

Protective effect of β -Lactoglobulin against heat induced loss of antioxidant activity of resveratrol

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Guo, Y. and Jauregi, P. (2018) Protective effect of β -Lactoglobulin against heat induced loss of antioxidant activity of resveratrol. *Food Chemistry*, 266. pp. 101-109. ISSN 0308-8146 doi: <https://doi.org/10.1016/j.foodchem.2018.05.108>
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To link to this article DOI: <http://dx.doi.org/10.1016/j.foodchem.2018.05.108>

Publisher: Elsevier

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1 Protective effect of β -Lactoglobulin against heat induced loss of antioxidant

2 activity of resveratrol

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6

7 **ABSTRACT**

8 Resveratrol exhibits many health benefits however, low water-solubility and instability to
9 processing conditions such as heating can be some of the main challenges for its processing and
10 formulation. Here the complexation of β -lactoglobulin (β -Lg) with resveratrol was investigated to
11 improve its solubility and stability. The solubility of resveratrol in water was determined as
12 7mg/100ml. Resveratrol- β -Lg nanoparticles (181.8 nm) were produced at pH 6 and 75°C for 45
13 min. Heating resveratrol solutions at 75°C for 45 min resulted in isomerization of resveratrol and
14 reduced antioxidant activity. However, resveratrol- β -Lg nanocomplexes which had undergone the
15 same heat treatment exhibited improved antioxidant activity. Heating at pasteurisation conditions
16 led to similar results and both native β -Lg and nanoparticles exhibited a protective effect against
17 heat induced chemical changes in resveratrol resulting in enhanced antioxidant activity.

18 Fluorescence measurements revealed strong interactions of resveratrol with both, native protein
19 and nanoparticles.

20 Keywords: Resveratrol; β -lactoglobulin; nanoparticles; antioxidant activity; heating

21

22 1. Introduction

23 Nowadays, consumers are attracted to dietary supplements instead of drugs to improve health.
24 ~~and~~ Oral administration is the most convenient and commonly applied method to develop new
25 nutraceuticals. Based on many studies, the predominant problem of nutraceuticals is poor
26 bioavailability due to their low aqueous solubility which, affects over 40% of the new chemical
27 entities produced by high throughput screening processes (Lipinski, 2002). Several reasons
28 contribute to the solubility problem: 1) high molecular weight; 2) high Log of the octanol-water
29 partition coefficient (log P), which is a measure of a drug's lipophilicity. In order to improve the
30 aqueous solubility, numerous researchers have developed many approaches such as particle size
31 reduction, crystal engineering, salt formation, solid dispersion and surfactant complexation
32 (Savjani, Gajjar., & Savjani, 2012). New techniques like nanoparticles, nanotubes,
33 nanosuspensions and nanocomplexes are employed to improve the solubility of poorly soluble
34 drugs and nutraceuticals (Rabinow, 2004; Tran, Tran, & Lee, 2013). Proteins like zein, whey
35 proteins, soy proteins and other natural polymers like cyclodextrins have been applied not only in
36 pharmaceutical but also in food applications (Duarte et al., 2015; Gorji et al., 2015; Pujara,
37 Jambhrunkar, Wong, McGuckin, & Popat, 2017). Simple and safe carrier candidates, which meet
38 the GRAS (Generally Recognised As Safe) qualification and possibly have essential health
39 benefits, should be investigated to improve aqueous solubility and stability of bioactives.

40

41 Resveratrol (3,5,4'-Trihydroxystilbene), is a typical non-flavonoid phenol that belongs to the
42 stilbene family and has various properties including antioxidant, anti-cancer, anti-inflammatory,
43 and antineoplastic (Bhat & Pezzuto, 2002; Jang et al., 1997; Soleas, Diamandis, & Goldberg,
44 1997). Resveratrol is thought to be one of the main contributors to the famous “French paradox”:
45 despite the French people’s high-fat intake, a low cardiovascular diseases incidence was found. It
46 has been ascribed partly to the red wine consumption (Catalgol, Batirel, Taga, & Ozer, 2012).
47 Since then, the studies of resveratrol have been carried out broadly in the food and pharmacy field.
48 There are 72 different natural resources which contain resveratrol, especially grapes, peanuts and
49 blueberries (Burns, Yokota, Ashihara, Lean, & Crozier, 2002; Jimenez-Garcia et al., 2012; Lyons
50 et al., 2003). Even though resveratrol is widely distributed in various plants and fruits, there are
51 some pharmacokinetic limitations which lead to its low bioavailability: The low water solubility,
52 labile properties, and rapid metabolism (Amri, Chaumeil, Sfar, & Charrueau, 2012; Pund, Joshi,
53 & Patravale, 2016). The low solubility of resveratrol in oral administration is due to the chemical
54 structure of resveratrol: two phenolic rings bonded together by a double styrene bond which leads
55 to two isometric forms ,cis- and trans-resveratrol (Gambini et al., 2015). It is believed that the
56 trans-isomeric form is more abundant than cis-resveratrol, while the trans-isomer is able to
57 transform into cis-isomer under light exposure and heating. Light exposure allows a rapid
58 isomerization of trans-resveratrol, especially in the ultraviolet radiation at 366nm or at low
59 concentration (Flieger, Tatarczak-Michalewska, & Blicharska, 2017; Gambini et al., 2015).
60 Heating not only leads to increased solubility but also results in degradation, isomerization and
61 reduction of antioxidant activity. The effect of heating on resveratrol can be complex depending
62 on temperature (Flieger et al., 2017; Lee et al., 2014). In order to overcome its low solubility and
63 low stability, encapsulation might be a solution. The encapsulation of resveratrol with a number

64 of biopolymers has been investigated including chitosan and γ -poly (Jeon, Lee, & Lee, 2016), soy
65 protein isolate (Pujara et al., 2017), milk protein (Gorji et al., 2015), zein (Penalva et al., 2015)
66 and β -lactoglobulin (Zhang, Liu, Subirade, Zhou, & Liang, 2014). The main whey protein, β -
67 lactoglobulin, plays an interesting role in transporting retinol molecules and binds small
68 hydrophobic molecules. Resveratrol has been found to bind to the surface of the hydrophobic
69 pocket of β -lactoglobulin (Liang & Subirade, 2010; Liang, Tajmir-Riahi, & Subirade, 2008). Thus,
70 β -lactoglobulin can be an attractive candidate as a carrier to offer a solution to both, poor solubility
71 and low stability to processing /storage conditions such as heating and light exposure.

72 In previous work of our group, β -lactoglobulin nanoparticles were found to complex with
73 caffeine (Guo, Harris, Kaur, Pastrana, & Jauregi, 2017). Higher binding affinity of
74 β -lactoglobulin in nanoparticles form rather than in the native form was found. The aim of the
75 present work was to apply this method to produce resveratrol- β -lactoglobulin nanoparticles and
76 investigate the effect of these nanoparticles and native β -lactoglobulin on the solubility and
77 stability of resveratrol under different thermal processing conditions. The main hypothesis tested
78 here was that complexation of protein (native and/or nanoparticles) with resveratrol in aqueous
79 solution can improve its solubility and/or stability. The stability was measured in terms of
80 antioxidant activity. In addition, fluorescence measurements were carried out to gain an insight
81 into the interactions between these two molecules.

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87 **2. Materials and methods**

88 **2.1. Material**

89 Trans-resveratrol (98% w/w) was supplied by Evolva RES140332 . The β -Lactoglobulin from
90 bovine milk ≥ 85.0 % (PAGE), lyophilized powder L2506 was purchased from Sigma.

