Optimization of the extraction process of phenolic compounds from the brown algae Sargassum fluitans Børgesen (Børgesen)

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TECHNIQUE

ABSTRACT

Sargassum fluitans is a brown seaweed species that annually arrive to Cuban shores in extensive biomass amounts. It contains considerable content of total polyphenols as structural constituents of vegetal cell walls, which can be extracted from this species for therapeutic applications as antioxidants, antiviral and anti-inflammatory agents. In previous research, there were implemented the extraction method by maceration with heat under agitation, but yields of total polyphenols were found lower than required for an efficient extraction process. Therefore, this work was aimed to optimize the extraction conditions of total polyphenol-rich compounds. For this, a complete factorial design 3^2 with replicates in the center of the plane was studied, together with the resulting effect of extraction time and percentage of ethanol in the extraction solution as independent variables. The response variable used was total polyphenols' content, as determined by the Folin-Ciocalteu method. Optimal conditions were successfully established, improving yields of total polyphenols. Increaing ethanol concentration was detrimental for yields, while enlarging the extraction time was not so relevant for the efficiency of the process. The resulting regression model coefficients provided optimal design conditions, which were experimentally corroborated: 17.75 % ethanol hydroalcoholic solution (10:1 mL/g of dry seaweed) and incubation at 50 °C under stirring at 800 rpm for 123.5 min. These conditions allowed obtaining up to 8.66 mg of total polyphenols per g of dry seaweed. These conditions are relevant to implement efficient processes for polyphenols extraction from these species, as raw material for the biopharmaceutical industry.

Keywords: Sargassum fluitans, extraction, total polyphenols, optimization, factorial design

RESUMEN

Optimización del proceso de extracción de compuestos fenólicos del alga parda *Sargassum fluitans Børgesen (Børgesen)"*. *Sargassum fluitans* es una alga marina parda, de la cual arriban anualmente cantidades de biomasa considerables a las costas cubanas. Entre los compuestos de interés encontrados en este tipo de algas están los polifenoles totales. Estos son componentes estructurales de la pared celular vegetal del alga y poseen propiedades funcionales y bioactivas como antioxidantes, antivirales y anti-inflamatorios de interés biofarmacéutico. En este trabajo se modelaron y desarrollaron las condiciones óptimas para la extracción de compuestos ricos en polifenoles totales mediante el método de maceración con agitación y calor. Para ello se aplicó un diseño factorial completo 3^2 con réplicas en el centro del plano y se estudió el efecto de dos factores (variables independientes) durante la extracción: el tiempo de extracción y el porcentaje de etanol en el disolvente. El contenido de polifenoles totales se determinó como variable de respuesta mediante el método de Folin-Ciocalteu. Se estableció que el incremento de las concentraciones de etanol disminuía proporcionalmente los rendimientos, sin que el tiempo de exposición de la biomasa afectara de forma notable la eficiencia del proceso. Los coeficientes de los modelos de regresión permitieron el diseño de las condiciones óptimas, las cuales fueron corroboradas experimentalmente: solución hidroalcohólica de etanol al 17.75 % (10:1 mL/g de biomasa seca) e incubación a 50 °C durante 123.5 min con agitación a 800 rpm. Esto permitió aumentar el rendimiento de polifenoles totales hasta 8.66 mg por g de biomasa seca, con vistas a implementar procesos industriales más eficientes para la extracción de estos compuestos a partir de esta especie de alga.

Palabras clave: Sargassum fluitans, extracción, polifenoles totales, optimización, diseño factorial

Introduction

Over the past twenty years, the sea has become the main natural source of bioactive molecules [1], with marine macroalgae as source of substances with novel structures and different biological activities.

Among them, brown algae produce a wide variety of secondary metabolites, which include terpenoids, oxilipins, florotanins, volatile compounds, phenolic compounds and products of mixed biogenetic origin [2, 3]. Phenolic compounds are an important group of secondary metabolites that exhibit antioxidant activity, among other biological functions. They play an important role in cellular defense of algae against abiotic and biotic stress. In fact, phenolic compounds are found in brown macroalgae such as: phenolic acids; florotanins [4] (oligomeric structures of floroglucinol (1,3,5- trihydroxybenzene) and their polymerized derivatives)[5]; polysaccharides like alginic acid, mannitol and fucans, some of them sulfated and with anticoagulant, anti-inflammatory and antitumor activities [6].

