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Optimization of Bioactive Compounds with Antioxidant Activity of *Himanthalia elongata* by Microwave-Assisted Extraction Using Response Surface Methodology †

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Abstract: *Himanthalia elongata* is a brown alga used in applications in the food, pharmaceutical and nutraceutical industries due to its biological properties, such as antioxidant, anti-inflammatory, and antimicrobial, among others. These effects are attributed to the high content of nutrients and secondary metabolites, especially phenolic compounds. The objective of this study is to optimize the microwave-assisted extraction method to recover phenolic compounds and flavonoids, considering three extraction parameters: the concentration of ethanol in water, the extraction time and pressure. The total phenolic content and the total flavonoid content were evaluated, and two biological tests were performed to assess the antioxidant properties.

Keywords: macroalgae; microwave-assisted extraction; *Himanthalia elongata*; bioactive compounds; antioxidant

1. Introduction

Traditionally, algae have been used as food and for medicinal purposes, mainly in eastern countries. However, its popularity is increasing in western countries, due to the search for healthier and more natural products by consumers, including food, cosmetics, pharmaceutical products, etc. [1,2]. Numerous studies indicate the good nutritional value of algae: they provide proteins and essential amino acids, and they are rich in non-digestible carbohydrates and polyunsaturated fatty acids, vitamins, and minerals. Furthermore, they are a source of compounds with various biological activities (e.g., antioxidants, antivirals, antimicrobials, antifungals, etc.) [3–5], which has attracted the attention of researchers hoping to study them and develop new industrial applications [6–8]. The antioxidant activity of some species of algae has been attributed to the presence of phenolic compounds such as polyphenols, hydroquinones and flavonoids. *Himanthalia elongata* is a brown alga of the order Fucales, found mainly in the N-W Atlantic Ocean and the North Sea.

Its antioxidant properties have been described previously [4], and it is reported that the amount of polyphenolic content is higher than in other algae [9].

Bioactive compounds from algae were commonly extracted using organic solvents (methanol, ethanol, acetone) with application temperatures between 45 and 60 °C, for hours or days, which implies high energy and environmental costs [10]. In contrast, non-conventional or green extraction techniques, such as ultrasound-assisted extraction (EAU), high-pressure-assisted extraction (HPAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE), pulsed electrified field extraction (PEF), pressurized-liquid-assisted extraction (PLE) and surfactant-assisted extraction (SAE), have proven to be a valid alternative in the recovery of bioactive compounds from algae [11–13]. Among them, microwave-assisted extraction (MAE) is an efficient and environmentally friendly technique, which reduces the extraction time and the amount of organic solvents and, in the best of cases, uses less polluting solvents such as water [10,13]. Different variables, such as the type of solvent, time and pressure, influence the recovery efficiency of bioactive compounds. Optimal extraction parameters can be estimated with statistical optimization methods. In this sense, the response surface methodology (RSM) uses quantitative data from an experimental design to solve the multivariate equation and maximize the results of the selected response variables. The objective of this study is to establish the most favorable conditions for MAE, in terms of the type of solvent, time and pressure required to produce extracts of *H. elongata* rich in bioactive compounds that present antioxidant activity.

2. Material and Methods

2.1. Sample Preparation

H. elongata samples were provided by the company Algas Atlánticas Algamar S.L located in Pontevedra, Spain. The algae were collected from the coasts of the province of Pontevedra, and they were washed with distilled water, frozen at −80 °C and later lyophilized. Next, the samples were crushed and ground to obtain a homogeneous matrix, which was stored at −20 °C until use.

2.2. Microwave-Assisted Extraction (MAE)

The process for obtaining bioactive compounds was carried out by MAE, using the multiwave-3000 equipment (Anton-Par). The extraction was carried out using 0.6 g of the lyophilized alga and 20 mL of solvent (solute/solvent ratio of 30 g/L). The variables studied were the ethanol concentration ($\%Et$), pressure (P) and time (t), as critical extraction parameters. Specifically, the $\%Et$ varied between 0 and 100% v/v, the P from 2 to 20 bar and t from 3 to 25 min. The power and frequency of the microwave were fixed for this batch of experiments and set at the maximum value of 1400 W for power and 2.45 GHz for frequency. Once the extraction was completed, the samples were placed in an ice bath for 5 min in order to rapidly lower the temperature and avoid degradation of the thermolabile compounds. Finally, the samples were centrifuged at 9000 rpm for 15 min and filtered to separate the supernatant from algae debris. These extracts were stored in a freezer at −80 °C.

