTDYK		
	92 – 100	1064.6	1064.6	VLVLDTDYK		✓
	92 – 101	1192.6	1192.7	VLVLDTDYKK	✓	✓
	94 – 100	852.4	852.4	VLDTDYK		
	94 – 101	980.5	980.5	VLDTDYKK	✓	✓
	95 – 101	881.4	881.5	LDTDYKK	✓	
	92 – 99	936.4	936.5	VLVLDTDY	✓	✓
	96 – 101	768.3	768.4	DTDYKK	✓	✓
	108 – 113	645.3	645.3	ENSAEP	✓	✓
	110 – 115	659.3	659.3	SAEPEQ		
	125 – 135	1244.6	1244.6	TPEVDDEALEK		✓
	125 – 136	1391.6	1391.6	TPEVDDEALEKF		✓
	125 – 138	1634.6	1634.8	TPEVDDEALEKFDK		✓
	127 – 135	1046.5	1046.5	EVDDEALEK		✓
	127 – 138	1436.4	1436.7	EVDDEALEKFDK		
	142 – 148	837.4	837.5	ALPMHIR	✓	✓
	149 – 155	805.4	805.4	LSFNPTQ	✓	✓
	149 – 156	918.5	918.5	LSFNPTQL	✓	✓
	149 – 159	1304.6	1304.6	LSFNPTQLEEQ		✓
α–La	1 – 5	617.3	617.3	EQLTK	✓	✓
	12 – 16	615.4	615.4	LKDLK	✓	✓
	15 – 24	989.5	989.6	LKGYGGVSLP		
	32 – 36	563.2	563.2	HTSGY		✓
	37 – 43	773.4	773.4	DTQAIVQ	✓	✓
	37 – 44	887.4	887.4	DTQAIVQN		✓
	45 – 50	727.1	727.3	NDSTEY		✓
	51–58	932.5	932.5	GLFQINNK	✓	✓
	63–68	724.2	724.3	DDQNPH	✓	✓
	94–98	615.4	615.4	KILDK		
	99–104	750.3	750.4	VGINYW	✓	✓

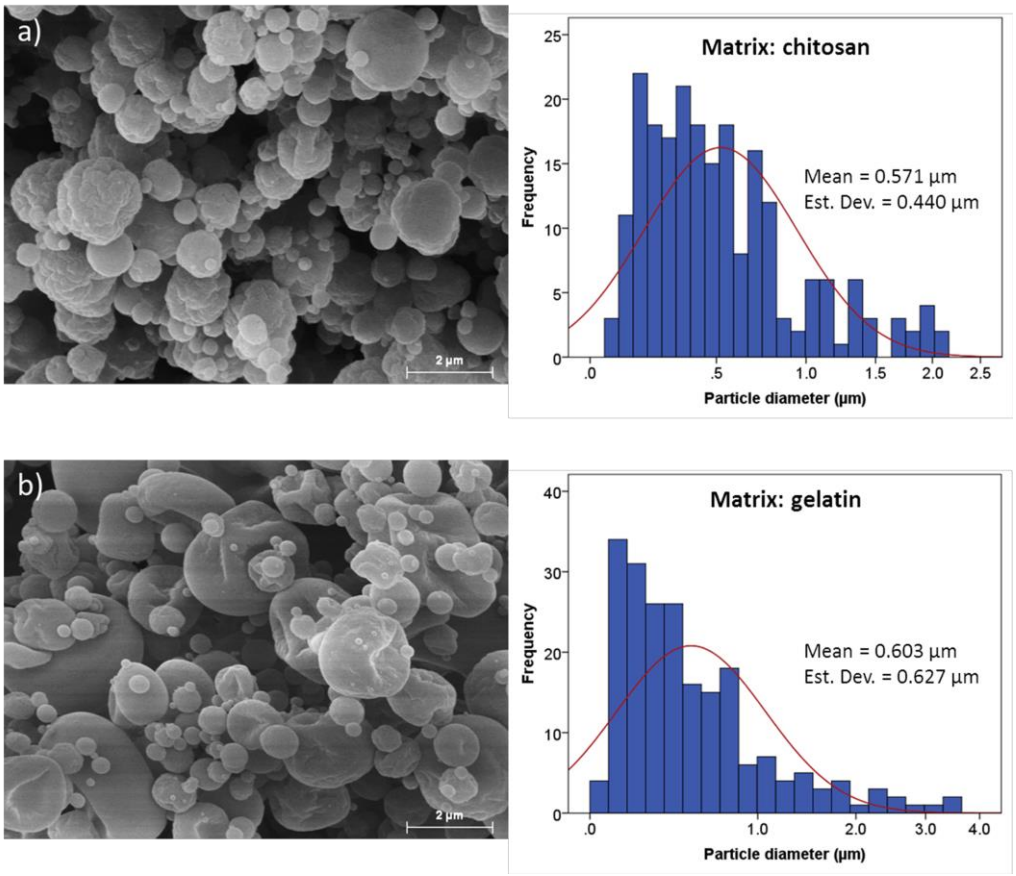


Figure 1. SEM images of hydrolysate-loaded spray-dried chitosan (a) and gelatin (b) particles, together with their size distributions. Scale bars correspond to 2 µm.

4.3. FT-IR analysis of the microencapsulated hydrolysate

The spray-dried materials, together with the free hydrolysate, were characterized using FT-IR spectroscopy, and the obtained spectra are shown in Fig. 2.

The spectrum of spray-dried chitosan showed a broad band with a maximum at 3386 cm^{-1}, attributed to the –OH and –NH stretching vibration, and other characteristic bands at 2929 and 2885 cm^{-1} (stretching of C–H bonds), 1643 cm^{-1} (Amide I, C=O stretching), 1561 cm^{-1} (Amide II, $–NH_2$ bending) and 1076 cm^{-1} (C–O stretching of sugar rings) [22, 38]. The spectrum of spray-dried gelatin also exhibited its most characteristic bands at 3307 cm^{-1} (Amide A), 3078 cm^{-1} (Amide B), 1653 cm^{-1} (Amide I), 1542 cm^{-1} (Amide II) and 1244 cm^{-1} (Amide III) [39]. On the other hand, given the protein nature of the hydrolysate, its spectrum showed similar bands as the gelatin one, although centred at slightly different wavenumbers: 3293 cm^{-1} (Amide A),

3079 cm^{-1} (Amide B), 1649 cm^{-1} (Amide I), 1545 cm^{-1} (Amide II) and 1243 cm^{-1} (Amide III).

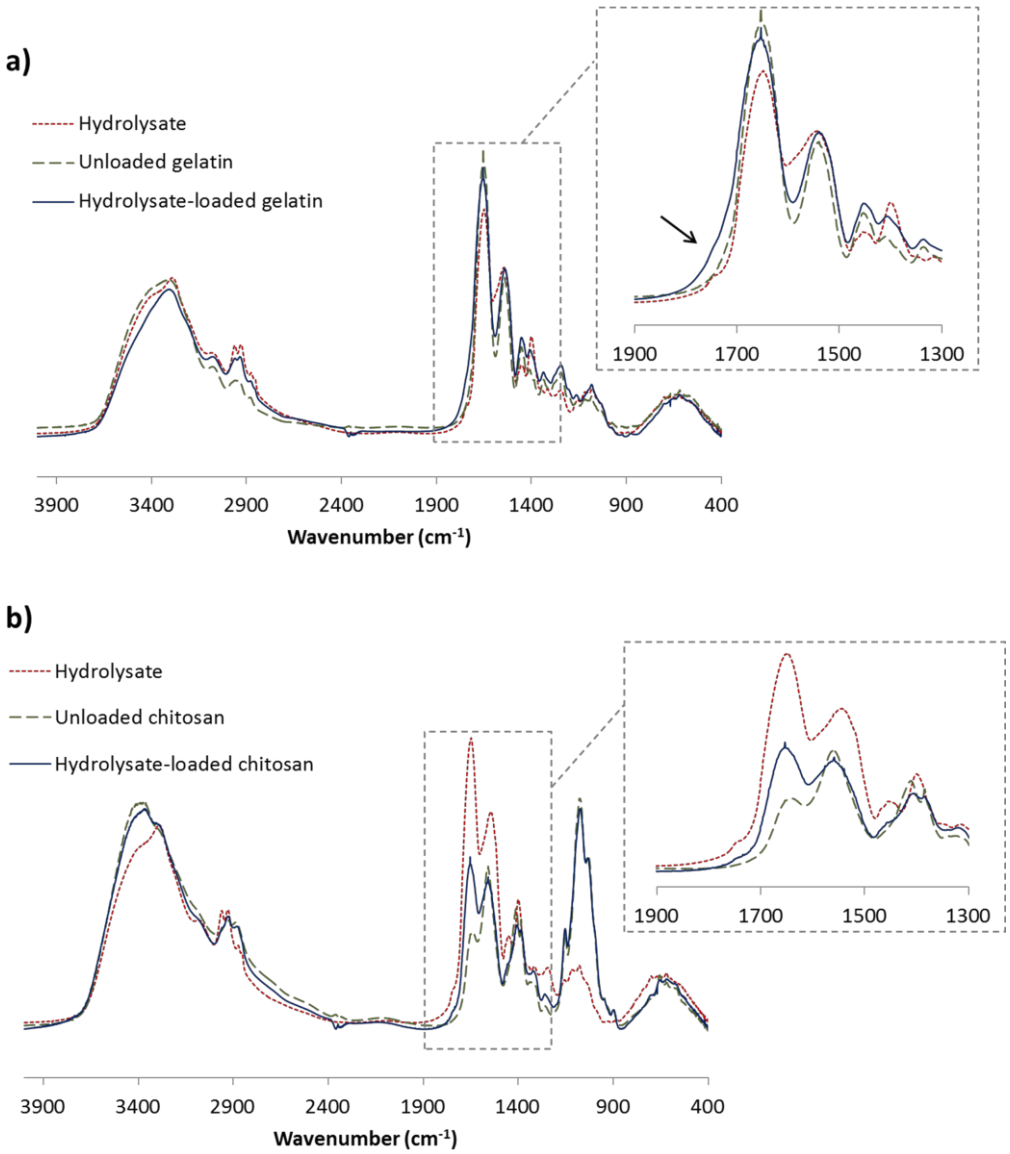


Figure 2. Infrared spectra of the hydrolysate together with the (a) gelatin and (b) chitosan spray-dried materials. Insets show magnification of the Amide I and II area of the spectra.

The spectra of the microencapsulated hydrolysate showed the characteristic bands of either chitosan or gelatin and the hydrolysate, generally at intermediate wavelengths due to the contribution of both materials present in the capsules. For instance, one of the bands ascribed to the stretching of C–H bonds had its maximum at 2939 cm^{-1} in gelatin and at 2930 cm^{-1} in the free hydrolysate, being centred at an intermediate wavelength of 2935 cm^{-1} in the hydrolysate-loaded gelatin microparticles. Similarly, the band centred at 1413 cm^{-1} in chitosan and 1400 cm^{-1} in the free hydrolysate had its maximum at 1407 cm^{-1} in the hydrolysate-loaded chitosan capsules. However, certain bands of the encapsulated hydrolysate shifted to higher or lower wavenumbers as compared to both components of the particles. Although it is difficult to draw conclusions given the overlapping of most spectral bands and the highly coupled modes in the Amide I and II regions, interactions between the peptides from the hydrolysate and the encapsulation matrices seem to have taken place during the encapsulation process, as inferred from the spectral changes in this area observed in the hybrid capsules (see insets in Fig. 2a and 2b). These interactions could, in fact, explain why certain peptides were not detected after dissolving the capsules (Table 1). Crosslinking reactions of proteins have been described upon thermal treatments, as high temperatures lead to protein denaturation, leaving internal thiol and hydrophobic groups exposed and available to form intermolecular disulphide bonds and hydrophobic interactions [40, 41]. The spray drying process used in this work for the encapsulation of the protein hydrolysate, involving the use of high temperatures, might have thus contributed to promoting this type of crosslinking reactions between the gelatin and the hydrolysate. In fact, an increase in the intensity of the amide band towards greater wavenumbers, related to antiparallel β-sheet interactions [42, 43] was clearly observed in the hybrid capsules (arrow in inset of Fig. 2a).

4.4. Identification of peptides after encapsulation

In order to corroborate the effective encapsulation of the hydrolysate within the two biopolymer matrices, the loaded chitosan and gelatin capsules were subjected to extraction in acidic conditions, dissolving the encapsulation matrices and thus favouring the release of the peptides. The comparison of the total ion current (TIC) chromatograms obtained by HPLC-MS/MS showed little differences indicating an effective release of the hydrolysate from the capsules (Fig. 3). Most of the peptides present in the initial hydrolysate were identified, which demonstrated that the encapsulation procedure did not affect the peptide profile to a great extent (Table 1). Even then, 13 peptides out of 58 from the hydrolysate could not be identified after capsule disruption, probably due to interactions of the peptides with the encapsulation matrices as suggested by the FT-IR results. On the other hand, some peptides were newly found after dissolution, 11 in the case of chitosan capsules and 9 in the case of gelatin capsules, 4 of which were common sequences. Peptide-matrix interactions might have affected peptide identification, specially taking into account that the purification of the samples prior to HPLC-MS/MS analysis included an ultrafiltration step to remove high molecular weight molecules, probably affecting the recovery of

the peptides interacting with the matrices. Despite the observed exceptions, it was confirmed that the peptides in the hydrolysate could be released from the microcapsules under suitable conditions. These results are consistent with previous works which had demonstrated that model proteins (such as bovine serum albumin), peptides (e.g. RGVKGPR, KLGPKGPR or SSPGPPVH) or protein hydrolysates (such as atlantic salmon protein hydrolysates) could be effectively released from gelatin and chitosan-based encapsulation structures, respectively, in aqueous systems [44-46].

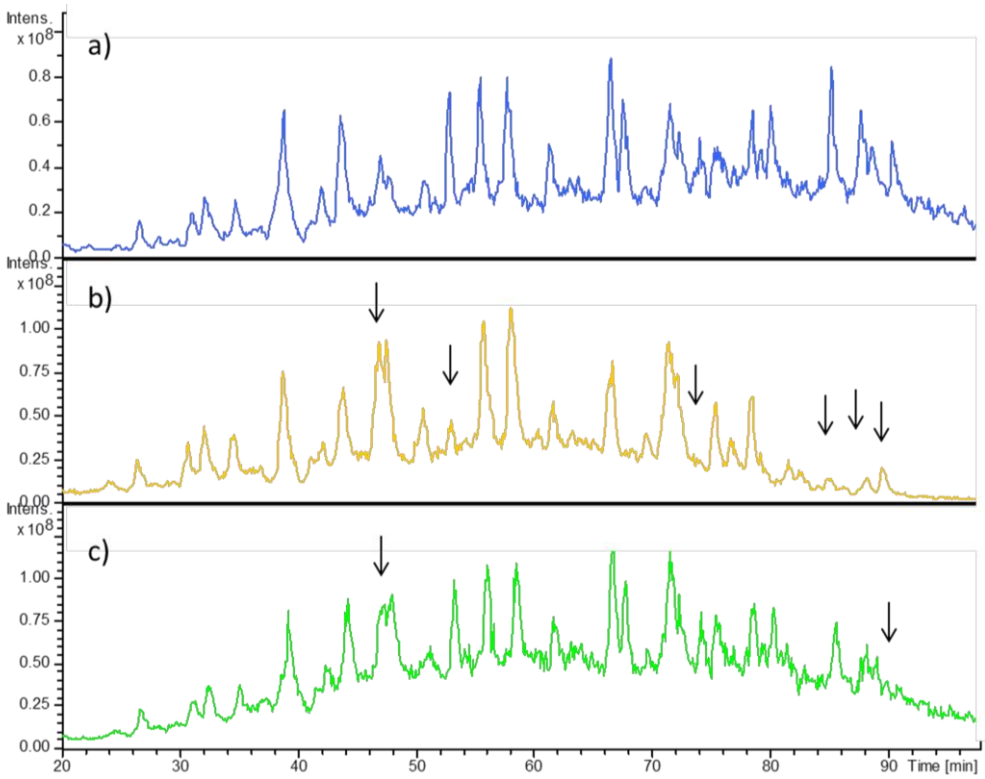


Figure 3. Total ion current (TIC) chromatograms of the free WPC hydrolysate (a), chitosan-encapsulated hydrolysate (b) and gelatin-encapsulated hydrolysate (c) after matrix dissolution. Arrows indicate differences in the chromatographic profile.

4.5. Simulated digestion of the microencapsulated hydrolysate

Although encapsulation may be effective in protecting functional ingredients, it has also been reported that their entrapment within certain microstructures may decrease their bioaccessibility to a certain extent after ingestion [47]. Thus, the microstructures obtained in this work were subjected to *in-vitro* digestion to assess

whether the peptides from the hydrolysate would be effectively released during passage through the gastrointestinal tract.

Simulated digestion of the free hydrolysate resulted in a remarkable change in the identified peptides. Their number was reduced by half in the case of β-Lg fragments (Fig. 4a, b). In the case of α-La, with a lower number of peptides, a similar tendency was found. Only two complete sequences from β-Lg (fragments 108-113, ENSAEP and 110-115, SAEPEQ) and one from α-La (fragment 63-68, DDQNPH) were resistant to the simulated gastrointestinal digestion. In most cases, peptides identified in the digesta corresponded to fragments from those found in the non-digested sample. The lower number of peptides can be attributed to their degradation to form di- or tri-peptides or free amino acids. Besides, the digesta contained enzyme autolytic fragments and bile salts, giving rise to a much more complex matrix which complicated peptide detection.

In the digesta from the hydrolysate-loaded chitosan microparticles, 23 peptides could be identified, 17 of which were similar to those found in the digested free hydrolysate (Fig. 4b, c). On the other hand, the digesta from the hydrolysate-loaded gelatin capsules produced a very complex chromatogram where only two peptides from the whey proteins could be identified. The proteinaceous origin of the encapsulation matrix, which was also digested into peptides by the enzymes added during the assay was most probably causing this interference.

Summarizing, the results indicated that digestion of the samples modified the peptide profile of the hydrolysate towards lower number of peptides and reduced molecular weight. Even though a protective effect during digestion was not evidenced, the encapsulation within chitosan microparticles did not alter to a great extent the peptide profile of the digests. Therefore, the peptide bioaccessibility was not expected to be substantially affected by the encapsulation. In fact, previous works have shown the potential of chitosan-based encapsulation structures as effective carriers for oral peptide delivery. Specifically, *in vivo* assays in rats demonstrated an enhanced bioactivity for salmon calcitonin after oral administration of the chitosan-encapsulated peptide [48, 49]. Biostability and bioavailability of the peptides are essential to achieve physiological benefits, as they need to reach their targets in an active form in order to exert their bioactivity [8].

4.6. Fermentation assays

Peptide-enriched yoghurts were produced by lactic acid fermentation of UHT low fat milk supplemented with the free and microencapsulated hydrolysate. In the yoghurts where free hydrolysate had been added, a total of 30 β-Lg and α-La peptide sequences, out of the 51 original, were identified. Thus, a large part of the peptides in the hydrolysate were lost during lactic acid fermentation. It is known that the susceptibility of peptides to living starter cultures depends on the amino acids sequence [26], and thus only some of the peptides were degraded during the

fermentation process. None of the peptide sequences identified in the original hydrolysate were detected in a blank yoghurt prepared in the absence of hydrolysate.

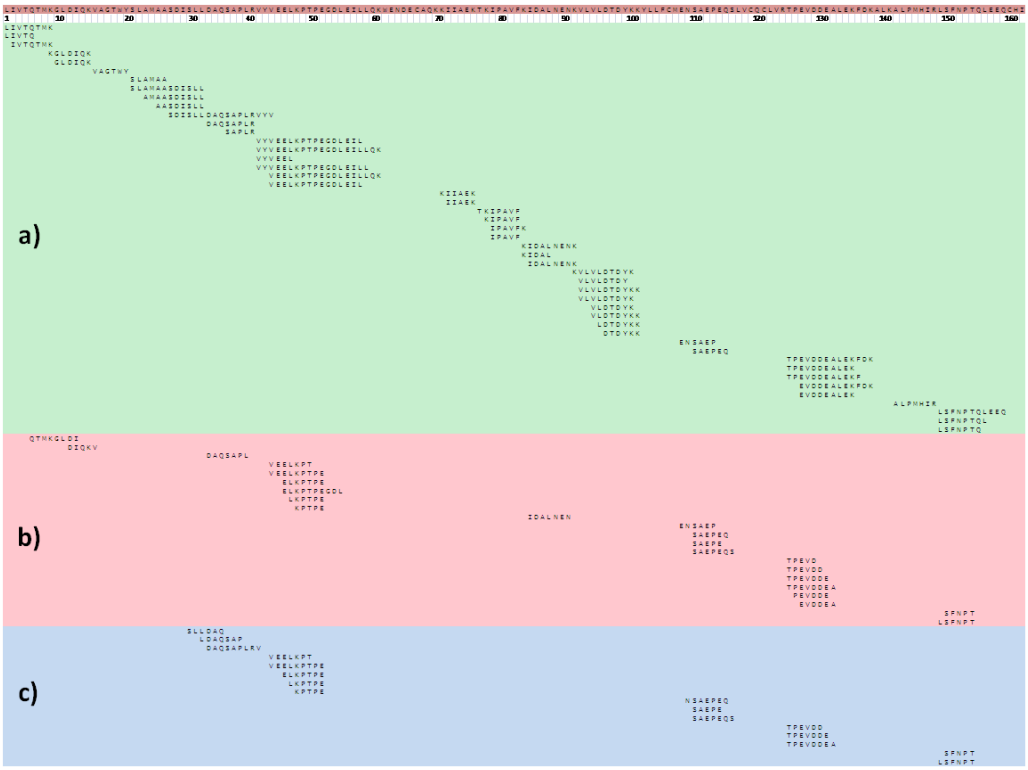


Figure 4. Peptides from β-Lactoglobulin identified in the hydrolysate before digestion (a), after digestion of the free hydrolysate (b) and after digestion of the hydrolysate-loaded chitosan microcapsules (c).

