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

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ABSTRACT

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

KEYWORDS

Microencapsulation; gelatin; chitosan; flavonoids; EGCG; bioaccessibility

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, Shankar, & Srivastava, 2011; Singh, Akhtar, & Haqqi, 2010; Steinmann, Buer, Pietschmann, & Steinmann, 2013), being (–)-epigallocatechin gallate (EGCG) the most abundant polyphenol in green tea possessing the greatest biological activity (Barras et al., 2009). However, EGCG is sensitive to heat (Wang, Zhou, & Wen, 2006), oxygen (Valcic, Burr, Timmermann, & Liebler, 2000) and light (Scalia, Marchetti, & Bianchi, 2013) and, in general, chemically unstable (Dube, Ng, Nicolazzo, & Larson, 2010a), especially in aqueous solutions (Dube, Nicolazzo, & Larson, 2010b; Li, Lim, & Kakuda, 2009). Thus, encapsulation of this bioactive compound has been widely explored to improve its stability during food processing and storage (Dube et al., 2010b; Fang & Bhandari, 2010; Gómez-Mascaraque, Lagarón, & López-Rubio, 2015; Li et al., 2009; Shutava, Balkundi, & Lvov, 2009a; Shutava et al., 2009b). Several techniques have been explored for this purpose, being costs and the use of food permitted solvents and matrices the limiting factors for the practical use of encapsulation structures in functional food applications. In this sense, spray-drying is the most widely used encapsulation technique in the food industry (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2014), as it is a straightforward and cheap procedure which allows the processing of a wide range of food-grade materials with accessible equipment (Gharsallaoui,

60 Roudaut, Chambin, Voilley, & Saurel, 2007). This technique involves the initial atomization of a
61 formulation containing the wall matrix and the bioactive into fine droplets, followed by their
62 rapid drying using a hot gas stream, which leads to solvent evaporation and rapid formation of
63 the microparticles. Regarding the encapsulation matrices, food-grade biopolymers capable of
64 forming physical hydrogels are of particular interest. Physical hydrogels are polymer networks
65 characterized by the presence of physical crosslinks, entanglements and/or rearrangements of
66 hydrophobic and hydrophilic domains (Gómez-Mascaraque, Méndez, Fernández-Gutiérrez,
67 Vázquez, & San Román, 2014). Thus, hydrogel-forming biopolymers can be processed in
68 aqueous solutions, while preventing dissolution of the obtained microparticles in aqueous foods
69 under certain conditions. While the protection exerted by the microparticles has obvious benefits
70 during the commercialization stage, an important aspect is the bioaccessibility of the functional
71 compounds after ingestion, as it has been recently observed that encapsulation may decrease it to
72 a certain extent (Roman, Burri, & Singh, 2012). Another desirable property of potential
73 encapsulating materials for the protection of EGCG is their processability in acidic pHs, as this
74 antioxidant molecule exhibits higher stability in acidic media (Dube et al., 2010a; Shpigelman,
75 Cohen, & Livney, 2012). Chitosan and gelatin are two edible, naturally-derived biopolymers
76 satisfying both requirements.

77 Chitosan is a linear polysaccharide obtained by deacetylation of naturally occurring chitin
78 and consists of β-1,4 linked 2-acetamido-2-deoxy-β-D-glucopyranose units and 2-amino-2-
79 deoxy-b-D-glucopyranose units in a proportion which depends on the degree of deacetylation of
80 chitin (Khor & Lim, 2003) (cf. Figure 1). Chitosan is soluble in acidic aqueous solutions, where
81 its amino groups are protonated, but gels at neutral or alkaline solutions because of the strong
82 intermolecular interactions, being considered a pH-sensitive hydrogel (Lim, Hwang, Kar, &

83 Varghese, 2014). Several beneficial properties such as antimicrobial activity (Kong, Chen, Xing,
84 & Park, 2010), lipid-lowering effects (Kerch, 2015), and wound healing (Ueno et al., 1999) have
85 been attributed to this polysaccharide, which has been previously used to stabilize EGCG in
86 nanoparticles by the ionic gelation method with tripolyphosphate (Dube et al., 2010a; Dube et
87 al., 2010b). Through spray-drying, it has also been used to successfully encapsulate other
88 polyphenolic extracts like olive leaf extract (Kosaraju, D'ath, & Lawrence, 2006) or yerba mate
89 extract (Harris, Lecumberri, Mateos-Aparicio, Mengíbar, & Heras, 2011).

90 Gelatin is a protein obtained from partial hydrolysis of naturally occurring collagen and
91 contains repeating sequences of glycine-aa_1-aa_2, where amino acids aa_1 and aa_2 are mainly
92 proline and hydroxyproline (Lai, 2013) (cf. Figure 1). Gelatin forms thermoreversible hydrogels
93 in aqueous solutions due to the formation of collagen-like triple helices below the so-called
94 helix–coil transition temperature, leading to chains entanglement and subsequent network
95 formation (Peña, de la Caba, Eceiza, Ruseckaite, & Mondragon, 2010) and, thus, it is considered
96 as a thermo-responsive hydrogel-forming biopolymer. Gelatin has been traditionally used for the
97 production of macro-capsules by the pharmaceutical industry (Roussenova et al., 2012), and
98 more recently as a microencapsulation material. Particularly, gelatin has been previously used to
99 protect EGCG in microcapsules produced by layer-by-layer assembly (Shutava et al., 2009a;
100 Shutava et al., 2009b), coacervation in combination with different polysaccharides (Fang et al.,
101 2010) or by electrospraying (Gómez-Mascaraque et al., 2015). Through spray-drying, it has also
102 been used to microencapsulate other flavonoids like naringin (Sansone, Aquino, Gaudio,
103 Colombo, & Russo, 2009).

104 In the present work, spray-dried edible micro-hydrogels based on the polysaccharide
105 chitosan and the protein gelatin were produced and used to microencapsulate EGCG as a model

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

113

114 INSERT FIGURE 1 ABOUT HERE

115

116 **2. EXPERIMENTAL SECTION**

117 **2.1. Materials**

118 Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, low
119 molecular weight chitosan (Ch), with reported Brookfield viscosity of 20.000 cps, (–)-
120 epigallocatechin gallate (EGCG), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
121 diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), buffer solutions of pH 7.4 (phosphate
122 buffered saline system, PBS) and pH 6.1 (2-(N-morpholino)ethanesulfonic acid hemisodium salt,
123 MES), potassium bromide FTIR grade (KBr), pepsin from porcine gastric mucosa, pancreatin
124 from porcine pancreas and bile extract porcine were obtained from Sigma-Aldrich. Acetic acid
125 (96% v/v) was purchased from Scharlab and Pefabloc® from Fluka. All inorganic salts used for
126 the *in-vitro* digestion tests were used as received.

127 Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized
128 water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus
129 from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose
130 filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity >
131 98%) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain).
132
133 **2.2. Preparation of biopolymer microparticles**
134 Gelatin stock solutions with a concentration of 10% (w/v) were prepared by dissolving
135 the protein in acetic acid 20% (v/v) at 40 ºC under magnetic agitation. The stock solutions were
136 then cooled down to room temperature and further diluted 50-fold in distilled water before
137 processing.
138 Chitosan stock solutions with a concentration of 2% (w/v) were prepared by dissolving
139 the polysaccharide in acetic acid 20% (v/v) at room temperature under magnetic agitation. The
140 stock solutions were further diluted 50-fold in distilled water before processing.
141 When EGCG was incorporated for its encapsulation, it was added to the corresponding
142 biopolymer stock solutions at room temperature under magnetic stirring, at a concentration of 10
143 (w/w) of the total solids content.
144 The biopolymer solutions were fed to a Nano Spray Dryer B-90 apparatus (Büchi,
145 Switzerland) equipped with a 7.0 μm pore diameter cap. The inlet air temperature was set at 90
146 ºC, as it proved to be enough to achieve complete drying of the particles at an inlet air flow of
147 146 ± 4 L/min and a reduced pressure of 50 ± 3 mbar. Under these conditions, the outlet air
148 temperature varied between 44 and 50 ºC. The spray-dried powders were deposited on the
149 collector electrode by means of an applied voltage of 15 kV.

150

151 **2.3. Characterization of the microparticles**

152 Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-
153 4100) at an accelerating voltage of 10 kV and a working distance of 9-16 mm. Samples were
154 sputter-coated with a gold-palladium mixture under vacuum prior to examination. Particle
155 diameters were measured from the SEM micrographs in their original magnification using the
156 ImageJ software. Size distributions were obtained from a minimum of 200 measurements.

