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Antioxidant properties of green tea extract incorporated to fish gelatin films after simulated gastrointestinal enzymatic digestion

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ABSTRACT

A green tea aqueous extract was prepared and blended at different percentages (2, 4 and 8%) with a commercial fish-skin gelatin in order to provide gelatin films with antioxidant capacity. This green tea extract proved to be an efficient antioxidant at non-cytotoxic concentrations. Gelatin films with green tea extract were subjected to enzymatic digestion with pepsin (gastric digestion) and with pepsin, trypsin and chymotrypsin (gastrointestinal digestion). The gelatin matrix was efficiently hydrolysed during gastrointestinal digestion and protein hydrolysates composed of low molecular weight peptides, regardless the content of green tea extract, were obtained in all the formulations. High percentages of total polyphenols were recovered from the films with green tea extract after gastrointestinal digestion, although a significant degradation of the major catechins of the green tea (EGCG and EGC) was observed. The increase of the content of green tea extract in the film formulation gave an increase in the antioxidant activity released from the film samples after enzymatic digestion. 85–100% of the maximum expected antioxidant activity was recovered after both gastric and gastrointestinal digestion in spite of the degradation observed of EGCG and EGC.

1. Introduction

Green tea (*Camellia sinensis* L.) is a source of polyphenolic compounds having strong antioxidant and antimicrobial activity (Almajano, Carbó, Jiménez, & Gordon, 2008; Chan, Lim, & Chew, 2007). Catechins are the major polyphenols in tea leaves. Besides flavanols, various flavonols and flavones are also present (Chan et al., 2007).

A number of recent studies have dealt with extending the functional properties of biodegradable films by adding different compounds with antioxidant or antimicrobial activities in order to yield a biodegradable active packaging material. Green tea extracts have been already incorporated in food products to extend its shelf life (Martín-Diana, Rico, & Barry-Ryan, 2008), and more recently they have been used as active compound in packaging films (López-de-Dicastillo et al., 2011). Although films generally represent only a minor portion of the food they cover, the study of their digestibility properties may provide useful information for extending potential uses for this type of material in the design of functional foods. In spite of the abundant information on physicochemical and technological features of edible packaging films, research on their digestibility is scarce. Furthermore, most of these studies are focused on the evaluation of changes in the nutritional properties of the biopolymers that constitute the films during and after film preparation (Hernández, Emaldi, & Tovar, 2008; Ou, Kowk, & Kang, 2004); but are not dealing with the residual activity of active compounds incorporated to the edible films after a simulated digestion. When a green tea extract is incorporated in edible packaging films, besides improving shelf life and quality of foods, green tea may have potential health benefits for the consumer when the edible films are consumed and the polyphenols are released. The health benefits of green tea consumption may include improving blood flow, preventing cardiovascular disease or improving resistance to various diseases (Afaq, Adhami, Ahmad, & Mukhtar, 2004).

Fish gelatin films with green tea extract were prepared in this study. Total polyphenol content, antioxidant capacity and cytotoxicity of the green tea extract were determined in order to establish the potential functionality in food packaging applications. The effect of enzymatic digestion on the gelatin matrix as well as on the residual antioxidant activity of tea polyphenols was evaluated.

Abbreviations: EGC, (−)-epigallocatechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGCG, (−)-epigallocatechin gallate.

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Furthermore, the recovery of total polyphenols as well as the major catechins after gastrointestinal digestion was also determined.

2. Materials and methods

2.1. Materials

(−)-epigallocatechin (EGC), (+)-catechin, (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin gallate (EGCG), rutin, hyperoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside were purchased from Extrasynthese (Genay, Cedex, France). Type A warm-water fish gelatin was supplied by Rousselot S.A.S. (Puteaux, France). TPTZ (2,4,6-tripyridyl-s-triazine), $FeCl_3$, $FeSO_4 \cdot 7H_2O$, ABTS radical [2, 2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid)], Folin–Ciocalteu reagent, gallic acid, sodium carbonate, potassium persulphate, L-ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), porcine gastric mucosa pepsin (3300 U/mg of protein using haemoglobin as substrate), bovine α-chymotrypsin and porcine trypsin (40 U/mg and 13,800 U/mg of protein using benzoyl-L-tyrosine ethyl ester as substrate, respectively) were purchased from Sigma–Aldrich (St. Louis, Mo., USA). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) salt and PMS (phenazine methosulfate) were supplied by Promega Biotech Ibérica (Madrid, Spain).

