BARLEY AND YEAST β-GLUCANS AS NEW EMULSIFIER AGENTS FOR THE DEVELOPMENT OF AQUEOUS NATURAL ANTIFUNGAL FORMULATIONS

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ABSTRACT

Barley and yeast β-glucans were selected, together with lecithin, to encapsulate resveratrol by emulsification-evaporation method to develop new and safer antifungal formulations. Different emulsification techniques were used: high-shear, high pressure and high pressure and temperature emulsification. Morphology, crystallinity, encapsulation efficiency and *in vitro* antifungal activity against *Botrytis cinerea* of the different formulations were evaluated. No significant differences between each emulsification procedure in particle size (below 90 nm) and in encapsulation efficiency (70-100%) were observed; only barley β-glucan emulsions showed lower efficiency due to the formation of a gel that retained most of the active compound. A great influence of the emulsification method and the encapsulating material on the crystallinity of the particles was observed. The highest antifungal activity (up to 53% growth inhibition) was obtained by the formulations with yeast β-glucans, indicating an enhanced absorption of encapsulated resveratrol through the cell wall of the fungus at the presence of (1-3, 1-6)-β-glucans.

Keywords: β-glucan, lecithin, resveratrol, encapsulation, high-pressure emulsification, *Botrytis cinerea*.

25 **1. Introduction**

26 Fungal and bacterial infections are a major concern in agriculture since they produce great losses
27 (Spadaro & Gullino, 2004). On one hand, many fruits and vegetables must be discarded because
28 they cannot be recovered once they are affected, raising an ethical issue due to the increasing
29 global demand of food derived from a bigger population (Bebber & Gurr, 2015). On the other
30 hand, producers must carry out a big investment in order to keep infections under control and
31 to minimize the damages on their crops. Therefore, it is important to develop effective products
32 which reduce the growth of pathogens and preserve the quality of food. For many years, several
33 chemical products have been used with this purpose. However, they can leave toxic residues on
34 the plant, which afterwards could affect human health and contaminate the environment (Cruz
35 Cabral, Fernández Pinto, & Patriarca, 2013). Furthermore, fungi and bacteria can develop
36 resistant strains after prolonged application (Panebianco et al., 2015). Thus, in recent years
37 there is a tendency towards the development of alternative antifungal and antibacterial
38 products from non-toxic, natural origin substances (Mari, Bertolini, & Pratella, 2003).

39 In this sense, essential oils have been extensively studied because of their antifungal properties
40 (Soylu, Kurt, & Soylu, 2010; Stević et al., 2014). The biocide activity of essential oils is mainly due
41 to their content in polyphenolic compounds that increases cell membrane permeability and
42 promotes its disruption (Tiwari et al., 2009); they also trigger defense mechanisms in the
43 infected plant: produce alkalinization of the medium, stimulate oxidative burst and induce
44 defense genes (Chang, Heene, Qiao, & Nick, 2011). Therefore, many works analyze plant
45 extracts, focusing on their phenolic content and their activity against different fungi, like
46 *Aspergillus, Penicillium, Botrytis* or *Candida* (Gatto et al., 2011; Martins, Barros, Henriques, Silva,
47 & Ferreira, 2015; Rashed, Ćirić, Glamočlija, & Soković, 2014).

48 The aim of this work is to develop a liquid antifungal formulation with a model phenolic
49 compound, resveratrol, from an oil-in-water emulsion and removal of the organic solvent.

50 Resveratrol is included among phytoalexins, secondary metabolites that are synthesized by
51 plants as protective agents against bacterial and fungal attacks (Adrian & Jeandet, 2012).
52 Resveratrol is present in many plants, although grapes have the highest concentration
53 (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012). Antifungal activity of
54 resveratrol has been previously reported (Jung et al., 2005), also against *Botrytis cinerea* (Adrian,
55 Jeandet, Veneau, Weston, & Bessis, 1997), which is a widely extended fungus that affects several
56 fruits and plants in postharvest stage, causing great losses (Williamson, Tudzynski, Tudzynski, &
57 Van Kan, 2007). Besides, structural changes in conidia upon resveratrol application were
58 observed (disrupted plasma membrane, disorganized cytoplasm withdrawn from conidial wall),
59 which could explain the mode of action of resveratrol leading to cell death (Adrian & Jeandet,
60 2012). However, fungal laccases produce extracellular oxidation of resveratrol and its
61 degradation (Chang et al., 2011; Rivera-Hoyos et al., 2013; Timperio, D'Alessandro, Fagioni,
62 Magro, & Zolla, 2012). Therefore, a proper formulation is required in order to encapsulate
63 resveratrol and protect it from laccases until the target. In our previous work, solid particles of
64 resveratrol encapsulated on barley β-glucans were obtained by emulsification-evaporation and
65 further drying of the suspensions (Salgado, Rodríguez-Rojo, Alves-Santos, & Cocero, 2015).
66 These particles showed antifungal activity against *B. cinerea.* However, a liquid formulation
67 would be preferred because it is easier to apply *in situ*. Resveratrol has been previously
68 formulated as liquid suspension in different materials such as lipids, and synthetic and natural
69 polymers (proteins and polysaccharides), mainly for pharmaceutic, cosmetic or food-related
70 applications. These suspensions were created by different methods, including evaporation
71 (Pujara, Jambhrunkar, Wong, McGuckin, & Popat, 2017), antisolvent precipitation and
72 electrostatic deposition (Xulin Huang et al., 2017), ionic gelation (Jeon, Lee, & Lee, 2016), or
73 ultrasounds (Caddeo et al., 2016), among others.