91 Folin-Ciocalteu reagents: Folin-Ciocalteu (F9252), and Sodium carbonate BioXtra, $\geq 99.0\%$
92 (S7795) were purchased from Sigma.

93 Reagents of ABTS method: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium
94 salt (ABTS) ≥ 98.0 % (HPLC) (A1888), Potassium persulfate (K2S2O8) ACS reagent, $\geq 99.0\%$
95 (21622, (\pm)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 97% (238813),
96 and ethanol, $\geq 99.8\%$ (GC) (32221) were purchased from Sigma-Aldrich

97 Reagents of the ferric reducing antioxidant power (FRAP) method reagents:

98 TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (T1253); Ferric Chloride Hexahydrate(207926) were
99 purchased from Sigma-Aldrich.

100

101 **2.2 Determination of resveratrol solubility in water solution**

102 A series of resveratrol solutions/suspensions were prepared from 1mg/100ml, 3mg/100ml,
103 5mg/100ml, 7mg/100ml, 8mg/100ml, and 9mg/100ml to determine the solubility of resveratrol.

104 All the solutions/suspensions were covered by tissue paper and were stirring with a magnetic stirrer
105 at room temperature for 2 hours before any measurements. Two methods were applied to determine
106 resveratrol solubility in water solution: the direct spectrophotometric method and total phenolic
107 content by Folin-Ciocalteu method.

108 **The direct spectrophotometric method:** The λ_{\max} value of resveratrol aqueous solution
109 (2mg/100ml) was determined by using a Lambda 20 spectrophotometer (Perkin-Elmer®) and

110 scanning UV absorption from wavelength 190nm to 600nm with 1 nm spectral bandwidth. The
111 absorbance of resveratrol solutions/suspensions was measured at the maximum wavelength by the
112 Spectrophotometers (Ultrospec ® 1100 pro) using a quartz cuvette with a path length of 1cm and
113 deionized water was used as the blank. The solubility of resveratrol at room temperature was
114 determined from the plot of the absorbance against concentration as the concentration after which
115 no further significant increases were observed.

116 **Folin-Ciocalteu method:** The total phenolic content of all concentrations of resveratrol
117 solutions/suspensions were measured by Folin- Ciocalteu method at 760nm. In brief, 0.2 ml of
118 resveratrol solution was added to around 6.0ml of deionized water into a 10ml volumetric flask.
119 Folin-Ciocalteu reagent (0.5ml) was added and mixed. After 1 minute and no longer than 8
120 minutes, 1.5 ml 20% sodium carbonate solution was added and the volume adjusted to 10ml. After
121 2 hours incubation in the dark, the absorbance was recorded at 760nm using a UV-Vis
122 Spectrophotometer (Ultrospec ® 1100 pro). Deionised water was used as the blank. All the
123 experiments were carried out in triplicates, and the results were expressed in gallic acid equivalents
124 (GAE; mg/L) using a gallic acid (10-120mg/L) standard curve. The solubility of resveratrol was
125 determined as the concentration at which the GAE value reached a peak and remained constant
126 afterwards.

127 **2.3 Production of resveratrol- β -lactoglobulin nanoparticles**

128 Native β -lactoglobulin powder (0.01 g) was added into 5ml resveratrol standard solutions
129 /suspensions of varying concentrations in a 7ml Sterile container. Then the pH of the sample was
130 adjusted to 6.0 using a pH meter (Mettler Toledo, Switzerland) with 0.1M HCl and 0.1M NaOH.
131 After this, the sample was introduced into a water bath (Grant Instrument Ltd., Cambridge, United
132 Kingdom) that have been previously heated at 75 °C. The sample was kept for 45 minutes at this

133 temperature; it must be noted that the temperature of the sample reached the set temperature after
134 about 13 minutes. On completion of the incubation time, samples were transferred to an ice bath
135 for 10 minutes to terminate incubation. After 10 minutes in the ice bath, the pH of the sample was
136 measured. The particle size was determined by dynamic light scattering (DLS).

137 **2.4 The effect of heating and native β -lactoglobulin and β -lactoglobulin nanoparticle on** 138 **the stability of resveratrol**

139 After determination of solubility of resveratrol, all the following studies were carried out in a
140 range of concentrations of resveratrol at the solubility or below. A set of resveratrol aqueous
141 solutions in the range of solubility were used as a control and compared against samples of the
142 same concentration of resveratrol that underwent different treatments. There were three different
143 treatments: heating (see details below), the addition of native β -lactoglobulin (details below) and
144 β -lactoglobulin nanoparticles (see section 2.3).

145 When studying the effect of heating, each of the resveratrol solutions/suspensions were heated
146 at 75°C in a water-bath for 45 minutes and moved to an ice bath for 10 minutes afterwards. Then,
147 samples were taken for analysis. This temperature was chosen as this was the temperature at which
148 the nanoparticles were formed.

149

150 When exploring the effect of adding native β -lactoglobulin, 0.01g native β -lactoglobulin powder
151 was added to 5ml resveratrol standard solutions/suspensions of varying concentrations in a 7ml
152 Sterile container. Then samples were subjected to heat treatment.

153 The effect of heating, adding native β -lactoglobulin and β -lactoglobulin nanoparticles on the
154 stability of resveratrol were determined by total phenolic content and the total antioxidant activity.
155 For the total phenolic content, the Folin-Ciocalteu method mentioned above was applied, and when

156 measuring the samples with native β -Lactoglobulin and β -Lactoglobulin nanoparticles, the
157 interference of protein was eliminated by subtracting the GAE value of protein from the GAE
158 value of samples. All the samples were prepared and determined in duplicate.

159 For the total antioxidant activity, the [2,2'-Azinobis(3-Ethylbenzothiazoline-6-Sulphonic Acid)]
160 (ABTS) Free Radical Scavenging Activity Assay and Ferric ion Reducing Antioxidant Power
161 assay (FRAP) were used which are described in detail below.

162 **ABTS method:** The total antioxidant activity of all samples was measured by ABTS assay at
163 734nm, which was modified from Re et al. (1999). In general, the ABTS⁺⁺ stock solution was
164 prepared by mixing solution 5ml ABTS solution (7mM, 50ml volume, stored in an amber flask
165 and kept under refrigeration at 0-4°C up to one month) and 88 μ l Potassium Persulfate (K₂S₂O₈)
166 solution (140mM, 10ml volume, stored in an amber flask and dark place at room temperature up
167 to one month) together. Then the mixture was kept in the dark and room temperature for at least
168 16h prior to use. For the study of phenolic compounds, the working solution of the ABTS⁺⁺ was
169 obtained by diluting the ABTS⁺⁺ stock solution with phosphate buffered saline (PBS pH 7.4) to an
170 absorbance of 0.70 \pm 0.02 at 734nm. 20 μ l of samples was added into 2ml ABTS⁺⁺ working solution,
171 and the mixture was homogenised by 1mins vortex. The mixture was then incubated in the dark
172 for 6 minutes, and the absorbance (ABS_{sample}) was recorded at 734nm using a UV-Vis
173 Spectrophotometers (Ultrospec ® 1100 pro). The absorbance of ABTS⁺⁺ working solution was
174 measured at the same wavelength and used as control (ABS_{control}). The PBS was to blank the
175 spectrophotometer. The percentage of scavenging activity of each sample on ABTS⁺⁺ was
176 calculated as the inhibition% (I%) using the following equation (Shah & Modi, 2015): Eq.(1)

177
$$I\% = \frac{(ABS_{control} - ABS_{sample})}{ABS_{control}} \times 10 \quad \underline{\underline{(1)}}$$

178 When measuring the samples with native β -Lactoglobulin and β -Lactoglobulin nanoparticles,
179 the interference of protein was eliminated by subtracting the I% of protein from the I% of samples.
180 All the samples were prepared and determined in duplicate.

