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Quercetin loaded gelatin films with modulated release and tailored anti-oxidant, mechanical and swelling properties

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Rubini K., Boanini E., Menichetti A., Bonvicini F., Gentilomi G.A., Montalti M., et al. (2020). Quercetin loaded gelatin films with modulated release and tailored anti-oxidant, mechanical and swelling properties. FOOD HYDROCOLLOIDS, 109, 1-9 [10.1016/j.foodhyd.2020.106089].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/772373> since: 2020-09-21

*Published:*

DOI: <http://doi.org/10.1016/j.foodhyd.2020.106089>

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This is the final peer-reviewed accepted manuscript of:

Rubini, K.; Boanini, E.; Menichetti, A.; Bonvicini, F.; Gentilomi, G. A.; Montalti, M.; Bigi, A. Quercetin Loaded Gelatin Films with Modulated Release and Tailored Anti-Oxidant, Mechanical and Swelling Properties. *Food Hydrocolloids* **2020**, *109*, 106089.

The final published version is available online at:  
<https://doi.org/10.1016/j.foodhyd.2020.106089>

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1        **Quercetin loaded gelatin films with modulated release and tailored**  
2        **anti-oxidant, mechanical and swelling properties**

3  
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19        **Abstract**

20        Quercetin, a flavonoid widely diffused in fruits and vegetables, is known for its good  
21        pharmacological qualities, such as anti-oxidant and anti-inflammatory properties. In this  
22        work, we loaded quercetin on gelatin films with the aim to develop materials with  
23        tailored anti-oxidant, mechanical and stability properties. To this purpose, gelatin films  
24        at increasing flavonoid content were prepared using two different solvents, namely  
25        H<sub>2</sub>O/EtOH (EtOH films) and DMSO (DMSO films). Quercetin content increased up to  
26        about 3.8 and 1.8 wt% in DMSO and EtOH films, respectively. The use of DMSO as  
27        solvent prevents the partial regain of collagen triple helix structure during gelling of  
28        gelatin sols and results in remarkable extensibility of the films. At variance, EtOH films  
29        display X-ray diffraction patterns and DSC plots in agreement with the presence of  
30        triple helix structure, and exhibit reduced swelling and increasing mechanical properties  
31        on increasing quercetin content. Moreover, their values of denaturation enthalpy  
32        indicate the presence of chemical interaction between the flavonoid and gelatin, which  
33        can be responsible of their lower quercetin release in PBS in comparison to DMSO  
34        films. The flavonoid release is sustained for both series of films and occurs through  
35        anchorage to gelatin nanoparticles. Moreover, both DMSO and EtOH functionalized  
36        films exhibit relevant anti-oxidant properties, in agreement with their RSA levels, which  
37        are comparable to that of pure quercetin.

38

39        **Keywords:** gelatin; quercetin; anti-oxidant properties; mechanical properties;  
40        fluorescence; swelling

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## 1. Introduction

Gelatin is a renewable and biodegradable material (Chiellini, Cinelli, Corti, & Kenawy, 2001) which is obtained by chemical or thermal degradation of collagen. Its characteristics, including abundance, biocompatibility, low cost and absence of antigenicity, make gelatin an ideal raw material for food processing, packaging, as well as for pharmaceutical and biomedical applications. On the other hand, this biopolymer exhibits poor mechanical and water vapor barrier properties. In fact, gelatin is highly soluble in aqueous solutions, which limits most of its potential applications. Improvement of gelatin films functionality is usually achieved through crosslinking with chemical agents or through physical treatments. Dehydrothermal treatment, U.V. and gamma irradiation are the most frequent utilized physical methods, whereas chemical agents include dialdehydes, diisocyanates, genipin, carbodiimides, acyl azide, polyepoxy compounds, oxidized alginate and transglutaminase (Amadori et al., 2015; Boanini, Rubini, Panzavolta, & Bigi, 2010; Kolodziejaska, & Piotrowska, 2007; Kuijpers et al., 1999; Rault, Frei, Herbage, Abdul-Malak, & Huc, 1996; Sung, Huang, Chang, Huang, Hsu, 1999; van Wachem et al., 1999).

Generally speaking, protein crosslinking can also be achieved through reaction of their side chain amino groups with polyphenols (Hosseini, & Gómez-Guillén, 2018; Strauss, & Gibson, 2004). These compounds, widespread in fruits, vegetable and seeds, are potent anti-oxidants and are receiving increasing interest thanks to their health beneficial properties, including anti-carcinogenic, anti-thrombotic, anti-inflammatory, anti-microbial, vasodilatory effects (Ozidal, Capanoglu, & Altay, 2013). Furthermore, polyphenols can stimulate osteoblast proliferation and activity and, as a consequence, promote bone formation and mineralization (Shavandi et al., 2018). Their interactions with proteins have been suggested to involve hydrogen bonding, ionic and hydrophobic interactions and covalent bonding (Hosseini et al., 2018; Zhang et al., 2010). Several

72 studies indicate that crosslinking gelatin films with polyphenolic compounds, including  
73 green tea polyphenols, rosemary and oregano extracts, caffeic, ferulic and gallic acids,  
74 provide materials with improved mechanical parameters and anti-oxidant properties  
75 (Chen et al., 2017; Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009;  
76 Zhang et al., 2010). Herein, we studied the modifications of the properties of gelatin  
77 films induced by one of the most abundant flavonoids, namely quercetin (3,3',4',5,7-  
78 pentahydroxy-flavone). Quercetin has remarkable anti-oxidant, anti-inflammatory, anti-  
79 bacterial and anti-cancer effects. In particular, it has been shown to inhibit the  
80 proliferation of different types of cancer cells (Wang et al., 2016). On the other hand, its  
81 low water solubility and poor oral bioavailability limits its efficacy in dietary and  
82 pharmaceutical applications. These drawbacks have stimulated the research of possible  
83 systems for local delivery of the flavonoid (Forte et al., 2016; Forte et al., 2017; Patel,  
84 Heussen, Hazekamp, Drost, & Velikov, 2012; Zhang, Yang, Tang, Hu, & Zou, 2008).  
85 In this study, we developed gelatin films added with increasing amounts of quercetin,  
86 with the purpose to enrich the films with the peculiar functionalities of quercetin and, at  
87 the same time to modulate the mechanical and swelling properties of the films. Since  
88 quercetin is moderately soluble in ethanol and highly soluble in dimethyl sulfoxide  
89 (DMSO), the films were prepared following different routes, which imply the use of  
90 water/ethanol or DMSO, as solvents.

