

# Physico-chemical, antimicrobial and antioxidant properties of gelatin-chitosan based films loaded with nanoemulsions encapsulating active compounds

Pérez-Córdoba, Luis J.; Norton, Ian T.; Batchelor, Hannah K.; Gkatzionis, Konstantinos; Spyropoulos, Fotios; Sobral, Paulo J.A.

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37 bis(3-ethylbenzothiazoline-6-sulphonic acid) (PubChem CID: 16240279); 1,1-Diphenyl-2-  
38 picrylhydrazyl (PubChem CID: 2735032).

39

## 40 **1. Introduction**

41 The development of biodegradable packaging has been the focus of recent research, as an  
42 alternative to plastic material derived from petroleum, which due to their poor biodegradation generate  
43 a massive accumulation of plastic waste in the environment (Arancibia, Giménez, López-Caballero,  
44 Gómez-Guillén, & Montero, 2014; Rubilar et al., 2013). Films based on biopolymers do not have the  
45 same physical properties as synthetic plastics, but they present a promising application because they  
46 generally are from renewable sources, non-toxic, biodegradable, biocompatible, and sometimes could  
47 become edible material (Chen et al., 2016; Kurek, Galus, & Debeaufort, 2014; Pérez-Córdoba & Sobral,  
48 2017). Furthermore, these films are excellent vehicles for incorporating a wide variety of active agents,  
49 such as antioxidant and antimicrobial compounds, and thus, these biodegradable materials can be used  
50 for active packaging (Abdollahi, Rezaei, & Farzi, 2012; Rhim & Ng, 2007).

51 According to Gennadios, McHugh, Weller, & Krochta (1994), gelatin (G) was one of the first  
52 materials used as a carrier of bioactive components. Gelatin is a protein obtained by hydrolyses of the  
53 collagen from bones and skin via exposure to acidic (type-A) or alkaline (type-B) pre-treatment  
54 conditions (Gómez-Guillén et al., 2009). Gelatin has excellent film-forming properties and can  
55 generally form films with good mechanical characteristics that also act as barriers to oxygen, carbon  
56 dioxide, and volatile compounds (Tongnuanchan, Benjakul, & Prodpran, 2012); they form however a  
57 relatively poor barrier to moisture mainly due to the hydrophilic nature of the gelatin molecules (Ahmad  
58 et al., 2012). Moreover, gelatin has the ability to blend well with others biopolymers, such as chitosan  
59 (Bonilla & Sobral, 2016; Pérez-Córdoba & Sobral, 2017).

60 Chitosan (Ch) is a linear polysaccharide consisting of  $\beta$ -(1-4)-2-acetamido-D-glucose and  $\beta$ -  
61 (1-4)-2-amino-D-glucose units, derived from chitin through deacetylation in alkaline media, and it is  
62 the second most abundant polysaccharide found in nature, after cellulose (Baron, Pérez, Salcedo, Pérez-  
63 Córdoba, & Sobral, 2017; Elsabee & Abdou, 2013). Similar to gelatin, chitosan has excellent film-  
64 forming properties and offers great potential as the basis for active packaging material due to its intrinsic  
65 antimicrobial activity (Kanatt, Rao, Chawla, & Sharma, 2012). Blending chitosan with gelatin can  
66 produce films with improved properties, showing antimicrobial or antioxidant activity due to the  
67 presence of chitosan, or following the incorporation of hydrophilic bioactive agents (Benbettaïeb,  
68 Kurek, Bornaz, & Debeaufort, 2014; Bonilla & Sobral, 2016; Hosseini, Rezaei, Zandi, & Ghavi, 2013;  
69 Jridi et al., 2014; Pereda, Ponce, Marcovich, Ruseckaite, & Martucci, 2011; Rivero, García, & Pinotti,  
70 2009).

71 More recently, a number of studies have reported biopolymer films loaded with lipophilic  
72 compounds that are dispersed within the hydrophilic film structure as nanodroplets (nanoemulsions)  
73 (Acevedo-Fani, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2015; Alexandre, Lourenço, Quinta

74 Bittante, Moraes, & Sobral, 2016; Chen et al., 2016; Otoni, Avena-Bustillos, Olsen, Bilbao-Sáinz, &  
75 McHugh, 2016; Sasaki, Mattoso, & de Moura, 2016). In parallel to these studies, other works have  
76 focused on the encapsulation of essential oils within a nanoemulsion microstructure (Sasaki et al.,  
77 2016), flavonoids, such as rutin (Dammak & Sobral, 2017), curcumin (Sari et al., 2014) and other  
78 compounds like  $\alpha$ -tocopherol (Cheong, Tan, Man, & Misran, 2008; Yang & McClements, 2013),  
79 cinnamaldehyde (Donsì, Annunziata, Vincensi, & Ferrari, 2012) or garlic oil (Wang, Cao, Sun, &  
80 Wang, 2011). Potential applications of nanoemulsions for the encapsulation of bioactive components,  
81 either as a viable and efficient approach to increase their physical stability or in order to minimize their  
82 potentially detrimental sensorial effects, have been well documented within the food sciences research  
83 arena (Donsì, Annunziata, Sessa, & Ferrari, 2011; Fathi, Mozafari, & Mohebbi, 2012).

84         Among such bioactive compounds recently studied,  $\alpha$ -tocopherol ( $\alpha$ -t), cinnamaldehyde (Cin),  
85 and garlic oil (GO) have been shown to exhibit a wide range of biological effects including  
86 antimicrobial and/or antioxidant properties (Donsì et al., 2012; Wang et al., 2011; Yang & McClements,  
87 2013).  $\alpha$ -tocopherol is an isomer and the most naturally abundant and biologically active form of  
88 vitamin E in humans (Yang & McClements, 2013) and it has been shown to have high antioxidant  
89 activity in both biological and food systems (Saber, Fang, & McClements, 2013). Cinnamaldehyde is  
90 a hydrophobic aromatic compound with a benzene ring and an aldehyde group. It is the main active  
91 component of cinnamon oil (Chen et al., 2016) and it has been shown to be active against a broad range  
92 of foodborne pathogens bacteria, fungi and viruses (Wei, Xiong, Jiang, Zhang, & Wen Ye, 2011). Garlic  
93 oil is an essential oil extracted from garlic bulbs, which contains a range of compounds; mainly diallyl  
94 disulfide (60%), diallyl trisulfide (20%), allyl propyl disulfide (16%), a small quantity of disulfide and  
95 possibly diallyl polysulfide (Pranoto, Rakshit, & Salokhe, 2005). It is also used as a food preservative  
96 and it has been shown to inhibit the growth of a wide range of pathogens and spoilage microorganisms,  
97 including bacteria, mold, fungi, parasites and viruses (Sung, Sin, Tee, Bee, & Rahmat, 2014). All three  
98 of these active compounds have been categorized as safe (GRAS) for use in food by the US Food and  
99 Drug Administration (FDA) (Chen et al., 2016; Wei et al., 2011) and have been independently used as  
100 active additives within a range of packaging formulations (Noronha, De Carvalho, Lino, & Barreto,  
101 2014; Otoni et al., 2016; Pranoto et al., 2005). However, they are poorly soluble in water and as such  
102 extremely difficult to incorporate within film formulations, which are usually hydrophilic/aqueous  
103 systems (Alexandre et al., 2016).

104         The present study reports on a microstructural approach that involves the encapsulation of  
105 active compounds within oil-in-water (O/W) nanoemulsions, before incorporating these into a  
106 biopolymer film formulation, in order to facilitate dispersion of the bioactive species into the  
107 biopolymer matrix (Chen et al., 2016). To the best of the authors' knowledge, the joint incorporation of  
108 nanoencapsulated active compounds (NAC), such as  $\alpha$ -t, plus Cin and/or GO, within gelatin-chitosan  
109 (G-Ch) based films, in order to improve the films' physicochemical, antimicrobial and antioxidant  
110 properties, has not been previously reported. The objective of this work was to successfully produce G-

111 Ch based films loaded with O/W nanoemulsions containing the encapsulated  $\alpha$ -t, and Cin and/or GO  
112 active compounds and then characterize these formulations in terms of moisture content, solubility in  
113 water, swelling, light transmission, opacity, crystallinity, mechanical and thermal properties,  
114 microstructure, as well as their antioxidant and antimicrobial activities, thus enabling future  
115 development and application of such composite systems as food packaging material.