181 **FRAP method:** The total antioxidant activity of all samples was also assessed by FRAP method
182 at 595nm, which was modified from Benzie and Strain (1996). In principle, The stock solution of
183 FRAP method included: 300 mM Acetate buffer (pH 3.6, 2.699 g sodium acetate trihydrate and
184 16ml (16.8g) glacial acetic acid dissolved in 1L deionised water), 2,4,6-tripyridyl-s-triazine
185 (TPTZ) (10mM) in 10ml HCl (40mM), and 20mM Ferric Chloride Hexahydrate aqueous solution.
186 The FRAP reagent was mixed with the 25ml Acetate buffer, 2.5ml TPTZ and 2.5ml Ferric chloride
187 solutions. Then, 10 μ l of the sample/standard was added into 300 μ l FRAP reagent in a
188 microcentrifuge tube and vortexed for 10 seconds. Then 100 μ l of this mixture, in triplicates, was
189 transferred into the microwell plate (96well, NUNC, FB) and absorbance was measured at 595nm
190 by a computer controlled Tecan Microplate reader. Results were express as the ascorbic acid
191 equivalents (AAE) using an ascorbic acid (0.001761mg/ml-0.1761mg/ml) standard curve.
192 Increased absorbance of the reaction mixture indicated greater reduction capability. When
193 measuring the samples with native β -Lactoglobulin and β -Lactoglobulin nanoparticles, the
194 interference of protein was eliminated by subtracting the AAE value of protein from the AAE
195 value of samples. All the samples were prepared and determined in triplicate.

196

197 **2.5 The effect of native β -lactoglobulin and β -lactoglobulin nanoparticle on resveratrol** 198 **under pasteurisation conditions**

199 Different concentrations of resveratrol standard solutions/suspensions, resveratrol-native β -
200 lactoglobulin solutions and resveratrol- β -lactoglobulin nanoparticles solutions prepared using the

201 methods above were incubated at pasteurisation conditions, 63°C for 30 minutes. Then the total
202 phenolic content was determined by Folin-Ciocalteu method. The total antioxidant capacity of
203 samples was measured by the ABTS method. All the samples were determined in duplicate.

204 **2.6 Fluorescence measurement of resveratrol- β -lactoglobulin solutions**

205 The concentrations of resveratrol solutions were 0.23mg/100ml, 0.46mg/100ml, 0.92mg/100ml,
206 1.84mg/100ml, and 2.48mg/100ml. The protein concentration of resveratrol-native β -Lg solutions
207 and resveratrol- β -Lg nanoparticles solutions was kept constant at 0.2% w/v. According to Liang,
208 L., & Subirade, M. (2012), the protein intrinsic fluorescence due to tryptophan (Trp) and tyrosine
209 (Tyr) residues is sensitive to the fluorophore environment and thus potentially an indicator of
210 changes in protein conformation. Trp in the protein emits fluorescence when excited at a
211 wavelength (λ_{ex}) of 295 nm. The protein conformational changes as a result of interactions with
212 the quencher can be studied by the changes of the intensity of emission. Therefore, the degree of
213 β -Lg conformational changes was determined based on fluorescence emission of tryptophan (Trp),
214 which was measured by fluorescence spectrophotometer with the temperature controller (Varian
215 Cary Eclipse, United Kingdom). Fluorescence spectra were obtained after excitation at 295nm,
216 scanning an emission wavelength range between 300 nm to 510 nm, using 5nm excitation and
217 emission slits wavelength. The data was collected by Cary Eclipse software version 2 (Varian Cary
218 Eclipse, United Kingdom) and samples were analysed at a constant temperature of 20°C.
219 According to Liang and Subirade (2012), the fractional residual fluorescence or relative
220 fluorescence intensity ($\mathbf{RFI}=\mathbf{F}_{\max}/\mathbf{F}_0\times\mathbf{100}$) was the fraction of the total protein fluorescence that
221 was not quenched, and thus the fraction of β -Lg not bound to the ligand; where, \mathbf{F}_0 is fluorescence
222 intensity of pure β -Lg; \mathbf{F}_{\max} is the intensity at the emission maximum (λ_{\max}). A low ratio of RIF
223 demonstrated a strong binding of the ligand, while a ratio of 100% indicated no binding.

224 For the calculation of binding constant and binding number, the fluorescence quenching data
225 were analysed by fitting to the Stern-Volmer equation (Liang et al., 2008) in dynamic quenching.

226 Eq. (2):

$$227 \quad F_0/F_{\max} = 1 + K_q \times \tau_0 \times [\text{Resveratrol}] = 1 + K \times [\text{resveratrol}] \quad (2)$$

228 F_0 and F_{\max} are the fluorescence emission intensities without and with resveratrol; [resveratrol]
229 is resveratrol concentration; K_q ($M^{-1}s^{-1}$) is the fluorescence quenching rate constant; τ_0 (s) is the
230 fluorophore fluorescence lifetime without quencher, and K ($K_q \times \tau_0$) is defined as the Stern-
231 Volmer quenching constant. By plotting F_0/F_{\max} as a function of [resveratrol] and according to
232 Eq. (2) K was obtained from the slope of the straight line.

233 For the static quenching, the binding constant K_s and binding number n can be calculated
234 according to the double logarithmic equation (He et al., 2016) Eq. (3):

$$235 \quad \log[(F_0 - F_{\max})/F_{\max}] = \log K_s + n \log[\text{resveratrol}] \quad (3)$$

236

237 From a plot of $\log (F_0 - F_{\max})/F_0$ as a function of $\log [\text{resveratrol}]$ and according to Eq. 3 the K_s
238 value was obtained from the intercept of the resulting straight line and n from the slope.

239

240 **2.7 The stability of resveratrol to light**

241 Resveratrol solutions/suspensions were exposed to overhead light (Philips, TL-D 840, 70w, and
242 distant was 1 meter) in the laboratory during 0, 1, 3, 6 and 12 hours. The total phenolic content
243 was measured at 303nm by a spectrophotometer (Ultrospec[®] 1100 pro) and the Folin-Ciocalteu
244 method as described above. The total antioxidant capacity of samples was measured by ABTS as
245 described above. All the samples were determined in duplicate.

246 **2.8. Statistical analysis**

247 Figures were plotted using MS Excel, MS Office 2013 (Microsoft Corporation, Redmond, WA,
248 USA). The results were statistically analysed by analysis of variance using IBM® SPSS®
249 Statistics version 20.0. Means and standard deviations from at least two measurements carried out
250 on two samples repeated. The significance level was set at 0.05. Data fitting of experimental data
251 to models was performed with Excel MS Office 2013 (Microsoft Corporation, Redmond, WA,
252 USA)

253 **3. Results and discussion**

254 **3.1. The solubility of resveratrol in aqueous solutions**

255 The solubility of resveratrol in water was determined by measuring total phenolic content using
256 the direct spectrophotometric method and Folin-Ciocalteu method (Fig S1).