74 Lipids (e.g. fatty acids, lecithin) are commonly used as surfactants because they can incorporate
75 both hydrophilic and lipophilic active compounds, are non-toxic and easily absorbed through

76 biological membranes due to the formation of liposomes, which are very similar to cell
77 membranes (Varona et al., 2013). Polysaccharides are also used because they enhance the
78 stability of the emulsion through an increase of viscosity that reduces the movement of the
79 droplets within the emulsion (García, Alfaro, Calero, & Muñoz, 2014). Among polysaccharides,
80 β-glucans, polymers of D-glucose linked by glycosidic bonds in positions (1-3), have been
81 selected because they are present in several fungi, bacteria and some kind of plants, including
82 barley or oat. Therefore, it is hypothesized that, since β-glucans are present in the cell wall of *B.*
83 *cinerea* (Tenberge, 2007), they may improve the absorption of the encapsulated active
84 compound and thus increase its effectiveness, besides acting as surfactant. β-glucans have
85 different structure depending on their origin. For instance, cereal β-glucans have (1-4)
86 branching, whereas yeast β-glucans (YBG) have (1-6) branching (Zhu, Du, & Xu, 2016). Also, the
87 variation on their structure provides them different physical and biological properties. For
88 instance, branched (1-3),(1-6)-β-glucans have stronger immune modulatory properties than (1-
89 3),(1-4)-β-glucans (Mikkelsen, Jespersen, Mehlsen, Engelsen, & Frøkiær, 2014). Moreover, many
90 works report the ability of β-glucans as elicitors in plants to induce the formation of secondary
91 metabolites and trigger defense responses (Almagro, García-Pérez, Belchí-Navarro, Sánchez-
92 Pujalte, & Pedreño, 2016; Aziz et al., 2003).

93 β-glucans have been used as encapsulating material for anthocyanins by coacervation (Xiong,
94 Melton, Easteal, & Siew, 2006), for proteins through the production of cryogels (Lazaridou,
95 Kritikopoulou, & Biliaderis, 2015) and for resveratrol by spray-drying and PGSS-drying (Salgado
96 et al., 2015). In emulsion, they have been previously used as stabilizers (Burkus & Temelli, 2000;
97 Kontogiorgos, Biliaderis, Kiosseoglou, & Doxastakis, 2004; Thammakiti, Suphantharika,
98 Phaesuwan, & Verduyn, 2004). Barley β-glucans (BBG) were found to reduce surface tension in
99 aqueous solutions, reaching a constant value of 50-55 mN/m at 2 g/L (unpublished work).
100 However, to the authors' knowledge, there is just one work in which they were used as
101 surfactant of emulsions, but it was found out that the active compound (quercetin) precipitated

102 in crystals inside the polymer, so to avoid this, lecithin was added as co-surfactant (Gonçalves et
103 al., 2015).

104 In this work, β-glucans were used as encapsulating material for the production of liquid
105 formulations of resveratrol by emulsification-evaporation. Also lecithin was used, both alone
106 and mixed with the β-glucans, in order to enhance the formation of the emulsion. Different
107 emulsion techniques were used: high-shear, high pressure and high pressure and temperature
108 emulsification. The latter methods were performed because they were reported to provide
109 higher encapsulation efficiencies (de Paz, Martín, Mateos, & Cocero, 2013). Finally, the
110 antifungal activity of the formulations with resveratrol was tested against *B. cinerea*.

111

112 **2. Materials and methods**

113 *2.1. Materials*

114 As encapsulating materials, two different types of β-glucans were used: barley (1-3, 1-4)-β-
115 glucans (75% purity; 125 kDa, determined as indicated in (Salgado et al., 2015) Glucagel, kindly
116 supplied by DKSH, France) and (1-3, 1-6)-β-glucans from yeast *Saccharomyces cerevisae* (64%
117 purity, determined as indicated in section 2.2.1. β-glucan content; L-Naturae Nutraceutical,
118 kindly supplied by Naturae, Spain). Soybean lecithin (Glama-sot, SOTYA S.A., Spain) was also
119 used as encapsulating material. Ethyl acetate (99%), malt extract agar, acetic acid glacial and
120 sodium acetate were purchased from Panreac (Spain). Resveratrol with 98% purity was
121 purchased from Pure Bulk (USA). Pullulan standards were purchased from Shodex.

