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1 **Probing the stability of the food colourant R-phycoerythrin from dried Nori**  
2 **flakes**

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17 **DECLARATION OF INTEREST**

18 Declarations of interest: none.

19 **ABSTRACT**

20 This study aims to characterise the stability of R-phycoerythrin (R-PE) spectroscopically, a  
21 vivid natural colourant with emerging potential for application in the food industry. High-  
22 quality ( $A_{560}/A_{280} \geq 5$ ), native ( $\alpha$ -helix content 75%) R-PE was purified from commercial dried  
23 Nori (*Porphyra* sp.) flakes. The thermal unfolding revealed two transitions (at 56 and 72°C),  
24 ascribed to different protein subunits. Contrary to temperature, high-pressure (HP) treatment  
25 showed a significant advantage under applied conditions: the R-PE unfolding is partly  
26 reversible, and the colour bleaching is minimised. The binding of  $\text{Cu}^{2+}$  ( $6.3 \times 10^5 \text{ M}^{-1}$ ) and  $\text{Zn}^{2+}$   
27 ( $1.7 \times 10^3 \text{ M}^{-1}$ ) influenced conformational changes in protein tetrapyrrole chromophore without  
28 affecting R-PE structure and stability (colour). Results give new insights into the stability of  
29 R-PE with a good use-value in replacement of toxic synthetic dyes, preservation of red colour  
30 of this phycobiliprotein in fortified food and beverages by HP processing, and as a biosensor  
31 for  $\text{Cu}^{2+}$  in aquatic systems.

32 **Keywords:** R-phycoerythrin, High-pressure, Temperature, Binding, Stability, Metal ions.

## 33 1. INTRODUCTION

34 Cultivation of marine macroalgae is of significant economic and cultural importance  
35 for providing food, medicine, cosmetics, and biofuel (Gao, Gao, Bao, Xu, & Li, 2019). Red  
36 microalgae *Porphyra* sp., commonly known as Nori, has been used as food for centuries in  
37 many Asian countries, but its consumption has extended to Western consumers (Cermeno, et  
38 al., 2019; Gao et al., 2019). It is usually used to prepare sushi, soup, cakes, consumed as  
39 dried Nori flakes (Bito, Teng, & Watanabe, 2017). Dried Nori is an excellent choice of  
40 many nutrients, such as dietary fibres, polyunsaturated fatty acids, minerals, vitamins, and a  
41 large amount of proteins (approx. 40% on a dry basis).

42 Numerous studies have shown antioxidant, immunomodulatory, anticancer and  
43 antihypertensive effects of *Porphyra* sp. extracts and components (Venkatraman & Mehta,  
44 2019). The health benefits are mainly attributed to sulfated polysaccharides, bioactive  
45 peptides, microsporine-like amino acids and R-phycoerythrin (R-PE) (Bito, et al., 2017). R-  
46 phycoerythrin is one of the most abundant proteins in *Porphyra* sp., representing between  
47 1.3–1.5% of the dry biomass (Cao, Wang, Wang, & Xu, 2016). It is a strongly fluorescent  
48 (quantum yield ~98%) and water-soluble molecule belonging to the phycobiliprotein (PBP)  
49 family, with the primary function to transfer excitation energy to reaction centres during  
50 photosynthesis (Apt, Hoffman, & Grossman, 1993; Gaigalas, et al., 2006). The intensive  
51 reddish-pink colour of R-PE arises from its covalently attached (*via* thioether bond) linear  
52 tetrapyrrole chromophores phycoerythrobilin (PEB) and phycourobilin (PUB). It is composed  
53 of three subunit types forming a hexamer complex  $[(\alpha\beta)6\gamma]$ :  $\alpha$  (18–20 kDa),  $\beta$  (19–21 kDa),  
54 and  $\gamma$  (30–33 kDa) (Apt, et al., 1993; Wang, et al., 2020). Two PEB chromophores are bound  
55 to both  $\alpha$  and  $\beta$  subunits, while one PUB chromophore is found attached to the  $\beta$  subunit (Fig.  
56 1A). Gamma subunit binds one PEB and three PUB chromophores (Gaigalas, et al., 2006).  
57 Besides R-PE, *Porphyra* sp. contains other PBPs: blue R-phycoerythrin (R-PC) and light-blue  
58 allophycoerythrin (APC). The distinction in the colour of phycobiliproteins, e.g. in its VIS  
59 absorption spectra, is the consequence of differences in the type and number of bound  
60 chromophores in protein oligomers (Apt, et al., 1993).

61 Studies have shown that R-PE exhibits significant antioxidant (Yabuta, Fujimura,  
62 Kwak, & Watanabe, 2010), anticancer (Cai, et al., 2014), and immunomodulatory effects  
63 (Wang, et al., 2020), mainly originating from the covalently attached chromophores. The  
64 health benefits of R-PE, its brilliant and intensive fluorescence and vivid colour, and a huge  
65 need to replace the synthetic colourants, give R-PE a great potential to be used for food

66 fortification and colouring. Indeed, it has been already used as a food colourant in Asia  
67 (**Fleurence, et al., 2012**). A bright yellow fluorescence of R-PE providing opportunities to  
68 prepare visually attractive fluorescent sweets and soft drinks (**Sekar & Chandramohan,**  
69 **2008**).

70 One of the major factors for selecting a suitable food colourant is its stability. R-  
71 phycoerythrin has good functional stability through a wide pH range (from 3 to 10) (**Liu, et**  
72 **al., 2009**), but the thermal treatment of protein above 40°C significantly reduce its colour  
73 intensity (**Munier, et al., 2014**). Roasting dry Nori flakes induces a change of colour from red  
74 to green due to the thermal sensitivity of R-PE (**Bito, et al., 2017**). High-pressure (HP) food  
75 processing could preserve nutrients, colours, and flavours upon processing, giving it a good  
76 potential as an alternative approach for food treatment (**Wgiorgis, 2019**). Another critical  
77 factor affecting PBPs colour stability is microelements in the food due to the high propensity  
78 of tetrapyrrole chromophores to bind metal ions. Previous studies have shown the ability of  
79 various bioactive metal ions ( $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ , etc.) to influence the colour and  
80 fluorescence properties of PBPs (e.g., **Minic, et al., 2016**; **Suresh, Mishra, Mishra, & Das,**  
81 **2009**). Furthermore, the binding of microelements to R-PE could affect their bioavailability in  
82 the gastrointestinal tract and (patho)physiological processes involving these metal ions.

