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Optimization of Extraction Conditions of Total Flavonoid Content from *Cystoseira. amentacea var. Stricta* Using Response Surface Methodology

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ABSTRACT

This work was focused on the optimization of extraction conditions for total flavonoid content (TFC), from brown marine macroalgae *Cystoseira. amentacea var. Stricta* using response surface methodology (RSM). Methanol, an aqueous organic solvent were used, with different concentrations (20%, 50%, 80%, v/v), liquid to solid ratio (1/5, 2.5/5, 4/5), temperatures (20˚C, 40˚C, 60˚C), extraction times (30 - 150 min). Results showed that the optimal value of total flavonoids (23.80 mg of quercetin equivalents/g) from *C. amentacea var. Stricta* was obtained with 80% aqueous methanol, liquid to solid ratio (1/5) at 20˚C for 123.29 min. Fitting experimental data and prediction of the response was done with a second-order equation model which produced a good fit: R^2 = 0.91 for TFC.

KEYWORDS: Macroalgae, *Cystoseira. amentacea var. Stricta,* Total flavonoid content, Response surface methodology

1.INTRODUCTION

Among the class of polyphenols, flavonoids are the most important, their chelating and antioxidant properties, have a valuable health effects, this class of polyphenol contribute greatly to the plants antioxidant capacity [24]. They proceed by free radical scavenging, or blocking the generation of hypervalent metal forms, or by preventing lipid peroxidation [36].
Marine seaweeds can produce a large range of secondary metabolites with wide spectrum of pharmaceutical and biological propriety such as anticancer, anticoagulant, antioxidant, antibacterial and antiviral activities [32] [3]. *Cystoseira* is a genus of brown seaweed, in Algeria, it consist mainly of *Cystoseira amentacea var. Stricta*, a Fucophyceae algae endemic to the Mediterranean, which is subordinate to the infralittoral fringe (level 0 to 0.5 m deep) [6]. The presence of many molecules such as steroids, terpenoids, and alkaloids have been reported by authors in different species of genus Cystoseira, a Mediterranean brown algae, however, little research on pharmacological properties has been done [13][4] [2] [21].
The one-factor-at-a-time method is commonly used for optimization of the extraction processes, nevertheless this approach is time-consuming, expensive and cannot predict optimal conditions in addition to the fact that, it underestimate interactions between factors [1].
RSM a statistical and mathematical tools, are usually used for optimization of extraction parameters, in which response depends on different independent factors [23]. Response surface methodology, generate a mathematical model in a statistical way [5], after processing the quantitative data from an experimental model to create a second order polynomial equation [30].
In the present work, RSM was used to optimize four extraction parameters for TFC, of brown marine macroalgae *C. amentacea var. Stricta* harvested from the western coastline of Algeria.

2. MATERIAL AND METHODS

2.1. Plant materials

The samples of *C. amontaceae var stricta* were collected in spring (april) from an exposed intertidal rocky shore (during low tide) at Ain Defla site (35º50’ 40 N/ 0º28’ 59 O) located on the east of Oran (a coastal town in west of Algeria). Epiphytes, microorganisms, salts, and other suspended materials were removed from fresh samples with fresh water and air dried, then, the dried thalli of *C. amontaceae var stricta*were were grinded by an electric grinder, and kept in a dark place at room temperature.

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2.2. Extraction procedure

Methanol is frequently used for the extraction of secondary metabolite from seaweeds [12] [33] [9]. In order to extract most of the components from seaweed, a solvent with optimum polarity is required [24]. In our study, the grounded sample was submitted to extraction with 100% methanol. Approximately one gram of algal material was extracted in 100 ml of solvent and allowed to agitation during 24h with a rotary shaker. The filtered extract (Whatman no.1 filter paper) was kept at 4°C.

2.3. Total Flavonoid Content (TFC).

The Aluminum chloride ($AlCl_3$) method described by Djeridane et *al*. [16] was used to determine total flavonoid content (TFC) with some modification. About one1 ml of extract was added to the same volume of $AlCl_3$ (2% Methanol) and well mixed using a vortex. The solution was incubated at room temperature in the dark, for 10 min under shaking. The absorbance of the blank against standard and samples was recorded in triplicates at 430 nm with a spectrophotometer. The standard curve was calibrated by Quercetin. The results were showed as miligram of quercetin equivalents/g

2.4. Experimental design

For the extraction optimization of TFC from *C. amontaceae var stricta* sample, Response Surface Methodology was used. It is reported that the yield of total polyphenol and total flavonoids content is affected by numerous parameters [28] [31] [19] [38] [27], it is difficult to identify all the parameters influencing the response, then, factors with an important effects should be selected, [26].