With the aim of using compounds as mentioned above for therapeutic and pharmaceutical purposes, several groups have linked the demonstrated bioactivities of brown algae to the presence of different chemical compounds such as polyphenols [7]. There is great interest in phenolic compounds due to their antioxidant properties and their possible implications in cardiovascular disease, cancer cell inhibition, cholesterol,

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among others [8]. Several articles report on fast, reproducible and selective techniques for the extraction of phenolic compounds from species of the genus *Sargassum*. Some of them comprise Ultrasound-Assisted Extraction (AUE), Microwave Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE), Enzyme Assisted Extraction (EAE), among others [9]. In Cuba, extraction processes implemented for these purposes are in line with more traditional procedures, such as solid-liquid extraction (SLE) methods employing stainless-steel reactors adequate for the extraction of these metabolites, without using more modern equipment which implies higher production costs. Moreover, despite reports on phenolic compounds content in Sargassum sp. algae [10], there were not found techniques able to improve the extraction efficiency of total polyphenols in this particular genus with that traditional methods.

Previous studies run at the Center for Marine Bioproducts, in Havana, Cuba, showed that up to 0.23 mg/g dry extract of phenolic compounds could be obtained from a 50 % ethanol hydroalcoholic extract [11]. Therefore, this work was aimed to optimize the extraction conditions of this method, to improve yields of total polyphenols in the extract, using brown seaweed *Sargassum fluitans Børgesen (Børgesen)* drifts to Cuban shores as starting material.

Materials and methods

Collection

The specimens of *S. fluitans* were collected in February 2015 from drifts on Guanabo Beach (23º10'44 "N and -82º07'01" W), Havana, Cuba, identified by Beatriz Martínez Daranas, Ph.D. (Center for Marine Research). They were stored in the collection of the National Aquarium of Cuba, labeled as HANC AC001. The algae were washed with tap water to remove salts, sand and other debris, and subsequently dried (15 % maximum humidity) in a ventilation oven at 50-60 °C until constant weight. Later on, they were milled in a hammer mill to a particle size of less than 6-mm fragments and stored in black polyethylene containers on shelf under controlled humidity (65-70 %) and temperature (28-30 ºC) conditions.

Preparation of extracts

Extractions were made by applying hydroalcoholic solutions (20, 35 and 50 % ethanol concentration) to algae material at a 10:1 v/w ratio. Each test was run with 10 g of dry and milled algae, which was mixed with 100 mL of hydroalcoholic solution and placed on a water bath at 50 °C, under constant stirring at 800 rpm for 90, 120 or 140 min. Afterwards, the resulting extract was cooled to 25 °C and collected by filtration through qualitative Whatman filter paper, 180 mm in diameter (Whatman, USA), and stored in amber flasks at 4 °C until analysis.

Determination of total polyphenols

Total polyphenols concentrations were determined from extracts by interpolating the absorbance of samples in the standard curve obtained from increasing concentrations (0.01-0.06 mg/mL) of pyrogallol reagent (BDH, USA). Standard curve determinations

were made by the Folin-Ciocalteu spectrophotometric method [12]. Absorbance of samples and the standard was measured at 760 nm with a UV-Vis spectrophotometer (Shimadzu UV-1201, Shimadzu, Japan). The results were expressed as milligrams of pyrogallol (standard) per gram of dry extract.

Experimental design

A complete 3^2 factorial design with replicates in the center was performed to optimize and evaluate the dependence of the content of total polyphenols as response variable (dependent variable). It was analyzed as a function of variation of extraction time (90, 120 and 140 min) and ethanol concentration (20, 35 and 50 %, v/v) as independent variables.

Then, the quadratic model regression equation was derived for each independent variable, equaled to zero, and each relative or local optimum value was calculated. Additionally, the response variable was correlated with independent variables by adjusting the prediction of the response variable by using a quadratic polynomial equation, which general formula is as follows:

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum \sum b_{ij} x_i x_j \quad (1)$$

Where: Y is the dependent variable (response variable); Xi is the independent variable influencing the response variable; b_0 is the linear coefficient; bii is the quadratic coefficient and bij is the interaction coefficient [13].

Subsequently, the second derivative was evaluated with optimal values, verifying that a local maximum of the function for the study area was attained. Then, the regression curve was obtained with the significant coefficients from the values.

Statistical analysis

The response variable (total polyphenol concentration) was analyzed by the response surface methodology. The experimental design, the statistical analysis of data and the regression model were generated with the STATISTICA software (version 4.0; Stat-Soft Inc., USA).