After analysis of the fermented products, five peptides were protected by encapsulation, since they were present in the hydrolysate prior fermentation but not in the yoghurt enriched with free hydrolysate (Fig. 5). Four of these sequences were only found when the hydrolysate was encapsulated within chitosan microparticles (β-Lg fragments 25-32, AASDISLL, 70-75, KIIAEK, 95-101, LDTDYKK, and 45-50, NDSTEY), while only one sequence (α-La fragment 37-44, DTQAIVQN) was protected by both types of encapsulation matrices. Two peptides, β-Lg fragments 21-32, SLAMAASDISLL, and 36-40, SAPLR, were not observed in the fermented milks containing the encapsulated hydrolysate, fact which could be ascribed either to a low concentration of the peptides in the products or to interactions with the encapsulation matrices, thus hindering release and subsequent identification.

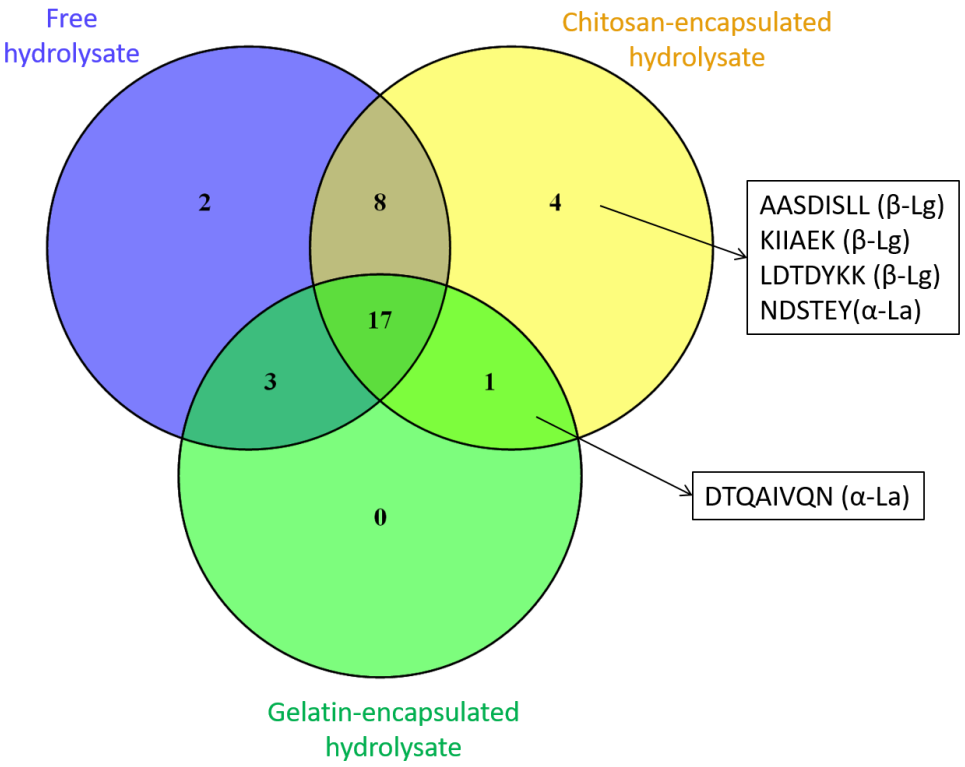


Figure 5. Venn diagram of the number of peptides identified in fermented milk fortified with the hydrolysate in its free form, encapsulated in chitosan and encapsulated in gelatin.

As the chemical species within the protein hydrolysates are characterized by their heterogeneity, the protection effect that encapsulation exerted on the protein hydrolysate during milk fermentation was sequence-dependent. Not all the fermentation-susceptible peptides could be stabilized through encapsulation. On the other hand, encapsulation within chitosan protected a greater number of peptides as compared to gelatin. Thus, selecting the most appropriate encapsulation matrix is of utmost importance in order to achieve the protection of selected protein fragments with regard to the intended purpose of the hydrolysate.

5. Conclusions

A whey protein hydrolysate was microencapsulated by spray-drying using two different encapsulation matrices, i.e. chitosan and gelatin, obtaining pseudo-spherical particles in both cases. Most of the hydrolysate peptides could be effectively released

from the microcapsules by simply dissolving the biopolymeric matrices under acidic conditions. However, 13 peptides could not be identified after capsule disruption, probably due to peptide-matrix interactions which affected peptide recovery during the purification process. *In-vitro* digestion assays were carried out to further assess the release of the peptides during passage through the gastrointestinal tract, given the importance of the bioavailability of the compounds in order to exert their bioactivities. Although no protective effect during digestion was evidenced upon encapsulation within chitosan microparticles, this encapsulation did not substantially alter the peptide profile of the digest as compared to the free hydrolysate, and therefore, peptide bioaccessibility was not expected to be compromised by the encapsulation. Regarding the use of gelatin matrix, the complexity of the chromatogram obtained for the digested samples precluded the identification of the peptides from the hydrolysate and the results were not conclusive. On the other hand, the protection exerted by the encapsulation during milk fermentation was sequence- and matrix-dependent. Only 5 out of the 21 fermentation-susceptible peptides could be stabilized through encapsulation within chitosan, one of which was also protected using gelatin. Overall, chitosan yielded improved results when compared to gelatin regarding peptide protection during milk fermentation, although the most appropriate encapsulation matrix should be selected individually based on the specific target protein fragments, that is, the potentially bioactive peptides present in a hydrolysate.

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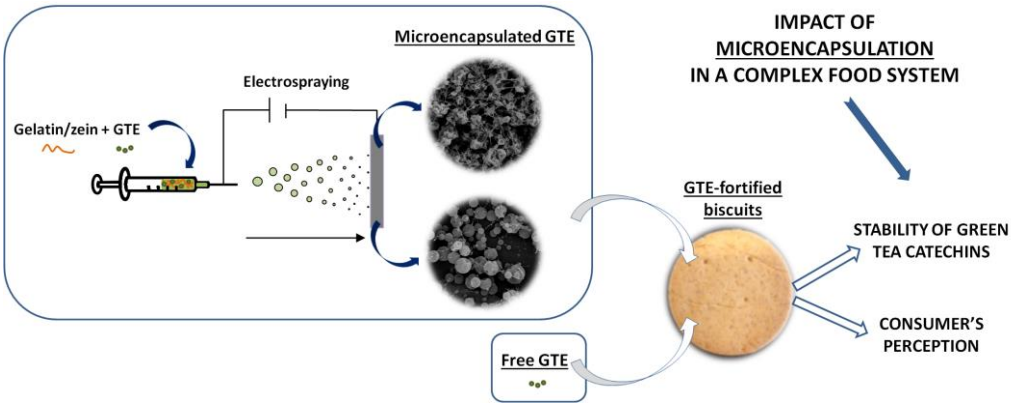
5.2

IMPACT OF MICROENCAPSULATION WITHIN ELECTROSPRAYED PROTEINS ON THE FORMULATION OF GREEN TEA EXTRACT-ENRICHED BISCUITS



This section is an adapted version of the following published research article:

Gómez-Mascaraque, L. G., Hernández-Rojas, M., Tarancón, P., Tenon, M., Feuillère, N., Vélez Ruiz, J. F., Fiszman, S., & López-Rubio, A. (2017). *Impact of microencapsulation within electrosprayed proteins on the formulation of green tea extract-enriched biscuits*. LWT - Food Science and Technology, 81, 77-86. (DOI: http://dx.doi.org/10.1016/j.lwt.2017.03.041).

1. Abstract

In this work, a green tea extract (GTE) was encapsulated within electrosprayed protein (i.e. gelatin and zein) microparticles, and the protective ability of both systems on the green tea catechins was assessed. The microparticles (with encapsulation efficiencies ~90 g/100g), proved to be very effective in stabilizing the catechins during a thermal treatment at 180 ºC (12 min), preserving 85-90 g/100g of their initial catechins content, while free GTE lost almost 40 g/100g of its catechins content. In order to assess the impact of microencapsulation in a real food system, the GTE-loaded electrosprayed microparticles were added to biscuits dough. Results showed that microencapsulation did not significantly protect during biscuit processing and emphasized the need of assessing the behaviour of microencapsulation systems in real food processing conditions. The sensorial analysis of the biscuits indicated that addition of the GTE-loaded microparticles did not impact the acceptability of the biscuits, as perceived by consumers.

2. Introduction

Green tea catechins are a group of polyphenolic antioxidants which have received increasing research attention in recent years because of their reported health promoting properties, such as anti-carcinogenic [1] and antimicrobial effects [2]. Among them, (−)-epigallocatechin gallate (EGCG) is the most abundant and bioactive flavonoid in green tea [3]. Together with (−)-epicatechin gallate (ECG) it is accepted as an indicator of the quality of green tea extracts (GTE) [4]. These products are being increasingly added to a variety of foods in order to improve their nutritional value [5] and make them more appealing to consumers [6], whose interest towards functional foods is increasing [7].

Despite the promising benefits of supplementing food products with GTE, there are a number of technological concerns due to the poor stability of tea catechins, which are thermosensitive and highly unstable in alkaline conditions, especially EGCG and ECG [8], so their bioactivity could be compromised upon food processing and storage. Moreover, GTE has been reported to negatively impact aroma, flavour and overall acceptability of bread and, thus, the quality of the final food products [9]. Being one of the most popular bakery products consumed by nearly all levels of society and all age groups [10], biscuits are regarded as good candidates to be fortified with functional ingredients [11]. They are convenient snacks with a long shelf life and appealing sensory attributes [12]. In fact, the supplementation of biscuits with GTE [4, 13] or grounded tea leaves [14] has already been attempted with different purposes. However, biscuit doughs generally have high pH values, and high

temperatures are used to bake them, threatening the stability of tea catequins if supplemented with GTE [4].

Microencapsulation, or entrapment of an ingredient within a protective micron-sized matrix, is a plausible approach to protect sensitive bioactive molecules against degradation [15]. Electrospraying has recently emerged as an alternative to traditional microencapsulation techniques in the food industry such as spray-drying [16], as it can be performed under mild temperatures [17], being especially adequate for the encapsulation of thermosensitive functional ingredients [18]. This technology, based on electrohydrodynamic processing, consists of subjecting a polymer solution to a high voltage electric field in such a way that a charged polymer jet is ejected towards a grounded collector, generating dry polymeric particles as the jet breaks down into fine droplets during its flight and the solvent evaporates [19]. For food applications, solutions or suspensions of edible biopolymers such as proteins or polysaccharides containing the ingredient to be encapsulated can be electrosprayed using food-grade solvents [20]. Furthermore, these food macromolecules are known to interact with polyphenols [21, 22], which may contribute to the stabilization of these bioactive compounds when encapsulated within them [23].

In previous work, we demonstrated the potential of electrosprayed gelatin submicroparticles to protect EGCG against degradation in alkaline conditions [23]. Zein fibers produced through electrohydrodynamic processing have also been reported to be effective in stabilizing EGCG in aqueous environment [24]. The aim of the present study was to evaluate GTE-containing microparticles produced through electrospraying using both protein matrices when applied to a real food product. Specifically, they were added to biscuit dough. The protection exerted by the encapsulation matrices on the catechins upon biscuit preparation was evaluated by comparing the loss of these compounds and their antioxidant activity in comparison with the direct addition of the non-encapsulated GTE to the biscuit dough. A sensorial analysis was also conducted to assess whether the incorporation of the GTE and/or the microparticles to the biscuit formulation had an impact on the consumers' acceptability.

3. Materials and methods

3.1. Materials and ingredients

Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, and zein prolamine from corn, grade Z3625, were both purchased from Sigma-Aldrich and used as received without further purification. A green tea extract (GTE) with high oxygen radical absorbance capacity (ORAC) was kindly donated by Naturex (Avignon, France). Potassium persulfate ($K_2O_8S_2$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium bromide FTIR grade (KBr),

phosphoric acid (H_3PO_4), pyrogallol and ascorbic acid were obtained from Sigma-Aldrich. Acetic acid (96 mL/100mL) was purchased from Scharlab, ethanol (96 mL/100mL) from Panreac and acetonitrile from Biosolve Chimie (Dieuze, France). (+)-catechin (C), (-)-gallocatechin (GC), EGCG, ECG, (-)-epigallocatechin (EGC), (-)-epicatechin (EC) and (-)-gallocatechin gallate (GCG) standards were purchased from Sigma Aldrich.

The ingredients used to prepare the biscuits were 350 g soft wheat flour (Belenguer, S.A., Valencia) with 11 g/100g protein, 0.6 g/100g ash and alveograph parameters of P/L = 0.27 and W = 134, as provided by the supplier, where P is the maximum pressure required, L the extensibility and W the baking strength of the dough; 112.52 g vegetable margarine (Unilever, Spain), 103.07 g sugar (Azucarera Ebro, Madrid, Spain), 37.62 g reconstituted skimmed milk (from 6.12 g skimmed milk powder, Central Lechera Asturiana, Spain), 3.67 g salt, 1.22 g sodium bicarbonate (A. Martínez, Cheste, Spain) and 0.70 g ammonium hydrogen carbonate (Panreac Quimica, Barcelona, Spain).

3.2. Catechins profile of the GTE

High-performance liquid (HPLC) was used to identify the catechins profile of the GTE. The LC system used was an Agilent 1200 series (Agilent Technologies, Santa Clara, USA) equipped with a binary pump and a diode-array-detection (DAD) system. Separation of the different catechins (i.e. C, GC, EGCG, ECG, EGC, EC and GCG) was performed using a Zorbax SBC_8 (150 x 4.6 mm, 3.5 µm of particle size) LC-column. The flow rate was set to 0.9 mL/min and the oven temperature was 30 ºC. Eluent A was acetonitrile and eluent B water slightly acidified with 0.1 g/100 g H_3PO_4. The elution gradient started with 100 mL/100 mL of eluent B, decreasing to 90 mL/100 mL B in 20 min and to 85 mL/100 mL in 60 min. The injection volume was 2 µL. Acquisition was done using the ChemStation software (Agilent Technologies). The detection wavelength was set at 280 nm. The catechins were identified based on their retention times, as compared with their standards.

3.3. Microencapsulation of the GTE

The GTE was microencapsulated through electrospraying within two different protein matrices: gelatin and zein. For this purpose, solutions of the proteins in adequate solvents were first prepared. Gelatin aqueous solutions (8 g/100 mL) were prepared in diluted acetic acid (20 mL/100 mL) as described in Gomez-Mascaraque et al. (2015) [23], and cooled down to room temperature before addition of the GTE. On the other hand, zein (12 g/100 mL) was dissolved in ethanol (85 mL/100 mL) at room temperature (concentrations selected from preliminary tests in order to obtain electrosprayed particles as free of fibres as possible). The GTE was then added to the protein solutions under magnetic stirring at a concentration of 20 g/100 g of the total solids content in both cases.

The GTE-protein solutions were processed using a homemade electrospraying apparatus, equipped with a variable high-voltage 0–30 kV power supply. The solutions were introduced in a 5 mL plastic syringe and pumped at a constant flow-rate through a stainless-steel needle (0.9 mm inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a grounded stainless-steel plate, placed facing the syringe in a horizontal configuration, at a distance of 10 cm, as illustrated in Fig.1. The voltage applied to the needle and the pumping flow-rate, respectively, were 15 kV and 0.2 mL/h for the gelatin solutions [23], and 13 kV and 0.15 mL/h for the zein solutions (conditions selected from preliminary tests).

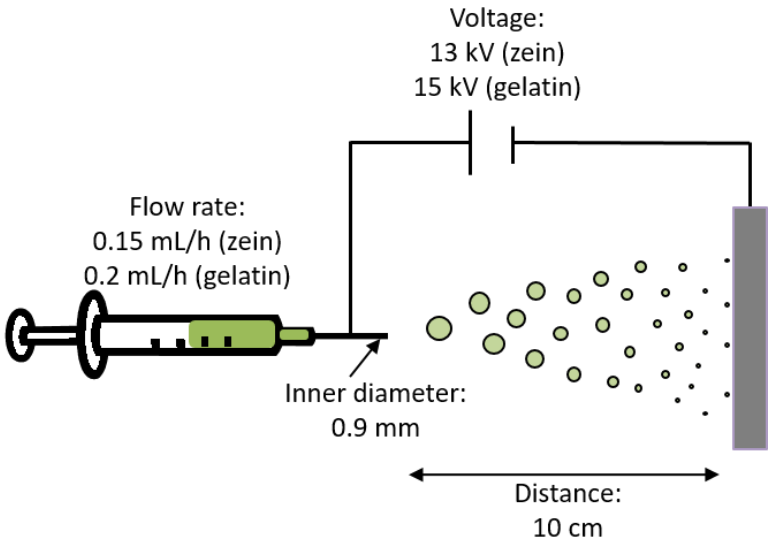


Figure 1. Schematic representation of the electrospraying setup used to produce the microparticles.

3.4. Characterization of the microparticles

Scanning electron microscopy (SEM) was conducted on a Hitachi S-4800 microscope (Hitachi High-Technologies, Tokyo, Japan) at an accelerating voltage of 10 kV and a working distance of 8–9 mm. Samples were sputter-coated with a gold–palladium mixture under vacuum prior to examination. Particle diameters were measured from the SEM micrographs in their original magnification using the ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

The samples were also subjected to Fourier transform infrared (FT-IR) analysis. Unloaded and GTE-containing particles of ca. 1 mg were grounded and dispersed in 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa. FT-IR spectra were collected in transmission

mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1/cm resolution. The spectrum of raw GTE was also obtained.

3.5. Microencapsulation efficiency

In order to estimate the amount of catechins in the microparticles, these were previously dissolved to release their contents. Ethanol (85 mL/100 mL) and diluted acetic acid (20 mL/100 mL) were used to dissolve the zein and gelatin particles, respectively. The obtained solutions were analysed by HPLC using the method described in Section 3.2 to quantify the amount of recovered catechins from the particles.

The microencapsulation efficiency (MEE) of the systems was calculated in terms of the fraction of catechins from the GTE that was effectively incorporated within the capsules, according to Eq. (1).

$$MEE\ (\%) = \frac{Catechins\ content\ in\ the\ electrosprayed\ capsules}{Catechins\ content\ added\ to\ the\ protein\ solutions} \times 100 \qquad \text{Eq. (1)}$$

3.6. Thermal stability of GTE upon microencapsulation

Free and microencapsulated GTE were subjected to a thermal treatment at 180 ºC for 12 minutes under inert atmosphere (N_2). The microparticles were then dissolved following the procedure described in Section 3.5 and analysed by HPLC according to the method described in Section 3.2.

3.7. Biscuits preparation

The shortening, sugar, milk powder, leaving agents, salt, and water were mixed in a mixer (Kenwood Ltd., UK) for 1 min at low speed (60 rpm). The bowl was scraped down and the mixture was mixed again for 3 min at higher speed (255 rpm). The flour was subsequently added and mixed at 60 rpm for 20 s. After scraping down the bowl, the mixture was mixed again at 60 rpm for 40 s. The dough was sheeted with a lab-scale sheeting machine (Parber, Vizcaya, Spain) and cut into discs of 64 mm in diameter and 4 mm in thickness using a mold. Twenty biscuits were placed on a perforated tray and baked in a conventional oven (De Dietrich, France) for 14 min at 180 °C. The same oven and trays were used to bake all the biscuits, keeping the number of biscuits and the position of the trays constant. After cooling (1 h), the biscuits were packed in metalized polypropylene bags and heat-sealed (Sealboy, Audion Elektro, Kleve, Germany) until further use. For the fortified biscuits, a ratio of 2 g/100g of GTE with respect to flour was previously mixed with the flour by manual mechanical action before being added to the dough.