2.2. Preparation and characterization of green tea extract

2.2.1. Preparation of green tea extract

Chinese green tea known as *Lung Ching* (*C. sinensis* L.) was purchased from a specialized tea store (Madrid, Spain). The dry green tea was ground into powder using a blender (Oster, Madrid, Spain). The powder (35 g) was mixed with distilled water (350 mL) and heated at 80 °C for 30 min with continuous stirring. The slurry was centrifuged (12,000 g/10 min, 5 °C). The supernatant was filtered twice (Whatman n° 1) and stored at −20 °C.

2.2.2. Determination of phenols compounds

2.2.2.1. Determination of total phenolic. The total phenolic content in tea extract was determined spectrophotometrically (UV-1601, model CPS-240, Shimadzu, Japan) using Folin–Ciocalteau reagent according to a modified method of Slinkard and Singleton (1977). An aliquot of extract (20 μL) was mixed with 1.58 mL distilled water and oxidized with 100 μL Folin–Ciocalteau reagent. The reaction was alkalinized with 300 μL of 20% sodium carbonate solution and incubated for 2 h at room temperature. The absorbance was measured at 765 nm. Gallic acid (GA) was used as standard and total phenolic content was expressed as mg GA equivalent/mL of tea extract. All determinations were performed at least in triplicate.

2.2.2.2. Quantification by HPLC. Reverse phase high performance liquid chromatography was performed to analyse phenolic compounds in the green tea extract. The separation module consisted of a model SPE-MA10AVP HPLC–UV (Shimadzu, Kyoto, Japan), equipped with a C18 column (25 × 0.78 cm, 5 μm, C18 Tracer Excel 120 ODS-A column, Teknokroma, Spain) and a UV–Vis detector (SPD-10V). The samples were eluted with a gradient system consisting of a solvent A (Milli-Q water) and solvent B (acetonitrile) both containing 1% formic acid at a flow rate of 2.8 mL/min. The temperature of the column was 25 °C and the injection volume was 20 μL. The gradient system started at 95% solvent A and decreased to 75% A over 70 min, followed by a further decrease to 35% solvent A over 10 min. The final conditions were held for 10 min. The peaks of the phenolic compounds were monitored by absorbance at 210 nm and 350 nm. Individual compounds were quantified using a calibration curve of the corresponding standard compound. All analyses were performed in triplicate.

2.2.3. Antioxidant activity

2.2.3.1. Ferric reducing ability of plasma (FRAP). The FRAP assay was carried out according to the method described by Benzie and Strain (1996) with some modifications. The sample (30 μL) was incubated (37 °C) with 90 μL of distilled water and 900 μL of FRAP reagent, containing 10 mM TPTZ and 20 mM $FeCl_3$. Absorbance values were read at 595 nm after 30 min using an UV-1601 spectrophotometer (CPS-240, Shimadzu, Japan). Results were expressed as μmol Fe^{2+} equivalents/mL of sample based on a standard curve of $FeSO_4 \cdot 7H_2O$. All determinations were performed at least in triplicate.

2.2.3.2. ABTS. The ABTS radical scavenging capacity was carried out according to the method of Re et al. (1999). Results were expressed as mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC)/mL of sample based on a standard curve of L-ascorbic acid. All determinations were performed at least in triplicate.

2.2.3.3. DPPH. This assay was carried out as described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications (Fukumoto & Mazza, 2000). The green tea extract was analysed by triplicate, testing at least five concentrations ranging from 2.5 to 20 μg dry matter/mL. The radical scavenging activity was expressed as IC_{50} value, the concentration necessary to quench 50% of initial DPPH radical. Trolox was used as reference compound. The DPPH radical scavenging capacity of Trolox, expressed as IC_{50} value, was determined by testing concentrations ranging from 3 to 50 μg/mL.

2.2.4. Cytotoxic effect of green tea extract

2.2.4.1. Culture of cell lines. The mouse fibroblast cell line 3T3-L1 was grown in DMEM medium (4.5 g/L glucose) supplemented with 10% foetal bovine serum (FBS), at 37 °C under a humidified 5% CO_2 atmosphere. When the cells were approximately 70% confluent, they were split by mild trypsinization and seeded into 24-well plates (1 x 10^4 cells/well). The 24-well plates were incubated at 37 °C/24 h and 5% CO_2. Runs were performed in triplicate with different passage cells.