98 **2. Materials and Methods**

99 **2.1 Preparation of the films**

100 *2.1.1 Direct syntheses*

101 Gelatin from pig skin (280 Bloom, Italgelatine S.p.A.) was used: 5 g of gelatin were  
102 dissolved in 100 ml of a mixture of water/ethanol (50/50) or in 100 ml of pure dimethyl  
103 sulfoxide (DMSO) at 40°C for about 30 min. Films were obtained on the bottom of  
104 Petri dishes (diameter = 6 cm) from 7.4 ml of gelatin solution after solvent evaporation:  
105 water/ethanol at room temperature, DMSO at 45 °C. Complete drying was attained  
106 under laminar flow hood.

107 Gelatin-quercetin films were obtained following the above procedure, and adding  
108 quercetin powder to gelatin before solvent addition. Different amounts of quercetin  
109 were used: 0.5, 1, 1.5 and 2 g/l.

110 Therefore samples prepared in water/ethanol and in DMSO were respectively labeled  
111 as W 05, W 1, W 15, W 2 and DMSO 05, DMSO 1, DMSO 15, DMSO 2.

112 Similarly, reference samples with no quercetin were labeled W 0 and DMSO 0.

113 *2.1.2 Preparation by adsorption*

114 Since direct synthesis in water/ethanol (50/50) provoked precipitation of quercetin in  
115 solution, water/ethanol was used as a solvent following an adsorption procedure. In this  
116 case, 5 g of gelatin were dissolved in 100 ml of water at 40°C for about 30 min. Films  
117 were obtained on the bottom of Petri dishes (diameter = 6 cm) from 7.4 ml of gelatin  
118 solution after solvent evaporation at room temperature. Before complete drying, 7.4 ml  
119 of a solution of quercetin in water/ethanol (50/50) was placed into each Petri dish for 24  
120 hours. Afterwards films were washed with distilled water and completely dried under  
121 laminar flow hood.

122 Different amounts of quercetin were used: 0.5, 1, 1.5 and 2 g/l. Therefore samples  
123 prepared by adsorption of quercetin were respectively labelled as EtOH 05, EtOH 1,  
124 EtOH 15, EtOH 2, whereas reference sample with no quercetin was labeled EtOH 0.

## 125 ***2.2 Characterization of the films***

### 126 *2.2.1 Quercetin content and quercetin release*

127 Quercetin content was determined on samples dissolved in H<sub>2</sub>O/EtOH 1:1 solution at a  
128 concentration of 20 mg/mL and maintained at 40°C for one day. Absorption spectra of  
129 each series of films were collected on 1:50 diluted film solutions (0.4 mg/mL) .

130 Quercetin release was determined on samples (1cmx1cm) placed at the bottom of a vial  
131 and added with 5 mL of PBS 0.1M (pH 7.4). The release was monitored for 10 hours at  
132 room temperature, collecting absorption spectra on supernatant diluted 1:1 with  
133 Ethanol. The spectra were recorded at selected times and the PBS solution was  
134 refreshed after each absorbance measurement.

135 Absorption spectra were collected with a Perkin Elmer UV/Vis spectrometer Lambda  
136 45. Quercetin content of the films, as well as quercetin release, was obtained by means  
137 of Lambert-Beer law:

$$138 \text{Abs} = \varepsilon \cdot b \cdot c$$

139 where *Abs* is the Absorbance,  $\varepsilon$  [M<sup>-1</sup>cm<sup>-1</sup>] is the extinction molar coefficient, *b* [cm] is  
140 the optical path (1cm) and *c* [M] is the concentration of quercetin.

141 For the evaluation of quercetin content, the molar extinction coefficient (15360 M<sup>-1</sup>  
142 cm<sup>-1</sup>) was calculated by collecting absorption spectra of known quantities of quercetin  
143 dissolved in a water/ethanol 1:1 mixture containing gelatin (0.4 mg/mL).

144 For the evaluation of quercetin release, the molar extinction coefficient (14234 M<sup>-1</sup>  
145 cm<sup>-1</sup>) was calculated by collecting absorption spectra of known quantities of quercetin  
146 dissolved in a PBS 0.1M/ethanol 1:1 mixture.

147



148 *2.2.2 Dynamic light scattering analysis*

149 Dynamic light scattering DLS (Malvern Zetasizer Nano-ZS) was performed on DMSO  
150 15 and EtOH 15 samples, compared to the reference samples (DMSO 0 and EtOH 0).  
151 Samples of about 1 cm<sup>2</sup> were placed on the bottom of a vial, added with 5 mL of PBS  
152 and kept at 25°C for 1 hour. After 1 hour the supernatant solution was collected for DLS  
153 characterization. When possible, the solutions were filtered by means of an RC 0.45µm  
154 filter.

155 *2.2.3 Steady state fluorescence anisotropy measurements*

156 Steady state fluorescence anisotropy analysis was performed (Edinburgh Instrument)  
157 using a 380 nm LED light to excite quercetin. Emission spectra were recorded from 400  
158 nm to 700 nm.

159 *2.2.4 Fluorescence microscopy*

160 Transmission and fluorescence images were obtained by a fluorescence microscope  
161 (Olympus IX71) with a 10x objective. In order to collect the fluorescence images,  
162 excitation was performed at 378-400 nm and the fluorescence was observed at 510-542  
163 nm.

164 *2.2.5 Thermal analyses*

165 Calorimetric measurements were performed using a Perkin Elmer Pyris Diamond  
166 differential scanning calorimeter equipped with a model ULSP 90 intra-cooler.  
167 Temperature and enthalpy calibration was performed by using high-purity standards (n-  
168 decane and indium). The measurements were carried out on dried samples, hermetically  
169 sealed in aluminium pans. Heating was carried out at 5 °C min<sup>-1</sup> in the temperature  
170 range from 40 °C to 120 °C. Denaturation temperature was determined as the peak  
171 value of the corresponding endothermic phenomena. The value of denaturation enthalpy  
172 was calculated with respect to the sample weight.

173 Thermogravimetric analysis was performed using a Perkin–Elmer TGA-7, heating  
174 samples (5–10 mg) in a platinum crucible in air flow (20 cm<sup>3</sup>/min) at a rate of 10  
175 °C/min up to 800 °C.

#### 176 *2.2.6 X-ray diffraction*

177 X-ray diffraction analysis was carried out by means of a Panalytical XCellerator powder  
178 diffractometer. Cu K $\alpha$  radiation was used (40 mA, 40 kV). The 2 $\theta$  range was from 5° to  
179 40° with a step size of 0.1° and time per step of 200 s.

