Production of water soluble quercetin formulations by pressurized ethyl acetate-in-water emulsion technique using natural origin surfactants

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Abstract

Quercetin is a strong antioxidant flavonoid with several bioactive properties such as anti-inflammatory and anticarcinogenic activities, becoming an interesting compound to be incorporated into pharmaceutical, cosmetic or food products. However, these applications are limited by the low bioavailability of this flavonoid. Quercetin is poorly soluble in aqueous media, such as gastrointestinal fluids, being also degraded by gut flora. Thus, it is necessary the development of quercetin's formulations capable of improving its water solubility resulting in increased bioavailability and thus higher biological activity of this compound.

The aim of the present work was the formulation of quercetin using three distinct natural origin surfactants, namely OSA-starch, Lecithin and β-glucan, by precipitation from a pressurized ethyl acetate-in-water emulsion. Formulations of quercetin with encapsulation efficiencies up to near 76% and a micellar particle size in the range of nanometers were obtained using lecithin. An improved antioxidant activity (3-fold higher per unit mass of quercetin) was also observed in these formulations, demonstrating that lecithin is a good emulsifier for the encapsulation of quercetin. Furthermore, the addition of glycerol as co-

25 solvent increased the colloidal stability of the suspension and the encapsulation efficiency

26 of the flavonoid.

27

28 **Keywords:** Quercetin, OSA-starch, Lecithin, β-glucan, Encapsulation, High Pressure Emulsion

29 Technique, Nanosuspension

30

31 **1. Introduction**

32 Quercetin (3,3',4',5,7-Pentahydroxyflavone), one of the most representative member of the
33 flavonoid family with high antioxidant activity, is commonly found in several fruits and
34 vegetables like onions, apples, grapes or strawberries, as well as in red wine or green/black tea
35 [1,2]. This compound has attracted the interest of the pharmaceutical and nutraceutical
36 industries due to its bioactive properties, such as anti-inflammatory, anti-proliferative and
37 neuroprotective effects [1,3]. In order to achieve quercetin plasma's concentration above 10
38 mM required for obtaining pharmacological activity, the ingestion of quercetin-enriched foods
39 or supplements could not be enough due to the low bioavailability of this flavonoid [4]. The
40 low water solubility (2ppm at 25ºC to 60 ppm at 100 °C) allied with gastrointestinal
41 degradation limits quercetin's biological effects *in vivo* [2,5]. There are two main approaches to
42 increase the bioavailability of this compound, whether by chemical modification or by the
43 development of colloidal quercetin delivery systems [6].

44 Formulations of quercetin using different methods and distinct carrier materials have been
45 developed by several authors. Regarding polymers, Kumari and co-authors achieved a
46 controlled release of quercetin by its encapsulation into poly-D,L-lactide (PLA) nanoparticles
47 through solvent evaporation technique [7]. Wu et al. have produced quercetin-loaded
48 nanoparticles by a nanoprecipitation method using Eudragit® E and polyvinyl alcohol (PVA) as

49 carriers, obtaining a quercetin's release 74-fold higher in comparison with the pure compound
50 [8]. Quercetin was also encapsulated in Pluronic F127 through supercritical antisolvent method
51 by Fraile and co-authors, enabling an improved dissolution behaviour of the compound in
52 simulated physiological fluids [9]. Cyclodextrins (CD), such as α-CD, β-CD or HP-β-CD, have also
53 been used for the encapsulation of quercetin using the freeze-drying or co-evaporation
54 method [10,11]. Barras and co-authors used lipids to encapsulate quercetin, being able to
55 increase its apparent aqueous solubility by a factor of 100 [6]. Besides solid lipid nanoparticles,
56 nanostructured lipid carriers and lipid nanoemulsions were produced using a high pressure
57 homogenizer for the encapsulation of quercetin by Aditya et al., achieving encapsulation
58 efficiencies above 90% [12]. Quercetin-loaded liposomes have also been produced and are
59 reported in the literature [1,13]. Inorganic materials can also be an option as carriers for the
60 encapsulation of quercetin and, recently, quercetin-loaded silica microspheres were developed
61 by Kim and co-authors using polyol-in-oil-in-water (P/O/W) emulsion and sol-gel methods to
62 improve the flavonoid's stability as well as its properties [14].
63 In 2012, de Paz and co-authors [15] developed a novel method for the encapsulation of
64 hydrophobic compounds, based on the production and processing of ethyl acetate-water
65 emulsions at high pressure and temperature. The authors achieved stable aqueous
66 suspensions of β-carotene with micellar particle sizes down to 400 nm and encapsulation
67 efficiencies up to 80%. Moreover, the type of emulsifier used had been shown to affect the
68 final properties of the suspension [16]. This process is an attractive alternative to the
69 conventional emulsion evaporation process, since it enables the acceleration of the mass
70 transfer kinetics to the time scales of the precipitation processes. This intensification of the
71 process allows an improved control over the precipitation, at the same time that the
72 exposition of the product to degrading high-temperature conditions is decreased.
73 This work presents the development of water soluble formulations of quercetin through
74 pressurized ethyl acetate-water emulsion technique. Ethyl acetate has been chosen as organic