2.2.4.2. Experimental treatments. After 1 day of incubation, cultures were exposed to increasing concentrations of green tea extract (25–300 μg dry matter/mL) sterilized by filtration and diluted in DMEM medium with 10% FBS. Controls (containing only the culture medium) were included in each plate. The plates were incubated at 37 °C with 5% CO_2 for 24 h.

2.2.4.3. MTS assay. The viability of the 3T3-L1 cells treated with green tea extract for 24 h was determined by the MTS assay, composed of the tetrazolium salt MTS and an electron coupling reagent (PMS). A coloured aqueous soluble formazan product is formed from the tetrazolium compound by mitochondrial activity of viable cells at 37 °C. The amount of formazan produced is directly proportional to the number of living cells in culture. A combined MTS/PMS solution (20:1) was added to the culture medium in a ratio of 1:5 (reagent mixture:medium). After 1 h of incubation, absorbance was measured in a microplate reader at 485 nm (Appliskan, Thermo Scientific, Madrid, Spain).

2.3. Preparation of films

Gelatin control film (0% of green tea extract, C) was prepared by dissolving fish gelatin in distilled water (4 g/100 mL). Gelatin films containing green tea extract were obtained by dissolving gelatin

(4 g/100 mL) in a distilled water/green tea extract mixture with the following ratios: 98/2 v/v (2% of extract), 96/4 v/v (4% of extract) and 92/8 v/v (8% of extract) for F2, F4 and F8, respectively. Glycerol was used as plasticizer (15% w/w of gelatin). The mixtures were stirred to obtain a good blend and the films made by casting 7.85 mL on 28 cm^2 plates, drying afterwards at 40 °C in a forced-air oven for 16 h to yield a uniform thickness in all cases [200 μm ($p \leq 0.05$)]. Films were conditioned (22 °C, 58% relative humidity) for 2 days.

2.4. Enzymatic digestion of gelatin films

The whole films (0.41 ± 0.01 g) were suspended in 17 mL of acidic saline (150 mM NaCl, pH 2.5) and readjusted to pH 2.5 with HCl as required. Pepsin was added to a final concentration in the aqueous phase of 146 U/mL (Mandalari et al., 2008). The samples were incubated (37 °C/30 min) in a water bath with agitation (100 rpm/min) (OSL 200, Grant Instruments Ltd., Cambridge, England). To assay the effects of digestion with pepsin (gastric digestion) the enzyme was inactivated (90 °C/10 min), samples were centrifuged (4000 rpm/20 min) and the supernatants were stored at −20 °C until analysis. Simulated gastrointestinal digestion was performed by subjecting the gelatin films to digestion with pepsin followed by digestion with trypsin and chymotrypsin. After digestion with pepsin in the conditions described above, the pH was raised to 6.5 by addition of NaOH. Trypsin and chymotrypsin were dissolved in 150 mM/L NaCl and added so that the final concentrations were 104 units/mL trypsin and 5.9 U/mL chymotrypsin (Mandalari et al., 2008). The samples were digested (37 °C/2 h) in a water bath with agitation (100 rpm/min). After this step of digestion, the enzymes were inactivated (90 °C/10 min), samples were centrifuged (4000 rpm/20 min) and the supernatants were stored at −20 °C until analysis.

2.5. Characterization of film digestions

2.5.1. Molecular weight distribution

Gelatin and film digestions were mixed with the loading buffer (2% SDS, 5% mercaptoethanol and 0.002% bromophenol blue) achieving a final concentration of 1.6 mg gelatin or digested film/mL. Protein samples were heat-denatured (90 °C/5 min) and analysed by SDS-PAGE according to Laemmli (1970) using 5% stacking gel and 12% resolving gel in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA, USA) at 25 mA/gel. The loading volume was 15 μL. Protein bands were stained with Coomassie brilliant Blue R250. A prestained protein standard consisting of proteins between 2 and 250 kDa was used (Bio-Rad, Madrid, Spain).

2.5.2. Total phenolic compounds, EGCG and EGC

The total phenolic content in the film digestions was determined as described for green tea extract. The results were expressed as mg GA equivalent/g film. EGCG and EGC were determined in the film digestions as described in green tea extract. The results were expressed as μg EGCG and EGC/mL digestion, respectively.

2.5.3. Antioxidant activity: FRAP and ABTS

FRAP and ABTS radical scavenging capacity of film digestions were carried out as described for green tea extract. The results were expressed as μmol Fe^{2+} equivalents/g film and mg VCEAC/g film for FRAP and ABTS, respectively.