#### 180 *2.2.7 Swelling, water solubility and contact angle*

181 For swelling experiments films were cut into portion 1 cm x 1cm and were weighted in  
182 air-dried conditions. Then they were immersed in phosphate-buffered saline (PBS, 0.1  
183 M) solution for different periods of time. Wet samples were wiped with filter paper to  
184 remove excess liquid and weighed. The amount of absorbed water was calculated as

$$185 W(\%) = [(W_w - W_d) / W_d] \cdot 100$$

186 where  $W_w$  and  $W_d$  are the weights of the wet and the air-dried samples.

187 Water solubility was determined in triplicate (Giménez, Gómez-Estaca, Alemán,  
188 Gómez-Guillén, & Montero, 2009). Portions of the films (2x2 cm) were placed into  
189 glass containers with 15 ml of distilled water and subjected to gentle shaking at 65 rpm  
190 for 15 h at 22°C. Filtration on filter paper was used to recover the remainder of the  
191 undissolved film, which was desiccated at 105°C for 24 h. The film solubility was  
192 calculated as

$$193 FS(\%) = [(W_0 - W_f) / W_0] \cdot 100$$

194 where  $W_0$  is the initial weight of the film expressed as dry matter and  $W_f$  is the weight  
195 of the desiccated undissolved rest of the film.

196 Static contact angle measurements were performed on films using a KSV CAM-101  
197 instrument under ambient conditions. The side profiles of deionized water drops were

198 recorded for image analysis in a time range of 0–10 s, by collecting an image every 0.5  
199 s. At least three drops were observed for each sample.

#### 200 *2.2.8 Mechanical tests*

201 For mechanical tests, films prepared by direct synthesis in DMSO or by adsorption from  
202 water/ethanol were immersed into a mixture of water/ethanol (in the ratio 2:3) for 1 min  
203 or 72 h respectively.

204 Stress–strain curves of strip-shaped (3 mm x 30 mm, thickness around 0.12 mm) films  
205 were recorded using an INSTRON Testing Machine 4465, with a crosshead speed of 5  
206 mm min<sup>-1</sup>, and the Series IX software package. The thickness of the samples was  
207 determined using a Leitz SMLUX- POL microscope. The Young's modulus  $E$ , the  
208 stress at break  $\sigma_b$  and the strain at break  $\varepsilon_b$  of the strips were measured.

209 Statistical analysis was performed with the Student  $t$ -test considering a  $p$  value of less  
210 than 0.05 to be significantly different.

#### 211 *2.2.9 Radical Scavenging Assay*

212 Antioxidant activity was determined on the basis of quercetin ability to act as radical  
213 scavengers toward the stable 2,2-diphenyl-1-picrylhydrazyl free radical, (DPPH•)  
214 (Sigma). Solutions of EtOH 1, EtOH 2, DMSO 1 and DMSO 2 were prepared by  
215 dissolving the solid samples in MilliQ water/EtOH at 40 °C, thereafter they were  
216 diluted, basing on their known quercetin contents, in order to get 10, 30 and 50  $\mu$ M of  
217 quercetin concentrations. Dilutions of pure quercetin (Sigma), 10, 30 and 50  $\mu$ M, were  
218 prepared and used as reference samples. For the assay, 50  $\mu$ L of each solution (samples  
219 and references) were added to 3 mL of DPPH• saturated ethanol/water (20/80 V/V)  
220 solution, previously clarified by centrifugation (10000 rpm for 10 minutes). After an  
221 incubation of 10 minutes at room temperature in darkness, absorbance values ( $A$ ) were  
222 spectrophotometrically measured at 517 nm. The radical scavenging activity (RSA) was  
223 determined through the following equation:

224       % RSA = (A0 – Ax)/A0 × 100

225       where A0 is the absorbance of the control (containing DPPH• solution without  
226       quercetin), and Ax is the absorbance in the presence of quercetin (as reference) or of  
227       quercetin-containing samples.

228       Statistical evaluation of data was performed using GraphPad Prism version 5.00 for  
229       Windows (GraphPad Software). One-way analysis of variance (ANOVA) followed by  
230       Dunnett's Multiple comparison test was used to determine significance of differences  
231       (p<0.05) among experimental groups and reference samples.

#### 232       2.2.10 Antibacterial properties of films

233       The *in vitro* antibacterial activity of the films was evaluated against *Staphylococcus*  
234       *aureus* (ATCC 25923) and *Escherichia coli* (ATCC25922) selected as controls and  
235       representative strains for Gram positive and Gram negative bacteria. The effectiveness  
236       of samples to inhibits bacterial growth was assessed by a standardized Kirby-Bauer  
237       (KB) diffusion test on Mueller-Hinton agar plate and by measuring the bacterial-free  
238       zone around the disk-shaped samples (Ø = 6 mm) after 24h of incubation at 37°C.

239       In addition, the films were assayed for their antibacterial activity following incubation  
240       in PBS, at pH 7.4 and at 37°C to mimic physiological environment. For this purpose,  
241       each film was prepared in a vial and incubated with 200 µL of PBS. Then, the liquid  
242       samples, containing released quercetin from the different films, were evaluated against  
243       *S. aureus* and *E. coli* by means of a broth microdilution method using a 96-well plate  
244       (Boanini, et al. 2018). In parallel, pure quercetin dissolved in DMSO at 30 mg/mL was  
245       assayed against *S. aureus* and *E. coli* in the range 500 – 15.625 µg/mL. Bacterial growth  
246       was determined by measuring the absorbance value (A) at 630 nm (OD) and the  
247       effectiveness of the quercetin, as pure compound and released from the film samples,  
248       was expressed as percentage value relative to the positive growth control (bacterial  
249       suspension in regular medium).

250 All experiments were performed on duplicate in different days, using gentamicin disk  
251 (10 µg) or gentamicin solution (in the range 5 µg/mL to 0.005 µg/mL,) as reference  
252 drug.

253

254

### 255 **3. Results and discussion**

256 Quercetin functionalized gelatin films display different properties depending on the  
257 preparation process. The use of DMSO as solvent allowed to dissolve gelatin and  
258 quercetin in the same solution and to obtain films at different content of the flavonoid  
259 (DMSO films). At variance, the films prepared in water/ethanol (W films) were opaque  
260 and not homogeneous due to the precipitation of quercetin (Figure S1).