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

161 Empty and bioactive-containing capsules of ca. 1 mg were grounded and dispersed in 130
162 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing
163 the sample at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker
164 (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging
165 10 scans at 1 cm^{-1} resolution. The microencapsulation efficiency (MEE) of the EGCG-loaded
166 capsules was determined based on FTIR absorbance measurements. Calibration curves (R_{Gel}^2 =
167 0.995 and R_{Ch}^2 = 0.999) were obtained using Gel/EGCG and Ch/EGCG physical mixtures,
168 respectively, of known relative concentrations (0, 5, 10 and 15 % w/w of EGCG). For the
169 Gel/EGCG calibration curve, the relative maximum absorbances at 1409 cm^{-1} (corresponding to
170 gelatin) and 1039 cm^{-1} (attributed to EGCG) were plotted against the EGCG concentration in the
171 mixtures. For the Ch/EGCG calibration curve, the relative maximum absorbances at 2885 cm^{-1}
172 (corresponding to chitosan) and 1223 cm^{-1} (attributed to EGCG) were used. The EGCG content

in the capsules was interpolated from the obtained linear calibration equations, and the MEE of the systems was then calculated using Eq. (1):

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

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

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

The extracted aliquots were analyzed by UV-vis spectroscopy (Shanghai Spectrum model SP-2000UV, Shanghai, China) by measuring the absorbance at 274 nm (maximum of absorbance of EGCG). Calibration curves for EGCG quantification in MES solution by UV-vis absorbance were previously obtained (R^2 = 0.999). The EGCG release values were obtained from three independent experiments at the same conditions.

Experimental data were fitted to the Peppas-Sahlin semi-empirical model, whose general equation is Eq. 2, where M_t is the mass of EGCG released at time t, M_0 is the total mass of EGCG loaded in the particles, k_i are kinetic constants, and m is the Fickian diffusional exponent (Siepmann & Peppas, 2012).

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

2.5. Antioxidant activity of EGCG-containing micro-hydrogels

$ABTS^{\bullet+}$ radical scavenging assay was performed in order to quantify the antioxidant activity of both free and encapsulated EGCG, following the decolorization assay protocol described in a previous work (Gómez-Mascaraque et al., 2015). Briefly, a stock solution of $ABTS^{\bullet+}$ was prepared by reacting ABTS with potassium persulfate (7 and 2.45 mM in distilled water, respectively) and allowing the mixture to stand in the dark at room temperature for 24 h. The $ABTS^{\bullet+}$ stock solution was then diluted with acetic acid 20% v/v to an absorbance of 0.70±0.02 at 734 nm. Stock solutions of free and encapsulated EGCG (5 mM of EGCG in all cases) were prepared in acetic acid 20% v/v and subsequently diluted 20-fold. 10 μL of diluted sample solution were added to 1 mL of diluted $ABTS^{\bullet+}$, and the absorbance at 734 nm was measured 1 min after initial mixing. The radical scavenging activity (RSA), expressed as the percentage of reduction of the absorbance at 734 nm after sample addition, was calculated using Eq. (3):

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

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

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

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 ºC for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37 ºC for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37 ºC for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively, using 1M HCl or 1M NaOH solutions. After the duodenal phase, the protease inhibitor Pefabloc® (1 mM) was added. Aliquots were collected after the gastric and the duodenal phases and snap-frozen in liquid

242 nitrogen immediately. The antioxidant activity of the digestas was estimated after centrifugation
243 by means of the $ABTS^{\bullet+}$ assay, as an indirect assessment of the bioaccessibility of EGCG after
244 digestion. HPLC-MS was also used to analyze the digestas. The LC system used for this analysis
245 was an Agilent 1290 HPLC system. Separation of EGCG was performed using an Acquity BEH
246 C18 (Waters, 50 mm × 2.1 mm, 1.7 µm of particle size) LC-column. The flow rate was set to 0.4
247 mL/min and the oven temperature was 30 ºC, being eluent A water slightly acidified with 0.1%
248 of formic acid, and eluent B methanol slightly acidified with 0.1% of formic acid. The elution
249 gradient started with 5% of eluent B during 2 min, increasing to 95% B in 11 min. The injection
250 volume was 10 µl. A Triple TOF™ 5600 system with a DuoSprayTM source operating in the
251 negative ESI mode was used for detection (AB SCIEX, CA, USA). The following parameter
252 settings were used: ion spray voltage, - 4500 ISVF; ion source heater, 400 °C ; curtain gas, 25
253 psi; ion source gas 1.50 - 2.50 psi. For the full MS-IDA (information dependent acquisition)
254 MS/MS analysis, the full MS, the survey scan, and the MS/MS experiments were run in positive
255 mode with a scan range from m/z 100 to m/z 800 and a 250 ms accumulation time for the full
256 MS. The declustering potential was -150 eV and collision energy was -77 eV. The MS was using
257 an IDA acquisition method with two experiments: the survey scan type (TOF-MS) and the
258 dependent scan type (product ion). Data was evaluated using the XIC manager in the
259 PeakViewTM software.
260
261 **2.7. Protection ability of gelatin spray-dried microhydrogels**
262 The $ABTS^{\bullet+}$ assay was also used to evaluate the ability of the selected gelatin micro-
263 hydrogels to protect EGCG from degradation in aqueous media. For this purpose, the antioxidant
264 activity of free and encapsulated EGCG was measured after dissolution/suspension in PBS.

265 Solutions (5 mM) of EGCG in PBS were prepared. Suspensions of EGCG-loaded capsules in
266 PBS with theoretical EGCG concentrations of 5 mM were also prepared. After specific time
267 intervals, the solutions/suspensions were diluted 20-fold with acetic acid 20% v/v and their RSA
268 was calculated using eq. (2), after conducting the $ABTS^{\bullet+}$ assay.
269 In order to confirm the results obtained from the degradation assays, HPLC-MS was also
270 used to analyze the samples as described before.
271
272 **2.8. Statistical analysis**
273 A statistical analysis of experimental data was performed using IBM SPSS Statistics
274 software (v.23) (IBM Corp., USA). Significant differences between homogeneous sample groups
275 were obtained through two-sided t-tests (means test of equality) at the 95% significance level (p
276 < 0.05). For multiple comparisons, the p-values were adjusted using the Bonferroni correction.
277
278 **3. RESULTS AND DISCUSSION**
279 **3.1. Characterization of EGCG-loaded spray-dried biopolymeric microparticles**
280 Spray-dried powders were produced from a protein (Gel) and from a polysaccharide
281 (Ch), both derived from naturally occurring biopolymers, and both leading to micro-hydrogels
282 upon hydration in aqueous media. It is important to remark that the solutions were processed at
283 considerable lower temperatures than those described in other works for the production of
284 EGCG-loaded spray-dried biopolymeric particles (Peres et al., 2011), minimizing the
285 degradation of the bioactive molecule when present.
286 The morphology of the obtained powders is shown in Figure 2. Pseudo-spherical particles
287 were observed, but different morphologies could be distinguished. Some particles exhibited a

288 corrugated surface, while others revealed a smooth surface. These two types of morphology have
289 been previously described for spray-dried particles obtained from aqueous solutions (De Cicco,
290 Porta, Sansone, Aquino, & Del Gaudio, 2014; Fu et al., 2011; Kusonwiriyawong, Lipipun,
291 Vardhanabhuti, Zhang, & Ritthidej, 2013). Interestingly, a third morphology was also observed
292 only in the gelatin samples, where concave and considerably bigger particles were also
293 generated. This shape is typical of the so-called 'ballooning' effect which occurs at high drying
294 rates when the polymeric matrix is elastic enough to enable this dents formation due to the
295 thermal expansion of air or water vapors inside the drying particles, before solidification of the
296 matrix (Peres et al., 2011).
297
298 INSERT FIGURE 2 ABOUT HERE
299
300 The particle size distributions of the spray-dried powders, determined by laser diffraction,
301 are shown in Figure 3. The results were in agreement with the particle sizes observed in Figure 2,
302 and showed that Gel provided significantly bigger microparticles than Ch. This might be due to
303 differences in the packing structure and density of the two polymers, but also to the lower
304 polymer concentration in the Ch solutions, owing to the high viscosity of this polysaccharide.
305 The polydispersity was also lower for microparticles made of Ch. For Gel, the 'ballooning' effect
306 resulted in some particles being substantially bigger than others. Nevertheless, the apparent
307 bimodal distribution observed for this sample was attributed to multi-particle aggregation, as
308 particles having sizes about 100 μm were not observed at all in the SEM micrographs. The
309 EGCG-loaded particles showed similar morphologies and particle size distributions as compared
310 to their unloaded counterparts.

