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

Laura G. Gómez-Mascaraque¹, Marai Hernández-Rojas², Paula Tarancón¹, Mathieu Tenon³, Nicolas Feuillère³, Jorge F. Vélez Ruiz², Susana Fiszman¹, Amparo López-Rubio^{1*}

¹Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Agustín Escardino 7, 46980 Paterna, Valencia, Spain.

²Universidad de las Americas, Puebla, Santa Catarina Martir, Cholula, 72820, Puebla, Mexico.

³Naturex SA, Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France.

*Corresponding author: Tel.: +34 963900022; fax: +34 963636301

E-mail address: amparo.lopez@iata.csic.es (A. López-Rubio)

ABSTRACT

In this work, a green tea extract (GTE) was encapsulated within electrosprayed protein (i.e. gelatin and zein) microparticles, and the protective ability of both systems on the green tea catechins was assessed. The microparticles (with encapsulation efficiencies ~90 g/100g), proved to be very effective in stabilizing the catechins during a thermal treatment at 180 °C (12 min), preserving 85-90 g/100g of their initial catechins content, while free GTE lost almost 40 g/100g of its catechins content. In order to assess the impact of microencapsulation in a real food system, the GTE-loaded electrosprayed microparticles were added to biscuits dough. Results showed that microencapsulation did not significantly protect during biscuit processing and emphasized the need of assessing the behaviour of microencapsulation systems in real food processing conditions. The sensorial analysis of the biscuits indicated that addition of the GTE-loaded microparticles did not impact the acceptability of the biscuits, as perceived by consumers.

KEYWORDS

Electrospraying; encapsulation; green tea extract; catechin; biscuits

1. Introduction

Green tea catechins are a group of polyphenolic antioxidants which have received increasing research attention in recent years because of their reported health promoting properties, such as anti-carcinogenic (Larsen & Dashwood, 2010) and antimicrobial effects (Steinmann, Buer, Pietschmann, & Steinmann, 2013). Among them, (–)-epigallocatechin gallate (EGCG) is the most abundant and bioactive flavonoid in green tea (Barras et al., 2009). Together with (–)-epicatechin gallate (ECG) it is accepted as an indicator of the quality of green tea extracts (GTE) (Sharma & Zhou, 2011). These products are being increasingly added to a variety of foods in order to improve their nutritional value (Ananingsih, Gao, & Zhou, 2013) and make them more appealing to consumers (Yilmaz, 2006), whose interest towards functional foods is increasing (Siró, Kápolna, Kápolna, & Lugasi, 2008).

Despite the promising benefits of supplementing food products with GTE, there are a number of technological concerns due to the poor stability of tea catechins, which are thermosensitive and highly unstable in alkaline conditions, especially EGCG and ECG (Su, Leung, Huang, & Chen, 2003), so their bioactivity could be compromised upon food processing and storage. Moreover, GTE has been reported to negatively impact aroma, flavour and overall acceptability of bread and, thus, the quality of the final food products (Bajerska, Mildner-Szkudlarz, Jeszka, & Szwengiel, 2010). Being one of the most popular bakery products consumed by nearly all levels of society and all age groups (Tarancón, Fiszman, Salvador, & Tárrega, 2013), biscuits are regarded as good candidates to be fortified with functional ingredients (Choudhury, Badwaik, Borah, Sit, & Deka, 2015). They are convenient snacks with a long shelf life and appealing sensory attributes (Fradinho, Nunes, & Raymundo, 2015). In fact, the supplementation of biscuits with GTE (Mildner-Szkudlarz, Zawirska-Wojtasiak, Obuchowski, & Gośliński,

2009; Sharma & Zhou, 2011) or grounded tea leaves (Gramza-Michałowska et al., 2016) has already been attempted with different purposes. However, biscuit doughs generally have high pH values, and high temperatures are used to bake them, threatening the stability of tea catequins if supplemented with GTE (Sharma & Zhou, 2011).

Microencapsulation, or entrapment of an ingredient within a protective micron-sized matrix, is a plausible approach to protect sensitive bioactive molecules against degradation (Ye, Cui, Taneja, Zhu, & Singh, 2009). Electrospraying has recently emerged as an alternative to traditional microencapsulation techniques in the food industry such as spray-drying (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007), as it can be performed under mild temperatures (Lopez-Rubio & Lagaron, 2012), being specially adequate for the encapsulation of thermosensitive functional ingredients (Gómez-Mascaraque & López-Rubio, 2016). This technology, based on electrohydrodynamic processing, consists of subjecting a polymer solution to a high voltage electric field in such a way that a charged polymer jet is ejected towards a grounded collector, generating dry polymeric particles as the jet breaks down into fine droplets during its flight and the solvent evaporates (Bhardwaj & Kundu, 2010). For food applications, solutions or suspensions of edible biopolymers such as proteins or polysaccharides containing the ingredient to be encapsulated can be electrosprayed using food-grade solvents (Gómez-Mascaraque, Ambrosio-Martín, Fabra, Pérez-Masiá, Lagaron, & López-Rubio, 2016). Furthermore, these food macromolecules are known to interact with polyphenols (Jakobek, 2015; Le Bourvellec & Renard, 2012), which may contribute to the stabilization of these bioactive compounds when encapsulated within them (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015).