75 solvent because it is a Generally Recognized as Safe (GRAS) solvent with low toxicity (Lethal
76 Dose LD50 in rats: 11.3 g/kg) and it can be safely used as a flavouring agent [17]. Three natural
77 origin surfactants, namely modified n-octenyl succinate anhydride (OSA) starch, soybean
78 lecithin and barley β-glucan, were used in this work for the encapsulation of quercetin. Starch
79 is the second most abundant biomass material present in nature being the most abundant
80 storage polysaccharide in plants [18,19]. However, natural starch is mainly hydrophilic, which
81 could limit its application in the encapsulation of hydrophobic compounds. Nevertheless, OSA
82 (modified amphiphilic starch) is capable to overcome this drawback, and was already used for
83 the encapsulation and delivery of compounds with distinct polarities[19]. Lecithin is a mixture
84 of naturally occurring phospholipids, mainly phosphatidylcholine, which is usually available
85 from sources such as soybeans or eggs. Phospholipids are amphiphilic molecules composed by
86 hydrophobic tails and hydrophilic heads, being capable to rearrange themselves as liposomes,
87 spherical and closed structures composed of lipid bilayers [20,21]. Liposomes are interesting
88 carrier materials for the delivery of hydrophobic/hydrophilic compounds, and since they have
89 affinity to cellular membranes, they are capable to increase the absorption of several drugs
90 [22]. Besides the good properties as encapsulating agent and emulsifier, lecithin is also known
91 for its antioxidant activity [23]. β-glucans are soluble fibers present in cereal grains, especially
92 in barley, constituted by linear polysaccharides of glucose units, connected by (1 → 3) or (1 →
93 4)-beta linkages. These carbohydrates are known for their therapeutic effects on coronary
94 heart disease, diabetes and hypercholesterolemia, and have been used as encapsulating
95 agents [24–27]. The influence of the main process parameters has been studied, namely the
96 effect of quercetin and emulsifier's concentration, the effect of the flows of organic solvent,
97 suspension of quercetin and dissolution of emulsifier and also the organic to water ratio. By
98 comparing the results obtained with the three different emulsifiers, their roles on the
99 emulsion formation and quercetin's encapsulation can be established. Product analysis

100 included particle size, encapsulation efficiency, antioxidant activity and structural
101 characterisation.
102

103 **2. Materials and methods**

104 *2.1. Materials*

105 Quercetin hydrate (Q) with a (purity≥95%) was purchased from Sigma-Aldrich. Ethyl Acetate
106 with a purity of 99.5% and glycerol were purchased from Panreac Química (Barcelona, Spain).
107 Modified OSA-starch refined from waxy maize was kindly provided by National Starch Group
108 (Hamburg, Germany). Soybean lecithin (97% phospholipids) was obtained from Glama-Sot
109 (SOTYA, Madrid, Spain). Glucagel™ (barley β-Glucan) was kindly supplied by DKSH France
110 (purity 78%, MW : 125 -140 kDa).

111 *2.2. Precipitation from pressurized ethyl acetate-on-water emulsions*

112 The equipment used in this work, already described by De Paz et al[15,16], is represented in
113 Fig. 1 with a schematic flow diagram.