83 In this research, we study the thermal and HP stability of food-derived R-PE isolated  
84 from dried Nori flakes for the first time. We used a combination of precipitation and  
85 chromatography techniques to obtain the high-purity protein. Absorption, fluorescence, and  
86 CD spectra revealed that purified phycobiliprotein was a typical R-PE with high  $\alpha$ -helical  
87 content. High-pressure and high-temperature studies, established by absorption  
88 measurements, indicated that pressure treatment has substantially fewer adverse effects on R-  
89 PE stability than thermal processing. A fluorescence quenching approach characterises the  
90 binding of selected microelements ( $Zn^{2+}$  and  $Cu^{2+}$ ) to R-PE. At the same time, the effects of  
91 metal ions on the stability of R-PE were evaluated by absorption and CD spectroscopy.

## 92 **2. MATERIALS & METHODS**

### 93 **2.1. Materials**

94 Dried Nori flakes (*Porphyra purpurea* collected from off the coast of the Galicia  
95 region in Spain) were purchased from Porto-Muñños company (Spain). Hydroxyapatite (HA)  
96 resin and DEAE-Sepharose were bought from BioRad (CA, USA) and Sigma (MO, USA),  
97 respectively. Spectroscopic characterisation of R-PE, high-pressure, and metal-binding  
98 experiments were performed in 20 mM HEPES (pH 7.0), while 20 mM phosphate (pH 7.0)

99 was used for the R-PE thermal unfolding. All other chemicals were of analytical reagent  
100 grade, and Milli-Q water (Millipore, Molsheim, France) was used throughout the experiments.

## 101 **2.2. R-phycoerythrin isolation and purification**

102 Nori flakes were homogenised with liquid nitrogen, and obtained powder was  
103 resuspended in 20 mM phosphate buffer, pH 7.0. The suspension was mixed for 4 hours on  
104 the magnetic stirrer, followed by sonication with the ultrasonic probe at 30W (Branson  
105 Sonifier 150, Emerson, MO, USA) for 5 minutes (intervals of 20 seconds of sonification and  
106 10 seconds of pause). We centrifuged obtained suspension and precipitated the proteins from  
107 the supernatant with ammonium sulfate (65% of final concentration). The obtained pellet is  
108 resuspended and dialysed against 2.5 mM phosphate buffer, pH 7.0 overnight. We applied  
109 dialysed sample on HA resin (previously equilibrated with dialysis buffer) to separate R-PE  
110 from other PBPs (R-PC and APC). Non-bound fraction contains R-PE, while R-PC and APC  
111 are bound to HA resin. The phosphate buffer concentration in the R-PE fraction is adjusted to  
112 40 mM to obtain the same ionic strength as the equilibration buffer for IEC. R-phycoerythrin  
113 was further purified on DEAE-Sepharose at pH 7.0. Non-bound proteins are washed with  
114 equilibration buffer, while R-PE is eluted with 40 mM phosphate buffer (pH 7.0) containing  
115 150 mM NaCl. We analysed obtained fractions using UV/VIS absorption spectroscopy  
116 (**Section 2.3**) and SDS-PAGE under standard reducing conditions. Gels were first incubated  
117 in 1 M ZnSO<sub>4</sub> for 15 min (**Minic, et al., 2018**), then visualised fluorescent PBP bands on  
118 Typhoon FLA 7000 imager (GE Healthcare, Sweden). The same gels are then stained using  
119 Coomassie Brilliant Blue R-250.

## 120 **2.3. UV/VIS absorbance measurements**

121 UV/VIS absorption spectra were recorded on a NanoDrop 2000c spectrophotometer  
122 (Thermo Scientific, USA) in a quartz cuvette with a 1.0 cm pathlength. The spectra were  
123 collected in the range 190–750 nm at room temperature. R-PE concentration was determined  
124 using equation (**Nikolic, Minic, Macvanin, Stanic-Vucinic, & Velickovic, 2020**):

$$125 \quad R - PE \left( \frac{mg}{mL} \right) = 0,1247 \times ((A_{564} - A_{730}) - 0,4583 \times (A_{618} - A_{730})) \quad (1)$$

126 The absorption spectra of R-PE (0.25 μM) were measured in the absence or the  
127 presence of Zn<sup>2+</sup> or Cu<sup>2+</sup> metal ions (both 10 μM). Absorption of Cu<sup>2+</sup>/Zn<sup>2+</sup> solutions without  
128 R-PE was subtracted to correct background.

129 The effects of temperature on VIS absorption spectra of R-PE were studied on UV-  
130 1800 Shimadzu spectrophotometer (Japan), using Peltier element for temperature control. The

131 absorbance of R-PE solution (0.37  $\mu$ M) was recorded in a quartz cuvette with a 1.0 cm  
132 pathlength, between 450 and 650 nm, with a bandwidth of 1 nm and a data interval of 0.2 nm,  
133 at a scanning speed of 60 nm/min. Spectra were recorded at various temperatures (between 25  
134 to 85°C, with steps of 10°C). The temperature increased at a 5°C/min speed, while  
135 equilibration time was set to 3 minutes for each temperature point. Measurements of buffer  
136 solution were performed at the same conditions as above, and recorded spectra were  
137 subtracted from the corresponding protein spectra.