3.8. Content of GTE catechins after biscuit preparation

The stability of free and microencapsulated GTE during biscuit preparation was evaluated by measuring the content of GTE catechins in the biscuits after baking. The catechin recovery from the biscuits was calculated according to Eq. (2) after extraction with ethanol (85 mL/100 mL) for zein microparticles or diluted acetic acid (20 mL/100 mL) for gelatin microparticles and subsequent HPLC analysis using the method described in Section 3.2.

$$Recovery\ (\%) = \frac{Catechins\ content\ extracted\ from\ the\ biscuits}{Initial\ catechin\ content\ added\ to\ the\ biscuit\ dough} \times 100 \quad \text{Eq. (2)}$$

3.9. Antioxidant activity

The radical scavenging activity (RSA) of the GTE-enriched biscuits was measured after extraction with ethanol (85 mL/100 mL) for zein microparticles or diluted acetic acid (20 mL/100 mL) for gelatin microparticles, and compared to that of the GTE as is. For this purpose, the $ABTS^{\bullet+}$ decolorization assay was applied following the protocol described in Gómez-Mascaraque, Soler, & Lopez-Rubio (2016) [25]. Briefly, a stock solution of $ABTS^{\bullet+}$ was prepared by reacting ABTS with potassium persulfate (7 and 2.45 mmol/L in distilled water, respectively). After 24 h in the dark at room temperature, the $ABTS^{\bullet+}$ stock solution was diluted with the respective extraction solvent to an absorbance of 0.70±0.02 at 734 nm. Then 10 µL of the samples (theorical concentration of GTE adjusted to 0.2 mg/mL in all cases) were added to 1 mL of diluted $ABTS^{\bullet+}$, and the absorbance at 734 nm was measured 1 min after initial mixing. The RSA was calculated using Eq. (3), where A_0 and A_1 are the absorbance values at 734 nm before and 1 min after addition of the samples, respectively. The recovery of GTE from the biscuits in terms of antioxidant activity was estimated according to Eq. (4).

$$\text{RSA}\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{Eq. (3)}$$

$$Recovery_{RSA}\ (\%) = \frac{RSA\ of\ biscuit\ (0.2\ mg/mL\ GTE\ theoretical)}{RSA\ of\ raw\ GTE\ (0.2\ mg/mL)} \times 100 \quad \text{Eq. (4)}$$

Experiments were performed in independent triplicates on a VWR spectrophotometer model V-1200 (Pennsylvania, USA). The unloaded microparticles and biscuits without GTE were also evaluated to take into account the potential contribution of these materials to the total RSA. Significant differences were determined through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$) using IBM SPSS Statistics software (v.23) (IBM Corp., USA).

3.10. Biscuits' liking evaluation

A study was carried out in order to assess whether the addition of free and microencapsulated GTE in biscuits had an effect on consumers' sensory liking. Two hundred fourteen consumers participated in the study (149 women and 65 men aged from 18 to 65 years old). They were recruited according to their willingness to participate and with the requirement of eating biscuits at least once a week and not having intolerance to gluten. Three biscuits were presented to consumers: 'C' (control biscuit without additions), 'free GTE' (biscuit added with GTE as is) and 'Gel-GTE' (biscuit added with gelatin-encapsulated GTE) without any information about their composition. Consumers were asked to evaluate their global, texture and taste liking using nine-point hedonic scales ranging from 1 = "dislike extremely" to 9 = "like extremely". The evaluations were carried out in a standardized test room and the biscuits were served in small white plastic plates. The samples were identified with random three-digit codes and were presented monadically following a balanced design (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Mineral water was provided to consumers to rinse their mouths between samples.

All experiments were performed in compliance with the national legislation, and according to the institutional framework and practices established by CSIC Ethics Committee. All participants received written information about the study before giving their informed consent.

3.11. Data analysis

Analysis of variance was performed using the Statgraphics Plus 5.1 software package (Statistical Graphics Co., Rockville, Md., U.S.A.). Fisher's least significant difference (LSD) test was used to calculate mean value differences ($\alpha < 0.05$).

4. Results and discussion

Many different research works have evaluated the potential of microencapsulation for the protection of food bioactives. However, for the commercial success of this kind of technologies it is important to investigate how these microparticles would behave in real processing conditions and the way they can affect food sensorial properties. Therefore, in this work GTE-containing microencapsulation structures were prepared in sufficient amount to be incorporated within the biscuit dough as a model food product. In the first part of the work, the microparticles obtained through electrospraying using two different protein matrices were characterized and their potential ability for the thermal stabilization of the catechins present in the GTE was evaluated. Later on, the effect of GTE addition to biscuits (free vs. microencapsulated GTE) was assessed in terms of antioxidant, morphological and sensorial acceptability.

4.1. Characterization of the microencapsulation structures

The GTE was microencapsulated within gelatin and zein microparticles by electrospraying. Fig. 2 shows the images obtained by SEM for the obtained materials, together with their particle size distributions. GTE-containing gelatin particles were quasi-spherical and very similar to those obtained in a previous work where pure EGCG was microencapsulated within electrosprayed gelatin structures [23]. GTE-loaded zein particles, on the other hand, were smaller, had a rougher surface and a greater content of small fibrils, which involved a greater extent of aggregation of the particles. These morphological differences were the result of both the distinct molecular structure of each protein and the different electrospraying conditions (solvent, process parameters…) used to produce the microparticles [26]. In both cases, most of the particles had a size in the submicron range, so they were not expected to have a negative impact on the textural perception of the biscuits.

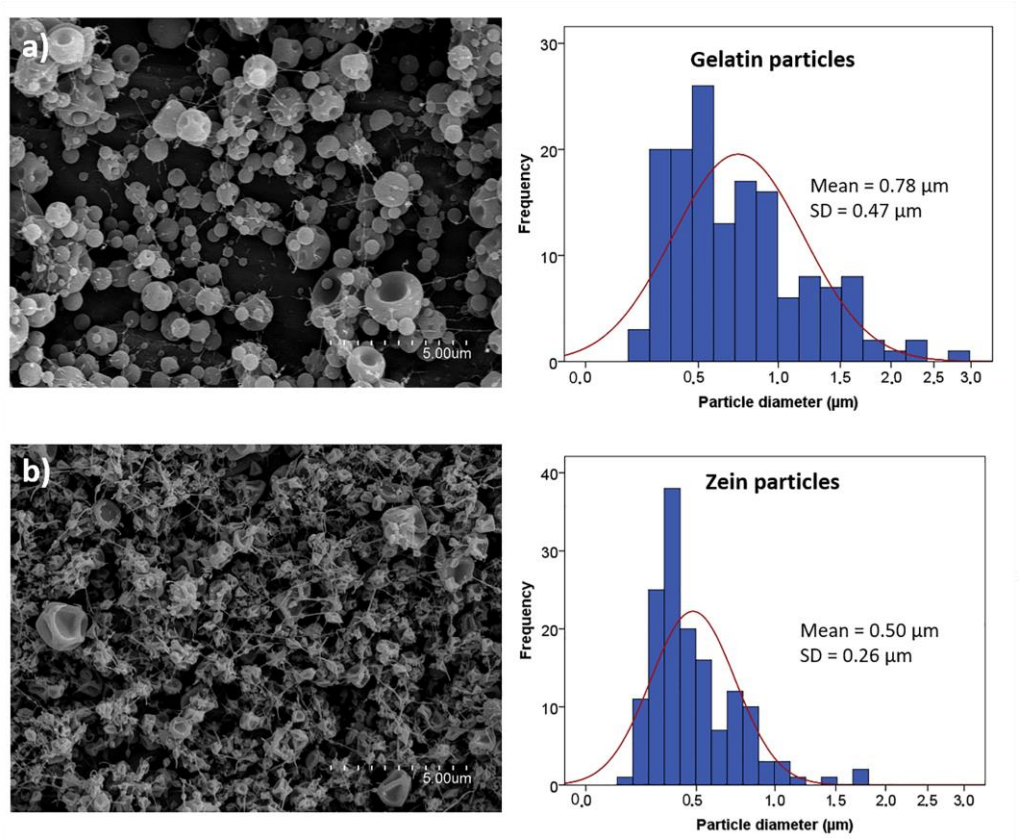


Figure 2. SEM images of electrosprayed microcapsules based on gelatin (a) and zein (b) containing green tea extract, together with their particle size distribution. SD means standard deviation.

The FT-IR spectra of the GTE-containing particles (Fig. 3) showed characteristic peaks from both the raw GTE and the proteins, but some spectral shifts were observed. Specifically, the bands attributed to the GTE which appeared in the range from 1300/cm to 900/cm shifted to higher wavenumbers in the encapsulation structures (cf. insets in Fig. 3), suggesting the presence of intermolecular interactions between the components of the GTE and its encapsulation matrices. Indeed, the ability of proteins to interact with polyphenols, mainly through non-covalent hydrophobic interactions and hydrogen bonds, is widely recognized [21]. The interactions with the encapsulation matrices are known to contribute to the stabilization of the encapsulated compounds [23].

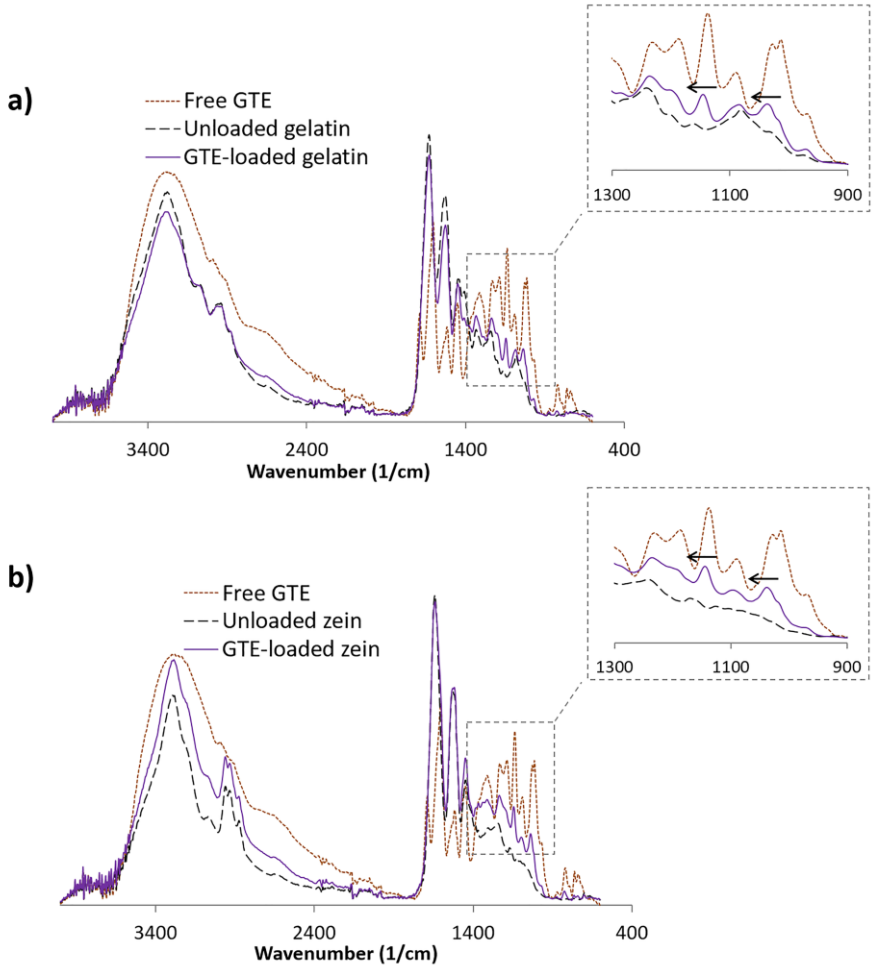


Figure 3. FT-IR spectra of electrosprayed microcapsules based on gelatin (a) and zein (b) containing green tea extract. The spectra of the raw green tea extract and unloaded capsules are also shown.

4.2. Microencapsulation efficiency

Initially, the GTE used in this work was characterized by HPLC in order to quantify the amount of each tea catechin present in the extract. The mass percentage of each catechin in the GTE was found to be as follows: EGCG = 41.8 g/100 g; ECG = 8.9 g/100 g; EGC = 9.6 g/100 g; EC = 6.3 g/100 g; GCG = 2.0 g/100 g; GC = 1.5 g/100 g; C = 1.0 g/100 g. According to these results, the most abundant catequin in the GTE is EGCG, reported as the most biologically active flavonoid in green tea [3].

Fig. 4b summarizes the catechin recovery obtained for the microencapsulated GTE by HPLC analysis after dissolution of the zein and gelatin particles, respectively. The chromatograms of the free and microencapsulated GTE are also shown (Fig. 4a), where the main tea catechins are identified based on their retention times. No catechins were detected in the chromatograms of the unloaded protein particles (cf. Supplementary Material). For each catechin, the recovery from the GTE-loaded particles was calculated according to Eq. (1), and the overall MEE was estimated as the weighted sum of the recoveries obtained for each catechin taking into account the relative proportion of each catechin in the original GTE.

According to the results, the electrospraying technique used for the micro-encapsulation of the GTE yielded very high encapsulation efficiencies, as observed previously for purified EGCG [23]. The MEE was slightly lower when gelatin was used as the encapsulating matrix, probably due to its processing in aqueous solution, given the instability of the catechins in aqueous media [27, 28]. The greatest differences were observed in the recovery of C, which was significantly lower from the gelatin microparticles. Nevertheless, the differences in the overall MEE were not significantly different. Moreover, the low pH of the gelatin solutions in diluted acetic acid managed to minimize catechins degradation, which is accelerated in alkaline pHs [27], thus obtaining a considerably high encapsulation efficiency too. The catechins profile of the GTE was slightly altered upon microencapsulation within both matrices, which could also be attributed to degradation and epimerization reactions taking place in the electrospraying solution [27]. For instance, while the EGC content decreased in the microencapsulated GTE compared to the free extract, the concentration of its epimer (GC) increased, suggesting that epimerization reactions took place during the encapsulation process. Still, the recovery of EGCG (the most abundant and bioactive catechin) from the microparticles was close to 90 g/100 g of its content in the original GTE, suggesting that electrospraying is an efficient technique for the micro-encapsulation of GTE within protein matrices such as gelatin and zein.

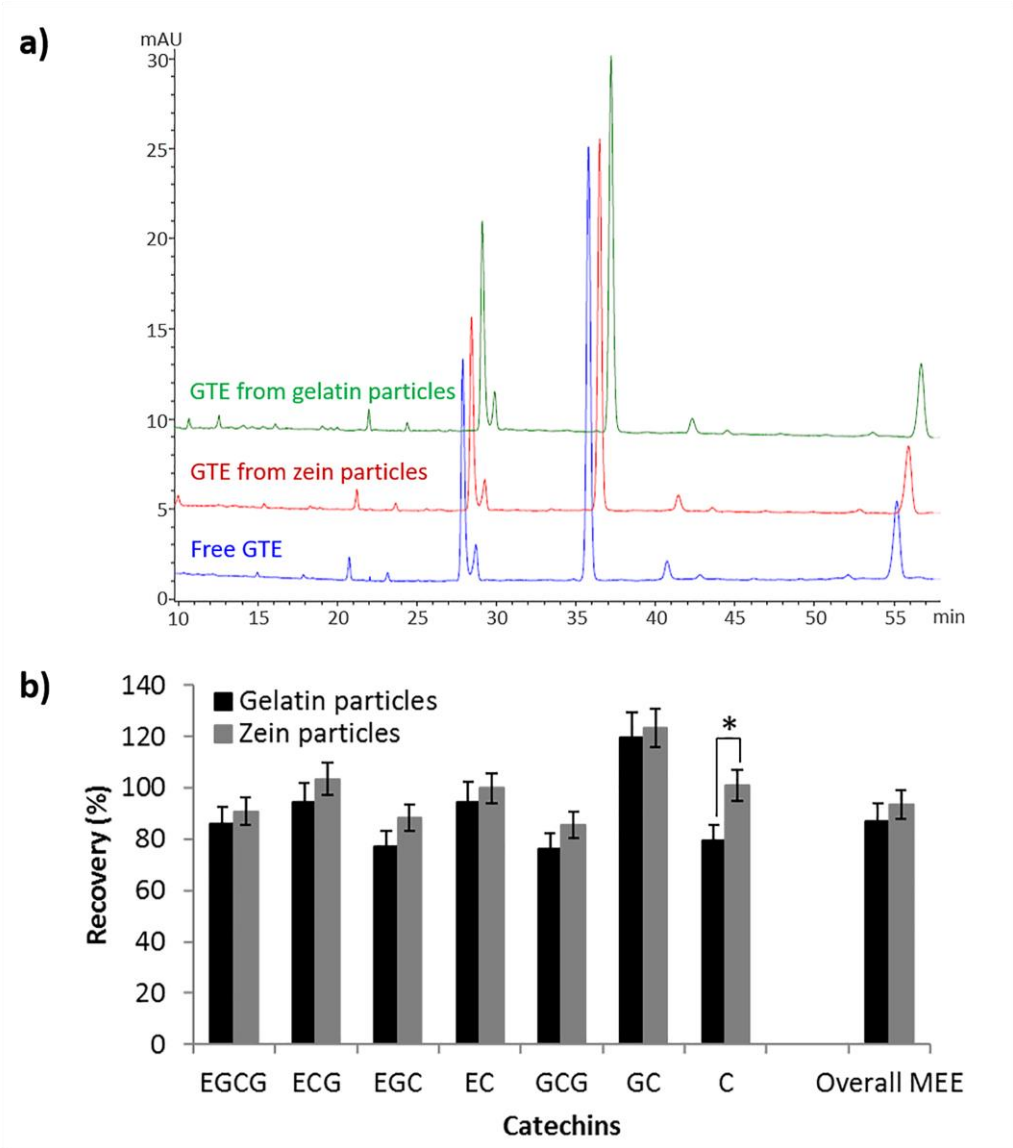


Figure 4. a) Chromatograms of free and microencapsulated green tea extract (GTE) after dissolution. Retention times of relevant peaks (as for the raw GTE): (-)-gallocatechin (GC, 15 min), (-)-epigallocatechin (EGC, 21 min), (+)-catechin (C, 23 min), caffeine (28 min), (-)-epicatechin (EC, 29 min), (-)-epigallocatechin gallate (EGCG, 36 min), (-)-gallocatechin gallate (GCG, 41 min), (-)-epicatechin gallate (ECG, 55 min). Note that the chromatograms of the GTE extracted from the microparticles have been slightly shifted for clarity purposes. b) Catechins recovery and overall microencapsulation efficiency (MEE) from encapsulated GTE. Asterisk (*) depicts significant differences among the samples ($p < 0.05$).

4.3. Thermal stability of GTE upon microencapsulation

It is well-known that the stability of tea catechins decreases with increasing temperatures and pH values [4], and given that the production of GTE-enriched biscuits involves the extract being subjected to high pHs and temperatures, suitable microencapsulation matrices for this ingredient should be capable of protecting catechins against degradation under these conditions. Electrosprayed biopolymers have already proven to stabilize EGCG against degradation in slightly alkaline solutions [29], but their protective effect on catechins against thermal degradation had not been demonstrated yet. Hence, the free and microencapsulated GTE was subjected to thermal treatment (i.e. the baking temperature, 180 ºC). Fig. 5a shows the obtained chromatograms and Fig. 5b summarizes the catechin recovery after the thermal treatment, calculated in the same way as done in the previous section for the as-prepared samples. Again, the recovery was calculated according to Eq. (1) for each catechin and the overall recovery was estimated taking into account the relative proportion of each catechin in the original GTE.

The total recovery of catechins from the GTE after the thermal treatment showed that microencapsulation was very effective in protecting the catechins against thermal degradation. While almost 40 g/100 g of the initial catechins content in the free GTE was lost, ~90 g/100 g and ~85 g/100 g of the total catechins content was recovered after the treatment from the gelatin and zein microparticles, respectively. However, the catechin profile of the GTE was affected by the thermal treatment, even when microencapsulated, although to a lesser extent in the latter case. While the content of epi-structured catechins (EGCG, ECG, EGC and EC) decreased, that of their non-epi-structured counterparts (GCG, CG and C) increased, suggesting that epimerization was one of the main phenomena taking place upon exposure to high temperatures. Indeed, it has been reported that epimerization and degradation reactions occur simultaneously for catechins and their relative reaction rate may vary depending on the temperature of the thermal treatment [27]. Microencapsulation was capable of precluding both epimerization and degradation processes, as the decrease in the content of epi catechins as well as the increase in non-epi catechins was limited. Small differences were observed between the two microencapsulation systems, which were only significant for EGC and GCG. Given the smaller size of the zein particles and the greater presence of fibrils, their increased surface area to volume ratio as compared to the gelatin particles might partially explain these small differences. Aditionally, new peaks were found in the chromatogram of the free GTE after thermal treatment (cf. circle in Fig. 5a), and were attributed to degradation products. The absence of these new peaks in the encapsulated samples together with the lower extent of epimerization, suggested that the selected electrosprayed matrices could be suitable for the protection of tea catechins during biscuit manufacturing, and were therefore evaluated in a real biscuit-making process.