311

312 INSERT FIGURE 3 ABOUT HERE

313

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

323

324 INSERT FIGURE 4 ABOUT HERE

325

326 The MEE of the EGCG-loaded capsules was 95% ± 6% and 82% ± 9% for Gel and Ch,
327 respectively. These high encapsulation efficiency values can be explained considering the great
328 solubility of EGCG in the feed solutions and Moreover, the EGCG-loaded particles experienced
329 some shifts of their infrared bands with respect to the components alone, such as the amide III
330 band of gelatin which moved to 1240 cm^{-1} or the displacement of the Amide I band of Ch to
331 lower wavenumbers, suggesting intermolecular interactions between the antioxidant molecule
332 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 Figure S1.

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

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

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

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). Figure 5 shows an initial burst release from both chitosan and gelatin micro-hydrogels, which was more abrupt for the chitosan matrix. The release from the

355 gelatin capsules was noticeably more sustained in the initial hours. While the maximum release
356 from chitosan was observed within the first 10 hours, the maximum EGCG release from gelatin
357 was attained more than 5 times later, suggesting that gelatin micro-hydrogels are more effective
358 in delaying the dissolution of the antioxidant in aqueous media than their chitosan counterparts.
359
360 INSERT FIGURE 5 ABOUT HERE
361
362 One of the most common semi-empirical models used to describe the release kinetics of
363 bioactive molecules from delivery systems is the Peppas-Sahlin equation (Siepmann et al.,
364 2012), which takes into account the combination of Fickian (diffusion) and non-Fickian
365 (polymer relaxation) release mechanisms. Thus, this model was used to fit the first points of the
366 experimental data (up to 10 h), when the sink assumption is valid (Ritger & Peppas, 1987). Table
367 2 shows the EGCG release kinetic parameters for both microencapsulation matrices in the MES
368 aqueous food simulant according to the Peppas-Sahlin equation, assuming a spherical
369 morphology (i.e. aspect ratio of 1) for the spray-dried micro-hydrogels and hence a Fickian
370 diffusional exponent (m) of 0.425.
371
372 **Table 2.** EGCG release kinetic parameters (k_i) and the linear correlation coefficients (R^2)

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

373
374 Both spray-dried matrices exhibited higher absolute values for k_1 than for k_2. Given that
375 the first term of the Peppas-Sahlin equation (k_1) is related to the contribution of the diffusion
376 phenomenon to the overall release kinetics, and the second term (k_2) accounts for the case-II

377 transport or relaxational phenomenon (Siepmann et al., 2012), the values in Table 2 suggest that
378 the predominant release mechanism for these micro-hydrogels was a diffusion phenomenon.
379 These results are in good agreement with those previously reported for EGCG-loaded gelatin
380 microparticles obtained by electrospraying (Gómez-Mascaraque et al., 2015). The negative
381 values obtained for k_2 in both cases indicated that the swelling (or relaxation) of the polymeric
382 matrices impeded the EGCG release in the initial burst release phase, due to the fast solvent
383 uptake. The Peppas-Sahlin model also confirmed the faster EGCG release kinetics from the Ch
384 hydrogels than from the Gel matrix, which could in part be attributed to their smaller particle
385 size and consequent higher specific area, besides the intrinsic differences in the release
386 mechanisms from both matrices.
387 Even though the release was slower from the gelatin matrix, it was still relatively fast for
388 these capsules to be directly applied to beverage foods, as the antioxidant molecule would be
389 released during their storage. Rather, they would be more appropriate for the formulation of dry
390 food products, which may require processing as a liquid or humid paste for a limited time but
391 dried before storage, such as pastry or bakery products.
392
393 **3.3. Antioxidant activity of EGCG-loaded micro-hydrogels**
394 The radical scavenging activity of both encapsulated and free EGCG was assessed by
395 means of the $ABTS^{\bullet+}$ decolourization assay to ascertain whether the microencapsulation process
396 had an impact on the antioxidant activity of the bioactive. There were no significant differences
397 between the inhibition of the absorbance caused by the solvent blank and the two unloaded
398 hydrogels (cf. Table 3), so the antioxidant activity of the matrices was indeed neglected. The
399 RSA of the encapsulates was thus attributed only to the contribution of their EGCG content.

While no significant differences were found between the RSA of the gelatin encapsulates and the free EGCG, suggesting that the antioxidant activity of the bioactive was fully retained during the encapsulation process, the chitosan encapsulates showed a lower RSA than EGCG in its free form, supporting the lower encapsulation observed when chitosan was used as the encapsulating matrix. In fact, the antioxidant activities measured are in close agreement with encapsulation efficiencies estimated from the infrared spectra of the materials, as the gelatin micro-hydrogels retained 97% of the antioxidant activity of free EGCG, whereas chitosan encapsulates showed only 84% of its RSA.

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

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

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

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 outmost importance in the design of novel functional foods, given that the Regulation (EC) 1924/2006 on nutrition and health claims made on foods declares (in Section 15) that "In order to ensure that the claims made are truthful […] The substance should also be available to be used by the body" (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 $ABTS^{\bullet+}$ assay, which provided an indirect estimation of the EGCG content released from the matrix during digestion. The unloaded spray-dried micro-hydrogels, as well as blank samples (water) were also digested in order to disregard possible contributions of the encapsulation matrices and/or the enzymes added during digestion to the total antioxidant activity of the digestas. The value of RSA obtained for the digestas of the unloaded hydrogels and the blanks were then substracted from the RSA of the corresponding EGCG-containing digestas in order to take into account only the contribution of their EGCG content to their total antioxidant activity. The results are summarized in Table 4.

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

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

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

446 The RSA of the digestas of free EGCG and, consequently, its bioaccessibility, was
447 significantly higher than the value obtained for encapsulated EGCG, suggesting that only part of
448 the EGCG content was released from the microcapsules during *in-vitro* GI digestion, while free
449 EGCG was already completely dissolved in water before digestion. This resulted in the partial
450 degradation of free EGCG, as observed from the decrease in the antioxidant activity after the
451 duodenal phase, while an increase in this antioxidant activity was observed for the soluble
452 fraction of digested EGCG-loaded Gel microcapsules during digestion. Therefore, even though
453 this fraction only represented the 27% of the value obtained for free EGCG after the gastric
454 phase, it increased up to a 68% of the activity of free EGCG after the duodenal phase. These
455 values are consistent with the EGCG release profile obtained for the gelatin matrix in an aqueous
456 buffer (cf. Figure 5). From these results it was seen that around 50% of the EGCG content was
457 released in MES after 3.3 hours. Given that digestion lasted 4 hours and that digestive enzymes
458 were present in the simulated fluids, plus differences in the pH and ionic strength, and a slightly
459 higher temperature, a release of a 68% of the EGCG content during digestion was in good
460 agreement with the release results obtained.
461 To further confirm these results, the supernatant of the digestas of EGCG-loaded Gel
462 micro-hydrogels were analyzed by HPLC-MS. The mass spectrum of EGCG is shown in
463 Supplementary Figure S2. The HPLC-MS experimental results obtained from the encapsulates
464 followed the same trend as in the antioxidant studies, i.e. a greater difference in EGCG content
465 was observed after the gastric phase, while only slight differences were observed in the digestas
466 from the free and Gel encapsulated EGCG after the duodenal phase (cf. Supplementary Figure
467 S3).
468

469 INSERT FIGURE 6 ABOUT HERE

470

471 Interestingly, the mass spectra shown in Figure 6 suggested that after the gastric phase,
472 the amount of dimer EGCG (m/z 915) in the digesta obtained from free EGCG was greater than
473 in the Gel-encapsulated counterpart, where the monomeric compound (m/z 457) was most
474 abundant. It is important to emphasize that while catechin monomers are readily absorbed in
475 human subjects and animals, there are controversies about the bioavailability of oligomeric
476 forms (Serra et al., 2010) thus suggesting that encapsulation could improve bioavailability by
477 hindering the formation of oligomeric species.

478 At the end of the intestinal digestion process, there was a marked decrease in the amount
479 of EGCG which, in agreement with previous studies, showed that the major degradation of
480 EGCG occurs in intestinal fluids (Serra et al., 2010).

