Development of gelatin-coated ι -carrageenan hydrogel capsules by electric field-aided extrusion. Impact of phenolic compounds on their performance

Laura G. Gómez-Mascaraque^{1*}, Marta Martínez-Sanz, María José Fabra, Amparo López-Rubio

Food Safety and Preservation Department, IATA-CSIC, Avda. Agustín Escardino 7,
46980 Paterna, Valencia, Spain

¹ Present address: Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork,
Ireland

*Corresponding author: Tel.: +353 76 111 22 41

E-mail address: laura.mascaraque@teagasc.ie (L. G. Gómez-Mascaraque)

ABSTRACT

The aim of this work was to develop edible encapsulation structures based on gelatin-coated ι-carrageenan hydrogel beads, and to study the impact of incorporating different phenolic compounds within them in their microstructure, physic-chemical properties and release performance. The developed capsules were produced in aqueous solutions and mild conditions (i.e. suitable for food applications and sensitive bioactive compounds) by electric field-aided extrusion of ι-carrageenan solutions onto a gelatin bath containing calcium chloride, and exhibited shape-memory behaviour upon re-hydration in water after freeze-drying. The impact of loading them with gallic acid, catechin, chlorogenic acid and tannic acid was evaluated, and the results showed that only tannic acid could be efficiently incorporated within the hydrogel structures, while it limited the binding of the gelatin coating and altered the structure and thermal properties of the capsules. Reducing the concentration of tannic acid minimized its impact on the structure of the beads without negatively affecting its release, which was found to be however affected by the capsule size. The potential of these structures for the encapsulation of hydrophobic compounds was also anticipated.

KEYWORDS

Gelatin, carrageenan, polyphenol, electrospraying, encapsulation, tannic acid

1. Introduction

The commercial interest in functional foods has grown in the last decades in response to the increased consumer awareness of the relationship between nutrition and health (Day, Seymour, Pitts, Konczak, & Lundin, 2009; Ozen, Pons, & Tur, 2012; Siró, Kápolna, Kápolna, & Lugasi, 2008). One of the main strategies to design new functional food products is the addition of bioactive ingredients to conventional food matrices (Kaur & Das, 2011). However, direct incorporation of functional ingredients to foodstuffs is challenging, as many of them are very sensitive and prone to lose activity during manufacturing, storage or even passage through the gastrointestinal tract (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015). Moreover, some of these compounds have poor bioavailability, some may raise miscibility issues, and some can even alter the organoleptic properties of the food products in which they are incorporated (Deng, Chen, Huang, Fu, & Tang, 2014; Lafarga & Hayes, 2017). In this context, encapsulation has been proposed as a promising strategy to overcome these limitations, since it consists of coating or embedding an ingredient of interest within a protective matrix which acts as a physical barrier between the ingredient and the food matrix (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2014). Encapsulation has been successfully used for the stabilization of biologically active ingredients in food systems (Munin & Edwards-Lévy, 2011; Santhanam, Lekshmi, Chouksey, Tripathi, & Gudipati, 2015), preserving their bioactivity (Nickerson, Yan, Cloutier, & Zhang, 2014), avoiding off-flavours (Khor, Ng, Kanaujia, Chan, & Dong, 2017; M. X. Liu, et al., 2016), enhancing the compatibility of hydrophobic compounds with aqueous food matrices (Braithwaite, et al., 2014; Kuang, Oliveira, & Crean, 2010), and even improving their bioavailability (Paolino, et al., 2016; Uddin, et al., 2013).

Numerous encapsulation methods have been developed, many of which have already been used for food applications. Hydrogel capsules are of particular interest for this purpose, since the hydrogel-forming biopolymers can be readily processed in aqueous solutions and in mild conditions, while their subsequent crosslinking prevents the disruption of the gelled capsules once they are formed (Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016). One of the most commonly used processes to produce hydrogels for encapsulation purposes is through extrusion, whose basic principle consists of extruding a liquid mixture of the bioactive ingredient with its encapsulating matrix through an orifice and subsequently gelling the obtained droplets, usually by dripping them onto a gelling bath (Whelehan & Marison, 2011). This technique has been extensively exploited for the production of alginate-based hydrogel beads (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Ramos, et al.; Zeeb, Saberi, Weiss, & McClements, 2015), although the use of other biopolymers (e.g. gums, whey proteins or κ -carrageenan) for this purpose has also been explored to a lesser extent (Ainsley Reid, et al., 2005; Dinakar & Mistry, 1994; Rokka & Rantamäki, 2010; Sun & Griffiths, 2000). Apart from the conventional extrusion technique, in which the nozzle consists of a simple orifice, several improved designs have been proposed in order to increase the productivity of the technique and/or decrease the capsule size (McClements, 2017; Prüsse, et al., 2008; Zuidam & Shimoni, 2010). One of them is the so-called electric field-aided extrusion, also known as electrostatic extrusion and often considered a variant of the electrospraying technique, which allows the reduction of the capsule size by subjecting the polymeric solution to a high-voltage electric field (Tapia-Hernández, et al., 2015). This electric field generates repulsive electrostatic interactions within the charged polymeric liquid causing the initial droplets to break into smaller droplets. Again, this technology has mainly been exploited for the preparation of alginate-based

microcapsules using calcium chloride as the gelling agent (Balanč, et al., 2016; Gryshkov, et al., 2014; Li, Kim, Chen, & Park, 2016; Prüsse, et al., 2008; Zhu, et al., 2009).