114 (FIGURE 1)

115 Briefly, it consists of three small storages at ambient pressure, corresponding to the feed of
116 pure ethyl acetate (V-1), quercetin suspension in the same organic solvent (V-2) and the
117 aqueous solution of the emulsifier (V-3). The installation also counts with two piston pumps
118 GILSON 305 (maximum flow rate: 25 mL/min; flow rate control with an a accuracy of 0.1
119 mL/min) used to feed the aqueous dissolution of the emulsifier and the quercetin suspension
120 (pumps P-3 and P-2, respectively) and a piston pump JASCO PU-2080 plus (maximum flow rate:
121 10 mL/min; flow control with an a accuracy of 0.1 mL/min) used to feed the pure organic
122 solvent (pump P-1). An oven (KNK-2000-C series GAS CHROMATOGRAPH) is used to preheat
123 the organic solvent stream.

124 This process starts with the total dissolution of quercetin in hot and pressurized ethyl acetate,
125 where by increasing temperature, it is possible to increase the solubility of quercetin in this
126 solvent, which is around 1g/L at ambient conditions. In this work, a temperature, typically,
127 between 125-140ºC was used, keeping a constant pressure between 6.0 and 6.5 MPa in order
128 to maintain the solvent in liquid state. The dissolution is achieved by mixing a flavonoid's
129 suspension in pressurized ethyl acetate at ambient temperature, with a stream of preheated
130 and pressurized ethyl acetate, using a T- mixer (M-1 in Fig. 1). In order to reduce the exposure
131 of quercetin to high temperatures, this hot and pressurized solution is mixed with the
132 ambient-temperature aqueous solution of emulsifier using the second T-mixer of the
133 equipment (M2). Therefore, the contact time between quercetin and the hot solvent is
134 reduced to less than two seconds, avoiding the degradation of the product. The contact of the
135 hot solution of quercetin with the aqueous solution in mixer M-2 causes the emulsification of
136 the organic solvent and the precipitation of quercetin by a combined antisolvent and cooling
137 effect. The last step is the removal of the organic solvent from the formed emulsion using a
138 rotary evaporator, in order to obtain an aqueous dispersion of quercetin particles stabilized by
139 the surfactant.

140 A typical experiment started with the preparation of an aqueous solution of emulsifier and a
141 suspension of quercetin in ethyl acetate, which were pre-heated and stirred in order to obtain
142 homogeneous mixtures. Afterwards, pumps were switched on and the pressure in the system
143 was fixed between 6.0 and 6.5 MPa. The oven was switched on to heat the organic solvent
144 until the desired temperature (140-150ºC). When the required temperature in mixer M-1 was
145 reached, the pure solvents used until this moment were replaced with the quercetin
146 suspension, in continuous agitation, and with the aqueous solution of the emulsifier. In all
147 experiments, a volume of approximately 25 mL of suspension containing quercetin was
148 processed. Afterwards, the organic solvent of the obtained emulsion was removed from the

149 sample using a rotary evaporator (BÜCHI 011-BÜCHI 461 Water Bath) under vacuum (-0.06,
150 60ºC and 110min), to eliminate the organic solvent.

151 *2.3. Product characterization*

152 *2.3.1. Microscopy*

153 An automated upright microscope system for life science research (Leica DM4000 B, Wetzlar,
154 Germany) was used to obtain microscopic images of the emulsions and suspensions.

155 2.3.2. *Physical stability of emulsion*

156 The physical stability of prepared emulsions was studied through the optical analyser
157 Turbiscan™ Classic on the production day, by measuring the variations in backscattering.

158 *2.3.3. Particle size*

159 The particle size of the micelles were measured by a Laser Diffraction (LD) equipment model
160 Malvern Mastersizer 2000 following a centrifugation step at 7800rpm, 5min, in order to
161 precipitate the crystals of quercetin that were not encapsulated. Suspensions were dispersed
162 in distilled water and measurement was carried out after a gentle rotation of the suspension
163 container in order to obtain a better dispersion of the micelles. In this work, particle size
164 measurements are reported as volume distribution and defined as the average diameter ($d_{0.5}$).

165 *2.3.4. Encapsulation efficiency (EE)*

166 For the quercetin's water suspensions stabilized with starch the Encapsulation Efficiency (EE)
167 was determined through Ultraviolet-visible spectrophotometry, while in the suspensions
168 stabilized with lecithin or β-glucan, due to the interference of these carrier materials on UV
169 assays, this parameter was determined by HPLC.