481 Regarding the bioaccessibility of EGCG microencapsulated in Ch, it was considerably
482 lower than for the protein matrix. These results were unexpected considering the faster release of
483 EGCG in aqueous media from the polysaccharide than from gelatin. However, they could be
484 explained in light of the pH-responsive behavior of Ch hydrogels (Lim et al., 2014). In fact, the
485 short oral phase of the digestion, although frequently disregarded in many studies (Minekus et
486 al., 2014), seemed to be crucial for assessing the bioaccessibility of EGCG encapsulated in Ch.
487 The neutral pH of the simulated salivary fluid would have neutralized the previously protonated
488 amino groups of spray-dried Ch (as a consequence of its processing in acetic acid), favoring the
489 formation of strong intra- and intermolecular interactions through hydrogen bonding (possibly
490 involving the bioactive molecule) which are characteristic of Ch and are the main reason for the
491 poor solubility of this polysaccharide in aqueous media (Filion, Lavertu, & Buschmann, 2007).

492 This neutralization of the Ch molecules would have hindered swelling of the particles and release
493 of their EGCG contents. Previous studies had observed that neutralization of chitosan
494 membranes prepared from acetic acid solutions triggered a molecular rearrangement of its
495 polymer chains, modifying its physico-chemical properties and swelling behavior (Campos,
496 Nogueira Campos, Ferreira Grosso, Cárdenas, & Inocentinni Mei, 2005). In order to confirm this
497 neutralization during the oral phase, spray-dried Ch was subjected to the neutral pH found in the
498 salivary solution and dried under vacuum. The bands in the 1800 – 1500 cm^{-1} region of the
499 infrared spectrum of the resulting sample shifted back to the profile exhibited by the raw
500 chitosan (cf. Figures S4 and S5 in the Supplementary material), thus supporting our hypothesis.
501 With the aim of confirming that the differences observed in Table 4 were attributable to a
502 lack of EGCG release from the capsules during digestion, the duodenal digestas (including the
503 insoluble fraction) were subjected to treatment with acetic acid (20% v/v) and vigorous agitation
504 during 4 hours, with the aim of dissolving the encapsulation structures. The treated digestas were
505 then subjected to the $ABTS^{\bullet+}$ assay, observing no significant differences between the RSA
506 corresponding to free EGCG and both encapsulated EGCG samples (cf. Table S1 in the
507 Supplementary material). Hence, the presence of the antioxidant compound in the insoluble
508 fraction of the duodenal digestas of the encapsulates was corroborated.
509 Comparing both matrices, although the bioaccessibility of EGCG decreased upon
510 microencapsulation in both cases, it was significantly higher for Gel than for Ch, so the protein
511 was considered to be a more suitable encapsulation matrix than the polysaccharide. Moreover, as
512 commented on above, although a greater bioaccessibility was observed, the bioavailability of
513 free EGCG during digestion might be compromised by the formation of oligomeric species, as

514 deduced from the mass spectra in Figure 6, which was much more limited when EGCG was
515 encapsulated within the developed Gel micro-hydrogels.
516
517 3.5. **Protection ability of spray-dried gelatin micro-hydrogels**
518 As gelatin proved to be a more adequate matrix for the encapsulation of EGCG than
519 chitosan, not only exhibiting higher encapsulation efficiencies and, thus, higher retention of the
520 antioxidant activity, but also a more delayed EGCG release in aqueous solution and higher
521 bioaccessibility of the bioactive after digestion, this matrix was selected to carry out an *in-vitro*
522 degradation test. This test was also based on the $ABTS^{\bullet+}$ decolorization assay and was used to
523 compare the degradation profiles of free and gelatin-encapsulated EGCG in PBS, by monitoring
524 the decrease in their RSA value with time after dissolution or suspension in this medium.
525 Hence, solutions of EGCG (5 mM) and suspensions of EGCG-loaded gelatin micro-
526 hydrogels (theoretical EGCG concentration also 5 mM) in PBS were prepared. The fast
527 degradation of EGCG upon dissolution in this buffer could be visually observed by a change in
528 color from a light pink to an intense yellowish color, as seen in previous work (Gómez-
529 Mascaraque et al., 2015). After different degradation periods, the samples in PBS were diluted
530 20-fold with acetic acid 20% v/v to stop the degradation process by lowering the pH of the
531 medium, and to facilitate the complete dissolution of the Gel micro-hydrogels. The RSA of the
532 resulting solutions was then calculated by means of the $ABTS^{\bullet+}$ assay and the results are shown
533 in Figure 7.
534
535 INSERT FIGURE 7 ABOUT HERE
536

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).

INSERT FIGURE 8 ABOUT HERE

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

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

560

561 **4. Conclusions**

562 Spray-dried micro-hydrogels based on gelatin and chitosan were proposed as
563 encapsulating matrices for the model flavonoid EGCG. Although the polysaccharide gave rise to
564 smaller microparticles with narrower size polydispersity than the protein, the latter achieved
565 higher microencapsulation efficiencies (95% ± 6%) than the former (82% ± 9%), as estimated by
566 infrared spectroscopy. These results were confirmed by $ABTS^{\bullet+}$ assays, which corroborated that
567 the gelatin micro-hydrogels retained 97% of the antioxidant activity of free EGCG, while
568 chitosan showed only 84% of its radical scavenging activity. The TGA profiles of the samples
569 suggested that microencapsulation was successful in stabilizing the bioactive thermally, as only
570 the degradation steps attributed to the matrices were detected. The release of EGCG from the
571 spray-dried particles when suspended in slightly acidic aqueous solution was faster from the
572 chitosan matrix, so gelatin was more effective in delaying the solubility of the flavonoid in this
573 medium. Furthermore, the bioaccessibility of EGCG after digestion was higher when
574 microencapsulated in gelatin than in chitosan, as the latter pH-sensitive micro-hydrogel
575 precluded the release of the bioactive after being neutralized in the basic mouth conditions.
576 Moreover, encapsulation in gelatin hindered EGCG dimer formation, which may also have an
577 impact from the bioavailability viewpoint. The overall results suggested that gelatin-based
578 micro-hydrogels are more adequate as encapsulation matrices for flavonoids than chitosan for
579 application in the development of functional foods. The capability of gelatin to stabilize EGCG
580 against degradation in slightly alkaline aqueous solution was also demonstrated, thus broadening
581 the potential for food incorporation.

582

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740

741

742 **Figure Captions**

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

744 **Figure 2**. SEM images of spray-dried particles

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

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

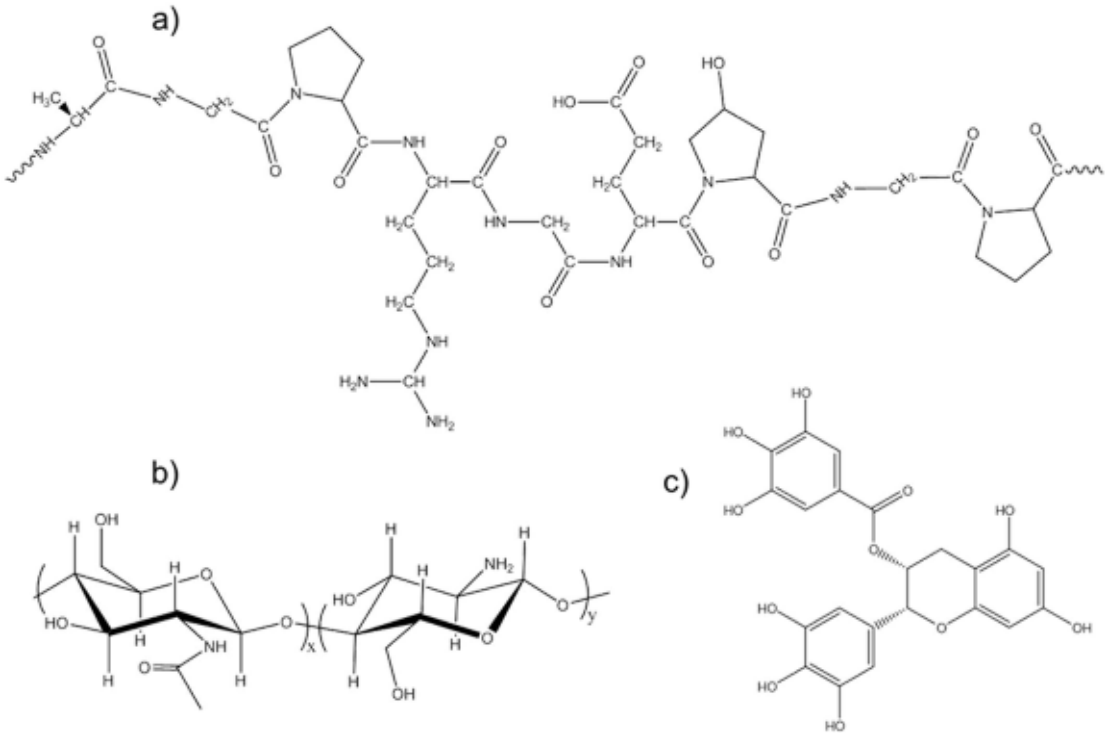
747 **Figure 5.** EGCG release profiles from the spray-dried micro-hydrogels. The inset shows an
748 extended release profile up to 350 hours.

