

Application of Response Surface Methodology for Extraction Optimization of Water-soluble Polysaccharides from *Pteridium aquilinum*

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Abstract : The optimum conditions for the extraction of the water-soluble polysaccharides from *Pteridium aquilinum* were determined using response surface methodology (RSM). A central-composite experimental design (CCD) was used to investigate the interaction effects of three independent variables, namely liquid-solid ratio, extraction temperature and extraction time. Also, the antioxidant activity of the crude polysaccharides obtained from *Pteridium aquilinum* was investigated in this study. The optimum conditions are as follows: liquid-solid ratio 18.8:1 (V/W), extraction temperature 62.5 °C and extraction time 5.9 h. The crude polysaccharide shows excellent antioxidant activity according to FARP antioxidative assay.

Key words: response surface methodology; polysaccharides; antioxidant activity; *Pteridium aquilinum*

响应曲面法优化蕨菜水溶性多糖提取工艺的研究

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摘 要: 在单因素试验的基础上, 利用响应曲面法(response surface methodology, RSM), 通过中心组合设计, 对蕨菜多糖提取工艺参数进行优化分析研究。选择料水比、提取温度和提取时间作为优化因子, 研究了各因子对蕨菜多糖得率的影响。通过分析得到优化多糖的提取条件: 水料比 18.8:1(V/W), 提取温度为 62.5℃; 时间 5.9h。在此条件下蕨菜多糖提取得理论值达到 2.06%, 实际最大多糖得率为 2.02% ± 0.16%。同时, 用 FARP 法测定了多糖的抗氧化活性。

关键词: 响应曲面法; 多糖; 抗氧化性; 蕨菜

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Fern is sporous plant with fascicular, and now exists approximately 12000 species around the globe, common in warm, darkness boondocks in torrid and subtorrid zone^[1]. In China, there is plenty of fern source, about 61 families, 223 genuses, 2600 species. Wild brake (*Pteridium aquilinum*(L) Kuhn.var.latiusculum) has been described as one of the most common ferns in China. The uses of its rhizomes and fronds as foods have been widespread. Various biologically active compounds such as flavonoids, terpenoids, and steroids, isolated from *P. aquilinum*, have been reported by many

researches^[2-3]. However, there are few studies on extraction conditions of water-soluble polysaccharides of *Pteridium aquilinum*.

Many plant polysaccharides have been reported to have immunological, anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV and hypoglycemic activities^[4-7]. The solubility of the water-soluble polysaccharide, as well as its functionality, may be affected by various parameters, such as pH, temperature, ionic force, solvent type, extraction time, solid-solvent ratio and presence of components causing

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linking.

Response surface methodology (RSM) was employed in the present study, as it is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses^[8]. This statistical technique has been successfully applied in many areas of biotechnology such as optimization of extraction conditions^[9-10], lipase-catalyzed reaction conditions^[11], xylanase production^[12], protease production^[13] etc. But it has not yet been reported as a means to study and optimize extraction conditions for the water-soluble polysaccharides from *Pteridium aquilinum*.

In this study, at first, the effect of liquid-solid ratio, extraction temperature and extraction time on the extraction of polysaccharides from *Pteridium aquilinum* were evaluated by response surface methodology. In the second part, the antioxidative activity of the water-soluble polysaccharides was assayed by FRAP antioxidative assay.

1 Materials and Methods

1.1 Chemicals and reagents

The following chemicals were used: TPTZ (2,4,6-tripyridyl-s-triazine, Sigma), ethanol (95%, Beijing Chemical Industry, Beijing, China) and chloroform (Beijing Chemical Industry, Beijing, China). The reagents used in experiments were of analytic reagent grade.

1.2 Materials and extraction of polysaccharides

The young croziers of *Pteridium aquilinum* were obtained from Beijing, China. The samples were thoroughly washed with tap water, dried at 50 °C and finely powdered with a mixer. The powder was subjected to successive extraction with 90 % ethanol to remove lipids and pigments. Defatted powder was then subjected to water extraction. The supernatant was subjected to the Sevag's method to remove free proteins and dialyzed for 72 h, and then concentrated by rotary evaporation at reduced pressure below 50 °C^[14]. Ethanol (3~4 times by volume.) was added to precipitate the crude polysaccharides. Finally the precipitation was collected and freeze-dried.

1.3 Analysis of polysaccharides

The polysaccharides were determined by the phenol-sulfuric acid reaction, using glucose as standard^[15].

