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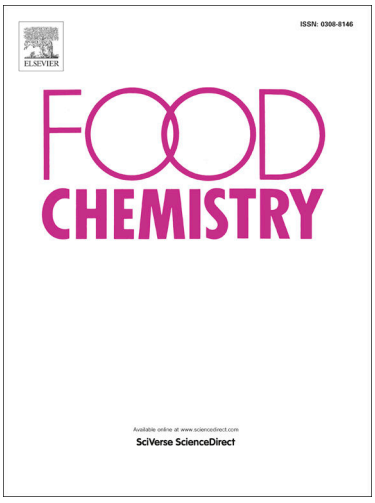

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Optimization of phycobiliprotein pigments extraction from red algae *Gracilaria gracilis* for substitution of synthetic food colorants

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Abstract

The extraction of phycobiliprotein (PBP) pigments from red algae *Gracilaria gracilis* was optimized using maceration, ultrasound-assisted extraction (ultrasonic water bath and ultrasonic probe), high pressure-assisted extraction, and freeze-thaw. The experimental conditions, namely homogenization time (t1), buffer concentration (C), treatment time (t2), biomass: buffer ratio (R), and pressure (P), were optimized using Response Surface Methodology (RSM). The yield of phycoerythrin (PE) extracted, determined spectroscopically, was used as the response variable. Maceration was the most efficient extraction method yielding 3.6 mg PE /g biomass under the optimal conditions (t1 = t2 = 10 min; C = 0.1 M; R = 1:50). Scanning Electron Microscopy (SEM) analysis of the biomass before and after the cell disruption treatments revealed a more efficient cell wall rupture with maceration.

Keywords: natural pigments; Phycobiliproteins; *Gracilaria gracilis*; Response Surface Methodology; extraction optimization.

1 Introduction

Phycobiliproteins (PBPs) are non-toxic water-soluble proteins mostly found in Rhodophyta (red algae), Cyanobacteria, and Cryptophyta. Due to their strong absorbance and fluoresce properties as well as antioxidant and free-radical scavenging activities, PBPs have been widely employed in food, cosmetics, pharmaceutical, and biomedical industries (Sonani, Rastogi, Patel, & Madamwar, 2016). Since purified PBPs are expensive, the search for more effective extraction and purification methods is desirable. PBPs are present in the thylakoid membrane in the stroma inside the seaweed's chloroplast so their efficient extraction typically requires the use of appropriate solvents and cell disruption methods (Beattie, Morançais, Déléris, Fleurence, & Dumay, 2018). Red algae of the *Gracilaria* genus are a rich source of PBPs, namely phycoerythrin (PE), being valuable resources for industrial and biotechnological applications (Francavilla, Franchi, Monteleone, & Caroppo, 2013).

The most widely used solvents in PBPs extraction are phosphate buffer, distilled water, and seawater (Sudhakar, Jagatheesan, Perumal, & Arunkumar, 2015). Several methods have been studied for protein extraction such as homogenization (Dumay, Morançais, Nguyen, & Fleurence, 2015), maceration in presence of liquid nitrogen (Munier et al., 2014), maceration with mortar and pestle (Sudhakar et al., 2015), freeze grinding (Fleurence, 2003; Galland-Irmouli et al., 2000), freezing and thawing (Senthilkumar, Kurinjimalar, et al., 2013), and ultrasonication (Le Guillard et al., 2015).

Among conventional extraction methods, maceration is one of the most used, due to its low economic impact, short extraction time, and easy setup (Beattie et al., 2018). Freeze-thaw is another conventional method widely used for cellular disruption with advantages such as high reproducibility and robustness, achieving higher purity extracts (Kannaujiya, Sundaram, & Sinha, 2017). As a non-conventional extraction methodology considered a 'green technology', ultrasound-assisted extraction presents a significant reduction in extraction time, solvent consumption, with higher extraction yields at lower temperatures, being suitable for thermolabile compounds (Juin et al., 2015; Mittal, Tavanandi, Mantri, & Raghavarao, 2017). Another modern, non-conventional alternative is high pressure-assisted extraction. It can be a fast and highly efficient extraction method, easily operated and highly mechanized, requiring

low amounts of solvent. Moreover, it can be operated at room temperature, protecting the bioactivity of compounds with low thermal stability, which is useful in the extraction of heat-sensible chromoproteins (Alexandre et al., 2017; Huang, Hsu, Yang, & Wang, 2013; Santos, Salvador, Domingues, Cruz, & Saraiva, 2013).

The extraction yield is highly influenced by numerous factors, such as the biomass: solvent ratio, the cellular disruption method, the solvent used, and the extraction time (Beattie et al., 2018). The optimization of the influence of such experimental conditions may be exhaustive and laborious (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). Response Surface Methodology (RSM) is a statistical tool that can be used to determine and optimize the optimal experimental conditions to achieve maximum yields with minimum time and resources consumption(Asfaram, Ghaedi, Abidi, et al., 2018; Asfaram, Ghaedi, Javadian, & Goudarzi, 2018; Khuri A. I., 2017; Taofiq et al., 2019). In the present study, *Gracilaria gracilis*, a red alga from the Portuguese coast, was used as a natural source for the extraction of PBP pigments for application as food colorant. Several extraction techniques were used, namely maceration, ultrasounds, freeze-thaw, and high pressure, and the experimental conditions optimized using RSM.

2 Materials and Methods

2.1 Biomass

Red macroalgae *Gracilaria gracilis* was collected in October 2018 from the intertidal zone of Buarcos, Figueira da Foz (40.1773° N, 8.8749° W), Portugal. The algae were washed with salt water (35% (w/v)), frozen at -80 ºC and freeze-dried. The freeze-dried biomass was ground using a mixer and stored in a dry recipient in the absence of light until further studies.