2.5.4. Evaluation of cytotoxic effect

Cytotoxicity of the film digestions was carried out by MTS assay as described for green tea extract. The digestions were lyophilized, dissolved in the same volume of culture medium (DMEM with 10% FBS) and sterilized by filtration. Cell cultures were exposed to the film digestions for 24 h at 37 °C with 5% CO_2. Results were expressed as % of cell viability.

2.6. Statistical analysis

Statistical tests were performed using the SPSS computer program (SPSS Statistical Software Inc., Chicago, IL, USA). One-way analysis of variance was carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey-b test. The level of significance was $p \leq 0.05$.

3. Results and discussion

3.1. Characterization of the green tea extract

The total polyphenol content of the green tea extract was approximately 12 g GA equivalents/L (Table 1). The phenolic content of tea extracts may depend on the tea type and processing method, together with the conditions used to prepare tea infusions (ratio leaves/water, extraction time, water temperature) (Peterson et al., 2005). The main classes of polyphenols in green tea are flavanols and flavonols (Komes, Horžić, Belščak, Ganić, & Vulić, 2010). Five major peaks of catechins (flavan-3-ol) were identified in the polyphenolic profile of the green tea extract obtained: EGC, (+)-catechin, EC, ECG, and EGCG; as well as five flavonols: rutin, hyperoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside. EGCG was the major phenolic compound in green tea extract (~6100 mg/L; Table 1), while catechin showed the lowest concentration (~100 mg/L). EGCG was followed by EGC (~3350 mg/L), EC and ECG, which were found at lower concentration (~1250–1300 mg/L). EGCG is usually found as the prevailing compound in green tea extracts (Almajano et al., 2008; Komes et al., 2010). In contrast to the catechins, none of the flavonols determined in this study was clearly predominant in the composition of the green tea extract. The most common flavonols described in green tea are quercetin, kaempferol and myricetin, commonly conjugated to a range of sugars (Peterson et al., 2005). The green tea extract showed a high antioxidant activity measured by DPPH, ABTS and FRAP assays (Table 2). When compared with Trolox, the green tea extract showed a significantly higher DPPH radical scavenging capacity, with IC_{50} values 1.64-fold lower than those obtained for Trolox. The antioxidant activity of a green tea extract is related to the type of tea together with the extraction conditions, which in turn may determine the type and concentration of catechins in the extract (Atoui, Mansouri, Boskou, & Kefalas, 2005). According to Toschi et al. (2000) the antioxidant activity of several green tea extracts in a lipid model system was

Table 1
Content of total phenolics, catechins and flavonols in green tea extract.

Total polyphenols[a]	12,092 ± 840
(−)-Epigallocatechin[b]	3350 ± 45
(+)-Catechin[b]	98 ± 6
(−)-Epicatechin[b]	1252 ± 27
(−)-Epicatechin-3-gallate[b]	1301 ± 11
(−)-Epigallocatechin gallate[b]	6133 ± 39
Rutin[b]	39.8 ± 3.1
Hyperoside[b]	17.6 ± 1.2
Quercetin-3-*O*-glucoside[b]	18.1 ± 0.6
Kaempferol-3-*O*-rutinoside[b]	8.17 ± 0.9
Kaempferol-3-*O*-glucoside[b]	14.3 ± 0.5

[a] Total phenolic content is expressed as equivalents of gallic acid (mg/L).
[b] Content of polyphenols is expressed as μg/mL.

Table 2
Antioxidant activity measured by FRAP, ABTS and DPPH, as well as cytotoxic effect of green tea extract.

	Green tea extract	Trolox
FRAP[a]	224.8 ± 4.0	
ABTS[b]	17.10 ± 0.21	
DPPH (IC_{50})[c]	10.74 ± 0.77	17.48 ± 0.36
Cytotoxicity (IC_{50})[d]	272 ± 33	

[a] FRAP, values are expressed as mmol Fe^{2+}equivalents/L.
[b] ABTS values are expressed as mg VCEAC/mL.
[c] DPPH values are expressed as μg dry matter/mL.
[d] Citotoxicity values are expressed as μg dry matter/mL.

related to the high content of EGCG and EGC, which were the most abundant catechins in our green tea extract (Table 1). Guo et al. (1999) suggested that the scavenging ability of EGCG and its epimer GCG was higher than that of EGC, GC, EC and C due to the gallate group at 3 position of the C ring, while the ability to scavenge free radicals for EGC and its epimer GC was stronger than EC and C because of a hydroxyl group at the 5′ position of B ring.