261 Therefore, we followed an alternative route where air-dried films prepared from gelatin  
262 aqueous solution were maintained in contact with quercetin dissolved in water/ethanol  
263 for 24 hours (EtOH films).

#### 264 ***3.1 Quercetin content***

265 The amount of quercetin incorporated in the functionalized films was determined  
266 through evaluation of absorption spectra of films dissolved in a water/ethanol mixture  
267 (Figure 1).

268 The data of quercetin concentration in the solutions of the different samples were  
269 utilized to calculate quercetin content of the films (Table 1).

270 The results reported in Table 1 show that the two film preparation methods lead to  
271 different amounts of quercetin loading. Quercetin content of DMSO films corresponds  
272 to that present in the preparation solution, as expected. On the contrary, the preparation  
273 procedure of EtOH samples implies quercetin adsorption from solution after preparation  
274 of the gelatin films, which yields a lower flavonoid content in the final films.

275

### 276 **3.2 Fluorescence microscopy**

277 Quercetin distribution inside the films was evaluated through fluorescence of the films  
278 (Figure S2). The results show that quercetin is homogeneously distributed in DMSO  
279 film, whereas its distribution in ETOH films seems not completely uniform, as shown in  
280 Figure S2: although the comparison of the image of EtOH 1 with that of EtOH 0 shows  
281 that the flavonoid is distributed all through the film, there are regions where it is more  
282 densely packed. Fluorescence microscope images confirm the particular distribution of  
283 quercetin in the films, as shown in Figure S3 for EtOH 1, which involves the formation  
284 of quercetin fluorescent aggregated structures of various sizes (from 20-30  $\mu\text{m}$  to 80-90  
285  $\mu\text{m}$ ).

### 286 **3.3 Thermogravimetric analysis**

287 The results of thermogravimetric analysis confirm the greater quercetin content of  
288 DMSO than EtOH films. The TG plots of all the samples are characterized by three  
289 weight losses: the first (between about 30 and 250°C) corresponds to water loss; the  
290 second (between about 250 and 500°C) is due to gelatin decomposition; the third one  
291 (centered around 600-700°C) is due to combustion of the residual components (Bigi,  
292 Panzavolta, & Rubini, 2004a). Quercetin decomposition occurs around 350°C  
293 (Borghetti et al., 2012), that is in the same region of gelatin decomposition. Figure 2  
294 reports the TG plots of the EtOH 2 and DMSO 2 samples compared with those of the  
295 relative controls. The comparison of the different weight losses in the range of  
296 temperature 250-500°C indicate that quercetin content in EtOH 2 and DMSO 2 films is  
297 about  $1\pm 1$  and  $3\pm 1$  wt% respectively (Table S1), in good agreement with absorbance  
298 results. Moreover, the results of thermogravimetric analysis demonstrate that the films  
299 do not contain any residual solvent. In particular, the absence of DMSO is supported  
300 also by the results of FTIR analysis: the spectrum of DMSO is characterized by a strong  
301 band at  $102\text{ cm}^{-1}$ , which is not present in the spectra of DMSO films, as shown in Figure

302 S4. It is also worth noticing that the temperature corresponding to complete combustion  
303 is higher for the samples containing quercetin (745 and 726°C respectively for EtOH 2  
304 and EtOH 0; 770 and 758°C for DMSO 2 and DMSO 0), suggesting a stabilizing effect  
305 of the flavonoid.

### 306 ***3.4 Swelling, water solubility and contact angle***

307 The incorporation of quercetin in EtOH films resulted in reduced values of swelling in  
308 phosphate buffer (Figure 3): swelling of pure gelatin films is about 1700% after 48 h  
309 immersion in PBS and decreases as a function of quercetin content down to 1000% for  
310 EtOH films at the maximum quercetin content. The degree of swelling of gelatin films  
311 prepared in DMSO is significantly lower: the maximum values reached by DMSO 0 and  
312 DMSO 2 films are about 700% and 600% respectively. However, the stability of the  
313 DMSO films in PBS is significantly reduced in comparison to that of EtOH films, so  
314 that after 48 h they are completely dissolved. Although also this series of films display a  
315 clear contribution of quercetin, the noticeable reduction of swelling induced by DMSO  
316 does not allow to appreciate a clear trend as a function of the content of the flavonoid.

317 At variance with swelling results, the values of contact angle and of water solubility of  
318 the different samples do not exhibit significant differences. In particular, contact angle  
319 displays mean values in the range 72°-77°, showing that all prepared films have a  
320 hydrophilic behavior and a good wettability (Table S2). Although DMSO films are less  
321 stable than EtOH films in PBS, they show similar water solubility, indeed FS (%)  
322 values do not vary even as a function of quercetin content, as reported in Table S4.

### 323 ***3.5 Differential scanning calorimetry***

324 DSC analysis provides information on the thermal stability of gelatin and on its content  
325 of triple helical structure. In fact, cooling of gelatin aqueous solutions results in a partial  
326 renaturation of collagen structure which implies a partial recovery of the triple helix  
327 structure (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). The DSC

328 plot of gelatin exhibits an endothermic peak due to the transition of the recovered  
329 portions of triple helix to random coils (denaturation), with an associated denaturation  
330 enthalpy which depends on the extent of triple helix structure (Bigi, Panzavolta, &  
331 Rubini, 2004b).

332 The DSC plot of EtOH 0 shows indeed the presence of an endothermic peak centered at  
333 about 100°C (denaturation temperature,  $T_d$ ) with an associated denaturation enthalpy  
334 ( $\Delta H_d$ ) of about 28 J/g (Table 2). Loading of quercetin in the EtOH films does not  
335 significantly affect their thermal stability, in agreement with the values of the  
336 denaturation temperature which do not vary significantly with composition (Table 1).  
337 At variance, the values of denaturation enthalpy of EtOH films containing quercetin are  
338 drastically reduced when compared with that of the unloaded sample, although it is not  
339 possible to appreciate variations as a function of quercetin content due to the broadening  
340 of the peaks (Figure 4, Table 2). Reduction of the value of gelatin denaturation enthalpy  
341 is usually observed on samples treated with crosslinking chemical agents and can be  
342 ascribed to a reduction of hydrogen bonds (which break endothermically) and/or to an  
343 increase of covalent crosslinks (which break exothermically) (Bigi et al., 2004b, Finch,  
344 & Ledward, 1972). On this basis, the observed decrease of denaturation enthalpy  
345 suggests possible chemical interactions between gelatin and quercetin molecules which  
346 stabilize the films, in agreement with the observed reduction of the degree of swelling.