Results

In order to establish the optimal conditions for improved yields of phenolic compounds extracted from *Sargassum fluitans Børgesen* (*Børgesen*) with ethanol, a complete 3^2 factorial design was applied with extraction time (90, 120 and 140 min) and ethanol concentration (20, 35 and 50 %) as independent variables. As shown in table 1, extraction values ranged 7.01-8.63 despite the variant used.

Total polyphenols concentrations were estimated from a Pyrogallol calibration standard curve, following the formula: y = 7.9449x + 0.0062 (R² = 0.9975)

The regression model coefficient obtained for this equation corresponds to variables having significant effects on the concentration of total polyphenols. It is observed that a high ethanol concentration in the extraction solution negatively influences on the content of polyphenols, and, conversely, a decrease in ethanol concentration leads to an increase in the total polyphenol content. On the other hand, extraction time

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does not have significant effects on the yields, determining that this variable was not further considered.

Regarding multiple interactions (ethanol concentration for each extraction time, (ethanol concentration)^2 and (ethanol concentration)^2), they showed an inverse dependence, their decrease leading to an increase in polyphenols content. Based on these results, an adjusted polynomial was generated as follows:

$$\text{Total polyphenols} = 8.437 - 0.405\,[\text{EtOH}]$$
$$- 0.195\,[\text{EtOH}] \times t - 0.191\,[\text{EtOH}]^2$$
$$- 0.606\,t^2 \qquad (2)$$

Where:

t: Extraction time; and [EtOH]: Ethanol concentration

The coefficients of the regression equation forming the gradient vector provided the clues to find optimal conditions. Variance analysis provided a determination coefficient (R^2) of 0.9719, with an optimal adjustment of the model for the independent variable in the range evaluated, as expected [14].

Subsequently, the response and contour surfaces were obtained (Figure), delineating the various effects independent variables have on the dependent variable. As shown in figure A, the response surface of total polyphenols content indicates that maximum values of total polyphenols can be obtained below and close to the center of the plane as a function of ethanol concentration and extraction time, respectively. In the case of the contour surface chart (Figure B), relating the ethanol concentration and extraction time to total polyphenols content, it depicted to what levels the operating point can move without leaving the optimum zone during extraction. This guarantees obtaining maximum concentration values of total polyphenols while adapting operational conditions to specific experimental settings.

Considering all these results, an optimal combination of both hydroethanol solvent concentration and extraction time was set for the extraction of phenolic compounds from *S. fluitans*, by adding 17.75 % ethanol hydroalcoholic solution (10:1 mL/g of dry seaweed) and incubation at 50 ºC under stirring at 800 rpm for 123.5 min. Hence, the function optimum combination was experimentally confirmed in three replicates (Table 2).

*D*iscussion

The extraction method by maceration with agitation and heat has proven effective and time saving to obtain phenolic compounds from seaweeds [15]. Nevertheless, few studies refer to its use with *S. fluitans* as starting material. Particularly, the time for extraction is an important factor to obtain polyphenols, with an excessive time regarded as detrimental. In fact, and according to the Fick's diffusion law, a final equilibrium is expected to be achieved at longer contact times between the solute of the solid matrix and the solvent during the extraction process [16]. This dynamics is fundamental to take advantage of the great potential of marine organisms as a significant source of secondary metabolites, with more complex structures as biomolecules which cannot be found in terrestrial organisms [17].

Table 1. Results of the 3^2 factorial design for the extraction of phenolic compounds from the brown algae *Sargassum fluitans Børgesen* (Børgesen) by the ethanol method*

Experiment	Ethanol concentration	Extraction time	Total polyphenols (mg/g of dry seaweed)
1	-1	-1	7.81 ± 0.58
2	-1	0	8.63 ± 0.81
3	-1	1	8.20 ± 0.72
4	0	-1	8.00 ± 0.23
5	0	0	8.30 ± 0.52
6	0	1	7.80 ± 0.84
7	1	-1	7.40 ± 0.37
8	1	0	7.90 ± 0.14
9	1	1	7.01 ± 0.65
10	0	0	8.20 ± 0.76
11	0	0	8.12 ± 0.54