A Box Behnken design was used in this study, with four independent factors coded at three levels (-1, 0 and +1): methanol concentration (X_1), liquid/solid ratio (X_2) temperature (X_3) and time (X_4). The chosen coded and decoded levels for the independent variables are shown in **Table 1:**

Table 1: coded and decoded independent factors

Independent variables	Symbols	Levels		
		-1	0	1
Methanol	X_1	20	50	80
Ratio	X_2	1/5	2.5/5	4/5
Temperature	X_3	20	40	60
Time	X_4	30	90	150

The number of selected experiments determined by RSM was a total of 27 sets and was performed to determine significant factors for the extraction of TFC.

The general second order polynomial model was:

$$Y = \beta 0 + \sum_{i=1}^{4} \beta iXi + \sum_{i=1}^{4} \beta itXi^2 + \sum_{i<j}^{4} \beta ij\ XiXj$$

The regression coefficients are β_0, β_i, β_{ij} and β_{ii} are for intercept, linearity, and interaction square terms, respectively, Y was the dependent response, X_i, X_iX_j and X^2_i represented the coded levels of linear, interaction and quadratic terms of independent factors, respectively.

2.5. Statistical analysis

For statistical analysis of quantitative data, ANOVA using a significant difference test (Fisher's least) at 5%.

3. RESULTS AND DISCUSSION

Drawing response surface plots of the design is the best way to show the effect of the independent variables on the dependent variables. This can be done by varying two factors and keeping constant the two others at the central point [35].

3.1. Fitting the models

The total flavonoid content dry matter extract of *C. amontaceae var stricta* obtained from the 27 sets are shown in **Table 2**. Experimental set of data were subjected to multiple regression analysis, the correlation between extraction factors (methanol, liquid/solid ratio, extraction time and temperature) and measured response (total flavonoid content) fitted the second-order multivariate equation.

Table 2: TFC experimental design.

Run	X_1 (%)	X_2	X_3 (C°)	X_4 (min)	TFC_{exp}	TFC_p
1	50	2.5/5	40	90	10,61	10,12
2	50	2.5/5	20	150	08,43	09,91
3	50	2.5/5	60	30	05,54	04,18
4	20	4/5	40	90	13,79	12,81
5	80	1/5	40	90	16,98	18,08
6	50	2.5/5	20	30	09,47	09,84
7	80	4/5	40	90	03,81	05,19
8	50	2.5/5	60	150	03,89	03,65
9	20	1/5	40	90	13,74	12,47
10	20	2.5/5	40	150	07,75	07,54
11	50	4/5	60	90	04,37	05,75
12	50	4/5	20	90	06,96	07,83
13	50	1/5	20	90	19,35	17,44
14	20	2.5/5	40	30	11,12	10,54
15	80	2.5/5	40	30	06,55	06,77
16	50	2.5/5	40	90	09,90	09,84
17	80	2.5/5	40	150	08,71	09,31
18	50	1/5	60	90	08,99	08,14
19	50	4/5	40	150	06,53	04,46
20	50	1/5	40	30	09,02	10,96
21	80	2.5/5	20	90	15,77	13,51
22	20	2.5/5	20	90	11,43	12,34
23	20	2.5/5	60	90	06,43	08,55
24	80	2.5/5	60	90	06,43	05,38
25	50	1/5	40	150	12,44	12,88
26	50	4/5	40	30	07,42	06,84
27	50	2.5/5	40	90	09,42	09,97

TFC_p : predicted value of TFC, TFC_{exp} : experimental value of TFC

To investigate the models adequacy and identify the significant factors, ANOVA was executed. The response and tested variables relationship are exhibited in the equation below:

$$Y_{(TFC)} = 0,471 - 0,02X_1 - 0,124\ X_2 - 0,117\ X_3 - 0,005\ X_4 - 0,130\ X_1X_2 - 0,043\ X_1X_3 + 0,077\ X_2X_3 + 0,055\ X_1X_4 - 0,043\ X_2X_4 - 0,006\ X_3X_4 + 0,038\ X^2_1 + 0,042\ X^2_2 - 0,039\ X^2_3 - 0,089\ X^2_4.$$