347 The DSC plot of quercetin films prepared from DMSO solution did not show any  
348 endothermic peak indicating absence of triple helix structure and suggesting that gelatin  
349 does not gelify in DMSO (Kozlov, & Burdigina, 1983).

### 350 ***3.6 X-ray diffraction analysis***

351 In agreement with DSC results, the X-ray diffraction patterns of DMSO films show  
352 only a broad peak centered at about 20° of  $2\theta$ , corresponding to a periodicity of about  
353 0.45 nm, which has been ascribed to the distance between adjacent polypeptide strands



354 of gelatin (Okuyama, 2008), confirming that DMSO prevents the partial renaturation of  
355 the protein. At variance, the XRD patterns of EtOH films exhibit also a sharp peak at  
356 about  $7^\circ$  of  $2\theta$  corresponding to a periodicity of about 1.1 nm and related to the  
357 diameter of the triple helix (Okuyama, 2008), as shown in Figure 5.

### 358 ***3.7 Mechanical properties***

359 The absence of triple helix structure in the DMSO films can be considered responsible  
360 of their mechanical behavior, which is quite different from that of EtOH films. At  
361 variance with EtOH films, DMSO films cannot be maintained in the mixture  
362 water/ethanol for the period of time (72 h) usually utilized to reach a constant relative  
363 humidity of 75% (Boanini et al., 2010), and must be removed from the mixture just  
364 after 1 min because they become sticky. Moreover, under tensile stress, they undergo a  
365 remarkable elongation and exhibit jagged stress-strain curves (Figure S5), which  
366 prevent a proper evaluation of their mechanical parameters.

367 On the contrary, the stress strain curves of the samples of the EtOH series are similar to  
368 those characteristic of gelatin films (Amadori, et al. 2015), as shown from the  
369 comparison of a few typical curves of films at different quercetin content reported in  
370 Figure 6. The values of the Young's modulus,  $E$ , the stress at break,  $\sigma_b$ , and the  
371 deformation at break,  $\epsilon_b$ , for the different samples are reported in Table 3. Both the  
372 values of stress at break and deformation increase significantly on increasing quercetin  
373 concentration up to 1.5 g/L, whereas the value of Young's modulus does not show any  
374 significant variation. On the other hand, quercetin concentration of 2 g/L, which means  
375 a flavonoid content of 1.8 wt% (Table 1), provokes a significant worsening of the  
376 mechanical parameters of the films, as shown in Table 3, most likely also due to the non  
377 homogeneous distribution of the flavonoid inside the films.

378

379

### 380 *3.8 Quercetin release*

381 Quercetin release in PBS from some selected functionalized films was determined from  
382 absorbance values at the quercetin maximum (375 nm) as a function of time.

383 The results reported in Figure 7 show that the release initially increases with time, but  
384 reaches a steady state in a few hours. The amount of quercetin released by EtOH films  
385 is significantly lower than that liberated by DMSO films (maximum value of release :  
386 about 45 and 70 % of the initial content from EtOH and DMSO samples, respectively).  
387 Moreover, the percentage of quercetin release from DMSO films generally increases  
388 with quercetin content (with the exception of DMSO 1 sample), whereas EtOH samples  
389 display an opposite trend. The decrease of quercetin release from EtOH films on  
390 increasing their pristine content is in agreement with their swelling behavior and  
391 confirms the stabilizing role of the flavonoid, as suggested by the results of denaturation  
392 enthalpy.

393 Quercetin is not soluble in aqueous solutions (Zhang et al., 2008); however, quercetin  
394 release in PBS aqueous solution yields very clear and transparent solutions, suggesting  
395 that the flavonoid is actually released anchored to gelatin nanoparticles, which enhances  
396 its solubility. This process was studied in DMSO 15 and EtOH 15 samples through DLS  
397 analysis and fluorescence anisotropy measurements on the supernatant solution after  
398 quercetin release for 1 hour.

399 DLS measurements allow to detect the presence of nanoparticles in solution during the  
400 release (Montalti, Battistelli, Cantelli, & Genovese, 2014). The results of DLS analysis  
401 (Table S4) indicate that functionalized films, as well as EtOH 0 film, release  
402 nanoparticles with a diameter of about 30 nm. The release solution of DMSO 0 sample  
403 cannot be filtered, most likely because of a significant presence of gelatin in solution,  
404 and gave a result in agreement with a polydisperse solution with average size of almost  
405 160 nm.

406 Fluorescence anisotropy measurements give information about the rotational freedom of  
407 the emitting molecules (Rampazzo et al., 2018). The supernatant solutions of the films  
408 (Figure S6) gave high values of mean anisotropy (0.21 and 0.15 for DMSO 15 and  
409 EtHO 15 respectively) indicating the presence of anisotropy in both DMSO 15 and  
410 EtOH 15 samples. This means that quercetin molecules in the solution experience  
411 limited rotational mobility and their fluorescence is thus partially polarized, which  
412 would confirm that quercetin is released through anchorage to gelatin nanoparticles

### 413 ***3.9 Radical scavenging activity***

414 Quercetin can quench reactive oxygen species (ROS) displaying antioxidant activity  
415 (Enoki et al., 2014). ROS are involved in a number of pathologies, including not just  
416 inflammation but also cardiovascular diseases and cancer (Enoki et al., 2014;  
417 Obrenovich et al., 2011). Therefore, it is important to verify if quercetin functionalized  
418 gelatin films display the antioxidant properties of the flavonoid. To this aim, we tested  
419 the radical scavenging activity (RSA) of the different samples by means of the 1,1-  
420 diphenyl-2-picryl-hydrazyl (DPPH<sup>•</sup>) assay (Berlier et al., 2013; Mohan, Birari,  
421 Karmase, Jagtap, & Kumar Bhutani, 2012). DPPH<sup>•</sup> exhibits a characteristic absorption  
422 band at 515 nm. The intensity of this band can be reduced by the scavenging action of  
423 the antioxidant material, which donates hydrogen to DPPH<sup>•</sup> to form the corresponding  
424 hydrazine. The test was carried out on different amounts of samples in order to get  
425 different concentration of quercetin. The results show that RSA increases as a function  
426 of quercetin concentration. In particular, pure quercetin exhibits RSA values up to about  
427 35% as its concentration increases up to 50  $\mu$ M. The data reported in Figure 8 show that  
428 all the examined samples display RSA values similar to those recorded for pure  
429 quercetin, indicating that the antioxidant properties is maintained in both series of  
430 functionalized films. No statistically significant differences were measured in RSA

431 values comparing the pure quercetin, set as reference control, and the tested samples at  
432 the same quercetin content, irrespective of the solvent used for preparation.