116

## 117 **2. Material and Methods**

### 118 **2.1 Material**

119 Garlic oil (purity >99%), cinnamaldehyde (>95%), and  $\alpha$ -tocopherol (>96%), Span 60, medium  
120 molecular weight chitosan (degree of deacetylation: 75–85% and viscosity: 200–800 cps), Trolox, TPTZ  
121 (2,4,6-tripyridyl-s-triazine), chloride acid, Iron trichloride, and ethanol were purchased from Sigma-Aldrich  
122 and Labsynth (São Paulo, Brazil). Pigskin gelatin (type A, bloom 260° and molecular weight  $\sim 5.2 \times 10^4$   
123 Da) was supplied by GELNEX (Itá, SC, Brazil). Acetic acid, glycerol, Tween 20, DPPH (2,2-diphenyl-  
124 1-picrylhydrazyl), potassium persulfate, ABTS<sup>•+</sup> [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic  
125 acid)], sodium bromide, sodium hydroxide, nutrient broth, and Mueller Hinton agar were obtained from  
126 Sigma-Aldrich (Dorset, England, UK). Canola oil was purchased from a local supermarket. Deionized  
127 Millipore water (Elix<sup>®</sup> 5UV, essential), tetracycline, and strains of bacteria *P. aeruginosa* (ATCC  
128 15692) and *L. monocytogenes* (ATCC 35152) were provided by the microbiology laboratory at the  
129 School of Biochemical Engineering of the University of Birmingham.

130

### 131 **2.2 Nanoemulsion preparation**

132 The  $\alpha$ -tocopherol and cinnamaldehyde and/or garlic oil were encapsulated in nanoemulsions  
133 using the microfluidization technique. Three oil-in-water (O/W) nanoemulsions containing a fixed  
134 amount of 3% (w/v)  $\alpha$ -t/Cin (N<sub>2</sub>),  $\alpha$ -t/GO (N<sub>3</sub>), or an equimolar mixture of  $\alpha$ -t/Cin and GO (N<sub>4</sub>) were  
135 prepared by firstly incorporating these active compounds into canola oil, using Span 60 (1.5 w/v) as the  
136 lipophilic emulsifier. This oil phase (10 % w/v) was then initially mixed with an aqueous phase  
137 containing water and Tween 20 (3.5% w/v) as the hydrophilic emulsifier in a 1:9 ratio using a magnetic  
138 stirrer (RH basic2, IKA, Germany) for 5 min at room temperature. Afterwards, a coarse emulsion was  
139 prepared using a high shear mixer (Silverson L5M, Buckinghamshire, UK) operating at 5000 rpm for  
140 5 min. These coarse emulsions were analyzed by optical microscopy (DFC 450C, Leica, Germany).  
141 Nanoemulsions were obtained by passing the coarse emulsion through a microfluidizer (M-110S,  
142 Microfluidics, USA) at different pressures (69 – 100 MPa) and 3 processing cycles, selected after  
143 previous optimization (data not shown).

144 An O/W nanoemulsion with the same oil:aqueous phase (1:9) ratio, without active compounds,  
145 was prepared following the same procedure, and it was considered as a control (N<sub>1</sub>). Samples were  
146 stored in amber glass containers at  $4 \pm 1^\circ\text{C}$  and their stability was monitored over a period of 90 days.

147 The encapsulation efficiency (EE) of all active species within the nanoemulsions was calculated  
148 immediately post-emulsification and after 90 days of storage (Equation 1).

$$149 \quad EE = (AC_R/AC_I) \times 100 \quad (1)$$

150 where  $AC_R$  is the amount of active compound ( $\alpha$ -t, Cin or GO) remaining within the droplets of the  
151 nanoemulsion, determined as described below, and  $AC_I$  is the amount of active compound initially  
152 added to the emulsion (Davidov-Pardo & McClements, 2015).

153 The amount of active compound ( $\alpha$ -t, Cin or GO) remaining within the droplets of the  
154 nanoemulsion was determined by using an UHPLC<sup>+</sup> (Dionex Ultimate 3000, Thermo scientific,  
155 Germany). Analyses were carried out by diluting the sample in methanol to facilitate the  $\alpha$ -tocopherol  
156 and garlic oil (0.01% v/v) or cinnamaldehyde (0,003% v/v) detection. The diluted samples were  
157 separated in a Phenomenex Luna 3a C18 column (150 x 4.6 mm, i.d. 3  $\mu$ m) with an elution system of  
158 methanol:acetonitrile:water (68:28:4) for  $\alpha$ -tocopherol or methanol:acetonitrile:phosphoric acid (1%  
159 v/v) (50:30:20) for cinnamaldehyde and garlic oil. The flow rate of the mobile phase solvents was 1  
160 mL/min, the injection volume was 25  $\mu$ L ( $\alpha$ -t) or 10  $\mu$ L (Cin and GO), and the detection wavelength  
161 was set at 208, 285 and 210 nm, for  $\alpha$ -t, Cin and GO respectively (Mao, Yang, Xu, Yuan, & Gao, 2010).

162 The nanoemulsions were characterized in terms of their mean particle size, polydispersity  
163 index, and  $\zeta$ -potential using a Zetasizer (Nanoseries, Malvern Instruments, UK), pH using a pHmeter  
164 (SevenCompact, Mettler Toledo, Switzerland), flow behavior using a rheometer (Kinexus Pro<sup>+</sup>,  
165 Malvern Instruments, UK), and microstructure and morphology using atomic force microscopy (Ntegra  
166 prima, NT-MDT Co., Russia). All measurements were performed at least in triplicate. These  
167 characterized nanoemulsions (N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, and N<sub>4</sub>) were then incorporated within the fabricated G-Ch  
168 based films.

169

### 170 **2.3 Film production**

171 Films were produced by blending G-Ch (4:1 ratio) using the casting technique. A film-forming  
172 solution (FFS) (5 g biopolymer/100 g FFS), loaded with nanoemulsions encapsulating active  
173 compounds (5 g/100 g biopolymer) and glycerol (30 g/100 g biopolymer) as the plasticizer, was used.  
174 Gelatin and chitosan solutions loaded with nanoemulsions were prepared separately, then, the FFS was  
175 mixed under stirring in a plate stirrer (SB162-3, Stuart, UK) for 10 min, and subsequently homogenized  
176 using a high shear mixer (Silverson L5M, Buckinghamshire, UK) at 5000 rpm for 5 min. During  
177 stirring, the pH was adjusted at 5.6 for complexation between chitosan and gelatin to take place; the  
178 selected pH value is above the isoelectric point of gelatin ( $P_i = 4.5-5.2$ ), where all the gelatin chains  
179 are negatively charged, and below pH 6.2 in order to prevent chitosan precipitating out of solution  
180 (Benbettaieb et al., 2014). FFS was sonicated and degassed in a Sonicator (ultrasonic cleaner QS18,  
181 Ultrawave, UK) at 50°C for 10 min. Finally, FFS was poured into a plastic Petri dish (14 cm diameter)

182 and placed in a forced air oven (GPS/50/CLAD/250/HYD, Leader, UK) at  $30 \pm 0.5$  °C for 24 h, in order  
183 to obtain the films.

184 After peeling from the petri dish, the films were conditioned inside desiccators containing a  
185 saturated solution of NaBr (relative humidity 58%) for 7 days, prior to the characterization of their  
186 physicochemical, antimicrobial, and antioxidant properties. For SEM and AFM analyses, the newly  
187 formed films were instead conditioned in silica gel (relative humidity 0 %) for the same period.  
188 Furthermore, two films were made using the same G-Ch blend (4:1). The first one was prepared without  
189 the incorporation of a nanoemulsion ( $N_0$ ), while the second one was loaded with a control nanoemulsion  
190 ( $N_1$ ) described in section 2.2. Both films were formed using glycerol as a plasticizer and they were  
191 produced and conditioned as described above; hereinafter referred to as control 1 and control 2 films,  
192 respectively.