In previous work, we demonstrated the potential of electrosprayed gelatin submicroparticles to protect EGCG against degradation in alkaline conditions (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015). Zein fibers produced through electrohydrodynamic processing have also been reported to be effective in stabilizing EGCG in aqueous environment (Li, Lim, & Kakuda, 2009). The aim of the present study was to evaluate GTE-containing microparticles produced through electrospraying using both protein matrices when applied to a real food product. Specifically, they were added to biscuit dough. The protection exerted by the encapsulation matrices on the catechins upon biscuit preparation was evaluated by comparing the loss of these compounds and their antioxidant activity in comparison with the direct addition of the non-encapsulated GTE to the biscuit dough. A sensorial analysis was also conducted to assess whether the incorporation of the GTE and/or the microparticles to the biscuit formulation had an impact on the consumers' acceptability.

2. Materials and Methods

2.1 Materials and ingredients

Type A gelatin from porcine skin (Gel), with reported gel strength of 175 g Bloom, and zein prolamine from corn, grade Z3625, were both purchased from Sigma-Aldrich and used as received without further purification. A green tea extract (GTE) with high oxygen radical absorbance capacity (ORAC) was kindly donated by Naturex (Avignon, France). Potassium persulfate ($K_2O_8S_2$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium bromide FTIR grade (KBr), phosphoric acid (H_3PO_4), pyrogallol and ascorbic acid were obtained from Sigma-Aldrich. Acetic acid (96 mL/100mL) was purchased from Scharlab, ethanol (96

mL/100mL) from Panreac and acetonitrile from Biosolve Chimie (Dieuze, France). (+)-catechin (C), (-)-gallocatechin (GC), EGCG, ECG, (-)-epigallocatechin (EGC), (-)-epicatechin (EC) and (-)-gallocatechin gallate (GCG) standards were purchased from Sigma Aldrich.

The ingredients used to prepare the biscuits were 350 g soft wheat flour (Belenguer, S.A., Valencia) with 11 g/100g protein, 0.6 g/100g ash and alveograph parameters of $P/L = 0.27$ and $W = 134$, as provided by the supplier, where P is the maximum pressure required, L the extensibility and W the baking strength of the dough; 112.52 g vegetable margarine (Unilever, Spain), 103.07 g sugar (Azucarera Ebro, Madrid, Spain), 37.62 g reconstituted skimmed milk (from 6.12 g skimmed milk powder, Central Lechera Asturiana, Spain), 3.67 g salt, 1.22 g sodium bicarbonate (A. Martínez, Cheste, Spain) and 0.70 g ammonium hydrogen carbonate (Panreac Quimica, Barcelona, Spain).

2.2 Catechins profile of the GTE

High-performance liquid (HPLC) was used to identify the catechins profile of the GTE. The LC system used was an Agilent 1200 series (Agilent Technologies, Santa Clara, USA) equipped with a binary pump and a diode-array-detection (DAD) system. Separation of the different catechins (i.e. C, GC, EGCG, ECG, EGC, EC and GCG) was performed using a Zorbax SBC₈ (150 x 4.6 mm, 3.5 μ m of particle size) LC-column. The flow rate was set to 0.9 mL/min and the oven temperature was 30 °C. Eluent A was acetonitrile and eluent B water slightly acidified with 0.1 g/100 g H₃PO₄. The elution gradient started with 100 mL/100 mL of eluent B, decreasing to 90 mL/100 mL B in 20 min and to 85 mL/100 mL in 60 min. The injection volume was 2 μ L. Acquisition was done using the ChemStation software (Agilent Technologies). The detection wavelength

was set at 280 nm. The catechins were identified based on their retention times, as compared with their standards.

2.3 Microencapsulation of the GTE

The GTE was microencapsulated through electrospraying within two different protein matrices: gelatin and zein. For this purpose, solutions of the proteins in adequate solvents were first prepared. Gelatin aqueous solutions (8 g/100 mL) were prepared in diluted acetic acid (20 mL/100 mL) as described in Gomez-Mascaraque et al. (2015), and cooled down to room temperature before addition of the GTE. On the other hand, zein (12 g/100 mL) was dissolved in ethanol (85 mL/100 mL) at room temperature (concentrations selected from preliminary tests in order to obtain electrosprayed particles as free of fibres as possible). The GTE was then added to the protein solutions under magnetic stirring at a concentration of 20 g/100 g of the total solids content in both cases.

The GTE-protein solutions were processed using a homemade electrospraying apparatus, equipped with a variable high-voltage 0–30 kV power supply. The solutions were introduced in a 5 mL plastic syringe and pumped at a constant flow-rate through a stainless-steel needle (0.9 mm inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump. Processed samples were collected on a grounded stainless-steel plate, placed facing the syringe in a horizontal configuration, at a distance of 10 cm, as illustrated in Fig.1. The voltage applied to the needle and the pumping flow-rate, respectively, were 15 kV and 0.2 mL/h for the gelatin solutions (Gomez-Mascaraque et al., 2015), and 13 kV and 0.15 mL/h for the zein solutions (conditions selected from preliminary tests).

2.4 Characterization of the microparticles

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

The samples were also subjected to Fourier transform infrared (FT-IR) analysis. Unloaded and GTE-containing particles of ca. 1 mg were grounded and dispersed in 130 mg of spectroscopic grade potassium bromide (KBr). A pellet was then formed by compressing the sample at ca. 150 MPa. FT-IR spectra were collected in transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1/cm resolution. The spectrum of raw GTE was also obtained.