Where X_1 (methanol concentration), X_2 (liquid/material ratio), X_3 (temperature) and X_4 (time) are the independent variables and Y_1 (the total flavonoid content) is the response;

Table 3: Analysis of variance ANOVA

Responses	*Source*	*Degree of freedom*	*Sum of squares*	*Mean square*	*F- value*	*p-Value*
TFC	**Model**	16	0,569	0,035	6,2609	0,0028*
	Residual	10	0,057	0,006		
	Total	26	0,626			
			R^2= 0.91			

3.2. Effect of extraction process on total flavonoid content

Phenolic compounds Isolation, identification, and quantification are influenced by extraction which is an important step in these processes [11]. The most frequently used methodes to prepare plant extracts are solvent extractions, as it is more efficient, easy to use and has large applications, the type of solvents with varying polarities, sample ratio, temperature and extraction time in addition to the physical and chemical characteristics of the samples have a great impact on the yield of extraction [14].

Tableau 4: Regression coefficients of the fitted polynomial equations for TFC

Terme	Estimation	Écart-type	Rapport t	Prob.>|t|
Constante	9,98	1,11	9,03	<,0001*
Methanol(20,80)	-0,5	0,55	-0,91	0,3861
Solvent/sample(1/5, 4/5)	-3,14	0,55	-5,68	0,0002*
Temperature(20,60)	-2,98	0,55	-5,39	0,0003*
Time(30,150)	-0,11	0,55	-0,21	0,8405
Methanol*Solvent/sample	-3,3	0,96	-3,45	0,0062*

Methanol*Temperature	-1,09	0,96	-1,13	0,2835
Solvent/sample*Temperature	1,94	0,96	2,03	0,0699
Methanol*Time	1,38	0,96	1,44	0,1793
Solvent/sample*Time	-1,078	0,96	-1,13	0,2866
Temperature*Time	-0,15	0,96	-0,16	0,8766
Methanol*Methanol	0,95	0,83	1,15	0,2767
Solvent/sample*Solvent/sample	1,07	0,83	1,28	0,2279
Temperature*Temperature	-0,98	0,83	-1,18	0,2656
Time*Time	-2,25	0,83	-2,72	0,0217*

The interaction effect of methanol with sample ration was statistically significant and negative (p = 0.0062) as shown in table 4. The highest value of TFC (23.80 mg of quercetin equivalents/g) was obtained with methanol concentration of 80% **Figure 1(A).** As the polarity of methanol decreased from 0% to 80% the value of TFC increased. In the study of Rajauria *et al.* [24], a highest value of flavonoid expresseed in mg quercetin equivalents/g was obtained with 60% methanol concentration which exhibited a significant *p* value ($p < 0.05$). By contrast, the best concentration for *Calendula officinalis* flowers was obtained with 80% of aqueous methanol [10].

The linear effect of sample ratio was statistically significant and negative with p = 0.0002 (Table 4). Extraction of TFC, as shown in Figure 1(B), was maximal when sample ratio was at 1/5. Normally, the rate of TFC augments with the raise of sample ratio, because more solvent can penetrate cells allowing a large amount of flavonoids to diffuse into the solvent under the higher solvent to sample ratio condition [25]. The high value of TFC obtained with a relatively low sample ratio, may be due to the conjugated effect of long extraction time as shown in Figure 1(B).