### 433 **3.10 Antibacterial activity**

434 The antibacterial properties of the gelatin-quercetin-films, and of the quercetin released  
435 from the films following incubation in PBS, were evaluated by means of a standardized  
436 Kirby-Bauer disk diffusion test and a broth microdilution methodology, respectively.  
437 Despite the antimicrobial activity of quercetin against different bacterial strains has  
438 been previously demonstrated (Bonvicini et al., 2017), the gelatin-quercetin films  
439 obtained in the present study show no activity towards *S. aureus* and *E. coli*,  
440 irrespective of the assay used. The result could be ascribed to the low quercetin content  
441 of the films. Considering the highest quercetin amount loaded on the film samples  
442 (3.8%, see Table 1), and the weight of gelatin-quercetin films ( $5.39 \pm 1.80$  mg), none of  
443 the samples contained the Minimum Inhibitory Concentration (MIC) of quercetin  
444 enabling bacterial growth arrest. Indeed, the maximum amount of quercetin in the PBS  
445 solution tested in the broth microdilution assay was  $20 \mu\text{g/mL}$  while the MIC values for  
446 pure quercetin measured  $125 \mu\text{g/mL}$  and  $500 \mu\text{g/mL}$  for *S. aureus* and *E. coli*,  
447 respectively.

448

## 449 **4. Conclusions**

450 The two different methods of preparation developed in this work provide functionalized  
451 films characterized by different properties. In particular, DMSO films are less stable in  
452 aqueous solution and display a very high extensibility, which hinders the measurement  
453 of their mechanical properties, most likely because they lack triple helix structure.

454 The increase of quercetin content in EtOH films provokes a reduction of swelling  
455 degree, an increase of the mechanical parameters and a reduction of the denaturation

456 enthalpy, in agreement with the presence of chemical interaction between gelatin and  
457 the flavonoid. Most importantly, both quercetin containing DMSO and EtOH films  
458 show a sustained, gelatin nanoparticles mediated, flavonoid release in PBS, as well as  
459 RSA values indicative of remarkable anti-oxidant properties, suggesting possible  
460 applications as local delivery systems of the functionalizing agent.

461

## 462 **Acknowledgments**

463 The authors are grateful to the support of the University of Bologna.

## 464 **Conflict of Interest**

465 The authors have no competing interests to declare.

466

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607

608 **Table 1.** Quercetin content of the functionalized films as a function of its theoretical  
609 content.

Theoretical (T) and Experimental (E) quercetin content					
Sample	T (wt%)	E (wt%)	Sample	T (wt%)	E (wt%)
DMSO 05	1.0	1.0	EtOH 05	1.0	0.4
DMSO 1	2.0	2.0	EtOH 1	2.0	0.8
DMSO 15	2.9	2.9	EtOH 15	2.9	1.2
DMSO 2	3.8	3.8	EtOH 2	3.8	1.8

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612 **Table 2.** Denaturation temperatures ( $T_D$ ) and enthalpies ( $\Delta H_D$ ) of EtOH films recorded  
613 through DSC measurements.

<i>Sample</i>	$T_D$ ( $^{\circ}C$ )	$\Delta H_D$ (J/g)
EtOH 0	100±1	28±1
EtOH 1	102±1	14±1
EtOH 15	98±1	18±1
EtOH 2	99±1	14±1

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615

616 **Table 3.** Strain at Break,  $\sigma_b$ , Stress at Break,  $\varepsilon_b$ , and Young's Modulus, E, of EtOH films. Each  
 617 value is the mean of at least seven determinations and is reported with its standard deviation.

618

sample	E (MPa)	$\sigma_b$ (MPa)	$\varepsilon_b$ (%)
EtOH 0	1.5 ± 0.2	0.28 ± 0.05 <sup>a</sup>	100 ± 20 <sup>a</sup>
EtOH 05	1.3 ± 0.4	0.54 ± 0.08	135 ± 20 <sup>b</sup>
EtOH 1	1.8 ± 0.6	1.1 ± 0.2 <sup>a</sup>	202 ± 29 <sup>a</sup>
EtOH 15	1.9 ± 0.6	1.4 ± 0.1 <sup>a</sup>	238 ± 10 <sup>a,b</sup>
EtOH 2	1.4 ± 0.8	0.4 ± 0.2 <sup>a</sup>	109 ± 30 <sup>a</sup>

619

620  $\sigma_b$  : <sup>a</sup> EtOH 0 vs EtOH 05, EtOH 1, EtOH 15; EtOH 1 vs EtOH 05; EtOH 15 vs EtOH 05; EtOH 2  
 621 vs EtOH 1, EtOH 15 ( p<0.001). <sup>b</sup> EtOH 15 vs EtOH 1 (p<0.05)

622  $\varepsilon_b$  : : <sup>a</sup> EtOH 0 vs EtOH 1, EtOH 15; EtOH 1 vs EtOH 05; EtOH 15 vs EtOH 05; EtOH 2 vs EtOH  
 623 1, EtOH 15 ( p<0.001). <sup>b</sup> : EtOH 05 vs EtOH 0; EtOH 15 vs EtOH 1 (p<0.05)

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628 **Figures Captions.**

629 **Figure 1-** Absorption spectra of DMSO (a) and EtOH (b) gelatin-quercetin film  
630 solutions (0.4 mg/mL in water/ethanol 1:1).

631 **Figure 2-** TGA plots of (a) EtOH films and (b) DMSO films. The insets show an  
632 enlargement of the final parts of the plots.

633 **Figure 3-** Swelling curves of (a) EtOH films and (b) DMSO films. Each value was  
634 determined in triplicate. Standard deviations are comprised into the size of the symbols.

635 **Figure 4-** DSC thermograms recorded from dried EtOH 0 and EtOH 1 films show the  
636 presence of an endothermic peak due to collagen triple helix denaturation.

637 **Figure 5-** X-rays diffraction patterns of EtOH films showing the presence of the 1.1 nm  
638 reflection; which is absent in DMSO films.

639 **Figure 6-** Typical stress-strain curves of EtOH gelatin films at different quercetin  
640 content.