2.5 Microencapsulation efficiency

In order to estimate the amount of catechins in the microparticles, these were previously dissolved to release their contents. Ethanol (85 mL/100 mL) and diluted acetic acid (20 mL/100 mL) were used to dissolve the zein and gelatin particles, respectively. The obtained solutions were analysed by HPLC using the method described in Section 2.2 to quantify the amount of recovered catechins from the particles.

The microencapsulation efficiency (MEE) of the systems was calculated in terms of the fraction of catechins from the GTE that was effectively incorporated within the capsules, according to Eq. (1).

$$MEE (\%) = \frac{\text{Catechins content in the electrosprayed particles}}{\text{Catechins content added to the protein solutions}} \times 100 \quad \text{Eq. (1)}$$

2.6 Thermal stability of GTE upon microencapsulation

Free and microencapsulated GTE were subjected to a thermal treatment at 180 °C for 12 minutes under inert atmosphere (N₂). The microparticles were then dissolved following the procedure described in Section 2.5 and analysed by HPLC according to the method described in Section 2.2.

2.7 Biscuits preparation

The shortening, sugar, milk powder, leaving agents, salt, and water were mixed in a mixer (Kenwood Ltd., UK) for 1 min at low speed (60 rpm). The bowl was scraped down and the mixture was mixed again for 3 min at higher speed (255 rpm). The flour was subsequently added and mixed at 60 rpm for 20 s. After scraping down the bowl, the mixture was mixed again at 60 rpm for 40 s. The dough was sheeted with a lab-scale sheeting machine (Parber, Vizcaya, Spain) and cut into discs of 64 mm in diameter and 4 mm in thickness using a mold. Twenty biscuits were placed on a perforated tray and baked in a conventional oven (De Dietrich, France) for 14 min at 180 °C. The same oven and trays were used to bake all the biscuits, keeping the number of biscuits and the position of the trays constant. After cooling (1 h), the biscuits were packed in metalized polypropylene bags and heat-sealed (Sealboy, Audion Elektro, Kleve, Germany) until

further use. For the fortified biscuits, a ratio of 2 g/100g of GTE with respect to flour was previously mixed with the flour by manual mechanical action before being added to the dough.

2.8 Content of GTE catechins after biscuit preparation

The stability of free and microencapsulated GTE during biscuit preparation was evaluated by measuring the content of GTE catechins in the biscuits after baking. The catechin recovery from the biscuits was calculated according to Eq. (2) after extraction with ethanol (85 mL/100 mL) for zein microparticles or diluted acetic acid (20 mL/100 mL) for gelatin microparticles and subsequent HPLC analysis using the method described in Section 2.2.

$$\text{Recovery (\%)} = \frac{\text{Catechins content extracted from the biscuits}}{\text{Initial catechin content added to the biscuit dough}} \times 100$$

Eq. (2)

2.9 Antioxidant activity

The radical scavenging activity (RSA) of the GTE-enriched biscuits was measured after extraction with ethanol (85 mL/100 mL) for zein microparticles or diluted acetic acid (20 mL/100 mL) for gelatin microparticles, and compared to that of the GTE as is. For this purpose, the ABTS^{•+} decolorization assay was applied following the protocol described in Gómez-Mascaraque, Soler, & Lopez-Rubio (2016). Briefly, a stock solution of ABTS^{•+} was prepared by reacting ABTS with potassium persulfate (7 and 2.45 mmol/L in distilled water, respectively). After 24 h in the dark at room

temperature, the ABTS^{•+} stock solution was diluted with the respective extraction solvent to an absorbance of 0.70±0.02 at 734 nm. Then 10 µL of the samples (theoretical concentration of GTE adjusted to 0.2 mg/mL in all cases) were added to 1 mL of diluted ABTS^{•+}, and the absorbance at 734 nm was measured 1 min after initial mixing. The RSA was calculated using Eq. (3), where A₀ and A₁ are the absorbance values at 734 nm before and 1 min after addition of the samples, respectively. The recovery of GTE from the biscuits in terms of antioxidant activity was estimated according to Eq. (4).

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

$$Recovery_{RSA} (\%) = \frac{RSA \text{ of biscuit } (0.2 \text{ mg/mL GTE theoretical})}{RSA \text{ of raw GTE } (0.2 \text{ mg/mL})} \times 100$$

Eq. (4)

Experiments were performed in independent triplicates on a VWR spectrophotometer model V-1200 (Pennsylvania, USA). The unloaded microparticles and biscuits without GTE were also evaluated to take into account the potential contribution of these materials to the total RSA. Significant differences were determined through two-sided t-tests (means test of equality) at the 95% significance level ($p < 0.05$) using IBM SPSS Statistics software (v.23) (IBM Corp., USA).

2.10 Biscuits' liking evaluation

A study was carried out in order to assess whether the addition of free and microencapsulated GTE in biscuits had an effect on consumers' sensory liking. Two hundred fourteen consumers participated in the study (149 women and 65 men aged from 18 to 65 years old). They were recruited according to their willingness to participate and with the requirement of eating biscuits at least once a week and not

having intolerance to gluten. Three biscuits were presented to consumers: ‘C’ (control biscuit without additions), ‘free GTE’ (biscuit added with GTE as is) and ‘Gel-GTE’ (biscuit added with gelatin-encapsulated GTE) without any information about their composition. Consumers were asked to evaluate their global, texture and taste liking using nine-point hedonic scales ranging from 1 = “dislike extremely” to 9 = “like extremely”. The evaluations were carried out in a standardized test room and the biscuits were served in small white plastic plates. The samples were identified with random three-digit codes and were presented monadically following a balanced design (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Mineral water was provided to consumers to rinse their mouths between samples.