Carrageenans and gelatins are two types of edible biopolymers capable of forming thermo-reversible hydrogels through coil/helix conformational transitions (Michon, Vigouroux, Boulenguer, Cuvelier, & Launay, 2000). Both gelatin, a protein obtained from partial hydrolysis of collagen (Lai, 2013), and carrageenans, a group of linear, sulphated polysaccharides extracted from marine red algae (Daniel-da-Silva, Ferreira, Gil, & Trindade, 2011), have been widely used in the food industry as gelling agents or texture modifiers, to enhance the elasticity, consistency or stability of food products (Liu, Chan, & Li, 2015; Okutan, Terzi, & Altay, 2014). Moreover, at pHs below the isoelectric point of the protein, they can interact with each other in aqueous solution through attractive electrostatic interactions, leading to the formation of polyelectrolyte complexes through complex coacervation (Michon, Cuvelier, Launay, Parker, & Takerkart, 1995). In a previous work, we developed novel ι -carrageenan/gelatin hydrogel capsules by extruding ι -carrageenan solutions onto a gelatin bath, which showed almost ideal release properties for intestinal targeted delivery purposes when they were used to encapsulate a polyphenol-rich grape juice extract (Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz, Fabra, & López-Rubio, 2018). ι -Carrageenan was selected over κ -carrageenan because its digestion has been reported to yield fragments of greater molecular weight, which have been associated to lower toxicities (Ekström, Kuivinen, & Johansson, 1983). Although the production of carrageenan hydrogel capsules through extrusion had already been reported using inorganic salts as gelling agents (Dinakar, et al., 1994), to the best of our knowledge, this was the first time that the protein-polysaccharide interactions between gelatin and

carrageenan were exploited to produce gelatin-coated ι -carrageenan beads by combining the extrusion and coacervation techniques. However, the size of the obtained capsules was too large for their practical application, which might be overcome by using an electric field-aided extrusion configuration.

On the other hand, biopolymers and, in particular, proteins, have been described to strongly interact with natural polyphenols through different mechanisms, including hydrophobic interactions and hydrogen bonding (Jakobek, 2015; Siebert, Troukhanova, & Lynn, 1996). These intermolecular interactions are responsible, at least partially, of the stabilization effect and the release properties of polyphenol-loaded encapsulation structures (Atay, et al., 2017; Gómez-Mascaraque, et al., 2018). Most research works focus on the impact of microencapsulation on the stabilization of the bioactive ingredients to be protected (Gómez-Mascaraque & López-Rubio, 2016; Kong, Bhosale, & Ziegler, 2018; Pérez-Masiá, Lagaron, & Lopez-Rubio, 2015; Torres-Giner, Martinez-Abad, Ocio, & Lagaron, 2010). However, the impact that adding bioactive ingredients has on the encapsulation matrices themselves has received considerably less attention. Since polyphenols can be used as natural crosslinkers for proteins (Anvari & Chung, 2016), the effect of incorporating different phenolic compounds within ι -carrageenan/gelatin hydrogel capsules on the morphology and properties of the encapsulation structures is also a topic of great interest.

Accordingly, the aim of this work was to apply the electric field-aided extrusion method to produce gelatin-coated ι -carrageenan hydrogel capsules with reduced sizes, and study the impact of adding different phenolic compounds to the formulations on the characteristics of the developed capsules. For this purpose, four representative phenolic compounds with different molecular structures and sizes (i.e. gallic acid, (+)-catechin, chlorogenic acid and tannic acid) were used, and the morphological, thermal, physic-

chemical and release properties of the obtained capsules were evaluated. The impact of the polyphenol concentration and the capsule size on the release properties was studied for tannic acid as the selected phenolic compound, and the potential of the developed hydrogel beads for the encapsulation of hydrophobic bioactive compounds was also shown.

2. Materials and Methods

2.1. Materials

Gelatin from porcine skin, with reported gel strength of 180 g Bloom, was supplied by Gelita AG (Germany). *ι*-Carrageenan, gallic acid (170.12 g/mol), (+)-catechin (290.27 g/mol), chlorogenic acid (354.31 g/mol), tannic acid (1701.2 g/mol), Sudan III, soybean oil (SBO), calcium chloride dehydrate, potassium bromide (spectroscopic grade, KBr) and phosphate buffered saline system (PBS, pH = 7.4) were obtained from Sigma-Aldrich (Spain). Acetic acid 96% (v/v) was supplied by Scharlab (Spain).

2.2. Preparation of the feed formulations

Gelatin and *ι*-carrageenan were separately dissolved in distilled water under magnetic stirring by mild heating at 40°C. A concentration of 1% (w/v) was selected for gelatin, as it was low enough to avoid gelation at room temperature. Regarding *ι*-carrageenan, a concentration of 0.75% (w/v) was selected after preliminary trials, as it was the maximum concentration which could be pumped through the PTFE wire of the electrospraying circuit (cf. Section 2.3). Lower concentrations were not sufficient to form spheres. When CaCl₂ was incorporated to the system, it was dissolved at a

concentration of 50 mM in the gelatin solution. This optimal concentration had been previously determined (Gómez-Mascaraque, et al., 2018).

For the capsules produced in the presence of the phenolic compounds, these (i.e. gallic acid, (+)-catechin, chlorogenic acid or tannic acid) were first dissolved in distilled water at room temperature and then mixed with concentrated ι-carrageenan solutions (1%, w/v) so that the final concentration of ι-carrageenan was adjusted to 0.75% (w/v) and the amount of phenolic compounds was 30% (w/w) with respect to the mass of ι-carrageenan.

Soybean oil (SBO), as a model hydrophobic ingredient, was also incorporated within the capsules at a concentration of 10% (w/w) with respect to the mass of ι-carrageenan. For this purpose, the oil was stained beforehand with Sudan III to facilitate its subsequent visualization and homogenized within the carrageenan solution using an IKA T-25 Digital ULTRA-TURRAX® equipped with a S 25N – 25F dispersing element whose stator diameter was 25 mm (IKA, Germany) at 6000 rpm during 2 min.