193

## 194 **2.4 Film Characterization**

### 195 **2.4.1 Thickness**

196 A digital micrometer (AK9635D, Sealey, UK) was used to measure the film thickness to the  
197 nearest 0.001 mm at 10 random positions on the surface of each film produced (Barón et al. 2017).

198

### 199 **2.4.2 Moisture content**

200 Moisture content (MC) was determined by cutting film samples into discs (20 mm in diameter)  
201 and measuring the reduction in the mass of a minimum of 3 discs (from each film) following oven  
202 drying (GPS/50/CLAD/250/HYD, Leader, UK) at 105 °C for 24 h. The results were expressed as g of  
203 water/100 g of wet material (Barón et al. 2017). Measurements were performed in triplicate.

204

### 205 **2.4.3 Solubility in water and swelling**

206 For solubility in water (SW) and swelling (S) measurements, film samples were cut in discs (20  
207 mm in diameter), weighed, and immersed in 50 mL of distilled water under stirring in a shaker (Incu-  
208 Shake MIDI, SciQuip, UK) at 60 rpm and at room temperature for 24 h. Film samples were then  
209 removed from the solution, re-weighed, and dried in an oven at 105°C for 24 h to determine their final  
210 dry matter. These values were then used to calculate SW and S, expressed as g of solubilized mass/100  
211 g of dried material and g of gained water/g of dried material, respectively (Gontard et al. 1994). All  
212 measurements were carried out in triplicate.

213

### 214 **2.4.4 Mechanical properties**

215 Tensile strength (TS), elongation at break (EB), and elastic modulus (EM) were measured  
216 according to the ASTM D 882/12 standard method (2001). Samples were cut into 15 mm x 100 mm  
217 strips, and tested using a texture analyzer (TA.XT2i, Stable Micro System, UK) with grip separation of  
218 50 mm and speed rate of 1 mm/s until breaking. TS and EB were obtained directly from the stress vs.

219 strain curves, which are produced from the force–deformation data, and the EM was determined as the  
220 angular coefficient in the linear part of the curve using the Exponent Lite v.4.0.13.0 software (Stable  
221 Micro System, UK) (Baron et al., 2017). Data were collected for at least 10 sample strips from each  
222 film.

223

#### 224 **2.4.5 Light transmission and transparency**

225 Light transmission of films against ultraviolet and visible light was determined in transmittance  
226 mode at selected wavelengths (200 to 800 nm) using a UV-VIS spectrophotometer (Orion AquaMate  
227 8000, Thermo Scientific, Germany), according to the procedure described by Bonilla & Sobral (2016).

228 The transparency value for each film was calculated using Equation 2.

229

$$230 \text{ Transparency value} = (-\log T_{600})/x \quad (2)$$

231

232 where  $T_{600}$  is the fractional transmittance at 600 nm, and  $x$  is the film thickness (mm). The higher  
233 transparency value represents the lower transparency of films (Ahmad et al., 2012). Five samples of  
234 each film were used for transmittance measurements.

235

#### 236 **2.4.6 X-ray diffraction (XRD)**

237 XRD was used to determine the film's crystallinity. Analyses were carried out using an X-ray  
238 diffractometer (Miniflex600, Rigaku, Japan) with Cu as the source. Samples were cut in squares of 20  
239 mm x 20 mm and placed on a glass plate, which was placed inside the chamber of the equipment.  
240 Measurements were recorded in triplicate at room temperature, 40 kV and 40 mA current, in the region  
241 of  $2\theta$  from  $8^\circ$  to  $70^\circ$  (with a constant speed of  $1^\circ \text{ min}^{-1}$ ) using the Miniflex Guidance software (Rigaku,  
242 Japan) (Chen et al., 2016).

243

#### 244 **2.4.7 Differential scanning calorimetry (DSC)**

245 Thermal properties of the films were determined using a differential scanning calorimeter (DSC  
246 TA2010, TA Instruments, USA), controlled by a TA5000 system (TA Instruments, USA) and a quench  
247 cooling accessory. Approximately 10 mg ( $\pm 0.01$  mg) of sample were weighed in a precision balance  
248 (AP 2500 Analytical Plus, Ohaus, Switzerland), were conditioned in a hermetically sealed aluminum  
249 pan and heated in double run at  $5^\circ\text{C}/\text{min}$  from  $-150$  to  $150^\circ\text{C}$  in an inert atmosphere ( $45 \text{ ml}/\text{min}$  of  $\text{N}_2$ ).  
250 An empty pan was used as the control. The results were analyzed using the instrument's software  
251 (V1.7F, TA Instruments, USA) in order to determine the glass transition temperature ( $T_g$ ), in the first  
252 and second scan, as well as the melting temperature ( $T_m$ ) and enthalpy ( $\Delta H_m$ ) of the sol-gel transition  
253 (Alexandre et al., 2016; Sobral, Menegalli, Hubinger, & Roques, 2001). DSC measurements were  
254 performed in triplicate.



#### 255 **2.4.8 Atomic force microscopy (AFM)**

256 AFM analyses were performed according to Ma et al. (2012), using the atomic force microscope  
257 (Topview optics™ Nanowizard, JPK Instruments, Germany) equipped with a DP17/GP/NAI  
258 (μMASCH) tip and operated in contact mode. Samples (2 cm × 2 cm) from each film were pasted on a  
259 glass slice using a double-sided adhesive tape. AFM images (with a scan size of 10 μm × 10 μm) were  
260 collected from the air side of the films at a fixed scan rate of 0.7 – 0.8 Hz. The surface roughness of the  
261 films was calculated based on the root mean square (RMS) deviation from the average height of peaks  
262 after subtracting the background using the JPK-SPM and JPK Data processing software (JPK,  
263 Germany) (Ma et al., 2012).

264

#### 265 **2.4.9 Scanning electron microscopy (SEM)**

266 Film microstructures were studied using an environmental scanning electron microscope (FEG-  
267 ESEM XL30, Phillips, Japan). Film samples were fixed on the support using double-sided adhesive  
268 tape and initially coated with Platinum in a Sputter coater (SC7640, Quorum Technologies, UK) to  
269 allow better observation of film surface and cross section. Micrographs of the films' surfaces and cross-  
270 sections were taken in triplicate at random positions on the films, at 10 kV and a magnification of 1000x.  
271 For cross-sectional analysis, samples were cryo-fractured after immersion in liquid nitrogen (Kurek et  
272 al., 2014).

273

#### 274 **2.4.10 Antimicrobial activity**

275 The antimicrobial activity of the films was assessed against *Pseudomonas aeruginosa* ATCC  
276 15692 and *Listeria monocytogenes* ATCC 35152 by the agar diffusion method based on the guidelines  
277 of the Clinical and Laboratory Standards Institute (CLSI, 2006) with slight modifications (Wayne,  
278 2006). Microbial cultures were grown overnight in nutrient broth (Sigma Aldrich, England, UK) at 37  
279 °C and 150 rpm. The cells were harvested by centrifugation at 2000 rpm for 10 min and washed in  
280 sterile phosphate buffer saline (pH 7.2) twice (Kadri, Devanthi, Overton, & Gkatzionis, 2017). Inocula  
281 with a turbidity equivalent to a McFarland 0.5 standard were prepared (10<sup>8</sup> cfu/mL), then diluted to a  
282 final concentration of 10<sup>5</sup> cfu/mL into Mueller Hinton agar (Merck, UK) and poured into petri plates  
283 after mixing (Kavoosi, Rahmatollahi, Mohammad Mahdi Dadfar, & Mohammadi Purfard, 2014). After  
284 solidification, discs (diameter 20 mm) of films containing the nanoemulsions N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub> and N<sub>4</sub> (or not,  
285 N<sub>0</sub>), were placed in plicate on the medium, and the plates were incubated at 37 °C for 24 h. The area of  
286 the whole zone was calculated, then subtracted from the film disc area, and this difference in area was  
287 reported as the zone of inhibition (Seydim & Sarikus, 2006).