All experiments were performed in compliance with the national legislation, and according to the institutional framework and practices established by CSIC Ethics Committee. All participants received written information about the study before giving their informed consent.

2.11. Data analysis

Analysis of variance was performed using the Statgraphics Plus 5.1 software package (Statistical Graphics Co., Rockville, Md., U.S.A.). Fisher’s least significant difference (LSD) test was used to calculate mean value differences ($\alpha < 0.05$).

3. Results and discussion

Many different research works have evaluated the potential of microencapsulation for the protection of food bioactives. However, for the commercial success of this kind of

technologies it is important to investigate how these microparticles would behave in real processing conditions and the way they can affect food sensorial properties. Therefore, in this work GTE-containing microencapsulation structures were prepared in sufficient amount to be incorporated within the biscuit dough as a model food product. In the first part of the work, the microparticles obtained through electrospraying using two different protein matrices were characterized and their potential ability for the thermal stabilization of the catechins present in the GTE was evaluated. Later on, the effect of GTE addition to biscuits (free vs. microencapsulated GTE) was assessed in terms of antioxidant, morphological and sensorial acceptability.

3.1 Characterization of the microencapsulation structures

The GTE was microencapsulated within gelatin and zein microparticles by electrospraying. Fig. 2 shows the images obtained by SEM for the obtained materials, together with their particle size distributions. GTE-containing gelatin particles were quasi-spherical and very similar to those obtained in a previous work where pure EGCG was microencapsulated within electrosprayed gelatin structures (Gomez-Mascaraque et al., 2015). GTE-loaded zein particles, on the other hand, were smaller, had a rougher surface and a greater content of small fibrils, which involved a greater extent of aggregation of the particles. These morphological differences were the result of both the distinct molecular structure of each protein and the different electrospraying conditions (solvent, process parameters...) used to produce the microparticles (Chakraborty, Liao et al. 2009). In both cases, most of the particles had a size in the submicron range, so they were not expected to have a negative impact on the textural perception of the biscuits.

The FT-IR spectra of the GTE-containing particles (Fig. 3) showed characteristic peaks from both the raw GTE and the proteins, but some spectral shifts were observed. Specifically, the bands attributed to the GTE which appeared in the range from 1300/cm to 900/cm shifted to higher wavenumbers in the encapsulation structures (cf. insets in Fig. 3), suggesting the presence of intermolecular interactions between the components of the GTE and its encapsulation matrices. Indeed, the ability of proteins to interact with polyphenols, mainly through non-covalent hydrophobic interactions and hydrogen bonds, is widely recognized (Jakobek, 2015). The interactions with the encapsulation matrices are known to contribute to the stabilization of the encapsulated compounds (Gomez-Mascaraque et al., 2015).

3.2 Microencapsulation efficiency

Initially, the GTE used in this work was characterized by HPLC in order to quantify the amount of each tea catechin present in the extract. The mass percentage of each catechin in the GTE was found to be as follows: EGCG = 41.8 g/100 g; ECG = 8.9 g/100 g; EGC = 9.6 g/100 g; EC = 6.3 g/100 g; GCG = 2.0 g/100 g; GC = 1.5 g/100 g; C = 1.0 g/100 g. According to these results, the most abundant catechin in the GTE is EGCG, reported as the most biologically active flavonoid in green tea (Barras, et al., 2009).

Fig. 4b summarizes the catechin recovery obtained for the microencapsulated GTE by HPLC analysis after dissolution of the zein and gelatin particles, respectively. The chromatograms of the free and microencapsulated GTE are also shown (Fig. 4a), where the main tea catechins are identified based on their retention times. No catechins were detected in the chromatograms of the unloaded protein particles (cf. Supplementary Material). For each catechin, the recovery from the GTE-loaded particles was calculated

according to Eq. (1), and the overall MEE was estimated as the weighted sum of the recoveries obtained for each catechin taking into account the relative proportion of each catechin in the original GTE.

According to the results, the electrospraying technique used for the microencapsulation of the GTE yielded very high encapsulation efficiencies, as observed previously for purified EGCG (Gomez-Mascaraque et al., 2015). The MEE was slightly lower when gelatin was used as the encapsulating matrix, probably due to its processing in aqueous solution, given the instability of the catechins in aqueous media (Wang, Zhou et al. 2008, Sang, Lambert et al. 2011). The greatest differences were observed in the recovery of C, which was significantly lower from the gelatin microparticles. Nevertheless, the differences in the overall MEE were not significantly different. Moreover, the low pH of the gelatin solutions in diluted acetic acid managed to minimize catechins degradation, which is accelerated in alkaline pHs (Wang, Zhou, & Jiang, 2008), thus obtaining a considerably high encapsulation efficiency too. The catechins profile of the GTE was slightly altered upon microencapsulation within both matrices, which could also be attributed to degradation and epimerization reactions taking place in the electrospraying solution (Wang et al., 2008). For instance, while the EGC content decreased in the microencapsulated GTE compared to the free extract, the concentration of its epimer (GC) increased, suggesting that epimerization reactions took place during the encapsulation process. Still, the recovery of EGCG (the most abundant and bioactive catechin) from the microparticles was close to 90 g/100 g of its content in the original GTE, suggesting that electrospraying is an efficient technique for the microencapsulation of GTE within protein matrices such as gelatin and zein.