2.3. Preparation of the hydrogel capsules

ι-Carrageenan/gelatin hydrogel capsules were prepared using a Spraybase® (Dublin, Ireland) electrospraying apparatus. The ι-carrageenan solutions (containing the phenolic compounds or the SBO when these were incorporated) were introduced in a 10 mL plastic syringe and pumped at a constant flow-rate (15 mL/h) through a stainless-steel needle (0.838 mm inner diameter), placed vertically above a stainless-steel bath containing the gelatin solution and 50mM CaCl₂, at a distance of 9 cm. The needle was connected through a PTFE wire to the syringe, which was placed on a digitally

controlled syringe pump, and the bath was grounded. The applied voltage was 13 kV, as selected in preliminary trials to obtain the most uniform, spherical hydrogels. The obtained capsules were subsequently filtered using muslin cloth and intensively rinsed with distilled water to remove the excess gelatin. Selected hydrogel capsules were also produced in the absence of electric field for comparison purposes.

2.4. Morphological characterization of the hydrogel capsules

Optical microscopy images were taken at 4x magnification using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

2.5. Freeze-drying

ι -Carrageenan/gelatin hydrogel capsules were freeze-dried using a Genesis 35-EL freeze-dryer (Virtis). ι -Carrageenan and gelatin solutions in the absence and in the presence of the phenolic compounds, and ι -carrageenan/gelatin blends prepared by mixing the individual polymer solutions under continuous stirring using a Micra D-9 high-speed homogenizer (Epica S.L., Spain), were also freeze-dried for comparison purposes and subsequently ground using a IKA A11 Basic grinder (Germany), prior soaking in liquid nitrogen, to obtain powdery materials.

2.6. Fourier transform infrared (FT-IR) analysis of the materials

Freeze-dried samples (*ca.* 1–2 mg) were grounded and dispersed in about 130 mg of spectroscopic grade potassium bromide. A pellet was then formed by compressing the

samples at ca. 150 MPa and FT-IR spectra were collected in transmission mode using a Thermo Nicolet Nexus equipment. The acquisition time was 128 s at 4 cm⁻¹ resolution, and the average spectra are reported.

2.7. Determination of protein content in the capsules

Samples were analysed for total nitrogen content and equivalent protein concentration using an Elemental Analyser Rapid N Exceed (Paralab S.L., Spain). About 100 mg of each of the powdered samples were pressed to form a pellet which was then analysed using the Dumas method, which is based on the combustion of the sample and subsequent detection of the released N₂ (Wiles, Gray, & Kissling, 1998).

2.8. Thermogravimetric analysis (TGA) of the materials

TGA was performed with a TA Instruments model Q500 TGA following the method described in (Gómez-Mascaraque, et al., 2015). Briefly, the freeze-dried samples (ca. 8 mg) were heated from room temperature to 600 °C at a heating rate of 10 °C/min under dynamic nitrogen, and the weight loss of the samples was monitored.

2.9. Differential scanning calorimetry (DSC)

DSC measurements of the hydrated materials were performed on a Perkin-Elmer DSC 8000 thermal analysis system using N₂ as the purging gas. Freeze-dried capsules (ca. 5–6 mg) were accurately weighted and placed into hermetically sealable stainless steel sample pans. 50 µL of distilled water were then added to hydrate the samples, and the pans were hermetically sealed. The samples were subjected to a heating ramp from 20

°C to 150 °C at a heating rate of 10 °C/min, after equilibration at 20 °C for 2 min. An empty sample pan was also subjected to the same heat treatment and the obtained thermogram was subtracted from all the thermograms of the samples. The DSC equipment was calibrated using indium as a standard.

2.10. Release of phenolic compounds from the capsules

The release of the phenolic compounds from the capsules in different food simulants was assessed following a method adapted from Gómez-Mascaraque, et al. (2018). Briefly, the freeze-dried capsules (5 mg/mL) were suspended in 20 mL of release medium and the absorbance of the supernatant at the maximum of absorbance of each phenolic compound was measured after different time intervals using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Calibration curves were previously build for each phenolic compound in each release medium ($R^2 > 0.995$). The samples were gently shaken to homogenize them before analysis of the supernatant. Experiments were performed in independent triplicates. Ethanol 10% and 50% (v/v), and acetic acid 3% (v/v) were selected as food simulants, according to the Commission Regulation 10/2011 EU (10/2011/EC). Moreover, PBS (pH=7.4) was also used as release medium in order to assess the impact of the pH on the release behavior of the capsules.

2.11. Quantification of phenolic compounds

The amount of phenolic compounds diffused out to the gelatin bath during the production of the hydrogel capsules was quantified by means of the Folin-Ciocalteu

colorimetric assay (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, Folin-Ciocalteu reagent was diluted 1:10 with distilled water and 1 mL of the final dilution was mixed with 0.2 mL of gelatin bath used to produce the encapsulation structures. Subsequently, 0.8 mL of Na₂CO₃ (75 mg/mL) were added and the samples were heated up to 50°C for 30 minutes, before measuring their absorbance at a wavelength of 750 nm. Calibration curves were built for each particular polyphenol, and all determinations were carried out in triplicate.

2.12. Statistical analysis

Significant differences in Table 1 were obtained through two-sided t-tests at $p < 0.05$ using IBM SPSS Statistics software (v.24) (IBM Corp., USA).

3. Results and discussion

3.1. Development of ι -carrageenan/gelatin hydrogel capsules

In our previous work, quasi-spherical ι -carrageenan/gelatin capsules were obtained by extrusion of ι -carrageenan onto a gelatin solution in the presence of a divalent salt, and were used to encapsulate a polyphenol-rich grape juice extract, showing almost ideal release properties for intestinal targeted delivery purposes (Gómez-Mascaraque, et al., 2018). However, the size of the obtained capsules (~ 5-6 mm) was too large for their practical application. Therefore, electric field-aided extrusion was used in this work to obtain ι -carrageenan/gelatin hydrogel capsules with reduced sizes, using the same biopolymers employed in our previous work, which had been previously characterized (Gómez-Mascaraque, et al., 2018). The obtained capsules are shown in Figure 1A. A

more general view of the developed ι -carrageenan/gelatin hydrogel capsules can also be observed in Figure S1 of the Supplementary Material.