257 In the direct spectrophotometric method, the λ_{\max} of 2mg/100ml resveratrol aqueous solution was
258 determined as 303 nm. Therefore, solubility was measured by measuring absorbance of a range of
259 concentrations at this wavelength . The absorbance of resveratrol reached a peak and levelled off
260 at resveratrol concentration 7mg/100ml after an approximately linear increase. Results from Folin-
261 Ciocalteu method showed the same trend, and at 7mg/100ml the GAE also remained constant at
262 about 35.5 mg/L. Both these results indicated that 7mg/100ml corresponded to the limit of
263 solubility of resveratrol in water. Surprisingly, it was in disagreement with the reported solubility
264 value (3mg/100ml), but this was determined by high-performance liquid chromatography (HPLC)
265 (Jeon et al., 2016; Pujara et al., 2017). Moreover, the solubility value reported by Camont et al.
266 (2009) using the UV spectrophotometric method was 6.85 mg/100ml, but they claimed that this
267 increased solubility was due to sonication which helped to dissolve resveratrol. Another possible
268 explanation of this different result was that, in our method, samples were not filtered prior to
269 absorbance measurements, so any insoluble particles of resveratrol in suspension could have led

270 to an overestimation of the solubility. However, in the Folin assay, 0.5ml samples were taken and
271 20 times dilution was applied so any interferences by suspended particles would be minimal. In
272 any case filtration was not an option in this study as filtration of aqueous solutions of resveratrol
273 with a 0.45 μm Polyethersulfone (PES) membrane proved to be very difficult and all resveratrol
274 was retained (results obtained by HPLC but not shown here). Only the 0.22 μm Polypropylene
275 (PVDF) filters showed high permeability for resveratrol but still, a reduction in absorbance
276 (7.69%) was observed. Moreover, with this filter high retention of β -Lg nanoparticles (about 80%)
277 was obtained and that also resulted in retention of resveratrol. Therefore, as filtration is a key step
278 before injecting the samples into the column, HPLC was not used in this study to determine the
279 concentration of resveratrol.

280 To sum up, in this study, the solubility of resveratrol in deionized water at room temperature
281 was 7mg/100 ml. As our solubility result was higher than some of the reported values, it was
282 assumed that at 7mg/100 ml, a nearly saturated solution of resveratrol was obtained. Therefore,
283 the rest of experiments were carried out at the range of 1mg/100ml to 7mg/100ml.

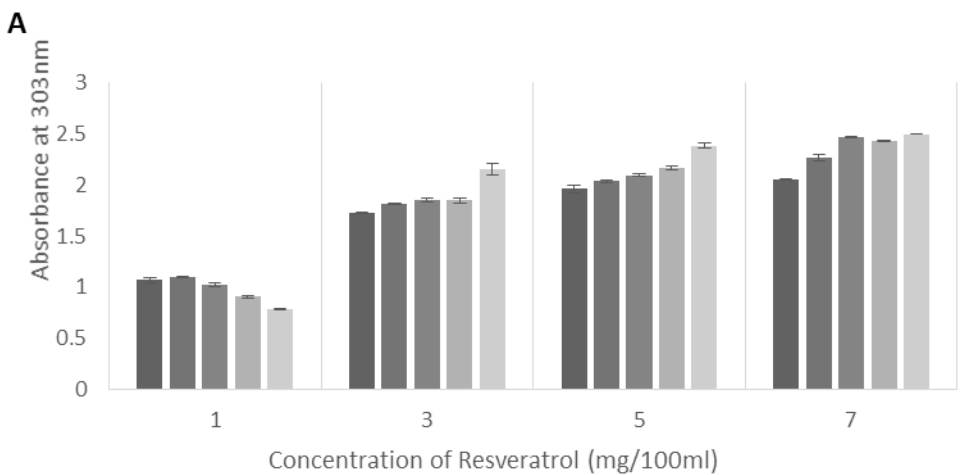
284

285 **3.2 The effect of light on resveratrol solubility and stability**

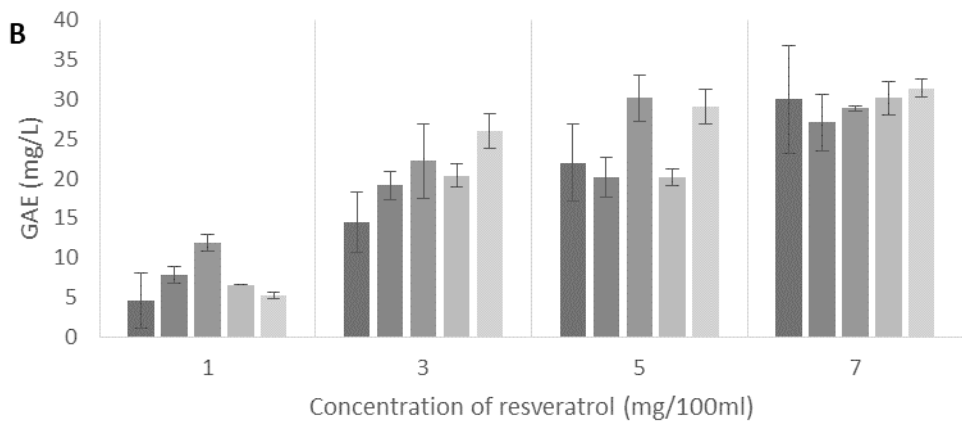
286 For the stability to light, the resveratrol suspensions were exposed to the lab light for 12 hours.
287 Based on the measurement of the direct spectrophotometric method, the concentration of
288 resveratrol had an increasing trend with one exception at 1mg/100ml (Fig 1). Also, according to
289 Figure 1, there was a reduction of GAE value at 1mg/100ml, but no significant difference at
290 3mg/100ml-7mg/100ml during 12 hours light exposure with a wide variance. Corresponding to
291 the total phenolic content, inhibition% had the same increasing trend at 3mg/100ml-7mg/100ml.
292 A significant positive correlation between total phenolic content (the direct spectrophotometric

293 method) and total antioxidant capacity was observed as Pearson value was 0.844. Therefore, it was
294 concluded that the resveratrol solution was not stable when exposed to lab light. Simimalrly,
295 Pinelo, Rubilar, Sineiro, and Núñez (2005) found that resveratrol solution achieved maximum
296 antioxidant activity at 22°C in water bath after three days.

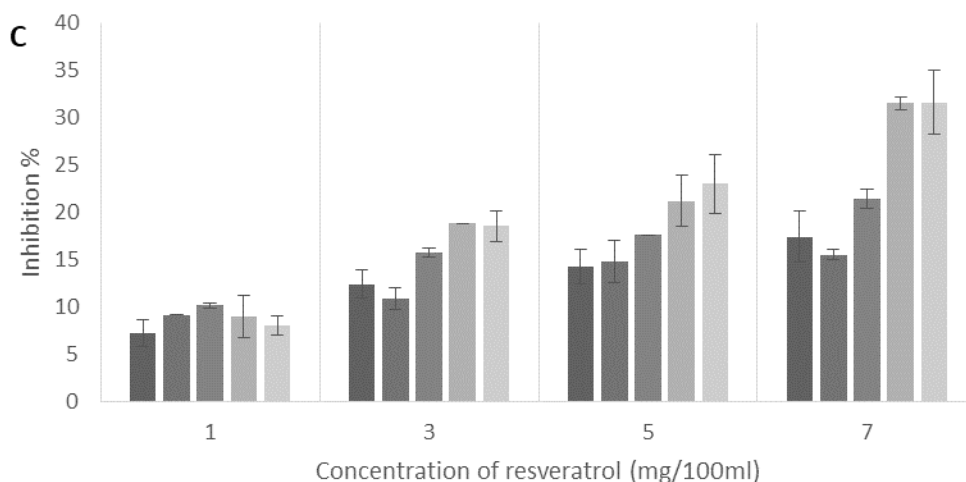
297 Also, according to Orgován, Gonda, and Noszál (2017), the trans-resveratrol is more lipophilic
298 than its isomer. Moreover, they also found that the cis-resveratrol has over ten times more
299 solubility than trans-resveratrol. Another potential explanation could be that at high concentration,
300 the trans-resveratrol converted to cis-resveratrol under the light condition and more cis-resveratrol
301 dissolved in the solution leading to an increase in antioxidant activity.