122 *2.2. Solubilization and characterization of YBG*

123 BBG were soluble in water, so they could be directly dissolved in water and used in
124 emulsification. However, YBG were insoluble in water, therefore a pretreatment was required
125 in order to solubilize them. For that purpose, a 170 mL stainless steel vessel was used. Beside

126 YBG, sodium acetate (1.41 mg/g of β-glucan) and glacial acetic acid (12.2 µL/g of β-glucan) were
127 charged into the vessel at concentrations reported in (Cox, 2008), with a solid to liquid ratio of
128 1:10 (w:w). The vessel was heated with an electric external resistance and kept at 135° C and
129 0.28 MPa for 4.5 hours. Samples of the product were obtained at 3, 3.5, 4 and 4.5 h. Further
130 description of the equipment can be found in (Sánchez-Bastardo, Romero, & Alonso, 2017) .

131 *2.2.1. β-glucan content*

132 The quantity of β-glucan on the solubilized YBG was analyzed by means of the *"β-Glucan Assay*
133 *Kit (Yeast & Mushroom)"* from Megazyme (Ireland). Following the described procedure in the
134 kit, the percentage of total glucans and α-glucans was determined spectrophotometrically
135 (Shimadzu UV-2550), and the percentage of β-glucans was calculated as the difference between
136 the total and the α-glucans.

137 *2.2.2. Molecular weight of the β-glucans*

138 Molecular weight of YBG was measured by size exclusion chromatography, with a guard column
139 (Shodex SB-G), a column (Shodex SB-804 HQ, particle size 10 µm, 7.8x300 mm) and a differential
140 refractive index detector (410, Waters Corporation). The column was kept at 35º C and flow rate
141 of the mobile phase (0.1 M $NaNO_3$ and 0.02% NaN_3) was set at 0.4 mL/min. Pullulan standards
142 (5.9-708 kDa) were used.

143 *2.3. Preparation of emulsions*

144 *2.3.1. High shear emulsification*

145 An oil-in-water emulsion was formed, containing resveratrol dissolved in ethyl acetate (7.5 g/L)
146 as organic phase and the encapsulating material dissolved in the aqueous phase, previously
147 saturated with ethyl acetate. Different concentrations were tested in the aqueous phase (10, 15
148 and 20 g/L) for each of the encapsulating materials used: BBG, solubilized YBG and lecithin, and
149 mixtures of lecithin and each of the glucans (50% in weight).

150 Both liquid phases were mixed in a ratio 1:9 (v:v) at 800 rpm for 5 min and then the emulsion
151 was fed into a 200 mL rotor-stator machine (IKA LABOR PILOT 2000/4) and processed for 4
152 minutes at 4200 rpm, as described in (Salgado et al., 2015).

153 *2.3.2. Precipitation from a pressurized emulsion*

154 High pressure emulsification was also performed with the same aqueous and organic phases
155 aforementioned, at 6 MPa and ambient temperature. The aqueous solution was pressurized
156 with a Dosapro Milton Royal D (18 mL/min, 20 g surfactant/L), whereas a Jasco PU-2080 plus
157 pump was used for feeding the organic phase (2 mL/min, 7.5 g resveratrol/L). After
158 pressurization, both streams were put in contact in a T-mixer, where the emulsion was formed,
159 and afterwards the product was recovered.

160 *2.3.3. Precipitation from a hot pressurized emulsion*

161 This method of encapsulation was previously used (de Paz et al., 2012) to accelerate the mass
162 transfer kinetics to the time scales of the precipitation process, thus improving the control over
163 the precipitation. Briefly, a solution or suspension of the active compound in the organic phase
164 is put in contact with another stream of pure organic solvent at high temperature and
165 immediately afterwards this flow is mixed with the cold water phase which contains the
166 surfactant. This causes the emulsification of the organic phase and the precipitation of the active
167 compound into the polymer. The emulsion is thus formed by the combined anti-solvent and
168 cooling effect of the water, and the active compound does not undergo thermal degradation
169 during the process because due to the configuration of the equipment, the exposition time to
170 high temperature is lower than 2 seconds. A detailed explanation about the equipment used
171 can be found in (de Paz et al., 2012).

172 The operation starts with the pressurization of the 3 streams up to 6.0-6.5 MPa and heating up
173 of the organic solvent to the desired temperature (in this case, 85-90ºC). When the operating
174 conditions were reached, the feed was changed from pure water and ethyl acetate to the

175 solutions used in this work: a solution of ethyl acetate with 7.5 g resveratrol/L at 2.75 mL/min,
176 hot ethyl acetate at 4.25 mL/min and a solution of 20 g surfactant/L in water at 20 mL/min. The
177 operating conditions were selected based on a previous work in which quercetin was
178 encapsulated on starch, lecithin and β-glucans (Gonçalves et al., 2015).

179 *2.4. Formation of suspensions*

180 Suspensions were obtained for all the different emulsification methods by removing the organic
181 solvent by vacuum evaporation (Heidolph) at 60º C, 75 rpm and a vacuum of 0.08 MPa.
182 Afterwards, big agglomerates of polymer and non-encapsulated crystals of resveratrol were
183 removed by centrifugation at 7800 rpm (6461 g) for 10 min.