Figure 1. Optical micrographs of the developed ι -carrageenan/gelatin hydrogel capsules. A) As-prepared capsules. B) Freeze-dried capsules. C) Freeze-dried capsules after re-hydration in distilled water.

It is worth mentioning that ι -carrageenan/gelatin capsules could also be prepared in the absence of CaCl_2 through this technique, since the interaction between both biopolymers through different mechanisms (i.e. electrostatic, hydrophobic interactions and hydrogen bonding (Antonov & Goncalves, 1999; Fang, Li, Inoue, Lundin, & Appelqvist, 2006; Michon, et al., 2000; Voron'ko, Derkach, Vovk, & Tolstoy, 2017; Wang, et al., 2015)) promotes the formation of complexes. However, the addition of the divalent salt (50 mM) to the gelatin bath gave rise to stiffer capsules, which could be easily filtered to remove the excess gelatin and freeze-dried to increase their shelf stability (cf. Figure 1B). On the contrary, the capsules prepared in the absence of the salt were softer, and collapsed and aggregated upon filtration (cf. Figure S2 of the Supplementary Material), giving rise to a continuous cotton-like material upon freeze-drying. This was attributed to the additional crosslinking promoted by the salt, which

strengthened the biopolymeric network at the ι -carrageenan/gelatin interface, and possibly to the diffusion of Ca^{2+} cations through the external coating layer inducing the gelation of the ι -carrageenan core, as previously reported (Gómez-Mascaraque, et al., 2018).

Additionally, the developed capsules, which had a size in the range of 1-2 mm and exhibited a structure typical of the coated matrix type of capsules, were capable of fully recovering their shape upon rehydration in aqueous media after freeze-drying (cf. Figure 1C). This shape-memory behaviour emphasized the possibility of dehydrating the encapsulation structures for their enhanced preservation without significantly altering their morphology.

3.2. Impact of the phenolic compounds on the morphology of the hydrogel capsules

The developed capsules were loaded with different phenolic compounds, i.e. gallic acid, (+)-catechin, chlorogenic acid and tannic acid, by adding them to the core ι -carrageenan solution, in a concentration of 30% with respect to the mass of carrageenan. Figure 2 shows micrographs of the obtained capsules.

Biopolymers, especially proteins, can strongly bind to phenolic compounds through hydrophobic interactions and hydrogen bonding (Jakobek, 2015; Siebert, et al., 1996). In fact, polyphenols have recently attracted increasing attention as natural cross-linkers for proteins (Anvari, et al., 2016). In our previous work, polyphenols from grape juice extract showed a preferential interaction with gelatin rather than ι -carrageenan (Gómez-Mascaraque, et al., 2018). These biopolymer/polyphenol interactions, which may be different depending on the molecular structure of the phenolic compounds (Le Bourvellec & Renard, 2012), are thus expected to compete with the gelatin/ ι -

carrageenan interactions which trigger the self-assembly of the hydrogel capsules (Gómez-Mascaraque, et al., 2018), potentially affecting their structural organization. Indeed, Figure 2 shows that the type of phenolic compound incorporated within the capsules had a great impact on the morphology of the obtained capsules. Particularly, the incorporation of tannic acid within the encapsulation structures caused the greatest structural changes (cf. Figure 2D).

Figure 2. Optical micrographs of as-prepared ι -carrageenan/gelatin hydrogel capsules loaded with gallic acid (A), (+)-catechin (B), chlorogenic acid (C) and tannic acid (D).

In general, phenolic compounds with higher molecular weights have been reported to bind to macromolecules to a higher extent, although other factors such as the degree of galloylation or the stereochemistry of the polyphenols may also be relevant (Gómez-Mascaraque, Dhital, López-Rubio, & Gidley, 2017; Le Bourvellec, Bouchet, & Renard, 2005; Le Bourvellec, Guyot, & Renard, 2004; Le Bourvellec, et al., 2012). Tannic acid has the highest molecular weight from all the phenolic compounds assayed in this work. Moreover, the great affinity of tannic acid towards proteins, and in particular gelatins, and its ability to crosslink them, has been previously demonstrated (Aewsiri, Benjakul, Visessanguan, Wierenga, & Gruppen, 2010; Anvari, et al., 2016; Zhang, Pan, & Chung, 2011). Anvari and Chung (2016) reported that the great affinity of tannic acid towards fish gelatin was capable of disrupting the electrostatic interactions between the protein and gum arabic in complex coacervates prepared from both biopolymers. Moreover, the interactions between fish gelatin and tannic acid led to reduced molecular mobility and conformational flexibility of the protein (Anvari, et al., 2016). A similar phenomenon might have taken place in the hydrogel capsules developed in this work. In contrast to the unloaded capsules or those loaded with the lower molecular weight phenolic compounds, which exhibited a soft surface, those prepared in the presence of tannic acid revealed a rather rigid and fragile skin-like surface (cf. Figure 2D). Figure S3 of the Supplementary Material shows a detail of the somewhat peeled-off superficial film of the capsules, obtained using a phase contrast condenser to take the micrographs. This suggested that tannic acid rapidly crosslinked gelatin upon contact of the ι -carrageenan/polyphenol droplet with the protein solution bath, presumably preventing the effective interaction between both biopolymers. Indeed, the typical structure of coated matrix clearly observed for the rest of the samples was not obtained when tannic acid was added.