170 For UV-Vis measurements, the suspensions were previously centrifugated at 7800rpm (5min)
171 and diluted in water. The concentration of quercetin was determined using a UV-Visible

172 Spectrophotometer (Shimadzu UV-2550, λ= 375 nm). Calibration was obtained by using
173 standard samples with concentrations between 3 and 12µg/mL, using ethanol as solvent.

174 HPLC analysis of quercetin was performed in accordance with a method previously reported
175 [28]. A Waters 515 HPLC pump, equipped with In-Line Degasser AF (Waters), 717 plus
176 Autosampler (Waters) and 2487 Dual ʎ Absorbance detector (Waters) were used.
177 Chromatographic separation was carried out with a Symmetry C18 Column (5 µm, 4.6 mm X
178 150 mm, Waters) coupled with a Bio-Sil C18 precolumn (5 µm, 4.6 mm X 30 mm, Bio-Rad) with
179 mobile phase of acetonitrile/5% acetic acid solution (27:73, v/v) at 30 ◦C. The detection
180 wavelength was set at 373 nm and the flow rate was 1.0 mL/min. The calibration curve was
181 linear within the range of 25–200µg/mL (R^2= 0.9997) using methanol:water (70:30) solution as
182 solvent. The quercetin suspension samples were detected by HPLC following centrifugation at
183 7800rpm (5min) and dilution in methanol. The run time for the assay was 13 min and the
184 retention time for quercetin was 8.32 min.

185 The absorbance is proportional to the amount of quercetin dispersed in solution which
186 corresponds to the encapsulated quercetin. Non-encapsulated quercetin can correspond
187 either to quercetin precipitated as big crystals and not stabilized in the suspension, or to
188 quercetin that has undergone degradation during the process. The encapsulation efficiency is
189 reported as the ratio between the amount of flavonoid dispersed in solution and total
190 quercetin amount in the initial feed.

191 *2.3.5. Structural characterisation*

192 Infrared spectra of the suspensions and pure materials were recorded on a Bruker ALPHA FT-IR
193 apparatus equipped with a Platinum ATR sampling module including a diamond crystal. The
194 suspensions were centrifuged at 13300rpm during 30min. The supernatant was removed and
195 the sample pellet's was submitted to further evaporation of water at -0.09MPa and 35ºC for
196 48h. The pellet was then used to perform FTIR. The spectra in the range from 4000 to 400 cm^{-1}

197 were the average of 64 scans at a resolution of 2cm^{-1}. The ATR signal was transformed to
198 Transmittance and the obtained spectra was normalised after the baseline correction.

199 *2.3.6. Cryo-TEM*

200 Cryo-TEM analysis were performed through a method already described [29]. Briefly, 4µl of
201 sample was deposited on a rack C-Flat 1.2/1.3, which was previously hydrophilized by a plasma
202 cleaner. A blotting is performed on either side of the filter paper grid during 4s where,
203 afterwards, liquid ethane is introduced in order to freeze the samples, avoiding the formation
204 of crystals (Gatan Cryoplunge 3). Samples are maintained in liquid nitrogen until their transfer
205 to the holder (Gatan Cryotransfer 626). JEOL JEM-FS2200 HRP 200 kV TEM equipment with
206 electron filtering was used to perform the TEM images.

207 *2.3.7. Stability of suspensions*

208 One of the aqueous suspensions with the best relation between EE and final quercetin's
209 concentration was chosen for stability examination. Some authors demonstrated that it's
210 possible to increase the stability of lecithin, reducing the appearance of its agglomerates and
211 increase drug's solubility by adding glycerol (G), as co-solvent, to aqueous lecithin dispersions
212 [30,31]. Thus, an equal suspension with 2.5% (v/v) of glycerol as co-solvent in the water phase
213 was prepared in order to compare the physicochemical properties of the suspension without
214 it. The stability examination was performed through the assessement of micellar particle size,
215 presence of crystals, quercetin's concentration and antioxidant activity of the suspensions
216 after 75 days of storage in a refrigerated dark room (10ºC).

217 The assessment of antioxidant activity was performed through oxygen radical absorption
218 capacity (ORAC) assay, which was carried out by a method previously described [32]. This assay
219 measures the ability of the antioxidant species present in the sample to inhibit the oxidation of
220 disodium fluorescein (FL) catalysed by peroxyl radicals generated from AAPH.