184 *2.5. Characterization of emulsions and suspensions*

185 *2.5.1. Droplet size distribution*

186 Droplet and particle size distribution of the emulsions and suspensions was measured by laser
187 diffraction (Mastersizer 2000, Malvern) by diluting the emulsions on a solution of water
188 saturated with ethyl acetate and the suspensions only in water. Particle size was measured both
189 before and after centrifugation of the suspensions. Results are expressed as % in number. The
190 droplet and particle sizes reported correspond to the value of d(0.5).

191 *2.5.2. CryoTEM*

192 CryoTEM analysis of the aqueous solutions of the different β-glucans and their mixtures with
193 lecithin were performed to check how their chains associated themselves, in a JEOL JEM-FS2200
194 HRP 200 kV TEM with electron filtering. In brief, 4 µL of sample was deposited on a rack C-Flat
195 1.2/1.3, which was previously hydrophilized by a plasma cleaner. A blotting is performed on
196 either side of the filter grid during 4 s and then liquid ethane is introduced there in order to
197 freeze the samples, avoiding the formation of crystals (Gatan Cryoplunge 3). Samples are
198 maintained in liquid nitrogen until their transfer to the holder (Gatan Cryotransfer 626).

199 *2.5.3. X-ray diffraction*

200 X-ray diffraction (XRD) measurements were performed on a Bruker Discover D8 diffractometer
201 to check the crystallinity of the particles in the final suspensions, using the Cu Kα radiation (λ =
202 0.15406 nm). The suspensions were further centrifuged at 20000 rpm for 30 min (Beckman) in
203 order to recover the encapsulated particles. Afterwards, this sediment was dried in a freeze-
204 drier (LyoQuest -55, Telstar) for 48 hours and the final powder was analyzed by XRD. The
205 scattering intensities were measured over an angular range of $5 < 2\theta < 70$ for all the samples,
206 with a step size of 0.02°.

207 *2.5.4. Encapsulation efficiency*

208 Quantification of encapsulated resveratrol was done by HPLC with a guard column (Bio-Sil C18
209 HL 90-5, 4.6 x 30 mm, particle size 5 µm, Bio-Rad), a column (Symmetry C18, 4.6 x 150 mm,
210 particle size 5 µm, Waters) and a UV detector (λ = 306 nm). The column was kept at 25º C and
211 flow rate of the mobile phase (acetonitrile and water in ratio 1:3, with 0.2% formic acid) was set
212 at 0.8 mL/min. Encapsulation efficiency was determined as the ratio between the quantity of
213 resveratrol in the centrifuged suspension and the amount of resveratrol in the initial emulsion.
214 Calibration range of resveratrol was between 10 and 40 ppm. Prior to analysis, the centrifuged
215 suspensions were diluted (0.6 mL of sample in 10 mL) and filtered (0.22 µm). The analysis was
216 performed in triplicate, and the data were analyzed by t-Student´s test (unpaired samples,
217 unequal variances) with a significance p-value of 0.05.

218 *2.6. B. cinerea culture*

219 For the *in vitro* culture of *B. cinerea*, the fungus was isolated from vines in our university (Campus
220 La Yutera, Universidad de Valladolid, Palencia, Spain) and it was grown on malt extract agar.
221 After autoclaving, the agar was poured into Petri dishes containing the corresponding sample or
222 control, and when it solidified, the fungus was placed in the center of the surface. The quantity
223 of sample on each plate was determined so as to have a concentration of resveratrol of 100

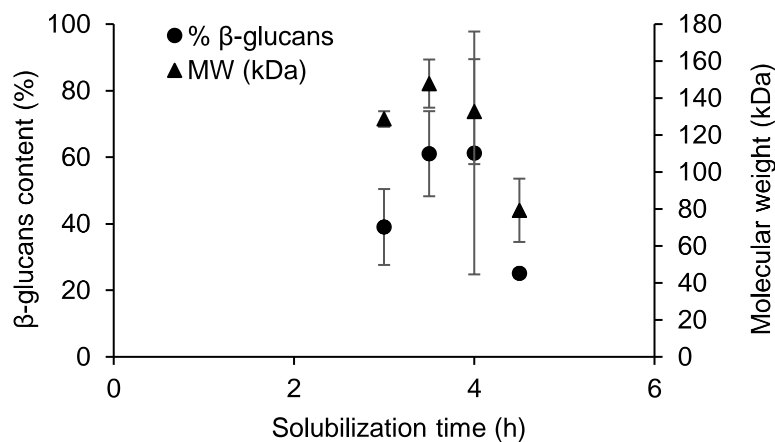
224 mg/L, which was reported to be within the range of growth inhibition for *B. cinerea* in previous
225 works (Adrian et al., 1997). Five replicates were grown for every test and the growth area was
226 calculated by measuring the diameter in two perpendicular directions after incubation at 22º C
227 for one week. Once measured, the average value and standard deviation were evaluated, and
228 Student's t-test was performed as mentioned before in order to check whether there were
229 significant differences between each sample.