288

#### 289 **2.4.11 Determination of antioxidant activity**

290 The films' antioxidant activity was measured using the 2,2'-azino-bis(3-ethylbenzothiazoline-  
291 6-sulphonic acid) (ABTS<sup>•+</sup>) and 1,1-diphenyl-2-picrylhydrazyl (DPPH<sup>•</sup>) free radical scavenging

292 methods, and the ferric reducing ability of plasma (FRAP) assay, as described by Re et al. (1999),  
293 Brand-Williams, Cuvelier, & Berset (1995) and Ferreira, Nunes, Castro, Ferreira, & Coimbra (2014),  
294 respectively. For ABTS<sup>•+</sup> and DPPH<sup>•</sup> analyses, 0.1 g samples from each film were immersed into 10 ml  
295 of a hydroalcoholic mixture (1:1) and kept under agitation overnight at 80 rpm and 20°C to encourage  
296 the extraction of the encapsulated compounds. All antioxidant analyses were performed in triplicate.

#### 298 **2.4.11.1 ABTS<sup>•+</sup> method.**

299 A solution containing ABTS<sup>•+</sup> radical (7 mM) and potassium persulfate (2.45 mM) was initially  
300 mixed (1:0.5) and kept in the dark for 16 h. Subsequently, an aliquot of this solution was diluted with  
301 ethanol in order to prepare the ABTS<sup>•+</sup> working solution with an absorbance value of  $0.70 \pm 0.02$ , as  
302 measured using a UV-Vis spectrophotometer at 734 nm. An aliquot (100  $\mu$ L) of the solubilized and  
303 centrifuged (4000 rpm, 30 min) samples was added to the ABTS<sup>•+</sup> working solution (900  $\mu$ L), and the  
304 mixture was kept in the dark within 6 min (Bonilla & Sobral, 2016; Re et al., 1999). Antioxidant activity  
305 is calculated and expressed as Trolox equivalent TE ( $\mu$ mol/g dried film).

#### 307 **2.4.11.2 DPPH<sup>•</sup> method.**

308 A centrifuged (4000 rpm, 30 min) aliquot of the solubilized film (1.5 mL) was added to 1.5 mL  
309 of DPPH<sup>•</sup> radical solution (60  $\mu$ M), and it was kept in the dark for one hour. After this period, the  
310 absorbance was determined at 515 nm using a UV-Vis spectrophotometer (Brand-Williams et al., 1995).  
311 Antioxidant activity is calculated and expressed as Trolox equivalent TE ( $\mu$ mol/g dried film).  
312 Antioxidant activity is expressed as TE ( $\mu$ mol/g dried film).

#### 314 **2.4.11.3 FRAP assay**

315 A solution of FeCl<sub>3</sub> (20 mM) was prepared in distilled water and TPTZ was prepared in 40 mM  
316 HCl. To prepare the FRAP reagent, 25 mL acetate buffer (0.3 M, pH 3.6) were mixed with 2.5 mL of  
317 TPTZ and 2.5 mL FeCl<sub>3</sub>. Film samples of 50 mm x 50 mm (~ 2.5 mg) were placed in 3 mL of FRAP  
318 solution and 0.3 mL of distilled water for 24 h. Following this period, the absorbance of the film-  
319 containing solution was measured at 593 nm using a UV-Vis spectrophotometer. The absorbance of the  
320 FRAP solution (without the film) was also measured as a blank (Ferreira et al., 2014). Antioxidant  
321 activity is expressed as TE ( $\mu$ mol/g dried film).

### 324 **2.5 Statistical analysis**

325 Analysis of variance (ANOVA) was conducted using the Statgraphics® centurion XV  
326 (StatPoint, Inc., 2006) software. The obtained mean values were subjected to Duncan's multiple-range  
327 test, and in all cases, values with  $p < 0.05$  were considered to be significant.

328

### 329 **3. Results and Discussion**

#### 330 **3.1 Nanoemulsion characterization**

##### 331 **3.1.1 Encapsulation efficiency**

332 The results presented in Table 1 show that Cin and GO had higher EE than  $\alpha$ -t during the  
333 encapsulation process and nanoemulsion storage. Nevertheless, all of them had a slight reduction in EE  
334 during storage. This loss could be associated with the high pressure and cycle number used in the  
335 nanoemulsion preparation or could be due to the partial volatility of those compounds, principally the  
336 Cin and GO. Furthermore, the harsh processing conditions, as well as the presence of heat, light, and  
337 oxygen during processing, could explain the active compound loss. These extreme conditions might  
338 have caused chemical degradation of  $\alpha$ -tocopherol, resulting in a reduction of the quantified  $\alpha$ -t  
339 concentration (Anarjan, Mirhosseini, Baharin, & Tan, 2011; Cheong et al., 2008). When comparing the  
340 EE for Cin or GO between N<sub>2</sub> or N<sub>3</sub> and N<sub>4</sub>, which contain the three joint mixed compounds (Table 1),  
341 a clear reduction in the encapsulated compound quantified immediately post-emulsification and also a  
342 significant difference ( $p < 0.05$ ) between the EE values after 90 days of storage for both Cin and GO was  
343 seen. Hence, the fact that encapsulating three compounds instead of two, clearly affected their EE. On  
344 the other hand, the EE for  $\alpha$ -t did not show significant difference ( $p > 0.05$ ) after post-emulsification  
345 regardless of the nanoemulsion. However the storage time had a significant ( $p < 0.05$ ) effect on the EE  
346 for this active compound in all nanoemulsions, which was expected due to the high sensitivity of this  
347 molecule (Nhan & Hoa, 2013).

348 Despite the obtained EE during the nanoemulsion preparation and the slight loss of the active  
349 compounds after 90 days under refrigeration, it was proven that the remaining NAC was sufficient to  
350 guarantee a very good antimicrobial and antioxidant properties for the prepared emulsions (data not  
351 shown).

352

##### 353 **3.1.2 Droplet size, polydispersity, $\zeta$ -potential and pH measurements**

354 The nanoemulsions were also evaluated in terms of their physicochemical properties (Table 1).  
355 The control nanoemulsion (N<sub>1</sub>) without encapsulated actives, presented the highest ( $p < 0.05$ ) droplet  
356 size, polydispersity index (PDI),  $\zeta$ -potential, and pH values, among all tested formulations (Table 1).  
357 For nanoemulsions loaded with active compounds, mean particle size, PDI, and  $\zeta$ -potential values  
358 remained between 111.0 and 130.0 nm, 0.14 – 0.20 and -12.0 to -16.0 mV, respectively, with all  
359 characteristics remaining unchanged over the 90 days storage (Table 1). All emulsions were found to  
360 possess droplet sizes within the desired nano-scale region with a monomodal size distribution (Figure  
361 1). Moreover, it could be confirmed that those nanoemulsions presented an excellent physical stability  
362 across the 90-day storage at 4 °C.

363 The nanoemulsions were also analyzed using an atomic force (AFM) microscope. The size,  
364 homogeneity and spherical morphology of the oil nanodroplets were confirmed by the AFM data and  
365 images, which revealed uniformly sized spherical particles with sizes from 110 to 150 nm for all  
366 nanoemulsions (Figure 2), as measured by the dynamic light scattering (DLS) in Zetasizer (Table 1).

367

368 **Insert Table 1**

369 **Insert Figure 1**

370 **Insert Figure 2**

371

372 With regard to their polydispersity, only nanoemulsions with encapsulated active compounds  
373 had PDI values lower than 0.20 over the 90-days storage (Table 1), displaying a monodisperse droplet  
374 size distribution (Figure 1) and showing a visual and physical stability, perhaps as a result of the optimal  
375 pressure and number of processing cycles used throughout the homogenization process, as reported in  
376 previous works by Tan & Nakajima (2005); Troncoso, Aguilera, & McClements (2012), and Pérez-  
377 Córdoba & Sobral, (2017). Although the PDI value for the control nanoemulsions was 0.20 upon  
378 formation, this shifted slightly to higher values as a small shoulder at size ranges of approximately  $8\mu\text{m}$   
379 developed during storage (Figure 1a). These results suggested that the microfluidizer was able to  
380 produce nanoemulsions from coarse emulsions containing polydisperse micrometers droplets  
381 (Supplementary Figure S1). Nanoemulsions with  $\zeta$ -potential values greater than +30 mV or lower than  
382 -30 mV are expected to be highly stable since droplets are sufficiently charged to enable inter-particle  
383 repulsive forces to dominate (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003; Salvia-Trujillo, Rojas-  
384 Graü, Soliva-Fortuny, & Martín-Belloso, 2013). As can be observed in Table 1, the negative  $\zeta$ -potential  
385 values for all nanoemulsions were above this -30 mV threshold, potentially as a result of the adsorption  
386 of hydroxyl ions at the oil-water interface and subsequent development of hydrogen bonds between  
387 these ions and the ethylene oxide groups of the surfactant (Dias et al., 2014; Jo & Kwon, 2014).  
388 Nevertheless, despite their moderate magnitude, the resulting net charge differences in the tested  
389 nanoemulsions were able to contribute to the systems' high stability against creaming and/or  
390 flocculation phenomena during storage (Jo & Kwon, 2014).

