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

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11
12 **Abstract**

13 The aim of this research was to develop and characterize gelatin-chitosan (4:1) based films that
14 incorporate nanoemulsions loaded with a range of active compounds; N₁: canola oil; N₂: α -
15 tocopherol/cinnamaldehyde; N₃: α -tocopherol/garlic oil; or N₄: α -tocopherol/cinnamaldehyde and garlic
16 oil. Nanoemulsions were prepared in a microfluidizer with pressures ranging from 69 to 100 MPa, and
17 3 processing cycles. Films were produced by the casting method incorporating 5g N_{1,2,3,4}/100 g
18 biopolymers and using glycerol as a plasticizer, and subsequently characterized in terms of their
19 physico-chemical, antimicrobial and antioxidant properties. No differences (p>0.05) were observed for
20 all films in terms of moisture content (18% w/w), and thermal properties. The films' solubility in water
21 and light transmission at 280 nm were considerably reduced as compared to the control, N₁ (15% and
22 60% respectively) because of the nanoemulsion incorporation. The film loaded with N₁ showed the
23 greatest (p<0.05) opacity, elongation at break and stiffness reduction, and was the roughest, whilst the
24 lowest tensile strength and ability to swell were attained by films loaded with N₃ and N₄, respectively.
25 DSC and X-ray analyses suggested compatibility among the biopolymeric blend, and a good
26 distribution of nanodroplets embedded into the matrix was confirmed by AFM and SEM analyses. Films
27 loaded with nanoencapsulated active compounds (NAC) were very effective against *Pseudomonas*
28 *aeruginosa*, and also showed high antioxidant activity. Overall, the present study offers clear evidence
29 that these active-loaded films have the potential to be utilized as packaging material for enhancing food
30 shelf life.

31 **Keywords:** biopolymer, active films, emulsion, α -tocopherol, cinnamaldehyde, garlic oil.
32

33 **Chemical compounds studied in this article:**

34 Cinnamaldehyde (PubChem CID: 637511); alpha-tocopherol (PubChem CID: 14985); Garlic oil
35 (PubChem CID: 6850738); Tween 20 (PubChem CID: 443314); Span 60 (PubChem CID: 14928);
36 Chitosan (21896651); Acetic acid (PubChem CID: 176); Glycerol (PubChem CID: 753); 2,2'-azino-

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 β -(1-4)-2-acetamido-D-glucose and β -
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 α -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, α -tocopherol (α -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). α -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 α -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 α -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 α -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^{•+} [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[®] 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 α -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) α -t/Cin (N₂), α -t/GO (N₃), or an equimolar mixture of α -t/Cin and GO (N₄) 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 (N₁). 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 (α -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 (α -t, Cin or GO) remaining within the droplets of the
154 nanoemulsion was determined by using an UHPLC⁺ (Dionex Ultimate 3000, Thermo scientific,
155 Germany). Analyses were carried out by diluting the sample in methanol to facilitate the α -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 μ m) with an elution system of
158 methanol:acetonitrile:water (68:28:4) for α -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 μ L (α -t) or 10 μ L (Cin and GO), and the detection wavelength
161 was set at 208, 285 and 210 nm, for α -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 ζ -potential using a Zetasizer (Nanoseries, Malvern Instruments, UK), pH using a pHmeter
164 (SevenCompact, Mettler Toledo, Switzerland), flow behavior using a rheometer (Kinexus Pro⁺,
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 (N₁, N₂, N₃, and N₄) 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 ± 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 2θ from 8° to 70° (with a constant speed of 1° 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 (± 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°C in an inert atmosphere ($45 \text{ ml}/\text{min}$ of 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 (ΔH_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 (10⁸ cfu/mL), then diluted to a
282 final concentration of 10⁵ 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 N₁, N₂, N₃ and N₄ (or not,
285 N₀), 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^{•+}) and 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) 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^{•+} and DPPH[•] 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^{•+} method.**

299 A solution containing ABTS^{•+} 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^{•+} working solution with an absorbance value of 0.70 ± 0.02 , as
302 measured using a UV-Vis spectrophotometer at 734 nm. An aliquot (100 μ L) of the solubilized and
303 centrifuged (4000 rpm, 30 min) samples was added to the ABTS^{•+} working solution (900 μ 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 (μ mol/g dried film).

307 **2.4.11.2 DPPH[•] 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[•] radical solution (60 μ 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 (μ mol/g dried film).
312 Antioxidant activity is expressed as TE (μ mol/g dried film).

314 **2.4.11.3 FRAP assay**

315 A solution of FeCl₃ (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 FeCl₃. 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 (μ 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 α -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 α -tocopherol, resulting in a reduction of the quantified α -t
339 concentration (Anarjan, Mirhosseini, Baharin, & Tan, 2011; Cheong et al., 2008). When comparing the
340 EE for Cin or GO between N₂ or N₃ and N₄, 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 α -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, ζ -potential and pH measurements**

354 The nanoemulsions were also evaluated in terms of their physicochemical properties (Table 1).
355 The control nanoemulsion (N₁) without encapsulated actives, presented the highest ($p < 0.05$) droplet
356 size, polydispersity index (PDI), ζ -potential, and pH values, among all tested formulations (Table 1).
357 For nanoemulsions loaded with active compounds, mean particle size, PDI, and ζ -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 ζ -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 ζ -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 ± 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 α -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 ± 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 2θ
529 = 10° , 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° 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° 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 (ΔH_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 ΔH_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 ΔH_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 mm²) 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 (N₀ and N₁) 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 \bullet and ABTS \bullet^{+} 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 \bullet or ABTS \bullet^{+} 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 \bullet or blue-coloured radical ABTS \bullet^{+}
660 converted to yellow-coloured DPPH-H or ABTS \bullet , respectively (Brand-Williams et al., 1995; Re et al.,
661 1999). The film loaded with the nanoemulsion encapsulating α -t/Cin (N_2) exhibited the greatest
662 antioxidant activity for both DPPH \bullet and ABTS \bullet^{+} radicals, with values of 0.22 ± 0.02 and 2.63 ± 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 \bullet^{+} 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 α -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, α -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 α -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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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)** α -
960 tocopherol/cinnamaldehyde; **(c)** α -tocopherol/garlic oil; and **(d)** α -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 * α -t: α -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. N₀ - Control 1: film without nanoemulsion; N₁ - Control 2: film with
969 control nanoemulsion (no encapsulated species); N₂: α -tocopherol/cinnamaldehyde; N₃: α -
970 tocopherol/garlic oil; N₄: α -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. N₀ - Control 1: film without nanoemulsion; N₁ - Control 2: film with
974 control nanoemulsion (no encapsulated species); N₂: α -tocopherol/cinnamaldehyde; N₃: α -
975 tocopherol/garlic oil; N₄: α -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. N₀ - Control 1: film without
980 nanoemulsion; N₁ - Control 2: film with control nanoemulsion (no encapsulated species); N₂: α -
981 tocopherol/cinnamaldehyde; N₃: α -tocopherol/garlic oil; N₄: α -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. N₀ - Control 1: film without
986 nanoemulsion; N₁ - Control 2: film with control nanoemulsion (no encapsulated species); N₂: α -
987 tocopherol/cinnamaldehyde; N₃: α -tocopherol/garlic oil; N₄: α -tocopherol/cinnamaldehyde and garlic
988 oil-loaded nanoemulsion.

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