230

231 **3. Results and discussion**

232 *3.1. Solubilization and characterization of (1-3;1-6)-β-glucans*

233 The results of the molecular weight and the quantity of β-glucans in the samples at increasing
234 solubilization time are shown in Figure 1. It can be noticed that both parameters have the same
235 trend: they increase with time, showing a maximum at 3.5 h, and they decrease again with
236 longer times.



237

238 **Figure 1.** Evolution of β-glucan content and their molecular weight with the time of
239 solubilization under acidic treatment at 135°C and 0.28 MPa.

240

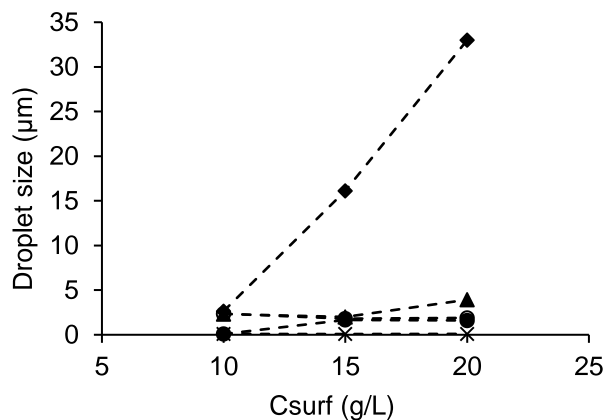
241 This behavior is due to the degradation of the product. Initially, the yield of dissolution increases,
242 but after some time in the acidic solution, the β-glucans are broken into smaller compounds,

243 which have lower molecular weight, and the presence of β-glucans is reduced. In order to have
244 a better quality product (higher purity of β-glucans and less degradation) the time for dissolution
245 of the YBG is 3.5 h.

246 *3.2. Characterization of the emulsions and suspensions*

247 *3.2.1. Droplet and particle size*

248 The influence of the type and concentration of surfactant was evaluated by high-shear
249 emulsification. Unimodal droplet size distributions were obtained in all cases, with narrow peaks
250 (results not shown here). Regarding average droplet size, there were significant variations
251 depending on the surfactant used and its concentration (Figure 2).

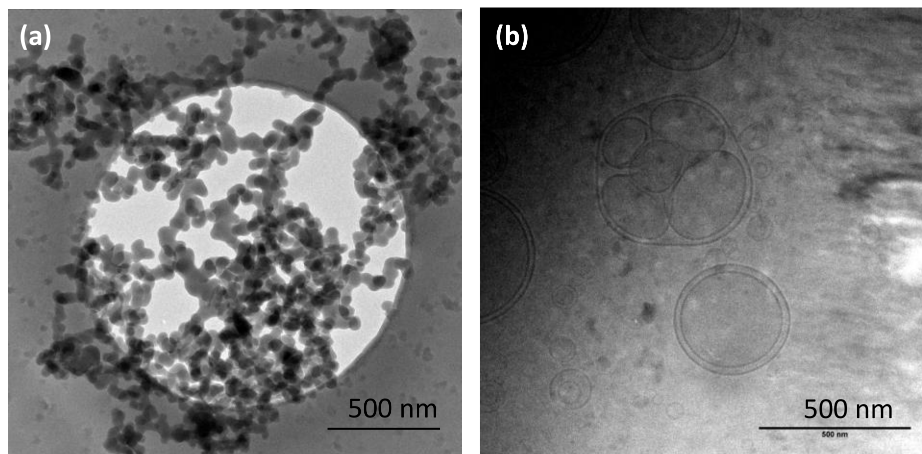


252

253 **Figure 2.** Average droplet size of the emulsions by high-shear emulsification with different
254 encapsulating materials at a range of surfactant concentration. ● lecithin; ▲ lecithin and
255 barley β-glucan; ◆ barley β-glucan; X lecithin and yeast β-glucan; ○ yeast β-glucan
256

257 The emulsions with lecithin had small droplet size in the concentration range tested, although it
258 increased with concentration from 90 nm to 1.6 µm. Also with YBG small droplets were achieved,
259 between 1.8 and 2.4 µm. However, with BBG, the higher the concentration of glucan, the greater
260 the droplet size, up to 33 µm. Some authors suggest that an increase on the viscosity of the
261 aqueous solution worsens the homogenization process, thus leading to the formation of bigger
262 droplets (X. Huang, Kakuda, & Cui, 2001). By cryo-TEM it was observed that BBG in solution tend
263 to form a network instead of single droplets (Figure 3a), which could explain the increase in

264 droplet size. This structure was not observed with YBG (not shown), and therefore smaller
265 droplet sizes were obtained in the emulsions. When lecithin was mixed with BBG, droplet size
266 was greatly reduced in comparison with the emulsions that had only β-glucans. In these cases,
267 the behavior was similar to that of pure lecithin, thus improving the emulsification properties of
268 the BBG. Also, the network of polymer obtained with BBG was not noticed if lecithin was added
269 to BBG (Figure 3b).