2.2 Chemicals

Sodium phosphate buffer pH 6.8 (Na_2HPO_4/NaH_2PO_4) was prepared using disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and sodium phosphate monobasic anhydrous (NaH_2PO_4) purchased from Scharlau and AMRESCO, respectively.

2.3 Extraction procedure

Different extraction methods and conditions were tested. The extraction methods used were both conventional, such as maceration and freeze-thaw, and non-conventional, like ultrasound-assisted extraction (ultrasonic water bath and ultrasonic probe) and high pressure-assisted extraction. The extraction conditions optimized were homogenization time (t1 = homogenization of biomass in phosphate buffer), buffer concentration (C), treatment time (t2 = time of application of the extraction method), biomass: buffer ratio (R) and pressure (P) (for high pressure-assisted extraction).

Extractions were performed using procedures adapted from Beattie et al. (2018), Mittal et al. (2017) and Alexandre et al. (2017). For all the runs, freeze-dried *G. gracilis* (100 mg) was suspended in different phosphate buffer concentration (0.01 M < C < 1 M, pH 6.8), at different biomass: buffer ratios (R) (V_{buffer} = 0.5-5 mL, 1:5 < R < 1:50) and homogenized, with the help of a magnetic stirrer, at room temperature for different lengths of time (5 min < t1 < 30 min). Then, an extraction treatment was applied for different periods of time (defined below for each method) to the suspension to promote/increase cell disruption and pigment release. Extractions were carried out using sodium phosphate buffer at pH 6.8 as several studies have shown that it is efficient for phycoerythrin extraction (Sharmila Banu, Santhosh, Hemalatha, Venkatakrishnan, & Dhandapani, 2017; Sudhakar et al., 2015). The diverse combinations of conditions were defined using the Response Surface Methodology (RSM) as described below. After extraction, the samples were centrifuged (Eppendorf Centrifuge 5810R, Billerica, EUA) at 10.000 rpm for 20 min at 4 ºC and the pellet discarded. The supernatant was further filtered through a PTFE 0.45 µm membrane (VWR, North America) and analyzed in a UV-Vis equipment (Thermo Scientific Evolution 201, Thermo Fisher Scientific Inc.).

2.3.1 Maceration

The biomass/buffer suspension was ground with a mortar and pestle for different periods of time (10 s < t2 < 10 min).

2.3.2 Ultrasound-assisted extraction

The biomass/buffer suspension was placed either in an ultrasonic bath (Ultrasonic Cleaner, VWR USC 600 TH, Radnor, USA; 45 kHz, 400 W) or subjected to sonication with an ultrasonic

probe (S2500 Branson Digital Sonicator, Danbury, CT, USA; 50/60 kHz, 200 W) for different periods of time (10 s < t2 < 10 min). In the last case, the probe was inserted in the sample container at about 0.5 cm from the bottom. The sample was cooled in an ice bath to avoid overheating and an ultrasonic pulse 'on and off' cycle of 30/20 seconds was used.

2.3.3 Freeze-thaw

Samples of the biomass/buffer suspension (C = 0.1 M, R = 1:50, and t1 = 10 min) were frozen for 18h at -20 or -80 ºC and thawed for 24h at 4 ºC or at room temperature (approximately 19 ºC).

2.3.4 High Pressure-assisted extraction

The biomass/buffer suspension (C = 0.1 M) was poured into PA/PE vacuum sealing bags and the extraction was carried out on a pilot-scale high-pressure equipment (Model 55, Hyperbaric, Burgos, Spain) for different periods of time (5 min < t2 < 30 min) at different pressures (0.1 MPa < P < 600 MPa). In this case, the homogenization time was the time necessary to prepare the suspension in the PA/PE bags (t1 = 2-5 min), prior to high-pressure treatment.

2.4 Phycobiliprotein quantification

The absorption spectra of the extracts were measured between 200 and 900 nm in a UV-Vis spectrophotometer (Thermo Scientific Evolution 201, Thermo Fisher Scientific Inc.).

The quantification of R-phycoerythrin (PE) and R-phycocyanin (PC) extracted was performed using the Beer & Eshel equations (1985):

$$PE = [(A_{564}-A_{592}) - (A_{455}-A_{592}) * 0.2] * 0.12 \quad \text{(Eq. 1)}$$

$$PC = [(A_{618}-A_{645}) - (A_{592}-A_{645}) * 0.5] * 0.15 \quad \text{(Eq. 2)}$$

where A564 is the maximum absorbance of PE, A618 is the maximum absorbance of PC, and A592, A455 and A645 are absorbance minimums for baseline correction. 0.12 and 0.15 are the absorption coefficients for PE and PC, respectively, and 0.2 and 0.5 are correction constants (Beer & Eshel, 1985).

141 PE and PC yields are expressed in mg PE/g biomass and mg PC/g biomass, respectively.

142 The purity of the extracts was determined using the purity index (Mensi & Romdhane, 2014):

143 $$PI = A_{564}/A_{280} \quad \text{(Eq. 3)}$$

144 where A564 is the maximum absorbance of PE and A280 is the absorbance of total proteins.

145 **2.5 Experimental design and Response Surface Methodology (RSM)**

146 Response Surface Methodology (RSM) considering a central composite rotatable design
147 (CCDR) was employed for the evaluation of variables effect on the PE yield extracted with
148 maceration and ultrasonic waves. The independent variables tested were homogenization time
149 (t1, 5-30 min), treatment time (t2, 10 s-10 min), biomass/buffer ratio (R, 1:5-1:50) and buffer
150 concentration (C, 0.01-1 M), as defined in section 2.3. The independent variables and their levels
151 are presented in Table S1 (Supplementary material). For the high pressure-assisted extraction,
152 a Box-Behnken design was employed, using the treatment time (t2, 5-30 min), biomass/buffer
153 ratio (R, 1:5-1:50), and pressure (P, 0.1-600 MPa) as independent variables in order to evaluate
154 the relationship between the measured responses and the individual and combined effects of the
155 conditions. The independent variables and their levels are presented in Table S2 (Supplementary
156 material). The ranges of the independent variables were defined considering similar work
157 described in the literature (Marinho-Soriano, 2012; Sudhakar et al., 2015) and experimental
158 limitations (time of manual maceration, minimum amount of biomass possible to suspend in
159 buffer, time of uninterrupted equipment operation).

