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

Laura G. Gómez-Mascaraque a, Carla Soler b, Amparo Lopez-Rubio a, * 

a Food Preservation and Food Quality Department, IATA-CSIC, Avda. Agustin Escardino 7, 46980, Paterna, Valencia, Spain 
b Institute of Materials Science, University of Valencia, C/ Catedrático Jose Beltrán 2, 46980, Paterna, Valencia, Spain

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.

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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; Dube, Ng, 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...
hydrophilic domains (Gómez-Mascaraque, Méndez, Fernández-Gutiérrez, Vázquez, & San Román, 2014). 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 (Roman, Burri, & Singh, 2012). 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 (Dube et al., 2010a; Shpigelman, Cohen, & Livney, 2012). 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-β-D-glucopyranose units in a proportion which depends on the degree of deacetylation of chitin (Khor & Lim, 2003) (cf. Fig. 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 (Lim, Hwang, Kar, & Varghese, 2014). Several beneficial properties such as antimicrobial activity (Kong, Chen, Xing, & Park, 2010), lipid-lowering effects (Kerch, 2015), and wound healing (Ueno et al., 1999) have been attributed to this polysaccharide, which has been previously used to stabilize EGCG in nanoparticles by the ionic gelation method with tripolyphosphate (Dube et al., 2010a, 2010b). Through spray-drying, it has also been used to successfully encapsulate other polyphenolic extracts like olive leaf extract (Kosaraju, D’ath, & Lawrence, 2006) or yerba mate extract (Harris, Lecumberri, Mateos-Aparicio, Mengíbar, & Heras, 2011).

Gelatin is a protein obtained from partial hydrolysis of naturally occurring collagen and contains repeating sequences of glycine-aa1-aa2, where amino acids aa1 and aa2 are mainly proline and hydroxyproline (Lai, 2013) (cf. Fig. 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 (Peña, de la Caba, Eceiza, Ruseckaite, & Mondragon, 2010) 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 (Rousenova et al., 2012), and more recently as a microencapsulation material. Particularly, gelatin has been previously used to protect EGCG in microcapsules produced by layer-by-layer assembly (Shutava et al., 2009a, 2009b), coacervation in combination with different polysaccharides (Fang & Bhandari, 2010) or by electrospraying (Gómez-Mascaraque et al., 2015). Through spray-drying, it has also been used to microencapsulate other flavonoids like naringin (Sansone, Aquino, Gaudio, Colombo, & Russo, 2009).

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 thermoresponsive 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.

![Fig. 1. Schematic chemical structures of raw materials: a) gelatin, b) chitosan and c) EGCG.](image-url)
2. Experimental section

2.1. 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 \((K_2SO_3)\), buffer solutions of pH 7.4 (phosphate buffered saline system, PBS) and pH 6.1 (2-(N-morpholino)ethanesulfonic acid hemisodium salt, MES), potassium bromide FTIR grade (KBr), pepsin from porcine gastric mucosa, pancreatic from porcine pancreas and bile extract porcine were obtained from Sigma-Aldrich. Acetic acid (96% v/v), sodium acetate (dihydrate), 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 Scharlab (Barcelona, Spain). Gelatin stock solutions with a concentration of 10% (w/v) were prepared by dissolving the protein in acetic acid 20% (v/v) at 40 °C 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 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 \((R^2_{Gel} = 0.995 \text{ and } R^2_{Ch} = 0.999)\) 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):

\[
\text{MEE}(\%) = \frac{\text{Actual EGCG content in the capsules}}{\text{Theoretical EGCG content in the capsules}} \times 100
\]

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

2.2. 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 °C 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 mm cellulose filter purchased from Scharlab (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

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 deposited on the microencapsulation efficiency (MEE) of the systems was then calculated using Eq. (1):

\[
\text{MEE}(\%) = \frac{\text{Actual EGCG content in the capsules}}{\text{Theoretical EGCG content in the capsules}} \times 100
\]

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

2.4. 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 °C 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 spectrophotometry (Shanghai Spectrum model SP-2000UV, Shanghai, China) by measuring the absorbance at 274 nm (maximum of absorbance of EGCC). 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, \(k_1\) are kinetic constants, and \(m\) is the Fickian diffusion exponent \((\text{Siepmann & Peppas, 2012})\).

\[
\frac{M_t}{M_0} = k_1 \cdot t^m + k_2 \cdot t^{2m}
\]

2.5. Antioxidant activity of EGCG-containing micro-hydrogels

ABTS⁺ radical scavenging assay was performed in order to quantify the antioxidant activity of both free and encapsulated EGCC, following the decolorization assay protocol described in a previous work \((\text{Gómez-Mascaraque et al., 2015})\). Briefly, a stock solution of ABTS⁺ 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$^+$ 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 \mu L of diluted sample solution were added to 1 mL of diluted ABTS$^+$, 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):

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

where $A_0$ and $A_1$ are the absorbances at 734 nm of ABTS$^+$ 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.