270 

271 **Figure 3.** Cryo-TEM images of aqueous solutions of BBG (a) and a mix of BBG and lecithin (b)
272

273 The same trend was observed for the emulsions produced by high pressure and high-pressure
274 and temperature emulsification: particle size for the experiments with BBG was much bigger
275 than with all the other encapsulating materials, but when they were mixed with lecithin, it was
276 greatly reduced (results not shown).

277 After centrifugation and removal of big agglomerations of polymer and crystals of non-
278 encapsulated resveratrol, all of the suspensions had narrow particle size distribution, with
279 average particle size between 60 and 90 nm, regardless of the emulsification process and the
280 encapsulating material (Table 1). These values are in the range of those obtained for resveratrol
281 suspensions by different encapsulation methods such as formation of liposomes using
282 ultrasounds (Caddeo et al., 2016) or microencapsulation in synthetic polymers by vortex mixing
283 (Tsai et al., 2016).

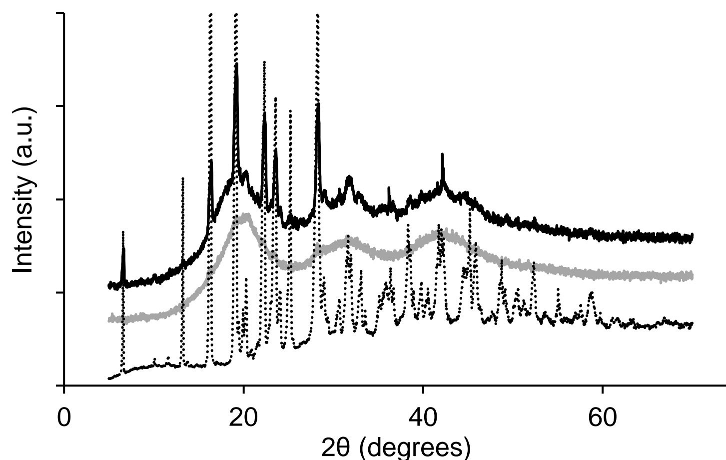
284 **Table 1** Particle size and encapsulation efficiency in the final centrifuged suspensions for the
285 different emulsification methods (HS: high shear; HP: high pressure; HPT: high pressure and
286 temperature) and encapsulating materials at 20 g/L.

Surfactant	Emulsification method	Particle size (nm)	Encapsulation efficiency (%)
Lec	HS	69	84 ± 11
	HP	88	100 ± 8
	HPT	75	78 ± 9
BBG	HS	63	31 ± 9
	HP	65	54 ± 7
	HPT	63	39 ± 2
Lec-BBG	HS	84	91 ± 11
	HP	62	79 ± 17
	HPT	64	66 ± 9
YBG	HS	65	84 ± 16
	HP	64	74 ± 10
	HPT	64	79 ± 7
Lec-YBG	HS	62	93 ± 14
	HP	61	79 ± 5
	HPT	66	71 ± 5

287

288 *3.2.2. Crystallinity of encapsulated resveratrol*

289 The crystallinity of the particles in the suspensions was analyzed by means of XRD. Different
290 results were obtained depending on the encapsulating material and the emulsification method.
291 Regarding the emulsification methods, a complete amorphous structure was obtained for all the
292 experiments by HPT, whereas with the 2 other methods the main characteristic peaks of pristine
293 resveratrol were noticed with some of the encapsulating materials. As an example, Figure 4
294 shows the XRD pattern for the particles of the suspension of resveratrol in lecithin by high-
295 pressure emulsification (black line) and high pressure and temperature emulsification (gray line),
296 compared to pure resveratrol (dots).



297

298 **Figure 4.** XRD patterns for pure resveratrol (dots) and for the suspension of resveratrol
299 encapsulated in lecithin by high-pressure (black) and by high-pressure and temperature
300 emulsification (gray).

301

302 According to these observations, the application of pressure to the emulsification does not
303 influence the crystallinity of the final particles at ambient temperature. However, by subjecting
304 the organic phase containing the active compound to a heating step followed by a sudden
305 cooling, the active compound has not time to form crystals and grow. Instead, it precipitates in
306 amorphous phase inside the encapsulating material.

307 Focusing on the different encapsulating materials, in the experiments with BBG, both alone and
308 mixed with lecithin, no peaks corresponding to crystals of resveratrol were noticed, indicating
309 that there was not presence of non-encapsulated crystals of resveratrol and that the
310 encapsulated resveratrol was on amorphous phase inside the particles. Among the other
311 encapsulating materials, lecithin showed the highest intensity of crystalline resveratrol peaks,
312 meaning worse encapsulation capacity for resveratrol. However, when it was mixed with any of
313 the β-glucans, the crystallinity of the particles was reduced (Figure 5).

