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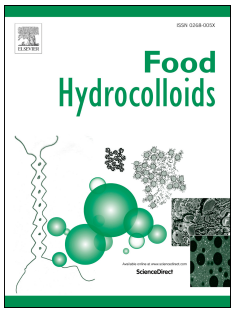

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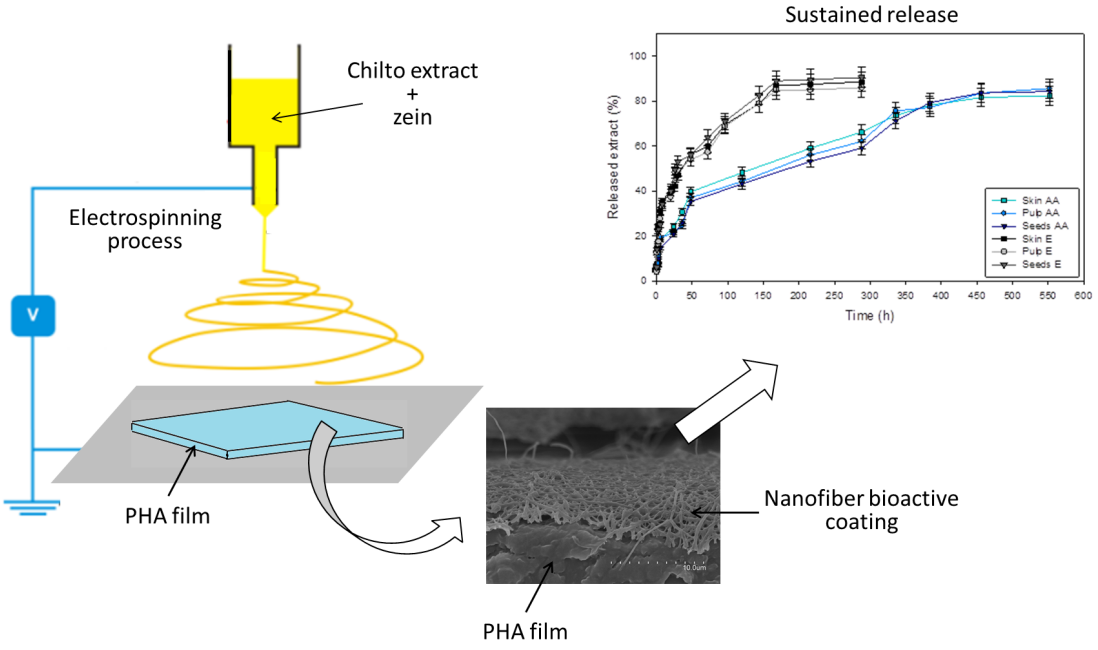

Crosslinked electrospun zein-based food packaging coatings containing bioactive chilto fruit extracts

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Abstract

In this work, zein fibers loaded with phenolic-enriched extracts from pulp, seed and skin of orange chilto were collected on polyhydroxyalkanoate (PHA) films through the electrospinning technique, for their potential use as bioactive internal coatings for food packaging applications. The zein fibers were characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and thermogravimetric analysis (TGA). The water stability of the zein fibers was improved by crosslinking with glutaraldehyde vapors. The encapsulation efficiency of all bioactive phenolic-enriched extracts was greater than 90%. Encapsulation in the zein fibers improved the thermostability of the extracts. Two food simulants (50% ethanol and 3% acetic acid) were used to evaluate the release of the extracts from the crosslinked zein fibers. It was observed that crosslinking delayed the release of phenolic compounds (rosmarinic acid, caffeic acid and its derivates) in both solvents (80% released after 7 days of contact in 50% ethanol and 23 days in 3% acetic acid) and their antioxidant properties were kept. Therefore, this work demonstrates the potential of the developed zein-based encapsulation structures containing chilto extracts to be applied as antioxidant coatings in food packaging structures to contribute to the preservation of both hydrophilic and lipophilic food products.

1. Introduction

Chilto (*Solanum betaceum* Cav) is a native fruit that grows in the Northwest region of Argentina. These fruits are popularly consumed (in salads, juices, jams, liquors, and other regional products) by different aboriginal and rural communities and their virtues are currently being rediscovered (Orqueda et al., 2017).

Solanum betaceum, known as "tamarillo", "chilto" or "tree tomato", produces edible fleshy fruits with a growing market in its native Andean countries, as well as in North America, Europe and Oceania (Prohens & Nuez, 2000; Samuels, 2015). The main types of fruit are recognized depending on the color of the skin, with dark purple, orange and red fruits (Prohens, Ruiz, & Nuez, 1996). Currently, the sustainable cultivation of orange chilto in Argentina is taking place in the Yungas region (Orqueda et al., 2017).

In a previous work, the interest of the pulp, seed and skin of chilto to produce functional foods was demonstrated. Specifically, the nutritional and phytochemical composition, as well as the antioxidant activity and the inhibitory capacity of fruit fractions on key enzymes involved in the metabolic syndrome (α-glucosidase, α-amylase and pancreatic lipase) were described (Orqueda et al, 2017). In fact, these bioactive properties were demonstrated both before and after simulated gastroduodenal digestion and were ascribed to the phenolic compounds present in the fruit biomass (Orqueda et al, 2017).

The main phenolic compounds of chilto seed, pulp and skin extracts were rosmarinic acid and its derivatives (Orqueda et al., 2017). Rosmarinic acid (RosA), a naturally water-soluble phenolic compound is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid. RosA has gained a great deal of attention due to its various biological activities, such as antibacterial, antiviral, antioxidant and anti-inflammatory activities (Ngo, Lau, & Chua, 2018; Gonçalves et al., 2019).

Moreover, the safety of the polyphenol-rich extracts from chilto were evaluated, showing no toxic and mutagenic effect on *Artemia salina* and *Salmonella typhimurium* strains, respectively (Orqueda et al., 2017).

The interesting health-promoting properties of chilto, make these fruits excellent sources of natural functional food ingredients. For this reason, pulp, skin and seed extracts of chilto, previously characterized by Orqueda et al. (2017), were considered for inclusion in bioactive food packaging structures.

Electrospinning has recently gained significant interest in the fields of bioactive encapsulation and functional food development as it is a relatively simple, versatile, low

cost, non-thermal process and does not require the use of organic solvents as aqueous-based solutions have shown their potential as starting solutions for electrospinning (Quek, Hadi, & Tanambell, 2019). This non-mechanical technique involves the use of a high voltage electrostatic field to charge the surface of a polymer solution droplet, thereby inducing the ejection of a liquid jet through a spinneret (Mendes, Stephansen, & Chronakis, 2017).