221 Briefly, in a 96-well microplate, 25 µL of diluted sample and 150 µL of disodium fluorescein
222 (2×10^{-7} mM) are added. Then, 25 µL of AAPH (153 mM) are added to start the reaction. The
223 microplate is put in a fluorescent reader (FLUOstar OPTIMA, BMG Labtech) at 37ºC.
224 Fluorescence emitted by the reduced form of fluorescein is measured and recorded every 1
225 min at the emission wavelength of 530±25 nm and excitation wavelength of 485±20 nm for a
226 period of 90 min. Phosphate buffer (75 mM, pH=7.4) is used to prepare AAPH and fluorescein
227 solutions and is used as blank. Solutions of 13.5, 25, 50, 100, and 200 µM/L of Trolox are used
228 as standards. Standards and blank are analyzed as triplicates, while the samples and one
229 control of 50 µM/L of Trolox are analysed 6 times. Final ORAC values are calculated by a
230 regression equation between the Trolox concentration and the net area under the FL decay
231 curve and are expressed as µM Trolox Equivalents (TE) per L of quercetin's suspension (µM
232 TE/L).

233 **3. Results and discussion**

234 *3.1. Starch*

235 As already mentioned, De Paz et al [15] studied β-carotene formulation using modified OSA-
236 starch as the emulsifier, showing that it is possible to obtain micelle's particle size in the range
237 of 300-600nm with a high percentage of encapsulated pigment (over 70%). This emulsifier has
238 a critical micelle concentration around 5g/L [33], and thus equal or higher concentration of
239 starch should be used in order to guarantee the formation of micelles. In the first experiments
240 performed in this work, the encapsulation of quercetin in modified OSA-starch was studied.
241 Table 1 presents the operating conditions of the experiments performed and the results
242 obtained.

243 (TABLE 1)

244 Regarding experiments E1-E8, and according to results obtained in the work of De Paz et al.
245 [16], a constant organic-water volume ratio (calculated as flow of organic solvent + flow of

246 quercetin suspension/flow of the dissolution of modified starch) of 0.7 was used, and the
247 concentration of surfactant and quercetin were changed. In experiments E9-E11, a constant
248 concentration of quercetin (5 g/L) and modified starch (20g/L) was used, and the organic-
249 water volume ratio was changed from 0.6 to 0.9.

250 Overall, the encapsulation efficiency was not higher than 10%, while the particle size was in
251 the range of micrometer, showing that modified starch is not an appropriate emulsifier to
252 encapsulate quercetin.

253 The highest percentage of encapsulated quercetin was reached when low starch
254 concentrations were used, while particle size increased when the concentration of the
255 emulsifier increased. In general, in a successful encapsulation the opposite trends of variation
256 with the concentration of carrier are expected, as observed by de Paz et al in the
257 encapsulation of β-carotene in starch [16]. Therefore, this result confirms that the OSA-
258 modified starch is not a suitable carrier for quercetin, as it does not favor the encapsulation or
259 reduction of particle size of the compound, and on the contrary it even precludes it, probably
260 due to the higher viscosity and increased mass transfer resistances in the samples with higher
261 starch concentrations.

262 Regarding the influence of the concentration of quercetin, in general high concentrations of
263 quercetin implied lower encapsulation efficiency and higher particle size. This could be
264 explained owing to the fact that at high concentrations of quercetin it gets more difficult for
265 the flavonoid to dissolve after M-1, leading to lower encapsulation efficiency and higher
266 particle size due to the presence of quercetin crystals.

267 An increase of the organic-water ratio led to an increase of both particle size and
268 encapsulation efficiency. Probably the higher initial volume of ethyl acetate used led to the
269 formation of an emulsion with larger droplet size and thus the formation of bigger particles

270 inside the micelles. A higher percentage of the organic solvent may also lead to increased
271 dissolution of quercetin, resulting in higher final EE.

272 *3.2. Lecithin*

273 The encapsulation of quercetin by using lecithin as the emulsifer was also studied in this work.
274 Since this emulsifier has a critical micelle concentration around 20g/L (experimentally
275 determined, data not shown), equal or higher concentration of lecithin was used in order to
276 guarantee the formation of micelles [34,35]. In Table 2 the operating conditions of the
277 experiments performed and the results obtained are displayed.