160 A statistical model for the condition's optimization was determined by RSM. For maceration
161 and ultrasounds-assisted extractions 24 experimental runs with different combinations of four
162 factors and two central point repetitions were carried out. For high pressure-assisted extraction
163 14 experimental runs with different combinations of three factors were performed along with
164 the repetition of central point six times.

165 **2.6 Biomass analysis by Scanning Electron Microscopy**

166 The *G. gracilis* biomass, before and after the extraction process, were analyzed by scanning
167 electron microscopy (SEM; Vega3 Tescan, Brno, Czechia, microscope operated at 15 kV.) to
168 evaluate the effect of the extraction conditions on the solid matrix and correlate it with the

extract retention. After extraction, the biomass samples were subjected to sequential dehydration with ethanol (70, 85, and 100%) followed by incubation in a drying oven (Memmert, Schwabach, Germany) at 35 ºC. Prior to the examination, samples were coated with a gold/palladium (Au/Pd) thin film, by sputtering, using the sputter coater equipment (Quorum Technologies). The cellular structure was compared to a pre-treatment lyophilized sample (control) at a magnification of 600×.

2.7 Statistical analysis

The experimental data were analyzed by regression analysis. Analysis of variance (ANOVA) generated the regression coefficients of linear, quadratic, and the interaction involved in the model, with a significance level of 95% ($p \leq 0.05$). The adequacy of the models was determined using the lack-of-fit test and R^2 (coefficient of determination) analysis.

Statistical analysis was performed using the software Statistica 10 (StatSoft, Inc., Minneapolis, USA). Where applicable the results are presented as mean ± standard deviation (SD).

3 Results and Discussion

3.1. Extraction Results

The procedure followed for the extraction of PBPs from *G. gracilis* is schematized in Figure 1 and a picture of the pinkish supernatant and respective absorption spectrum obtained after extraction with maceration can be found in Figure S1 (Supplementary material).

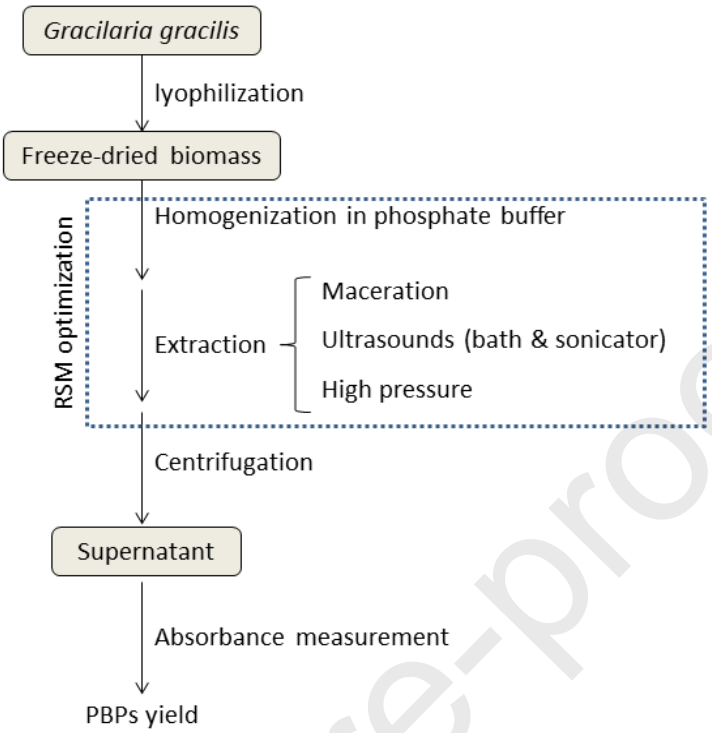

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Figure 1. Summarizing scheme of the procedure for the extraction of PBPs from *Gracilaria gracilis*.

Extraction conditions were optimized through RSM applied to several extraction methods as described above. The experimental results obtained using the conditions defined by RSM are presented in Tables 1 and 2.

All the methods successfully extracted PE from freeze-dried *G. gracilis*. The higher PE yields were obtained using maceration, with yields ranging from 1.65 to 3.77 mg PE/g biomass (Table 1). These values are in accordance with the data reported by Francavilla et al. (2013). The authors showed the existence of seasonal variations in PBPs concentration with higher contents of allophycocyanins (APC, 3.5 mg/g), phycoerythrins (R-PE, 7 mg/g), and phycocyanins (R-PC, 3 mg/g) being obtained from samples collected in January when compared with samples from October (APC, 1.5 mg/g; PE, 3.6 mg/g; R-PC, 0.7 mg/g)). The seasonal differences in solar radiation and nutrient concentration in the lagoon could justify the differences in PE concentrations. PBPs absorb sunlight in the wavelength range of 470-660 nm, being more active

at low light intensities when chlorophyll-*a* becomes inefficient in light absorption, thus allowing the survival of the species (Beattie et al., 2018). This leads to months with higher sunlight intensity resulting in lower PBPs contents. Regarding other *Gracilaria* species, previous studies with mortar maceration of *Gracilaria crassa* yielded 0.50 mg/g of R-PE, 0.28 mg/g of R-PC and 0.34 mg/g of APC (Sudhakar et al., 2015), while using a table mixer extracted 0.78 mg/g of R-PE, 0.49 mg/g of R-PC and 0.52 mg/g of APC from *Gracilaria corticata* (Sudhakar, Saraswathi, & Nair, 2014) and 0.067 mg/g of R-PE and 0.38 mg/g of R-PC from *Gracilaria verrucosa* (Jayasinghe, Pahalawattaarachchi, & Ranaweera, 2016).