Zein has demonstrated to be an excellent material for electrospinning and has been successfully used to encapsulate diverse bioactive compounds (Gómez-Mascaraque et al., 2017; Gómez-Mascaraque, Pérez-Masiá, González-Barrio, Periago, & Lopez-Rubio, 2017; Gomez-Mascaraque, Tordera, Fabra, Martínez-Sanz, & López-Rubio, 2019; Quek et al., 2019) and to generate biodegradable food packaging layers (Fabra, López-Rubio, & Lagaron, 2016). Zein is a plant protein that has found use in adhesive and coating materials for pharmaceutical, biomedical, and food applications, because of its non-toxicity, biodegradability, biocompatibility and economic reasons (Moradkhannejhad, Abdouss, Nikfarjam, Mazinani, & Heydari, 2018; Schmidt, Hamaker, & Wilker, 2018). It is a hydrophobic protein (prolamin) with high thermal stability and oxygen-barrier properties (Neo et al., 2013). Electrospun zein nanofibers are easily produced giving raise to homogeneous and flexible structures, but with poor mechanical properties and poor water stability, fact which restricts their applications. When immersed in water, zein fibers swell and collapse into films with a considerable decrease in surface area, also decreasing the number of interconnected pores and tensile strength (Jiang, Reddy, & Yang, 2010), fact which could hamper its use as bioactive food packaging coating. However, this can be significantly improved by applying crosslinking processes (Quek et al., 2018). Glutaraldehyde is a crosslinking agent that has been widely used to modify biodegradable films based on polyvinyl alcohol (Mansur, Sadahira, Souza, & Mansur, 2008), polyvinyl alcohol/methyl cellulose (Park, Park, & Ruckenstein, 2001), gelatin and zein (Fan, Duquette, Dumont, & Simpson, 2018; Matsuda, Iwata, Se, & Ikada, 1999; Shahbazi, Ahmadi, Seif, & Rajabzadeh, 2016). Some advantages of this crosslinking agent are the low cost, fast reaction time, and ability to crosslink many amino acid groups present in the protein molecules (Huang et al., 2014). Glutaraldehyde is more effective than other aldehydes as a crosslinking agent and its application to crosslink nanofibers upon exposure to its vapors has demonstrated lower or no cytotoxic effect (Destaye, Lin, & Lee, 2013). In fact, the use of glutaraldehyde in the vapor phase as crosslinking agent of natural and artificial polymer blends has proven to be an

effective procedure that avoids the presence of toxic residues into the materials, as no effect on cell viability and proliferation, neither increase in the cytoplasmic lactate dehydrogenase release, nor damages on mitochondrial and lysosomal functions were observed (Ramires & Milella, 2002; Shahbazi et al., 2016).

Therefore, the aim of this work was to develop and characterize crosslinked electospun zein fibers loaded with phenolic-rich orange chilto extracts with potential as bioactive food packaging coatings. The base material chosen in this work was a commercial polyhydroxyalkanoate, given the increased interest of these materials for biodegradable food packaging applications.

2. Materials and methods

2.1. Reagents

Zein from corn (grade Z3625), with reported molecular weight of 22–24 kDa, was purchased from Sigma-Aldrich (Spain). 96% (v/v) acetic acid was supplied by Scharlab (Spain). Absolute ethanol (> 99.9%) was purchased from VWR (UK). Glutaraldehyde solution and hydrochloric acid were obtained from Sigma-Aldrich.

The polyhydroxyalkanoate (PHA) film used as base film to deposit the electrospun zein layer was a polyhydroxybutyrate/polyhydroxyvalerate 8% - Biopolymer PHB92/PHV 8 film produced by Goodfellow GmbH (Germany) and distributed by MicroPlanet Laboratorios, S.L. (Spain).

2,2 -azino-bis (3-ethylbenzo thiazoline-6-sulphonic acid) (ABTS), 2,2-azo-bis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

Fruits of *Solanum betaceum* Cav. (orange cultivar) were collected in Parque Sierra de San Javier, Tucumán, Argentina, during February and March 2014 and 2015. The fruits were harvested at the ripening stage in which they are consumed and were transported immediately to the laboratory at 4ºC. The fresh fruits were washed with water. Skin, pulp and seeds (without jelly portion) were separated, freeze-dried and powdered.

2.3. Phenolic enriched extracts preparation

The powders of seed, pulp and skin were extracted at room temperature with 95° ethanol (1 g of powder per 5 mL of ethanol) assisted by ultrasound during 30 min under

stirring (40 cycles/min). Subsequently, the samples were centrifuged at 12,000 g for 10 min. The supernatants were taken to dryness under reduced pressure to give the phenolic-enriched extracts (PEE).

2.4. Preparation of zein fibers through electrospinning

Zein solutions 20% (w/v) were prepared by dissolving the protein in 80% (v/v) ethanol at room temperature under magnetic stirring. PEE (10% w/w of the total solids content) were added to the zein solutions and the mixtures were stirred until complete dissolution. Then, the solutions were electrospun following a procedure adapted from Gómez-Mascaraque, Sanchez, & López-Rubio (2016), using an electrospinning apparatus assembled in-house, equipped with a variable high-voltage 0-30 kV power supply, at a steady flow-rate of 0.15 mL/h, an applied voltage of 11 kV and a syringe tip-collector distance of 10 cm. The zein fibers were directly collected onto the PHA films for 40 min. The amount of electrospun extract loaded zein was calculated by weighing the film before and after the collection of fibers (Fabra, Sánchez, López-Rubio, & Lagaron, 2014).

2.5. Zein fibers Post-Treatment

Once the zein fibers were collected onto PHA films, they were exposed to 25% v/v glutaraldehyde (GA) vapors as a crosslinker and to 35% v/v hydrochloric acid (HCl) solution to create the necessary conditions for catalysis (Lee, Li, Chen, & Park, 2016). The crosslinking container was divided into two parts by a grid. On the bottom, a Petri dish was placed with the glutaraldehyde solution and another with the HCl solution. On top, PHA films containing the zein fibers were placed inside Petri dishes. In this way, the films were exposed to the vapors, in the closed container. The crosslinking was performed for 5h (Treatment A) and 24 h (Treatment B). All the crosslinked samples were rinsed for 30 min in phosphate buffer saline (PBS) to eliminate the residual glutaraldehyde and then they were dried in a desiccator at 23 ± 2 °C (Ramires & Milella, 2002).

Then, the films were spread between two Teflon layers and compressed in a hot press (Carver 4122, USA) at 45 °C for 2 min. The two hot plates from the press were put into contact at the specified conditions, but no extra pressure was applied. These conditions were sufficient to guarantee the adhesion between the zein coating and the PHA film.

206 **2.6. Characterization of films**

207 **2.6.1. Morphological characterization of the particles**

208 Samples were sputter-coated with a gold-palladium mixture under vacuum and analyzed
209 by scanning electron microscopy (SEM) on a Hitachi microscope (Hitachi S-4800),
210 following the method described in Gómez-Mascaraque et al. (2016). Particle diameters
211 were measured from the SEM micrographs using the ImageJ software. Size
212 distributions were obtained from a minimum of 200 measurements.

213

214 **2.6.2. Analysis of the particles by attenuated total reflectance Fourier transform**
215 **infrared spectroscopic (ATR-FTIR) and Fourier transform infrared (FT-IR)**

216 FT-IR spectra were collected following the method described in Gómez-Mascaraque et
217 al. (2016). PEE (*ca*. 1–2 mg) were dispersed in about 130 mg of potassium bromide and
218 a pellet was formed by compressing at ca. 150 MPa.
219 FT-IR spectra were collected in the transmission mode using a Bruker (Rheinstetten,
220 Germany) FT-IR Tensor 37 equipment. Zein fibers with and without PEE were
221 analyzed without further processing in ATR mode. All spectra were obtained by
222 averaging 10 scans at 1 cm^{-1} resolution.