278 (TABLE 2)

279 The experiments started with the determination of the organic-water ratio and lecithin's
280 concentration most suitable to produce stable emulsions (E12-E15), using a concentration of
281 quercetin soluble in ethyl acetate at ambient temperature (1g/L). In fact, it was found that the
282 experiment performed with organic-water ratio and lecithin's concentration at higher values
283 (E13) led to the formation of an unstable emulsion, with separation of phases immediately
284 after its production. Overall, the emulsions produced proved to be more stable at lower
285 lecithin concentration and lower organic solvent:water ratio tested. Moreover, in experiments
286 E12-E14, aggregates of lecithin precipitated during the evaporation step, perhaps due to the
287 higher concentration of the emulsifier in these experiments. Finally, by analysing the results
288 obtained in experiments E12 – E13, it is possible to see that an increase of the lecithin's
289 concentration led to an increase of particle size and decrease of EE. The same occurred while
290 comparing the results from E14 –E15, despite the organic-water volume ratio being lower in
291 these experiments.

292 Thus, experiments from E16-E19 were performed using a concentration of lecithin fixed at
293 25g/L, further reduced organic solvent:water ratio fixed at 0.35 and variable concentrations of
294 quercetin. The emulsion's internal phase diameter was determined by turbiscan, revealing that

295 the diameter of the discontinuous phase was in general below 5µm. Moreover, none of the
296 emulsions showed the presence of quercetin's crystals, revealing a good encapsulation of this
297 flavonoid. Nevertheless, crystals of quercetin precipitated in the flask during the evaporation
298 of ethyl acetate from the emulsions E18 and E19, revealing that quercetin's concentration of
299 7.5 and 10g/L, respectively, may be too high for its complete dissolution in the hot, pressurized
300 organic solvent in T-mixer M1. The presence of crystals in these experiments were confirmed
301 by optical microscopy. Microscope pictures of the emulsions and suspensions can be seen in
302 figure 2.

303 (FIGURE 2)

304 The final particle of the quercetin-loaded micelles was in the nanometer range. Regarding
305 encapsulation efficiency, almost 76% was achieved for experiment E16, where the final
306 quercetin's concentration in the aqueous suspension was 260ppm. Experiments E17, E18 and
307 E19 show lower EE, probably due to the higher initial concentration of quercetin used, yet
308 showing higher final quercetin's concentration (E19 shows a concentration of 630ppm). In
309 Figure 3 it is possible to verify that the EE decreased as the initial concentration of quercetin
310 used increased. However, final quercetin's concentration in the suspension increased as the
311 initial concentration of quercetin used increased, though this increase was first proportional,
312 yet reaching a plateau at 630ppm. This value could represent a saturation limit point, where
313 no more quercetin could be solubilised in the lecithin-aqueous suspension.

314 (FIGURE 3)

315 Lecithin showed to be a good emulsifier for the encapsulation of quercetin. In fact, the ability
316 of these two compounds to form chain-like structures linked by hydrogen bonds has been
317 already reported and demonstrated with nuclear magnetic resonance spectroscopy [36]. These
318 structures were called phenolipids and, unlike liposomes, they result from the reaction of
319 phospholipids with selected phenolic compounds. Furthermore, there seems to exist a

320 synergism between lecithin and quercetin due to the formation of these bonds, resulting in an
321 increased antioxidant potential [37,38].

322 In Fig.4 it is possible to see the Cryo-TEM pictures taken to the suspension resulting from
323 experiment E16.

324 (FIGURE 4)

325 By observing the Cryo-TEM pictures of lecithin's water suspension (Fig 4. a) it is possible to see
326 multivesicular and multilamellar vesicular formulations of the emulsifier with particle size
327 around 100nm [34]. Nevertheless, regarding the quercetin aqueous suspensions, it is possible
328 to verify the presence of small vesicles around 100nm with dark double layers (Fig. 4 b) and c))
329 probably due to presence of quercetin in this area. Moreover, in Fig.4 d), it is possible to
330 observe some larger vesicles with beehive-like structure, probably resulting from the
331 formation of quercetin-enriched lecithin structures linked by hydrogen bonds.