1.4 Ferric-reducing antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using a ferric reducing ability of plasma (FRAP) assay of Benzie and Strain^[16] as a measure of "antioxidant power".

FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6 with 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloric acid and with 1 volume of 20 mmol/L ferric chloride. Freshly prepared FRAP reagent (1.5 ml) was warmed to 37 °C and a reagent blank reading was taken at 593 nm. Subsequently, 50 μl of sample and 150 μl of deionized water was added to the FRAP reagent. Final dilution of sample in reaction mixture was 1:34. The sample was run in triplicate. After addition of sample to the FRAP reagent, a second reading at 593 nm was performed after 8 min. The initial blank reading with just FRAP reagent was the subtracted from the final reading of FRAP reagent with sample to determine the FRAP value of the sample. Standard curve was prepared at different concentrations (100~1000 μmol/L) of FeSO₄ • 7H₂O. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 μmol/L FeSO₄.

1.5 Experimental design

The combination effects of liquid-solid ratio, extraction temperature and extraction time were studied by response surface methodology. For this purpose, a central-composite design (CCD) with three variables at five levels was used to study the response pattern and to determine the optimum combination of variables. The effects of the independent variables X₁ (liquid-solid ratio, A), X₂ (extraction temperature, B) and X₃ (extraction time, t) at five variation levels (Table 1) in the extraction process are shown in Table 2. Six replicates (treatment 15~20) at the center of the design were used to predict whether the models gave significant lack-of-fit. The reliability of the models was evaluated by calculating the R² value. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

The variables were coded according to Eq. (1):

$$x_i = \frac{X_i - \bar{X}_i}{\Delta X_i} \quad (1)$$

Where x_i is dimensionless value of an independent variable; X_i is real value of an independent variable; \bar{X}_i is real value of an independent variable at the center point; ΔX_i is step change.

The specific codes are:

$$x_1 = \frac{A-20}{5} \quad (2)$$

$$x_2 = \frac{B-70}{10} \quad (3)$$

$$x_3 = \frac{t-8}{2} \quad (4)$$

Table 1 Independent variable values of process and their corresponding levels

Independent variables	Symbol		Levels					
	Uncodified	Codified	-2	-1	0	1	2	
Liquid-solid ratio (V/W)	X ₁	x ₁	10:1	15:1	20:1	25:1	30:1	
Extraction temperature(°C)	X ₂	x ₂	50	60	70	80	90	
Extraction time(h)	X ₃	x ₃	6	8	10	12	14	

1.6 Statistical analyses

The absorbance at 490 nm was measured, after xyloglucan-containing fractions were mixed with the Gramstain. A second-order polynomial equation was used to fit the experimental data given in Table 2. The model proposed for the response (Y_i) was given below:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where Y_i is the predicted response, β₀ is the value of the fitted response at the center point of the design, and β₁, β₁₁ and β₁ are the linear, quadratic and cross-product terms, respectively. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination, R². The significance of each coefficient was determined using the Student F-test and p-value. The behavior of the surface was investigated for the response function (Y_i) using the regression equation (5). A graphic technique was used to deduce workable optimum conditions by fixing one variable at predetermined optimum condition. The optimum conditions were verified by conducting experiments under these conditions. Responses were monitored and results were compared with model predictions.

In order to visualize the relationship between the response and experimental level of each factor and to deduce the optimum conditions, the fitted polynomial equation was expressed as surface and contour plots. The computer software used for this study was Design-Expert, version 7.0, by Etat-ease Inc.

Table 2 CCRD three variables with the observed responses and predicted values

Treatment	Variable levels			Experimental(Y ₀)	Predicted(Y ₁)	Y ₀ - Y ₁
	X ₁	X ₂	X ₃			
1	-1	-1	-1	1.898	1.928	-0.03
2	1	-1	-1	1.879	1.866	0.013
3	-1	1	-1	1.751	1.747	0.004
4	1	1	-1	2.235	2.31	-0.075
5	-1	-1	1	1.779	1.766	0.013
6	1	-1	1	1.853	1.919	-0.066
7	-1	1	1	2.102	2.177	-0.075
8	1	1	1	2.920	2.952	-0.032
9	-2	0	0	1.655	1.642	0.013
10	2	0	0	2.406	2.357	0.049
11	0	-2	0	1.598	1.594	0.004
12	0	2	0	2.506	2.448	0.058
13	0	0	-2	1.658	1.645	0.013
14	0	0	2	2.175	2.126	0.049
15	0	0	0	2.356	2.427	-0.071
16	0	0	0	2.456	2.427	0.029
17	0	0	0	2.469	2.427	0.042
18	0	0	0	2.463	2.427	0.036
19	0	0	0	2.432	2.427	0.005
20	0	0	0	2.445	2.427	0.018