#### 138 **2.4. *In situ* high-pressure VIS absorbance measurements**

139 Visible (VIS) absorption spectra under *in situ* HP were recorded on a Cary 3E  
140 spectrometer (Varian, Palo Alto, CA, USA) using an HP optical bomb with sapphire windows  
141 and HP generator as previously described (Minic, et al., 2020). A square quartz cell (with an  
142 optical pathlength of 5 mm) containing the sample was positioned within the HP optical  
143 bomb. A plastic membrane on the top of the cell separated the sample from the pressure-  
144 transmitting liquid (H<sub>2</sub>O). The absorbance of R-PE solution (0.37  $\mu$ M) was recorded between  
145 450 and 650 nm, with a bandwidth of 1 nm and a data interval of 0.2 nm, at a scanning speed  
146 of 60 nm/min. Spectra were recorded at various pressures (between 0.1 to 450 MPa, with  
147 steps of 10 or 30 MPa) at 20°C. The pressure was increased at 10 MPa/min speed, while  
148 equilibration time was set to 3 min for each pressure point. Measurements of buffer solution  
149 were performed at the same conditions as above, and recorded spectra were subtracted from  
150 the corresponding protein spectra. An increase of pressure induces compression of the sample  
151 and, consequently, measured absorbances are higher. Therefore, we corrected the spectra by  
152 multiplying the experimental absorbance values by the relative solvent volume ( $V$   
153 ( $P_{\text{measured}})/V$  (0.1 M Pa)). The dependence of the relative volume of water was taken from  
154 (Vedam & Holton, 1967). Data analyses are provided in the Supplementary material.

#### 155 **2.5. Fluorescence measurements**

156 The fluorescence spectra were recorded with a FluoroMax<sup>®</sup>-4 spectrofluorometer  
157 (HORIBA Scientific, Japan) under thermostable conditions (25°C), using 5 nm excitation and  
158 5 nm emission slit widths. The excitation wavelength was set at 488 nm, and the emission  
159 spectra were recorded between 510–650 nm. The synchronous fluorescence spectra were  
160 collected with interval  $\Delta\lambda = 10$  nm, where  $\Delta\lambda = \lambda_{\text{em}} - \lambda_{\text{ex}}$ .

161 The binding of metal ions to R-PE was studied by the fluorescence quenching titration  
162 method using intrinsic fluorescence of R-PE. The experimental details are provided in the  
163 Supplementary material.

## 164 **2.6. CD spectroscopy measurements**

165 CD measurements were carried out on Jasco J-815 spectropolarimeter (Jasco, Japan)  
166 under constant nitrogen flow. All spectra were recorded at 25°C. For the near-UV and visible  
167 region (250–600 nm), the concentration of R-PE was 0.25 µM, while the concentration of  
168 Cu<sup>2+</sup>/Zn<sup>2+</sup> was 10 µM. Buffer and metal ion spectra were subtracted from spectra of R-PE and  
169 R-PE/metal complexes, respectively. Scan speed and number of accumulations were set to  
170 200 nm/min, and two accumulations. A cell with an optical path length of 1 cm was used.

171 Far-UV CD spectra of 0.67 µM R-PE in the absence or presence of 27 µM Zn<sup>2+</sup> or  
172 Cu<sup>2+</sup> were recorded in the range 185–260 nm, at a scan speed of 50 nm/min, using a cell with  
173 an optical path length of 0.1 mm and with an accumulation of two scans. Buffer and metal ion  
174 spectra were subtracted from spectra of R-PE and R-PE/metal complexes, respectively.  
175 Secondary structure content was calculated by CD Pro software, using the SELCON  
176 algorithm and SP37 database.

177 Thermal unfolding of 75 nM R-PE was performed at the temperature range of 32–  
178 95°C, with a temperature increasing rate 2°C/min, while equilibration time was set to 1 min  
179 before each measurement. Ellipticity was measured in the far-UV region at 222 nm with  
180 pathlength cells of 1 cm. Results were normalised between 0 and 100 and expressed as a  
181 dependence of the percentage of R-PE unfolding on temperature. We fitted obtained data  
182 using the model, which comprises two transitions described previously (Greenfield, 2006),  
183 with slight modifications (see Supplementary Materials for further details on data analysis).

## 184 **3. RESULTS**

### 185 **3.1. Purification of R-phycoerythrin**

186 We performed a combination of precipitation and chromatography techniques to  
187 obtained pure R-PE from Nori flakes. Purification of R-PE has been monitored using UV/VIS  
188 absorption spectroscopy and SDS-PAGE. The raw extract exhibited strong absorption in the  
189 220–400 nm region due to various pigments. Ammonium sulfate precipitation (65%) induced  
190 a substantial increase in ratios A<sub>560</sub>/A<sub>280</sub> (from 0.2 to 1.0; **Fig. 1B**) by removing most of the  
191 non-protein impurities absorbing in the region 220–400 nm. This step did not distinguish  
192 between different PBPs, as could be concluded from the presence of R-PC and APC  
193 absorption bands in the region 600–700 nm (**Fig. 1B**). The bands on SDS-PAGE at 18 kDa  
194 after visualisation of PBPs fluorescence (**Fig. 1D**) and CBB staining (**Fig. 1D**) confirmed the  
195 presence of R-PC and APC. R-phycoerythrin has been mostly separated from R-PC and APC  
196 using hydroxyapatite chromatography in the batch mode (**Fig. 1**). The non-bound fraction



197 with light reddish-pink colour (**Fig. 1C**) contained R-PE with a remarkable increase of  
198  $A_{560}/A_{620}$  ratio (8.9) in comparison to the sample before chromatography (2.1). At the same  
199 time, R-PC and APC remained bound to the HA matrix (**Fig. 1C**), and the band's absence at  
200 18 kDa was observed (**Fig. 1D**). Although R-PC and APC were removed in this step, the  
201  $A_{560}/A_{280}$  ratio was still low (1.1), indicating other proteins not belonging to the PBP family.  
202 Therefore, we have performed anion-exchange chromatography on DEAE-Sepharose to  
203 purify R-PE further. R-phycoerythrin has been eluted with 150 mM NaCl, and a significant  
204 increase in  $A_{560}/A_{280}$  (5.1) has been observed, indicating that pure R-PE is obtained.  
205 Additionally, SDS-PAGE of IEC fraction of R-PE showed only bands arising from R-PE: a  
206 strong band at ~20 kDa, corresponding to  $\alpha$  and  $\beta$  subunits, and two discrete bands between  
207 30 and 35 kDa corresponding to  $\gamma$  subunits (**Fig. 1D**). Overlapped  $\alpha$  and  $\beta$  subunits at 20 kDa  
208 resulted from similar molecular masses (18.9 and 20.2 kDa, respectively) that could not be  
209 separated under applied conditions.

