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Proceeding Paper

Exploring the Bioactive Potential of *Gracilaria gracilis*: An Extraction Optimization Study Using Response Surface Methodology †

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Abstract: Extraction of bioactive compounds from the seaweed *Gracilaria gracilis* was optimized for food use, using a Response Surface Methodology. Two designs, Central Composite Face-centered (CCD) and Box-Behnken (BBD) assessed the effects of extraction time, temperature, and seaweed-to-solvent ratio using water as the solvent. The extraction yield was assessed by Total Phenolic Content (TPC). BBD's best model was a Reduced Quadratic (R^2 = 0.9356), predicting 3.336 mg GAE/L at 74 °C in 1.4 h, with a 1:75 ratio. CCD's top was Reduced Cubic (R^2 = 0.9091), forecasting 4.278 mg GAE/L at 46 °C in 1.1 h, same ratio. Actual obtained TPC values were 4.35 mg GAE/L for BBD and 4.25 mg GAE/L for CCD.

Keywords: algae; extraction optimization; total phenolic compounds (TPC)

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1. Introduction

Algae are diverse, photosynthetic organisms found in aquatic environments, crucial for ecosystem health [1]. They are broadly categorized into microalgae and macroalgae, with the latter known as seaweeds. Seaweeds are vital in human nutrition, offering fiber, minerals, omega-3 fatty acids, and proteins [2]. Their nutrient composition depends on type, season, and growth location [2].

Algae contain diverse bioactive compounds: phenolic acids, flavonoids, carbohydrates, proteins, vitamins, carotenoids, and minerals. They are especially rich in iodine, with some varieties surpassing the daily intake recommendations [3]. Algae also produce antioxidants to combat environmental stressors like UV radiation [4]. These antioxidants are beneficial, neutralizing free radicals and reducing oxidative stress. Algae antioxidants also prevent lipid peroxidation and are potential food industry additives [5].

Gracilaria species, known for their significance in agar production, have gained attention as a promising source of bioactive compounds with potential applications in the food, feed, and pharmaceutical sectors [6]. Studies have revealed that *Gracilaria gracilis*, in particular, exhibits noteworthy antioxidant properties and radical scavenging activity. Its antioxidant capacity rivals commercially available antioxidant compounds, especially when the highest concentration of total phenols is present [7].

Nevertheless, extracting algae compounds is complex due to the variety of algae and target compounds. Optimal extraction depends on the species, solvent, solid-liquid ratio, duration, and temperature [8]. Efficient extraction involves pre-treatment, extraction (single/multiple steps), separation, and concentration of compounds [8].

Response Surface Methodology (RSM) is a statistical method for process optimization, applied in extracting bioactive compounds from seaweeds [9]. RSM identifies optimal extraction conditions, enhancing the yield efficiency of compounds like polysaccharides and proteins. Compared to conventional methods, RSM is more efficient [9]. RSM also models multiple factors and their interactions. For example, it discerns how extraction time affects temperature. Another RSM benefit is fewer required experiments, saving resources [9]. RSM's validation step ensures optimized parameter accuracy [10].

However, RSM has limitations. It may produce local optima, and its polynomial equation assumptions might not always fit, potentially resulting in inaccuracies [10].

Optimizing the extraction conditions for algae antioxidants is of utmost importance to achieve the maximum benefits of algae bioactives. The main objective of this study is to determine the optimal extraction conditions for *G. gracilis*, aiming to maximize the extraction efficiency of antioxidants using RSM. By optimizing the extraction conditions, we aim to enhance the yield and quality of the extracted antioxidants from this algae species.

2. Materials and Methods

2.1. Algae Preparation

G. gracilis were acquired from Alga+ (Aveiro, Portugal), and after being rehydrated in a 35 g/L NaCl solution for 5 min, it was washed with deionized water to eliminate excess salt. Then seaweeds were dehydrated at 42 °C (Excalibur 9 Tray Dehydrator, Model 4926 T, USA) during 6–8 h and ground in a Moulinex grinder (Paris, France). The powdered samples were stored away from the light and humidity until further use.

2.2. Extraction Process

The extraction process was conducted using a solid-liquid extraction method and it was carried out using deionized water (40 mL) as the solvent.