223

224 **2.6.3. Thermogravimetric analysis (TGA)**

225 Thermogravimetric analysis (TGA) was performed with a TG-STDA Mettler Toledo
226 model TGA/STDA851e/LF/1600 analyzer. The samples (ca. 10 mg) were heated from
227 25 °C to 600 °C at a heating rate of 10 °C/min under dynamic nitrogen flow.
228 Thermogravimetric curves express the weight of the sample as a function of
229 temperature.

230

231 **2.7. Fiber stability assessment**

232 Stability of the films containing zein fibers with and without PEE, was assessed using a
233 protocol adapted from Kiechel & Schauer (2013). Briefly, the zein fibers collected on
234 PHA films were cut into pieces of 5 x 5mm. Each piece was immersed in 20 mL of
235 distilled water. After different time intervals, the zein coatings were removed from the
236 tubes and dried in a desiccator (0% relative humidity). The surface morphology of the
237 dried zein coating was analyzed by SEM as described in Section 2.6.1. The test was
238 carried out using films with and without crosslinking and with and without press
239 treatment. All experiments were performed in triplicates.

240

241 **2.8. Phenolic enriched extract encapsulation efficiency**

242 The total amount of each PEE incorporated within the fibers was estimated by UV–Vis
243 spectroscopy according to a protocol adapted from Atay et al. (2018). For this purpose,
244 zein fibers (10 mg/mL) were dissolved in ethanol 80% (v/v). The absorbance at 280 nm
245 was measured using a NanoDrop ND1000 spectrophotometer (Thermo Fisher
246 Scientific, USA). Calibration curves for each PEE in ethanol 80% were previously
247 obtained by its absorbance at 280 nm (R^2>0.997). The contribution of zein to the
248 absorbance at 280 nm was also considered.

249 The encapsulation efficiency (EE) was then calculated according to Eq. (1). Three
250 independent replicates of each sample were analyzed.

251 $$EE\ (\%) = \frac{\text{Total extract content in the fibers}}{\text{Theoretical extract content in the fibers}} \times 100 \quad (1)$$

252

253 **2.9. *In vitro* release assays**

254 The release of phenolic compounds from the zein fibers was assessed in two different
255 food simulants following a method adapted from Alehosseini, Gómez-Mascaraque,
256 Martínez-Sanz, & López-Rubio (2019). 50% ethanol and 3% acetic acid (w/v) were
257 selected as food simulants, according to the Commission Regulation 10/2011 EU on
258 plastic materials and articles intended to come into contact with food (10/2011/EC). For
259 this analysis, the fibers (20 mg / mL) were immersed in the release medium during
260 different times. The absorbance of each system was measured at 280 nm using a
261 NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), according to
262 an already established methodology (Moreno et al., 2018). With the absorbance values,
263 the concentration of phenolic components released over time was determined using
264 calibration curves (considering that the fibers contained 10% extract) of each PEE in
265 both media (R^2> 0.9975).

266 The contribution of zein to the absorbance at 280 nm was also considered and
267 subtracted from the total absorbance of the release medium. Experiments were
268 performed at room temperature (20 °C) in independent duplicates.

269

270 **2.10. High performance liquid chromatography (HPLC) analysis**

271 The phenolic compounds of each extract and the phenolic components released from
272 zein fibers (2 mg DW/mL) were identified by HPLC-DAD. The HPLC system

consisting of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 μL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA) were used to analyze the extracts eluted from the zein fibers and the individual extracts. An XbridgeTM 135 C18 column (4.6 x 150 mm, 5 μm; Waters Corporation, Milford, MA). The solvent system for the separation of components was composed of 0.1% acetic acid in water (A) and 0.1% methanol in acetic acid as follow: 10% to 57% B over 45 min and increasing to 100% B at 60 min. The flow rate was 0.5 mL/min and the volume injected was 20 μL. The compounds were monitored at 254 nm.

The identification of phenolic compounds was carried out by comparing the retention times and spectral data (220–600 nm) of each peak with those of standards from Sigma-Aldrich (MO, USA) and Fluka Chemical Corp. (USA). Polyphenols quantification was based on external calibration curves from available phenolic standards. Plots were built by comparison of the area and concentration in the range of 1-500 ppm. Results were expressed as μg equivalents of the standard compounds per milligram of dry weight. Experiments were performed in independent triplicates.

2.11.1. Antioxidant activities

In order to determine if the encapsulated extracts in fibers retained antioxidant activity, ABTS and AAPH assays were performed. For these tests, equilibrium concentrations of phenolic compounds previously eluted from the zein fibers (between 0.2 and 20 μg GAE/mL) were used.

2.11.1.1. ***Total antioxidant capacity assay***: The antioxidant power of the extracts loaded fibers was assayed by the improved ABTS radical cation ($ABTS^{\cdot+}$) method as described by Re et al. (1999). Results were expressed as SC_{50} (concentration of extract necessary to scavenge 50% of ABTS radical). In all cases, the antioxidant capacity of zein was considered.

2.11.1.2. ***Protection of oxidative hemolysis assay:*** The method reported by Mendes, de Freitas, Baptista, & Carvalho (2011) was used to determine the protective activity of oxidative hemolysis of red blood cells (RBC) using AAPH as reagent generator of peroxyl radicals. The reaction mixture (RBC, APPH and phenolic compounds

previously eluted from the zein fibers) was incubated at 37°C for 1 h in water bath, and then was centrifuged (4000xg) for 3 min. The absorbance of supernatant was measured at 545 nm in spectrophotometer, and the percent of oxidative hemolysis was calculated. IC_{50} values were determined as the concentration of extracts necessary to protect the RBC from oxidative hemolysis by 50%. The antioxidant capacity of zein was also determined.

2.12. Statistical analysis

Each experimental value is expressed as the mean ± standard deviation (SD). The statistical analysis of experimental data was performed using InfoStat software (Student Version, 2011). The one-way ANOVA with Tukey post-test at a confidence level of 95% was used to evaluate the significance of differences between groups. The criterion of statistical significance was taken as $p \leq 0.05$.

3. Results and discussion

The aim of this work was to develop bioactive food packaging coatings through electrospinning containing PEE of different parts from orange chilto fruits. The low compatibility of hydrocolloids like zein and hydrophobic materials such as polyhydroxyalcanoates (PHAs) used in this work, prevented the use of casting methods to develop multilayer systems or to combine both materials through compression moulding, since adhesion between the layers was very poor and partial or even complete delamination between the different layers occurred, as previously reported for similar systems (Martínez-Sanz, López-Rubio, & Lagaron, 2013; Fabra, López-Rubio, & Lagaron, 2016). Thus, in this work, the polyphenolic extract of pulp and even of waste material such as seed, and skin were used. These extracts mainly contained caffeoyl derivates, RosA and its derivatives and showed several functional properties principally antioxidant, and antimicrobial that could be useful for active food packaging applications (Orqueda et al., 2017). A preliminary optimization of the electrospinning parameters was carried out in order to select the best conditions for zein fibers production. Subsequently, the chilto PEE loaded zein fibers were produced and characterized.