3.3 Thermal stability of GTE upon microencapsulation

It is well-known that the stability of tea catechins decreases with increasing temperatures and pH values (Sharma & Zhou, 2011), and given that the production of GTE-enriched biscuits involves the extract being subjected to high pHs and temperatures, suitable microencapsulation matrices for this ingredient should be capable of protecting catechins against degradation under these conditions. Electrosprayed biopolymers have already proven to stabilize EGCG against degradation in slightly alkaline solutions (Gómez-Mascaraque, Sanchez, & López-Rubio, 2016), but their protective effect on catechins against thermal degradation had not been demonstrated yet. Hence, the free and microencapsulated GTE was subjected to thermal treatment (i.e. the baking temperature, 180 °C). Fig. 5a shows the obtained chromatograms and Fig. 5b summarizes the catechin recovery after the thermal treatment, calculated in the same way as done in the previous section for the as-prepared samples. Again, the recovery was calculated according to Eq. (1) for each catechin and the overall recovery was estimated taking into account the relative proportion of each catechin in the original GTE.

The total recovery of catechins from the GTE after the thermal treatment showed that microencapsulation was very effective in protecting the catechins against thermal degradation. While almost 40 g/100 g of the initial catechins content in the free GTE was lost, ~90 g/100 g and ~85 g/100 g of the total catechins content was recovered after the treatment from the gelatin and zein microparticles, respectively. However, the catechin profile of the GTE was affected by the thermal treatment, even when microencapsulated, although to a lesser extent in the latter case. While the content of epi-structured catechins (EGCG, ECG, EGC and EC) decreased, that of their non-epi-structured counterparts (GCG, CG and C) increased, suggesting that epimerization was

one of the main phenomena taking place upon exposure to high temperatures. Indeed, it has been reported that epimerization and degradation reactions occur simultaneously for catechins and their relative reaction rate may vary depending on the temperature of the thermal treatment (Wang et al., 2008). Microencapsulation was capable of precluding both epimerization and degradation processes, as the decrease in the content of epi catechins as well as the increase in non-epi catechins was limited. Small differences were observed between the two microencapsulation systems, which were only significant for EGC and GCG. Given the smaller size of the zein particles and the greater presence of fibrils, their increased surface area to volume ratio as compared to the gelatin particles might partially explain these small differences. Additionally, new peaks were found in the chromatogram of the free GTE after thermal treatment (cf. circle in Fig. 5a), and were attributed to degradation products. The absence of these new peaks in the encapsulated samples together with the lower extent of epimerization, suggested that the selected electrosprayed matrices could be suitable for the protection of tea catechins during biscuit manufacturing, and were therefore evaluated in a real biscuit-making process.

3.4 Catechins profile of GTE in biscuits

GTE-fortified biscuits were prepared by mixing the extract, either in its free form ('Free GTE biscuits') or microencapsulated within gelatin ('Gel-GTE biscuits') or zein ('Zein-GTE biscuits'), with the flour for the preparation of the biscuits dough. The recovery of catechins from the prepared biscuits was calculated according to Eq. (2) after extraction with an adequate solvent to dissolve the encapsulation matrices, and subsequent HPLC analysis. Results are shown in Fig. 6.

The total recovery of catechins was greater from the biscuits containing free GTE than for those enriched with microencapsulated GTE. These results were unexpected, given that the microparticles had proven to protect the catechins from thermal degradation in the preliminary tests (Section 3.4) and from degradation in slightly alkaline conditions in previous works (Gomez-Mascaraque et al., 2015). In addition, not only did the total recovery not increase upon microencapsulation of the extract, but it significantly decreased. These observations suggested that the catechins extraction from the encapsulation matrices might have not been complete when the microparticles were embedded within the biscuit matrix. Potential interactions between gelatin and zein and the biscuit components might have precluded complete dissolution of the microparticles and hence the complete release of the catechins. One possibility is that Maillard reactions might happen between the encapsulation matrices (proteins) and the sugars present in the biscuit formulation, potentially crosslinking the proteins (Ifeduba & Akoh, 2016) and thus hindering the release of GTE (Duffy, David et al. 2015). It would then be expected that the remaining complexes/aggregates would have been retained upon filtration of the samples prior to HPLC analysis and, thus, could not be quantified. Protein-lipid interactions might have also occurred in the complex food matrix upon baking, which could have also contributed to decrease the solubility of the proteins due to aggregation or complex formation (Hęś, 2017).

In order to confirm this hypothesis, the antioxidant activity of the extracted samples (without filtration) was measured by means of the ABTS^{•+} decolorization assay.

3.5 Antioxidant activity of GTE in biscuits

The antioxidant properties of green tea polyphenols have shown to be medium-dependent (Sharma & Zhou, 2011; Wang et al., 2008). Therefore, the radical scavenging activity (RSA) of the samples in the extraction medium, calculated according to Eq. (3), was compared to that of the free GTE extracted from the biscuits in the same extraction medium. The recovery of GTE from the biscuits in terms of antioxidant activity was calculated according to Eq. (4) and the results are summarized in Table 1. The antioxidant activity of the GTE-loaded microparticles before their incorporation within the biscuits was also measured as a control, and expressed as percentage of recovery in Table 1.