### 210 **3.2. Spectroscopic characterisation of purified R-phycoerythrin**

211 The absorption spectra of purified R-PE showed the typical peak at 560 and shoulder  
212 at 545 originating from the PEB chromophore. In comparison, the PUB chromophore gave a  
213 characteristic peak at 496 nm (**Fig. 1B**). Excitation of R-PE at 488 nm revealed the broad,  
214 distinct peak at 573 nm with the shoulder at 620 nm (**Fig. 2A**). The absence of symmetry in  
215 emission spectra of R-PE has been already described as the consequence of multiple  
216 interacting PEB and PUB chromophores in the molecule (**Gaigalas, et al., 2006**). A  
217 synchronous fluorescent spectrum could overwhelm challenges in interpreting classical  
218 fluorescence spectra of molecules with different fluorophores. Indeed, the synchronous  
219 spectrum of R-PE gave a narrow, symmetric peak at 573 nm without shoulder at 620 nm (**Fig.**  
220 **2B**). The asymmetric environment of tetrapyrrole chromophores within R-PE induced strong  
221 signals in its near-UV/VIS CD spectra. Consistent with absorption spectroscopy, three peaks  
222 were observed in the VIS region originating from both PEB (540 and 570 nm) and PUB (498  
223 nm) chromophores (**Fig. 2C**). Additionally, a strong peak at 306 nm is observed from a higher  
224 excitation state of the PEB chromophore (**Fig. 2C**) (**Glazer & Hixson, 1975**).

225 Secondary structures of R-PE was characterised by CD spectroscopy. Far-UV CD  
226 spectrum of R-PE at pH 7.0 showed negative ellipticity between 200 and 240 nm, with two  
227 minima at 208 and 221 nm and a positive peak at 193 nm (**Fig. 2D**), a typical characteristic of  
228  $\alpha$ -helical proteins. The calculated  $\alpha$ -helical content (75%) in R-PE is in agreement with  
229 previously published data, where the percentage of  $\alpha$ -helix varies in the range 60–79%

230 (D'Agnolo, Rizzo, Paoletti, & Murano, 1994; MacColl, et al., 1999; Ritter, Hiller,  
231 Wrench, Welte, & Diederichs, 1999).

### 232 3.3. R-phycoerythrin thermal stability

233 The decrease in R-PE ellipticity at 222 nm following heating, as a consequence of  $\alpha$ -  
234 helical loss, helps to study protein stability. The representative melting curve of R-PE is  
235 shown in **Fig. 3A**. Interestingly, the unfolding curve of R-PE showed two transitions with  
236 melting points at 56.6 and 72.6°C, and corresponding unfolding enthalpies of 263.6 and 575.4  
237 kJ/mol. This phenomenon could be ascribed to the heteromeric structure of R-PE.

238 In the second approach, we studied the effects of temperature on VIS absorption  
239 spectra of R-PE. An increase of temperature up to 45°C induced a slight decrease in  
240 absorbance in the whole spectrum, without changes in the shape of spectra (**Fig. 3B**). Further  
241 heating of the R-PE sample caused a significant reduction of absorption intensity, followed by  
242 the complete disappearance of the PEB band (545-560 nm) at 85°C (**Fig. 3B**). In comparison,  
243 the PUB band (496 nm) at 85°C still existed, but its spectral shape was significantly changed  
244 as the peak became broader (**Fig. 3B**). Absorbances at 560 and 496 nm were decreased by 86  
245 and 52%, respectively, at 85°C. Therefore, PUB chromophore exhibits better thermal stability  
246 in comparison to PEB chromophore. Lowering the temperature from 85 to 25°C, the PEB  
247 band could not recover, while the PUB band was still broad (**Fig. S1**), indicating irreversible  
248 denaturation of R-PE.

### 249 3.4. R-phycoerythrin high-pressure stability

250 We performed *in situ* HP VIS absorption spectroscopy to probe R-PE stability at HP  
251 conditions. Changes in a well-defined VIS absorption spectrum of R-PE could give valuable  
252 information on the effect of the treatment on protein dissociation and unfold. A moderate  
253 increase of pressure, up to 90 MPa, did not induce significant changes in the absorption  
254 spectra of R-PE, except that shoulder at 545 nm became more visible, and the slight redshift  
255 (from 496 to 498 nm) of the PUB band has been observed (**Fig. 3C**). Further increase of  
256 pressure, up to 150 MPa, induced a slight decrease of absorption intensity at 560 and 498 nm,  
257 while an additional increase in absorbance at 545 nm has been observed. Above 150 MPa,  
258 there was a significant decrease in absorbance in the whole spectrum. The most noticeable  
259 change in the protein spectrum was the disappearance of the fine peak structure at 560 nm: at  
260 360 MPa, it is wholly merged with the rest of the PEB band. An increase of pressure to 450  
261 MPa induced an additional decrease in absorption intensities: at 560 and 498 nm, absorbances  
262 were lower for 37 and 26%, respectively, than 0.1 MPa (**Fig. 3C**). This result indicated that

263 the PUB band is better preserved at HP conditions, in line with high-temperature  
264 measurements.