Given the potential application of green tea extracts as active compounds in edible packaging materials in contact with food and the high concentration of the extracts obtained in this study (10%), it is of great interest to study their possible cytotoxic effects. The cytotoxic effects of the green tea extract on fibroblast 3T3 cells are shown in Table 2. The IC_{50} value obtained was 272 ± 33 μg/mL. Only a few studies have reported the cytotoxic effects of tea extracts, while most of them are dealing with the cytotoxicity of individual catechins such as EC, EGCG, CG or ECG, both in cancer and normal cells (von Staszewski et al., 2012; Ugartondo, Mitjans, Lozano, Torres, & Vinardell, 2006; Weisburg, Weissman, Sedaghat, & Babich, 2004). Weisburg et al. (2004) reported that green tea was more cytotoxic than black tea, probably related to the higher content of EGCG. In our study, the green tea extract showed higher IC_{50} values than those reported by Weisburg et al. (2004) for a green tea extract in normal fibroblasts from human oral cavity (70–110 μg/mL), although these values were obtained after 72 h of exposure. The cytotoxic effect of green tea extracts and the most abundant polyphenol in these extracts (EGCG) has been related to the production of hydrogen peroxide in the cell culture medium (Chai, Long, & Halliwell, 2003), suggesting that the cytotoxic effects are an artefact of hydrogen peroxide generation under the assay conditions used. However, other studies suggest that other factors are involved in their cytotoxicity (Dashwood, Orner, & Dashwood, 2002; Weisburg et al., 2004). When the cytotoxic potential of the green tea extract was related to their antioxidant activity, specifically to the DPPH radical scavenging capacity, it could be observed that the effective antioxidant concentrations were noticeably lower than the cytotoxic ones (approximately 25-fold lower), so this extract showed antioxidant properties at concentrations below those found as IC_{50}, therefore at non-cytotoxic concentrations.

3.2. Enzymatic digestion of gelatin films

Fig. 1 shows the electrophoretic profile of the C films and the films with tea extract (F2, F4 and F8) after gastric and gastrointestinal digestion. The electrophoretic profile of the fish gelatin (Gn) used as biopolymer for film preparation was also shown. Films without tea extract were also subjected to the digestion conditions (pH and temperature) without enzymes. No differences were observed in the electrophoretic profile between fish gelatin and gelatin films subjected to the experimental conditions at which digestion was carried out (data not shown). Therefore, the changes described in the electrophoretic profile of the gelatin films can be entirely attributed to the enzymatic activity. All the films were

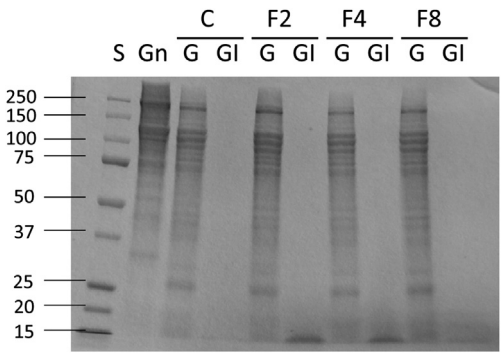

Fig. 1. Electrophoretic profile of gelatin and gelatin films after gastric and gastrointestinal digestion. S: standard; Gn: gelatin; G: gastric digestion; GI: gastrointestinal digestion; C: control gelatin film; F2: gelatin film with 2% green tea extract; F4: gelatin film with 4% green tea extract. F8: gelatin film with 8% green tea extract.