As expected from the high microencapsulation efficiencies obtained for both systems (cf. Section 2.5.) the antioxidant activity of the GTE-loaded microparticles was very similar to that of the free GTE. When incorporated within the biscuits, the recovery after extraction estimated by means of radical scavenging assays was slightly greater when the GTE was microencapsulated prior to its incorporation within the biscuits, suggesting that the extent of degradation during biscuit preparation was somewhat lower upon encapsulation. These results support the hypothesis of incomplete GTE extraction from the microparticles-containing biscuits previous to HPLC analysis. However, the observed differences were not statistically different, precluding the statement of assertive conclusions. As the degradation products from catechins also exhibit antioxidant activity (Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999), their RSA could contribute to the overall antioxidant activity of the samples masking the reduction in tea catechins upon degradation.

3.6 Sensory evaluation

The impact of adding free or microencapsulated GTE to the biscuits on their sensorial properties was also evaluated. Gelatin was selected as encapsulation matrix for this analysis, as it yielded less fibrillar structures and, thus, less aggregated particles easier to disperse in the flour. The consumers' evaluations are summarized in Table 2. According to the ANOVA results, no significant differences were found between the liking values of the three types of biscuits evaluated (control, free GTE, and microencapsulated GTE). This indicated that consumers rated all samples alike, so they did not perceive differences neither globally nor regarding their texture or taste affecting their biscuits' liking. Hence, the addition of free and microencapsulated GTE did not vary consumers' preferences. These results differed from those obtained by Bajerska et al. (2010), who reported that GTE had a negative impact on the aroma, flavour and acceptability of bread. However, their study is not directly comparable to the present work since the sensory profiles of bread and biscuits are very different. The information about sensory properties conferred by green tea extract to biscuits is scarce. Mildner-Szkudlarz and co-workers (2009) evaluated the protective effect of GTE on rancid odor development during storage of the GTE-added biscuits, but not the impact of the addition of GTE itself. Very recently, Ahmad, Baba et al. (2015) reported that the addition of green tea powder to biscuits slightly decreased their overall acceptability. However, in their work they used ten semi-trained panelists, which were more likely to perceive slight differences in the sensory properties than the two hundred fourteen non-trained consumers used in this study, which are more representative of the general consumers' population.

4. Conclusions

An EGCG-rich GTE was microencapsulated within gelatin and zein microparticles by electrospraying, achieving very high encapsulation efficiencies (~90 g/100g). Both protein matrices proved to be very effective in protecting the catechins during a thermal treatment (180 °C), preserving 85-90 g/100g of their initial content in catechins, while almost 40 g/100g was lost in the free GTE. Therefore, the proposed microencapsulation systems were expected to be effective in protecting the catechins during the manufacturing of bakery products. GTE-fortified biscuits were then prepared by adding the extract, both in its free form and microencapsulated, and the catechins recovery after baking was estimated by HPLC analysis and radical scavenging activity assays. While the first method was not adequate for catechin quantification due to incomplete extraction of the GTE from the biscuit-microparticles systems, the latter showed no significant differences among the recoveries from the free and microencapsulated GTE-containing biscuits, suggesting a poor protective effect of microencapsulation in the model food system. These results emphasize the need of assessing the protective effect of microencapsulation in real food products. On the other hand, the sensorial analysis carried out in this work showed that no significant differences in consumers' liking was found between biscuits fortified with electrosprayed protein microparticles and the control, so the addition had no impact on the acceptability. Future work should be conducted to study the impact of microencapsulation on the bioavailability of the catechins upon consumption of GTE-enriched biscuits.

Acknowledgements

Laura G. Gómez-Mascaraque is recipient of a predoctoral contract from the Spanish Ministry of Economy and Competitiveness (MINECO), Call 2013. The authors would like to thank the Spanish MINECO project AGL2015-63855-C2-1 for financial support.

Authors would also like to thank the Central Support Service for Experimental Research (SCSIE) of the University of Valencia for the electronic microscopy service.

REFERENCES

- Ahmad, M., W. N. Baba, T. A. Wani, A. Gani, A. Gani, U. Shah, S. M. Wani & F. A. Masoodi (2015). Effect of green tea powder on thermal, rheological & functional properties of wheat flour and physical, nutraceutical & sensory analysis of cookies. *Journal of Food Science and Technology*, 52, 5799-5807.
- Ananingsih, V. K., Gao, J., & Zhou, W. (2013). Impact of green tea extract and fungal alpha-amylase on dough proofing and steaming. *Food and Bioprocess Technology*, 6, 3400-3411.
- Bajerska, J., Mildner-Szkudlarz, S., Jeszka, J., & Szwengiel, A. (2010). Catechin stability, antioxidant properties and sensory profiles of rye breads fortified with green tea extracts. *Journal of Food and Nutrition Research*, 49, 104-111.
- Barras, A., Mezzetti, A., Richard, A., Lazzaroni, S., Roux, S., Melnyk, P., Betbeder, D., & Monfilliet-Dupont, N. (2009). Formulation and characterization of polyphenol-loaded lipid nanocapsules. *International Journal of Pharmaceutics*, 379, 270-277.
- Bhardwaj N., & Kundu, S. C. (2010). Electrospinning: A fascinating fiber fabrication technique. *Biotechnology Advances*, 28, 325-347.
- Chakraborty, S., I. C. Liao, A. Adler & K. W. Leong (2009). Electrohydrodynamics: A facile technique to fabricate drug delivery systems. *Advanced Drug Delivery Reviews*, 61, 1043-1054.