In order to study the influence of MAE conditions ($\%Et$, P and t), the RMS was applied using circumscribed central composite design (CCCD), which allows one to identify the operating conditions for maximizing five response variables: extraction yield (EY), total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of *H. elongata*. The interaction between the different variables generates a total of 28 experiments. The least squares regression method was used to fit the data obtained in the 28 experiments to a quadratic model shown in the following equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the predicted responses (Y_1: EY, Y_2: TPC, Y_3: TFC, Y_4: DPPH assay, Y_5: ABTS assay), b_0 is the constant of the model, b_i is the linear coefficient, b_{ii} is the coefficient quadratic, b_{ij} is the coefficient of the interaction and X_i is the dimensionless coded value of the independent variables (X_1: $\%Et$, X_2: P and X_3: t).

2.3. Determination of Bioactive Compounds and Antioxidant Capacity

The *EY* was evaluated based on the dry weight (dw) obtained according to Equation (2).

$$EY\ (\%) = (P_2 - P_1)/P_0 \times 100 \quad (2)$$

where P_0 is the mass of lyophilized algae prior to extraction (mg), P_1 is the mass of the empty crucible (mg), P_2 is the mass of the dry extract in the crucible (mg).

The TPC was determined using the Folin-Ciocalteu reagent, while the TFC was evaluated according to the methodology proposed by Cassani et al. [14]. The results were expressed as mg of phloroglucinol equivalents (PGE)/g of dw and mg of quercetin equivalents (QE)/g of dw, respectively. Regarding the antioxidant capacity, it was determined using two assays: the diphenyl-2-picryl-hydrazyl radical (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging assays. The results of both assays are expressed in mg of scavenged compound/mL of extract.

3. Results and Discussions

The experimental results of the RSM of CCCD for the optimization of *H. elongata*′ MAE for the five considered response variables are presented in Table 1.

Table 1. Experimental parameters of the optimization process.

	Independent Variables			Response Variables				
Run	***t* (min)**	***P* (bar)**	***Et* (%)**	***EY***	**TPC**	**TFC**	**DPPH**	**ABTS**
1	7.5	5.6	20.3	452	24.58	2.60	5.64	12.24
2	7.5	5.6	79.7	355	28.26	6.14	6.59	80.08
3	7.5	16.4	20.3	529	41.30	4.28	19.97	21.38
4	7.5	16.4	79.7	376	25.52	5.31	5.33	20.41
5	20.5	5.6	20.3	543	19.24	2.36	4.47	9.43
6	20.5	5.6	79.7	384	23.90	5.11	6.29	22.01
7	20.5	16.4	20.3	489	38.86	5.59	19.68	19.70
8	20.5	16.4	79.7	361	19.75	4.40	3.84	15.87
9	3	11	50	459	25.56	2.92	4.70	22.12
10	25	11	50	370	37.70	4.02	7.86	35.70
11	14	2	50	358	28.40	4.62	8.56	59.45
12	14	20	50	479	35.49	5.51	14.32	30.33
13	14	11	0	491	25.50	11.31	11.28	15.12
14	14	11	100	109	11.89	4.19	5.66	22.58
15	3	2	0	373	7.53	1.35	1.01	16.08
16	3	2	100	60	12.87	0.73	4.11	16.08
17	3	20	0	409	27.64	7.44	10.34	23.34
18	3	20	100	99	10.14	4.61	3.57	7.22
19	25	2	0	459	8.10	1.92	1.05	74.57
20	25	2	100	67	5.36	2.73	2.77	7.44
21	25	20	0	443	29.75	9.20	13.43	37.66
22	25	20	100	133	3.89	7.78	3.95	8.18
23	14	11	50	377	35.62	2.62	9.58	61.86
24	14	11	50	377	32.35	4.26	9.11	60.83
25	14	11	50	474	47.73	9.64	25.29	95.13
26	14	11	50	425	21.61	3.29	5.60	24.28
27	14	11	50	439	21.89	3.70	4.50	22.10
28	14	11	50	435	21.97	3.44	4.97	23.59

Abbreviations: extraction yield (*EY*), total phenolic content (TPC), total flavonoid content (TFC), antioxidant assays (DDPH and ABTS).