partially digested by pepsin. A decrease in the intensity of the protein bands corresponding to β-components (~200 kDa) and α_1-chains (~100 kDa) of gelatin could be observed as consequence of pepsin digestion (lanes 3, 5, 7 and 9). Furthermore, some new protein bands could be observed in all samples digested by pepsin, especially below 50 kDa, attributed to the protein fragments from the hydrolysis. In contrast, the films were easily digested by pepsin followed by trypsin and chymotrypsin. The resulting hydrolysates were composed of low molecular weight peptides since no protein bands could be observed and the major area of staining corresponded to protein material that run with the migration front (≤15 kDa). In previous studies performed in our laboratory, pepsin has shown a low efficiency for gelatin hydrolysis when compared to trypsin, Alcalase or Esperase (data not shown). This low efficiency may be favoured in the case of gelatin film digestion, since the film formation involves inter and intramolecular interactions between polymer chains as well as between polymer chains and the plasticizers that could impair the enzymatic hydrolysis. On the other hand, it is well known that polyphenols have a significant affinity for proteins. Protein-polyphenol complexes are formed by multiple weak interactions, mainly hydrophobic and hydrogen bonds (He, Lv, & Yao, 2006; von Staszewski, Pilosof, & Jagus, 2011). In sight of these studies, it could be expected that the incorporation of green tea extract in the gelatin film formulation could impair the enzymatic hydrolysis due to the polyphenol–gelatin interactions, and as a consequence, some differences in the electrophoretic profiles could be observed. However, no differences were observed between the C films and the films with green tea extract (F2, F4 and F8) in the distribution of molecular weights, largely suggesting that the presumptive polyphenol–gelatin interactions were not of covalent nature.

3.3. Characterization of the film digestions

3.3.1. Total phenolic compounds, EGCG and EGC

Fig. 2 shows the amount of equivalents of GA released from the films after gastrointestinal digestion. Although the C films did not contain green tea extract in the formulation, these samples showed values of ~3 mg GA equivalents/g film after gastrointestinal digestion due to the known reaction between Folin–Ciocalteu reagent and proteins (Lowry, Rosebrough, Farr, & Randall, 1951). This value was subtracted from the total phenolic compounds measured in the films with green tea extract after gastrointestinal digestion, as the film matrix was composed by 4% of gelatin in all the samples. The increase of the green tea extract in the film formulation gave a proportional increase in the amount of phenolic compounds

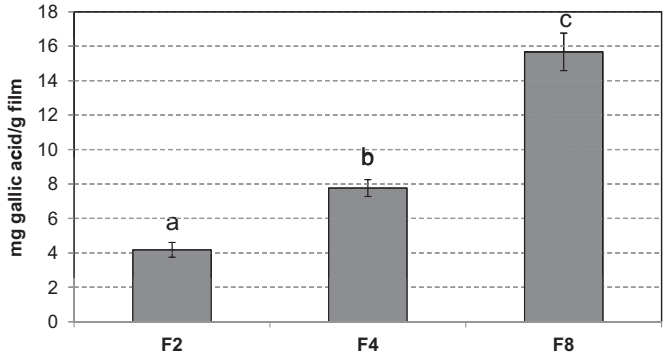

Fig. 2. Recovery of polyphenolic compounds after gastrointestinal digestion. F2: gelatin film with 2% green tea extract; F4: gelatin film with 4% green tea extract. F8: gelatin film with 8% green tea extract. Different letters (a, b, c) indicate significant differences among samples.

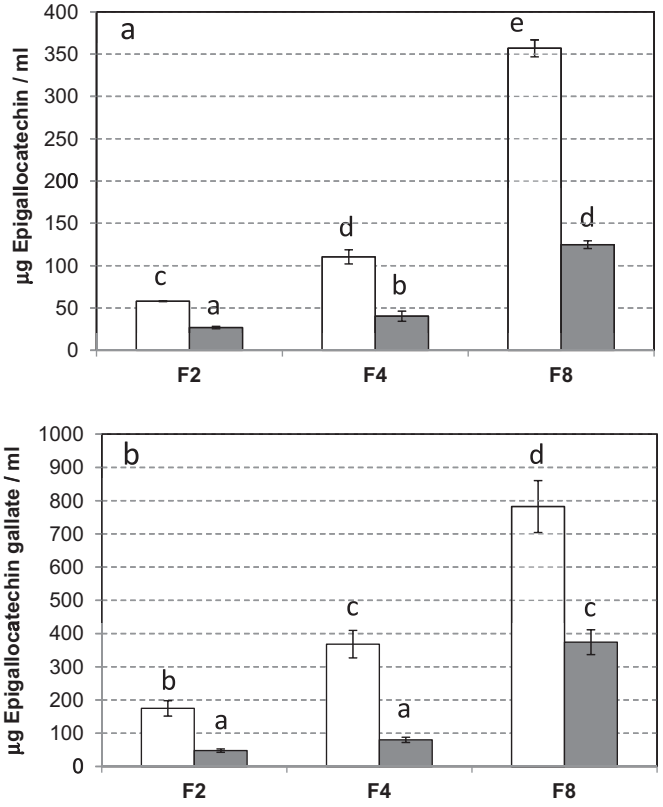

Fig. 3. Recovery of EGC (3a) and EGCG (3b) (µg/mL) after gastric (□) and gastrointestinal digestion (■). F2: gelatin film with 2% green tea extract; F4: gelatin film with 4% green tea extract. F8: gelatin film with 8% green tea extract. Different letters (a, b, c, d, e) indicate significant differences among samples.