Three different extraction times were employed: 1 h, 3 h, and 5 h. Algal mass to solvent ratios of 1:25, 1:50, and 1:75 (grams of algal mass to milliliter of solvent) were also tested. Three temperature levels: 25 °C, 50 °C, and 75 °C were key factors in the extraction procedure. Each algae sample was placed inside an Erlenmeyer flask, which was covered with tin foil to prevent solvent vaporization at higher temperatures. To guarantee full homogenization, a magnetic stirring bar was used at a speed of 250 rpm. For extractions performed at 50 °C and 75 °C, a hot plate magnetic stirrer was used to maintain the desired temperature. Samples were filtered through TNT filters, and the obtained liquid was frozen at −20 °C until further use.

2.3. Total Phenolic Compounds (TPC) Analysis

The total phenolic compounds (TPC) analysis was conducted using a microplate reader at 765 nm (Synergy HT W/TRF multimode microplate reader, BioTek Instruments, Winooski, VT, USA) using a Gen5 2.0 software (BioTek Instruments), and the results presented in mg of gallic acid equivalents per g of dry weight seaweed (mg GAE/g dw).

2.4. Experimental Design

The Box-Behnken Design (BBD) suits response surface methodology, estimating quadratic model parameters for in-depth variable analysis. It supports sequential designs, lack of fit detection, and block utilization [11]. Central Composite Design (CCD) incorporates factorial, axial, and center runs for optimization in RSM. Center points gauge experimental error, and axial points ensure repeatability [12]. Face-centered CCD was adopted, using star points on the domain's faces [13]. Experimental data were assessed in Design-

Expert 11.0.0 software, considering factors: temperature, biomass:solvent ratio, and time. Adequate sample size in the factorial design ensured meaningful, statistically valid results. TPC results were used as response for RSM designs.

3. Results

3.1. Results Obtained from Experimental Design: Box-Behnken Design

The BBD was employed to establish the optimal model for *G. gracilis*. Specific TPC (mg GAE/g dw) values were obtained through varying extraction conditions, enabling the determination of the best-fitting model for the algae. Several models were generated using the BBD, but the one that exhibited superior fit statistics and significant *p*-values was the reduced quadratic model. Table 1 presents the derived results using ANOVA for the Reduced Quadratic model, and the parameters of the best model are presented in Table 2.

Table 1. ANOVA for Reduced Quadratic model for *G. gracilis.*

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	5.45	4	1.36	43.57	<0.0001	significant
A	0.0216	1	0.0216	0.6918	0.4218	
B	5.14	1	5.14	164.25	<0.0001	
AB	0.2233	1	0.2233	7.14	0.0203	
B^2	0.0687	1	0.0687	2.20	0.1640	
Residual	0.3752	12	0.0313			
Lack of Fit	0.3002	8	0.0375	2.00	0.2625	not significant
Pure Error	0.0750	4	0.0187			
Cor Total	5.82	16				

Table 2. Fit Statistics of ANOVA for Reduced Quadratic model for *G. gracilis.*

Std. Dev.	0.1768	R^2	0.9356
Mean	2.43	Adjusted R^2	0.9141
C.V. %	7.29	Predicted R^2	0.8326
		Adeq Precision	21.6373

The final equation in terms of actual factors was:

$$TPC = 1.42383 - 0.020980A + 0.033532B + 0.000378AB - 0.000204B^2$$

Constraints were systematically applied during the BBD process to ensure that the extraction conditions remained within the specified boundaries. This allowed exploring the most favorable combinations of variables to maximize the TPC value while keeping the extraction variables within the initial ranges. The optimal conditions are presented in Table 3.

Table 3. Selected optimal conditions by ANOVA for Reduced Quadratic model for *G. gracilis.*

Number	Temperature	Ratio	Time	TPC	Desirability	
1	74.993	74.684	*3.507*	3.335	1.000	
2	74.354	74.855	*1.425*	3.336	1.000	Selected
3	74.142	74.979	*2.851*	3.338	1.000	
4	74.803	74.881	*3.954*	3.340	1.000	
5	74.516	74.876	*1.563*	3.338	1.000	

3.2. Results Obtained from Experimental Design: Central Composite Design

The CCD was also utilized to determine the optimal model for *G. gracilis*. Varying the extraction conditions allowed for specific TPC values (mg GAE/g dw), aiding the

identification of the most suitable model. Although multiple models were generated, the reduced cubic model showed superior fit statistics and significant *p*-values, as evidenced by ANOVA (Tables 4 and 5).