265 Based on the disappearance of the fine structure of the PEB peak at 560 nm, we  
266 constructed a dissociation/unfolding curve (**Fig. 3D**), and the obtained result is well fitted  
267 with the two-transition model (equation 2 in Supplementary Materials). This model enables us  
268 to extract the volume changes ( $\Delta V$ ), Gibbs free energies of dissociation/unfolding ( $\Delta G$ ) and  
269 the values of half-denaturation/dissociation pressure ( $P_{1/2}$ ) (**Table 1**). There were significant  
270 differences in  $\Delta G$  and  $P_{1/2}$  values, while the difference in the volume change was not so  
271 pronounced between the two transitions (**Table 1**). Interestingly, plotting the dependence of  
272 the redshift of the PUB band on pressure gave the one-transition curve (**Fig. S2**) with the  
273 fitting parameters very similar to the values obtained by fitting the first transition of the PEB  
274 band (**Table 1**; Equation 5 in Supplementary Materials). R-phycoerythrin unfolding is  
275 followed by a significant decrease in its PUB and PEB bands absorbance (**Liu, et al., 2009**).  
276 At ~110 MPa, where the first transition of the PEB band and the redshift of the PUB band  
277 occur, there was a negligible decrease in absorbance. Therefore, it is unlikely that the first  
278 transition is due to protein unfolding. A more plausible explanation is that a change in  
279 interactions between chromophores and subunits occurs at lower pressures (~110 MPa) during  
280 the first transition. Indeed, the previous studies proved that the fine structure of peak at 560  
281 nm arises from dimers of PEB chromophores at the subunit interfaces within R-PE hexamer  
282 (**Gaigalas, et al., 2006; Womick, Liu, & Moran, 2011**). Therefore, partial dissociation of R-  
283 PE hexamer induced the loss of fine structure of PEB band at 560 nm through dissociation of  
284 PEB dimers at the subunit interfaces. The second transition at ~250 MPa was followed by the  
285 disappearance of the fine structure of the PEB peak at 560 nm and with a significant decline  
286 in absorption intensity, suggesting that both proteins unfolding and dissociation occur.

287 Decreasing the pressure from 450 to 0.1 MPa induced recovering the fine structure of  
288 the peak at 560 nm. However, the absorption intensity was only partially recovered: R-PE's  
289 absorbances after HP treatment at 560 and 496 nm were lower for 24 and 16%, respectively,  
290 compared to its spectrum before treatment (**Fig. S3**). Therefore, similar to *in situ* study, *ex-*  
291 *situ* measurement showed better preservation of the PUB band than the PEB band upon HP  
292 treatment.

### 293 **3.5. The binding of Cu<sup>2+</sup> and Zn<sup>2+</sup> ions to R-phycoerythrin**

294 R-Phycoerythrin exhibited a strong fluorescence at 573 nm, but adding Zn<sup>2+</sup> and Cu<sup>2+</sup>  
295 ions strongly quenched its fluorescence intensity (**Figs. 4A and 4B**). In general, there are two

296 types of quenching: static (contact) and dynamic (collisional). Stern-Volmer (SV) plots (**Fig.**  
297 **S4**) for both metal ions were linear ( $r^2$  is 0.993 and 0.989 for  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$ , respectively),  
298 proving that only one type of quenching was present under applied conditions. The slope of  
299 these curves represented an SV quenching constant of  $1.7 \times 10^3 \text{ M}^{-1}$  ( $\text{Zn}^{2+}$ ) and  $0.83 \times 10^6 \text{ M}^{-1}$   
300 ( $\text{Cu}^{2+}$ ), with a bimolecular quenching rate constant of  $2.4 \times 10^{11}$  ( $\text{Zn}^{2+}$ ) and  $1.2 \times 10^{14} \text{ M}^{-1} \text{ s}^{-1}$   
301 ( $\text{Cu}^{2+}$ ), one and four orders of magnitude higher than the diffusion rates of biomolecules ( $10^{10}$   
302  $\text{M}^{-1} \text{ s}^{-1}$ ). These data suggest that static (contact) quenching of R-PE fluorescence by  $\text{Zn}^{2+}$  and  
303  $\text{Cu}^{2+}$  ions occurs through the stable complex formation between R-PE fluorophores and metal  
304 ions. Changes in absorption and near-UV/VIS CD spectra of R-PE (**Figs. 5A** and **5B**) in the  
305 presence of these metal ions also confirmed a static mechanism of quenching of protein  
306 fluorescence.  $\text{Cu}^{2+}$  shows a more substantial quenching effect on R-PE fluorescence than  $\text{Zn}^{2+}$   
307 (**Figs. 4A** and **4B**). Consequently, the binding constant of  $\text{Cu}^{2+}$  to R-PE ( $6.3 \times 10^5 \text{ M}^{-1}$ ; **Fig.**  
308 **4D**) is much higher than the affinity of  $\text{Zn}^{2+}$  ( $1.74 \times 10^3 \text{ M}^{-1}$ ; **Fig. 4C**).

309 The effects of metal ions binding on the conformation of tetrapyrrole chromophores of  
310 R-PE were evaluated by absorption and CD spectroscopy. The presence of  $\text{Cu}^{2+}$  induces a  
311 moderate decrease of absorbances at 496 nm (13%), 560 nm (10%), and 545 nm (14%) in  
312 comparison to free R-PE (**Fig. 5A**). On the contrary, there was a discrete effect of  $\text{Zn}^{2+}$  on the  
313 absorption spectrum of protein: the slight decrease of band intensity at 496 nm was observed  
314 (5%). The changes of absorbances at 560 and 545 nm were negligible, except for the slight  
315 change in the shape of the shoulder at 545 nm. The binding of  $\text{Cu}^{2+}$  significantly influenced  
316 near-UV/VIS CD spectra of R-PE (**Fig. 5B**). The most considerable reduction in band  
317 intensities was at 306 nm (33%) and 545 nm (25%), suggesting the preferential  $\text{Cu}^{2+}$  binding  
318 to PEB chromophore. In opposite to absorption spectra, near-UV/VIS CD spectra of R-PE  
319 were sensitive to the presence of  $\text{Zn}^{2+}$  (**Fig. 5B**). The intensity of the PUB band (498 nm) was  
320 reduced by 23%, while the shape of the PEB band at 545 nm was changed entirely, becoming  
321 more narrow and redshifted by 5 nm, followed by a 6% decrease of intensity. The changes in  
322 the shape and intensities of PEB bands at 306 and 570 nm upon  $\text{Zn}^{2+}$  addition are almost  
323 negligible. Therefore, zinc ions are preferably bound to the PUB chromophore, although  $\text{Zn}^{2+}$   
324 binding to PEB chromophore could also be observed, with an absorption/ellipticity peak at  
325 545 nm. The binding constant of R-PE for  $\text{Zn}^{2+}$ , based on synchronous fluorescence spectra  
326 data (**Fig. S5**) when PEB chromophore is selectively excited, was lower by 36% ( $1.1 \times 10^3 \text{ M}^{-1}$ )  
327 compared to the value using classical fluorescence spectra (**Figs. 4A** and **C**;  $1.7 \times 10^3 \text{ M}^{-1}$ ).  
328 Zinc ions have a higher preference to bind PUB chromophores, and selective excitation of

329 PEB chromophores disabling the energy transfer from PUB to PEB, which seems to produce a  
330 less pronounced quenching effect  $Zn^{2+}$  on PEB chromophore in synchronous spectra.