As can be observed, the five response variables were favored by different extraction conditions. Regarding *EY*, the most favorable conditions were 7.5 min, 16.4 bar and 20% *Et*. The TPC was favored by an extraction time of 14 min, 11 bars and a 50% *Et*. In contrast, TFC was favored under the same conditions of time and pressure, but differed with respect to TPC in the solvent, with 0% Et achieving the best results. Similar results have been reported previously [15]. On the other hand, higher TPC and TFC usually corresponded with higher antioxidant activity in ABTS and DPPH assays. In general terms, the time and pressure parameters with intermediate values favored the EY and the obtaining of TPC and TFC. On the other hand, the parameter with the greatest influence was the %*Et*, showing differences in obtaining bioactive compounds. This can be explained by considering the polarity of the solvent and the compounds [16].

In order to obtain a *H. elongata* extract rich in phenolic compounds and flavonoids, with the maximum antioxidant capacity, all the response variables were simultaneously optimized by means of RSM. The operational conditions that simultaneously optimize all the considered response variables are presented in Table 2. These optimal extraction conditions give rise to an *EY* of 502.28 ± 25.11 mg/g of dw, a TPC of 37.43 ± 3.74 mg PGE/g dw and a TFC of 9.93 ± 0.99 mg QE/g dw. Regarding the antioxidant assays, the radical elimination activity of DPPH and ABTS was 16.37 ± 0.82 and 65.77 ± 1.97 mg/mL, respectively (Table 2).

Table 2. Effect of *H. elongata* extract by MAE under optimal conditions on antioxidant activity.

Best Operating Conditions	**%*Et***	***P* (bar)**	***T* (min)**	
	0.00 ± 0.00	20.00 ± 0.50	16.01 ± 4.80	
EY	**TPC**	**TFC**	**Antioxidant Activity** DPPH	ABTS
502.28 ± 25.11	37.43 ± 3.74	9.93 ± 0.99	16.37 ± 0.82	65.77 ± 1.97

The optimized operating conditions are consistent with the study presented by Magnusson et al. [16], who obtained the best TPC using water as solvent and an extraction time between 3 and 15 min, but very high temperatures were required (160 °C). In this sense, Zhang et al. [17] stated, using terrestrial plants, that water is a solvent with good solubility and has an excellent ability to absorb microwave energy and lead to efficient heating of the sample. Regarding TPC, the results of previous studies are variable. For example, Jiménez-Escrig et al. [9] reported a similar TPC around 30 mg PGE/g dw; however, when using aqueous methanol (50%) and extraction times longer than 2 h, TPC was around 10 mg PGE/g dw when using water but also with longer extraction times (1 h). Fernández et al. [18] reported values of 18 mg gallic acid equivalents/g dw, but again they required the use of organic solvents and extraction times longer than one hour and with different steps. Therefore, our data present a rapid, simple and green method to effectively extract a different kind of biomolecule from *H. elongata*. Nevertheless, further microwave parameters, such as temperature, power and frequency need to be further analyzed to obtain the most efficient extraction method. Furthermore, it is noticeable that the differences observed between studies could be due to the great variability of the content and phytochemical profile of algae, which can be larger and affected by different climatic and intrinsic factors, such as season, age, geographical location and environmental conditions [19].

4. Conclusions

H. elongata is an alga species with reported antioxidant activity, which has been attributed to the presence of phenolic compounds and flavonoids. In this study, MAE resulted in a suitable technique to extract those compounds and obtain extracts with antioxidant activity. Furthermore, the RSM was a suitable statistical method to determine the optimal conditions that maximize the content of polyphenols and total flavonoids, the antioxidant capacity and the extraction performance using microwaves. According to the optimization results, the best operational conditions that allowed us to produce extracts rich in bioactive compounds and displayed significant antioxidant effects on DPPH and ABTS assays were 0% Et, 20.00 bar and an extraction time of 16 min. Considering the growing interest in algae compounds, this extract could be used in the development of functional food, cosmetic and pharmaceutical applications.

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