3.1. Characterization of films

3.1.1. Morphological characterization of the fibers

The SEM images and particle size distribution graphs of zein fibers (with and without PEE from seed, pulp and skin of orange chilto) are displayed in **Fig. 1**. This figure shows the average, maximum and minimum diameter values for the different electrospun fibers (µm). The diameter of the zein fibers without extracts was similar to those previously reported for electrospun zein fibers (Alehosseini et al., 2019; Li, Lim, & Kakuda, 2009; Neo et al., 2013), showing an ultrathin fibrillar structure, of cylindrical shape and homogeneous morphology (**Fig. 1**). No significant differences were observed in the average diameter between the extract-free zein fibers and those loaded with PEE. In all cases, the fiber diameters were between 0.064-0.274 µm. The obtained medium values were similar for all the developed structures, independently of the type of extract encapsulated.

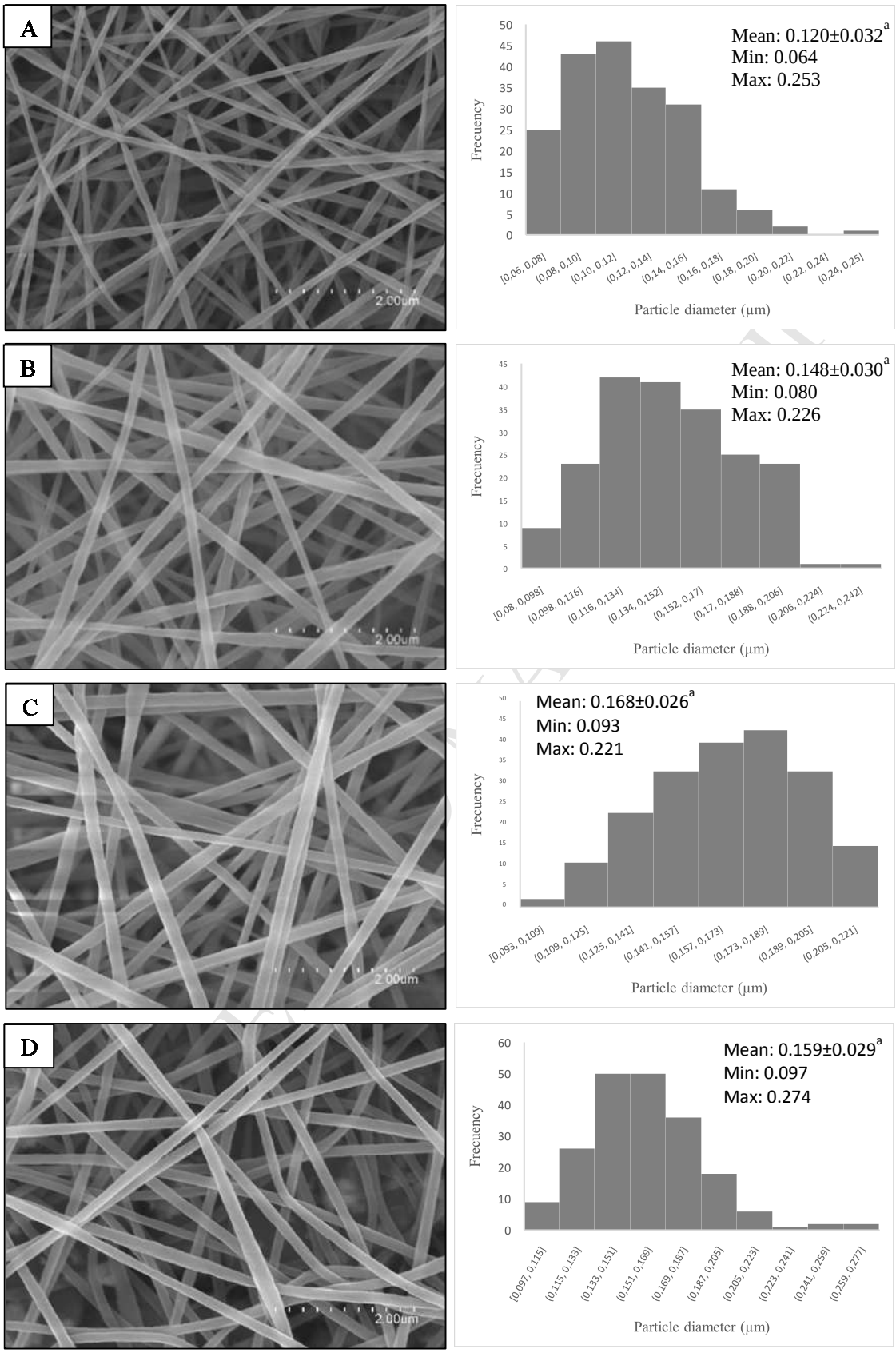


Figure 1. SEM images and particle size distributions graphs of zein fibers containing phenolic enriched extracts from: **A.** Orange chilto seeds; **B.** Orange chilto pulp; **C.** Orange chilto skin; and **D.** without extract.

3.1.2. Analysis of the fibers by FT-IR

FT-IR analysis was performed to evaluate the interactions between zein and the phenolic compounds from chilto PEE. **Fig. 2** shows, as an example, the FT-IR spectra of the zein fibers containing chilto seed extract together with the spectrum of the zein fibers without extracts, analyzed in ATR mode. The FT-IR spectrum of the chilto seed extract is also shown in **Fig. 2**. The spectra of the pulp and skin extracts and zein fibers with and without extracts are included in the **Supplementary Material** (**Fig. S1**). Typical zein bands were present in the different fiber coatings at 3290, 2950, 1640, 1530, 1440 and 1245 cm^{-1} that are derived from Amide A (N-H stretching vibration), Amide B (asymmetric stretching vibration of =C-H and $–NH_3^+$), Amide I band (80% C=O stretching, 10% C–N stretching), Amide II band (60% N–H bending, 30% C–N stretching and 10% C–C stretching), and Amide III band (complex band resulting from several coordinate displacements), respectively (Alehosseini et al., 2019; Costamagna et al., 2017; Deng, Kang, Liu, Feng, & Zhang, 2018; Sivam, Sun-Waterhouse, Perera, & Waterhouse, 2012).

The spectra of the PEE were characterized by the presence of absorption bands at 3325-3395 cm^{-1}, 2920 cm^{-1}, 1740-1700 cm^{-1} and 1600–800 cm^{-1} region attributed to the stretching, bending and deformation vibrations of polyphenolic compounds (Gannasin, Adzahan, Hamzah, Mustafa, & Muhammad, 2015; Moreno et al., 2018; Sivam et al., 2012). The peak at 1710 cm^{-1} is assigned to the stretching vibration of carbonyl groups (Stehfest, Boese, Kerns, Piry, & Wilhelm, 2004). The peaks at 1612 cm^{-1}, 1520 cm^{-1} and 1463 cm^{-1} are ascribed to benzene ring stretching vibrations (Stehfest, et al., 2004). The spectral vibrations of the PEE loaded zein fibers and unloaded zein fibers showed small spectral variations. For instance, a displacement to greater wavenumbers (~ 10-30 cm^{-1}) was observed in the Amide I and Amide II bands. In addition, a slight narrowing in the Amide A band was observed, which is generally due to changes in the hydrogen bonding structure of the protein (Doyle, Bendit, & Blout, 1975). These changes could be attributed to possible intermolecular interactions between the zein and the hydroxyl groups of rosmarinic acid and caffeic acid and its derivatives present in the chilto extracts or between free carboxyl group of phenolic compounds with the free ε-amino groups from the protein to form amide bands.