Compared to conventional maceration process, lower PE yields (1.48-1.99 mg/g, about 55% less than maceration) were obtained using ultrasound-assisted extraction. The ultrasonic bath, although having the advantages of being cheap, readily available and allowing the treatment of many samples simultaneously, also has drawbacks such as low reproducibility and low power delivery directly to the samples (Chemat et al., 2017). This could have accounted for the low extraction yield achieved using this extraction method. Previous studies reported the successful extraction of PBPs using ultrasonic waves from other types of algae, such as *Porphyridium cruentum* and *Heterosiphonia japonica* (Benavides & Rito-Palomares, 2006; Bermejo Román, Alvárez-Pez, Fernández, & Grima, 2002; Sun et al., 2009). Theoretically, higher concentrations could be achieved by extending the time in which the samples are subjected to the ultrasonic bath. (Beattie et al., 2018; Kannaujiya et al., 2017). However, when larger periods of treatment were used (10-20 min), a degradation of PBPs was observed (decrease in absorbance maximums), which is in agreement with other studies (Rodrigues, de Castro, Santiago-Aguiar, & Rocha, 2018).

In literature, it is suggested that the ultrasonic probe has advantages over the ultrasonic bath such as the ultrasonic intensity and the direct delivery of the ultrasounds to the samples with minimal ultrasonic energy loss (Chemat et al., 2017). Theoretically, the use of an ultrasonic probe should result in higher extraction yields when compared with an ultrasonic bath, but this was not verified. However, even lower PE Yields (1.27-1.79 mg/g) were obtained using the ultrasound probe. Also, the use of an ultrasonic probe led to the simultaneous extraction of chlorophylls along with PBPs. In nature, the PBPs complement other pigments such as chlorophylls allowing the transfer of light energy during photosynthesis (Fleurence, 2003).

However, in PBPs extraction assays, the simultaneous (and unintentional) extraction of chlorophylls is considered a contaminant (Laureci, Bresciani, Lami, & Morabito, 2017), which results in the change of the color of the extracts to a more greenish coloration (as opposed by the characteristic pinkish color of PEs).

For high pressure-assisted extraction, the C (buffer concentration) and t1 (homogenization time) variables were fixed at 0.1 M and 2-5 min (the necessary time to prepare the suspension prior to high-pressure treatment), respectively. The results, presented in Table, show that high pressure-assisted extraction was the least effective extraction method compared with the other extraction processes, giving PE yields of 0.25-1.27 mg/g (about 65% less than maceration). Although high pressure-assisted extraction is widely applied in bacteria and yeast, few studies have applied this process in algae (even less for the extraction of PBPs). Jubeau et al. (2012) extracted 3.6 mg of B-PE/g of dry weight biomass applying a two-step high-pressure process in *Porphyridium cruentum.*

Upon extraction optimization with RSM (section 3.2) a model to predict the optimum extraction conditions was obtained. The predicted optimum extraction conditions were then tested experimentally in triplicate (Table 1). Optimum predicted extraction conditions for maceration (t1 = t2 = 10 min, C = 0.1 M, and R = 1:50) yielded 3.58 ± 0.03 mg/g of PE. The amounts of R-phycocyanin were also determined at the optimal conditions (0.62 ± 0.02 mg/g) confirming that PE is the major PBP found in *G. gracilis*. Regarding the ultrasound-assisted extraction, the predicted optimum extraction conditions (t1 = 17.5 min, t2 = 7.5 min, C = 0.26 M, and R = 1:27) yielded 1.60 ± 0.12 mg PE/g biomass and 0.37 ± 0.03 mg PC/g biomass. On the other hand, the ultrasonic probe extracted 1.57±0.10 mg PE/g biomass and 0.44 ± 0.01 mg PC/g biomass using the predicted optimum extraction conditions (t1 = t2 = 10 min, C = 0.26 M, and R = 1:50). In the three extraction methods, the experimental PE yields were lower than the predicted values, at optimal conditions.

Considering the results obtained with RSM optimization, the following optimal conditions have been selected for the freeze-thaw extraction: C = 0.1 M, R = 1:50, and t1 = 10 min. Two freezing temperatures (-80 ºC and -20 ºC) and two thawing temperatures (4 ºC and RT (approximately 19 ºC)) were tested (Figure S2, Supplementary material). The maximum extraction was obtained

when freezing at -80 ºC and thawing at 4 ºC (1.51 ± 0.03 mg PE/g biomass). In general, the PE yields obtained did not present significant differences between them ($p > 0.05$), except for when the samples were frozen at -20 ºC and thawed at RT, in which case lower yields were obtained. Longer thawing times may lead, therefore, to higher yields. In freeze-thaw method, ice crystals form during the freezing step (Soni, Kalavadia, Trivedi, & Madamwar, 2006) that upon thawing break down the cellular walls and release the intracellular content directly (Hardouin, Bedoux, Burlot, Nyvall-Collén, & Bourgougnon, 2014). Therefore, repeated cycles of the freeze-thaw are often used to achieve higher yields (Li et al., 2019; Mittal et al., 2017; Senthilkumar, Suresh, et al., 2013) although some studies have shown that the increase in PBPs yield is not significant (Kannaujiya et al., 2017; Lawrenz, Fedewa, & Richardson, 2011; Thoisen, Hansen, & Nielsen, 2017).

Table 1. R-phycoerythrin (PE) yields (mg PE/g biomass) for maceration, ultrasonic bath, and ultrasonic probe extractions. Experimental PE yields were calculated using Eq. 1 and predicted values were calculated by the model.