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352 3.3. FT-IR analysis of the materials

353 Figure 3 shows the FT-IR spectra of the freeze-dried capsules, which evidenced the
354 presence of both gelatin and ι -carrageenan in the unloaded capsules. The four most
355 characteristic bands of proteins were observed at ca. 3440 cm^{-1} (Amide A), 1662 cm^{-1}
356 (Amide I), 1552 cm^{-1} (Amide II) and 1242 cm^{-1} (Amide III) (Gómez-Mascaraque, et al.,
357 2015). On the other hand, the characteristic bands of carrageenans were observed at
358 1242 cm^{-1} , attributed to the ester sulphate groups (overlapped with the Amide III band
359 of the protein), 937 cm^{-1} , ascribed to the anhydride galactose group, 856 cm^{-1} ,
360 corresponding to the galactose-D-sulphate group, and the hydroxyl band centred at 3440
361 cm^{-1} (overlapped with the Amide A band of the protein) (Derkach, Ilyin, Maklakova,
362 Kulichikhin, & Malkin, 2015). Figure S4 of the Supplementary Material shows the FT-
363 IR spectrum of the unloaded capsules in comparison with the spectra of their individual
364 biopolymeric constituents for a more detailed comparison. The interactions between
365 both biopolymers were already deeply discussed in our previous work (Gómez-
366 Mascaraque, et al., 2018).

Figure 3. FT-IR spectra of *ι*-carrageenan/gelatin capsules: A) unloaded, B) catechin-loaded, C) gallic acid-loaded, D) chlorogenic acid-loaded and E) tannic acid-loaded.

The spectra of the capsules prepared in the presence of the phenolic compounds were all very similar to the spectrum of the unloaded capsules, not showing strong evidence of distinct bands directly attributable to the polyphenols (cf. Figure S5 of the Supplementary Material for FT-IR spectra of the different phenolic compounds), except those containing tannic acid (discussed below). In general, extrusion techniques to prepare hydrogel capsules have been reported to obtain low microencapsulation efficiencies for small water soluble compounds (Đorđević, et al., 2015), as they can easily diffuse to the solution bath. Moreover, due to the high viscosity of the biopolymers commonly used to produce the capsules, their concentration in the feed solutions must be low to allow processing through extrusion, and, thus, low density gel networks are generally obtained, which do not provide sufficient barrier effects (Gómez-Mascaraque, 2017). As a result, the lowest molecular weight phenolic

compounds (i.e. gallic acid, (+)-catechin and chlorogenic acid) might not have been efficiently incorporated within the capsules developed in this work. Instead, they must have diffused out of the hydrogel network during the production process and subsequent washing with distilled water.

On the contrary, the spectrum of the tannic acid-loaded capsules exhibited a distinct band attributable to tannic acid (cf. Figure S5 of the Supplementary Material) at 1700 cm^{-1} , evidencing the presence of this polyphenol within the materials. The molecular size of tannic acid is considerably bigger than that of the other phenolic compounds. Moreover, this compound has a great affinity towards gelatin, being able to establish strong interactions with the protein as previously reported (Aewsiri, et al., 2010; Anvari, et al., 2016; Zhang, et al., 2011). These two factors might have contributed to the effective retention of tannic acid within the developed encapsulation structures.

Interestingly, substantial differences were also observed for the sample containing tannic acid in the region of the Amide I and II bands of the spectrum, which were not as clearly observed as for the rest of the samples. In this case, it seemed that the Amide I band shifted to lower wavenumbers and the Amide II band was considerably less intense. Taking into account that *ι* -carrageenan and tannic acid also exhibited characteristic bands in those regions (cf. Figures S4 and S5 of the Supplementary Material), these might have masked the contribution of the protein. This hypothesis would imply the presence of a lower gelatin content in the tannic acid-loaded capsules than in the rest of the materials. To further investigate this possibility, a solution containing only *ι* -carrageenan and tannic acid (30%) was freeze-dried and analysed through FT-IR spectroscopy, together with a blend of *ι* -carrageenan and gelatin containing tannic acid (30%). Both spectra are provided in the Supplementary Material (Figure S6). Indeed, the spectrum of the tannic-acid containing capsules was fairly

similar to that of the mixture of *ι*-carrageenan and tannic acid in the absence of protein, while the Amide I and II bands of proteins were clearly observed in the blend containing gelatin, supporting the hypothesis of a lower protein content in the tannic acid-loaded capsules.

3.4. Protein content in the capsules

The amount of gelatin bound to the surface of the developed *ι*-carrageenan/gelatin capsules was quantified through nitrogen analysis of the samples, and the results are summarised in Table 1. Gelatin/*ι*-carrageenan blends with known protein contents were used to calculate the nitrogen/protein conversion factor.

Table 1. Protein content in the *ι*-carrageenan/gelatin capsules

Sample	Nitrogen content (%)	Protein content (%)	pH of <i>ι</i> -carrageenan solutions
Unloaded capsules	8.9±0.2	40.9±1.0	7.8 ± 0.1
Gallic acid-loaded capsules	7.4±0.3 ^(*)	34.2±1.4 ^(*)	3.4 ± 0.1 ^(*)
(+)-Catechin-loaded capsules	9.0±0.1	41.4±0.6	7.2 ± 0.1 ^(*)
Chlorogenic acid-loaded capsules	7.1±0.1 ^(*)	32.4±0.2 ^(*)	2.5 ± 0.1 ^(*)
Tannic acid-loaded capsules	0.9±0.1 ^(*)	4.2±0.1 ^(*)	4.6 ± 0.1 ^(*)

The superscript asterisk ^(*) in each column indicates significant differences between that sample and the reference (unloaded capsules)

The results showed that the interactions between gelatin and *ι*-carrageenan facilitated the binding of a considerable amount of protein to the polysaccharide-based core in the developed encapsulation structures, as significant amounts of protein were present in the unloaded capsules. Indeed, the formation of polyelectrolyte complexes through complex coacervation of gelatin and *ι*-carrageenan in aqueous solutions occurs at pHs below the isoelectric point of the protein (Michon, et al., 2000). The pH of the gelatin and *ι*-carrageenan solutions used to produce the capsules was 4.7 ± 0.1 and 7.8 ± 0.1,

respectively, which were below the isoelectric point of type A gelatins (Michon, et al., 2000). High protein contents were also obtained when the low molecular weight phenolic compounds were incorporated to the formulations, as anticipated from their FT-IR spectra. However, as expected from the spectrum of the tannic acid-loaded capsules, a substantial decrease in the protein content was observed for this formulation.