released after digestion. Taking into account the amount of green tea extract added to each film forming solution and the total polyphenol content of this extract, the percentage of phenolic compounds recovered from F2, F4 and F8 was 90.9%, 84.4% and 90.4%, respectively. These high recovery percentages indicate the weak nature of the presumptive polyphenol–gelatin interactions in the film matrix, regardless the polyphenol concentration. The slight reduction in the content of phenolic compounds as a consequence of the gastrointestinal digestion (9–16%) could be due to certain instability of catechins to near neutral pH (>6) (Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999). In connection with this, EGCG and EGC have been reported to be the most sensitive catechins to elevated pH (Neilson et al., 2007). In the present study, these catechins were the most abundant catechins in the green tea extract (Table 1). EGC was significantly degraded by intestinal phase enzymatic conditions, with losses of 53–65% with respect to the amount recovered after the gastric phase (Fig. 3a), whereas losses of 52–78% were observed in the case of EGCG under intestinal phase conditions (Fig. 3b). The elevated pH (6–8), residual dissolved oxygen, and likely presence of reactive oxygen species may facilitate catechin degradation in intestinal lumen by auto-oxidation or epimerization reactions (Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007). It has been reported the formation of catechin dimers from EGCG, ECG and EGC subjected to *in vitro* digestion by auto-oxidation reactions (Neilson et al., 2007; Yoshino et al., 1999). Yoshino et al. (1999) also found that the content of total phenols slightly decreased (6.5–13.5%) when EGCG was treated with mild alkaline solution although 75% of this compound was degraded, since the resultant dimerization products maintained in their structures almost all the phenol groups which were originated from EGCG.

3.3.2. Antioxidant activity

The antioxidant activity released from the films after gastric and gastrointestinal digestion is shown in Fig. 4. The antioxidant activity of C films after gastrointestinal digestion was noticeably higher than that observed for C films digested only with pepsin, especially in the case of ABTS radical scavenging ability. This higher antioxidant activity can be attributed to the gelatin hydrolysate obtained after digestion with these enzymes (Fig. 1). Fish gelatin hydrolysates have exhibited a high antioxidant activity in numerous reports (Giménez, Alemán, Montero, & Gómez-Guillén, 2009). The incorporation of green tea extract at 2% to the film forming solution (F2) gave a significant increase of antioxidant activity after digestion when compared to the C films, with about 66-fold and 8-fold increases in FRAP and ABTS, respectively. As observed for total phenolic compounds (Fig. 2), the increase of the content of green tea in the film forming solution gave a proportional increase of both FRAP (Fig. 4a) and ABTS values (Fig. 4b). In the case of FRAP, similar values were reached after gastric and gastrointestinal digestion of each formulation ($p > 0.05$). However, in the case of ABTS, the radical scavenging capacity of the digested samples was slightly lower after gastrointestinal digestion, and significant differences between gastric and gastrointestinal digestion were observed in the case of F2 and F8 films. Taking into account the antioxidant capacity of the green tea extract (Table 2) and the amount of extract added to each film forming solution, 85–100% of the maximum antioxidant activity that could be expected was recovered after both gastric and gastrointestinal digestion in all the samples, in spite of the degradation observed of the main catechins (EGCG and EGC; Fig. 3a and b). These catechins have been reported to be largely responsible for the antioxidant activity of green tea in numerous reports. However, a high antioxidant activity has been also described for the degradation products that are potentially formed during intestinal digestion phase. Thus, according to Yoshino et al. (1999), some of the catechin autoxidation dimers formed at conditions near-neutral pH, such as those during simulated digestion, have Fe^{2+} chelation and $O_2^{\bullet -}$ scavenging activities equal to or greater than EGCG.