	Variables				PE yield (mg PE/g biomass)					
	R	C	t1	t2	Maceration		Ultrasonic bath		Ultrasonic probe	
Run	(mL)	(M)	(min)	(min)	Exp.[a]	Pred.[b]	Exp.[a]	Pred.[b]	Exp.[a]	Pred.[b]
1	1.7	0.3	11.43	2.69	2.52	2.46	1.69	1.67	1.51	1.49
2	1.7	0.7	11.43	2.69	2.65	2.82	1.68	1.68	1.51	1.52
3	1.7	0.3	11.43	7.47	3.44	3.58	1.74	1.77	1.59	1.63
4	1.7	0.3	23.57	7.47	3.41	3.10	1.64	1.79	1.58	1.51
5	1.7	0.3	23.57	2.69	2.54	2.33	1.60	1.63	1.42	1.39
6	1.7	0.7	23.57	2.69	2.48	2.28	1.48	1.64	1.36	1.44
7	1.7	0.7	11.43	7.47	3.78	3.71	1.59	1.62	1.42	1.48
8	1.7	0.7	23.57	7.47	2.67	2.81	1.56	1.63	1.28	1.38
9	3.8	0.3	11.43	2.69	2.54	2.45	1.69	1.72	1.61	1.52
10	3.8	0.3	23.57	2.69	2.52	2.63	1.56	1.58	1.67	1.56
11	3.8	0.3	11.43	7.47	3.26	3.41	1.85	1.83	1.79	1.71
12	3.8	0.3	23.57	7.47	3.25	3.23	1.72	1.74	1.77	1.73
13	3.8	0.7	11.43	2.69	1.65	1.91	1.74	1.72	1.27	1.35
14	3.8	0.7	23.57	2.69	1.65	1.67	1.58	1.57	1.49	1.42
15	3.8	0.7	11.43	7.47	2.37	2.64	1.59	1.66	1.28	1.35
16	3.8	0.7	23.57	7.47	1.92	2.05	1.51	1.58	1.43	1.40
17 (c)	2.8	0.5	17.5	5.08	2.86	2.76	1.99	1.90	1.55	1.53
18	0.5	0.5	23.57	5.08	2.25	2.48	1.73	1.59	1.51	1.42

19	5	0.5	17.5	5.08	2.31	1.98	1.59	1.59	1.47	1.58
20	2.8	0.01	17.5	5.08	3.08	3.25	1.68	1.65	1.47	1.62
21	2.8	1	17.5	5.08	2.41	2.17	1.51	1.44	1.36	1.23
22	2.8	0.5	17.5	0.17	1.95	2.00	1.63	1.61	1.37	1.43
23	2.8	0.5	17.5	10	3.68	3.52	1.85	1.71	1.60	1.56
24	2.8	0.5	5	5.08	3.46	3.17	1.79	1.79	1.54	1.48
25	2.8	0.5	30	5.08	2.32	2.45	1.78	1.65	1.32	1.43
26 (c)	2.8	0.5	17.5	5.08	2.68	2.76	1.94	1.90	1.52	1.53
Optimal [c]	10	5	10	0.1	**3.58**	**4.15**	-	-	-	-
Optimal [c]	17.5	2.8	7.5	0.26	-	-	**1.60**	**1.88**	-	-
Optimal [c]	10	5	10	0.26	-	-	-	-	**1.57**	**1.89**

[a] Experimental values of response.
[b] Predicted values of response (by RSM proposed model).
[c] Optimal conditions (maximum response) obtained by RSM model
t1 - homogenization time; t2 - treatment time; R – biomass: buffer ratio; C- buffer concentration; (c) – central point.

Table 2. R-phycoerythrin (PE) yields (mg PE/g biomass) for high pressure-assisted extraction. Experimental PE yields were calculated using Eq. 1 and predicted values were calculated by the model.

	Variables			**PE yield (mg PE/g biomass)**	
	t2	P	R	High pressure	
Run	(min)	(MPa)	(mL)	Exp.[a]	Pred.[b]
1	10	122	1.41	0.91	0.91
2	25	122	1.41	1.14	1.06
3	10	479	1.41	0.66	0.59
4	25	479	1.41	0.51	0.51
5	10	122	4.09	1.27	1.24
6	25	122	4.09	1.06	1.10
7	10	479	4.09	0.84	0.88
8	25	479	4.09	0.54	0.51
9	5	300	2.75	1.14	1.16
10	30	300	2.75	0.95	0.97
11	17.5	0.1	2.75	1.02	1.04
12	17.5	600	2.75	0.25	0.28
13	17.5	300	0.50	0.65	0.72
14	17.5	300	5.00	1.03	1.00
15 (c)	17.5	300	2.75	1.10	1.05
16 (c)	17.5	300	2.75	1.05	1.05
17 (c)	17.5	300	2.75	1.05	1.05
18 (c)	17.5	300	2.75	1.03	1.05

19 (c)	17.5	300	2.75	1.12	1.05
20 (c)	17.5	300	2.75	0.96	1.05
Optimal [c]	5	300	5	-	**1.32**

[a] Experimental values of response.
[b] Predicted values of response (by RSM proposed model).
t2 - treatment time; P – pressure; R – biomass: buffer ratio; C- buffer concentration; (c) – central point.

Figure 2 compares the PE yields obtained with the different extraction methods tested. The values used for comparison were the PE yields obtained at optimal conditions for maceration and ultrasound-assisted extraction (bath and probe) and the best experimental values for freeze-thaw and high pressure-assisted extraction. Although all the methods tested succeeded in the extraction of pigments from freeze-dried macroalgae, maceration was the most advantageous method affording yields of 3.58 mg PE/g biomass, approximately 55-65% more than the other methods tested. Maceration also afforded PE with the higher purity index (PI). Phycobiliproteins are known to be temperature sensitive, which may account for the higher yields obtained with maceration, as the ultrasounds and high pressure induce an increase in the extract temperature that can cause pigment denaturation. No statistically significant differences ($p > 0.05$) were found between ultrasound (bath and probe) and high pressure-assisted extractions and between freeze-thawing and high pressure-assisted extraction.