332 Fourier transform infrared spectroscopy measurements were performed in order to study the
333 possible formation of a complex or chemical association between quercetin and lecithin, since
334 such interaction bonds can be detected by this technique [9]. In figure 5, the FTIR spectrum of
335 unprocessed quercetin hydrate presents its characteristic bands, like the broad band at
336 3500−3000 cm−1, assigned to a free -OH bond vibration, bands at 1660 and 1600 cm−1,
337 assigned to the stretching vibration of the C=O group, the band at 1515 cm−1, assigned to
338 aromatic groups, the bands at 1310 and 1160 cm−1, assigned to the C-O-C vibration, and
339 finally the band at 1010 cm−1, assigned to aromatic C-H groups [9,39].

340 (FIGURE 5)

341 By analyzing the FTIR spectra of the encapsulated quercetin (E16) it is possible to see some
342 complexation between lecithin and quercetin, since it presents different band shapes observed
343 between 1,500 and 1,660 cm^{-1} when compared with unprocessed quercetin and physical

344 mixture spectra. It must be noticed that quercetin's crystals show a characteristic carbonyl
345 absorption band around 1600 cm^{-1}, assigned to aromatic ketonic carbonyl stretching [10,40],
346 which is almost no detectable in the spectrum of encapsulated quercetin, revealing a good
347 encapsulation of this flavonoid.

348 *3.3. β-Glucan*

349 The encapsulation of quercetin through the use of β-Glucan as emulsifier was also performed
350 in this work.

351 Since this emulsifier has a critical micelle concentration around 2g/L (experimentally
352 determined, data not shown), higher concentration of β-Glucan (15g/L) was used in order to
353 guarantee the formation of micelles. Moreover, β-Glucan has low oral bioavailability, due to its
354 poor absorption, and its complexation with lecithin was suggested to overcome this
355 undesirable characteristic [41]. For that purpose, lecithin was also used in some experiments,
356 with a mass ratio between β-Glucan and Lecithin varied according to Veverka et al [41].
357 Quercetin's concentration of 1g/L was chosen for these experiments. In Table 3 it is displayed
358 the operating conditions of the experiments performed and the results obtained.

359 (TABLE 3)

360 Emulsions from experiments performed with mixtures of surfactants, E21-E23, presented
361 some carrier's agglomerates. Nevertheless, emulsion from experiment E20 and stabilized just
362 with β-glucan, did not show these aggregates. It seems that mixtures of β-glucan and lecithin
363 form complexes with tendency to precipitate without improving the stability of the emulsions.
364 In fact, in experiments performed with both emulsifiers, the precipitation of the lecithin:β-
365 glucans increased during the evaporation step. Nevertheless, none of the emulsions showed
366 the presence of quercetin's crystals, being the diameter of the discontinuous phase in general
367 below 4μm. According to turbiscan measurements, the sample that contained higher
368 concentration of lecithin, E23, showed higher diameter of organic solvent droplets. Still, it is

369 important to remember that turbiscan only provides mean particle size value but not the
370 dispersion of this value, hence the differences between turbiscan's values and optical
371 microscope images.
372 Regarding experiment E20, crystals of quercetin precipitated in the flask during the
373 evaporation of the emulsions, which was confirmed by optical microscopy. β-Glucan did not
374 seem to be a good carrier/emulsifier to encapsulate this flavonoid, and so, higher
375 concentrations of quercetin were not tested. The final particle of the quercetin-loaded
376 micelles was in the micrometer range. In the case of E20, the presence of quercetin's crystals
377 contributed for the micrometer size, whereas in the samples E21-E23 the presence of carrier's
378 agglomerates contributed to this particle size. Comparing E20 and E22, it is possible to see that
379 the addition of lecithin increased the particle size due to the formation of lecithin:β-glucan
380 complexes [41]. Nevertheless, the addition of more lecithin (E21 and E23) led to a reduction of
381 particle size, probably due to the formation of higher amount of small sized quercetin
382 encapsulated particles.
383 Regarding the encapsulation efficiency, it is possible to verify that comparing E20 and E22, the
384 addition of lecithin to the system led to a decrease of EE. The addition of lecithin led to the
385 complexation of the carriers, and their further precipitation. Perhaps there is a competition
386 between quercetin and lecithin for the complexation with β-glucan, resulting in less EE in E22
387 comparing with E20.
388 Nevertheless, as the amount of the lecithin increased in β-glucan:lecithin mixtures
389 (E22<E21<E23), so as increased the EE of the system. These values are in agreement with the
390 reduction of particle size in these samples, as discussed previously. Perhaps in experiment E23
391 there is a higher portion of free lecithin, not complexed with β-glucan, to interact with
392 quercetin, increasing its encapsulation. This fact reinforces the capacity of lecithin to
393 encapsulate quercetin