331 Finally, far-UV CD spectra were recorded to estimate the effects of metal ions binding  
332 on secondary structures of R-PE. The binding of metal ions did not crucially change the  
333 spectral region between 208–222 nm (**Fig. 5C**). The only change was observed in band  
334 intensity at 193 nm upon adding  $Cu^{2+}$  (**Fig. 5C**). As described before, the principal secondary  
335 structure in free R-PE is  $\alpha$ -helix (75%), while the presence of  $Cu^{2+}$  (74%) and  $Zn^{2+}$  (77%) has  
336 a discrete effect on the  $\alpha$ -helical content in protein.

#### 337 **4. DISCUSSION**

338 In the present study, we combined different experimental approaches to probe the  
339 stability of food-derived nutraceutical R-PE. We report that high-pressure treatment had  
340 significantly less detrimental effects on R-PE stability and colour than thermal treatment. The  
341 binding of selected bioactive metal ions ( $Cu^{2+}$  and  $Zn^{2+}$ ) to R-PE induced substantial  
342 conformational changes in covalently bound tetrapyrrole chromophores without affecting  
343 protein colour and only minor changes in protein secondary structure content.

344 R-phycoerythrin is purified from commercial dried Nori flakes, obtained from red  
345 algae *Porphyra purpurea* collected at the Atlantic coast of Spain. In the algal cells, R-  
346 phycoerythrin with other homologous PBPs (R-PC and R-PE) forms phycobilisomes, large  
347 photosynthetic complexes (**Apt, et al., 1993**). Therefore, the significant obstacle in R-PE  
348 purification is to remove R-PC and APC from the extract. We tackle this challenge by simple  
349 sample application of HA resin in batch mode, under the condition where R-PE is the only  
350 PBP not bound to the matrix. IEC removed the other proteins. The obtained R-PE's purity  
351 ratio of 5.1 ( $A_{560}/A_{280}$ ) was considerably higher than the commonly accepted criterion for R-  
352 PE purity of 3.2 (**Nikolic, et al., 2020**). SDS-PAGE justified the heteromeric structure of R-  
353 PE with two bands arising from  $\gamma$  subunit ( $\gamma_{33}$  and  $\gamma_{31}$ ), belonging to the isoforms of R-PE  
354 containing two different  $\gamma$  subunits (**Apt, et al., 1993**).

355 We further utilised absorption, fluorescence and CD spectroscopy to characterise  
356 purified phycobiliprotein. It exhibited a typical characteristic of R-PE: well-defined  
357 absorption and near-UV/VIS CD bands, and strong fluorescence detectable at pM  
358 concentration (**Gaigalas, et al., 2006; Liu, et al., 2009; Munier, et al., 2014**). The high  $\alpha$ -  
359 helical content confirmed that the native R-PE structure is preserved in dried Nori flakes. An  
360 increase in a temperature-induced gradual decrease of  $\alpha$ -helix ellipticity, giving the unfolding  
361 R-PE curve with two transitions: at 56°C and 72°C. A previous CD study on the thermal

362 unfolding of B-PE from *Porphyridium cruentum* also demonstrates two transitions  
363 (**Gonzalez-Ramirez, et al., 2014**). The thermal unfolding of co-isolated R-PC exhibited only  
364 transition at  $\sim 55^{\circ}\text{C}$  (data not shown). R-phycoerythrin only contains  $\alpha$ - and  $\beta$ -subunits  
365 (homologous to R-PE subunits) but not  $\gamma$  subunit. Therefore, the second transition ( $72^{\circ}\text{C}$ ) in  
366 the R-PE unfolding curve seems to result from a more stable  $\gamma$  subunit in the protein oligomer  
367 structure. The BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>)  
368 found high homology only between  $\alpha$  and  $\beta$  subunits, implying the different stability of the  $\gamma$   
369 subunit. Absorption measurements confirm findings from previous studies (e.g., **Munier, et**  
370 **al., 2014**) that the R-PE colour is not stable above  $40^{\circ}\text{C}$  and that the PEB band in the  
371 spectrum was much more sensitive to thermal treatment than the PUB band. With three PUB  
372 chromophores,  $\gamma$ -subunit mainly contributes to the overall absorption of the PUB band  
373 (**Gaigalas, et al., 2006**). Therefore, combining results from absorption and far-UV CD  
374 thermal unfolding measurements, it is evident that the  $\gamma$  subunit of R-PE is more stable than  
375 the  $\alpha$  and  $\beta$  subunits.