391 In terms of pH, the control nanoemulsions were able to maintain a value of pH 6 for the duration  
392 of storage, whilst a significant ( $p<0.05$ ) pH reduction was observed for all nanoemulsions with  
393 encapsulated active compounds. This behavior could be attributed to the production of acidic  
394 compounds (carboxylic acids) after the decomposition of hydroperoxides from the oxidation of the  
395 encapsulated lipophilic compounds (Cheong, Tan, & Nyam, 2017; Grill, Ogle, & Miller, 2006).  
396 Cheong et al., (2017) also observed the same pH reduction behavior and very close pH values for kenaf  
397 seed (*Hibiscus cannabinus* L.) oil-in-water nanoemulsion stored at 4 °C. Hsu & Nacu (2003) affirm that  
398 an ideal pH value for O/W emulsions should be greater than 4.0 to ensure stability. Similarly,

399 Nejadmansouri et al. (2016) reported that, at higher pH values ( $\text{pH} > 4$ ), nanoemulsions remain relatively  
400 stable against droplet aggregation as a result of sufficient electrostatic repulsions between negatively  
401 charged droplets (Nejadmansouri et al., 2016).

402

### 403 **3.1.3 Flow behavior of nanoemulsions**

404 In this study, the viscosity was not dependent on the shear rate used for the sample test when  
405 measured at ambient temperature ( $20^\circ\text{C} \pm 2^\circ\text{C}$ ). All prepared nanoemulsions presented viscosity values  
406 of approximately  $10^{-3}$  mPa.s, being closer to the viscosity of water, and showed Newtonian behavior.  
407 This behavior could be attributed to that those nanoemulsions were prepared with an oil phase of 10%  
408 w/w. According to Flourey, Desrumaux, Axelos, & Legrand, (2003), emulsions containing less than 20%  
409 (w/w) of the dispersed phase always show a Newtonian behavior, regardless of the homogenization  
410 pressure or another condition applied in their preparation. Alexandre et al. (2016) obtained similar flow  
411 behavior when preparing O/W nanoemulsion loaded with ginger essential oil. This rheological behavior  
412 can be considered as interesting because water is the solvent usually used in the biopolymer-based film  
413 preparation (Alexandre et al. 2016).

414

## 415 **3.2 Film characterization**

416 Films prepared without ( $N_0$ ) or with nanoemulsions ( $N_1$ ,  $N_2$ ,  $N_3$ , or  $N_4$ ) were visually  
417 homogeneous with no cracks, scratches, bubbles, or visible phase separation. Film thickness was well  
418 maintained by controlling the mass ratio of FFS/dish area and thus remained constant at  $0.080 \pm 0.002$   
419 mm ( $p > 0.05$ ) across all film formulations (Table 2). According to Benbettaieb et al. (2014), controlling  
420 thickness is key for ensuring the films' physical and barrier properties.

421

### 422 **Insert Table 2**

423

#### 424 **3.2.1 Moisture content, solubility in water and swelling**

425 No significant difference ( $p > 0.05$ ) was observed in the moisture content (MC) of all samples  
426 (Table 2), which was maintained at approximately 18%. It is therefore evident that the oil phase fraction  
427 in the nanoemulsions was relatively low and did not affect the hygroscopicity of the produced films,  
428 which was predominantly dictated by the biopolymer matrix (Pérez-Córdoba & Sobral, 2017).

429 Solubility is another important film characteristic that can affect film integrity as well as the  
430 migration of the encapsulated bioactive compounds into the foodstuff (Mihaly Cozmuta et al., 2015).  
431 All films loaded with nanoemulsions ( $N_1$ ,  $N_2$ ,  $N_3$ , or  $N_4$ ) presented slightly lower ( $p < 0.05$ ) solubility in  
432 water (SW) than the control 1 film ( $N_0$ ); SW values for the former were between 43.1 and 48.9%, with  
433 films loaded with  $N_2$  and  $N_3$  exhibiting the lowest SW ( $p > 0.05$ ) (Table 2).

434 Ahmad et al. (2012) reported a reduction on the water solubility of gelatin-based films  
435 following the incorporation of bergamot and lemongrass oil. This was presumably due to the non-polar

436 components in the used oils, which resulted in a substantial physical interference in the entanglement  
437 of gelatin polypeptide chains within the film matrix. Such interference, which might have led to a  
438 significant blockade on the capacity of gelatin to interact with water molecules, would be mainly  
439 responsible for reducing the water solubility of the composite films (Hosseini et al., 2013; Mihaly  
440 Cozmuta et al., 2015).

441 These SW values were similar to those reported by Ma et al. (2012) (44.7 %) and Gómez-  
442 Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero (2010) (41.1%) for gelatin or  
443 gelatin-chitosan based films loaded with nanoemulsified olive or clove oil droplets in water,  
444 respectively. This was attributed to the establishment of protein-polyphenol interactions which weaken  
445 the interactions that stabilize the protein network (Gómez-Estaca et al., 2010). On the other hand, Jridi  
446 et al. (2014) reported higher SW (85.6%), and Benbettaïeb et al. (2014), Hosseini et al. (2013), and  
447 Gómez-Estaca et al. (2010) obtained lower SW values for G-Ch (37.8 – 39.1%) or G-Ch films loaded  
448 with essential clove oil (29.5%) than those obtained in this work. This evidence demonstrates that SW  
449 does not correspond to a simple rule of mixing and may result from interactions between both gelatin  
450 and chitosan caused by electrostatic forces, hydrogen bonding, etc, or by the presence of droplets oil  
451 that stabilize the film structure (Jridi et al., 2014; Pereda et al., 2011), as will be discussed in section  
452 3.2.4 and seen in the X-ray diffractograms (Figure 3).

453 Despite its highest SW, the control 1 film ( $N_0$ ) displayed the lowest ability to swell (26.9 g/g)  
454 as well as the greatest ( $p < 0.05$ ) surface hydrophobicity amongst all tested samples; the latter was  
455 evaluated by contact angle measurements (data not shown). Although film swelling (S) was found to  
456 vary between different systems ( $p < 0.05$ ), this was not dependent on the incorporation (or not) of the  
457 nanoemulsion, with the  $N_1$  and  $N_4$  films, displaying the highest (30 g/g) and lowest (25.3 g/g) swelling,  
458 respectively. Nonetheless it is expected that these films would exhibit a high degree of swelling due to  
459 the great water uptake capacity of gelatin and also the porous structure of its polymeric network  
460 (Kavoosi, Mohammad, Dadfar, Purfard, & Mehrabi, 2013).

461

### 462 **3.2.2 Mechanical properties**

463 The  $N_0$  films displayed the highest ( $p < 0.05$ ) tensile strength (TS) and the lowest elongation at  
464 break (EB) values among all samples (Table 2); 19.0 MPa and 89.1%, respectively. In comparison to  
465  $N_0$  films, films loaded with nanoemulsions showed a considerable reduction in TS, as well as an increase  
466 in their EB values, a typical behavior of plasticized films (Sobral et al., 2001). This is in agreement with  
467 previous studies reporting that addition of lipophilic species (e.g. essential oils or fatty acids) decreases  
468 the TS values of biopolymer-based films; e.g., films from gelatin (Limpisophon, Tanaka, & Osako,  
469 2010; Tongnuanchan, Benjakul, & Prodpran, 2013), chitosan (Martins, Cerqueira, & Vicente, 2012;  
470 Rubilar et al., 2013) or whey protein (Soazo, Rubiolo, & Verdini, 2011), etc. This has been attributed  
471 to the inability of lipids to form continuous and cohesive matrices (Péroval, Debeaufort, Despré, &  
472 Voilley, 2002; Rubilar et al., 2013).