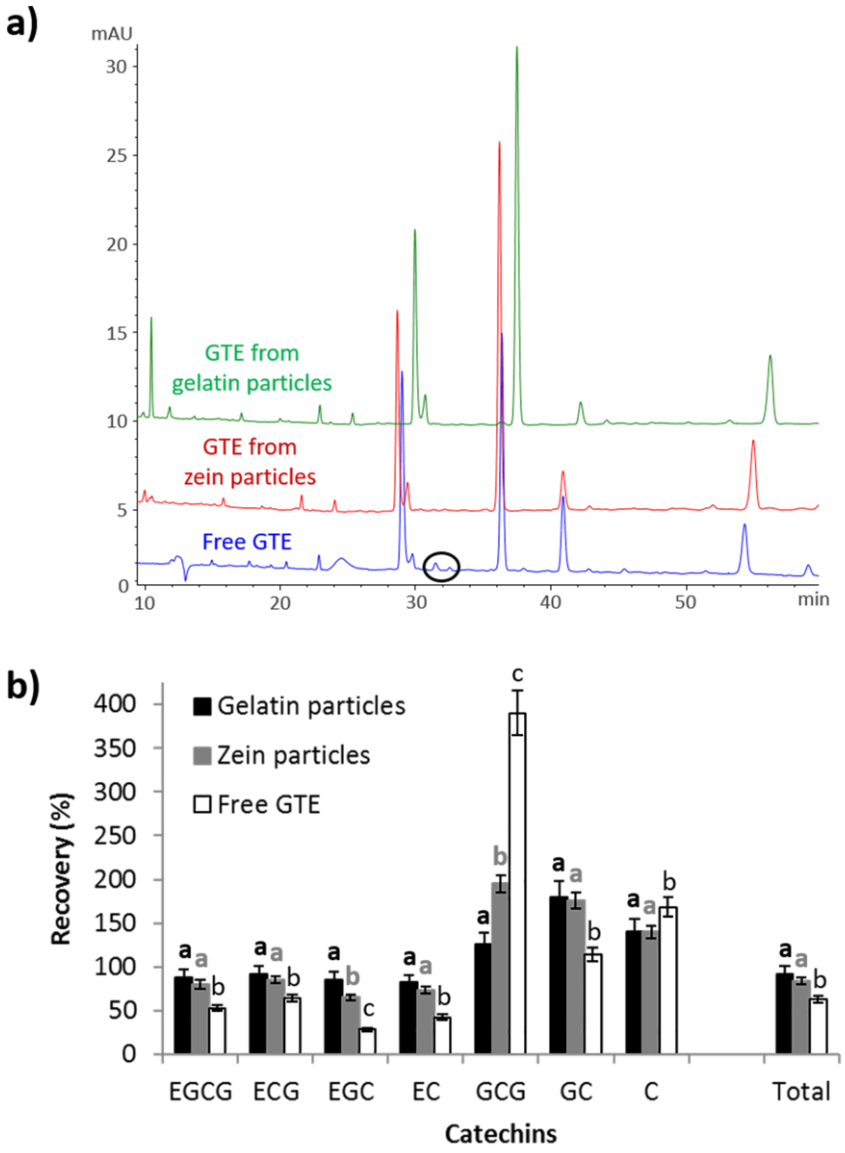


Figure 5. a) Chromatograms of catechins recovery from free and microencapsulated green tea extract (GTE) after thermal treatment. Retention times of relevant peaks (as for sample 'GTE-zein'): (-)-gallocatechin (GC, 14 min), (-)-epigallocatechin (EGC, 20 min), (+)-catechin (C, 22 min), caffeine (27 min), (-)-epicatechin (EC, 28 min), (-)-epigallocatechin gallate (EGCG, 35 min), (-)-gallocatechin gallate (GCG, 40 min), (-)-epicatechin gallate (ECG, 54 min). The circle in the chromatogram corresponding to free GTE highlights the new peaks found in this sample after the thermal treatment. b) Catechins individual and total recoveries after thermal treatment from free and microencapsulated GTE. Different letters (a–c) indicate significant differences among the samples ($p < 0.05$).

4.4. Catechins profile of GTE in biscuits

GTE-fortified biscuits were prepared by mixing the extract, either in its free form ('Free GTE biscuits') or microencapsulated within gelatin ('Gel-GTE biscuits') or zein ('Zein-GTE biscuits'), with the flour for the preparation of the biscuits dough. The recovery of catechins from the prepared biscuits was calculated according to Eq. (2) after extraction with an adequate solvent to dissolve the encapsulation matrices, and subsequent HPLC analysis. Results are shown in Fig. 6.

The total recovery of catechins was greater from the biscuits containing free GTE than for those enriched with microencapsulated GTE. These results were unexpected, given that the microparticles had proven to protect the catechins from thermal degradation in the preliminary tests (Section 4.3) and from degradation in slightly alkaline conditions in previous works [23]. In addition, not only did the total recovery not increase upon microencapsulation of the extract, but it significantly decreased. These observations suggested that the catechins extraction from the encapsulation matrices might have not been complete when the microparticles were embedded within the biscuit matrix. Potential interactions between gelatin and zein and the biscuit components might have precluded complete dissolution of the microparticles and hence the complete release of the catechins. One possibility is that Maillard reactions might happen between the encapsulation matrices (proteins) and the sugars present in the biscuit formulation, potentially crosslinking the proteins [30] and thus hindering the release of GTE [31]. It would then be expected that the remaining complexes/aggregates would have been retained upon filtration of the samples prior to HPLC analysis and, thus, could not be quantified. Protein-lipid interactions might have also occurred in the complex food matrix upon baking, which could have also contributed to decrease the solubility of the proteins due to aggregation or complex formation [32].

In order to confirm this hypothesis, the antioxidant activity of the extracted samples (without filtration) was measured by means of the $ABTS^{\bullet+}$ decolorization assay.

4.5. Antioxidant activity of GTE in biscuits

The antioxidant properties of green tea polyphenols have shown to be medium-dependent [4, 27]. Therefore, the radical scavenging activity (RSA) of the samples in the extraction medium, calculated according to Eq. (3), was compared to that of the free GTE extracted from the biscuits in the same extraction medium. The recovery of GTE from the biscuits in terms of antioxidant activity was calculated according to Eq. (4) and the results are summarized in Table 1. The antioxidant activity of the GTE-loaded microparticles before their incorporation within the biscuits was also measured as a control, and expressed as percentage of recovery in Table 1.

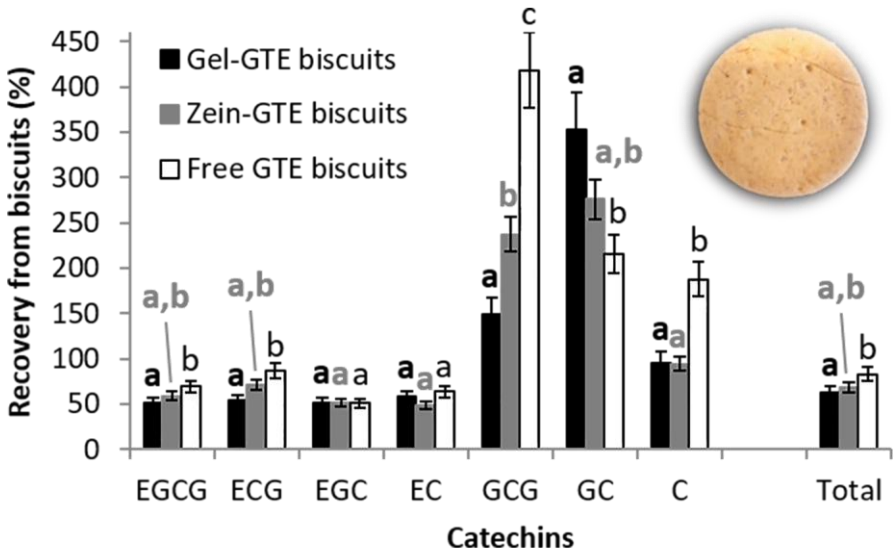


Figure 6. Catechins recovery from biscuits for free and microencapsulated green tea extract. Different letters (a–c) indicate significant differences among the samples ($p < 0.05$). Acronyms: green tea extract (GTE), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-gallocatechin gallate (GCG), (-)-gallocatechin (GC) and (+)-catechin (C).

Table 1. GTE recovery from biscuits in terms of antioxidant activity.

Extraction media	Sample	Recovery $_{RSA}$ (%)
Acetic acid (20 mL/100mL)	Gel-GTE particles	93 ± 4 ^{a}
	Biscuits without GTE	9 ± 1 ^{b}
	Free GTE biscuits	52 ± 3 ^{c}
	Gel-GTE biscuits	58 ± 4 ^{c}
Ethanol (85 mL/100mL)	Zein-GTE particles	95 ± 3 ^{a}
	Biscuits without GTE	8 ± 1 ^{b}
	Free GTE biscuits	65 ± 7 ^{c}
	Zein-GTE biscuits	68 ± 2 ^{c}

The same letter (a-c) within the same medium indicates non-significant differences at $p<0.05$ between samples. GTE: green tea extract, Gel-GTE: gelatin-encapsulated GTE; Zein-GTE: zein-encapsulated GTE.

As expected from the high microencapsulation efficiencies obtained for both systems (cf. Section 4.2.) the antioxidant activity of the GTE-loaded microparticles was very similar to that of the free GTE. When incorporated within the biscuits, the recovery after extraction estimated by means of radical scavenging assays was slightly greater when the GTE was microencapsulated prior to its incorporation within the biscuits, suggesting that the extent of degradation during biscuit preparation was somewhat lower upon encapsulation. These results support the hypothesis of incomplete GTE extraction from the microparticles-containing biscuits previous to HPLC analysis. However, the observed differences were not statistically different, precluding the statement of assertive conclusions. As the degradation products from catechins also exhibit antioxidant activity [33], their RSA could contribute to the overall antioxidant activity of the samples masking the reduction in tea catechins upon degradation.

4.6. Sensory evaluation

The impact of adding free or microencapsulated GTE to the biscuits on their sensorial properties was also evaluated. Gelatin was selected as encapsulation matrix for this analysis, as it yielded less fibrillar structures and, thus, less aggregated particles easier to disperse in the flour. The consumers' evaluations are summarized in Table 2.

Table 2. Consumers' sensorial evaluation of the prepared biscuits.

Biscuit	Texture liking	Flavour liking	Overall liking
Control (without GTE)	7.0 ± 1.5 [a]	7.0 ± 1.3 [a]	6.9 ± 1.3 [a]
Free GTE	6.9 ± 1.3 [a]	6.9 ± 1.3 [a]	6.8 ± 1.2 [a]
Microencapsulated GTE	6.7 ± 1.5 [a]	7.0 ± 1.3 [a]	6.9 ± 1.3 [a]

The same letter (a) within the same column indicates non-significant differences at $p<0.05$ between biscuit samples. GTE: green tea extract.

According to the ANOVA results, no significant differences were found between the liking values of the three types of biscuits evaluated (control, free GTE, and microencapsulated GTE). This indicated that consumers rated all samples alike, so they did not perceive differences neither globally nor regarding their texture or taste affecting their biscuits' liking. Hence, the addition of free and microencapsulated GTE did not vary consumers' preferences. These results differed from those obtained by Bajerska et al. (2010) [9], who reported that GTE had a negative impact on the aroma, flavour and acceptability of bread. However, their study is not directly comparable to the present work since the sensory profiles of bread and biscuits are

very different. The information about sensory properties conferred by green tea extract to biscuits is scarce. Mildner-Szkudlarz and co-workers (2009) evaluated the protective effect of GTE on rancid odor development during storage of the GTE-added biscuits, but not the impact of the addition of GTE itself [13]. Very recently, Ahmad, Baba et al. (2015) reported that the addition of green tea powder to biscuits slightly decreased their overall acceptability [34]. However, in their work they used ten semi-trained panelists, which were more likely to perceive slight differences in the sensory properties than the two hundred fourteen non-trained consumers used in this study, which are more representative of the general consumers' population.

5. Conclusions

An EGCG-rich GTE was microencapsulated within gelatin and zein microparticles by electrospraying, achieving very high encapsulation efficiencies (~90 g/100g). Both protein matrices proved to be very effective in protecting the catechins during a thermal treatment (180 ºC), preserving 85-90 g/100g of their initial content in catechins, while almost 40 g/100g was lost in the free GTE. Therefore, the proposed microencapsulation systems were expected to be effective in protecting the catechins during the manufacturing of bakery products. GTE-fortified biscuits were then prepared by adding the extract, both in its free form and microencapsulated, and the catechins recovery after baking was estimated by HPLC analysis and radical scavenging activity assays. While the first method was not adequate for catechin quantification due to incomplete extraction of the GTE from the biscuit-microparticles systems, the latter showed no significant differences among the recoveries from the free and microencapsulated GTE-containing biscuits, suggesting a poor protective effect of microencapsulation in the model food system. These results emphasize the need of assessing the protective effect of microencapsulation in real food products. On the other hand, the sensorial analysis carried out in this work showed that no significant differences in consumers' liking was found between biscuits fortified with electrosprayed protein microparticles and the control, so the addition had no impact on the acceptability. Future work should be conducted to study the impact of microencapsulation on the bioavailability of the catechins upon consumption of GTE-enriched biscuits.

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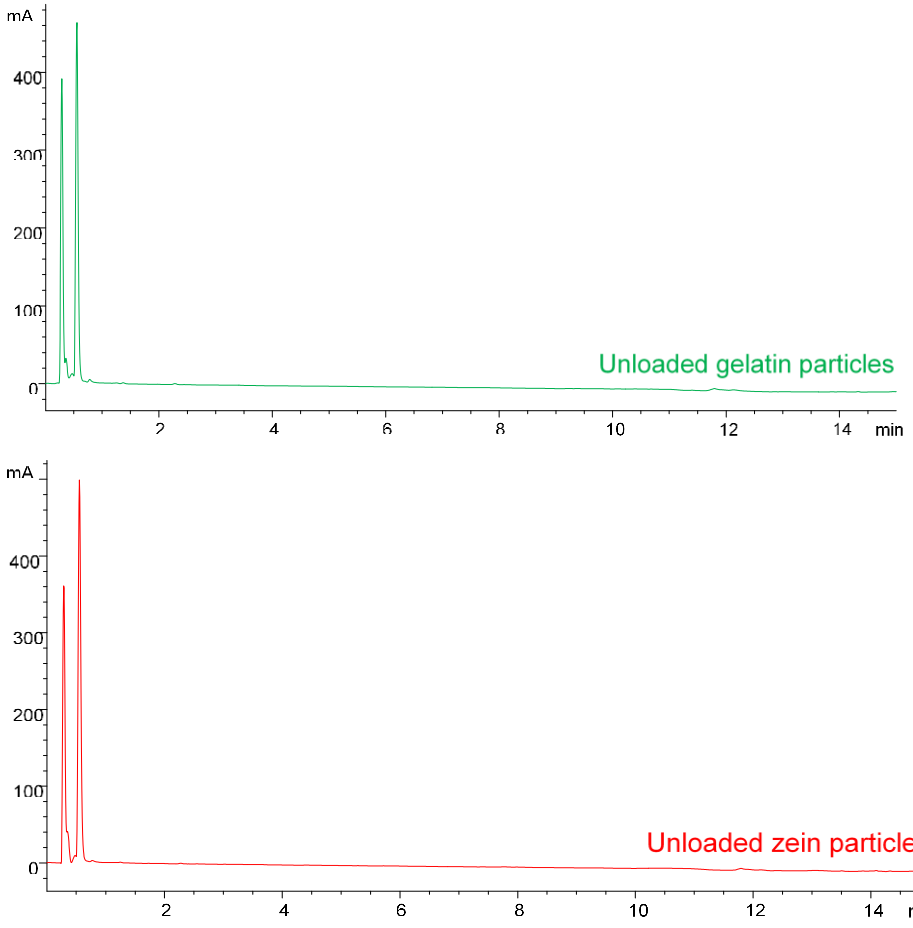
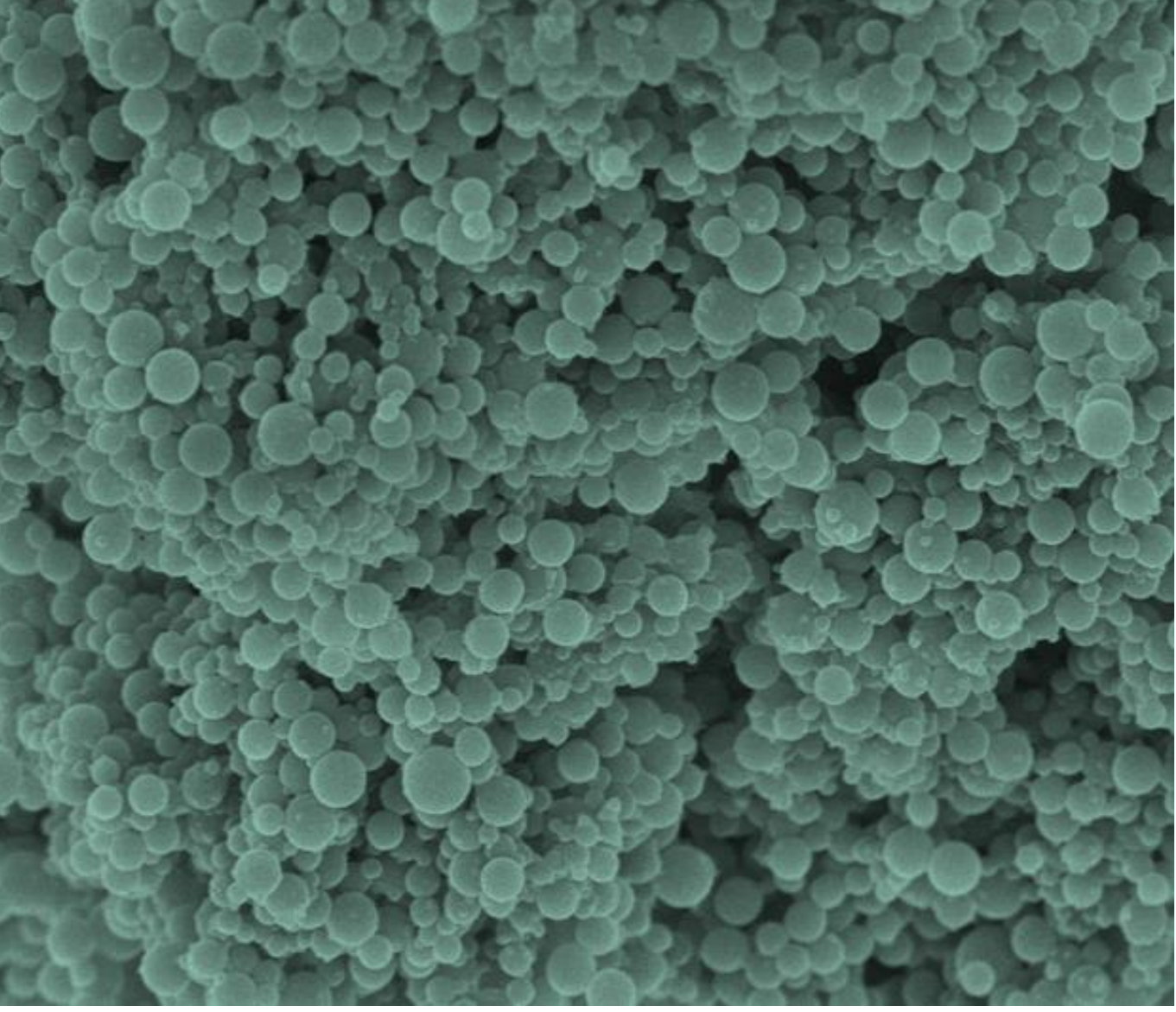
8. Supplementary Material

Figure S1. Chromatograms of unloaded gelatin and zein microparticles after dissolution.





IV. General Discussion

General discussion of the results

1. Microencapsulation of hydrophilic bioactive ingredients

In the first chapter of this thesis, the antioxidant (–)-epigallocatechin gallate (EGCG) was used as a model water-soluble bioactive ingredient. Two different biopolymers, namely gelatin and chitosan, were assessed as encapsulation matrices using two microencapsulation techniques: electrospraying and spray-drying.

Despite the advantages of electrospraying as a microencapsulation technique for food applications, one of its main challenges is the need for process optimization for each particular encapsulation system. Electrospraying from biopolymer aqueous solutions is intricate in many cases, especially when they have a polyionic nature and low chain flexibility. Hence, the first step in the design of electrosprayed gelatin and chitosan encapsulation structures was to optimize the processing conditions in order to obtain particulate encapsulation structures as free of fibrils as possible. This was successfully accomplished by adjusting the solution properties of the feed. The polymer concentration and/or molecular weight and the solvent used were the key factors which determined the morphology of the obtained materials.