2.6. 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 (Minekus et al., 2014). Solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized compositions (Minekus et al., 2014). In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at 37 °C 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 °C 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 °C for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively, using 1 M HCl or 1 M 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$^+$ assay, as an indirect assessment of the bioaccessibility of EGCG after digestion. HPLC-MS was also used to analyze the digestas, HPLC-MS was also used to analyze the samples as described before.

2.7. Protection ability of gelatin spray-dried microhydrogels

The ABTS$^+$ 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$^+$ assay.

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

2.8. 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.

3. Results and discussion

3.1. 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 (Peres et al., 2011), minimizing the degradation of the bioactive molecule when present.

The morphology of the obtained powders is shown in Fig. 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 (De Cicco, Porta, Sansone, Aquino, & Del Gaudio, 2014; Fu et al., 2011; Kusonwiriyawong, Lipipun, Vardhanabhuti, Zhang, & Ritthidej, 2013). 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 vapors inside the drying particles, before solidification of the matrix (Peres et al., 2011).

The particle size distributions of the spray-dried powders, determined by laser diffraction, are shown in Fig. 3. The results were in agreement with the particle sizes observed in Fig. 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.

The presence of absorption bands attributed to EGCG in the infrared spectra of the loaded particles (cf. Fig. 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 concentration in the mixtures. The thermogravimetric (TG) profiles of pristine materials and their corresponding spray-dried particles, both unloaded and EGCG-loaded, were analyzed to ascertain possible thermostability effects (Dube et al., 2010b).

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 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 (Siebert, Troukhanova, & Lynn, 1996), the absence of partitioning effects (Dube et al., 2010b).

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 Fig. S1.

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.

3.2. 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 (Tola & Ramaswamy, 2014). Fig. 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 h, 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 (Siepmann & Peppas, 2012), 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 (Ritger & Peppas, 1987). 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.

Both spray-dried matrices exhibited higher absolute values for k₁ than for k₂. Given that the first term of the Peppas-Sahlin equation (k₁) is related to the contribution of the diffusion phenomenon to the overall release kinetics, and the second term (k₂) accounts for the case-II transport or relaxational phenomenon (Siepmann & Peppas, 2012), 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 (Gómez-Mascaraque et al., 2015). The negative values obtained for k₂ 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.

3.3. Antioxidant activity of EGCG-loaded micro-hydrogels

The radical scavenging activity of both encapsulated and free EGCG was assessed by means of the ABTS⁺ decolorization assay to
ascertain whether the microencapsulation 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.

3.4. 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 (Roman et al., 2012). Thus, the assessment of the bioaccessibility of the encapsulated functional ingredients is of utmost 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” (Commission, 2006). 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” (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009), that is, “the fraction that is soluble in the gastrointestinal environment” (Cardoso, Afonso, Lourenço, Costa, & Nunes, 2015).

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−• 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 subtracted 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.

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

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**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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_{onset}$ (°C)</th>
<th>$T_{max1}$ (°C)</th>
<th>$T_{max2}$ (°C)</th>
<th>$WL_1$ (%)</th>
<th>$WL_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>228.8</td>
<td>235.4</td>
<td>483.9</td>
<td>33.7</td>
<td>62.3</td>
</tr>
<tr>
<td>Gel</td>
<td>265.6</td>
<td>301.3</td>
<td>537.7</td>
<td>45.2</td>
<td>36.9</td>
</tr>
<tr>
<td>Ch</td>
<td>268.9</td>
<td>293.5</td>
<td>542.7</td>
<td>53.8</td>
<td>40.4</td>
</tr>
<tr>
<td>Spray-dried Gel</td>
<td>244.0</td>
<td>267.0/322.2</td>
<td>534.9</td>
<td>62.2</td>
<td>31.9</td>
</tr>
<tr>
<td>Spray-dried Gel + EGCG</td>
<td>238.1</td>
<td>268.7/329.3</td>
<td>518.4</td>
<td>65.53</td>
<td>32.1</td>
</tr>
<tr>
<td>Spray-dried Ch</td>
<td>218.1</td>
<td>257.8</td>
<td>458.4</td>
<td>62.1</td>
<td>25.76</td>
</tr>
<tr>
<td>Spray-dried Ch + EGCG</td>
<td>222.6</td>
<td>262.3</td>
<td>471.9</td>
<td>59.0</td>
<td>28.5</td>
</tr>
</tbody>
</table>

**Table 2**

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k_1$ (h$^{-0.425}$)</th>
<th>$k_2$ (h$^{-0.856}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray-dried Gel + EGCG</td>
<td>0.51 ± 0.06</td>
<td>-0.12 ± 0.02</td>
<td>0.996</td>
</tr>
<tr>
<td>Spray-dried Ch + EGCG</td>
<td>0.82 ± 0.01</td>
<td>-0.21 ± 0.01</td>
<td>0.996</td>
</tr>
</tbody>
</table>