Since the pH of the solutions has a strong impact on the protein-polysaccharide interactions, the pH of the polyphenol-containing ι -carrageenan solutions was measured, and the results are also shown in Table 1. While the addition of (+)-catechin had a little impact on the pH of the ι -carrageenan solutions, which resulted in no significant differences in the protein content of the (+)-catechin-loaded capsules with respect to the unloaded capsules, the incorporation of the other three phenolic compounds considerably decreased the pH of the solutions, which correlated with significantly lower protein contents in the corresponding capsules. However, this reduction in the protein content, although statistically significant for gallic acid- and chlorogenic acid-loaded capsules, was not as dramatic for these two phenolic compounds as it was for tannic acid, which suggested that the pH was not the only factor affecting the amount of gelatin incorporated in the capsules. As commented above, the great affinity of tannic acid towards gelatin (Anvari, et al., 2016) might have limited the effective interactions between the protein and ι -carrageenan through its rapid crosslinking. The resulting rigid gelatin film formed on the surface of the tannic acid-containing capsules (cf. Section 3.2) must have prevented the diffusion of the biopolymers through the interface, resulting in a lower amount of protein binding to the ι -carrageenan droplets. This limited diffusion might have also contributed to the more effective retention of tannic acid within the encapsulation structures as compared to the other phenolic compounds.

3.5. Thermal and physicochemical properties of the materials

DSC thermograms were obtained for the polyphenol-loaded and the unloaded capsules, as well as for the individual biopolymers and the results are shown in Figure 4. For this purpose, the freeze-dried materials were previously rehydrated in water. The DSC thermogram of the re-hydrated freeze-dried ι-carrageenan showed a wide endothermic peak at 81.1 °C, which has been previously ascribed to the gel-sol or helix-coil transition of carrageenan hydrogels upon heating (Iijima, Hatakeyama, Takahashi, & Hatakeyama, 2007; Liu & Li, 2016). On the other hand, the freeze-dried gelatin showed an exothermic peak at 36.0, which was attributed to a structural relaxation phenomenon during heating of the re-hydrated samples, probably due to the incorporation of water molecules within its structure (known as structural water (Correia, et al., 2013)). As a result, the thermogram of the ι-carrageenan/gelatin capsules prepared in the absence of phenolic compounds exhibited both an exothermic and an endothermic transition, attributed to their gelatin and ι-carrageenan components, respectively. However, both transitions took place at higher temperatures in the capsules (75.1 and 96.7 °C, respectively) than in the pristine biopolymers. This was attributed to the intermolecular interactions between the protein and the carbohydrate in the encapsulation structures, which delayed the aforementioned transitions. Shifts of thermal transitions to higher temperatures upon complexation of proteins and polysaccharides have been previously observed and attributed to the rearrangement of the biopolymer chains and/or the formation of new network structures (Niu, et al., 2018; Zhao, et al., 2018).

Figure 4. DSC thermograms of re-hydrated freeze-dried materials. The thermograms have been offset for clarity purposes.

Interestingly, when the phenolic compounds were incorporated, no exothermic transition was observed, suggesting that the gelatin coating of these capsules did not experience the structural relaxation observed for their unloaded counterparts. Figure 4 shows the thermograms of the capsules prepared in the presence of tannic acid and catechin, as representative examples of an effectively incorporated polyphenol and a phenolic compound which was not as effectively incorporated, respectively. Although the incorporation of some of the phenolic compounds within the capsules was not very effective (cf. Section 3.3), small quantities of these molecules (not detectable by FT-IR) must have been retained within the encapsulation structures, affecting their thermal properties. The thermal transition of gelatin in those materials was most probably impeded by intermolecular interactions established between gelatin and the phenolic compounds, the newly formed hydrogen bonds hindering the binding of structural water to the protein. This hypothesis was further confirmed through a thermogravimetric analysis of the materials (see below), whose thermograms are shown in Figure 5.

Regarding the capsules containing tannic acid, the endothermic transition attributed to ι-carrageenan took place over a broader temperature range as compared to the rest of the capsules (see the thermogram of the catechin-loaded capsules in Figure 4 as a representative example), evidencing again the particular behavior of this formulation.

Figure 5. TGA thermograms of unloaded ι-carrageenan/gelatin capsules and their individual constituent biopolymers (A), and ι-carrageenan/gelatin capsules loaded with representative phenolic compounds (B).

As shown in Figure 5A, the TG curves of the unloaded ι-carrageenan/gelatin capsules showed a delayed degradation with respect to freeze-dried ι-carrageenan, which experienced a fast degradation at 200 °C. Although a gradual weight loss was experienced by the capsules up to 250 °C, due to both water loss and, presumably, a slower degradation of its constituent ι-carrageenan (due to its interactions with gelatin

and Ca^{2+}), their major weight loss was observed between 340 and 400 °C, that is, at a higher temperature than the temperature of maximum degradation rate of freeze-dried gelatin too (306 °C). Therefore, the obtained structures were more thermally stable than their individual biopolymer constituents, due to the structural changes caused by the existing intermolecular interactions.

The addition of phenolic compounds also affected the thermal stability of the capsules. Figure 5B shows the thermograms of two representative samples, i.e. those containing catechin and tannic acid, in comparison with the unloaded capsules. One of the main changes that occurred upon addition of these compounds was related to the loss of water molecules from the materials. While this first weight loss stage extended up to 250 °C for the unloaded capsules, the temperature of maximum weight loss for the phenolics-containing capsules was close to 100 °C. It has been described that proteins bind to water molecules at two different levels, i.e. absorbed and structural water (Correia, et al., 2013). Whilst absorbed water is removed from the samples up to 100 °C, structural water needs more energy to unbind and, thus, it is eliminated at higher temperatures (Gómez-Mascaraque, et al., 2015). Accordingly, these results suggested that only absorbed water was present in the samples prepared in the presence of the phenolic compounds, which was consistent with the lack of structural relaxation transition in their DSC thermograms.