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

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

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

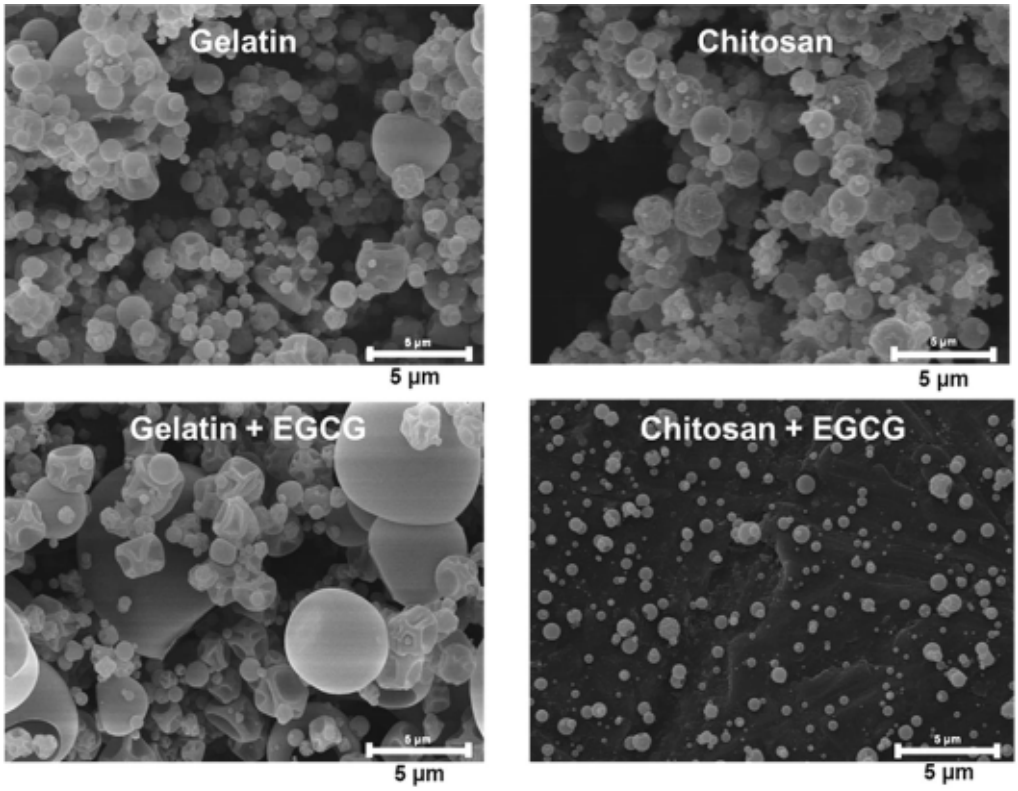
756



757

758 **FIGURE 1.**

759



760

761 **FIGURE 2.**

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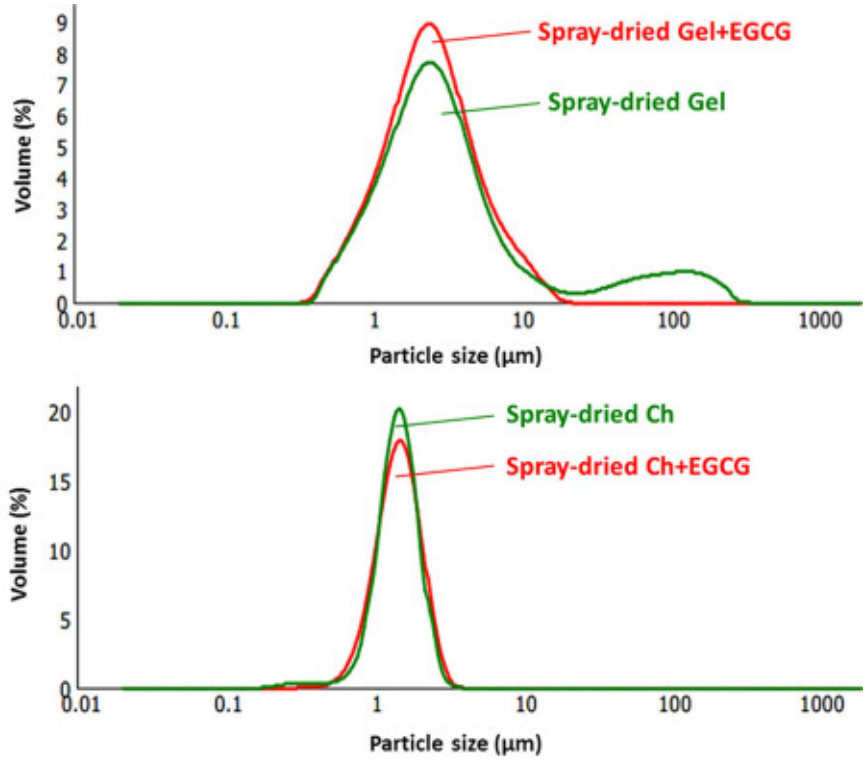

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764 **FIGURE 3.**

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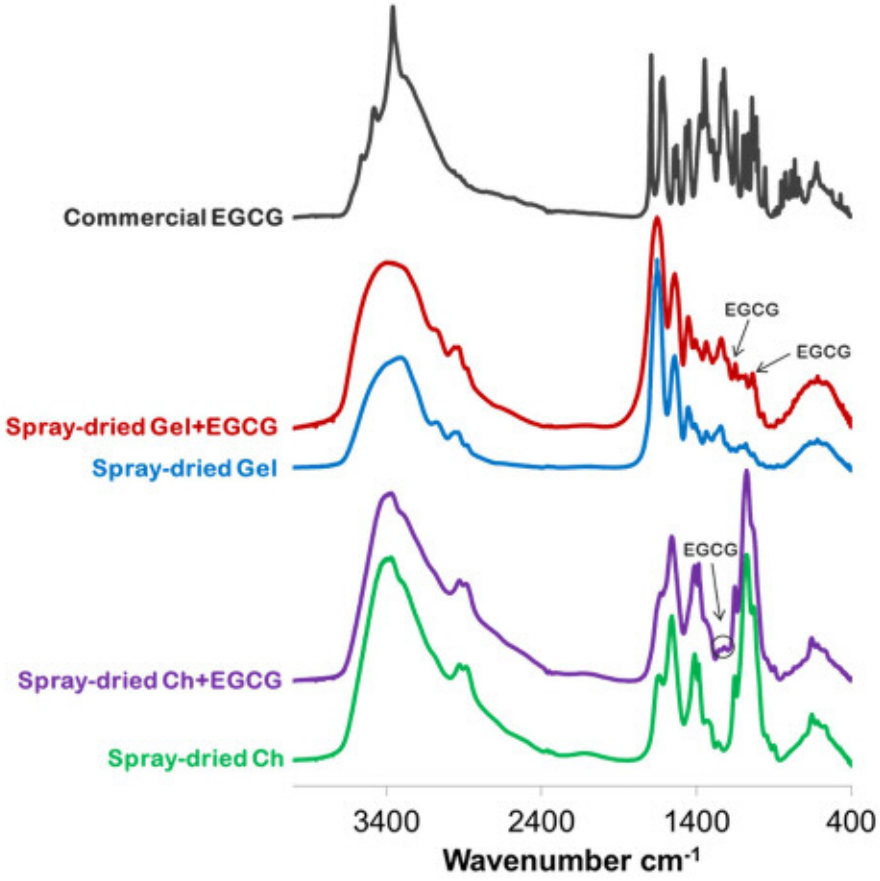