Electrosprayed gelatin particles with sizes in the submicron range were successfully produced from aqueous solutions prepared in diluted acetic acid (20% v/v), which avoided premature gelation of the protein during processing. The optimal gelatin concentration of 8% w/v yielded pseudo-spherical and almost fibre-free particles, while higher concentrations led to fibre formation and lower concentrations resulted in some dripping of the solutions. These differences were mainly attributed to the changes in the rheological properties of the gelatin solutions with the protein concentration, as their conductivity and surface tension were hardly affected.

For chitosan, given the great impact of its molecular weight on its properties in solution, this parameter was also adjusted in combination with the polymer concentration. Indeed, there was only a small range of molecular weight-concentration combinations which yielded fibre-free particles. In this case, not only the rheological properties but also the conductivity and the surface tension were altered with the chitosan concentration and molecular weight, and the combination of these three solution properties determined the morphology of the obtained materials. From the different fiber-free electrosprayed samples, the chitosan with the lowest molecular weight was selected as optimum (i.e. 25 kDa at 5% w/v concentration), as it allowed the greatest productivity.

The developed electrosprayed structures were used to microencapsulate EGCG, by simply dissolving the polyphenol (10% w/w of the total solids content) in the optimized polymer solutions and processing them through electrospraying in the same conditions. The addition of EGCG did not significantly affect the solution properties

and, thus, the morphology of the encapsulates was very similar to their unloaded counterparts. Very high microencapsulation efficiencies were obtained in both cases (80-100%), due to the high solubility of the bioactive molecule in the polymer aqueous solutions.

Additionally, the proposed microencapsulation systems proved to be capable of stabilizing EGCG against degradation in slightly alkaline aqueous solution (pH = 7.4). The antioxidant and antiviral activities of EGCG were used to assess the protective effect of the electrosprayed gelatin and chitosan matrices, respectively. In both cases, the *in-vitro* bioactivity of EGCG was better preserved when microencapsulated than in its free form. This could be attributed to both its delayed release in aqueous media when microencapsulated, and to the intermolecular interactions which were established between the active molecule and its encapsulating matrices, as inferred from the infrared spectroscopy analysis. Therefore, the developed electrosprayed gelatin and chitosan particles are considered promising encapsulation vehicles for water-soluble bioactive ingredients with application in the development of novel functional foods.

EGCG-loaded gelatin and chitosan microparticles were also produced using the well-established spray-drying technique, under the same processing conditions, and the performance of both encapsulation matrices was compared. The encapsulation efficiencies obtained through this method were very close to those obtained by electrospraying. Again, the encapsulation efficiency was higher for the protein matrix, confirmed both by infrared spectroscopy and antioxidant activity measurements. The release of the polyphenol in slightly acidic conditions was also more sustained from the gelatin microgels than from the chitosan matrix. These results suggest that the interactions between the bioactive ingredient and its encapsulation matrices were more intense when the protein was used.

The spray-dried capsules and the free EGCG were also subjected to *in-vitro* digestion to ascertain whether microencapsulation had an impact on the bioaccessibility of the polyphenol. The results showed that its bioaccesibility decreased when it was microencapsulated, due to incomplete release from the matrices, and the reduction was greater for the chitosan matrix. Although gelatin also precluded a complete release of EGCG during digestion, it partially prevented its degradation during the intestinal phase and limited the formation of oligomeric derivatives of EGCG, whose bioavailability is lower than that of the monomeric EGCG. Overall, gelatin exhibited a better performance as encapsulation matrix for EGCG than chitosan in terms encapsulation efficiency, release properties and bioaccessibility after *in-vitro* digestion.

2. Microencapsulation of hydrophobic bioactive ingredients

The encapsulation of hydrophobic ingredients using aqueous biopolymer solutions involves an additional challenge due to their immiscibility. Different strategies were used in the second chapter to disperse lipophilic bioactive ingredients within protein

solutions or dispersions prior to their microencapsulation by drying techniques such as electrospraying or spray-drying. Oil-in-water emulsions and liposome dispersions were found to be proper vehicles for this purpose, being their gravitational stability a key factor to achieve adequate encapsulation efficiencies.

Proteins were the encapsulation matrices of choice in this case due to their amphiphilic nature and subsequent superior performance as emulsifiers than polysaccharides. On the other hand, three model lipophilic ingredients with recognized health-promoting properties were used: α-linolenic acid, β-carotene and curcumin.

Firstly, the feasibility of electrospraying protein-stabilized oil-in-water emulsions was assessed using gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) as encapsulation matrices, and two different emulsification procedures: one consisted of a simple high-speed homogenization treatment, and the other included a second ultrasonication step. For these preliminary assays, an inexpensive oil (a soybean oil) was used. The appearance of the emulsions was dramatically different for each protein and emulsion procedure. In general, ultrasonication decreased droplet size and aggregation, thus resulting in increased gravitational stability and improved morphology of the electrosprayed materials subsequently obtained.

The soybean oil was then substituted by the bioactive α-linolenic acid, and microencapsulation structures were produced from the prepared emulsions both by electrospraying and spray-drying. Being α-linolenic acid a thermosensitive compound, the well-established spray-drying technique was shown to be inappropriate for its microencapsulation, as the ω-3 fatty acid was completely degraded during processing. Conversely, the proposed electrospraying technique proved to be a satisfactory alternative, achieving microencapsulation efficiencies of up to 67% ± 5%. However, the low pH required for processing gelatin through electrospraying was found to be detrimental for α-linolenic acid, and this bioactive was also affected by the local heating resulting from the ultrasonication treatment when using the second emulsification procedure. Hence, the best encapsulation efficiencies were achieved using WPC and SPI as encapsulation matrices and the simple homogenization procedure for the preparation of the emulsions. Additionally, the microcapsules obtained in these conditions significantly delayed the oxidation of α-linolenic during accelerated degradation assays at 80ºC, in comparison with the free bioactive compound. Thus, electrospraying of protein-stabilized emulsions proved to be a plausible alternative to traditional microencapsulation technologies used in the food industry such as spray-drying for the stabilization of thermosensitive and hydrophobic bioactive ingredients.

The emulsion-electrospraying technique was also used to microencapsulate β-carotene with the aim of increasing its low bioaccessibility, using WPC and zein as encapsulation matrices. In this case, as the bioactive ingredient was solid at room temperature, a carrier oil (specifically soybean oil) was used to previously dissolve β-carotene. This involved increasing the oil ratio in the emulsions with respect to the previous work, which precluded obtaining stable emulsions using WPC by the simple

high-speed homogenization method. Therefore, although ultrasonication reduced the total β-carotene content in the zein capsules, due to thermal degradation as previously observed for α-linolenic acid, this treatment was necessary for the stabilization of the WPC emulsions. It is worth mentioning that the gravitational stability of the emulsions dramatically impacted the encapsulation efficiency of the technique. Remarkably, all the microencapsulation structures obtained from stable emulsions increased the bioaccessibility of β-carotene after *in-vitro* digestion, which was negligible in its free form.

Liposome dispersions are an interesting alternative to emulsions to be used as vehicles for lipophilic bioactive ingredients. A novel concept of food-grade encapsulation structures based on the entrapment of ingredient-loaded liposomes within a protein matrix through electrospraying was also explored in this thesis. Similarly to the emulsion-electrospraying technique, ultrasonication of the liposome dispersions before electrospraying improved their subsequent processability by reducing their size. This allowed the incorporation of liposomes produced at high lipid concentration (up to 80 g/L) within electrosprayed WPC capsules.

The potential of this hybrid liposome-electrospraying microencapsulation technique to stabilize and increase the bioaccessibility of lipophilic bioactive ingredients was assessed using curcumin as a model compound. The entrapment efficiency of curcumin within the liposomes decreased with increasing curcumin concentrations. Interestingly, the global encapsulation efficiency within the hybrid electrosprayed capsules was higher than within the simple liposome dispersions, as part of the non-entrapped curcumin remaining in suspension could be incorporated within the protein capsules. Interestingly, the developed hybrid microstructures were more effective in delaying the degradation of curcumin in phosphate buffered saline solution (pH = 7.4) than the simple entrapment within liposomes, due to the stabilization effect of the whey protein coating. Moreover, microencapsulation of curcumin within the proposed hybrid structures significantly increased its bioaccessibility after *in-vitro* digestion compared to the free compound.

A semi-continuous manufacturing process was also developed to produce these hybrid liposome/protein microencapsulation structures by combining the microfluidic and electrospraying technologies. Specifically, an assembled micromixer-electrospraying setup was built to mix the protein and liposome dispersions in-line before electrospraying. The next step towards the design of a fully continuous manufacturing process would involve the incorporation of additional microchannels in the microfluidic devices to continuously produce the liposomes by flow focusing before their mixture with the protein.

Overall, the emulsion-electrospraying and liposome-electrospraying encapsulation techniques are two promising strategies for the microencapsulation of sensitive hydrophobic bioactive compounds of interest in the development of functional foods.

3. Microencapsulation of probiotic microorganisms

Compared to the previous bioactive ingredients studied, which were chemical compounds, the encapsulation of probiotic microorganisms involves additional challenges, since their viability must be preserved throughout the encapsulation process.

Although electrospraying is an attractive technique for the microencapsulation of probiotic bacteria given the possibility of working in mild conditions, preliminary experiments showed that certain viability loss occurred when sensitive strains were subjected to electrohydrodynamic processing (probably as a consequence of the abrupt osmotic change caused during the rapid drying of the structures when applying the voltaje). This highlighted the need of thoroughly optimizing the electrospraying parameters when attempting to microencapsulate live microorganisms, in order to minimize this viability loss while maintaining high productivities. This was attempted in chapter 3 of this thesis using *Lactobacillus plantarum* as a model probiotic strain and WPC as the encapsulation matrix.

The results showed that lower viability losses and greater process productivities were obtained when fresh cultures instead of freeze-dried bacteria were used to prepare the feed suspensions for electrospraying. This could be attributed to the poorer bacterial dispersion achieved within the protein suspension when using freeze-dried bacteria, which hindered the proper microencapsulation of the bacteria. Also, the addition of a surfactant (Tween20®) considerably improved the product yield, as it enhanced bacterial dispersion and led to lower surface tensions of the feed suspensions.

On the other hand, an increase in the concentration of surfactant and/or prebiotic (Fibersol®) added to the formulation, as well as an increase in the applied voltage, had a favourable effect on the product yield within the assayed limits. These correlations between the different factors and the product yield could be successfully modelled using a Box-Behnken experimental design. However, the model could not explain the experimental viability losses with statistical significance. Regardless, the viability loss was lower than 1 $\log_{10}$ CFU/g in all cases, and average bacterial counts of ~9 $\log_{10}$ CFU/g in the final electrosprayed materials were obtained, showing the potential of the electrospraying technique for the effective microencapsulation of probiotic bacteria.

Moreover, the electrosprayed microcapsules conferred *L. plantarum* enhanced protection during storage of the food ingredient at high relative humidity conditions, as compared to conventional freeze-drying, which is a more widely-used and much more expensive preservation method. This was attributed to a more effective barrier obtained in the compact electrosprayed capsules than in the highly porous structure of the freeze-dried materials, which allowed extended shelf-lives. However, similar protection against digestion was obtained through both drying techniques, as WPC is water-dispersible and thus the microcapsules were disrupted in the simulated digestion fluids. Future works should address microencapsulation of probiotic bacteria

within hydrogel-forming electrosprayed matrices in order to assess whether this encapsulation technique may provide them with enhanced protection during digestion if the produced microcapsules are not disrupted in simulated digestion fluids.

4. Potential of plant cells as delivery vehicles for functional ingredients

An alternative encapsulation approach based on the use of plant cells as carriers of functional ingredients was also proposed in this thesis. Specifically, the potential of cells isolated from potato tubers to bind and entrap phenolic compounds was demonstrated.

Intact potato cells and their individual cell components (i.e. starch granules and cell walls), which mainly consist of polysaccharides, showed variable absorption capacities towards the assayed phenolic compounds ((+)-catechin, phloridzin and vanillic acid) depending both on the molecular structure of these phenolics and the structure of the polysaccharide-based substrates. In general, the binding affinities followed the order: (+)-catechin > phloridzin > gallic acid. This was attributed to the greater number of hydroxyl groups in the structures of (+)-catechin and phloridzin than in gallic acid, given that hydroxyl groups are potential interaction sites with polysaccharides via hydrogen bonding. However, factors such as the steric hindrance also played an important role, limiting the binding capacity of the most compactly structured cell components (starch granules) towards phloridzin, presumably due to its glycosylated form. Conversely, electrostatic interactions did not significantly affect the binding capacities, which were similar at different pH conditions.

Interestingly, the polyphenols were able to penetrate the intact cells and bind to the intracellular starch granules, which acted as sinks for the bioactive molecules. Moreover, the absorption of the phenolic compounds and, thus, the loading capacity of the proposed vehicles could be considerably increased if the potato cells were previously boiled, due to the gelatinization of starch and subsequent increase in the accessibility of its potential binding sites.

Given the preliminary results obtained in chapter 4 of this thesis, intact potato cells are very promising as selective delivery vehicles. Since cell walls are not digestible, the contents of the intact cells are expected to be delivered to the large intestine where the fermentation by bacteria would allow their release, provided the cells remain intact upon passage through the gastrointestinal tract. Future works should address these aspects in order to assess the release behavior of the bioactive compounds during digestion.

5. Application of microencapsulation in real food systems

Despite the promising results discussed above, which highlight the potential of microencapsulation to improve the stability and/or bioaccessibility of diverse functional

ingredients, these aspects must be tested when incorporated within complex food matrices in order to demonstrate their benefits for the development of real food products. This was attempted in the last chapter of this thesis, in which the impact of microencapsulation in the enrichment of yogurts and biscuits with a peptide hydrolysate and a green tea extract, respectively, was studied.

For practical and economic reasons, enriched functional foods are more likely to be supplemented with a peptide hydrolysate instead of a highly purified bioactive peptide, or with a catechin-rich green tea extract rather than with an individual, purified catechin. This aspect adds up to the intrinsic complexity of real food matrices and their possible interactions with the functional ingredients and/or their microencapsulation matrices, complicating the analysis.

One of the consequences of the complexity of these systems was the difficulty for the accurate detection and/or quantification of the compounds of interest. However, the obtained results allowed the extraction of, at least, qualitative conclusions.

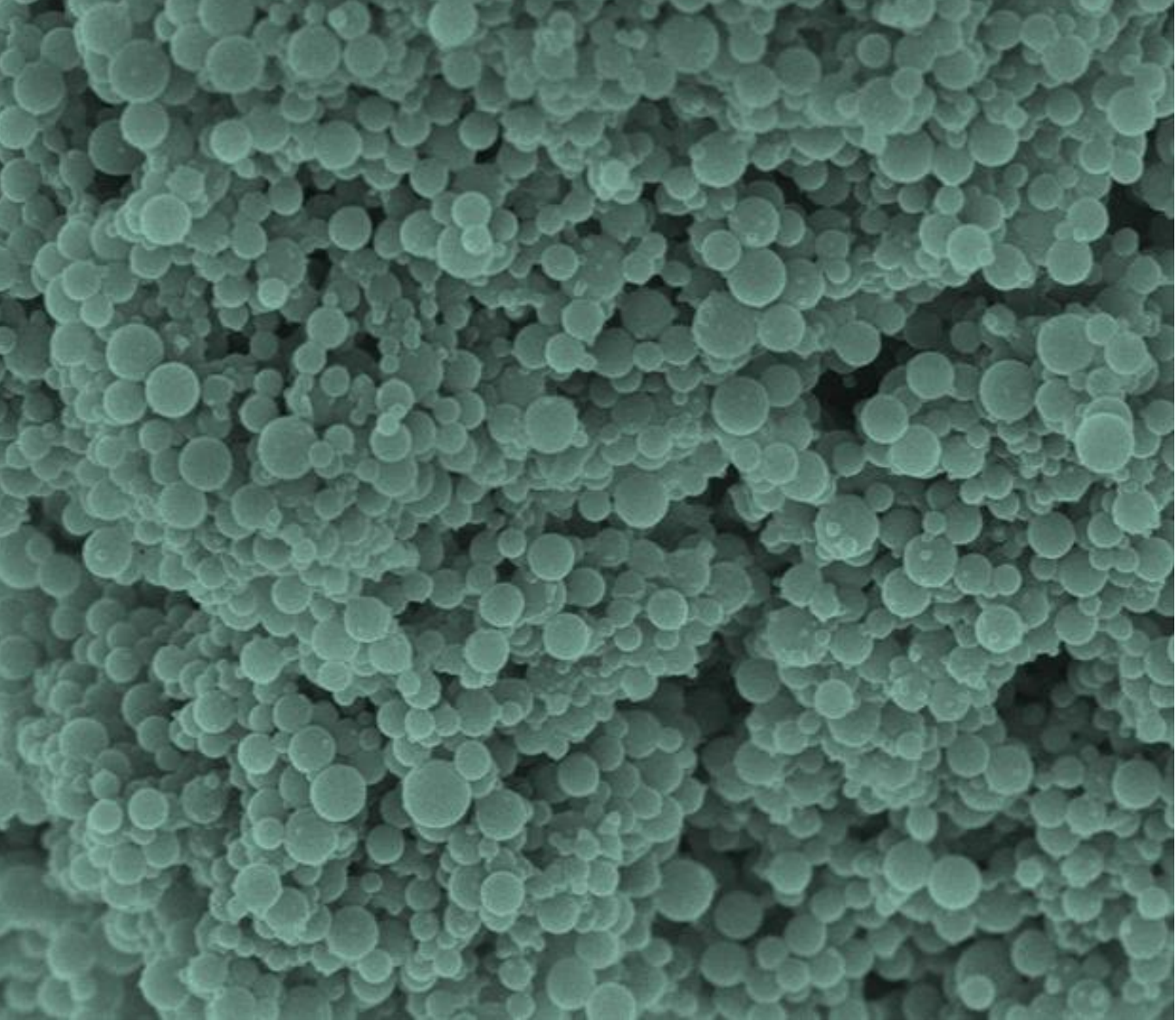
In the case of the peptide-enriched yogurts, the protection exerted by the microencapsulation matrices during milk fermentation was sequence- and matrix-dependent. From the total 58 peptides detected in the hydrolysate, 21 were completely susceptible to fermentation and could not be detected in the peptide-enriched yogurts when the hydrolysate was incorporated in its free form. In contrast, 5 of these 21 peptides could be stabilized through encapsulation within the spray-dried chitosan microparticles, while only one of them was protected using gelatin. The overall results of the study emphasized the need of evaluating the protective effect of microencapsulation within a particular matrix for a particular peptide of interest, even if it is incorporated as part of a complex mixture of peptides, since each of them may establish different interactions with the microencapsulation matrix and/or other food components. For the particular whey protein hydrolysate used, chitosan showed better protective ability than gelatin. Moreover, microencapsulation within spray-dried chitosan matrix did not substantially alter the peptide profile after *in-vitro* digestion of the hydrolysate and, thus, peptide bioaccessibility was not expected to be compromised by encapsulation.

Regarding the green tea extract-enriched biscuits, although both electrosprayed gelatin and zein microparticles greatly improved the thermal stability of the tea catechins during an *in-vitro* thermal treatment (180 ºC) and had previously proven to stabilize catechins in alkaline conditions, these specific encapsulation systems did not demonstrate a statistically significant protective capacity during the production of biscuits. Again, these results stress the need of assessing the protective effect of microencapsulation in real food products.

Nevertheless, the sensorial analysis carried out for the green tea extract-enriched biscuits suggested that the addition of electrosprayed microcapsules did not have an impact on the consumers' acceptability of the final product.

Overall, microencapsulation has a great potential to help overcoming some of the challenges encountered in the development of novel functional foods. However, it is of

outmost importance to select the most adequate encapsulation matrices and techniques for each specific bioactive ingredient, and to assess whether the desired effect is also achieved when incorporated within the food matrix.