302



303



304

305 Figure 1: the stability of resveratrol to light by total phenolic content at 303nm (A), total phenolic
 306 content by Folin-Ciocalteu method (B) and total antioxidant capacity by ABTS (C). ■ 0h ■ 1h ■
 307 3h ■ 6h ■ 12h.

308 3.3. The characterization of resveratrol- β -lactoglobulin nanoparticles

309 After heating the mixture of resveratrol and β -lactoglobulin (0.2% w/v) to form nanoparticles,
 310 the size of nanoparticles was measured by dynamic light scattering. The average size of
 311 resveratrol- β -lactoglobulin nanoparticle was $181.80 \text{ nm} \pm 1.48 \text{ nm}$ with polydispersion index 0.048.
 312 The nanoparticle size was close to that reported by Fonseca, Khalil, and Mainardes (2017) who
 313 encapsulated resveratrol with bovine serum albumin nanoparticles (175nm) by desolvation method
 314 with ethanol. There was no significant difference in size among different concentrations of
 315 resveratrol nanoparticles and nanoparticles alone. So the nanoparticles (200nm) produced were not
 316 affected by the concentrations of resveratrol. On the other hand, the size of nanoparticles was
 317 smaller than the nanoparticles we produced in our previous work (Guo et al 2017) and this is
 318 possibly because no hydration step (at 4 °C overnight) was applied prior to the production of

319 nanoparticles. Here no hydration step was applied in order to reduce any risk of resveratrol
320 degradation during storage.

321

322 **3.4. Effect of heating, native β -lactoglobulin, and resveratrol- β -lactoglobulin nanoparticles** 323 **on the stability of resveratrol**

324 The effect of heating was investigated under the same conditions as those used in the β -
325 lactoglobulin nanoparticles preparation: heating at 75°C for 45 mins. After heating, 10 minutes in
326 the ice bath allowed rapid reduction of samples temperature to room temperature. The
327 concentration of native β -lactoglobulin added to the resveratrol solution was the same as that used
328 in the preparation of nanoparticles. The stability of resveratrol solutions was assessed after: (i)
329 heating, (ii) protein addition and (iii) nanoparticles production; this was assessed by comparing
330 the total phenolic content (Folin-Ciocalteu method), and total antioxidant activity (ABTS method
331 and the FRAP method) of treated samples with those of the control (resveratrol aqueous solution
332 1mg/100ml-7mg/100ml).

333 **3.4.1. Total phenolic content by Folin-Ciocalteu method**

334 A clear increment of GAE value was observed after heating at all concentrations (Fig 2),
335 indicating that the heating has a significant influence on resveratrol. Even though heating could
336 improve the solubility of resveratrol in water dramatically (Filipa et al., 2003) since in this study
337 all samples were subjected to an ice bath after heating, the increase of solubility due to heating
338 would have been reduced or eliminated. So the increase in GAE observed at all concentrations,
339 particularly those below the solubility (< 7 mg/100 ml) could be due to chemical changes in the
340 molecule which resulted in changes in its oxidative status and/or oxidation power and led to
341 enhanced blue colour production. However, at 7 mg/ 100 ml, the big increase in GAE from 30.01

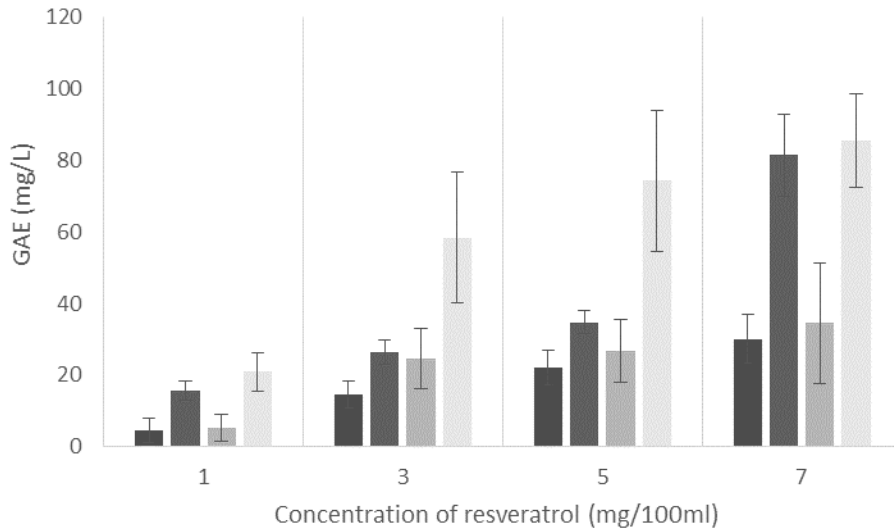
342 to 81.46 mg/L may also be partly due to an increase in solubility since near saturation might have
343 been reached at this concentration of resveratrol. Flieger et al. (2017) pointed out that when the
344 temperature reached 75°C or above, heating could induce the isomerization of resveratrol from
345 trans-resveratrol to cis-resveratrol even without light. Also, Orgován et al. (2017) found that the
346 solubility of cis-resveratrol was over ten times more than that of trans-resveratrol in water as trans-
347 resveratrol was more lipophilic than its isomer. Thus, a possible explanation for the increase in
348 GAE observed here could be that some of the trans-resveratrol isomerized to cis-resveratrol and
349 that led to an increase in solubility.

350 When adding native β -lactoglobulin, an increase in total phenolic content happened although
351 this increase was not significant ($P>0.05$) compared with the control sample at all concentrations.
352 The interference of protein on total phenolic content measurement has been eliminated by
353 subtracting the GAE value corresponding to the protein to the total GAE value (see Methods in
354 2.3). So it can be assumed that any increases in GAE as a result of protein addition were due to
355 interactions between native β -lactoglobulin and resveratrol.

356 Moreover, the samples of resveratrol with β -lactoglobulin nanoparticles had the largest
357 increment in phenolic content among these three treatments, and it was around three times the
358 phenolic content of the control sample at all concentrations (Fig 2). Interestingly the nanoparticles
359 (combined effect of heat and protein addition) led to a much higher increase than the heating alone
360 for those concentrations of RSV below solubility whereas for the highest concentration it had
361 almost the same effect than heating alone.

362 Overall these results suggest that at concentrations below saturation heating enhanced
363 resveratrol and β -lactoglobulin interactions as well as inducing isomerisation thus, these combined

364 effects led to an increase in total phenol content. For the highest resveratrol concentration which
365 was about saturation point heating had the strongest effect and led to a further increase in solubility.
366



367
368 Figure 2: The total phenolic content results by Folin-Ciocalteu method of: ■ resveratrol aqueous
369 solution (control); ■ heated resveratrol; ■ resveratrol with native β -Lg; ■ resveratrol with
370 nanoparticles of β -Lg. Results were expressed as Gallic Acid Equivalents GAE mg/L.