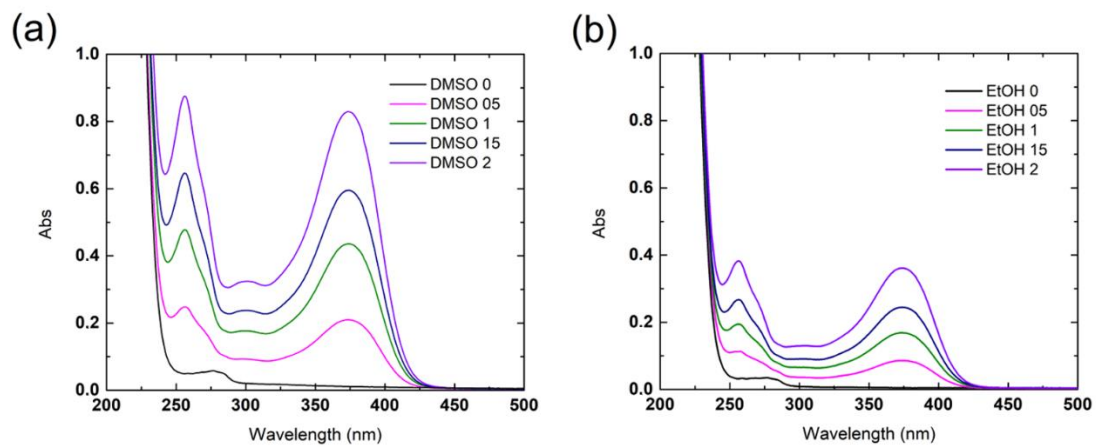
641 **Figure 7-** Quercetin release for each refresh (a), and cumulative release (b) expressed in  
642 mg. (c, d) cumulative release expressed as % of the initial content from DMSO (c) and  
643 EtOH (d) films.

644 **Figure 8-** Antiradical activity, expressed as % RSA, of the different samples and pure  
645 quercetin toward DPPH•. Bars represent the mean  $\pm$  SD of two independent  
646 measurements.

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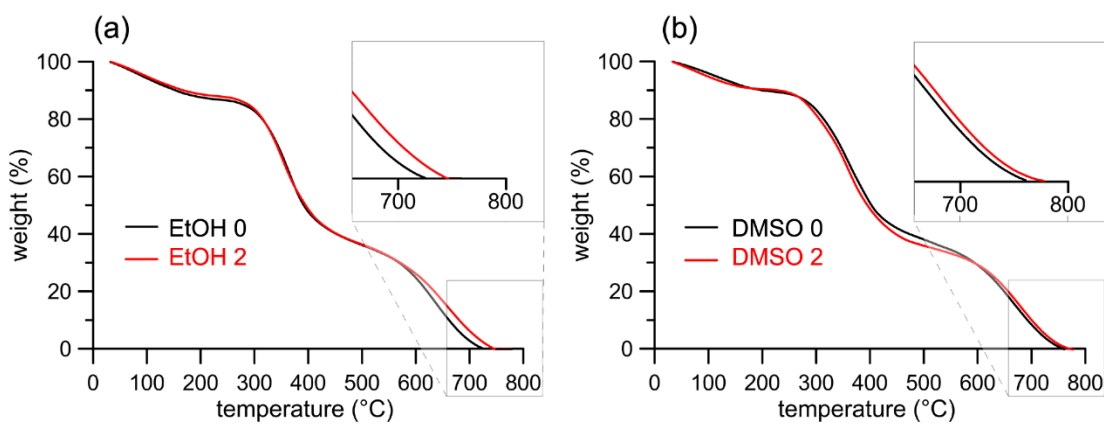
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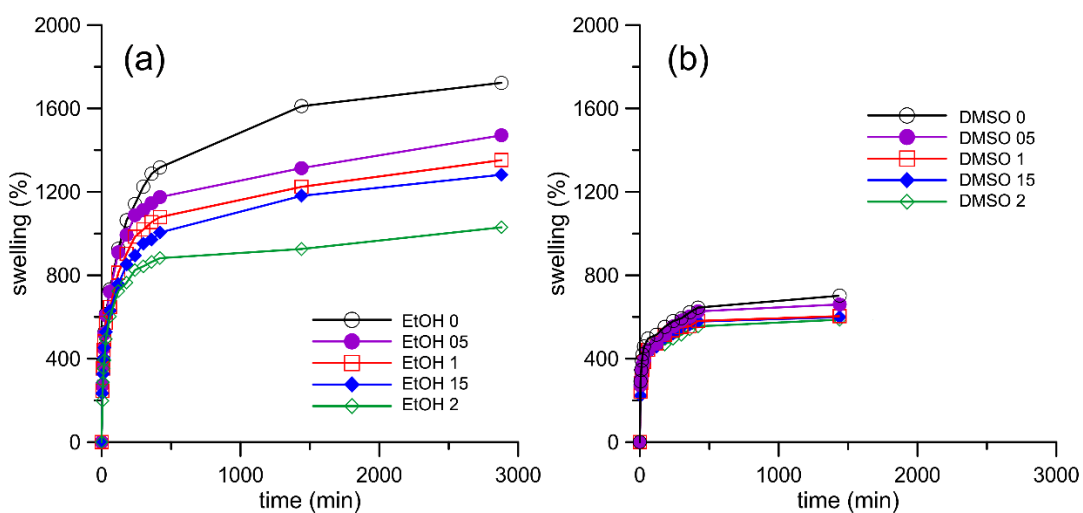
Figure 1



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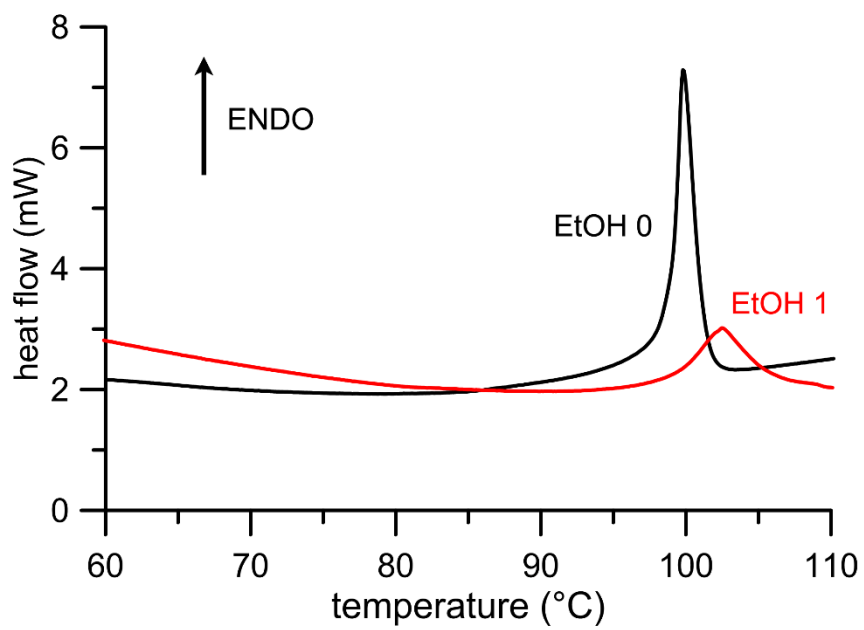
Figure 2