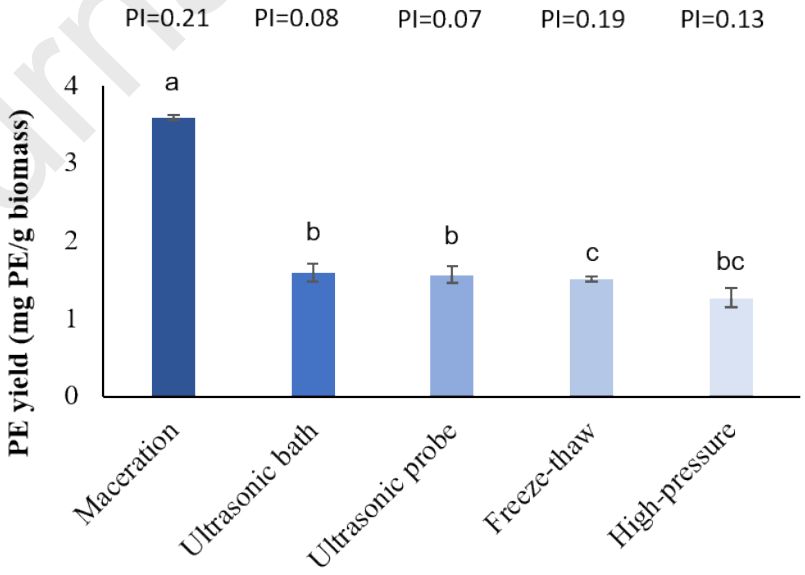

Figure 2. Comparison of PE yields obtained using the different extraction methods. The corresponding purity indexes (PI), calculated with equation 3, are also presented for each method. PE yields are expressed as mean ± SD (n = 3). Values with unlike letters differ significantly ($p < 0.05$).

3.2 Response surface methodology and statistical analysis

Analysis of the maceration results showed that there were statistically significant differences ($p < 0.05$) in all variables tested, as well as in the interaction between concentration and solid-liquid ratio, and the more impactful variables in the extraction yield were t2 and C (Table S3, Supplementary material). A coefficient of determination (R^2) of 0.90 indicated a good agreement of the model with the experimental results (Figure S3, Supplementary material). The lack of fit of 0.34 ($p > 0.05$ non-significant; supplementary material, Table S1) also indicated that, for the maceration process, the model was well adjusted to the experimental values representing the actual relationships of parameters well within the selected range (Bezerra et al., 2008).

The influence exerted by the variables on the extraction yield during the maceration process is displayed in the Pareto chart presented in the Supplementary material (Figure S4). At a 95% confidence level, the linear effect of t2 (L) (6.64) was the most significant in PE extraction, followed by the negative linear effect of C (L) (-3.96), linear effect of R (L) (-3.41), linear effect of R:C (L) (-3.25), and linear effect of t1 (L) (-3.21). The positive and linear effect of t2 indicates that the extraction yield increases with the increase of this variable while the negative and linear effect of the other variables suggests that the extraction will be more efficient using lower values of these conditions. This is shown in the response surface plots presented in Figure 3, which clearly illustrate the combined effects as well as visually describes these extraction tendencies.