Fig. 3 also shows a magnification of the regions of interest in which the arrows point out to the spectral differences. Moreover, a characteristic absorption peak of the extract

can be observed at 1050 cm^{-1} (see arrow), which confirms its incorporation into the zein fibers.

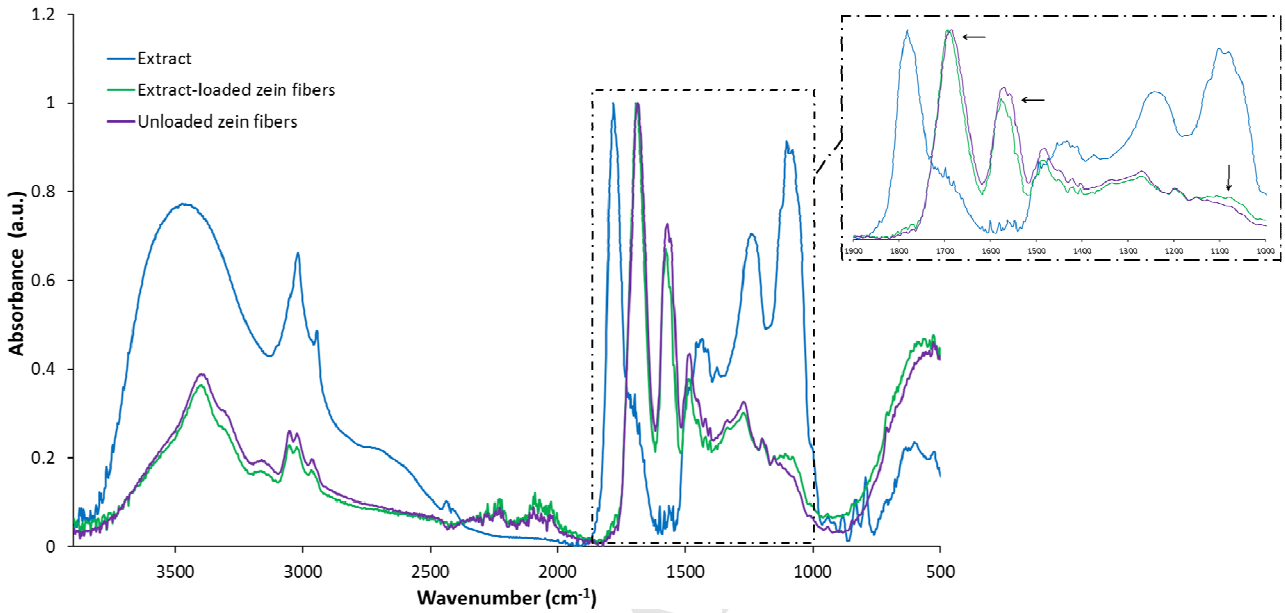


Figure 2. FT-IR spectra of chilto seeds extract, together with unloaded and extract-loaded zein fibers. Arrows in the magnified spectral range point out to the main spectral changes as a consequence of extract incorporation.

3.1.3. Thermal stability of PEE-loaded zein fibers

The thermal stability of the extracts-loaded zein fibers was also determined. **Fig. 3** shows, as an example, the thermogravimetric curves obtained for PEE from orange chilto skin and those for zein fibers with and without PEE.

The zein fibers were stable at 45° C, temperature used in the heat press treatment. An initial weight loss of 2-4% was observed in the zein fibers between 60° and 150°C, probably due to water evaporation. The main mass loss of zein fibers took place around 300°C, which could be ascribed to the thermal degradation of the polymer structure (Erdogan, Demir, & Bayraktar, 2015), only retaining around 17% of the initial mass.

The thermal degradation of the PEE from the chilto skin started at a lower temperature and showed a more gradual mass loss in the temperature range from ~100ºC to 450ºC.

When comparing the thermal stability of the electropun fibers containing the three different extracts from chilto, with that from the unloaded zein fibers, a very similar degradation profile was seen, which normally is ascribed to a thermal stabilization of

the encapsulated compounds. However, taking into account the small contribution of the extracts to the total mass of material, from **Figure 3** (as a representative example), it can only be stated that the incorporation of the PEE did not affect the thermal stability of the zein fibers.

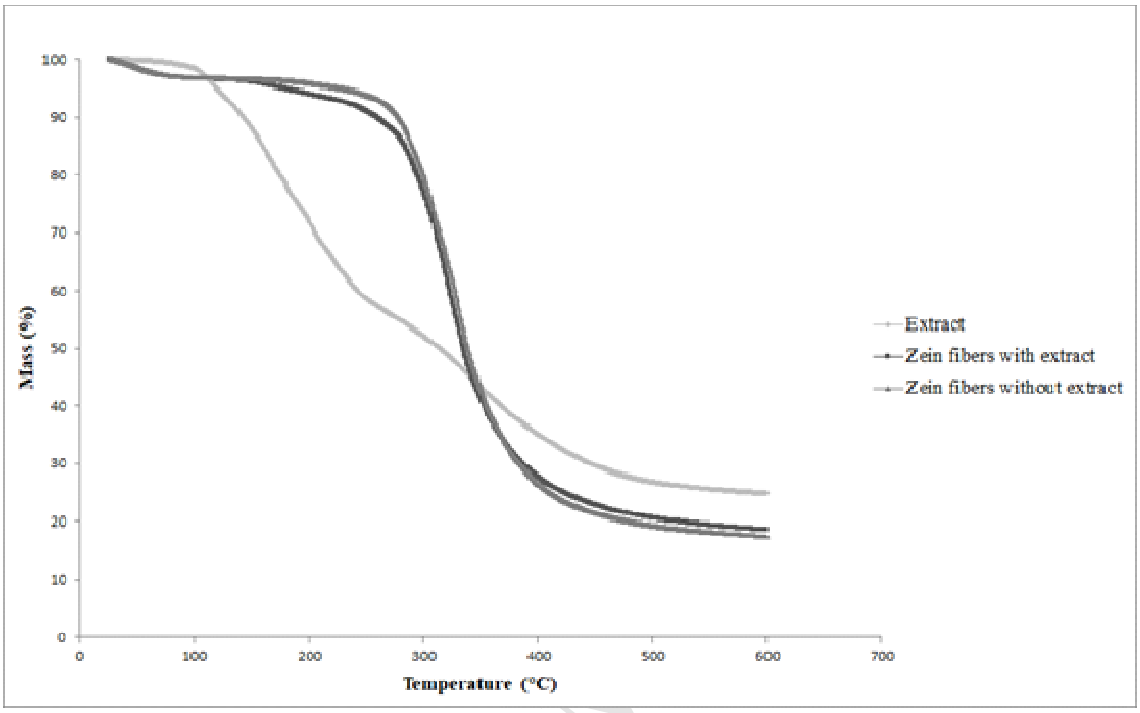


Figure 3. Thermogravimetric curves of PEE orange chilto skin and zein fibers with and without PEE.

3.2. Water resistance properties of the zein fibers

As most food products contain relatively high water contents, the integrity of the packaging coating upon water exposure was evaluated. The surface morphology of the coated PHA films was thus analyzed by SEM and the effect of crosslinking on the water resistance of the coatings was evaluated. The changes in the surface morphology at different water immersion time periods, are shown in **Fig. 4** for zein fibers containing orange chilto skin extract as an example.