- Choudhury, M., Badwaik, L. S., Borah, P. K., Sit, N., & Deka, S. C. (2015). Influence of bamboo shoot powder fortification on physico-chemical, textural and organoleptic characteristics of biscuits. *Journal of Food Science and Technology*, 52, 6742-6748.
- Duffy, C. V., L. David & T. Crouzier (2015). Covalently-crosslinked mucin biopolymer hydrogels for sustained drug delivery. *Acta Biomaterialia*, 20, 51-59.
- Fradinho, P., Nunes, M. C., & Raymundo, A. (2015). Developing consumer acceptable biscuits enriched with Psyllium fibre. *Journal of Food Science and Technology*, 52, 4830-4840.
- Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International*, 40, 1107-1121.
- Gómez-Mascaraque, L. G., Ambrosio-Martín, J., Fabra, M. J., Pérez-Masiá, R., Lagaron, J. M., & López-Rubio, A. (2016). Novel nanoencapsulation structures for functional foods and nutraceutical applications. In S. Sen & Y. Pathak (Eds.), *Nanotechnology in Nutraceuticals: Production to Consumption* (1st Edition, pp. 375-398). Boca Raton: Taylor & Francis Group LLC.
- Gómez-Mascaraque, L. G., Lagarón, J. M., & López-Rubio, A. (2015). Electrospayed gelatin submicroparticles as edible carriers for the encapsulation of polyphenols of interest in functional foods. *Food Hydrocolloids*, 49, 42-52.
- Gómez-Mascaraque L. G., & López-Rubio, A. (2016). Protein-based emulsion electrospayed micro- and submicroparticles for the encapsulation and stabilization of thermosensitive hydrophobic bioactives. *Journal of Colloid and Interface Science*, 465, 259-270.

Gómez-Mascaraque, L. G., Sanchez, G., & López-Rubio, A. (2016). Impact of molecular weight on the formation of electrosprayed chitosan microcapsules as delivery vehicles for bioactive compounds. *Carbohydrate Polymers*, 150, 121-130.

Gómez-Mascaraque, L. G., Soler, C., & Lopez-Rubio, A. (2016). Stability and bioaccessibility of EGCG within edible micro-hydrogels. Chitosan vs. gelatin, a comparative study, *Food Hydrocolloids*, 61, 128-138.

Gramza-Michałowska, A., Kobus-Cisowska, J., Kmiecik, D., Korczak, J., Helak, B., Dziedzic, K., & Górecka, D. (2016). Antioxidative potential, nutritional value and sensory profiles of confectionery fortified with green and yellow tea leaves (*Camellia sinensis*), *Food Chemistry*, 211, 448-454.

Hęś, M. (2017). Protein-Lipid Interactions in Different Meat Systems in the Presence of Natural Antioxidants—a Review. *Polish Journal of Food and Nutrition Sciences*, 67, 5-18.

Ifeduba, E. A., & Akoh, C. C. (2016). Microencapsulation of stearidonic acid soybean oil in Maillard reaction-modified complex coacervates. *Food Chemistry*, 199, 524-532.

Jakobek, L. (2015). Interactions of polyphenols with carbohydrates, lipids and proteins. *Food Chemistry*, 175, 556-567.

Larsen, C. A., & Dashwood, R. H. (2010). (–)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. *Archives of Biochemistry and Biophysics*, 501, 52-57.

Le Bourvellec, C., & Renard, C. M. G. C. (2012). Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms. *Critical Reviews in Food Science and Nutrition*, 52, 213-248.

- Li, Y., Lim, L. T., & Kakuda, Y. (2009). Electrospun zein fibers as carriers to stabilize (-)-epigallocatechin gallate. *Journal of Food Science*, 74, C233-C240.
- López-Rubio A., & Lagaron, J. M. (2012). Whey protein capsules obtained through electrospraying for the encapsulation of bioactives. *Innovative Food Science & Emerging Technologies*, 13, 200-206.
- Macfie, H. J., Bratchell, N., Greenhoff, K., & Vallis, L. (1989). Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. *Journal of Sensory Studies*, 4, 129-148.
- Mildner-Szkudlarz, S., Zawirska-Wojtasiak, R., Obuchowski, W., & Gośliński, M. (2009). Evaluation of antioxidant activity of green tea extract and its effect on the biscuits lipid fraction oxidative stability. *Journal of Food Science*, 74, S362-S370.
- Sang, S., J. D. Lambert, C.-T. Ho & C. S. Yang (2011). The chemistry and biotransformation of tea constituents. *Pharmacological Research*, 64, 87-99.
- Sharma, A, & Zhou, W. (2011). A stability study of green tea catechins during the biscuit making process. *Food Chemistry*, 126, 568-573.
- Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, 51, 456-467.
- Steinmann, J., Buer, J., Pietschmann, T., & Steinmann, E. (2013). Anti-infective properties of epigallocatechin-3-gallate (EGCG), a component of green tea. *British Journal of Pharmacology*, 168, 1059-1073.
- Su, Y. L., Leung, L. K., Huang, Y., & Chen, Z. Y. (2003). Stability of tea theaflavins and catechins. *Food Chemistry*, 83, 189-195.