473 EB results obtained here are comparable to those reported by Kavooosi et al. (2013) and  
474 Tongnuanchan, Benjakul, & Prodpran (2014) for gelatin based films; who obtained EB mean values of  
475 128% and 114%, respectively, and, similarly to the present study, a significant ( $p<0.05$ ) decrease in TS  
476 when carvacrol, and basil or lemon essential oils were incorporated into the gelatin films. Similarly,  
477 Hosseini, Rezaei, Zandi, & Farahmandghavi (2016) reported a significant ( $p<0.05$ ) increase in EB value  
478 (reaching a maximum value of 151.8%) for gelatin/chitosan based films emulsified with oregano oil  
479 (0.4% w/v) and also a reduction of 69% in its original tensile strength. This behavior has been attributed  
480 to the chemical nature of the films' biopolymeric components and the plasticizing role of the essential  
481 oil (loaded onto the matrix), resulting in the enhancement of their ductile properties (Hosseini et al.,  
482 2016; Tongnuanchan et al., 2012).

483 With regard to the EM results, the addition of nanoemulsions into the polymeric-blend matrix  
484 leads to a significant ( $p<0.05$ ) reduction of the films' stiffness. The highest (71.4%) and lowest (61.3%)  
485 EM reduction was observed for  $N_1$  and  $N_4$  films, respectively (Table 2). Hosseini et al. (2016) also  
486 reported a significant ( $p<0.05$ ) decrease on EM when different oregano oil concentrations were added  
487 into gelatin-chitosan based films. Similarly, Tongnuanchan et al. (2014) reported a significant ( $p<0.05$ )  
488 reduction of EM for gelatin based films loaded with different essential oils (basil, plai and lemon), in  
489 respect to the control film (without essential oils).

490

### 491 **3.2.3 Light transmission and opacity**

492 Incorporation of the  $N_1$  nanoemulsion within the gelatin-chitosan film (control 2) significantly  
493 reduces the transmittance values in the wavelength range of 250 - 280 nm (Table 3) in comparison to  
494 those of  $N_0$  films (control 1). These transmittance values are then further reduced by the incorporation  
495 of  $\alpha$ -t, Cin, and/or GO within the nanodroplets, thus indicating that the formulated films act as excellent  
496 barriers to radiation in the ultraviolet (UV) light region when compared with both control films ( $N_0$  and  
497  $N_1$ ). In addition to the aromatic rings of amino acid residues from the gelatin molecule, this protective  
498 capacity of the films is envisaged to be enhanced by the chemical structure of the encapsulated  
499 compounds which contain phenolic groups (Bonilla & Sobral, 2016; Dammak, Carvalho, Trindade,  
500 Lourenço, & Sobral, 2017). Good UV and visible light barrier properties in the 200 - 350 nm range  
501 were also found by Gómez-Estaca, Giménez, Montero, & Gómez-Guillén (2009) and Wu et al. (2013)  
502 in gelatin-based films containing oregano or green tea extracts, respectively. In the visible range (350 -  
503 800 nm), the  $N_0$  films showed the highest ( $p<0.05$ ) light transmission (80-97%) when compared to films  
504 loaded with  $N_1, N_2, N_3,$  or  $N_4$  (Table 3). These values were similar to those reported by Jridi et al. (2014)  
505 for gelatin-chitosan composite films (72.6-90.9%) and higher than those reported by Dammak et al.  
506 (2017) for pure gelatin-based films (45–56%). Hence, it can be seen that chitosan has a significant  
507 contribution in terms of light transmission in the visible range (Jridi et al., 2014).

508

509 **Insert Table 3**

510

511 On the other hand, the transparency of films differed significantly ( $p < 0.05$ ) among samples,  
512 when nanoemulsions were added, as evidenced in Table 3. This transparency values are directly  
513 associated with the film opacity (i.e, the  $N_1$  films presented the highest transparency value and the  
514 greatest opacity). In this case, the  $N_0$  films was the most transparent, however when adding the different  
515 nanoemulsions became opaque, maybe due to the nanoencapsulated active compounds (NAC), which  
516 were able to impede the light transmission through the films (Tongnuanchan et al., 2012) or due to the  
517 formation of poly-anion/cation complexes between the gelatin-chitosan matrix and the nanoemulsions  
518 (Jridi et al., 2014). Tongnuanchan et al. (2012) also reported that emulsified essential oil droplets  
519 incorporated into a gelatin based film lowered its transparency, likely due to the light scattering effect.  
520 The transparency values of the films loaded with  $N_1, N_2, N_3,$  and  $N_4$  were quite close to those opacity  
521 values previously reported by Rivero et al. (2009) for composite and bi-layer films based on gelatin and  
522 chitosan (0.68 – 0.99), while the  $N_0$  films showed a transparency value lower than that reported by Jridi  
523 et al. (2014) for gelatin-chitosan based films ( $0.99 \pm 0.12$ ).

524

#### 525 **3.2.4 X-ray diffraction**

526 The presence of a strong interaction between the biopolymer matrix and NAC was confirmed  
527 by X-ray diffraction (XRD) analysis. All films exhibited an X-ray diffraction pattern characteristic of a  
528 partially crystalline material (Figure 3), with two defined diffraction peaks, the first in the region of  $2\theta$   
529 =  $10^\circ$ , corresponding either to the crystalline triple helix structure of gelatin or the relatively regular  
530 crystal lattice of chitosan, and a second broader band at  $2\theta = 20^\circ$ , characteristic of an amorphous phase  
531 (Pereda et al., 2011; Valencia, Lourenço, Bittante, & Sobral, 2016). Peaks observed in the films at  
532 approximately  $32^\circ$  could be assigned to the (020) diffraction plane of hydrated chitosan crystals and  
533 relate to the films' preparation procedure (i.e. dissolution of chitosan in an acetic acid solution) or the  
534 chemical structure of the active compound incorporated (Pereda et al., 2011).

535 The incorporated active compounds through nanoemulsions  $N_2, N_3$  and  $N_4$ , slightly changed the  
536 highest peak intensity, but in general, the profile of diffraction spectra of these films was similar to  
537 those obtained for the control films ( $N_0$  and  $N_1$ ). The increase in the intensity of the peaks at  $10^\circ$  for the  
538  $N_3$  and  $N_4$  films, indicates that incorporation of nanoencapsulated GO into the biopolymer-blend matrix  
539 induces an increase in the films' crystallinity. A similar effect was observed by Rubilar et al. (2013)  
540 when incorporating carvacrol into chitosan based films. In contrast, Valenzuela, Abugoch, & Tapia  
541 (2013) reported that the introduction of sunflower oil into a quinoa protein–chitosan based film  
542 generated a structure less crystalline, whilst Alexandre et al. (2016) reported no effect on the  
543 crystallinity of gelatin based films when a ginger essential oil-loaded nanoemulsion was incorporated.

544

545 **Insert Figure 3**



546

### 547 **3.2.5 Thermal properties**

548 In general, all films exhibited similar differential scanning calorimetry (DSC) curves (Figure  
549 4). Curves from the first scan revealed a trace typical for partially crystalline material, with a glass  
550 transition, attributed to a fraction rich in gelatin, followed by a marked endothermic peak, associated to  
551 a helix-coil transition (Sobral et al., 2001; Valencia et al., 2016). In the second scan, a typical trace for  
552 amorphous material was observed, where a glass transition also occurred (Alexandre et al., 2016).

553

#### 554 **Insert Figure 4**

555

556 The glass transition temperatures ( $T_g$ ) of all films did not appear to be affected by formulation  
557 characteristics ( $p>0.05$ ), remaining at approximately 46°C and 10°C, in the first and second scan,  
558 respectively (Table 4).  $T_g$  values were in agreement to those reported by Gómez-Estaca et al. (2009) for  
559 films based on gelatin incorporated with extracts ( $T_g = 42 - 47^\circ\text{C}$ ) and by Hosseini et al. (2013) for a  
560 blend of gelatin-chitosan with no incorporated species ( $T_g = 45 - 56^\circ\text{C}$ ).