371 3.4.2. Total antioxidant activity by ABTS and FRAP methods

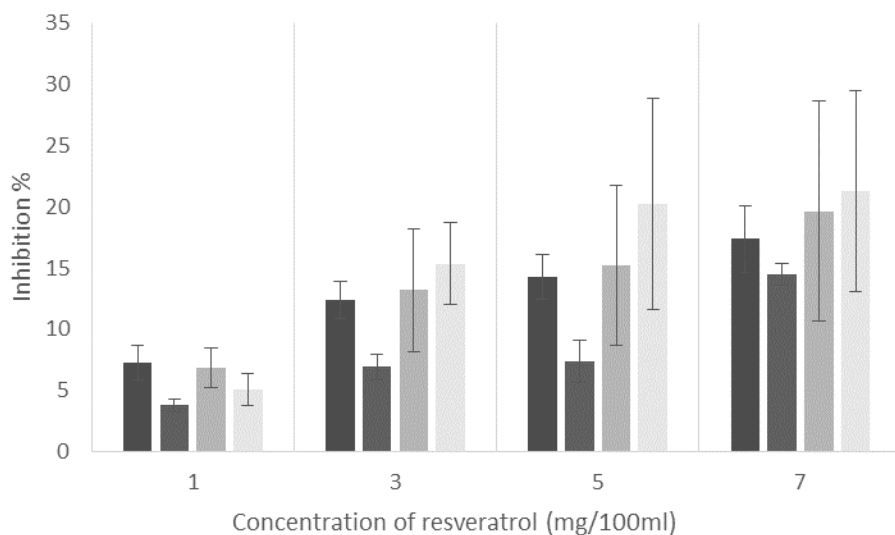
372 Besides total phenolic content, the total antioxidant capacity of resveratrol was measured to
373 assess changes as a result of the different treatments and in this way assess the stability of
374 resveratrol. In this work, two methods were applied to measure antioxidant capacity: ABTS
375 method and FRAP method. Results in Fig 3.demonstrated a reduction in antioxidant activity
376 (inhibition %) after heating at all concentrations ($P < 0.05$). According to Mikulski, Gorniak, and
377 Molski (2010), trans-resveratrol was a stronger antioxidant than cis-isomer due to the planar
378 conformation; this conformation would ensure the main parameters contributing to antioxidant

379 activity: favourable spin and unpaired electron distribution. Therefore, this reduction in antioxidant
380 activity supports further the hypothesis that trans-resveratrol changed to cis-isomer during heating
381 with the subsequent reduction in antioxidant activity and increase in total phenols (as shown in Fig
382 2).

383 The addition of native β -lactoglobulin led to no significant changes in antioxidant activity when
384 compared against the control samples, indicating that the antioxidant activity would not be masked
385 by β -lactoglobulin.

386 On the other hand, the resveratrol- β -lactoglobulin nanoparticles had higher activity than heated
387 samples ($P < 0.05$) at 1-5 mg/100ml. So the reduction in activity due to heating was counteracted
388 by the interactions of resveratrol with the nanoparticles which led to similar or higher activity than
389 the control. This suggests a protective effect of β -lactoglobulin nanoparticles against heat-induced
390 loss of antioxidant activity; possibly the interactions of β -Lg nanoparticles with resveratrol hinder
391 its isomerisation. Interestingly, at 7mg/100ml, the difference in antioxidant activity between the
392 heated sample (14.52%) and resveratrol- β -lactoglobulin nanoparticle samples (21.31%) was not
393 statistically significant ($p = 0.072$). At 7 mg/100 ml, the heating led to two counteracting effects:
394 (1) improving solubility which would increase activity and (2) isomerization which would result
395 in reduced activity, this is why both the heating effect and the nanoparticles did not have a
396 significant impact on antioxidant activity at this concentration

397



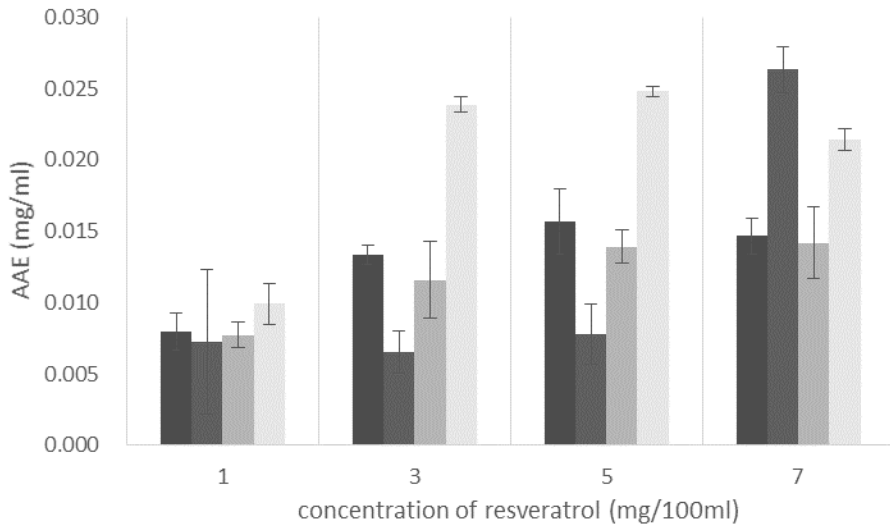
398

399 Figure 3: The total antioxidant activity results by ABTS method of: ■ resveratrol aqueous solution
 400 (control); ■ heated resveratrol; ■ resveratrol with native β -Lg ■ resveratrol with nanoparticles of
 401 β -Lg. Results were expressed as inhibition% (I%)

402 Another antioxidant activity measuring method, FRAP, was also applied to study the effects of
 403 different treatments on resveratrol. The antioxidant activity results by FRAP method expressed as
 404 AAE (mg/ml) are shown in Fig 4. It was noted that there was no significant difference ($P < 0.05$) in
 405 activity between 5mg/100ml and 7mg/100ml of control samples, which might suggest that
 406 solubility of resveratrol in water was 5mg/100ml instead of 7 mg/100ml.

407 A reduction in activity due to heating was observed for samples up to 5mg/100ml in agreement
 408 with ABTS measurements (Fig 3). Surprisingly, a sharp increase was observed at 7mg/100ml. This
 409 observation agrees with the sharp increase measured in total phenolic content at this concentration
 410 (Fig 2). Therefore, the increase in antioxidant activity at 7mg/100 ml could be explained by an
 411 increase in solubilized resveratrol in the form of cis-resveratrol as a result of heating.

412



413

414 Figure 4: The total antioxidant activity results by FRAP method of: ■ resveratrol aqueous solution
 415 (control); ■ heated resveratrol; ■ resveratrol with native β -Lg; ■ resveratrol with nanoparticles
 416 of β -Lg. Results were expressed as Ascorbic Acid Equivalents (AAE mg/ml)

417 In agreement with ABTS results the resveratrol- β -lactoglobulin nanoparticles had the highest AAE
 418 value at all concentrations among all treatments (except heated sample at 7mg/100ml), whilst
 419 resveratrol with native β -lactoglobulin samples had the same antioxidant activity as the control
 420 samples ($P > 0.05$) (Fig 4). So these results again confirm a protective effect of β -lactoglobulin
 421 nanoparticles against heat-induced loss of antioxidant activity. Moreover, according to the FRAP
 422 method, there was a significant effect of β -Lg nanoparticles as compared to native β -Lg (Fig 4).

423 To conclude, the results of both antioxidant capacity methods indicated that β -lactoglobulin
 424 nanoparticles had the ability to prevent chemical changes in resveratrol during heating and in this
 425 way protect its antioxidant capacity.

426

427

428 **3.5. The effect of pasteurization on stability of resveratrol with and without protein**

429 In order to study further the protective effect of β -lactoglobulin against thermal degradation of
430 resveratrol an industrial thermal process, pasteurisation, was applied to resveratrol with and
431 without protein. Pasteurisation is a common commercial method to minimise health hazards from
432 food pathogens and to extend shelf life for liquid food and drinks. Thus, it is important to examine
433 the effect of pasteurisation on resveratrol stability and how would adding protein affect this.