394 *3.4. Stability*

395 Experiment E17 was repeated with and without the inclusion of glycerol in order to evaluate
396 and compare their stability. For that purpose, the micellar particle size, presence of crystals,
397 quercetin's concentration and antioxidant activity of the resulting suspensions were measured
398 after 75 days of storage. During this period, the aqueous solutions were stored in a
399 refrigerated room at temperatures around 10ºC and protected from light. The refrigeration
400 condition was chosen for the stability study since it is recommended as the storage condition
401 for the phospholipids [42]. Results are reported in Table 4.

402 (TABLE 4)

403 As presented in this table, the sample prepared with glycerol as co-solvent maintained
404 colloidal stability at the end of one month and a half, with no significant change in its particle
405 size, indicating good stability during this period. In contrast, the sample without glycerol nearly
406 tripled its size after the same period, being this value even higher after two months and a half
407 after its preparation.

408 (FIGURE 6)

409 Evaluating the shape of the size distribution, shown in Fig. 6, the distribution of the sample
410 freshly prepared have main peak in the nanometers range, corresponding to quercetin
411 particles that were successfully encapsulated, and other peaks at sizes above 1 µm possibly
412 corresponding to quercetin's crystals and lecithin's agglomerates. During storage, the peaks
413 above 1 µm are displaced to considerably higher particle sizes, indicating that these bigger
414 particles are becoming more agglomerated and further destabilizing the suspension due to
415 their higher size. The addition of a co-solvent is essential for an adequate stability of the
416 formulation, avoiding not only the agglomeration of lecithin, but also delaying the appearance
417 of quercetin's crystals. In fact, in the sample prepared without glycerol, the appearance of
418 flavonoid's crystals was observed shortly after fifteen days of preparation. Regarding
419 encapsulation efficiency, the addition of a co-solvent appeared to slightly enhance the

420 solubility of quercetin in aqueous media. Nevertheless, the stability of quercetin over storage
421 was similar in the two tested samples.

422 Table 4 also shows the results achieved by ORAC antioxidant assay for the two suspensions
423 over storage. The results are expressed as micromoles of Trolox equivalents per gram of
424 quercetin. Antioxidant activity of pure quercetin are lecithin was also measured in this work,
425 revealing values of 6586 and 2.5 µmol of TE/g of product, respectively. The probable formation
426 of hydrogen bonds between lecithin and quercetin resulted in a 3-fold increased antioxidant
427 activity per unit mass of quercetin for both suspensions, being these values nearly preserved
428 after 75 days of storage.

429 **4. Conclusion**

430 The formulation of quercetin using three distinct emulsifiers, namely n-octenyl succinate
431 anhydride starch, soybean lecithin and barley β-glucan, was investigated in this work through
432 pressurized ethyl acetate-in-water emulsions. Results showed that OSA-starch was not suitable
433 for the encapsulation of quercetin due to the low encapsulating efficiencies achieved (below
434 10%) and micellar particle sizes in the range of micrometers. Experiments carried out with
435 lecithin yielded the best results, with encapsulation efficiencies up to near 76% and particle
436 sizes in the sub-micrometer range. Moreover, the aqueous suspensions of quercetin stabilized
437 with this surfactant presented concentrations of the flavonoid up to 630ppm, being 315-
438 fold higher compared with solubility of pure quercetin in water. The formation of
439 hydrogen bonds between lecithin and quercetin could explain the good results obtained
440 with this emulsifier, further comprising improved antioxidant activity (3-fold higher per
441 unit mass of quercetin) due to a synergistic effect. Furthermore, the addition of lecithin to
442 the emulsions stabilized with β-glucan improved the encapsulation efficiency and particle
443 size of the micelles, reinforcing the capacity of lecithin to encapsulate quercetin.

444 The results obtained in this work showed that high pressure emulsion technique is a versatile
445 method for the production of water soluble formulations of quercetin, avoiding quercetin's
446 degradation and increasing its solubility. Furthermore, the addition of glycerol as co-solvent
447 can increase the colloidal stability of the suspension and the encapsulation efficiency of
448 the flavonoid.

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