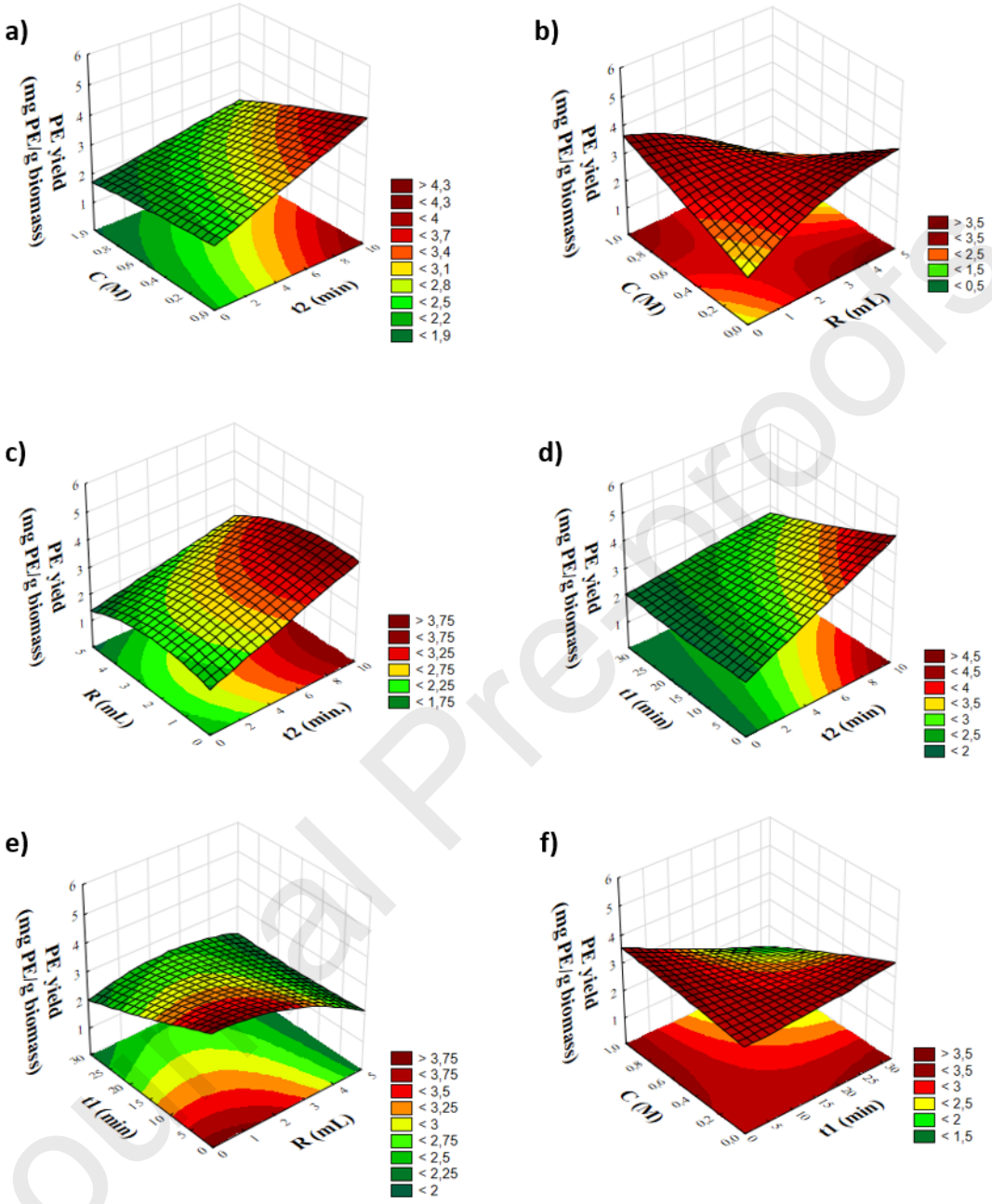

Figure 3. Response surface plots for PE yield (mg PE /g biomass) using maceration, with the combined effects of a) **C** (M) and **R** (mL of buffer); b) **C** (M) and **t2** (min); c) **R** (mL of buffer) and **t2** (min); d) **t1** (min) and **t2** (min); e) **t1** (min) and **R** (mL of buffer); and f) **C** (M) and **t1** (mL of buffer).

The same process was followed for the ultrasound-assisted extractions. The analysis of the results showed that using the ultrasonic bath there were only statistically significant differences ($p < 0.05$) in variables R and C with an R^2 of 0.67 and a lack of fit of 0.23 ($p > 0.05$ non-significant; supplementary material, Table S2). At a 95% confidence level, the most significant effect on PE extraction is the negative quadratic effect of C (-3.10), followed by the quadratic effect of R (-2.66) (Supplementary material, Figure S5). The quadratic and negative effects of C and R mean that the increase of these variables will increase the extraction of the pigment to a certain point, from which, even increasing these variables, the extraction decreases. The response surface plots shown in Figure S6 (Supplementary material) illustrate the combined effects of the different variables and visually describe the extraction trends.

As for the extractions using an ultrasonic probe, the ANOVA showed that there are only statistically significant differences ($p < 0.05$) in C and, like with the ultrasonic bath, an R^2 of 0.68 and a lack of fit of 0.15 ($p > 0.05$; supplementary material, Table S5). At a 95% confidence level, the only significant effect on the PE extraction is the negative linear effect of C (-3.40) (Supplementary material, Figure S7). This negative and linear effect suggests that the extraction yield decreases with the increase of the C. This effect can be seen in Figure S8 (Supplementary material), where the surface response plot illustrates the combined effect that the variables exert in the extraction tendency.

Finally, in the analysis of high pressure-assisted extraction, the ANOVA showed that there are statistically significant differences in all the conditions tested as well as in the interactions between t2 and P and between t2 and R, with an R^2 of 0.97 and lack of fit of 0.34 ($p > 0.05$ non-significant) (Supplementary material, Table S6). At a 95% confidence level, all variables were significant in PE extraction, as shown in Figure S9 (Supplementary material). The Pareto charts display the effect exerted by the variables on the extraction yield in the maceration process. The most significant effect on PE extraction is the negative linear (-13.42) and quadratic (-8.39) effect of P, followed by the positive linear (4.95) and negative quadratic (-4.07) effect of R, the negative combined effect of R and t2 (-3.34), negative linear effect of t2 (-3.23), and negative combined effect of t2 and P (-2.68). This suggests that the increase of the variables tested leads to a decrease of the extraction yield indicating that the extraction could be more efficient using lower values of these variables. Figure S10 (Supplementary material) represents the surface

response plots illustrating the effects that the combinations of variables have on the extraction yield.

Knowing the individual and combined effects that each variable exerts on the extraction of PEs, a model was constructed to predict the optimum conditions at which higher PE yields can be extracted. For maceration, the predicted optimum conditions (t1 = t2 = 10 min, C = 0.1 M, and R = 1:50) gave a PE yield lower than the one foreseen with the RSM (Table 1) but within the confidence interval. This proved the good prediction accuracy of the model to the optimal conditions of R-phycoerythrin extraction. Regarding the ultrasound-assisted extraction, the predicted optimum extraction conditions (t1 = 17.5 min, t2 = 7.5 min, C = 0.26 M, and R = 1:27) gave a lower PE yield than the one predicted by the model (Table 1), which falls out of the confidence interval predicted by the model. This discrepancy could be caused by the bad fit of the model for this method, with a low R^2 that only explains 66.5% of the experimental results. External factors could have contributed to the unfit adjustment of the model, such as the water temperature in the ultrasonic bath as well as the ultrasonic wave intensity that throughout the experiment were not controlled and could somehow have influenced the results. The ultrasonic probe, similarly to the results for the ultrasonic bath, gave a lower PE yield than the one predicted by the model (Table 1) at optimal conditions (t1 = t2 = 10 min, C = 0.26 M, and R = 1:50) but unlike the previous process one that fits within the confidence interval. This proves that the statistical model was reasonably adjusted to the experimental values and that the discrepancy could be caused by the low R^2, which only explains 67.9% of the experimental results. Overall, the response surface methodology resulted in accurate models capable of predicting the experimental values, proving to be a good procedure to optimize extraction methods.