Tarancón, P., Fiszman, S. M., Salvador, A., & Tárrega, A. (2013). Formulating biscuits with healthier fats. Consumer profiling of textural and flavour sensations during consumption. *Food Research International*, 53, 134-140.

Wang, R., Zhou, W., & Jiang, X. (2008). Reaction kinetics of degradation and epimerization of epigallocatechin gallate (EGCG) in aqueous system over a wide temperature range. *Journal of Agricultural and Food Chemistry*, 56, 2694-2701.

Ye, A., Cui, J., Taneja, A., Zhu, X., & Singh, H. (2009). Evaluation of processed cheese fortified with fish oil emulsion. *Food Research International*, 42, 1093-1098.

Yilmaz, Y. (2006). Novel uses of catechins in foods. *Trends in Food Science & Technology*, 17, 64-71.

Yoshino, K., Suzuki, M., Sasaki, K., Miyase, T., & Sano, M. (1999). Formation of antioxidants from (–)-epigallocatechin gallate in mild alkaline fluids, such as authentic intestinal juice and mouse plasma. *The Journal of Nutritional Biochemistry*, 10, 223-229.

Conflicts of interest

There are no conflicts of interest to declare.

Figure 1. Schematic representation of the electrospraying setup used to produce the microparticles.

Figure 2. SEM images of electrospayed microcapsules based on gelatin (a) and zein (b) containing green tea extract, together with their particle size distribution. SD means standard deviation.

Figure 3. FT-IR spectra of electrospayed microcapsules based on gelatin (a) and zein (b) containing green tea extract. The spectra of the raw green tea extract and unloaded capsules are also shown.

Figure 4. a) Chromatograms of free and microencapsulated green tea extract (GTE) after dissolution. Retention times of relevant peaks (as for the raw GTE): (-)-gallocatechin (GC, 15 min), (-)-epigallocatechin (EGC, 21 min), (+)-catechin (C, 23 min), caffeine (28 min), (-)-epicatechin (EC, 29 min), (-)-epigallocatechin gallate (EGCG, 36 min), (-)-gallocatechin gallate (GCG, 41 min), (-)-epicatechin gallate (ECG, 55 min). Note that the chromatograms of the GTE extracted from the microparticles have been slightly shifted for clarity purposes. b) Catechins recovery and overall microencapsulation efficiency (MEE) from encapsulated GTE. Asterisk (*) depicts significant differences among the samples ($p < 0.05$).

Figure 5. a) Chromatograms of catechins recovery from free and microencapsulated green tea extract (GTE) after thermal treatment. Retention times of relevant peaks (as for sample ‘GTE-zein’): (-)-gallocatechin (GC, 14 min), (-)-epigallocatechin (EGC, 20 min), (+)-catechin (C, 22 min), caffeine (27 min), (-)-epicatechin (EC, 28 min), (-)-epigallocatechin gallate (EGCG, 35 min), (-)-gallocatechin gallate (GCG, 40 min), (-)-epicatechin gallate (ECG, 54 min). The circle in the chromatogram corresponding to free GTE highlights the new peaks found in this sample after the thermal treatment. b) Catechins individual and total recoveries after thermal treatment from free and microencapsulated GTE. Different letters (a–c) indicate significant differences among the samples ($p < 0.05$).

Figure 6. Catechins recovery from biscuits for free and microencapsulated green tea extract. Different letters (a–c) indicate significant differences among the samples ($p < 0.05$). Acronyms: green tea extract (GTE), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-gallocatechin gallate (GCG), (-)-gallocatechin (GC) and (+)-catechin (C).

Table 1. GTE recovery from biscuits in terms of antioxidant activity

Extraction media	Sample	Recovery _{RSA} (%)
Acetic acid (20 mL/100mL)	Gel-GTE particles	93 ± 4 ^a
	Biscuits without GTE	9 ± 1 ^b
	Free GTE biscuits	52 ± 3 ^c
	Gel-GTE biscuits	58 ± 4 ^c
Ethanol (85 mL/100mL)	Zein-GTE particles	95 ± 3 ^a
	Biscuits without GTE	8 ± 1 ^b
	Free GTE biscuits	65 ± 7 ^c
	Zein-GTE biscuits	68 ± 2 ^c

The same letter (a-c) within the same medium indicates non-significant differences at $p < 0.05$ between samples. GTE: green tea extract, Gel-GTE: gelatin-encapsulated GTE; Zein-GTE: zein-encapsulated GTE

Table 2. Consumers' sensorial evaluation of the prepared biscuits

Biscuit	Texture liking	Flavour liking	Overall liking
Control (without GTE)	7.0 ± 1.5 ^a	7.0 ± 1.3 ^a	6.9 ± 1.3 ^a
Free GTE	6.9 ± 1.3 ^a	6.9 ± 1.3 ^a	6.8 ± 1.2 ^a
Microencapsulated GTE	6.7 ± 1.5 ^a	7.0 ± 1.3 ^a	6.9 ± 1.3 ^a

The same letter (a) within the same column indicates non-significant differences at $p < 0.05$ between biscuit samples. GTE: green tea extract.

Supplementary Material

Figure S1. Chromatograms of unloaded gelatin and zein microparticles after dissolution.