561 All films showed a crystal melting temperature ( $T_m$ ) at approximately 55°C ( $p>0.05$ ).  
562 Nevertheless, only films loaded with the nanoemulsions exhibited an additional marked endothermic  
563 peak at -18°C in both scans (Figure 5), which can be either attributed to the  $T_m$  of the canola oil (-10  
564 °C) used for encapsulating the active compounds in nanodroplets, or even to the  $T_m$  of the NAC  
565 themselves. Ma et al. (2012) also reported an extra endothermic peak at -8°C, attributed to the melting  
566 of olive oil that was emulsified into gelatin based films.

567 With regard to melting enthalpy ( $\Delta H_g$ ), this was significantly ( $p<0.05$ ) reduced from 12.1 J/g  
568 ( $N_0$  films) to approximately 9.0 J/g when the films were loaded with  $N_1, N_2, N_3$ , or  $N_4$  (Table 4). The  
569 higher enthalpy value for the  $N_0$  films indicated that they had a higher level of renaturation compared  
570 to the nanoemulsion-loaded films, leading to an improved strength value (Jridi et al., 2014), as  
571 demonstrated by the TS data (Table 2). It is possible that the inter-chain distances of the gelatin  
572 macromolecules increased with nanoemulsions-loaded films and this is expected to decrease the  
573 entanglement of the gelatin chains and to increase their molecular mobility, reducing the melting  
574 enthalpy. Alexandre et al. (2016) also observed a reduction in the  $\Delta H_g$  for films gelatin based films  
575 when ginger oil loaded-nanoemulsions were incorporated into the film matrix. However, Jridi et al.  
576 (2014) reported higher  $T_g$  (64.7°C) and  $\Delta H_g$  (66.4 J/g) values and no  $T_m$  for fish skin gelatin-chitosan  
577 based films, maybe due to a better level of blending after intermolecular interaction between the gelatin  
578 and chitosan (Jridi et al., 2014).

579

### 580 **3.2.6 Atomic force microscopy**

581 Atomic force microscopy (AFM) analyses were performed to observe the effect of  
582 nanoemulsions incorporation on the surface topography of the films. Typical 3-D and 2-D surface  
583 topographic AFM images are presented in Figure 5. The incorporation of the nanoemulsions into the  
584 biopolymeric matrix led to a marked increase in both the average ( $R_a$ ) and root-mean-square ( $R_q$ )  
585 roughness of the films (Table 4). The  $R_q$  increased drastically from 11.1 nm ( $N_0$  films) to a maximum  
586 value of 58.6 nm ( $N_1$  films) following the loading  $N_1, N_2, N_3$ , or  $N_4$  into the films. The  $R_a$  values showed  
587 a similar trend, increasing from 7.45 nm to 44.14 nm. Atarés, Bonilla, & Chiralt (2010), Hosseini et al.  
588 (2016), and Ma et al. (2012) have also reported an increase in terms of film roughness as a result of the  
589 incorporation of ginger oil, oregano oil, or olive oil into sodium caseinate, gelatin-chitosan blend, or  
590 gelatin based films, respectively. It has been proposed that this trend is potentially due to an  
591 enhancement in lipid aggregation and/or creaming phenomena, which are exacerbated by the drying  
592 step and ultimately result in an elevated level of irregularities on the films' surfaces (Ma et al., 2012).

593

594 **Insert Figure 5**

595 **Insert Table 4**

596

### 597 **3.2.7 Environmental scanning electron microscopy (ESEM)**

598 The environmental scanning electron microscopy (ESEM) micrographs of the surface and  
599 cross-sectional morphology of the films revealed a continuous and homogeneous microstructure,  
600 without the presence of scratches, phase separation, and/or porosity due to the presence of trapped air  
601 cells (Figure 6). Furthermore, no evidence of oil droplets separation from the biopolymer-blend matrix  
602 was observed in the films loaded with nanoemulsions. However, the previously determined roughness  
603 difference between the  $N_0$  film and the ones loaded with  $N_1, N_2, N_3$ , or  $N_4$  (Table 4) was also confirmed  
604 by the ESEM analysis (Figure 6). The marked roughness that was visible in the cross-sectional images  
605 of the films loaded with nanoemulsions has been previously reported by Hoque, Benjakul, & Prodpran  
606 (2011), Hosseini et al. (2016), and Pérez-Córdoba & Sobral (2017) for gelatin films or blends when  
607 these were loaded with some extract or essential oils (i.e. cinnamon, clove or star anise extracts and  
608 oregano or garlic oil).

609 Amongst the samples loaded with nanoemulsions, the  $N_1$  films appeared to possess the highest  
610 degree of surface and cross-sectional roughness, in agreement with the roughness data from AFM  
611 analyses (Figure 5). Then, this also suggests that NAC enhance the film roughness when incorporated  
612 into the matrix. Similarly, Acevedo-Fani et al. (2015), Chen et al. (2016), and Pérez-Córdoba & Sobral  
613 (2017) have reported an improvement in the microstructures of films based on biopolymer blends when  
614 mixed with nanoemulsified essential oils.

615

616 **Insert Figure 6**

617

### 618 **3.2.8 Antimicrobial Activity**

619 The inhibitory activity against both *P. aeruginosa* (Gram negative) and *L. monocytogenes*  
620 (Gram positive) was determined measuring the clear zone surrounding the disks (inhibition zone). Halo  
621 formation (65 - 138 mm<sup>2</sup>) around the active films was observed only in the case of *P. aeruginosa*, which  
622 exhibited greater sensitivity compared to *L. monocytogenes* (Table 5). Similar observations were  
623 reported by Hafsa et al. (2016) and Kavooosi et al. (2014) when tested chitosan and gelatin based films  
624 with incorporated Eucalyptus globulus or Zataria multiflora essential oils. Paparella et al. (2008)  
625 suggested that the antimicrobial activity of some essential oils, is due to their interaction with enzymes  
626 located on the cell wall or the breakdown of the phospholipids present in the cell membrane, which  
627 results to increased permeability and leakage of cytoplasm.

628 The antimicrobial effect against *P. aeruginosa* could have been enhanced by the presence of  
629 chitosan in the blend, which has been widely reported as an antimicrobial compound (Elsabee & Abdou,  
630 2013; Pranoto et al., 2005; Yuan, Chen, & Li, 2016). This has been ascribed to the presence of positively  
631 charged amino groups in the chitosan structure, which interact with the negatively charged microbial  
632 cell membranes and lead to the leakage of proteinaceous (and other intracellular) constituents from the  
633 microorganisms (Pereda et al., 2011, Pranoto et al., 2005). However, in this study all the G-Ch based  
634 films without active compounds (N<sub>0</sub> and N<sub>1</sub>) showed no activity against the tested bacteria (Table 5).

635 When active films were tested against *L. monocytogenes*, inhibition zones were not obvious  
636 ( $p>0.05$ ); however, a clear zone was observed underneath the films. This observation could be  
637 associated to the limited diffusion of NAC from the films to the media (Pereda et al., 2011; Ponce,  
638 Roura, del Valle, & Moreira, 2008) since in our case the active compounds were doubly encapsulated,  
639 into the nanodroplets and in the film matrix. Otoni et al. (2014), Seydim & Sarikus (2006) and Sung et  
640 al. (2014) have reported activity against *L. monocytogenes* when using nanoemulsified cinnamaldehyde  
641 or GO into pectin/papaya puree, whey protein and low-density-polyethylene/ethylene-vinyl-acetate  
642 based films. In our study, nanoemulsified active compounds when not tested in films, showed high  
643 activity against *L. monocytogenes* (data not shown), which could be considered a derivative of the  
644 antimicrobial compounds and their delivery through nano-sized droplets, as reported by Kadri et al.  
645 (2017).

646 Converse to expectation, the combined application of nanoencapsulated Cin and GO within the  
647 film did not enhance the antimicrobial properties of the G-Ch based film ( $p<0.05$ ), although both of  
648 them had the ability to induce an inhibitory effect as bulk agent on the microorganism tested, principally  
649 due to their chemical components, such as cinnamic aldehyde and diallyl trisulfide, diallyl disulphide,  
650 methyl allyl trisulfide, and diallyl tetrasulfide, which are able to disrupt and penetrate the lipid structure  
651 of the bacteria cell membrane, leading to its destruction (Peng & Li, 2014).