3.3.3. Cytotoxicity of film digestions

Cytotoxicity of film after gastrointestinal digestion is shown in Fig. 5. Only F2 gave cell viabilities over 50%. As previously described, the IC_{50} of the green tea extract was 272 ± 33 µg/mL. F2 subjected to digestion under these conditions contained about 252 µg dry matter of green tea/mL of digestion. Therefore, in sight of these

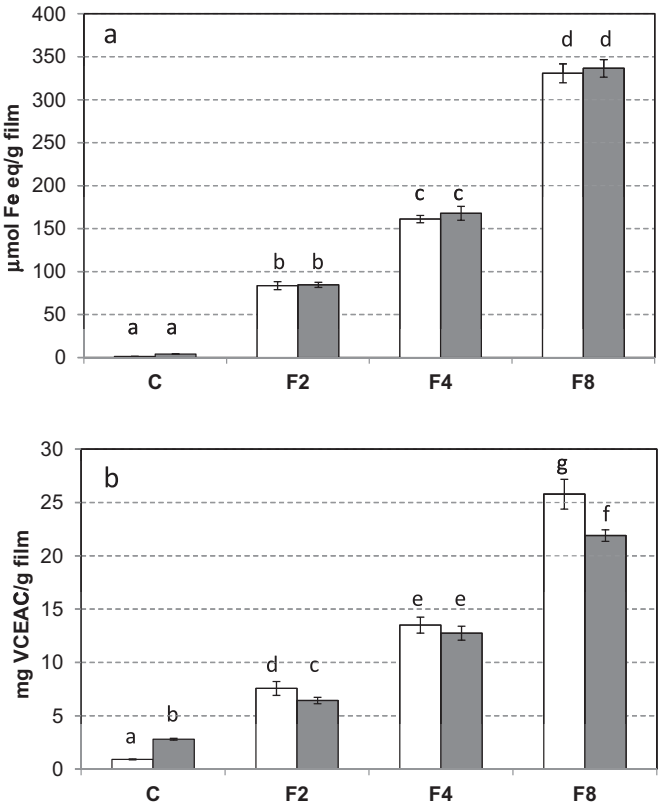

Fig. 4. FRAP (4a) and ABTS (4b) of gelatin films with green tea extract after gastric (□) and gastrointestinal digestion (■). C: control gelatin film; F2: gelatin film with 2% green tea extract; F4: gelatin film with 4% green tea extract. F8: gelatin film with 8% green tea extract. Different letters (a, b, c, d, e) indicate significant differences among samples.

results, it can be deduced that the cytotoxic level of the green tea extract was maintained after the gastrointestinal digestion in spite of the changes observed in the recovery of EGCG and EGC. The cytotoxicity of F4 and F8, despite showing cell viabilities below 50%, may not be considered physiologically significant, since the volume in which *in vitro* digestion was carried out (17 mL), while the volume that human stomach holds after a meal is from about 1–4 L) (Dressman & Yamada, 1991; Johnson, 1994). As a result, the films with high concentrations of extracts can be used safely because of the high dilution of the green tea extract inside the human stomach (50–200 times), which would avoid the possible side effects.

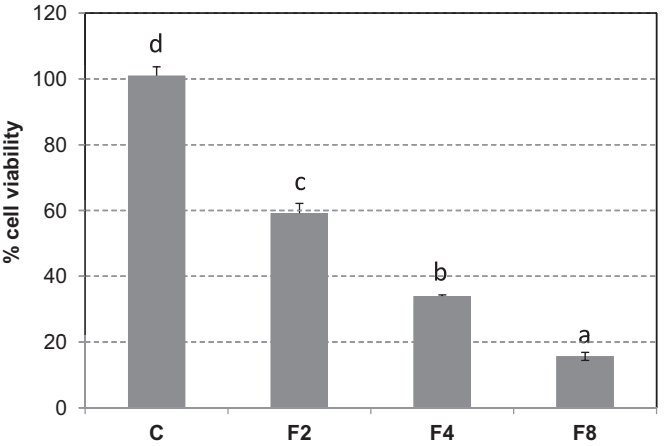

Fig. 5. Cytotoxicity of films after gastrointestinal digestion. C: control gelatin film; F2: gelatin film with 2% green tea extract; F4: gelatin film with 4% green tea extract. F8: gelatin film with 8% green tea extract. Different letters (a, b, c, d) indicate significant differences among samples.

4. Conclusion

The green tea extract obtained showed antioxidant properties at non-cytotoxic concentrations. The antioxidant properties of the green tea extract incorporated to gelatin films were maintained in spite of the desiccation process suffered during the film preparation as well as the degradation of its main polyphenolic components (EGCG and EGC) as a consequence of the enzymatic digestion of the films at near-neutral pH. Therefore, gelatin films may be a vehicle to deliver compounds which may have potential beneficial effects when these edible films are consumed.

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