V. CONCLUSIONS

Conclusions

In this thesis, novel encapsulation structures of interest in the development of functional foods were developed, using different microencapsulation techniques and encapsulation matrices for the protection of various bioactive substances. The main conclusions derived from this work are summarized below:

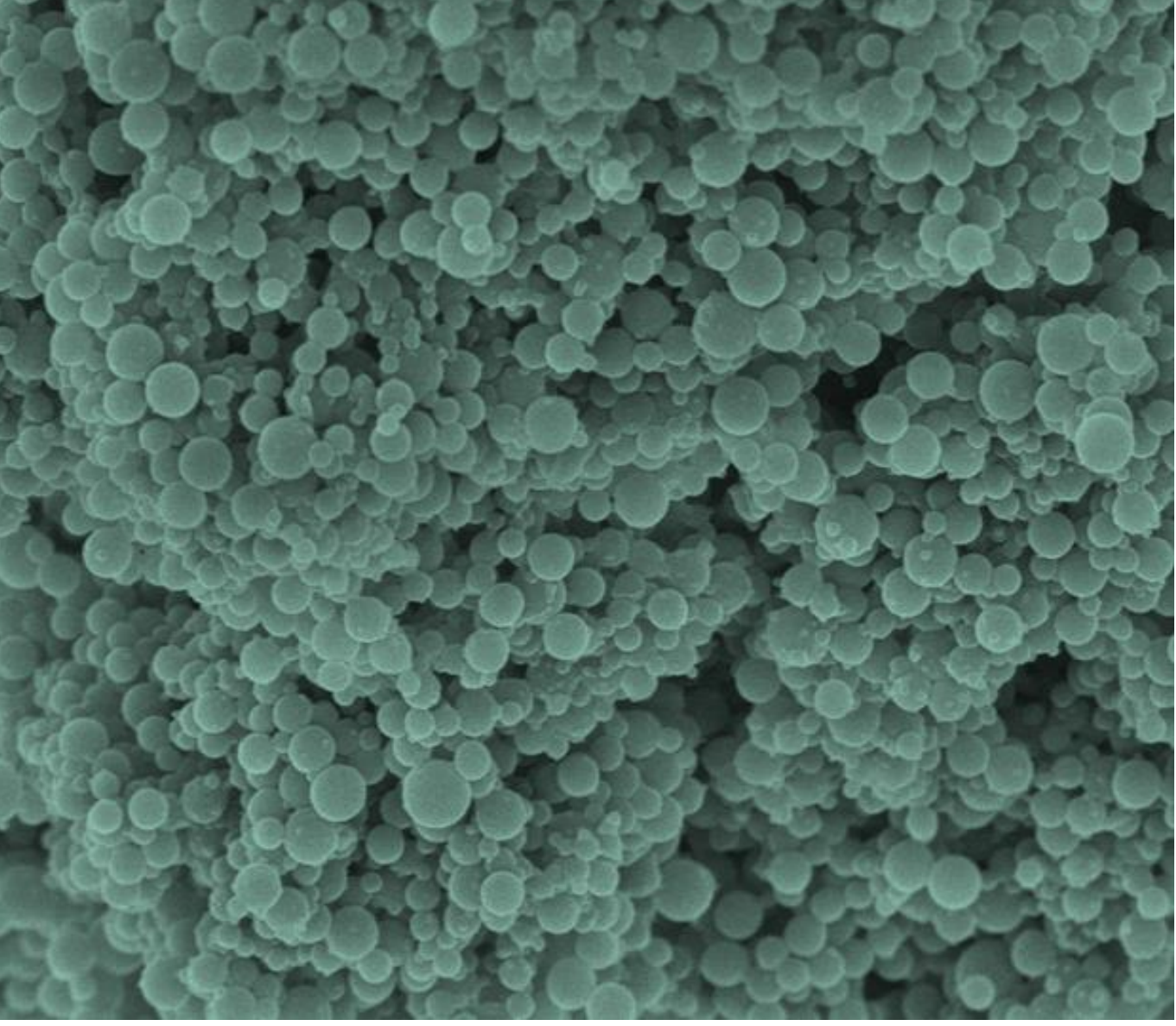
- Novel **electrosprayed microencapsulation structures** with particle sizes in the micron and sub-micron range were successfully produced from aqueous solutions and in mild conditions using food-grade proteins and polysaccharides as encapsulation matrices.
- In general, increasing the viscosity of the biopolymer solutions yielded more fibrillar structures, although the combined effect of their rheological properties, conductivity and surface tension had to be taken into account to accurately predict the morphology of the electrosprayed materials.
- Considerably high microencapsulation efficiencies were achieved through electrospraying. These were higher for water-soluble bioactive ingredients (80-100%), although reasonably high efficiencies were also achieved for hydrophobic compounds (up to ~70%).
- While hydrophilic ingredients could be directly dissolved in the feed biopolymer solutions prior to electrospraying, different strategies were optimized in order to microencapsulate other types of ingredients:
    - Emulsification or entrapment within liposomes before electrospraying were successful strategies for the microencapsulation of hydrophobic ingredients. The gravitational stability of these dispersions was a key factor determining the encapsulation efficiency, although treatments aimed at improving it, such as ultrasonication, partially degraded thermosensitive molecules. Hence, the convenience of applying these treatments should be considered for each particular system in order to maximize the encapsulation efficiency.
    - Regarding the microencapsulation of probiotic bacteria, preparing the feed suspensions from fresh culture was more effective than using freeze-dried bacteria in terms of obtaining lower viability losses and greater process productivities. The addition of a surfactant considerably improved the product yield too.
- The developed electrosprayed capsules were capable of stabilizing different bioactive ingredients in simulated stress conditions. For instance, (–)-epigallocatechin gallate (EGCG) and curcumin were stabilized against degradation in slightly alkaline solutions, the thermal oxidative stability of α-linolenic acid was

improved and the viability of *Lactobacillus plantarum* was significantly enhanced during storage at high relative humidity conditions.

- A continuous manufacturing process for the novel liposome/protein hybrid microencapsulation structures has also been proposed in this work by combining the microfluidic and electrospraying technologies. Part of the design has already been implemented.
- The **conventional spray-drying technique** was also used in this work for comparison purposes. Although similar encapsulation efficiencies to those obtained by electrospraying were achieved for EGCG, α-linolenic acid completely degraded when subjected to the spray-drying process, due to its great sensitivity to thermal oxidation. Therefore, the suitability of using a particular encapsulation technique should be considered for each individual ingredient. Electrospraying is a promising alternative to spray-drying, especially for thermosensitive compounds.
- The **selection of the most adequate encapsulation matrix** should also be studied for each particular ingredient of interest. Although better overall results were obtained for EGCG when gelatin instead of chitosan was used, chitosan provided enhanced stabilization to certain peptides during fermentation as compared to gelatin.
- **Intact plant cells** and, specifically, cells from potato tubers, are potential delivery vehicles for functional ingredients such as polyphenols, since these compounds are capable of penetrating their cell walls and bind to the intracellular starch granules. Their loading capacity can be considerably increased if the potato cells are previously cooked, as starch gelatinization is promoted, thus increasing the number of available binding sites.
- Regarding the impact of microencapsulation on the **bioaccessibility** of the functional ingredients, while it may decrease for hydrophilic compounds such as EGCG due to incomplete release from the matrix, it was enhanced for hydrophobic compounds such as curcumin or β-carotene, due to their improved dispersibility in aqueous fluids and, thus, their better incorporation into the mixed micelles fraction during digestion. It is also worth mentioning that microencapsulation was seen to protect EGCG from dimer formation during *in-vitro* digestion, which could also have a positive impact on the bioavailability of the compound.
- Although the benefits of microencapsulation may be preliminarily screened *in-vitro*, it is then essential to assess them in **real food systems**, since unexpected interactions with the components of the food matrix may deviate the results from those obtained in food simulants.
    - Preliminary results in yoghurts suggested that encapsulation within spray-dried microcapsules, particularly when chitosan was used as encapsulation matrix, could protect certain peptides from degradation by lactic acid bacteria during milk fermentation.

- The protective effect of electrosprayed protein microcapsules on tea catechins, which had been demonstrated *in-vitro*, could not be proved in a real biscuit manufacturing process.
- The addition of electrosprayed microcapsules to biscuits did not have an impact on the consumers' acceptability of the final product, aspect which is also of outmost importance for the success of new functional food products in the market.

Overall, **microencapsulation has a great potential to help overcoming some of the challenges encountered in the development of novel functional foods**. However, efforts must still be made to develop improved delivery systems, which must be optimized for each specific bioactive ingredient and assessed in real food systems.



VI. ANNEXES

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Electrosprayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods

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ABSTRACT

In this work, the potential of the electrospraying technique to obtain food-grade gelatin capsules in the submicron range for sensitive bioactive protection was explored, studying the influence of the protein concentration on the size and morphology of the obtained particles. Gelatin was selected as encapsulating material because, being commonly used as a food ingredient, it possesses unique gelation properties and is commercially available at a low cost. The electrosprayed matrices were used to encapsulate a model antioxidant molecule, (−)-epigallocatechin gallate (EGCG). Very high encapsulation efficiencies, close to 100%, were achieved, and the antioxidant activity of the bioactive was fully retained upon encapsulation. The EGCG release profiles showed a delayed release of the encapsulated antioxidant in aqueous solutions. Furthermore, while free EGCG in PBS lost a 30% of their antioxidant activity being completely degraded in 100 h, encapsulated EGCG retained its whole antioxidant activity within this time period.

1. Introduction

One of the main challenges in the development of functional foods is the preservation of the activity and bioavailability of the bioactive ingredients during food processing, storage and passage through the gastrointestinal tract. The development of edible nano- or microencapsulation matrices has been envisaged as a plausible option to protect these biologically active compounds against adverse conditions (Dube, Ng, Nicolazzo, & Larson, 2010). There are a number of encapsulation techniques which can be used to produce nano- or microparticulate systems, being emulsification-evaporation, spray-drying and coacervation the most extensively used (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012). However, some of these production methods involve exposure of the bioactives to high temperatures and/or the use of organic solvents, factors which can affect the stability of sensitive nutrients and preclude their use for food applications due to toxicity concerns associated with the residual traces of solvents (López-Rubio & Lagaron, 2011).

Electrospraying (e-spraying) has recently emerged as an alternative for the generation of polymeric particles incorporating bioactive agents (Bock, Dargaville, & Woodruff, 2012) with application in therapeutics, cosmetics and the food industry (Jaworek & Sobczyk, 2008). E-spraying, together with electrospinning (e-spinning), are versatile electrohydrodynamic fabrication methods which can generate encapsulation structures in a one-step process (Chakraborty, Liao, Adler, & Leong, 2009) without the need of employing high temperatures or toxic solvents (López-Rubio & Lagaron, 2012). A polymer solution flowing out from a nozzle is subjected to an external electrical field in such a way that when the electrical forces overcome the forces of surface tension, a charged jet is ejected towards a grounded collector. During the flight, the jet is elongated and the solvent evaporates, producing dry continuous fibres in the case of e-spinning (Bhardwaj & Kundu, 2010). In e-spraying, the jet breaks down into fine droplets which acquire spherical shapes due to the surface tension (Chakraborty et al., 2009), subsequently producing solid nano- or microparticles upon solvent evaporation. Apart from the feasibility of working at mild ambient conditions and using food-grade solvents, e-spraying

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Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds

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ABSTRACT

The molecular weight of chitosan is one of its most determinant characteristics, which affects its processability and its performance as a biomaterial. However, information about the effect of this parameter on the formation of electrosprayed chitosan microcapsules is scarce. In this work, the impact of chitosan molecular weight on its electrosprayability was studied and correlated with its effect on the viscosity, surface tension and electrical conductivity of solutions. A Discriminant Function Analysis revealed that the morphology of the electrosprayed chitosan materials could be correctly predicted using these three parameters for almost 85% of the samples. The suitability of using electrosprayed chitosan capsules as carriers for bioactive agents was also assessed by loading them with a model active compound, (−)-epigallocatechin gallate (EGCG). This encapsulation, with an estimated efficiency of around 80% in terms of preserved antioxidant activity, showed the potential to prolong the antiviral activity of EGCG against murine norovirus via gradual bioactive release combined with its protection against degradation in simulated physiological conditions.

1. Introduction

Micro- and nanoencapsulation, processes in which a compound is embedded within a protective matrix (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2014) which is organized in the form of micro- or nanosized structures, have attracted increasing research interest for the protection of sensitive bioactive compounds (Pérez-Masiá, López-Nicolás et al., 2015) and address current concerns related to their formulation, bioavailability or their delivery to specific sites (Zaki, 2014).

Among the different techniques used for microencapsulation, electrohydrodynamic spraying (electrospraying) is rapidly emerging as a promising technology for the production of polymeric microparticles containing bioactive molecules (Bock, Dargaville, & Woodruff, 2012), as it overcomes some of the limitations of conventional methods. Electrospraying can generate microencapsulation structures in a one-step process (Chakraborty, Liao, Adler, & Leong, 2009) under mild conditions (López-Rubio & Lagaron, 2012; Sosnik, 2014) and in the absence of organic/toxic solvents (Tapia-Hernández et al., 2015), limiting inactivation of the bioactive compounds (Zamani, Prabhakaran, & Ramakrishna, 2013), being adequate for both hydrophilic and hydrophobic drugs or ingredients (Gómez-Mascaraque & López-Rubio, 2016) and generally achieving high loading efficiencies (Sosnik, 2014; Zamani et al., 2013). Therefore, it has found a number of potential applications in various fields, including the pharmaceutical, cosmetic and food industries (Jaworek & Sobczyk, 2008). It basically consists on subjecting a polymer solution (containing the bioactive to be encapsulated) to a high voltage so that the electric field deforms the interface of the liquid drop and breaks it into fine charged droplets, which are ejected towards a collector while the solvent evaporates, generating dry polymeric microparticles (Anu Bhushani & Anandharamakrishnan, 2014; Sosnik, 2014).

Biopolymers are preferred as encapsulating matrices for most applications because of their biocompatibility, biodegradability and non-toxicity (Ghorani & Tucker, 2015). Specially, chitosan is a biorenewable, biocompatible and biodegradable polysac-

Abbreviations: ABTS2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DDA, degree of deacetylation; DFA, Discriminant Function Analysis; EGCG, (−)-epigallocatechin gallate; FT-IR, Fourier transform infrared spectroscopy; GRAS, Generally Recognised As Safe; MEE, microencapsulation efficiency; MNV, murine norovirus; Mw, molecular weight(s); PBS, phosphate buffer saline; RSA, radical scavenging activity; SEM, scanning electron microscopy.

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Stability and bioaccessibility of EGCG within edible micro-hydrogels. Chitosan vs. gelatin, a comparative study

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ABSTRACT

Micro-hydrogels are very promising systems for the protection and controlled delivery of sensitive bioactives, but limited knowledge exists regarding the impact of this encapsulation on their bioaccessibility. In this work, two different hydrogel-forming biopolymers (gelatin and chitosan) were compared as wall materials for the microencapsulation of a model flavonoid, (−)-epigallocatechin gallate (EGCG). Results showed that gelatin was more adequate as wall material for the encapsulation of EGCG than chitosan, achieving higher encapsulation efficiencies (95% ± 6%), being more effective in delaying EGCG release and degradation in aqueous solution and exhibiting a 7 times higher bioaccessibility of the bioactive compound (in terms of antioxidant activity) after *in-vitro* gastrointestinal digestion. A very low bioaccessibility of EGCG in chitosan was observed, due to the neutralization of the carbohydrate in the basic simulating salivary conditions, thus precluding subsequent flavonoid release. Moreover, gelatin micro-hydrogels also hindered dimer formation during *in-vitro* digestion, thus suggesting greater bioavailability when compared with free EGCG.

1. Introduction

The development of functional biopolymer nanoparticles or microparticles as encapsulation and delivery systems has enjoyed a great deal of interest in diverse academic fields such as foods, pharmaceutics or cosmetics, highlighting the potential of these structures to protect sensitive bioactives against degradation (Jones & McClements, 2011). Amongst the wide range of bioactive substances studied, green tea flavonoids are powerful antioxidants which have drawn much research attention because of their many attributed therapeutic benefits (Fu et al., 2011; Larsen & Dashwood, 2009, 2010; Singh, Akhtar, & Haqqi, 2010; Singh, Shankar, & Srivastava, 2011; Steinmann, Buer, Pietschmann, & Steinmann, 2013), being (−)-epigallocatechin gallate (EGCG) the most abundant polyphenol in green tea possessing the greatest biological activity (Barras et al., 2009). However, EGCG is sensitive to heat (Wang, Zhou, & Wen, 2006), oxygen (Valcic, Burr, Timmermann, & Liebler, 2000) and light (Scalia, Marchetti, & Bianchi, 2013) and, in general, chemically unstable (Dube, Ng, Nicolazzo, & Larson, 2010a), especially in aqueous solutions (Dube, Nicolazzo, & Larson, 2010b; Li, Lim, & Kakuda, 2009). Thus, encapsulation of this bioactive compound has been widely explored to improve its stability during food processing and storage (Dube et al., 2010b; Fang & Bhandari, 2010; Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Li et al., 2009; Shutava, Balkundi, & Lvov, 2009a; Shutava et al., 2009b). Several techniques have been explored for this purpose, being costs and the use of food permitted solvents and matrices the limiting factors for the practical use of encapsulation structures in functional food applications. In this sense, spray-drying is the most widely used encapsulation technique in the food industry (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2014), as it is a straightforward and cheap procedure which allows the processing of a wide range of food-grade materials with accessible equipment (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). This technique involves the initial atomization of a formulation containing the wall matrix and the bioactive into fine droplets, followed by their rapid drying using a hot gas stream, which leads to solvent evaporation and rapid formation of the microparticles. Regarding the encapsulation matrices, food-grade biopolymers capable of forming physical hydrogels are of particular interest. Physical hydrogels are polymer networks characterized by the presence of physical crosslinks, entanglements and/or rearrangements of hydrophobic and

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Protein-based emulsion electrosprayed micro- and submicroparticles for the encapsulation and stabilization of thermosensitive hydrophobic bioactives

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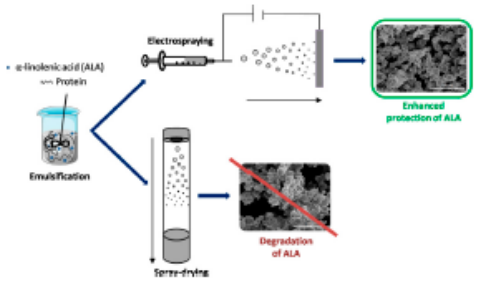
GRAPHICAL ABSTRACT



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ABSTRACT

This work shows the potential of emulsion electrospraying of proteins using food-grade emulsions for the microencapsulation and enhanced protection of a model thermosensitive hydrophobic bioactive. Specifically, gelatin, a whey protein concentrate (WPC) and a soy protein isolate (SPI) were compared as emulsion stabilizers and wall matrices for encapsulation of α-linolenic acid. In a preliminary stage, soy bean oil was used as the hydrophobic component for the implementation of the emulsion electrospraying process, investigating the effect of protein type and emulsion protocol used (i.e. with or without ultrasound treatment) on colloidal stability. This oil was then substituted by the ω-3 fatty acid and the emulsions were processed by electrospraying and spray-drying, comparing both techniques. While the latter resulted in massive bioactive degradation, electrospraying proved to be a suitable alternative, achieving microencapsulation efficiencies (MEE) of up to ~70%. Although gelatin yielded low MEEs due to the need of employing acetic acid for its processing by electrospraying, SPI and WPC achieved MEEs over 60% for the non-sonicated emulsions. Moreover, the degradation of α-linolenic acid at 80 °C was significantly delayed when encapsulated within both matrices. Whilst less than an 8% of its alkene groups were detected after 27 h of thermal treatment for free α-linolenic acid, up to 43% and 67% still remained intact within the electrosprayed SPI and WPC capsules, respectively.

Abbreviations: WPC, whey protein concentrate; SPI, soy protein isolate; MEE, microencapsulation efficiency; ALA, α-linolenic acid; O/W, oil in water; GRAS, generally recognized as safe; Gel, gelatin; SBO, soy bean oil; DLS, dynamic light scattering; CI, creaming index; SEM, scanning electron microscopy; FT-IR, Fourier transform infrared; TGA, thermogravimetric analysis; DTG, derivative thermogravimetric curves; HSH, high-speed homogenization; US, ultrasound.

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Potential of microencapsulation through emulsion-electrospraying to improve the bioaccesibility of β-carotene

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ABSTRACT

The development of carotenoid-enriched functional foods is limited by the low bioaccessibility of these bioactive compounds. The aim of this work was to improve the bioaccessibility of β-carotene after *in-vitro* digestion through its encapsulation within electrosprayed protein microparticles. Two different protein matrices (zein and a whey protein concentrate, WPC) and two emulsification procedures (high-speed homogenization and ultrasonication) were used to prepare the microcapsules through emulsion-electrospraying, using a soy bean oil as lipid carrier, and the impact of the emulsion properties on the microencapsulation efficiency (MEE) and the bioaccessibility of β-carotene was studied. Results showed that the stability of the prepared emulsions was the main factor affecting the microencapsulation efficiency. The application of an ultrasonic treatment was necessary to stabilize the WPC emulsions and increase the MEE of the WPC microcapsules, but had a slight negative impact on the total β-carotene content of the zein particles, due to thermal degradation of β-carotene, without significantly affecting their MEE. The highest MEE was achieved for the capsules obtained from zein emulsions (34 ± 7%). All the encapsulation structures, except those obtained from WPC emulsions prepared by high-speed homogenization, increased the bioaccessibility of β-carotene after *in-vitro* digestion, which was negligible in its free form.