2 Results and Discussion

2.1 Fitting of the models

The following regression equation, which is an empirical relationship between the absorbance and the test variable in coded unit as given in Eq. (5), was obtained with the application of RSM.

$$Y = 2.43 + 0.18x_1 + 0.21x_2 + 0.12x_3 + 0.16x_1x_2 + 0.053x_1x_3 + 0.15x_2x_3 - 0.11x_1^2 - 0.10x_2^2 - 0.14x_3^2 \quad (5)$$

A regression analysis (in Table 3) was carried out to fit mathematical models to the experimental data aiming at an optimal region for the responses studied. The significance of each coefficient was determined using the F-test and p-value in Table 3. The corresponding variables would be more significant if the absolute F-value becomes greater and the p-value becomes smaller^[17].

Analysis of variance (ANOVA) shows that the selected quadratic models adequately represented the data obtained for polysaccharides. It can be seen that the variables with the largest effect were the linear terms of extraction temperature (x₂), liquid-solid ratio (x₁) and the interaction effects of extraction temperature and liquid-solid ratio (x₂x₃). The factor F-test value (204.74) and p-value (p < 0.0001) correspond to x₂, while the F-test values for x₁ and x₂x₃ are smaller at 143.50 and 129.19, respectively, but the p values are still significant at p < 0.0001.

The total determination coefficient $R^2 = 91.58\%$ implies that the sample variations of 91.58% for the yield of polysaccharides are attributable to the independent variables, namely liquid-solid ratio, extraction temperature and extraction time.

The 'Lack of Fit F-value' of 3.09 implies the 'Lack of fit' is not significant relative to the pure error. There is a 12.08% chance that a 'Lack of Fit F-value' this large could occur due to noise.

Table 3 Significance of regression coefficient for yield of polysaccharides

Source	SS	DF	MS	F-value	p-value	Significance
Model	2.58	9	2.58	80.39	< 0.0001	Significant
X_1	0.51	1	0.51	143.50	< 0.0001	
X_2	0.73	1	0.73	204.74	< 0.0001	
X_3	0.23	1	0.23	65.06	< 0.0001	
X_1X_1	0.19	1	0.19	54.60	< 0.0001	
X_2X_2	0.023	1	0.023	6.40	0.02900	
X_3X_3	0.17	1	0.17	48.97	< 0.0001	
X_1X_2	0.29	1	0.29	80.48	< 0.0001	
X_1X_3	0.26	1	0.26	72.58	< 0.0001	
X_2X_3	0.46	1	0.46	129.19	< 0.0001	
Lack of fit	0.027	5	5.378×10^{-3}	3.09	0.1208	Not significant

Note: The coefficient of determination (R^2) of the predicted model was 0.9158.

2.2 Analysis of response surfaces

Since the models have shown lack of fit to be insignificant the responses were sufficiently explained by the regression equation. The regression models allowed the prediction of the effects of the three parameters on extraction of crude polysaccharides. The 3D surface curves were drawn to illustrate the main and interactive effects of the independent variables on the dependent one.

The effects of liquid-solid ratio, extraction temperature and extraction time on response are depicted (Table 3) by the coefficients of second-order polynomials. The response surfaces based on these coefficients are shown in Fig.1~3 with one variable kept at optimum level and the other two varied within the experimental range.

Fig. 2 shows the effect of liquid-solid ratio and extraction temperature on polysaccharides yield, and a quadratic effect for both liquid-solid ratio and extraction temperature on the response can be observed. The effect of liquid-solid ratio and extraction time is depicted with Fig.3, as both liquid-solid ratio and extraction time exerting a quadratic effect on polysaccharides production. Fig. 4 depicts the effect of extraction temperature and extraction time, as both them also exerting a quadratic effect. Interaction effect between two varies can be observed in Fig.1~3. In general, exploration of the response surfaces indicates a complex interaction be-

tween the variables.

In the application of the method, central-composite design data were taken into consideration and a formulation of the conditions were found as liquid-solid ratio of 18.8:1 (V/W), extraction temperature 62.5 h and extraction time 5.75 h.