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767 **FIGURE 4.**

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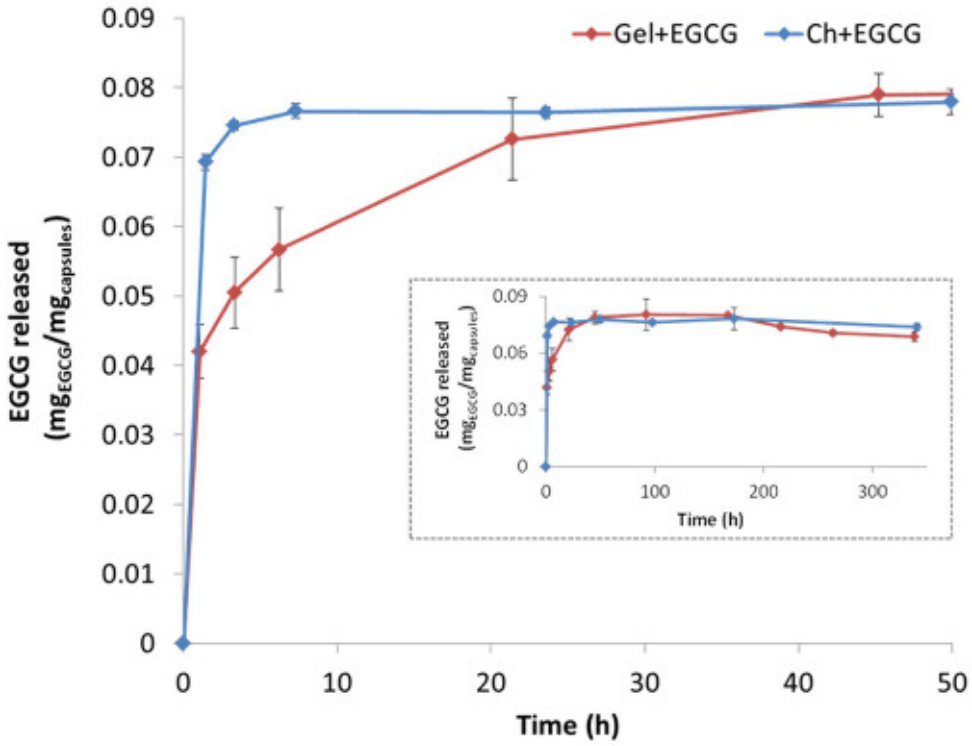

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770 **FIGURE 5.**

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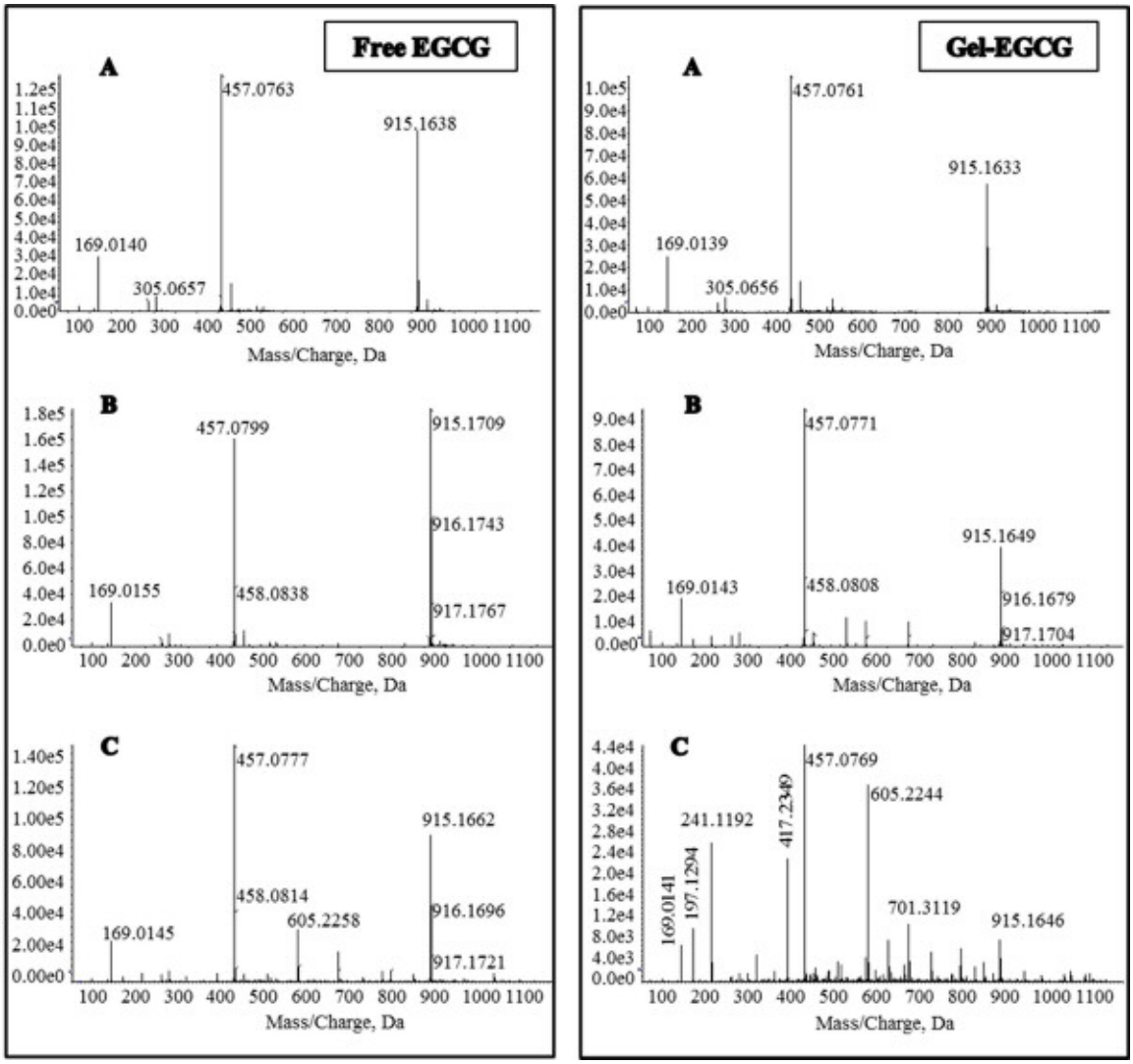

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773 **FIGURE 6.**

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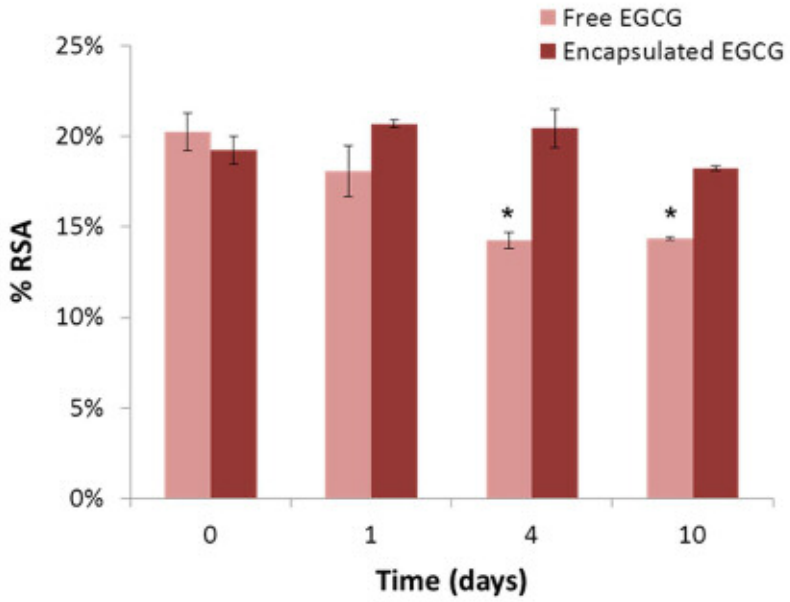