3.3. Biomass analysis by Scanning Electron Microscopy

To better understand the structural effects that the extraction methods have on the biomass, Scanning Electron Microscopy images of the biomass were acquired before and after the extraction processes. Figure 4 shows that there are significant differences in the biomass when compared with the control (freeze-dried biomass prior to treatment) in the extent to which the

394 cells are ruptured, facilitating the release of PBPs into the extraction buffer. Maceration (Figure
395 4, b) appears to promote the cell wall breakage to a higher extent than with the other extraction
396 methods (Figure 4, c-f). On the other hand, the use of an ultrasonic probe (Figure 4, c) improves
397 the exposure to the solvent, and thus enhances the simultaneous extraction of chlorophyll
398 compounds along with the PBPs. Although freeze-thawing and high-pressure seem to promote
399 a high extent of cell damage (Figure 4, e and f), it does not break the cell walls completely and
400 as efficiently as the maceration, leading to lower releases of PBPs. These observations were
401 consistent with the yields obtained during PE extraction optimization and may explain the better
402 results achieved with maceration.

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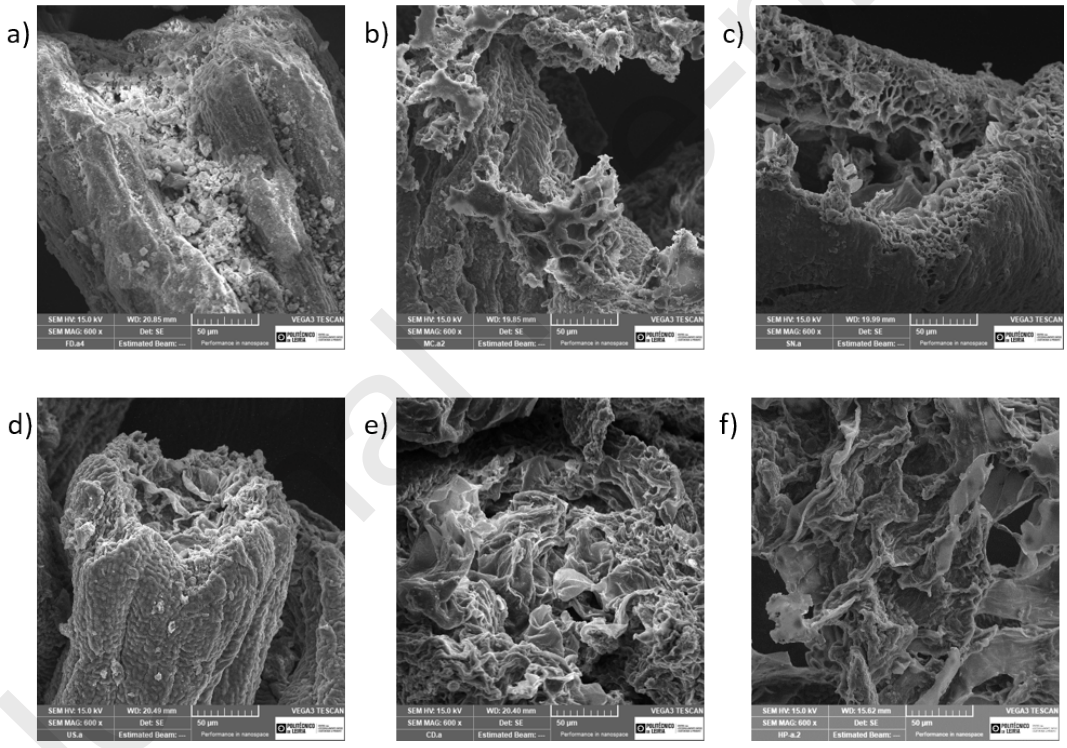

404

405 **Figure 4.** SEM images of the *Gracilaria gracilis* biomass cells a) before and b)-f) after the extraction
406 treatments (b – maceration, c – ultrasonic probe, d - ultrasonic bath, e - freeze-thaw, f - high pressure-
407 assisted extraction) at a magnification of 600x.

408

409

4 Conclusion

The extraction of PBP pigments from *Gracilaria gracilis* was optimized using different extraction methods. RSM proved useful in the optimization of PE extraction, providing a model with a good agreement between the experimental and predicted results. Maceration was the most efficient extraction method yielding 3.6 mg PE/g biomass at optimal conditions (t1 = t2 = 10 min, C = 0.1 M, and R = 1:50), which was 55-65% higher than the PE yields obtained with the other methods tested. The most impactful variable in the extraction process was treatment time (t2), with higher treatment times yielding higher concentrations of PE, whilst the least significant variable was homogenization time (t1) variable. SEM analysis showed the effect caused by the different extraction methods on the biomass, which agreed with PE yields obtained. *G. gracilis* revealed a good source of PE, that could be used as food colorant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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436 **Appendix:** Supplementary material

437

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Highlights

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- SEM analysis revealed a more efficient cell wall rupture using maceration

CRediT author statement

Tatiana Pereira: Investigation, Writing - Original Draft, Visualization; **Sónia Barroso:** Supervision, Writing - Review & Editing, Visualization; **Susana Mendes:** Formal analysis, Writing - Review & Editing, Validation; **Renata A. Amaral:** Investigation; **Juliana R. Dias:** Investigation; **Teresa Baptista:** Resources; **Jorge A. Saraiva:** Resources, Writing - Review

& Editing, Validation; **Nuno M. Alves:** Resources, **Maria M. Gil:** Conceptualization, Supervision, Funding acquisition, Project administration, Resources, Validation.

Conceptualization	Ideas; formulation or evolution of overarching research goals and aims
Methodology	Development or design of methodology; creation of models
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components
Validation	Verification, whether as a part of the activity or separate, of the overall replication/ reproducibility of results/experiments and other research outputs
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools
Data Curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse
Writing - Original Draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation)
Writing - Review & Editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or postpublication stages
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/ data presentation
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team
Project administration	Management and coordination responsibility for the research activity planning and execution
Funding acquisition	Acquisition of the financial support for the project leading to this publication

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