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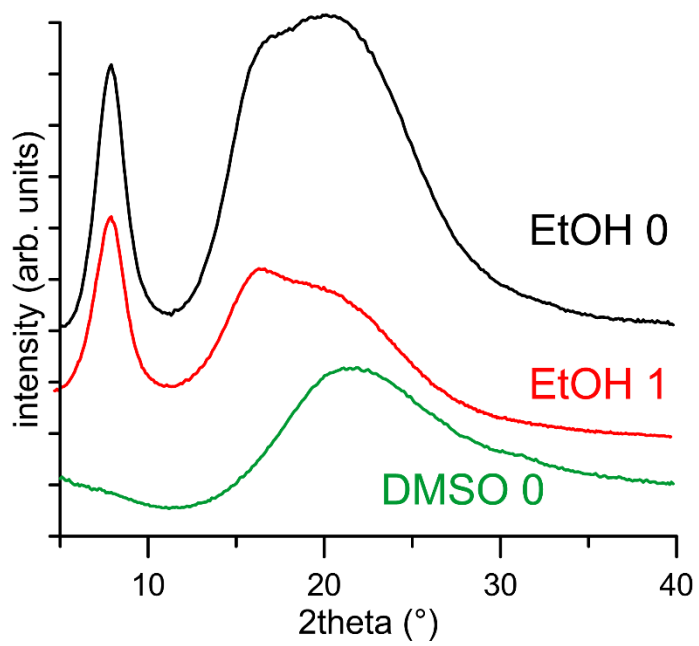
Figure 3



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Figure 4

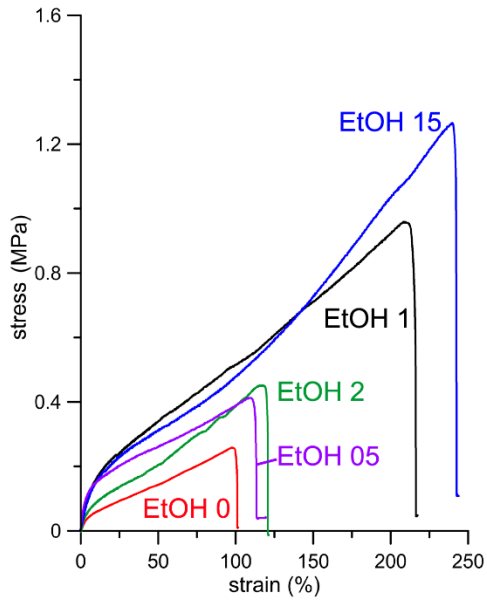


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Figure 5

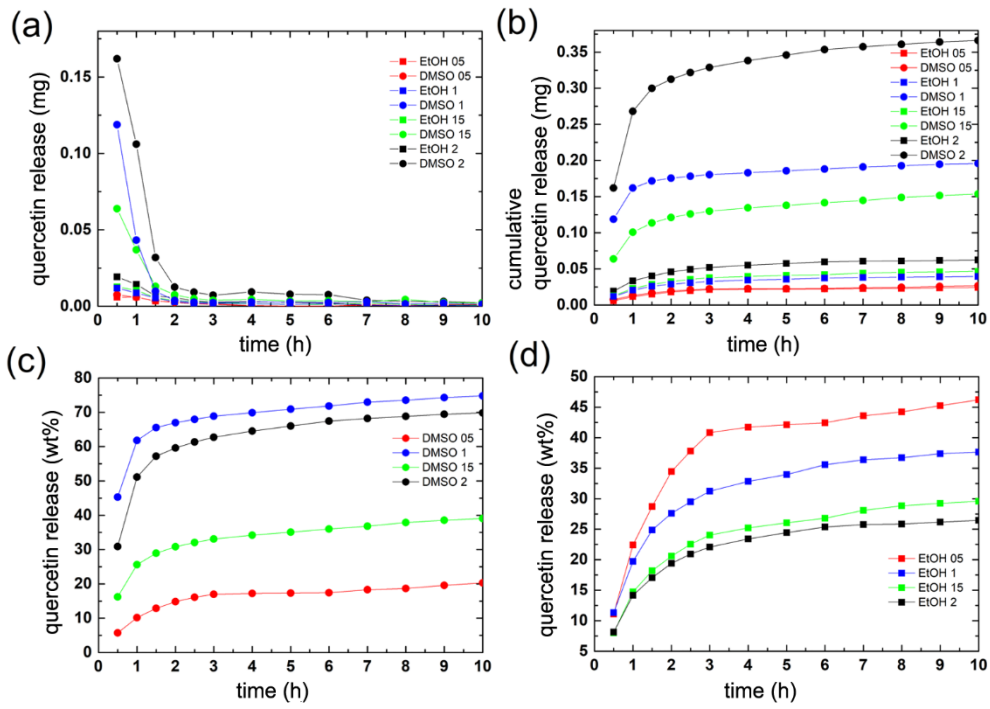




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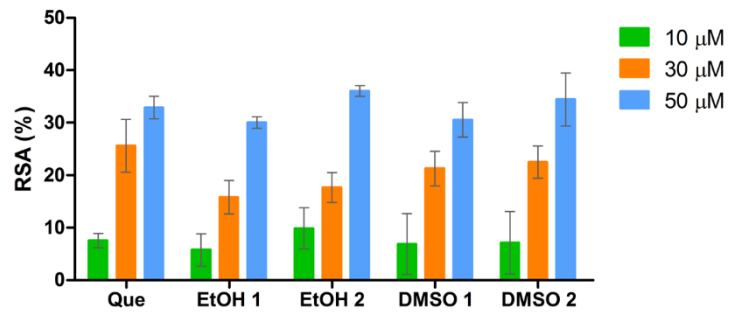
Figure 6



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Figure 7



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Figure 8