434 Firstly, as shown in Fig 5A, the total phenolic content of resveratrol after pasteurisation showed
435 the same results as the heating at 75°C: dramatic increase of GAE value after pasteurisation.
436 Secondly, it demonstrated that pasteurising resveratrol with native β -lactoglobulin had no
437 significant effect in total phenolic content at all concentrations as compared to control. However,
438 pasteurization of resveratrol with nanoparticles showed a significant decrease in total phenolic
439 content. A possible reason could be that the interaction with β -lactoglobulin suppressed the
440 isomerization undergone by resveratrol at 63°C This would agree with the observations above.

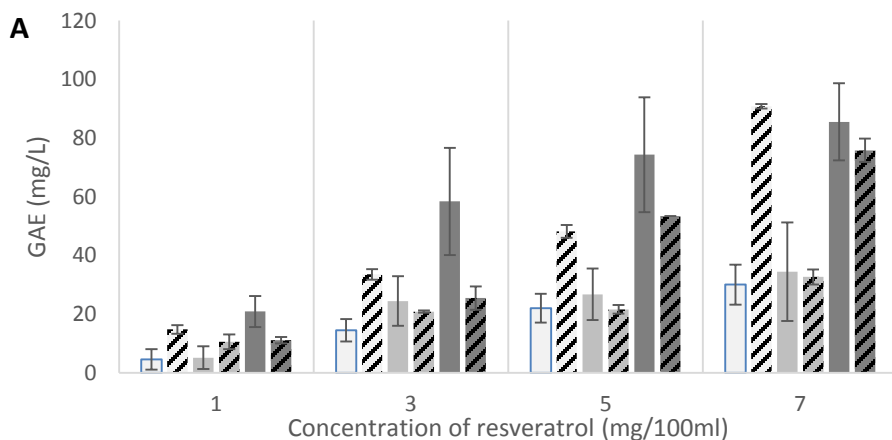
441 On the other hand, the antioxidant activity (%inhibition) of standard resveratrol slightly reduced
442 after pasteurisation at 1mg/100ml and 3mg/100ml but increased at 5mg/100ml and 7mg/100ml
443 (Fig 5B). These results were different to results of heating at 75°C, which indicated that heating at
444 75 °C led to more degradation than at 63°C, and here samples were not put in an ice bath after
445 pasteurisation so heating might have led to increased solubility.

446

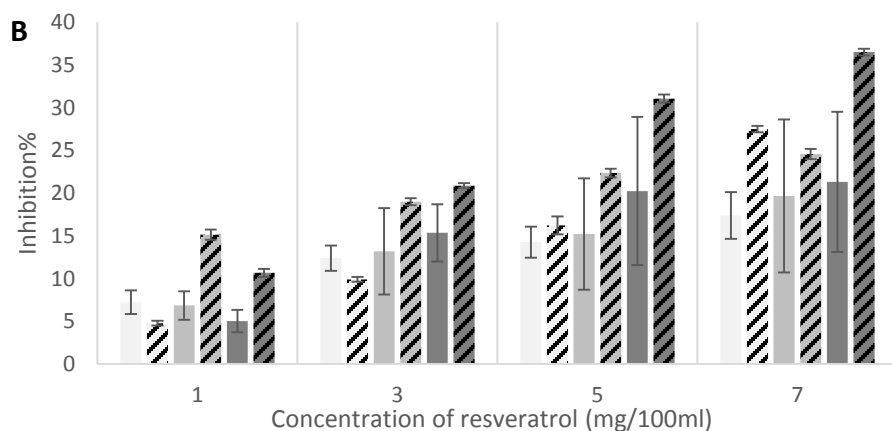
447 Moreover by comparing the pasteurised samples with and without protein it is clear that the
448 presence of protein led to higher antioxidant activity. The increment of total antioxidant activity
449 after pasteurisation in the samples containing protein could be explained as above, ie: the

450 interactions between resveratrol and protein hindered the isomerisation of resveratrol that led to
451 reduced activity.

452



453



454

455 Figure 5: The total phenolic content (A) and the total antioxidant activity (B) of :

456 resveratrol aqueous solution (control); resveratrol with native β -Lg; resveratrol with β -Lg

457 nanoparticles. The plane colour bars were samples before pasteurisation and the patterned bars the

458 same sample after pasteurisation.

459

460 To conclude, adding protein particularly in the nano form would suppress the heating effect on

461 resveratrol. Pasteurisation led to a small decrease in AOC at all concentrations (except at the

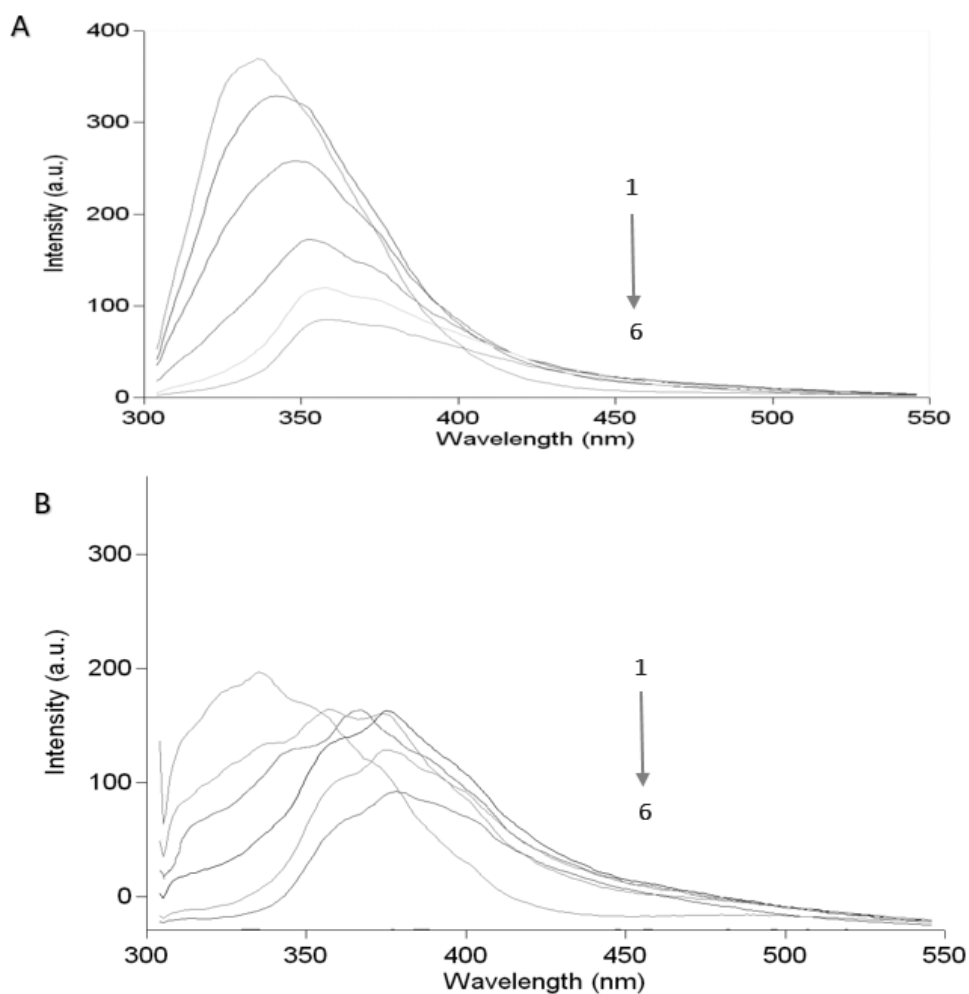
462 highest) whilst resveratrol samples with protein (both in native and nanoparticle form) showed an
463 increase in AOC.