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776 **FIGURE 7.**

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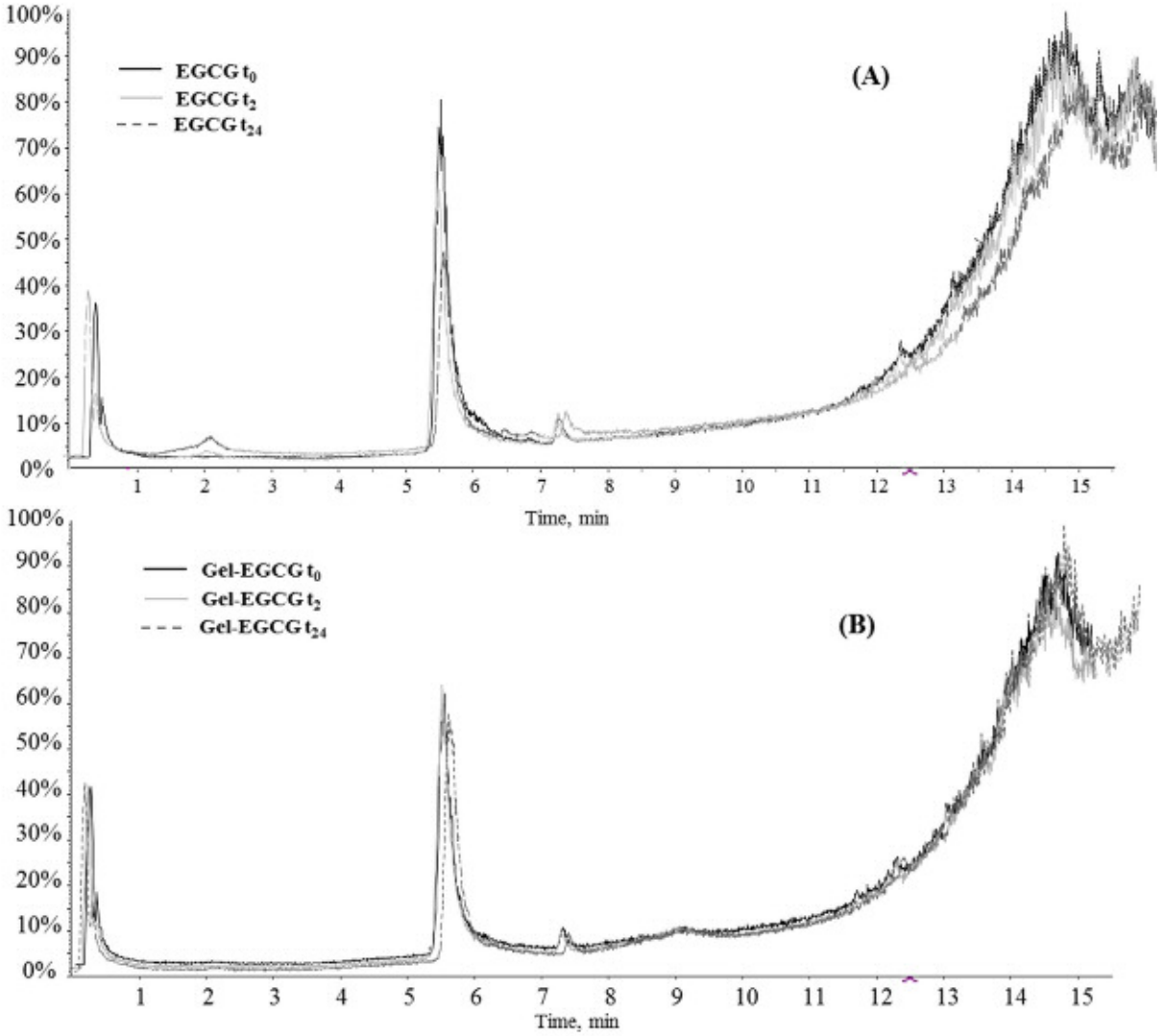

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779 **FIGURE 8.**

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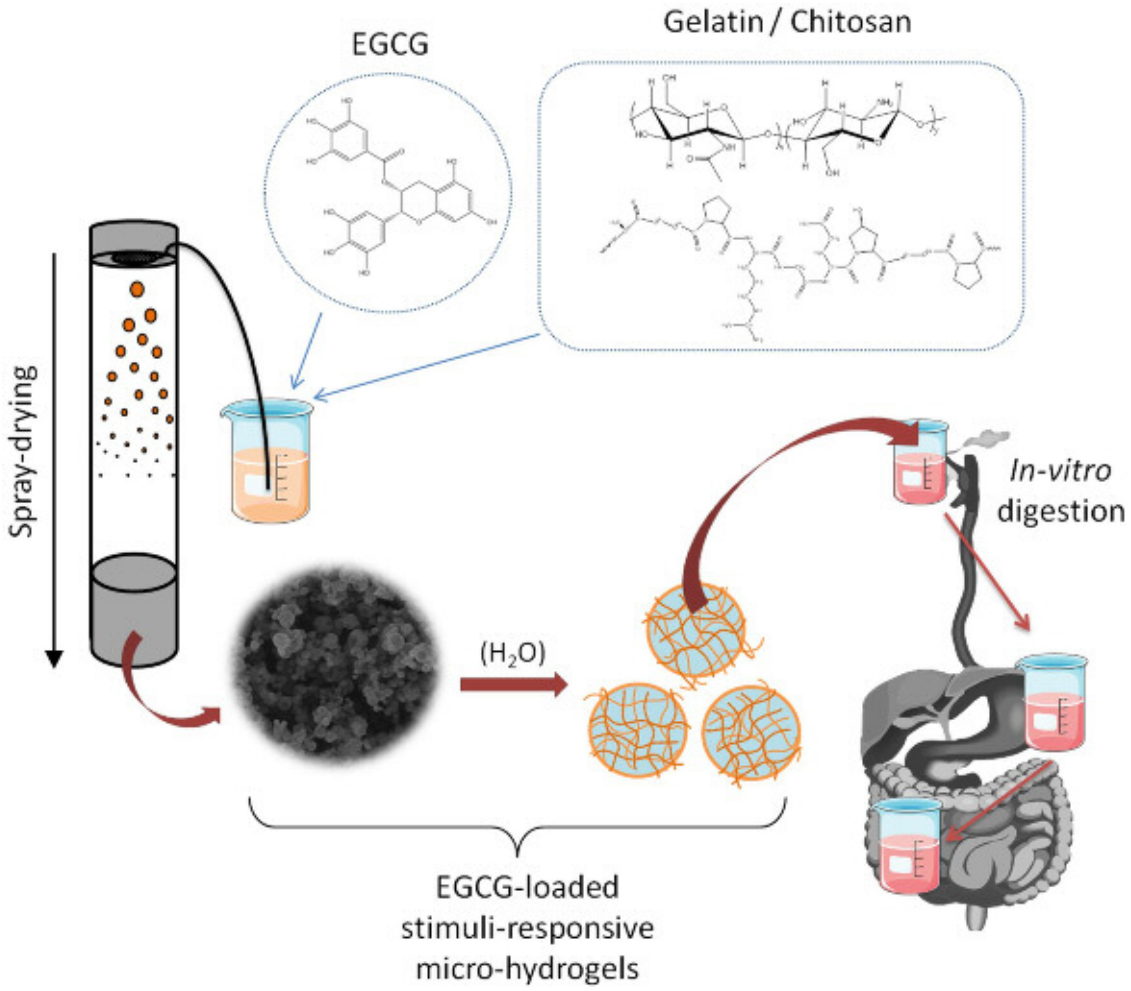

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782 **GRAPHICAL ABSTRACT**

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HIGHLIGHTS

- Gelatin retained the antioxidant activity of EGCG more effectively than chitosan
- Gelatin delayed the solubility of EGCG in aqueous medium to a greater extent
- Bioaccessibility of EGCG was greater when encapsulated in gelatin than in chitosan
- Microencapsulation in gelatin hindered EGCG dimer formation during digestion
- Gelatin stabilized EGCG against degradation in alkaline aqueous solution

Supporting Information

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

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Results and Discussion

Thermal stability of the microparticles

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

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

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

It is worth noting that the water loss during the first stage also changed after spray-drying the biopolymers. While the raw materials experienced a weight loss which extended well above

100ºC, especially in the case of gelatin, all spray-dried samples lost the water they contained before reaching 100ºC. This suggests that only absorbed water was present in the spray-dried samples, and that structural water was removed during processing due to the rapid drying of the materials.

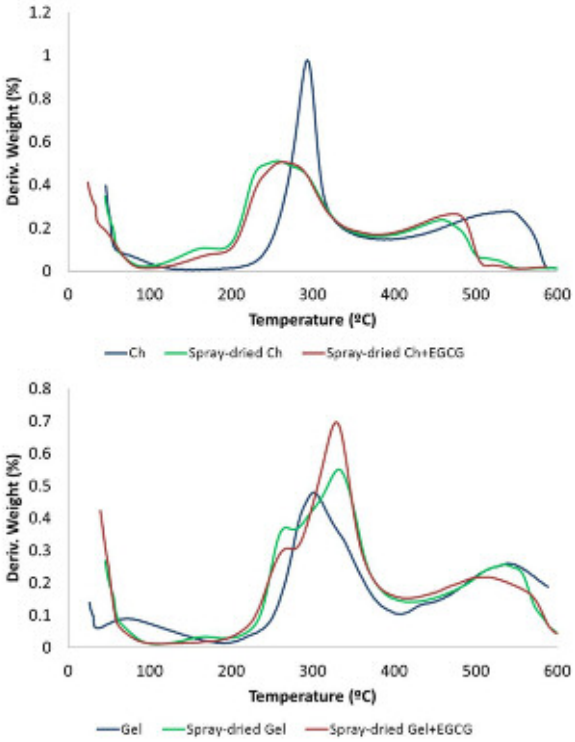

Figure S1. DTG curves of raw matrices and spray-dried particles of Ch (top) and Gel (bottom)

In-vitro GI digestion and bioaccessibility assessment

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

the mobile phase modifier, formic acid. Finally, the adduct ion at m/z 493, showed the chlorine adduct ion.