652

### 653 **3.2.9 Antioxidant properties**

654 The antioxidant activity of the films expressed as trolox equivalent ( $\mu\text{mol TE /g dried film}$ ) for  
655 the DPPH $\cdot$  and ABTS $\cdot^+$  radicals, and the FRAP reagent is shown in Table 5. As expected, the control 1  
656 film did not show any radical scavenging activity, in either of the DPPH $\cdot$  or ABTS $\cdot^+$  tested method, and  
657 possessed very low FRAP scavenging activity.

658 Films loaded with NAC were capable of acting as stronger donors of hydrogen atoms or  
659 electrons until reduction of the stable purple-coloured radical DPPH $\cdot$  or blue-coloured radical ABTS $\cdot^+$   
660 converted to yellow-coloured DPPH-H or ABTS $\cdot$ , respectively (Brand-Williams et al., 1995; Re et al.,  
661 1999). The film loaded with the nanoemulsion encapsulating  $\alpha$ -t/Cin ( $N_2$ ) exhibited the greatest  
662 antioxidant activity for both DPPH $\cdot$  and ABTS $\cdot^+$  radicals, with values of  $0.22 \pm 0.02$  and  $2.63 \pm 0.12$   
663  $\mu\text{mol TE/g film}$ , respectively. This activity corresponded to the highest radical scavenging effect of that  
664 nanoemulsion ( $N_2$ ) before incorporating in the film (data not shown). The results for ABTS $\cdot^+$  radical  
665 scavenging of the films were comparable to those reported by Bonilla & Sobral (2016) and Pérez-  
666 Córdoba & Sobral (2017) for gelatin-chitosan based films loaded with boldo or guarana extracts, and  
667 nanoemulsified active compounds, respectively.

668 On the other hand, the incorporation of  $\alpha$ -t/GO-loaded nanoemulsion ( $N_3$ ) into the film caused  
669 the highest ( $p < 0.05$ ) ferric reducing ability and, consequently, the best antioxidant activity measured by  
670 the FRAP assay with an increase of 91% and 51%, respectively, when compared with either of the two  
671 control films ( $N_0$  and  $N_1$ ). The FRAP assay gave the highest TE values, probably because of the direct  
672 contact of the film samples with the FRAP reagent during the reaction.

673 The antioxidant activity of the films is potentially attributed to the phenolic acids and terpenoids  
674 coming from the cinnamaldehyde, garlic oil, and principally,  $\alpha$ -tocopherol, which are able to quench  
675 free radicals by forming resonance-stabilized phenoxyl radicals (Dudonné, Vitrac, Coutière, Woillez,  
676 & Mérillon, 2009). In addition to this, the contribution from the residual free amino groups of the  
677 chitosan molecule, which also react with free radicals forming stable macromolecular radicals and  
678 ammonium groups, should also be taken into account in terms of antioxidant activity (Yen, Yan, &  
679 Mau, 2008; Yuan et al., 2016).

680

681 **Insert Table 5**

682

#### 683 **4. Conclusions**

684 O/W emulsions, with  $\alpha$ -toc, Cin and GO active compounds loaded within their dispersed phase  
685 droplets at high encapsulation efficiencies, were successfully formed at the nanoscale via a  
686 microfluidization technique. The formed nanoemulsions possessed a monomodal distribution and  
687 exhibited good physical stability over a 90 days storage and incorporation of the active species was not  
688 detrimental to either of these features. These nanoemulsions were subsequently incorporated into  
689 gelatin-chitosan (G-Ch) based films, which were shown to possess a homogeneous structure with a

690 good distribution of nanoencapsulated active compounds (NAC) throughout the biopolymer matrix and  
691 without any unfavorable effects ( $p>0.05$ ) on the films' original thickness, moisture content, glass  
692 transition, and melting temperature.

693 Nanoemulsion loading was found to enhance the films' resistance to water, reducing ( $p<0.05$ )  
694 their solubility, and increasing film elongation at break and light barrier properties, while also directly  
695 affecting their transparency, reducing their tensile strength and stiffness, and increasing their surface  
696 roughness. Therefore, nanoemulsions encapsulating active compounds are suitable to produce G-Ch  
697 based films, enhancing their physical and mechanical properties, antibacterial performance against *L.*  
698 *monocytogenes* and *P. aeruginosa*, and their radicals scavenging effect.

699 Films loaded with NAC have a potential applications in food packaging for food shelf-life  
700 improvement. Further studies on controlled release and foodstuff application are needed to know the  
701 real advantage of those active films when used on food.

702

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707

## 708 **Conflict of interest**

709 Authors declare that this work has not been published previously and there are no conflicts of interest.

710

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## Figures Captions

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957

958 **Figure 1.** Droplet size distributions of O/W nanoemulsions containing encapsulated active compounds as  
959 a function of storage time (all systems stored at 4 °C). **(a)** Control (no encapsulated species); **(b)**  $\alpha$ -  
960 tocopherol/cinnamaldehyde; **(c)**  $\alpha$ -tocopherol/garlic oil; and **(d)**  $\alpha$ -tocopherol/ cinnamaldehyde and garlic  
961 oil.

962

963 **Figure 2.** **(a)** 3-D AFM topographic images, and **(b)** profile of the height values along the sample in  
964 the marked area of 2D AFM images of O/W nanoemulsions containing encapsulated active compounds.  
965 \* $\alpha$ -t:  $\alpha$ -tocopherol, Cin: cinnamaldehyde, GO: garlic oil.

966

967 **Figure 3.** Diffractograms of gelatin-chitosan films loaded with O/W nanoemulsions containing  
968 encapsulated active compounds. N<sub>0</sub> - Control 1: film without nanoemulsion; N<sub>1</sub> - Control 2: film with  
969 control nanoemulsion (no encapsulated species); N<sub>2</sub>:  $\alpha$ -tocopherol/cinnamaldehyde; N<sub>3</sub>:  $\alpha$ -  
970 tocopherol/garlic oil; N<sub>4</sub>:  $\alpha$ -tocopherol/cinnamaldehyde and garlic oil-loaded nanoemulsion.

971

972 **Figure 4.** DSC thermograms of gelatin-chitosan films loaded with O/W nanoemulsions containing  
973 encapsulated active compounds. N<sub>0</sub> - Control 1: film without nanoemulsion; N<sub>1</sub> - Control 2: film with  
974 control nanoemulsion (no encapsulated species); N<sub>2</sub>:  $\alpha$ -tocopherol/cinnamaldehyde; N<sub>3</sub>:  $\alpha$ -  
975 tocopherol/garlic oil; N<sub>4</sub>:  $\alpha$ -tocopherol/cinnamaldehyde and garlic oil-loaded nanoemulsion. Straight  
976 traces correspond to the first scan and broken traces for the second scan.

977

978 **Figure 5.** AFM micrographs of (a) 3D topography and (b) 2D surface of gelatin-chitosan films loaded  
979 with O/W nanoemulsions containing encapsulated active compounds. N<sub>0</sub> - Control 1: film without  
980 nanoemulsion; N<sub>1</sub> - Control 2: film with control nanoemulsion (no encapsulated species); N<sub>2</sub>:  $\alpha$ -  
981 tocopherol/cinnamaldehyde; N<sub>3</sub>:  $\alpha$ -tocopherol/garlic oil; N<sub>4</sub>:  $\alpha$ -tocopherol/cinnamaldehyde and garlic  
982 oil-loaded nanoemulsion.

983

984 **Figure 6.** ESEM micrographs of the a) surface and b) cross section of gelatin-chitosan films loaded  
985 with O/W nanoemulsions containing encapsulated active compounds. N<sub>0</sub> - Control 1: film without  
986 nanoemulsion; N<sub>1</sub> - Control 2: film with control nanoemulsion (no encapsulated species); N<sub>2</sub>:  $\alpha$ -  
987 tocopherol/cinnamaldehyde; N<sub>3</sub>:  $\alpha$ -tocopherol/garlic oil; N<sub>4</sub>:  $\alpha$ -tocopherol/cinnamaldehyde and garlic  
988 oil-loaded nanoemulsion.

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