376 We have tested the effects of HP on R-PE stability up to 450 MPa, the value usually  
377 used for food processing (**Huang, Hsu, & Wang, 2020**). In situ HP VIS absorption study has  
378 shown that the PEB spectral band undergoes two transition processes. The first transition  
379 ( $\sim 110$  MPa) is ascribed to the R-PE oligomer dissociation, while the second ( $\sim 250$  MPa) is  
380 due to R-PE unfolding and additional dissociation. Subunit dissociation indeed preceded the  
381 protein unfolding (**Minic, et al., 2020**). Interaction between subunits in multimeric proteins  
382 (such as R-PE) causes cavities at subunit interfaces, which are eliminated at moderate  
383 pressures. Thermodynamic parameters of the first transition of the PEB band correspond with  
384 those obtained by studying the redshift of the PUB band. The HP-induced redshift of the PUB  
385 band indicates the changes in the level of the  $\gamma$  subunit, probably dissociating from the R-PE  
386 complex. It has been shown that the  $\gamma$  subunit lies in the central cavity of the R-PE disc,  
387 stabilising the  $\alpha\beta$  hexamer (**Chang, et al., 1996**). It looks that HP induces dissociation of  $\gamma$   
388 subunit from R-PE and destabilises  $\alpha\beta$  hexamer, dissociating to  $\alpha\beta$  trimers,  $\alpha\beta$  monomers or  
389 individual subunits. Further increase of pressure causes elimination of cavities within subunits  
390 and, as a final consequence, protein unfolding occurs. In accordance with temperature  
391 measurements, we found that the PUB spectral band is more resistant than the PEB band to  
392 HP treatment, confirming the higher stability of  $\gamma$  subunits compared to  $\alpha$  and  $\beta$  subunits.

393 High-pressure has a prominent advantage in various food treatments, preserving  
394 nutrients, colours and flavours. We have shown that after HP treatment at 450 MPa, the shape  
395 of the absorption spectrum of R-PE is completely recovered without detrimental loss of

396 absorption intensity. On the contrary, thermal treatment of R-PE induces irreversible,  
397 complete loss of the PEB band (colour bleaching). Therefore, HP treatment of R-PE  
398 containing food and beverages could be an excellent alternative to thermal processing to avoid  
399 losing vivid R-PE colour. It was already shown the absence of irreversible changes in B-PE  
400 structure after HP treatment of Porphyrinium cruentum extract at 400 MPa (**Tran, et al.,**  
401 **2019**). Further, the storage stability of the blue C-PC from Spirulina in beverages was  
402 obtained by HP processing at 450 MPa (**Zhang, Cho, Dadmohammadi, Li, &**  
403 **Abbaspourrad, 2021**).

404 The binding of metal ions could substantially influence the colour and fluorescence of  
405 PBPs (**Suresh, et al., 2009**). Significant quenching of the R-PE fluorescence at micromolar  
406 concentrations of  $\text{Cu}^{2+}$  in our study (**Fig. 4B**) indicates a strong interaction between protein  
407 and this essential microelement. The measured binding constant ( $6.3 \times 10^5 \text{ M}^{-1}$ ) was in the  
408 same order of magnitude as  $\text{Cu}^{2+}$  affinity for C-PC and phycocyanobilin from Spirulina  
409 (**Minic, et al., 2016**). The binding affinity of  $\text{Zn}^{2+}$  was significantly lower ( $1.7 \times 10^3 \text{ M}^{-1}$ ).  
410 According to near-UV/VIS CD spectra of R-PE, binding of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  induced significant  
411 changes in the chiral environment of tetrapyrrole chromophores. In the presence of these ions  
412 at  $10 \mu\text{M}$  ( $\sim 0.6 \text{ ppm}$ ), only minor (10% for  $\text{Cu}^{2+}$ ) and negligible ( $< 5\%$  for  $\text{Zn}^{2+}$ ) decrease in  
413 PEB band absorbance was observed. These microelements are usually present in beverages at  
414 similar concentrations (**Fernandez-Lopez, Gomez-Nieto, Gismera, Sevilla, & Procopio,**  
415 **2018**). Consequently, they should not affect the colour of drinks fortified with R-PE.  
416 Tetrapyrrole chromophores in extended conformations exhibit a large fluorescence quantum  
417 yield, while its cyclisation reduces the fluorescence of PBPs (**Bocker, et al., 2020**). It seems  
418 that the binding of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  caused conformational changes of chromophores from  
419 extended to cyclic ones, which are more prone to bind metal ions (**Suresh, et al., 2009**).

420 Near-UV/VIS CD and absorption measurements revealed that these metal ions have  
421 nonidentical preferences to different chromophores within R-PE:  $\text{Zn}^{2+}$  for PUB chromophore,  
422 while  $\text{Cu}^{2+}$  on the PEB at position 545. Both metal ions had the lowest binding to the PEB  
423 560. This chromophore in the R-PE structure makes a dimer at the interface between  $\alpha$  and  $\beta$   
424 subunits, introducing the steric hindrance and limiting metal ions binding potency (**Gaigalas,**  
425 **et al., 2006; Womick, et al., 2011**). Although the conformation of tetrapyrrole chromophores  
426 was changed, far-UV CD measurements did not show significant changes in the secondary  
427 structure content of R-PE upon metal ions binding, confirming that R-PE is still well folded in  
428 the presence of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ .

429           Herein presented results open up attractive possibilities for further investigation and  
430 application of R-PE. Many factors limit the bioavailability of dietary Zn and Cu in some food  
431 systems (**Wang, Zhou, Tong, & Mao, 2011**). R-phycoerythrin complexation of Zn<sup>2+</sup> and  
432 Cu<sup>2+</sup> could increase their bioavailability in the gastrointestinal tract. Indeed, it was shown that  
433 some proteins and peptides could form complexes with Zn<sup>2+</sup> and Cu<sup>2+</sup> ions, which improve  
434 their absorption and bioavailability in intestinal conditions (**Guo, et al., 2014; Wang, et al.,**  
435 **2011**). The free Cu<sup>2+</sup> ions generate reactive oxygen species in vivo (**Shin, et al., 2012**). The  
436 ability of R-PE to tightly bind cupric ions could prevent their pro-oxidative action.  
437 Fluorescent biosensors for detecting heavy metal ions in aqueous media (e.g., water and  
438 beverages) have recently gained popularity. They provide high specificity and low detection  
439 limits, fast response time, and technical simplicity (**De Acha, Elosua, Corres, & Arregui,**  
440 **2019**). The ability of Cu<sup>2+</sup> ions to significantly decrease fluorescence of R-PE even at 0.5 µM  
441 concentration (32 ppb) in our study indicates that R-PE could be exploited as a potential  
442 biosensor for Cu<sup>2+</sup> in aquatic life systems. Interestingly, the ability of C-phycoerythrin, PBP  
443 from *Spirulina*, to serve as possible biosensors for Hg<sup>2+</sup> ions was demonstrated (**Bhayani,**  
444 **Mitra, Ghosh, & Mishra, 2016**).