1. Introduction

Carotenoids are a group of natural pigments with many attributed health benefits when consumed in sufficient levels (Maiani et al., 2009; Qian, Decker, Xiao, & McClements, 2012). Especially, β-carotene has been described to exert protection against a number of severe health disorders, including cancer, cardiovascular diseases or macular degeneration (Albanes, 1999; Rock, 1997). Hence, there is increasing interest in the incorporation of β-carotene as a functional ingredient in food formulations. However, the poor solubility of this compound in aqueous media complicates its application in the food industry and causes its bioavailability to be extremely low (Deng, Chen, Huang, Fu, & Tang, 2014).

In order for β-carotene to be uptaken by the enterocytes in the small intestine, it has to be previously incorporated into mixed micelles during digestion (Kaulmann, André, Schneider, Hoffmann, & Bohn, 2016). Its simultaneous consumption with digestible lipids has shown to enhance its bioavailability (Borel, 2003; Thakkar, Maziya-Dixon, Dixon, & Failla, 2007; van het Hof, West, Weststrate, & Hautvast, 2000), since they can solubilize β-carotene and transport it to the mixed micelles via the free fatty acids which are released upon lipid digestion, thanks to the biliary salts and pancreatic lipase (Tyssandier, Lyan, & Borel, 2001). Hence, emulsion-based systems have been proposed as vehicles for the incorporation of β-carotene into aqueous food products in order to increase its bioaccessibility (Qian et al., 2012). However, the microbiological stability of oil-in-water emulsions is generally poor, especially when proteins are used as emulsifiers, since they favour the intensive growth of microorganisms (Glibowski, Kordowska-Wiater, & Glibowska, 2011). In order to improve their storage stability, these emulsions can then be dried to obtain microencapsulation structures (Deng et al., 2014) obtaining easy-to-handle powdery ingredients.

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Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin

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ABSTRACT

Novel food-grade hybrid encapsulation structures based on the entrapment of phosphatidylcholine liposomes, within a WPC matrix through electrospraying, were developed and used as delivery vehicles for curcumin. The loading capacity and encapsulation efficiency of the proposed system was studied, and the suitability of the approach to stabilize curcumin and increase its bioaccessibility was assessed. Results showed that the maximum loading capacity of the liposomes was around 1.5% of curcumin, although the loading capacity of the hybrid microencapsulation structures increased with the curcumin content by incorporation of curcumin microcrystals upon electrospraying. Microencapsulation of curcumin within the proposed hybrid structures significantly increased its bioaccessibility (~1.7-fold) compared to the free compound, and could successfully stabilize it against degradation in PBS (pH = 7.4). The proposed approach thus proved to be a promising alternative to produce powder-like functional ingredients.

1. Introduction

Curcumin, whose chemical structure is depicted in Fig. S1 of the Supplementary Material, is a multivalent compound with attributed antioxidant, antiinflammatory and antimutagenic activities (Chen et al., 2009) and, thus, an attractive bioactive ingredient for the development of functional foods. However, its poor solubility in water and its great chemical instability (Schneider, Gordon, Edwards, & Luis, 2015) result in very low bioavailability rates upon oral consumption (Liu et al., 2016). These drawbacks limit the direct application of curcumin not only in the food industry but also in the medical field (Chin, Huebbe, Pallauf, & Rimbach, 2013; Nelson et al., 2017). Hence, a number of strategies have been proposed to design appropriate delivery vehicles for this compound (Feng et al., 2016; Ndong Ntoutoume et al., 2016).

Liposome dispersions can facilitate the incorporation of lipophilic molecules into food products with a positive impact on their stability and bioavailability (Frenzel, Krolak, Wagner, & Steffen-Heins, 2015). Specifically, degradation of curcumin in alkaline conditions can be considerably reduced within liposome environments (El Khoury & Patra, 2013). However, these vehicles have been described to lose entrapped material during storage and to become instable due to osmotic pressure in contact with certain food components, such as sugars or salts (Karadag et al., 2013; Laye, McClements, & Weiss, 2008). Coating of liposomes with biopolymers has been proposed in a number of works as a plausible strategy to increase their stability and shelf-life (Monika Frenzel & Steffen-Heins, 2015; Gültekin-Özgüven, Karadağ, Duman, Özkal, & Özçelik, 2016; Tan, Feng, Zhang, Xia, & Xia, 2016).

On the other hand, the commercialization of powdery food ingredients is substantially more convenient than handling liquid ingredients, such as liposome dispersions, as dried powders are easier to handle and to preserve from contamination during storage and, moreover, they occupy reduced storage volumes (Garti & McClements, 2012). Therefore, biopolymer-coated liposome dry

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A step forward towards the design of a continuous process to produce hybrid liposome/protein microcapsules

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ABSTRACT

Microfluidics and electrospraying, two revolutionary technologies with industrial potential for the microencapsulation of lipophilic bioactive ingredients, have been combined to produce hybrid liposome/protein microencapsulation structures in a semi-continuous process, reducing the number of steps required for their manufacture. Three different microfluidic mixing devices, one of them consisting of a simple straight microchannel (cross junction design) and the other two exhibiting patterned microchannels with different geometries (Tesla and 'splitting and recombination' designs), were used to mix a liposome suspension with a whey protein concentrate dispersion. The Tesla design showed the best mixing performance, as observed by fluorescence microscopy, so it was selected to be assembled to an electrospraying apparatus. The proposed in-line setup was successfully used to produce the micron-sized encapsulation structures, as observed by scanning electron microscopy.

1. Introduction

Liposomes can be used as delivery vehicles for the incorporation of lipophilic bioactive compounds into food products, increasing their stability and bioavailability (Frenzel et al., 2015). However, the high semi-permeability of their membranes and low physical stability (Frenzel and Steffen-Heins, 2015) limit their practical application. Microencapsulation of the liposomes within dry biopolymeric matrices, obtaining convenient powdery ingredients, has been proposed as a strategy to overcome these limitations (Gültekin-Özgüven et al., 2016; Tan et al., 2016; Van Den Hoven et al., 2012; Wang et al., 2015).

Specifically, the electrospraying technique, a drying technology based on the electrohydrodynamic processing or atomization of polymeric fluids, has been very recently used to produce dry liposome/protein microencapsulation structures, which proved to successfully stabilize and improve the bioaccessibility of curcumin (Gómez-Mascaraque et al., 2017). The main advantage of electrospraying over other more commonly used drying techniques such as spray-drying is its operation under mild temperature conditions avoiding the thermal degradation of thermosensitive bioactive ingredients (Gómez-Mascaraque and López-Rubio, 2016).

On the other hand, conventional technologies used for the manufacturing of liposomes usually require numerous pre- and post-processing steps (Tien Sing Young and Tabrizian, 2015). Microfluidics, a technology which allows manipulation of tiny volumes of fluids inside micron-sized channels, has been proposed as an alternative for the continuous production of liposomes by flow focusing (Balbino et al., 2013a).

The combination of microfluidics and electrospraying would allow the design of an industrially attractive continuous process for the production of liposome/protein microencapsulation structures similar to those previously developed in batch (Gómez-Mascaraque et al., 2017). For this purpose, an intermediate mixing step would be necessary in order to blend the liposomes with the polymer prior to electrospraying the mixture. Due to the low flow rates used in both techniques, this mixing step should be also accomplished by means of microfluidics.

Several designs for microfluidic mixing devices ('micromixers') have been proposed. The most simple design is based on straight channels (Bothe et al., 2006). In these devices, mixing occurs through a diffusion mechanism due to the low Reynolds numbers

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Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and *in-vitro* digestion

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ABSTRACT

Electrospraying has recently emerged as a novel microencapsulation technique with potential for the protection of probiotics. However, research efforts are still needed to minimize the viability loss observed during the processing of sensitive strains, and to maximize productivity. The aim of the present work was the optimization of the electrospraying conditions for the microencapsulation of a model probiotic microorganism, *Lactobacillus plantarum*, within a whey protein concentrate matrix. In a pre-optimization step, the convenience of encapsulating fresh culture instead of freeze-dried bacteria was established. Additionally, a surface response methodology was used to study the effect of the applied voltage, surfactant concentration, and addition of a prebiotic to the formulation on cell viability and productivity. Viability losses lower than 1 $\log_{10}$ CFU were achieved and the bacterial counts of the final products exceeded 8.5 $\log_{10}$ CFU/g. The protection ability of the developed structures during storage and *in-vitro* digestion was also evaluated.

1. Introduction

Microencapsulation technologies constitute a plausible approach for the preservation of biologically active ingredients in food systems including probiotic bacteria (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Kailasapathy & Champagne, 2011; Krasaekoopt, Bhandari, & Deeth, 2003), not only during processing or storage, but also during gastrointestinal transit, improving the delivery of probiotic strains to the large intestine (Shori, 2015). Several methods have been reported to microencapsulate probiotic microorganisms, including spray-coating (Champagne, Raymond, & Tompkins, 2010), emulsion and/or spray-drying (Picot & Lacroix, 2003), extrusion (Doherty et al., 2012), and gel-particle technologies (Chandramouli et al., 2004). Being a well-established process that can produce large amounts of material, spray-drying is the most commonly used microencapsulation technique in the food industry (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). However, this technique involves the use of high temperatures, which results in significant cell mortality (Salar-Behzadi et al., 2013). On the other hand, spray-coating and entrapment in gel particles generate relatively big particles (90–250 μm and >200 μm, respectively) that may affect food sensory qualities (Augustin, 2003), and the latter is considerably expensive to be scaled up in the food industry (Champagne et al., 2010; Krasaekoopt et al., 2003). Recently, electrospraying has emerged as an alternative microencapsulation technique (Bock, Dargaville, & Woodruff, 2012) which can generate very fine particulate structures in a one-step process (Chakraborty, Liao, Adler, & Leong, 2009) under mild conditions (López-Rubio & Lagaron, 2012). It basically involves the application of a high voltage electrical field to a polymer solution, dispersion or melt which is sprayed towards a charged collector, where the dry nano- or microparticles are deposited (Bhardwaj & Kundu, 2010; Bhushani & Anandharamakrishnan, 2014; Chakraborty et al., 2009) (cf. Supplementary material). Some of the advantages of electrospraying include the possibility of working under mild

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Dietary polyphenols bind to potato cells and cellular components

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ABSTRACT

The ability of phenolic compounds to bind to dietary polysaccharides such as starch and plant cell wall components impacts their nutritional value. Here, we report interactions between potato cells and three different phenolic compounds (+)-catechin, phloridzin and vanillic acid. The binding interactions of the phenolic compounds with intact potato cells, as well as disrupted cells, cooked cells, isolated cell walls and starch granules was explored varying polyphenol concentration, pH and incubation time. Results showed that binding capacity depends on the type of phenolic compound ((+)-catechin > phloridzin > vanillic acid) as well as the type of substrate, to a maximum of ~50 μmol/g (dry weight). The observed differences ($p < 0.05$) were ascribed to the amount and accessibility of potential binding sites in both the phenolic compounds and the polysaccharides. Remarkably, polyphenols could penetrate intact cells and bind the starch within them, suggesting their potential as delivery vehicles, whose loading capacity more than doubled after cooking.

1. Introduction

The application of phenolic compounds as functional ingredients is widely studied (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Han et al., 2011; Kumpoun, Motomura, & Nishizawa, 2015; Shi et al., 2005; Sun-Waterhouse et al., 2009). In a complex food system, interactions between these bioactive compounds and the food matrix may affect the quality of polyphenol-rich food products, as these interactions impact both the bioavailability and bioactivity of the phenolic compounds (Jakobek, 2015; Le Bourvellec & Renard, 2012).

Recent studies using *in vitro* systems suggest that nutritionally-significant amounts of phenolic compounds can bind to plant cell walls and cell wall analogues (Padayachee et al., 2012a, 2012b; Phan et al., 2015). These bound phenolics are, therefore, delivered to the large intestine where both dietary fiber and polyphenols have been shown to exert positive effects on microbial populations, thereby promoting health benefits (Aleixandre & Miguel, 2016; Conlon & Topping, 2016; Kaulmann & Bohn, 2016; Klinder et al., 2016; Moreno-Indias et al., 2016). This and other practical implications have led to an increase in the research attention paid to the binding of phenolic compounds to plant cell walls (Bautista-Ortín, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Le Bourvellec, Guyot, & Renard, 2004; Renard, Baron, Guyot, & Drilleau, 2001). Apart from cell walls, the interactions between phenolic compounds and starch, another major plant-derived carbohydrate in human diet (Han, Choi, Kim, Kim, & Baik, 2015; Liu et al., 2016), may affect not only the nutritional properties of the phenolic compounds but also the processability as well as enzymic susceptibility of starches (Zhu, 2015).

It is generally agreed that the binding of polyphenols to macromolecules, and specifically polysaccharides, is mainly mediated by hydrogen bonds and hydrophobic interactions (Le Bourvellec & Renard, 2012; Le Bourvellec et al., 2004; Renard et al., 2001), although the exact mechanisms are not fully understood yet. Covalent bonding between phenolic compounds and polysaccharides has been reported as well (Rustioni, Fiori, & Failla, 2014). The occurrence of ionic interactions has also been proposed for charged molecules, such as the binding of anthocyanins to pectins (Padayachee et al., 2012a). Most works report that the binding

Abbreviations: CLSM, confocal laser scanning microscopy; FT-IR, Fourier transform infrared spectroscopy; ATR, attenuated total reflectance; PBS, phosphate buffered saline.

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Microencapsulation of a whey protein hydrolysate within micro-hydrogels: Impact on gastrointestinal stability and potential for functional yoghurt development

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ABSTRACT

Gelatin and chitosan micro-hydrogels containing a potentially bioactive whey protein hydrolysate were developed through spray drying and the impact of microencapsulation on protection during digestion and peptide stability against lactic acid fermentation during yoghurt manufacturing was assessed. The results showed that the protection exerted by the encapsulation structures during milk fermentation was sequence- and matrix-dependent, being chitosan more effective than gelatin in stabilising the peptides. However, only 5 out of the 21 fermentation-susceptible peptides identified could be protected through encapsulation within chitosan (1 of which was also protected by gelatin). Moreover, the encapsulation within chitosan microparticles did not substantially affect the peptide profile of the digested hydrolysate, and therefore, the peptide bioaccessibility was not expected to be compromised.

1. Introduction

Biologically active peptides are specific fragments of proteins with 2 to 20 amino acids that have desirable biological activities (de Castro & Sato, 2015). Specifically, bioactive peptides derived from milk proteins have attracted great interest in the field of functional foods (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Korhonen, 2009) because of their potential ability to promote human health by reducing the risk of chronic diseases or enhancing our natural immune system (Korhonen & Pihlanto, 2006; Nongonierma & FitzGerald, 2015). These peptides are inactive within the sequence of the precursor proteins and need to be released (by

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Abbreviations: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; FT-IR, Fourier transform infrared spectroscopy; Rt, retention time; SEM, scanning electron microscopy; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid; TIC, total ion current; WPC, whey protein concentrate

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Impact of microencapsulation within electrosprayed proteins on the formulation of green tea extract-enriched biscuits

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ABSTRACT

In this work, a green tea extract (GTE) was encapsulated within electrosprayed protein (i.e. gelatin and zein) microparticles, and the protective ability of both systems on the green tea catechins was assessed. The microparticles (with encapsulation efficiencies ~90 g/100 g), proved to be very effective in stabilizing the catechins during a thermal treatment at 180 °C (12 min), preserving 85–90 g/100 g of their initial catechins content, while free GTE lost almost 40 g/100 g of its catechins content. In order to assess the impact of microencapsulation in a real food system, the GTE-loaded electrosprayed microparticles were added to biscuits dough. Results showed that microencapsulation did not significantly protect during biscuit processing and emphasized the need of assessing the behaviour of microencapsulation systems in real food processing conditions. The sensorial analysis of the biscuits indicated that addition of the GTE-loaded microparticles did not impact the acceptability of the biscuits, as perceived by consumers.

1. Introduction

Green tea catechins are a group of polyphenolic antioxidants which have received increasing research attention in recent years because of their reported health promoting properties, such as anti-carcinogenic (Larsen & Dashwood, 2010) and antimicrobial effects (Steinmann, Buer, Pietschmann, & Steinmann, 2013). Among them, (−)-epigallocatechin gallate (EGCG) is the most abundant and bioactive flavonoid in green tea (Barras et al., 2009). Together with (−)-epicatechin gallate (ECG) it is accepted as an indicator of the quality of green tea extracts (GTE) (Sharma & Zhou, 2011). These products are being increasingly added to a variety of foods in order to improve their nutritional value (Ananingsih, Gao, & Zhou, 2013) and make them more appealing to consumers (Yilmaz, 2006), whose interest towards functional foods is increasing (Siró, Kápolna, Kápolna, & Lugasi, 2008).

Despite the promising benefits of supplementing food products with GTE, there are a number of technological concerns due to the poor stability of tea catechins, which are thermosensitive and highly unstable in alkaline conditions, especially EGCG and ECG (Su, Leung, Huang, & Chen, 2003), so their bioactivity could be compromised upon food processing and storage. Moreover, GTE has been reported to negatively impact aroma, flavour and overall acceptability of bread and, thus, the quality of the final food products (Bajerska, Mildner-Szkudlarz, Jeszka, & Szwengiel, 2010). Being one of the most popular bakery products consumed by nearly all levels of society and all age groups (Tarancón, Fiszman, Salvador, & Tárrega, 2013), biscuits are regarded as good candidates to be fortified with functional ingredients (Choudhury, Badwaik, Borah, Sit, & Deka, 2015). They are convenient snacks with a long shelf life and appealing sensory attributes (Fradinho, Nunes, & Raymundo, 2015). In fact, the supplementation of biscuits with GTE (Mildner-Szkudlarz, Zawirska-Wojtasiak, Obuchowski, & Gośliński, 2009; Sharma & Zhou, 2011) or grounded tea leaves (Gramza-Michałowska et al., 2016) has already been attempted with different purposes. However, biscuit doughs generally have high pH values, and high temperatures are used to bake them, threatening the stability of tea catequins if supplemented with GTE (Sharma & Zhou, 2011).

Microencapsulation, or entrapment of an ingredient within a

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20

Novel Nanoencapsulation Structures for Functional Foods and Nutraceutical Applications

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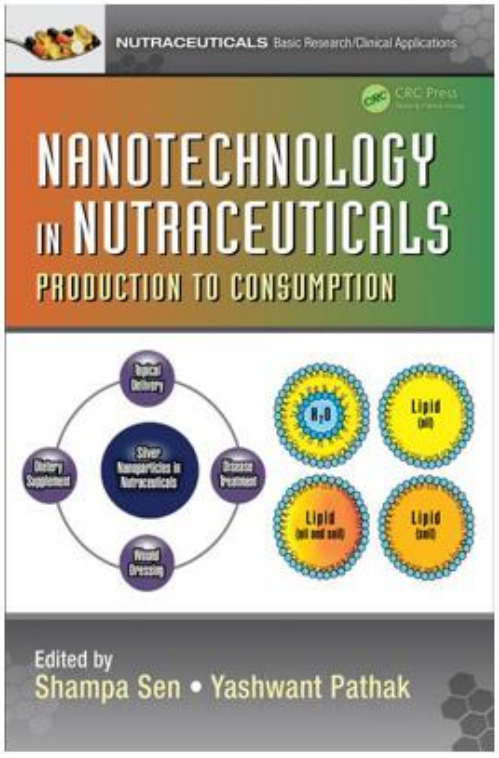
Contents



373

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Microencapsulated chañar phenolics: A potential ingredient for functional foods development

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ABSTRACT

In this work, a *Geoffroea decorticans* (chañar) extract very rich in polyphenols and with antioxidant and inhibitory activity against pro-inflammatory enzymes, was microencapsulated in a zein matrix using electrospraying to improve its stability and facilitate its handling as food ingredient. The electrosprayed microcapsules were characterized in terms of morphology, encapsulation efficiency, water sorption ability and release properties at different pH and during an *in vitro* digestion process. The bioactivity of the encapsulated extract in terms of inhibitory activity on enzymes related to the metabolic syndrome were also evaluated and compared to that of the non-encapsulated ingredient. Results showed that microencapsulation did not affect biological activity and effectively protected the bioactive polyphenols during the simulated digestion process. Therefore, this encapsulation structures could be considered a potential ingredient for functional food development.