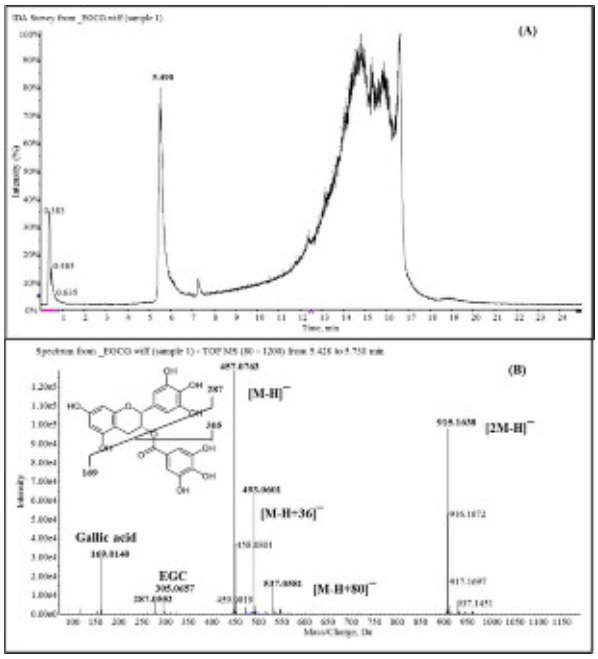

Figure S2. Information dependant acquisition (IDA) experiment of EGCG in PBS sample (t=0h) (A) Total Ion Chromatogram (B) EGCG spectrum and compound identification.

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

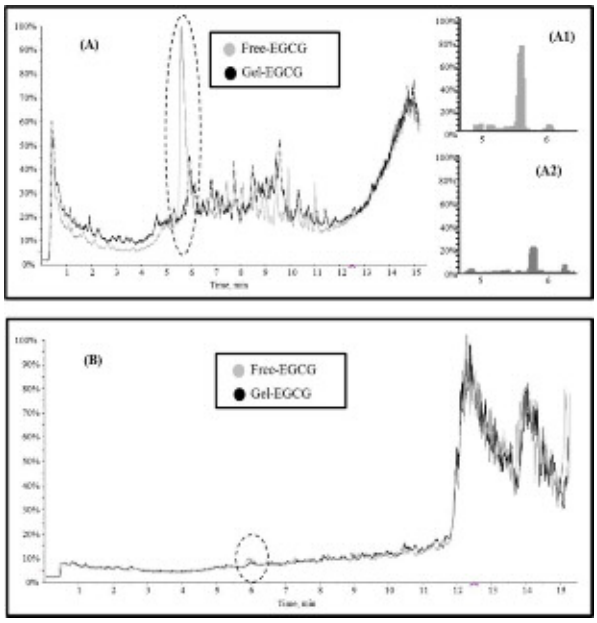

Figure S3: Information dependant acquisition (IDA) experiment of EGCG (A) TIC of gastric digesta extract (A1 corresponding to free-EGCG and A2 to Gel-encapsulated EGCG extracted chromatogram) and (B) TIC of intestinal digesta extract.

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

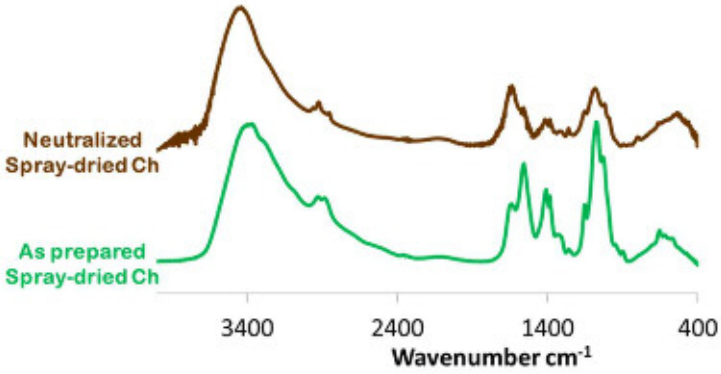

Figure S4. Spray-dried chitosan before (as prepared) and after contacting the simulated salivary fluid (neutralized).

Protection ability of spray-dried gelatin micro-hydrogels

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

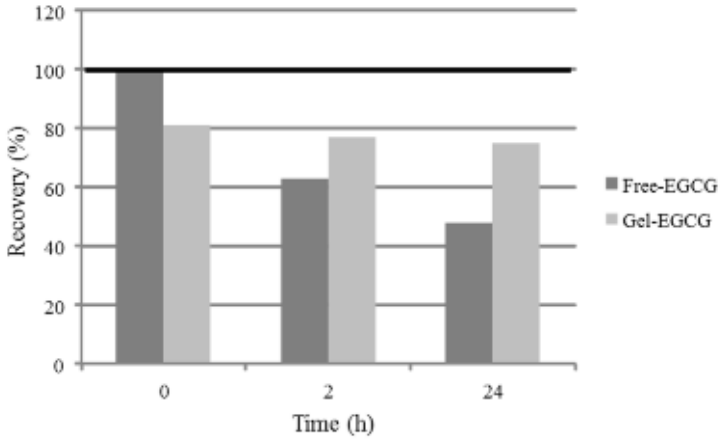

Figure S5: Stability studies of EGCG in PBS medium after 2 and 24 hours incubation.

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

As it can be observed in Figure S6, the mass spectrum of this peak exhibits the ions m/z 455 and m/z 473, which increased along incubation time. After an in-depth and exhaustive review of the published literature about EGCG degradation, these ions were identified as O-methyl

derivatives[8]. Since EGCG is a potent antioxidant, it tends to be oxidized within biological environments, thus leading to lower bioavailability and short half-life limiting its therapeutic efficiency[9]. Unfortunately, and as far as we know, no previous studies have been published regarding the antioxidant activity of EGCG O-methyl derivatives. However, Dueñas et al.[10] evaluated the antioxidant capacity of O-methylated metabolites of catechin, epicatechin and quecertin, concluding that despite the relative decrease of antioxidant capacity of these metabolites, they still presented relatively high antioxidant activity.

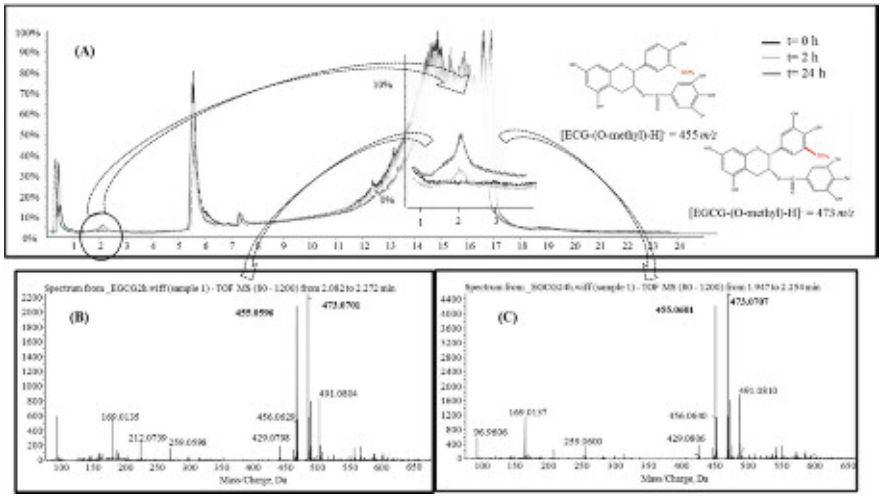

Figure S6. Information dependant acquisition (IDA) experiment of EGCG degradation after one day in PBS (A) Total Ion Chromatogram at different times (0 hour, 2 hours and 24 hours); (B) EGCG Spectrum at 2 hours and (C) EGCG Spectrum at 24 hours corresponding to 2.1 minutes peak.

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