## 445 **5. CONCLUSIONS**

446           In the present study, we examined the stability of R-PE obtained from commercial  
447 dried Nori flakes using spectroscopic methods. Thermal unfolding of R-PE exhibits a two-  
448 transition process due to the different stability of R-PE subunits. Protein heating at 85°C  
449 irreversibly denatures R-PE with detrimental effects on its colour. Moderate pressure (~110  
450 MPa) induces dissociation of R-PE hexamer, while the further increase of pressure (~250  
451 MPa) causes protein unfolding. Opposite to temperature, HP treatment at 450 MPa has far  
452 less destructive effects on R-PE colour intensity. At the same time, absorption bands are  
453 mostly recovered after a return to atmospheric pressure. R-phycoerythrin exhibited moderate-  
454 high- and low-affinity binding for Cu<sup>2+</sup> and Zn<sup>2+</sup> ions, respectively. Although both ions  
455 significantly influence the conformation of chromophores within R-PE, there was no  
456 substantial effect on protein colour or its secondary structure content.

457           Our study results suggest that HP processing of R-PE containing food and beverages  
458 could preserve protein red colour, a significant advantage compared to the thermal treatment.  
459 Further studies are needed for an additional improvement of the HP stability of R-PE. The  
460 presence of Cu<sup>2+</sup> and Zn<sup>2+</sup> ions does not have a high probability of impairing R-PE function as  
461 a food colourant. On the contrary, the binding to R-PE could improve their bioavailability in



462 the gastrointestinal tract. Therefore, food fortification with copper and zinc ions in the  
463 presence of R-PE could be a promising approach for the more efficient delivery of these  
464 microelements.

## 465 **AUTHOR CONTRIBUTIONS**

466 **Ana Simovic:** Conceptualisation, Formal analysis, Investigation. **Sophie Combet:**  
467 Investigation, Writing - review & editing. **Tanja Cirkovic Velickovic:** Funding acquisition,  
468 Writing - review & editing. **Milan Nikolic:** Conceptualisation, Formal analysis, Funding  
469 acquisition, Investigation, Supervision, Writing - review & editing. **Simeon Minic:**  
470 Conceptualisation, Formal analysis, Funding acquisition, Investigation, Supervision, Writing -  
471 original draft.

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## 475 **FIGURE LEGENDS**

476 **Figure 1.** (A) Ribbon model of the crystal structure of R-PE hexamer (PDB: 5B13).  $\gamma$  subunit  
477 occupies the central cavity of the R-PE hexamer. However, it is not visible in the electron  
478 density of R-PE crystal structure (Miyabe, et al., 2017); (B) UV/VIS absorption spectra of R-  
479 PE after each purification step, as indicated; (C) Separation of R-PE (light reddish pink  
480 solution) from R-PC and APC (light purple, bound fraction) using HA chromatography in  
481 batch mode; (D) SDS-PAGE profile of R-PE purification (14% gel) after staining with CBB  
482 (left) and Zn<sup>2+</sup>-induced fluorescence visualisation (right,  $\lambda_{ex}$ : 473 nm,  $\lambda_{em}$ : 580 nm): 1: raw  
483 extract; 2: ammonium sulfate precipitation (65%); 3: HA chromatography (supernatant); 4:  
484 IEC on DEAE Sepharose (150 mM NaCl); M-molecular size markers.

485 **Figure 2.** (A) Classical ( $\lambda_{ex}$ = 488 nm) and (B) synchronous fluorescence spectra ( $\Delta\lambda$ = 10  
486 nm) of R-PE (pH 7.0); Near-UV/VIS (C) and far-UV CD (D) spectra of R-PE (pH 7.0).

487 **Figure 3.** (A) The R-PE (75 nM) thermal unfolding curve with the corresponding fit (full red  
488 line), obtained by measuring ellipticity at 222 nm and pH 7.0; (B) The effects of temperature  
489 on VIS absorption spectra of 0.37  $\mu$ M R-PE at pH 7.0 (optical pathlength 1 cm); (C) The  
490 effects of HP on VIS absorption spectra of 0.37  $\mu$ M R-PE at pH 7.0 and 20°C (optical  
491 pathlength 0.5 cm): Inset represents the redshift of the band at 496 nm; (D) The R-PE

492 dissociation/unfolding curve with the corresponding fit (full red line) obtained by monitoring  
493 the disappearance of peak at 560 nm.

494 **Figure 4.** R-PE fluorescence quenching (excitation at 488 nm) in the presence of Zn<sup>2+</sup> (**A**)  
495 and Cu<sup>2+</sup> (**B**) ions at 25°C and pH 7.0; Fluorescence quenching based plots for determination  
496 of binding constants of Zn<sup>2+</sup> (**C**) and Cu<sup>2+</sup> (**D**) to R-PE.

497 **Figure 5.** The effects of Zn<sup>2+</sup> and Cu<sup>2+</sup> binding on VIS absorption (**A**), near-UV/VIS CD (**B**)  
498 and far-UV CD (**C**) spectra of R-PE at pH 7.0 and 25°C.

## 499 TABLES

Transition	$\Delta V$ (mL/mol)	$\Delta G$ (kJ/mol)	$P_{1/2}$ (MPa)
1 <sup>st</sup> transition in PEB band	$-79 \pm 9$	$8.2 \pm 0.5$	$107 \pm 7$
2 <sup>nd</sup> transition in PEB band	$-58 \pm 10$	$14.9 \pm 3.3$	$247 \pm 16$
The redshift of the PUB band	$-70 \pm 6$	$8.1 \pm 0.6$	$115 \pm 3$

**Table 1.** Thermodynamic parameters of pressure unfolding/dissociation for R-PE sample, obtained from HP-VIS absorption spectroscopy at 20°C and pH 7.0.

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