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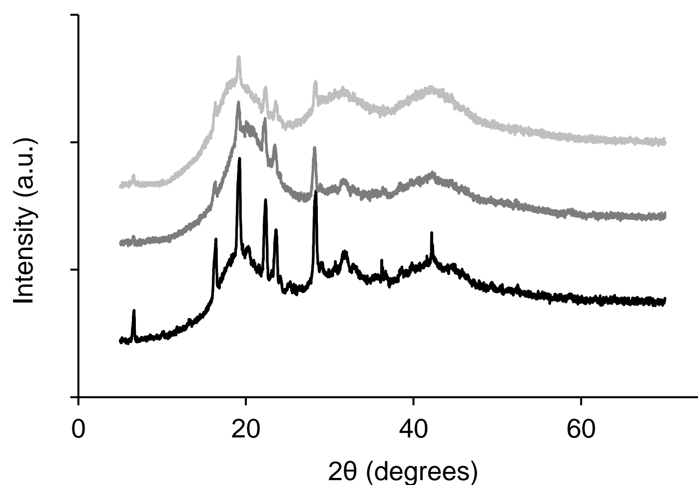


315 **Figure 5.** XRD patterns for the suspensions of resveratrol obtained by high-pressure with
316 lecithin (black), yeast β-glucan (light gray) and a mixture of lecithin and yeast β-glucan (dark
317 gray)

318

319 Thus, an interaction between β-glucans and resveratrol was found, which diminishes the
320 formation of crystals during emulsification, although this effect was mitigated when lecithin was
321 also applied. This complexation was previously noticed by (J. Wu, Deng, Tian, Wang, & Xie, 2008)
322 between calcofluor and oat β-glucan, through the formation of hydrogen bonding between the
323 hydroxyl groups of both compounds and afterwards Van der Waals interactions. Also (Z. Wu et
324 al., 2011) reported the formation of hydrogen bonding between oat β-glucan and tea
325 polyphenols composed mainly of (-)-epigallocatechin gallate, (-)-gallocatechin gallate and (-)-
326 epicatechin gallate.

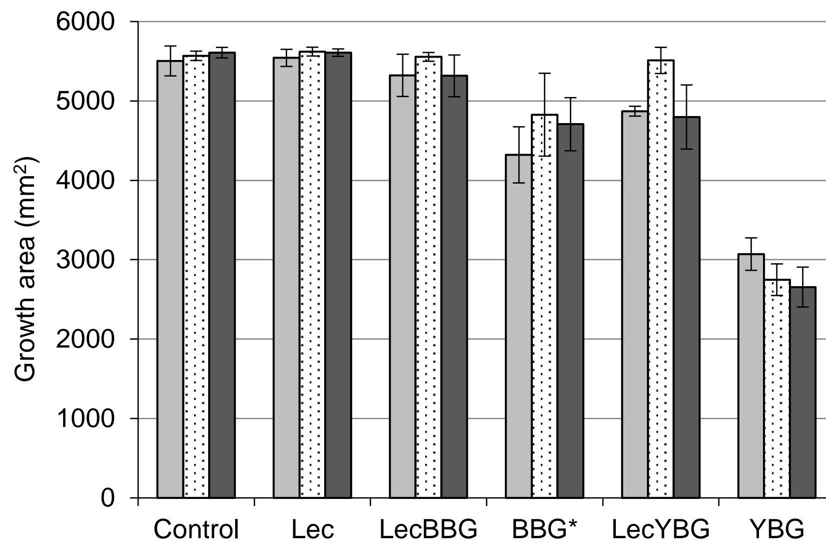
327 *3.2.3 Encapsulation efficiency*

328 Results of encapsulation efficiency for the suspensions at 20 g/L of encapsulating material are
329 shown on Table 1. In all cases, the concentration of resveratrol in the suspensions ranged from
330 550 to 800 ppm, except with BBG, that had between 240 and 440 ppm of resveratrol. This
331 supposes a concentration up to 20 times higher than the solubility of resveratrol in water (Filip
332 et al., 2003), an increase higher than those obtained for suspensions of resveratrol in some other
333 works (Pujara et al., 2017; Shi et al., 2008).

334 Encapsulation efficiency was much lower with BBG because during centrifugation a gel-like
335 structure was formed and it retained most of the compounds. No significant differences were
336 observed between each emulsification method with any of the encapsulating materials. In some
337 previous works much higher encapsulation efficiencies were achieved by high pressure and
338 temperature emulsification (80%) than by high-shear emulsification (8%) in the formulation of
339 β-carotene with modified starch (de Paz et al., 2013). However, in that case the increase was
340 due to the higher solubility of the active compound in the hot organic solvent, whereas in this
341 work the concentration of resveratrol in the organic phase was kept constant in all the
342 experiments.