On the other hand, the thermal degradation of the biopolymers in the capsules occurred more gradually but started at lower temperatures when the phenolic compounds were present, suggesting that the interactions between the phenolic compounds and gelatin, which competed with the stabilizing ι-carrageenan/gelatin interactions, had a negative impact on the thermal stability of the developed microencapsulation structures.

531

532 **3.6. Release of phenolic compounds from the capsules**

533 The release of the phenolic compounds from the developed capsules was assessed in
534 different media. Ethanol 10% and 50% (v/v) were selected as food simulants with
535 different hydrophilicity, according to the Commission Regulation 10/2011 EU
536 (10/2011/EC) (Comission, 2011). Acetic acid 3% (v/v) was also used as an acidic food
537 simulant, whose pH (2.9) was also close to that found in the human stomach.
538 Additionally, a PBS buffer (pH = 7.4) was used to mimic the pH found in the human
539 duodenum (Minekus, et al., 2014). The release profiles of all phenolic compounds in
540 ethanol 50% (the medium in which the release was maximal) are shown in Figure 6 as a
541 representative example. Figure 6 also shows the release profiles of one of the
542 polyphenols, i.e. tannic acid, in the different release media.

Figure 6. Release profiles of tannic acid from the developed capsules in different media (up) and release profiles of different phenolic compounds in ethanol 50% v/v (down).

The results showed that the release of the lowest molecular weight phenolic compounds (i.e. gallic acid, (+)-catechin and chlorogenic acid) from the capsules was negligible as compared to that of tannic acid. As mentioned above (cf. Section 3.3), the preparation of hydrogel capsules through extrusion techniques may yield low encapsulation efficiencies for small water soluble compounds which can easily diffuse out of the capsules towards the gelling bath. In light of the results from the release assays and in accordance with the findings observed from the FT-IR analysis, the lowest molecular weight phenolic compounds could not be efficiently incorporated within the capsules developed in this work and, as a result, their release in the food simulants was

negligible. In contrast, significant amounts of tannic acid were released from the capsules in all assayed media, revealing that this polyphenol was effectively retained within the capsules during the production process, as also confirmed by FT-IR spectroscopy.

In order to further confirm these results, the amount of phenolic compounds diffused out to the gelatin bath during the capsules production process was quantified by means of the Folin-Ciocalteu assay for catechin (as a representative low molecular weight phenolic compound) and for tannic acid. The results showed that while only $2.7 \pm 0.6\%$ of the tannic acid content in the carrageenan solution diffused out to the gelatin bath, up to $44.9 \pm 2.7\%$ of the catechin was detected in the gelling solution after production, confirming once more that tannic acid was more efficiently retained in the capsules. It is worth mentioning that significant amounts of the polyphenols might have also diffused out of the capsules during their filtration and washing with distilled water, implying that the actual loss of phenolic compounds was higher than that estimated in the assay.

The release profiles of tannic acid in the different release media are also shown in Figure 6. An initial burst release was observed in all food simulants, which is typical of hydrophilic matrices that rapidly swell in aqueous media (Atay, et al., 2017). Interestingly, differences in the amount of released polyphenol were observed depending on the food simulant used. Indeed, if a compound is bound to or interacting with its encapsulation matrix, its release will be the result of a balance between its affinity for this matrix *versus* its affinity for the solvent (Gómez-Mascaraque, et al., 2017), which varies for media with different polarities. The release of the polyphenol in PBS showed a particularly different trend, with a progressive decrease in the amount of

tannic acid detected after the initial burst release. This was attributed to the low stability of polyphenols (Gómez-Mascaraque, et al., 2015), and in particular tannic acid (Osawa & Walsh, 1993), in neutral or alkaline conditions, and its subsequent degradation during the assay. In all cases, the developed capsules were capable of releasing significant amounts of tannic acid in the different food simulants.

3.7. Impact of tannic acid concentration

The results from the previous sections showed that the intense intermolecular interactions between tannic acid and gelatin favoured the effective retention of this polyphenol within the developed capsules as compared to other lower molecular weight phenolic compounds used. However, this high affinity of tannic acid towards gelatin also caused the disruption of the nicely assembled coated matrix structure of the developed capsules. Anvari and Chung (2016) reported that, although high tannic acid concentrations ($> 0.2\%$) disrupted the interactions between biopolymers in fish gelatin-gum arabic coacervates, lower concentrations ($\leq 0.1\%$) of the polyphenol did allow the formation of the complex coacervates (Anvari, et al., 2016). Therefore, ι -carrageenan/gelatin hydrogel capsules were also produced in the presence of a lower concentration of tannic acid, i.e. 10% w/w with respect to ι -carrageenan, in order to study the impact of the polyphenol concentration on the developed encapsulation structures.

Figure 7A shows the structure of the obtained capsules, which exhibited a coated matrix structure more similar to that of the unloaded capsules, although considerably more irregular. This behaviour suggested that, although the lower concentration of tannic acid did not completely prevent the ι -carrageenan/gelatin capsule assembly, its high affinity

towards the protein did still compete to some extent with the intermolecular interactions between both biopolymers, somehow interfering with the assembling process. Indeed, the protein content of these capsules, estimated through nitrogen analysis, was 27.0 ± 0.5 %, which was significantly higher than for those loaded with a 30% tannic acid, but considerably lower than for the unloaded capsules (cf. Table 1).