When no crosslinking was applied, the fibrillar morphology of the electrospun zein coatings was completely lost after 6 h of immersion in water (**Fig 4A** and **Fig 4B**) similarly as previously reported by other authors (Jiang et al., 2010; Li et al., 2009). The fibers collapsed in the water and were released from the PHA base film.

In contrast, a better stability was observed for the coatings crosslinked using glutaraldehyde vapors in an acid environment. Glutaraldehyde has been previously used as a crosslinking reagent to produce electrospun zein fibers with improved physical

properties and solvent resistance (Selling, Woods, Sessa, & Biswas, 2008) and for the modification of biodegradable films (Shahbazi et al., 2016), demonstrating that the films crosslinked by this method did not display any cytotoxic effects on fibroblast cells. Ramires & Milella (2002) evaluated the harmful effects of glutaraldehyde residues released from crosslinked membranes through *in-vitro* cytotoxicity and cytocompatibility tests, showing that these do not have toxic effects. **Fig. 4C** and **4E** show the morphology of the fibers after the crosslinking treatment A and B, respectively, before water immersion, while **Fig. 4D** and **4F** show the same materials after 24 h immersion in water. It can be observed that while the shorter treatment A led to a certain degree of swelling after this immersion time, the fibers crosslinked for a longer period (treatment B), kept the integrity without significant morphological changes after being 24h in water (**Fig. 4F**). For this reason, treatment B was selected to check how the integrity of the fibers upon water immersion was kept at longer time periods (**Fig. S2, Supplementary Material**). It could be seen that the crosslinking treatment was very effective in protecting the integrity of the coating even after 12 days of immersing the coated PHA film in water, although from day 9 a significant swelling occurred which led to a partial collapse of the fibers.

Glutaraldehyde interacts with amino, carboxy, amido and other protein groups (Jayakrishnan, & Jameela, 1996). These bonds formed during crosslinking should be the main responsible for the best water stability of the crosslinked samples. In addition, crosslinking reduces the interstitial spaces between the protein chains, thus decreasing molecular motion and preventing swelling of the fibers (Fan et al., 2018; Jiang et al., 2010).

The cross-sections of the coated films were also analysed. **Fig. 5A** shows, as an example, the cross-section of coated PHA films, in this case with the zein fibers containing the chilto skin extract. A clear delamination of the coating was observed and, thus, a hot press treatment was applied to improve adhesion of the coating to the PHA base film. This treatment effectively improved the adhesion of the fibers to the PHA film, being even better when the fibers were previously crosslinked with treatments A and B **(Figs. 5B, 5C** and **5D)**.

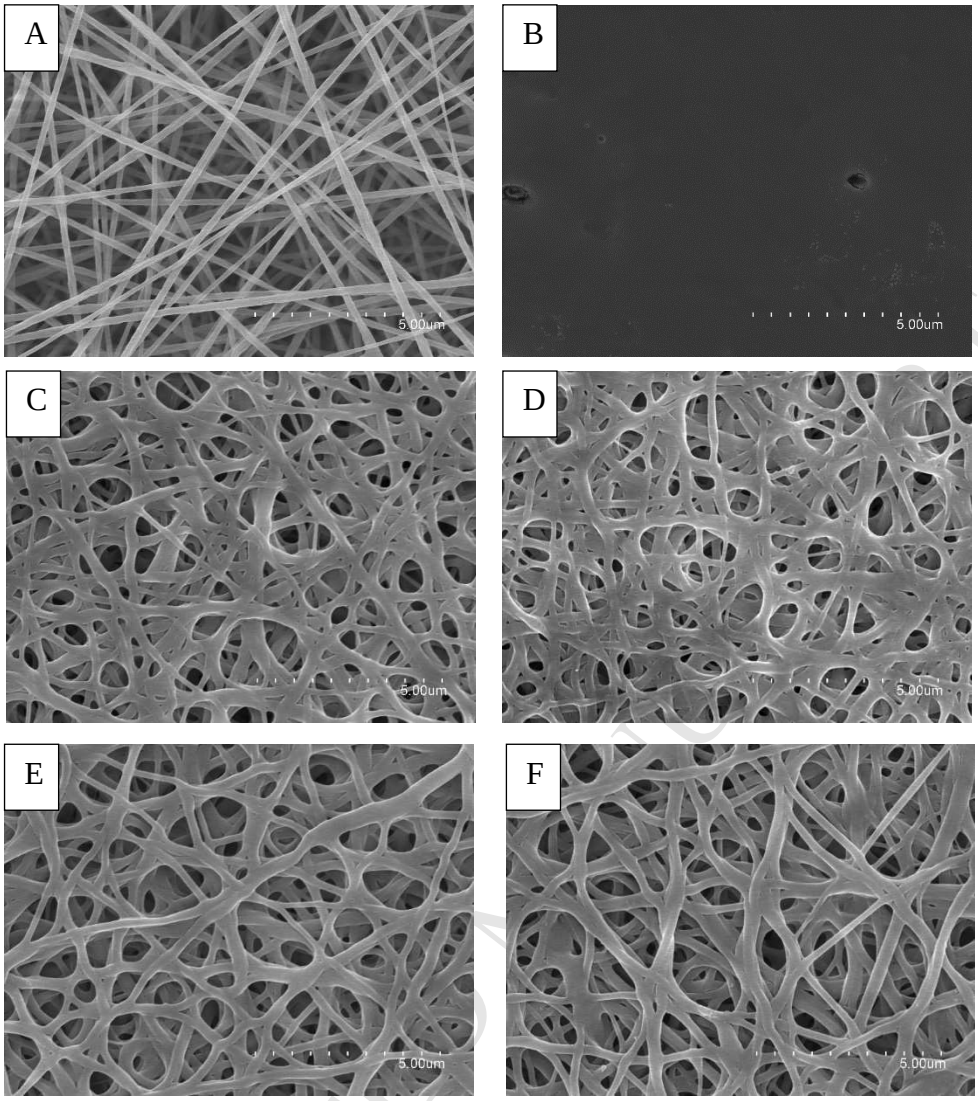


Figure 4. SEM micrographs of electrospun coatings containing orange chilto skin extract after immersion in water for different time periods: As-obtained fibers without crosslinking, before (A) and after immersion in water during 6 h (**B**); Fibers crosslinked for 5 h, before (C) and after immersion in water during 24 h (**D.**); Fibers crosslinked for 24 h, before (E) and after immersion in water for 24 h (**F**).