464

465 **3.6. Determination of β -lactoglobulin-resveratrol interactions by fluorescence**

466 Fluorescence measurements were carried out in order to obtain a further insight into the
467 resveratrol-protein interactions. The fluorescence measurements of resveratrol- β -lactoglobulin
468 solutions were conducted at constant protein concentration (for both native and nanoparticles) with
469 varying concentration of resveratrol from 0.23mg/100ml-2.5mg/100ml.

470



471

472 Figure 6: The fluorescence emission spectra of (A) resveratrol-native β -lg at 0mg/100ml-
473 2.48mg/100ml resveratrol (1-6); (B) resveratrol- β -lg nanoparticle at 0mg/100ml-2.48mg/100ml
474 resveratrol (1-6).

475 Fig 6 (A) showed the intrinsic fluorescence emission spectra of native β -Lg in presence of
476 different concentrations of resveratrol. As resveratrol concentration increased, a reduction in
477 intensity of fluorescence emission was observed. This was an indication of partial disruption of
478 the protein structure which led to a change in the polarity of the fluorophore (Trp groups in the
479 protein) and hence the reduced intensity. There was also a slight shift in λ_{\max} to longer wavelengths
480 (from 337.0 nm to 357.14 nm) which is in agreement with the protein conformational changes
481 induced by the resveratrol as reported by Liang et al. (2008). Interestingly the changes in
482 fluorescence intensity at increased resveratrol concentrations were not as big for the β -Lg
483 nanoparticles (Fig 6B) as for the native protein. The RFI value of native β -Lg (22.82%) was higher
484 than the RFI value of β -Lg nanoparticles (46.65%) indicating stronger interactions between these
485 two molecules when β -Lg is in native than in nanoparticle form. The nanoparticles were formed
486 by heat denaturation of protein; as the protein conformation had been already altered the addition
487 of resveratrol did not lead to important changes in fluorescence intensity. However, the λ_{\max} shift
488 was much bigger for the nanoparticles than for the native protein (from 335.08nm to 377.89nm)
489 suggesting a big change in the fluorophore environment towards a more hydrophilic environment.
490 This could be explained by hydrophobic interactions between the resveratrol and the unfolded
491 protein molecule which would lead to further exposure of the fluorophore to a more hydrophilic
492 media. Therefore, there was a large effect on the protein tertiary structure. Overall this seems to
493 agree with the stronger effect observed on the stability of resveratrol by the nanoparticles than the
494 native protein.

495 Furthermore, from the linear plot of F_{max}/F_0 as a function of resveratrol concentration and
496 according to Eq (2) the Stern-Volmer equation constant K of Resveratrol with native β -Lg was
497 $3.18 \times 10^4 \text{ M}^{-1}$ ($R^2 = 0.986$). Assuming that τ_0 was 1.28 ns for Trp of β -Lg (EricDufour, Genot, &
498 Haertlé, 1994; Stojadinovic et al., 2013) the K_q was determined as $2.5 \times 10^{13} \text{ M}^{-1}\text{s}^{-1}$, which was
499 higher than the maximal dynamic quenching constant ($1-2.0 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$). This suggests that
500 binding of resveratrol with β -Lg followed a static quenching mechanism where the fluorophore
501 formed a stable complex with another molecule that was non-fluorescent. This result was in
502 agreement with that reported by Liang and Subirade (2012) and Liang et al. (2008), who
503 confirmed that β -Lg fluorescence quenching induced by resveratrol is a static quenching. Thus,
504 from the linear equation of $\log(F_0 - F_{max})/F_{max}$ as a function of $\log[\text{resveratrol}]$ ($R^2 = 0.985$)
505 and according to Eq. 3 the binding constant K_s was determined from the intercept of the slope as
506 $1.67 \times 10^5 \text{ M}^{-1}$ and from the slope the binding number, n was obtained as 1.33. This means 1.33
507 resveratrol molecules bind one β -Lg molecule and also K_s was in the range of 10^5 and 10^6 M^{-1} ,
508 which was in agreement with binding constants reported for native β -Lg and a range of
509 substrates such as folic acid with $K_s = 4.3 \times 10^5 \text{ M}^{-1}$ (Liang & Subirade, 2010) and EGCG with
510 $K_s = 1.05 \times 10^5 \text{ M}^{-1}$ (Shpigelman, Israeli, & Livney, 2010). However, the Stern-Volmer quenching
511 equation might not be suitable to apply to the resveratrol- β -Lg nanoparticles binding since the β -
512 Lg fluorescence emission maximum changed with resveratrol concentration.

513 Overall from our work, it was demonstrated that more quenching by resveratrol occurred when
514 binding β -lactoglobulin in the native than in nanoparticle form. Resveratrol bound native β -Lg
515 following the Stern-Volmer static quenching model at ratio 1: 1 in agreement with previously
516 reported by Liang et al. (2008); most probably resveratrol bound at the surface of the protein. On
517 the other hand, the heat denaturation undergone by the protein during the formation of the

518 nanoparticles exposed new binding sites to which resveratrol bound. The quenching was not as
519 strong as with native β -Lg but these type of interactions (most probably of hydrophobic nature)
520 led to further structural changes in the protein as shown by the shift to longer wavelengths. This
521 may also explain the protective effect of nanoparticles against chemical changes in resveratrol
522 upon heating and the increased antioxidant activity.

523 Denaturation of the protein affects the binding of different ligands in different ways. For
524 example, Liang and Subirade (2012) found that thermal denaturation of β -Lg reduced the binding
525 affinity for tocopherol whilst it increased for resveratrol. Moreover, Shpigelman et al. (2010)
526 reported a higher association of heated β -Lg with Epigallocatechin-3-gallate (EGCG) than native
527 β -Lg whilst Perez, Andermatten, Rubiolo, and Santiago (2014) demonstrated no significant
528 differences between native and heat-induced β -Lg aggregates binding to linoleic acid. Protein
529 denaturation leads to structural changes which then can change access to binding sites in the
530 protein; polarity and size of the ligands influences which binding sites they bind to.

531

532 **4. Conclusions**

533 In this work, the solubility of resveratrol in water was determined as 7mg/100ml by the direct
534 spectrophotometric method and Folin-Ciocalteu method. Resveratrol was not stable to light, and
535 light exposure resulted in higher phenolic content and total antioxidant capacity. Nanoparticles of
536 β -lactoglobulin were produced in the presence of resveratrol with average size around 181.8nm
537 and the concentration of resveratrol had no significant difference in the size of nanoparticles. The
538 heat induced isomerisation of resveratrol at 75°C led to increased solubility but reduced
539 antioxidant capacity. β -lactoglobulin nanoparticles showed a protective effect against heat induced
540 loss of antioxidant activity. Moreover, when resveratrol with and without protein was treated at

541 pasteurisation conditions both, native and β -lactoglobulin nanoparticles showed a protective effect
542 against heat induced loss of activity and an increase in antioxidant activity. Fluorescence
543 measurements of resveratrol binding to native β -Lg showed strong fluorescence quenching of β -
544 Lg by resveratrol suggesting strong interactions between these two molecules. Interactions with β -
545 Lg nanoparticles led to smaller quenching but a significant shift of λ_{max} to longer wavelengths
546 which suggested also strong interactions with resveratrol. Overall it was found that the interactions
547 of protein and resveratrol in combination with heating led to improved antioxidant capacity. Thus,
548 complexation of protein with resveratrol proved to have a stabilization effect. These findings are
549 very relevant for the optimum processing and formulation of resveratrol.

550 Acknowledgements

551 The trans-resveratrol powder was supplied by Evolva. Special thanks to MSc student Napatsawan
552 Wonggotwarin, who initiate this work. This research did not receive any specific grant from
553 funding agencies in the public, commercial, or not-for-profit sectors.

554 Conflict of interest statement

555 The author declares that there are no conflicts of interest.

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