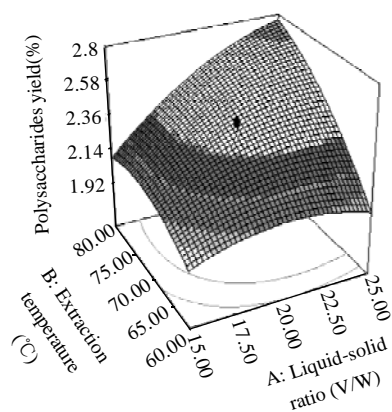


Fig.1 3D graphic surface optimization of polysaccharides yield versus liquid-solid ratio and extraction temperature

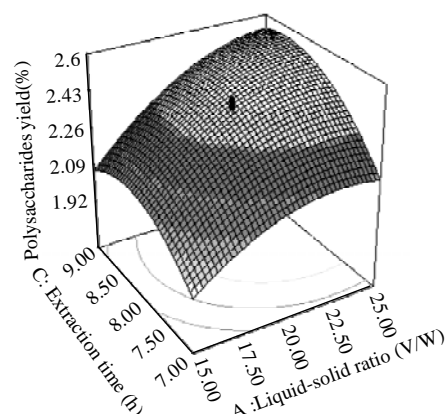


Fig.2 3D graphic surface optimization of polysaccharides yield versus liquid-solid ratio and extraction time

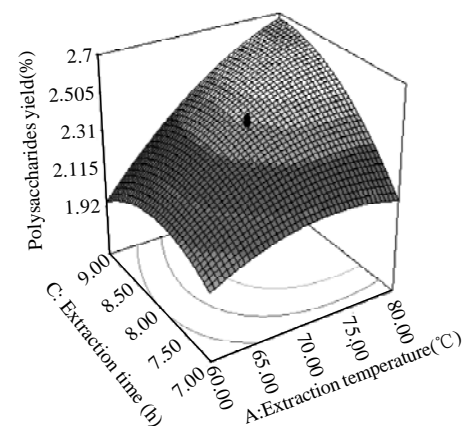


Fig.3 3D graphic surface optimization of polysaccharides yield versus extraction temperature and extraction time

2.3 Verification experiment

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. The experimental yield of polysaccharides is found to be in agreement with the predicted one (in Table 4).

Table 4 Predicted and experimental yield of polysaccharides under optimum conditions

Levels	Optimal conditions		Yield (%)		
	Liquid-solid ratio(V/w)	Extraction temperature(°C)	Predicted value	Experimental value	
				Mean	Range
Code levels	− 0.247	− 0.754			
Actual levels	18.8:1	62.5	2.06	2.02 ± 0.16	1.99~2.12

Note: Mean value of triplicate determinations.

2.4 Antioxidative activity analysis of crude polysaccharides

Natural anti-oxidative compounds from plants have aroused much attention, and more increasing efforts have been made to search for plant-derived antioxidants^[18-19]. The antioxidative activity of the water-soluble polysaccharide from wild brake was investigated by FRAP antioxidative assay. Before being mixed with the FRAP reagent, the samples was diluted to 0.04 μg/μl so that equal amounts of polysaccharides (2 μg) was added to the FRAP reagent. A dilution of 0.04 μg/μl was chosen because above the concentration the FRAP value measured was over the linear range of standard curve. The result showed that the mean FRAP value of 1 μg polysaccharides is equivalent to 435.0 ± 2.7 μmol/L FeSO₄ · 7H₂O.

3 Conclusions

RSM is effective for estimating the interaction effects of three independent variables. Both the extraction temperature and liquid-solid ratio have highly significant effects on the response value, and the interaction effects of extraction temperature and liquid-solid ratio also display significance. Based on the experimental results, the following conclusions have been drawn. The optimal predicted polysaccharides yield of 0.103 g from 5 g dried wild brake is obtained when the optimum extraction conditions of polysaccharides are liquid-solid ratio 18.8:1, extraction temperature 62.5 °C and extraction time 5.9 h. The water-soluble polysaccharides from *Pteridium aquilinum* have good antioxidative potency. 100 mg Vitamin C is equivalent to 1.51 mmol FRAP value as determined^[20]. The mean FRAP value for *Pteridium aquilinum* (435.0 ± 2.7 μmol/μg) is significantly higher than the FRAP

value for vitamin C (0.0151 μmol/μg).

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