1. Introduction

Geoffroea decorticans (Gill.ex Hook. et Arn.) Burk (Fabaceae), known as chañar, grows in the native forests of the Gran Chaco region in Argentina as well as in the Paraguayan and Bolivian Chaco and northern Chile (Scarpa, 2009). The ripe fruits (drupes) are still part of the diets of animals and aboriginal communities (Arena & Scarpa, 2007). The fruits, seeds and flour are used raw, roasted, boiled and/or fermented (beverage), in soups and in sweet jelly-like syrup called arrope. Chañar fruits flour and arrope can be used as functional foods, since apart from having high levels of sugar and fiber, they contain polyphenolic compounds with biological activities (Costamagna, Ordoñez, Zampini, Sayago, & Isla, 2013; Costamagna et al., 2016; Reynoso, Vera, Aristimuño, Daud, & Sánchez Riera, 2013). The compounds indentified in phenolic extracts from chañar were caffeic acid glycosides, simple phenolics (protocatechuic acid and vanillic acid), a glycoside of vanillic acid, *p*-coumaric acid and its phenethyl ester as well as free and glycosylated flavonoids (Costamagna et al., 2016).

The metabolic syndrome (MetS) is an emergent pathology that affects around 25% of the world population. The reduction of postprandial hyperglycemia by inhibition of enzymes involved in carbohydrate or lipid metabolism (α-glucosidase and α-amylase or lipase, respectively), inhibition of oxidative stress and delay of inflammatory processes are the most common therapeutic approaches (Prasad, Ryan, Celzo, & Stapleton, 2012). The chañar polyphenols enriched extract with and without gastroduodenal (GD) digestion inhibited enzymes associated with metabolic syndrome, exhibited antioxidant activity and inhibited the pro-inflammatory enzymes (Costamagna et al., 2016). These findings add evidence that the extract may be considered as a multipurpose bioactive ingredient. However, phenolic compounds are unstable in solution and, thus, it is necessary to eliminate the solvents present in the extracts in order to stabilize them prior to their incorporation in food products and facilitate their handling as food ingredients. Furthermore, polyphenols are highly susceptible to epimerization at high temperature and alkaline pH which also impact on the polyphenols activity (Kim et al., 2007). Additionally, polyphenols are very astringent and bitter in taste, making difficult their direct incorporation in food products or their use as nutraceuticals. Microencapsulation of polyphenols may help to overcome these problems. This process also facilitates the produc-

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Research Article

Impact of Acetic Acid on the Survival of *L. plantarum* upon Microencapsulation by Coaxial Electrospraying

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In this work, coaxial electrospraying was used for the first time to microencapsulate probiotic bacteria, specifically *Lactobacillus plantarum*, within edible protein particles with the aim of improving their resistance to in vitro digestion. The developed structures, based on an inner core of whey protein concentrate and an outer layer of gelatin, were obtained in the presence of acetic acid in the outer solution as a requirement for the electrospraying of gelatin. Despite the limited contact of the inner suspension and outer solution during electrospraying, the combination of the high voltage used during electrospraying with the presence of acetic acid was found to have a severe impact on the lactobacilli, not only decreasing initial viability but also negatively affecting the survival of the bacteria during storage and their resistance to different stress conditions, including simulated in vitro digestion.

1. Introduction

Probiotics can be incorporated into food products as bioactive ingredients for the development of functional foods. For this purpose, the microorganisms must be alive and metabolically active, and their concentration at the time of consumption should be high enough to exert their claimed health benefits [1]. Therefore, stabilization of bacteria is of outmost importance when supplementing food products with sensitive probiotic cultures whose survival can be compromised during their shelf life or digestion, and microencapsulation technologies are regarded as an effective approach to achieve it [2–4].

Electrospraying is a versatile electrohydrodynamic processing technique which can be used to generate ultrafine polymeric particles in a one-step process under mild conditions [5] by applying a high-voltage electric field to a polymer-containing fluid, causing its spraying towards a grounded collector where dry material is deposited [6–8]. This technology can be used for the microencapsulation of bioactive agents as an alternative to conventional techniques [9].

In a previous work, we showed the potential of this technique for the microencapsulation of *L. plantarum* within whey protein concentrate (WPC) capsules [10]. Although the obtained capsules proved to better protect the bacterial viability during storage under stress conditions than a traditional preservation technique such as freeze-drying, the protection exerted during simulated digestion was found to be similar using both techniques. This was attributed to the water-dispersible nature of the protein matrix used for encapsulation, which led to the disruption of the capsules in aqueous environments.

In an attempt to broaden the application range of the previously developed carriers to aqueous food products and enhance their protective effect during digestion, gelatin was selected in this work as a hydrogel-forming protein to coat the probiotic-loaded WPC particles. Gelatin can be electrosprayed from aqueous solutions while avoiding the complete disruption of the obtained capsules in aqueous environments

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Characterization, release and antioxidant activity of curcumin-loaded amaranth-pullulan electrospun fibers

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ABSTRACT

In this work, ultrathin electrospun fibers from two different blends of amaranth protein isolate (API) and the carbohydrate polymer pullulan (Pul) were loaded with two different concentrations of curcumin (0.05% y 0.075%). The loaded electrospun fibers were physicochemically characterized and the curcumin release profile as well as its antioxidant capacity under *in vitro* digestion conditions was evaluated. Round, uniform and homogenous fibers with diameters of around 224.5–248.6 nm were obtained for loaded API:Pul 50:50 fibers. The encapsulation efficiencies of curcumin varied between ~73% and ~93% for both loadings and fiber compositions. Moreover, a controlled and sustained released of curcumin was observed both in buffer solution (pH = 7.4) and during an *in-vitro* digestion process. The antioxidant activity of curcumin entrapped within the ultrathin fibers was maintained after the *in vitro* digestion process and it was superior in comparison with the non-encapsulated bioactive compound.

1. Introduction

Curcumin is a pleiotropic molecule (Fig. 1) that has been used as a remedy in traditional medicine in China and India (Hatcher, Planalp, Chob, Tortia, & Tortic, 2008). Several *in vitro* and *in vivo* studies have reported that curcumin has beneficial properties such as anti-inflammatory (Anand et al., 2008), antioxidant (Menon & Sudheer, 2007), and chemopreventive (Park, Ruhul, Georgia, & Shin, 2013). However, its poor water solubility and chemical instability reduce its bioavailability and, thus, its biological effect (Strimpakos & Sharma, 2008). Encapsulation represents an available and efficient approach to circumvent this problem. Therefore, curcumin has been encapsulated in nanoparticles (Bisht et al., 2007; Dadhaniya et al., 2011; Kumar, Kasoju, & Bora, 2010), superparamagnetic silica reservoirs (Chin et al., 2009) and ultrathin fibers (Fu et al., 2014). An ultrathin fiber is a one-dimensional flexible nano-element that can be processed by electrospinning (Ramakrishna, Fujihara, Teo, Lim, & Ma, 2005) and it has several advantages over other encapsulation systems such as high surface area per unit volume, high encapsulation efficiency and controlled release characteristics (Hu et al., 2014). Biopolymers can offer renewable nature, biodegradability, biocompatibility (Schiffman & Schauer, 2008), and they possess the necessary characteristics as specific visco-elastic properties, electric conductivities and surface tension values to produce electrospun fibers (Agarwal, Greiner, & Wendorff, 2009). Some types of proteins have been electrospun to obtain nanofibers such as casein (Xie & Hsieh, 2003), soy protein (Har-el, Gerstenhaber, Brodsky, Huneke, & Lelkes, 2014; Vega-Lugo & Lim, 2009), and more recently zein (Oliveira et al., 2014). Amaranth (*Amaranthus hypochondriacus*) is a pseudocereal with high protein content (17%) and amino acid composition close to the optimum amino acid balance required by human nutrition (Schnetzler & Breen, 1994). Amaranth protein contains a low proportion of prolamins which makes it a safe ingredient for people with celiac disease and recent studies have shown that amaranth peptides displayed antihypertensive and anti-inflammatory activity (Gorinstein et al., 2002; Orsini, Galleano, Añón, & Tironi, 2015; Silva et al., 2008). In our group, the ability of an amaranth protein isolate (API) combined with pullulan (Pul), a spinnable carbohydrate polymer (Fig. 1), to generate electrospun microstructures was demonstrated (Aceituno-Medina, Lopez-Rubio, Mendoza, & Lagaron, 2013a, 2013b). These structures can be used to encapsulate

Abbreviations: API, Amaranth protein isolate; Pul, pullulan; ATR-FTIR, Attenuated total reflectance infrared spectroscopy.

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Development of glucomannan-chitosan interpenetrating hydrocolloid networks (IHNs) as a potential tool for creating satiating ingredients

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ABSTRACT

The aim of this study was to understand the interactions taking place between konjak glucomannan (KGM), a soluble dietary fibre with extraordinarily high water-holding capacity, and chitosan, a pH-sensitive biopolymer, to promote the development of physically crosslinked interpenetrating hydrocolloid networks (IHNs). These IHNs could be useful for the design of satiating ingredients that would swell at gastric pH values. Initially, cast films from blends of KGM and chitosan were developed to study swelling ability and molecular organization through gravimetric studies, small-angle X-ray scattering (SAXS), wide-angle X-ray scattering (WAXS) and Fourier transform infrared spectroscopy (FTIR). The effects of chitosan molecular weight, KGM:chitosan ratio and neutralization with sodium carbonate were also analysed. Freeze-dried samples from the hydrocolloid blend solutions were also studied for comparison purposes. The rheological behaviour of these solutions was characterized and could be adequately explained by the interactions established during the formation of the IHNs, thus providing useful information for developing novel satiating ingredients. Interestingly, a different mechanism of Na_2CO_3-promoted interactions was observed when applied to the films (solid state) or incorporated into the hydrocolloid solutions.

1. Introduction

Obesity and overweight prevention is one of the main focuses of current global policies, as apart from being the main health concern (given the associated risk of developing chronic diseases such as diabetes mellitus or cardiovascular diseases), it has an enormous economic impact, having even been considered a "disability" by the European Court of Justice (European Court of Justice, 2014). In this sense, the development of the so-called satiating foods (those with an increased capacity to inhibit appetite in the period after consumption) has attracted a great interest from researchers and the food industry, being the latter one a major player for tackling this issue. Even though a number of foods which claim to be effective at staving off hunger have been commercialized during the last years, none of these products could be labelled as "satiating foods" according to the European Commission regulation (European Commission, 2007).

Currently, the only food ingredient having a positive opinion from the European Food Safety Authority (EFSA) for weight management is konjac glucomannan (KGM) (EFSA, 2010), the main polysaccharide from the tubers of the *Amorphophallus konjac* plant, a member of the family Araceae found in East Asia. This soluble fibre, consisting on β-(1 → 4)-linked D-mannose and D-glucose units in a ratio of 8:5 and having a small degree of branching, has an extraordinarily high water-holding capacity, forming highly viscous solutions when dissolved in water. This water-holding capacity would make KGM to swell in the stomach, providing satiety sensation and proving to be highly effective in the treatment of obesity (Walsh, Yaghoubian, & Behforooz, 1984). Moreover, it has been recommended as a remedy for constipation (Marzio, Del Bianco, Donne, Pieramico, & Cuccurullo, 1989), as it increases the faeces volume, for lowering cholesterol (Levrat-Verny, Behr, Mustad, Rémesy, & Demigné, 2000) since it interferes in the transport of cholesterol and of bile acids, and as hypoglycaemic and

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Effect of (−)-epigallocatechin gallate at different pH conditions on enteric viruses

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ABSTRACT

Epigallocatechin gallate (EGCG), a flavonoid from green tea, is said to have extensive antimicrobial activity in a wide range of food spoilage or pathogenic fungi, yeast and bacteria. In this work, the antiviral activity of EGCG was assessed against hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate, at different temperatures, contact times and pH conditions by cell-culture methods. EGCG was effective in reducing the titers of HAV and MNV in a dose-dependent manner at neutral pH and 25 and 37 °C, while no effect was reported at 4 °C. HAV and MNV infectivity was completely removed after overnight treatment with EGCG at 2.5 mg/mL at 37 °C. Furthermore, results also revealed that EGCG was very effective inactivating MNV and HAV at neutral and alkaline pH but was ineffective at pH 5.5. Results from cell-culture assays and viability RT-qPCR assays indicated that EGCG did not dramatically affect viral capsid, which instead may suffer subtle alterations of proteins. Moreover, HPLC/MS analysis of catechin solutions at different pHs indicated that antiviral activity was most likely due to catechin derivatives rather than EGCG itself, given the evolution of these compounds at the various pH conditions tested. These findings suggest that green tea catechins appear to be a suitable natural option for foodborne viral reduction.

1. Introduction

Nowadays, foodborne viral outbreaks are a growing concern for food safety authorities. Indeed, enteric viruses, in particular human noroviruses, which cause gastroenteritis, are the leading causes of foodborne illnesses in industrialized countries (Control & Prevention, 2013; EFSA, 2015). Moreover, hepatitis A virus (HAV) is considered as a re-emerging pathogen and is responsible for about half the total number of human hepatitis infections diagnosed worldwide (Sprenger, 2014). Norovirus and HAV can be transmitted directly from person-to-person, but also indirectly via virus-contaminated food (mainly associated with shellfish, soft fruits, leafy greens, and ready-to-eat meals), water, and surfaces.

Due to their low infectious dose (10–100 viral particles) (Teunis et al., 2008; Yezli & Otter, 2011) and to their stability and resistance to inactivation processes, the development of alternative methods for the viral decontamination of food has been recently promoted by public authorities (WHO, 2013). Amongst them, promising results have been reported for many natural compounds tested as antivirals *in vitro*, but when they were evaluated in food model systems or food applications, the viral decay was somewhat limited (Bozkurt, D'Souza, & Davidson, 2015; D'Souza, 2014; C. Sánchez, Aznar, & Sánchez, 2015). Many factors could be responsible for such decrease in efficacy such as the interaction of the active compound or the virus with food matrices, the pH, the water activity, etc.

From the commercially available natural extracts, green tea extract (GTE) has demonstrated inhibitory properties against foodborne bacteria (Perumalla & Hettiarachchy, 2011) and more recently against norovirus surrogates as well (Ueda et al., 2013). Chemically, GTE mainly contains catechins, a group of flavonoids with antioxidant properties (Yilmaz, 2006). Specifically, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin (GC), catechin (C) and epigallocatechin gallate (EGCG) have been found to be the main catechins present in GTE (Kajiya

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Procesado electrohidrodinámico de gelatina para la encapsulación de bioactivos con aplicación en alimentación funcional

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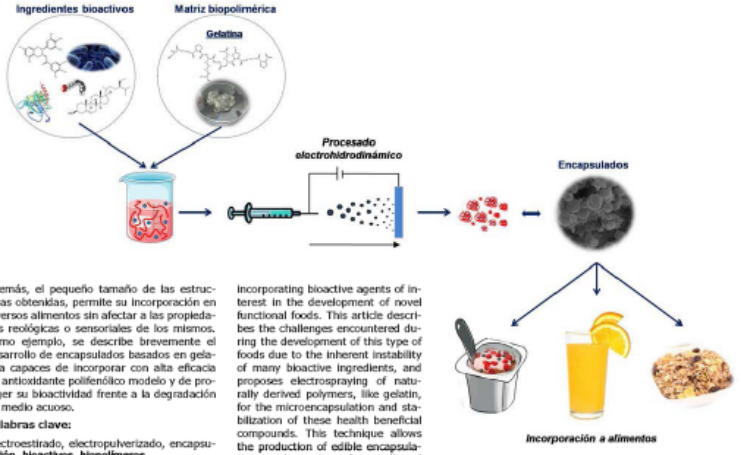


Resumen

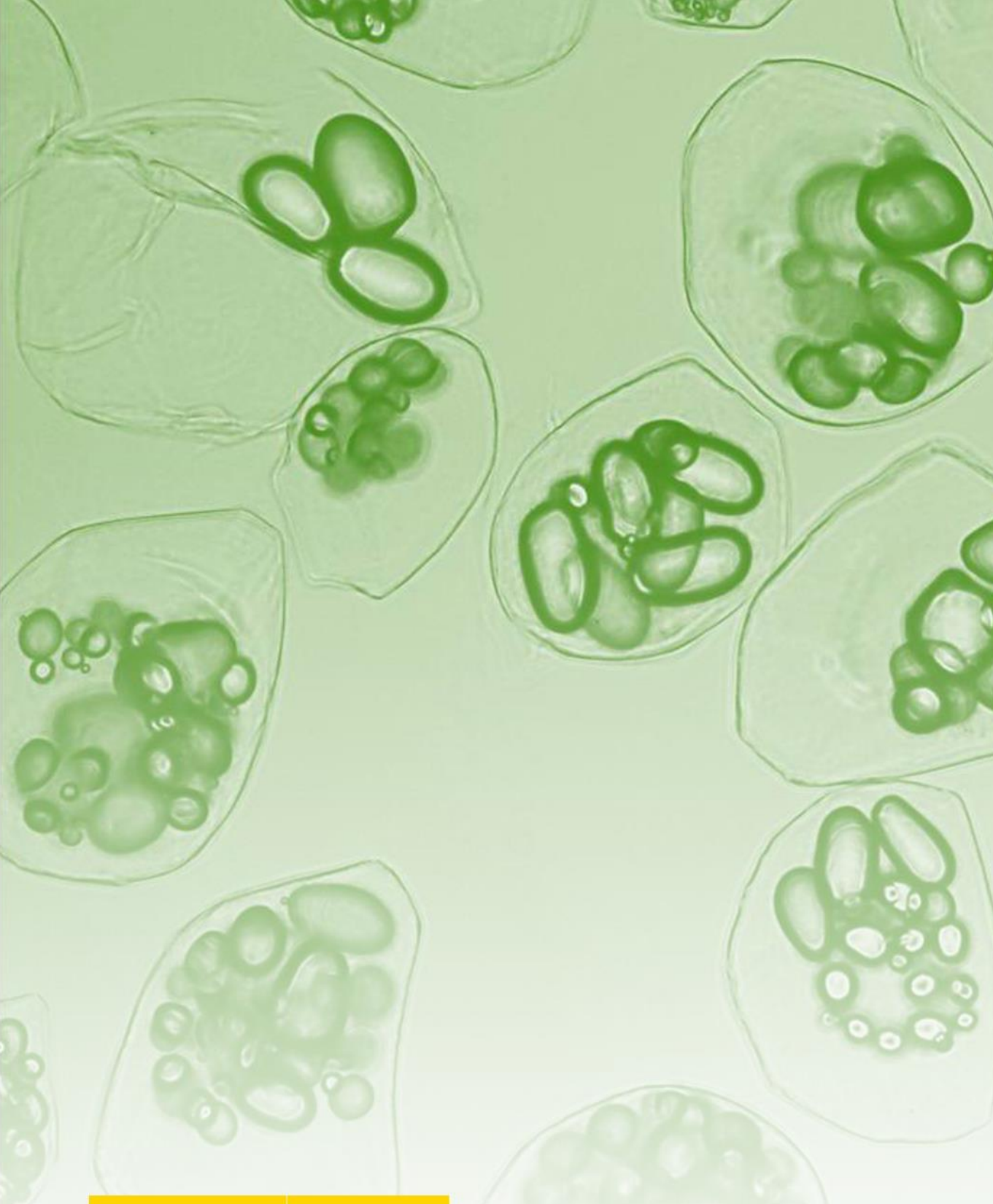
El procesado electrohidrodinámico de polímeros se presenta como una alternativa para la generación de nano- y micropartículas capaces de incorporar agentes bioactivos con aplicación en el desarrollo de nuevos alimentos funcionales. Este artículo expone la problemática que supone el desarrollo de este tipo de alimentos debido a la inherente inestabilidad de muchos ingredientes bioactivos, y propone el empleo del electropulverizado de polímeros de origen natural, como la gelatina, para la microencapsulación y estabilización de dichos compuestos con efecto beneficioso para la salud. De esta forma, se pueden producir matrices de encapsulación comestibles utilizando disolventes admitidos para contacto con alimentos, y en condiciones suaves, evitando el empleo de altas temperaturas. Además, el pequeño tamaño de las estructuras obtenidas, permite su incorporación en diversos alimentos sin afectar a las propiedades reológicas o sensoriales de los mismos. Como ejemplo, se describe brevemente el desarrollo de encapsulados basados en gelatina capaces de incorporar con alta eficacia un antioxidante polifenólico modelo y de proteger su bioactividad frente a la degradación en medio acuoso.

Palabras clave:

Electroestirado, electropulverizado, encapsulación, bioactivos, biopolímeros

Abstract

Electrohydrodynamic processing is proposed as an alternative technique for the production of nano- and microparticles capable of incorporating bioactive agents of interest in the development of novel functional foods. This article describes the challenges encountered during the development of this type of foods due to the inherent instability of many bioactive ingredients, and proposes electrospraying of naturally derived polymers, like gelatin, for the microencapsulation and stabilization of these health beneficial compounds. This technique allows the production of edible encapsulation matrices using solvents allowed for food contact, avoiding the need of high temperatures. Moreover, the small size of the structures obtained allows direct incorporation of the encapsulates within different food



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