343 *3.3. B. cinerea culture*

344 For the samples by high-shear emulsification, no significant differences were observed between
345 each product at the lowest concentrations of surfactant (10 and 15 g/L), since none of them had
346 effect on the fungal growth (results not shown). However, some inhibition was achieved with
347 the suspensions at 20 g/L of encapsulating material, also for the samples by high-pressure and
348 high-pressure and temperature emulsification. Figure 6 presents fungal growth area when
349 applying the suspensions of resveratrol formulated with lecithin (Lec), a mixture of lecithin and
350 BBG (LecBBG), BBG, a mixture of lecithin and YBG (LecYBG) and YBG. The results shown here
351 correspond to the centrifuged suspensions except in the case of BBG. These had much lower
352 encapsulation efficiency, so higher quantity of sample was required to perform the analysis and
353 therefore it interfered in the growth of the fungus due to the dilution of the agar. Therefore, the
354 bars named BBG in Figure 6 correspond to the suspensions without centrifugation.



355

356 **Figure 6.** Growth area of *B. cinerea* for the centrifuged suspensions of resveratrol. Light gray:
357 high-shear emulsification. Dots: high pressure emulsification. Dark gray: high pressure and
358 temperature emulsification. *: suspensions without centrifugation. Significantly different
359 results are considered for $p<0.05$.

360

361 The formulation of resveratrol with lecithin and with the mixture of lecithin and BBG had no
362 effect on fungal growth, although some inhibition was achieved with BBG without mixing (up to
363 23%). The greatest growth reduction corresponded to the suspensions of YBG, ranging the
364 inhibition between 44 and 53%. However, this effect was almost unnoticeable for the mixture
365 of YBG with lecithin: no inhibition was observed by high pressure emulsification, and up to 15%
366 for the other methods. Except in this case, no significant difference was noticed between each
367 emulsification method regardless of the encapsulating material. Pure resveratrol did not
368 produce any inhibition of fungal growth.

369 According to these results, the presence of β-glucans in the formulation enhances the fungicide
370 action of resveratrol with respect to the formulation with lecithin. Varona et al., 2013 studied
371 the penetration of liposomes formed by lecithin into cell membranes of different bacteria. In
372 that work, liposomes showed ability to cross cell walls when they are constituted by
373 phospholipid layers, as happens in gram-negative bacteria. However, they could not cross into
374 the cell when there were some other barriers, as in the case of the gram-positive bacteria. *B.*

375 *cinerea* has a two-layer cell wall composed of chitin and β-glucans (Tenberge, 2007). It is possible
376 that liposomes could not penetrate into that barrier and deliver resveratrol inside, and thus the
377 formulations with lecithin had no antifungal effect. On the contrary, resveratrol is better
378 absorbed within the cell wall of the fungus when it is encapsulated in β-glucans, especially YBG,
379 which contains the same β-glucan type as the fungal cell wall (Zhu et al., 2016), so the inhibitory
380 effect on fungal growth is bigger. Nevertheless, the inhibitory effect achieved with the liquid
381 formulations was not comparable to the one obtained with solid particles of the same products
382 reported in a previous work (Salgado et al., 2015). Some studies reported an elicitor effect of β-
383 glucans on the production of resveratrol by some plants, like *Vitis vinifera* (Vuong, Franco, &
384 Zhang, 2014). Thus, it is possible that when the formulations with β-glucans are applied *in vivo*,
385 the concentration of resveratrol increases, and this could cause a greater fungal growth
386 inhibition.

387

388 **4. Conclusions**

389 A liquid formulation of resveratrol was developed and tested against *B. cinerea*, by three
390 different emulsification methods: high-shear, high-pressure and high pressure and temperature
391 emulsification. As encapsulating materials, BBG and YBG were used, both alone and mixed with
392 lecithin. YBG showed good encapsulating properties regarding encapsulation efficiency (74-
393 84%), besides providing 50% inhibition of fungal growth, the best for all the products tested. On
394 the contrary, BBG were not as good as encapsulating material because their chains entangle and
395 form a net instead of single droplets, thus having low encapsulation efficiency (31-54%). Also
396 they showed lower antifungal activity (around 20% inhibition). The formulation with lecithin also
397 showed high encapsulation efficiency and small particle size, although it did not inhibit fungal
398 growth. When mixing β-glucans with lecithin, a reduction on particle size was observed,
399 although the mixing also induced the formation of crystals of resveratrol inside the encapsulated

400 particles in comparison with β-glucans alone. Therefore, it was concluded that the formulation
401 of resveratrol with β-glucans improved the action against *B. cinerea*, probably through an
402 enhanced absorption of the active compound by the fungus. Comparing the emulsification
403 methods developed in this work, there were not big differences between them on encapsulation
404 efficiency and particle size. Nevertheless, by high pressure and temperature emulsification,
405 amorphous resveratrol was obtained inside the particles, whereas it formed crystals with the
406 other methods. Despite this, the effect on the reduction of fungal growth was similar by all the
407 emulsification methods for each material, without significant differences between them. As β-
408 glucans appear as a promising effective encapsulating material to improve the antifungal activity
409 against *B. cinerea*, the formulations could be developed with other polyphenols in future works.
410 It would be also interesting to assess if they are also effective against other fungal species.

411

412

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