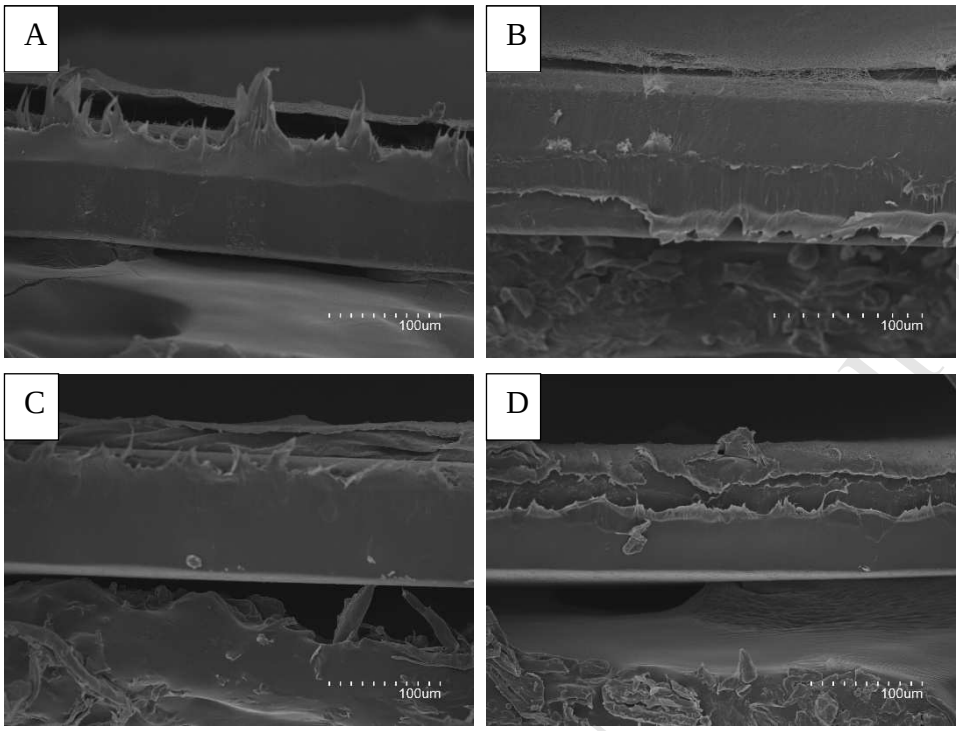


Figure 5. SEM Cross-section images of PHA films with the zein fiber coatings containing the chilto skin extract: **A.** Non-crosslinked coating without hot press treatment (delamination is clearly seen); **B.** Non-crosslinked coating after hot press treatment (better adhesion); **C.** Coating crosslinked for 5 h with hot press treatment; **D.** Coating crosslinked for 24 h with hot press treatment.

3.3. Extracts encapsulation efficiency

In order to estimate the amount of each extract effectively incorporated within the developed coatings, the encapsulation efficiency was calculated according to Eq. (1) and the results are summarized in **Table 1**.

All extracts showed similar encapsulation efficiencies (90.2 - 94.3%). These values were considerably high and similar to those previously reported for the microencapsulation of other natural compounds through electrohydrodynamic processing, using zein as an encapsulation matrix (Alehosseini et al., 2019; Fabra et al., 2016; Neo et al., 2013; Wang et al., 2017). The high encapsulation efficiency values obtained highlight the interest of the electrospinning technique for encapsulation purposes, not only derived from the high efficiency obtained, but also to additional advantages, such as the use of aqueous solutions and mild conditions (not requiring high temperatures for drying out the structures obtained). In this specific work, it is also

demonstrated that these type of structures can themselves constitute an alternative form of delivering bioactive compounds to foods in the form of inner coatings of packaging materials.

Table 1. Extracts encapsulation efficiency (%) for the developed coatings.

		Zein fibers
Solanum betaceum	Seeds	90.2±6.1[a]
Cav. (orange)	Pulp	94.3±4.2[a]
	Skin	92.4±4.4[a]

Values are reported as mean ± standard deviation of triplicates. The values in the same column with a common letter are not significantly different according to Tukey's test ($p \leq 0.05$).

3.4. Release of PEE from the electrospun coatings

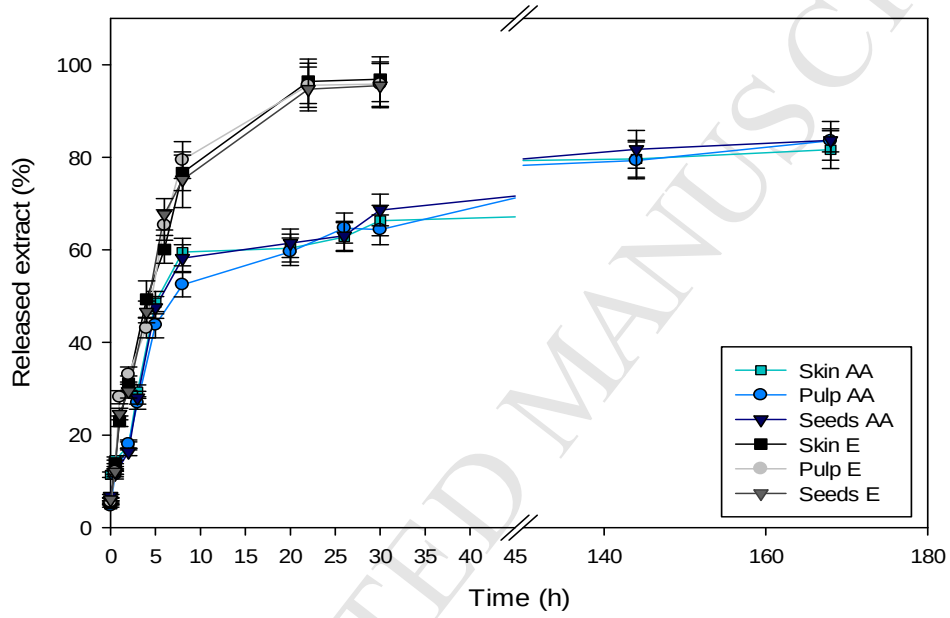
The objective of the developed coatings, as already mentioned, was to gradually release the extracts to the actual food product from the packaging structure. Therefore, the diffusion of the encapsulated bioactive compounds from the zein coatings into 50% ethanol and 3% acetic acid solutions used as food simulants was evaluated. The aqueous ethanolic solution can be used to simulate fatty food products, foods capable of extracting lipophilic substances, as well as alcoholic foods with an alcoholic strength greater than 20% or oil- in-water emulsions. On the other hand, 3% acetic acid is normally used as a simulant of hydrophilic foods or foods capable of extracting hydrophilic substances, with pH less than 4.5. Both solvents can simulate food type turbid drinks, juices, nectars, canned meats, yogurt, cream, cheese, etc (10/2011 / CE).

Given the previous results obtained, only the crosslinked materials were used for the release experiments. **Fig. 6** shows the release profiles obtained for the different formulations in both food simulants. The release was greater and faster with 50% ethanol, being superior to 90% after 24 h in the case of the coating crosslinked for lower time period (treatment A, **Fig. 6A**), and over 80% after 7 days of experiment for the different coatings crosslinked for 24h (treatment B, **Fig. 6B**). When 3% acetic acid was used as a release medium, a release higher than 80% was obtained for the crosslinking

treatment A after 7 days (**Fig. 6A**), while for treatment B, release values over 70% were obtained after 14 days, reaching 80-86% after 23 days of experiment (**Fig. 6B**).

The longer crosslinking (treatment B) effectively delayed the release of the extracts in both simulating solvents, which is desirable for the intended application. The release of phenolic compounds in ethanol 50% is faster because in this medium both the zein and the extracts are more soluble and thus, a partial dissolution of the fibers is expected, thus promoting a greater and quicker release. In contrast, only swelling took place when using 3 % acetic acid, thus allowing a slower extract release.