Table 4. ANOVA for Reduced Cubic model for *G. gracilis.*

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	8.73	7	1.25	17.14	<0.0001	significant
A	0.5080	1	0.5080	6.98	0.0215	
B	4.52	1	4.52	62.11	<0.0001	
AB	0.0017	1	0.0017	0.0227	0.8827	
A^2	0.0204	1	0.0204	0.2796	0.6066	
B^2	0.1342	1	0.1342	1.84	0.1996	
A^2B	1.26	1	1.26	17.35	0.0013	
AB^2	0.9287	1	0.9287	12.76	0.0038	
Residual	0.8734	12	0.0728			
Lack of Fit	0.7576	7	0.1082	4.67	0.0544	not significant
Pure Error	0.1158	5	0.0232			
Cor Total	9.61	19				

Table 5. Fit Statistics of ANOVA for Reduced Cubic model for *G. gracilis.*

Std. Dev.	0.2698	R^2	0.9091
Mean	2.61	Adjusted R^2	0.8560
C.V. %	10.32	Predicted R^2	0.6269
		Adeq Precision	17.6231

The final equation in terms of actual factors was:

$$TPC = 1218035 - 0.372110A - 0.357410B + 0.010539AB + 0.002715A^2 + 0.002766B^2 - 0.000057A^2B - 0.000049AB^2$$

As with BBD, constraints were applied to ensure that the TPC values were maximized, and the extraction variables were within the established ranges. The optimal conditions are presented in Table 6.

Table 6. Selected optimal conditions by ANOVA for Reduced Cubic model for *G. gracilis.*

Number	Temperature	Ratio	Time	TPC	Desirability	
1	46.496	75.000	*1.408*	4.278	0.963	
2	46.497	75.000	*3.388*	4.278	0.963	
3	46.470	75.000	*1.875*	4.278	0.963	
4	46.474	75.000	*1.120*	4.278	0.963	Selected

4. Discussion

In the BBD, *G. gracilis* extraction revealed a significant influence of the ratio factor (F-value 164.25, *p*-value < 0.0001), while temperature displayed low significance (F-value 0.6918, *p*-value 0.4218). The lack of fit was insignificant, validating the model (Table 1). Optimal TPC extraction conditions were 74.354 °C, 1:74.855 (algal biomass to solvent), 1.425 h, yielding 3.34 mg GAE/g dw, with a desirability of 1.000 (Table 3). Similarly, the CCD exhibited significant results for A (temperature) and B (biomass:solvent ratio) (Table 4). Optimal conditions were 46.474 °C, ratio 1:75, 1.120 h, producing 4.28 mg GAE/g dw, desirability 0.963 (Table 6). Both models were significant by F-values and *p*-values (Tables 1 and 4). BBD displayed higher predicted R^2 (0.8326), while the CCD model had better adjusted R^2 (0.8560), suggesting a superior overall performance (Tables 2 and 5). Notably, higher ratios enhanced TPC values in *G. gracilis* extraction, with the temperature's effect

as the less pronounced. In the case of BBD, the experimental value obtained was 4.35 ± 1.09 mg GAE/L (30% from the predicted value). In contrast, when utilizing the CCD, the experimental value was 4.25 ± 0.26 mg GAE/L (<1% from the predicted value). Although the BBD had limited success, the CCD demonstrated the validity of the experimental design model.

Quitério et al. [8] reported that cold water at room temperature yielded significantly more antioxidants from *G. gracilis* than hot water (60 °C). Our study aligns, revealing *G. gracilis*' better efficiency at 50 °C versus 75 °C. This result suggests *G. gracilis* may harbor heat-sensitive compounds which are extracted more effectively at colder temperatures [8]. Although TPC-specific data on *G. gracilis* is limited, Reboleira et al. [6] highlighted that by comparing aqueous and ethanolic extracts with similar antioxidant potential, a better yield was obtained using water. This study underscores *G. gracilis* as a promising source of versatile bioactive compounds.

5. Conclusions

In conclusion, this study focused on optimizing the extraction conditions for *G. gracilis*, aiming to maximize antioxidant extraction efficiency. The results highlighted the species-specific response to the extraction conditions, emphasizing the need to tailor parameters accordingly.

G. gracilis displayed improved extraction efficiency at lower temperatures, particularly at 50 °C, indicating the effectiveness of colder water temperatures for bioactive compound extraction.

Overall, the findings provide valuable insights into the influence of temperature, biomass:solvent ratio, and time on the extraction process for this algae species. This work contributes to developing standardized extraction protocols, vital for commercial applications in the food industry. However, variations arising from solvent choice, extraction method, and study objectives highlight the importance of the extraction optimization.

Future research should explore alternative extraction methods, assess other algae species, and investigate the integration of algae extracts into food products to enhance their nutritional and sensory attributes. Ultimately, this study advances the sustainable utilization of algae as a source of bioactive compounds in the food industry, contributing to innovative and environmentally conscious practices.

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