**Table 3**

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>RSA (%)</th>
<th>Standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>3.2$^a$</td>
<td>0.3</td>
</tr>
<tr>
<td>Spray-dried Gel</td>
<td>2.8$^a$</td>
<td>0.3</td>
</tr>
<tr>
<td>Spray-dried Ch</td>
<td>2.4$^a$</td>
<td>1.1</td>
</tr>
<tr>
<td>EGCG</td>
<td>26.9$^b$</td>
<td>0.9</td>
</tr>
<tr>
<td>Spray-dried Gel + EGCG</td>
<td>26.0$^b$</td>
<td>2.4</td>
</tr>
<tr>
<td>Spray-dried Ch + EGCG</td>
<td>22.7$^c$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Different letters (a–c) within the same column indicate significant differences among the samples.
zymes were present in the simulated matrix in an aqueous buffer (cf. Fig. 5). From these results it was consistent with the EGCG release pro-
activity of free EGCG after the duodenal phase. These values are for free EGCG after the gastric phase, it increased up to a 68% of the
though this fraction only represented the 27% of the value obtained
loaded Gel microcapsules during digestion. Therefore, even activity was observed for the soluble fraction of digested EGCG-
free EGCG, as observed from the decrease in the antioxidant activity
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. Fig. 5). From these results it was seen that around 50% of the EGCG content was released in MES after 3.3 h. Given that digestion lasted 4 h and that digestive en-
zymes 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 agree-
ment 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 Fig. 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 Fig. S3).

Interestingly, the mass spectra shown in Fig. 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 oligo-
meric forms (Serra et al., 2010) thus suggesting that encapsulation could improve bioavailability by hindering the formation of oligo-
meric 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 oc-
curs in intestinal fluids (Serra et al., 2010).

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 (Lim et al., 2014). In fact, the short oral phase of the digestion, although frequently disregarded in many studies (Minukus et al., 2014), seemed to be crucial for assessing the bio-
accessibility 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 poly-
saccharide in aqueous media (Filion, Lavertu, & Buschmann, 2007).

This neutralization of the Ch molecules would have hindered swelling of the particles and release of their EGCG contents. Pre-
vious studies had observed that neutralization of chitosan mem-
branes prepared from acetic acid solutions triggered a molecular rearrangement of its polymer chains, modifying its physico-
chemical properties and swelling behavior (Campos, Nogueira Campos, Ferreira Grosso, Cárdenas, & Inocentini Mei, 2005). 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. Figs. S4 and S5 in the Supplementary material), thus supporting our hypothesis.

With the aim of confirming that the differences observed in Table 4 were attributable to a lack of EGCG release from the caps-
ules during digestion, the duodenal digestas (including the insol-
uble fraction) were subjected to treatment with acetic acid (20% v/ v) and vigorous agitation during 4 h, with the aim of dissolving the encapsulation structures. The treated digestas were then subjected to the ABTS⁺ 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 signifi-
cantly 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 Fig. 6, which was much more limited when EGCG was encapsulated within the developed Gel micro-hydrogels.

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

As gelatin proved to be a more adequate matrix for the encap-
sulation 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⁺ 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 (Gómez-Mascaraque et al., 2015). After different degradation periods, the samples in PBS were diluted 20-fold with acetic acid 20% v/v to stop the degradation process by

### Table 4

Antioxidant activity of supernatants from gastric and duodenal digestas.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RSA (%) of gastric digesta</th>
<th>RSA (%) of duodenal digesta</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>84 ± 8²</td>
<td>52 ± 9²</td>
</tr>
<tr>
<td>Spray-dried Gel + EGCG</td>
<td>23 ± 2b</td>
<td>36 ± 3b</td>
</tr>
<tr>
<td>Spray-dried Ch + EGCG</td>
<td>15 ± 3³</td>
<td>5 ± 2³</td>
</tr>
</tbody>
</table>

* 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⁺ at 734 nm. Different letters (a–c) within the same column indicate significant differences among the samples.
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$^+$ assay and the results are shown in Fig. 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 (Barras et al., 2009; Li et al., 2009).

An HPLC-MS/MS analysis was carried out to confirm these results. As shown in Fig. 8A, after 24 h 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 h (cf. Fig. 8B and Supplementary Fig. S6 for more details).

Moreover, as observed from the chromatograms of non-encapsulated EGCG in Fig. 8, the decrease in the EGCG peak was accompanied by the appearance of a peak at 2.1 min which increased with incubation time and that corresponded to EGCG degradation products as shown in Supplementary Fig. 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 (Mizooki, Yoshikawa, Tsuneyoshi, & Arakawa, 2003). 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.

4. 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$^+$ 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 micro-encapsulated 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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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2016.05.009.

References


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