*The experimental design was randomized. Total polyphenols are expressed as average ± standard deviation of three replicates each. Experimental 3^2 factorial design conditions were: Etanol concentration, 20, 35 and 50 % (-1, 0 and 1, respectively); Time, 90, 120 and 140 min (-1, 0 and 1, respectively).
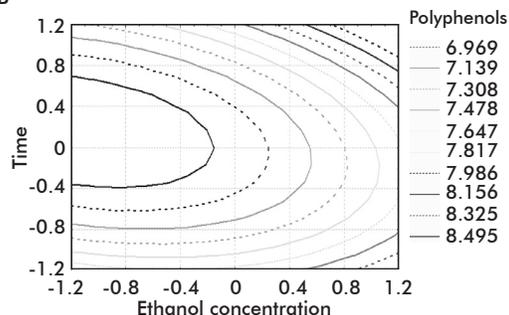
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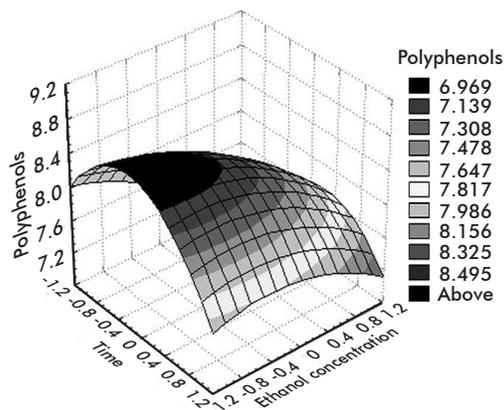
B

Figure. 3^2 factorial desing modeling of total polyphenols concentrations as response variable (dependent variable) to be extracted from *S. fluitans* seaweed. Independent variables were: Etanol concentration, 20, 35 and 50 % (-1, 0 and 1, respectively); Time, 90, 120 and 140 min (-1, 0 and 1, respectively). A) Surface response analysis. B) Contour analysis. Polyphenols concentration are expressed in mg per g of dry seaweed.

In this regard, polyphenols are among those molecules of therapeutic potential, with many properties derived from their radical scavenging activity as antioxidants, which can be isolated from seaweeds. For instance, Nakai *et al.* [18] identified and isolated

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bifuhalol (Phlorotannins) from a Sargassum species. But other groups have reported yields of total polyphenols, when using the maceration and heat extraction method, lower than the ones obtained with our process [19-21].

It was further observed that the higher the ethanol concentration, the lower the yields of total phenolic compounds extracted, with ethanol concentration as a significant variable during the process. This effect could be possibly related to the overall polarity of the compounds present in this species and their interaction or binding to glycosylated groups [22]. In other results, Valdés Iglesias *et al*. [11] showed that maceration with occasional agitation of extracts of *S. fluitans* rendered as few as 0.23 mg/g of dry seaweed material. Here we have shown that with optimized conditions and after the effective factorial engineering of the variables involved, yields can be readily improved to up to 8.66 mg/g of *S. fluitans* dry material. It is considered that the effective combination of incubation time and ethanol concentration at the assay temperature (50 ºC) facilitates the expansion of components of the vegetal seaweed cell wall and the passage of secondary metabolites into the extraction solution, this process accelerated by agitation [23].

Our optimized extraction conditions gain relevance when facing industrial scale-up, since it uses low costs reagents (ethanol) and standard equipment, and

Table 2. Experimental confirmation of optimized conditions for the extraction of phenolic compounds from the brown algae *Sargassum fluitans Børgesen* (*Børgesen*) by the ethanol method*

Experiment	Total polyphenols (mg/g of dry seaweed)
1	8.65 ± 0.33
2	8.68 ± 0.71
3	8.67 ± 0.54
Average	8.66

*Optimized conditions were: 17.75 % ethanol hydroalcoholic solution (10:1 mL/g of dry seaweed) and incubation at 50 ºC under stirring at 800 rpm for 123.5 min. Total polyphenols values are expreassed as average ± standard deviation of three replicates each.

the process is environmental friendly (low polluting gas emission). Moreover, the process employs natural materials regarded as waste (i.e., seaweeds seasonal banks) as raw material, rich in bioactive compounds, aiding on the national production of those compounds for its application in the pharmaceutical industry.

In summary, a relatively simple, inexpensive and scalable extraction process was implemented and optimized, for the extraction of phenolic compounds from the seaweed *S. fluitans*. It combines the maceration with agitation and heat method with adjusted concentrations of ethanol, providing a significant source of total polyphenols which can be used either for practical application as for characterization studies of the seaweed species arriving to Cuban shores.

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