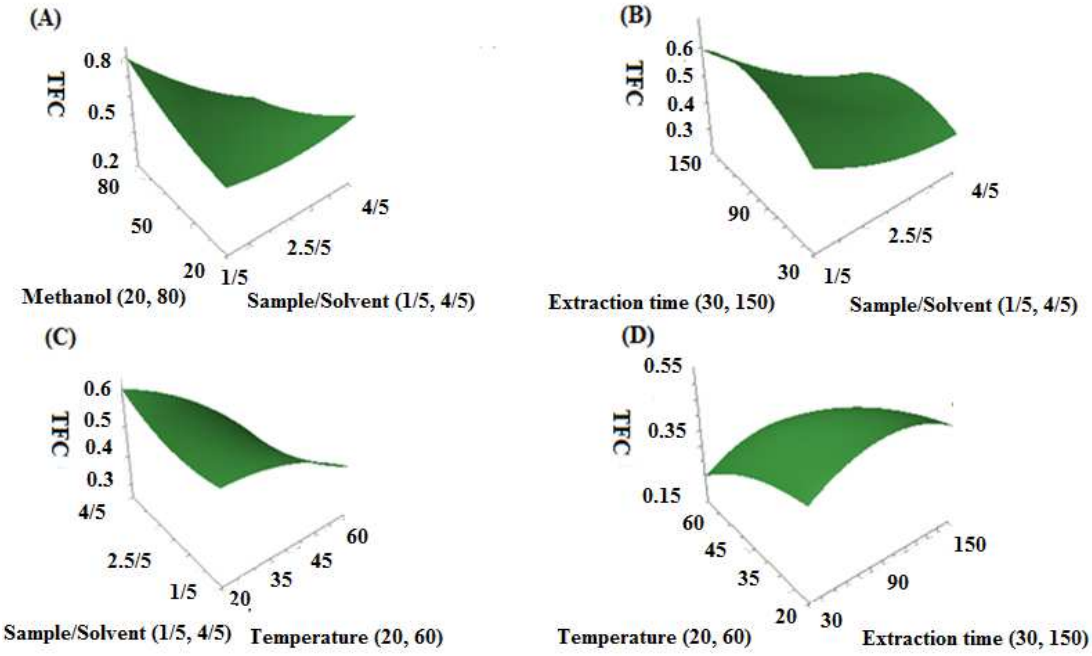

Fig 1: The RSM plots rschowing the effects of (A) methanol concentration and solvent to material ratio (B) Extraction time and solvent to material ratio (C) temperature and solvent to sample ratio (D) temperature and extraction time of TFC from *C. amontaceae var stricta.*

The linear effect of temperature was statistically significant and negative, p= 0.0003) as presented in Table 4. The maximum value of TFC was obtained with temperature of 20°C Figure 1(C). In the investigation of Bouterfas K *et al.* [8], on secondary metabolites from leaves of *Marrubium vulgare* L. (white Horehound), the

optimal value of TFC was obtained with temperature of 20°C. It is reported that, an important diminution of TFC can be caused by extraction temperature higher than 25˚C, then it is recommended to adjust temperature during extraction process to avoid thermal degradation of flavonoid derivatives, mainly hydroxyl groups [15] [7]. Furthermore, cell wall integrity are weaken and plant tissues are softened by mild heating which enhance the release of phenolic compounds [18] [29].
In the present work, the quadratic effect of extraction time was statistically significant and negative with *p value* equal to= 0.0217 (Table 4). The optimum extraction time of TFC in our study was with 123.29 min as shown in Figure 1(D). In other studies, the best extraction time for TFC from some thyme varieties and *Callicarpa nudiflora* leaves was tree hours [22] [37]. In the work of Liu *et al,* [19], on *Gynura medica* leaves, the most suitable extraction time of TFC was 30 min. It's well known that, prolonged extraction time allows the solvent and solute to be in contact during a longer time which favors the mass transfer [17].

3.3. Validation of the model

The aim of this work was the extraction optimization of parametters of flavonoid content from *C. amontaceae var stricta*. The desirability function procedure suggested the following optimal conditions: 80% of methanol concentration, 1/5 of solvent/sample ratio, 20°C of temperature and 123.29 min of time for TFC. A good R^2 value of 0.91 for TFC was obteined which means that, 0.91% can be explained by this model. However, the predicted results (23.80 mg of quercetin equivalents/g) were different to the experimental value (4.98 mg of quercetin equivalents /g). In fact, as reported by Liyana-Pathirana and Shahidi [20], optimisation and exploration of a fitted response surface may generate misleading or poor results, unless the design displays a good fit, hence, it is very important to check the model adequacy. In our study, the *p*-value of the model was 0.003 (Table 3), indicating a significant model fitness [20] [34].

4. CONCLUSION

The methanolic extract of *Cystoseira amentacea var. Stricta*, was analysed for its total flavonoid content following 27 different sets of four independent factors: methanol, solvent/material ratio, extraction time and extraction temperature. The optimum condition of these different factors was obtained using RSM. The second-order polynomial model proposed for *Cystoseira amentacea var. Stricta* extract exhibited a good R^2 value of 0.91 of total flavonoid content.
The present study is not exhaustive therefore, other experimental conditions may be tested as well as, the nature and the number of extractions procedures (ultrasound extractions, ultrafiltration, flash distillation, supercritical fluid). Also, other extraction solvents (ethanol, acetone, ethyl acetate and acetic acid etc.), will be taken into account in our future works.

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