A



B

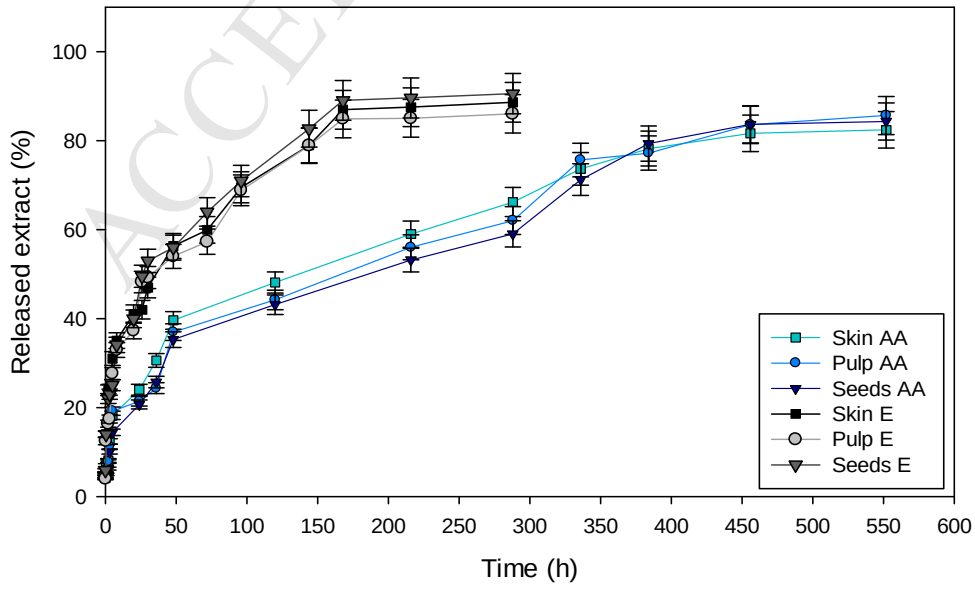


Figure 6. Release of PEE from the electrospun fibers after crosslinking during 5 h (**A**) and during 24 h (**B**) in 50% ethanol (E**)** and 3% acetic acid (AA**).**

3.5. HPLC analysis

HPLC-DAD was used to analyze the individual phenolic compounds released from the fibers, as well as to evaluate the recovery of phenolic compounds after the release experiments. The chromatography profile of each of them was similar to each extract used in this study.

Fig. 3 (Supplementary Material) shows the profile obtained for the eluate from the orange chilto skin fibers with the crosslinking treatment B, after 7 days of release in 50% ethanol. Peaks 1 and 2 were identified as 3-caffeoyl quinic acid and rosmarinic acid hexoside derivative, respectively. Previously, Orqueda et al. (2017) showed that the main phenolic compounds found in the chilto extracts were RosA and caffeic acid derivatives.

Under our experimental conditions, the percent recovery of the compounds 3-caffeoylquinic acid and rosmarinic acid hexoside derivative was 98 and 54%, respectively in 50% ethanol and 78% and 43%, respectively in 3% acetic acid (**Fig. 3, Supplementary Material**). Orqueda et al. (2017) reported for the orange chilto skin extract a content of 1724.1 ± 80.7 and 871.8 ± 3.5 mg / 100 g of dry weight of 3-caffeoylquinic acid and rosmarinic acid, respectively. Previously, Espin et al. (2016) reported values between 25.04 and 163.62 mg / 100 g of dry weight for 3-O-caffeoylquinic acid (CaQA) and between 12.22 and 121.89 mg / 100 g of dry weight for RosA, respectively, for samples of chilto from Ecuador. CaQA and RosA and its derivatives, are characterized as natural antioxidants and potential natural anti-diabetic and anti-obesity compounds, because they are glucosidase and amylase inhibitors (McCue & Shetty, 2004; Chen et al., 2014; Ngo et al., 2018; Gonçalves et al., 2019) and lipase inhibitors (Mohamed, 2014). Therefore, it is also feasible that the effect of chilto extracts on enzyme related to metabolic syndrome could be attributed to CaQA and RosA and its derivatives present in the extracts (Orqueda et al., 2017).

3.6. Antioxidant activity of extracts released from PEE loaded zein fibers

The antioxidant activity of orange chilto seed, skin and pulp extracts in cell free systems was previously reported (Orqueda et al., 2017). In this work, the antioxidant capacity of PEE was compared with the activity of the phenolics released from the fiber coatings.

The released phenolics were able not only to reduce ABTS but also to prevent and limit the release of lysosomal enzymes from human red blood cell to the extracellular matrix. In agreement with the results obtained for the non-encapsulated extracts, the antioxidant capacity of the phenolic compounds from skin released from the coatings was higher than that from the coatings containing seeds and pulp extracts (**Table 2**). In general, no significant differences were observed between the antioxidant activity of free and released extracts, thus confirming that encapsulation through electrospinning does not affect the bioactivity of the compounds.

Table 2. Antioxidant activity of *S. betaceum* polyphenolic extracts before and after encapsulating procedure.

Sample		SC_{50} (μgGAE/mL)	IC_{50} (μgGAE/mL)
S. betaceum		ABTS	AAPH
Seeds	PEE	1.38 ± 0.05^{a}	0.91 ± 0.05^{a}
	PEE loaded fiber	2.69 ± 0.01^{b}	1.12 ± 0.05^{a}
Pulp	PEE	1.09 ± 0.10^{A}	0.40 ± 0.03^{A}
	PEE loaded fiber	0.90 ± 0.02^{A}	0.53 ± 0.01^{A}
Skin	PEE	0.80 ± 0.10^{II}	0.50 ± 0.00^{I}
	PEE loaded fiber	0.49 ± 0.002^{I}	0.78 ± 0.02^{I}

SC_{50}: Concentration of polyphenolic extract necessary to scavenge 50% of ABTS.

IC_{50}: Concentration of polyphenolic extract necessary to inhibit 50% of oxidative hemolysis. Different letters or number in the same column for each extract indicated significant differences in the antioxidant activity according to Tukey's test ($p \leq 0.05$).

4. Conclusions

In this work, bioactive coatings consisting on zein fibers with orange chilto PEE were collected onto PHA films through the electrospinning technique, with encapsulation efficiencies greater than 90%.

Zein electrospun nanofiber coatings showed poor mechanical properties and stability in aqueous environments, which were improved by crosslinking with glutaraldehyde vapors.

The crosslinked zein fibers showed better morphological stability and were able to maintain their fibrous structure after immersion in water for more than 12 days. In addition, the crosslinking of the zein fibers for 24h (treatment B), allowed for a gradual release of the encapsulated extract in two different food simulants, showing a slower and more limited release in 3% acetic acid, with a total polyphenolic release of 80-86% after 23 days. Given the similar composition of the three different extracts evaluated, they all showed similar release behavior, and, thus, the proven health benefits ascribed to chilto fruits (skin, pulp and seeds) can be exploited for inclusion in bioactive packaging structures. This work has demonstrated that it is possible to develop bioactive coating structures based on zein fibers containing chilto PEE, which could result in added value applications of these fruits that are currently commercially underexploited, as well as in the valorization of the skin and the seeds from these fruits that are usually discarded. These coatings can be optimized for packaging structures in contact with more hydrophilic or lipophilic food products.

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Highlights

- Bioactive coatings for food packaging were obtained by electrospinning
- Chilto extracts with antioxidant capacity were included in the coatings
- The encapsulation efficiency of extracts within the coating was greater than 90%
- Cross-linking of zein fibers improved coating integrity upon water contact
- A sustained release of the extracts in food simulants was observed