Figure 7. A) Optical micrographs of t-carrageenan/gelatin hydrogel capsules loaded with 10% (w/w) tannic acid. B) Release profiles of tannic acid from the developed capsules produced with 10% and 30% (w/w) tannic acid in ethanol 50% v/v. The dotted line shows the profile for the 10% tannic acid samples multiplied by 3, to facilitate comparison with the 30% tannic acid samples.

The release of tannic acid from these capsules was also evaluated in ethanol 50% (v/v), i.e. the food simulant in which the release was maximal (cf. Figure 6), and the obtained profile is shown in Figure 7B in comparison to that of the capsules loaded with 30% w/w tannic acid. Naturally, the amount of tannic acid released at equilibrium from these capsules was lower, so the experimental release values were multiplied by 3 and the results were also plotted in Figure 7B to facilitate comparison between the samples.

The results showed that comparable relative amounts of tannic acid were released from both samples in the selected medium, so reducing the tannic acid load of the encapsulation structures was considered an effective approach to incorporate the polyphenol within the capsules without severely disrupting their structure.

3.8. Impact of capsule size on release

Electric field-aided extrusion is an effective approach to reduce the capsule size as compared to simple extrusion. Therefore, the impact of size on the release properties of the capsules was investigated. For this purpose, larger tannic acid-loaded capsules were also produced in the absence of electric field, and their release profiles in ethanol 50% (v/v) were evaluated after freeze-drying. The results are shown in Figure 8.

Figure 8. Release profiles of tannic acid from the capsules produced using the simple and electric field-aided extrusion methods with 30% w/w (up) and 10% w/w (down) tannic acid in ethanol 50% v/v.

The amount of tannic acid released from the capsules was higher for the larger capsules prepared by simple extrusion, regardless of the tannic acid load (10 or 30%). Since the smaller capsules had a higher surface to volume ratio, there are two possible explanations for these results. As mentioned earlier, the encapsulation efficiency for water-soluble compounds within hydrogel capsules prepared by extrusion may decrease do to their diffusion to the bath solution. Having a greater specific surface area, a faster diffusion of the polyphenol from the biopolymer matrix would be expected for the smaller capsules, resulting in a lower retention of tannic acid within the structures and, thus, a lower total release at equilibrium.

Another plausible explanation would be that part of the tannic acid content in the capsules was not released, due to its strong binding to the biopolymer matrix. This had been previously observed for grape juice extract polyphenols incorporated within ι -carrageenan/gelatin matrices in our previous work (Gómez-Mascaraque, et al., 2018). Since tannic acid strongly binds to gelatin, and given that the gelatin coating is located on the surface of the capsules, a greater specific surface area in the smaller capsules would imply a greater fraction of tannic acid molecules which could strongly bind to gelatin and, thus, not be released.

As a result, even though reducing the capsule size is generally desirable for food applications due to a reduced impact on the organoleptic properties of the final product, the impact of this size reduction on the release properties of the materials must be considered during the design of novel delivery vehicles.

3.9. Potential for the encapsulation of hydrophobic compounds

Although the previous results showed that the effective encapsulation of water-soluble compounds in the developed encapsulation structures is limited by their capacity to establish strong intermolecular interactions with the wall materials, especially for low molecular weight compounds, hydrogel beads usually yield considerably higher encapsulation efficiencies for hydrophobic bioactive ingredients (Gouin, 2004). Therefore, as a proof of concept, a model oil (soybean oil) was also incorporated within the structures by dispersing it in the ι -carrageenan solution before extrusion. The soybean oil was stained beforehand with Sudan III and an oil-in-water emulsion was prepared by intense homogenization of the oil within the carrageenan solution. Figure 9 shows microscopy images of the as-prepared and freeze-dried capsules containing soy

bean oil. These show the effective incorporation of the stained oil within the capsules, which are somewhat more irregular than those prepared in the presence of water-soluble compounds, and highlight the potential application of the proposed gelatin-coated ι-carrageenan hydrogel capsules for the effective encapsulation of hydrophobic compounds.

Figure 9. Optical micrographs of as-prepared (A) and freeze-dried (B) ι-carrageenan/gelatin hydrogel capsules loaded with stained soy bean oil.

4. Conclusions

This work showed that pseudo-spherical, millimetrical encapsulation structures based on gelatin-coated ι-carrageenan hydrogel beads can be produced through electric field-aided extrusion. These novel materials which, interestingly, exhibited shape-memory properties upon re-hydration in water, could be an alternative as encapsulation vehicles in the development of novel food products, since they were produced in aqueous solutions using edible biopolymers.

The ability of these capsules to entrap phenolic compounds within their structures was explored, concluding that they were not efficient in incorporating small molecular weight phenolic compounds such as gallic acid, (+)-catechin or chlorogenic acid within

their structures, as these easily diffused out of the hydrogel network. Nevertheless, tannic acid could be effectively incorporated within them, probably due to its higher molecular weight and its strong affinity for proteins. This great affinity, however, was found to compete with the gelatin/ι-carrageenan interactions, limiting the complexation between both biopolymers and causing significant structural changes on the capsules. These results highlight the need of studying the impact that adding bioactive ingredients has on the microstructure of the encapsulation vehicles themselves, fact that is often disregarded in the design of encapsulated ingredients. In this work, the negative impact that incorporating tannic acid within the capsules had on their microstructure could be minimised by decreasing its content.

Regardless of their tannic acid content, the developed capsules were capable of releasing significant amounts of tannic acid in different food simulants. This release was dependent on the size of the capsules, being higher for the larger beads prepared by simple extrusion than for the millimetrical capsules produced through electric field-aided extrusion. Therefore, despite the efficiency of the latter technique to achieve reduced capsule sizes, which are generally preferred to avoid undesirable effects on the textural properties of the food products, the impact of this size reduction on the release properties of the materials should also be evaluated when designing novel encapsulation structures.

Finally, the suitability of the developed materials for the encapsulation of hydrophobic food ingredients was also confirmed by effectively incorporating a stained soybean oil within the gelatin/ι-carrageenan hydrogel beads.

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