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Protein Extraction from Grape Seeds by Reverse Micelles: Optimization of the Forward Extraction

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Abstract

The optimization of the reverse micelles extraction of protein from grape seeds was carried out using response surface methodology (RSM). Based on the Plackett-Burman design and steepest ascent, CTAB concentration, pH, NaCl concentration and crude protein concentration were selected as the most extract conditions. Subsequently, the optimum combination of the selected factors was investigated by the Box-Behnken design. The final condition of extraction optimized with RSM was CTAB concentration 39 mmol/L, pH 5.6, NaCl concentration 0.01 mol/L, and crude protein concentration 2.1 mg/mL. The forward extraction yield of 82.3% in triplicate under optimal extraction condition was obtained.

Subject Areas

Food Science & Technology

Keywords

Reverse Micelles, Response Surface Methodology, Grape Seeds, Protein

1. Introduction

The grape is one of the major fruit crops worldwide and its harvest is about 60 million tonnes per year [1]. About 80% of the harvest is utilized for winemaking and the grape waste consists the 20% of the weight of processed grapes. However, winemaking leads to the generation of large quantities of wastes, which considerably increase the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD_5) due to a high pollution load (high content of organic substances such as sugars, tannins, polyphenols, polyalcohols, pectins and lipids)

with detrimental effects on the flora and fauna of discharged zones [2].

Therefore, treatment and disposal of winery waste are serious environmental problems and winery waste must find another use other than as animal feed or as fertilizers.

Grape seeds are the primary main byproducts of viniculture and fermentation. In the last few years, increased attention has been focused on industrial wastes that are rich source of polyphenolic compounds, flavonoids, protein and oil [3] [4] [5] [6]. Grape seeds extract in particular show interesting biological properties, such as antioxidant, anticancer, anti-inflammation, anti-aging and anti-bacterical activities [2] [7]-[12].

Grape seeds have relatively high content of protein (13% - 18%), which can be extracted by conventional procedures such as solvent extraction and isoelectric precipitation [13] [14] [15]. However, this method has some fatal defects: a great deal of wastewater is produced which causes serious environmental pollution and it is also limited capacity of raw material treatment and high consumption of acid and alkali. Moreover, it is easy to cause protein denaturation. Therefore, it is imperative to explore an alternative extraction approach of grape seeds proteins.

The reverse micelles extraction is a novel separation technology with prospect for separating bio-product. Reverse micelles are aggregates of surfactant molecules spontaneously in non-polar solvents. The aggregates of surfactant molecules contain an inner core of water molecules and are dispersed in a continuous organic solvent medium. The bio-molecules can be transferred from the aqueous phase to the polar core of reverse micelles without loss in activity [16], mainly because of the attractive electrostatic interaction between the inner micelle charge wall and the bio-molecules. Optimization of extraction conditions has been used in enhancing the yield of many proteins [17] [18] [19] [20] [21]. However, there is no literature reported to optimize the extraction conditions for grape seeds protein using reverse micelles. Thus, the main aim of the present work was to optimize the conditions for proteins extraction by reversed micelles from grape seeds. The objective of this study was to develop an alternative extraction method of grape seeds protein by reverse micelles, and to investigate the effects of factors (CTAB concentration, extraction time, crude protein concentration, temperature, NaCl concentration, pH, alkyl alcohol than) on the forward extraction efficiency of grape seeds protein. Response surface methodology (RSM) was used to optimize the extraction conditions for enhancing the forward extraction efficiency of grape seeds protein by implementing the Box-Behnken experimental design [22].

2. Materials and Methods

2.1. Materials

Grape seeds were obtained from Palieri grape cultivar. Ceryl-trimethyl-ammonium bromide (CTAB), sodium chloride were purchased from the 6th Chemical

Reagent Factory of Tianjin, Chin. Other materials used in this study were of analytical grade.

2.2. Sample Preparation

Grape seeds were selected manually and cleaned to remove contaminants. Grape seeds were milled using a small scale hammer mill (FZ-102, Hebei province, China), and the resulting flour was sieved through a 200-mesh screen. Grape seeds power was defatted with n-hexane for 10 h and air-dried at room temperature (about 20˚C) by Soxhlet extraction. The power was kept in polyethylene bags and stored at 4˚C until used.

2.3. Extraction of Crude Protein

Defatted grape seeds power was soaked by 0.2 mol/L citric acid-sodium hydrogen phosphate buffer solution at pH 6.0 for 1 h. The solution and residue were isolated by a centrifuge at a rolling speed of 4000 rpm and 4˚C for 10 min. The crude protein was collected.

2.4. Reversed Micellar Extraction

The reversed micelles systems were formed by Ceryl-trimethyl-ammonium bromide (CTAB), methenyl trichoride and butyl alcohol. The aqueous solutions were crude protein after centrifugation. Sodium chloride was added to the aqueous solution to adjust the ionic strength. For the forward extraction, equal volumes of the reverse micellar systems (the organic solution) and aqueous solution were mixed in a test tube in a reciprocating shaker bath for various time periods and temperatures. The mixture was then centrifuged at 1500 g for 5 min to separate the two phases. The aqueous phase was then taken for analysis. All the experiments were carried out in duplicate.

2.5. Protein Determination [23]

Protein concentration in water phase was determined by UV-Vis spectrophotometer (LabTech UV-2100, Beijing). BSA was used as standard, and the results were expressed as BSA equivalents. The forward-extraction efficiency was calculated as follows.

$$\text{Forward-extraction efficiency}(\text{Y}\%) = \frac{\text{total protein in the supernatant} - \text{total protein in aqueous solution}}{\text{total protein in the supernatant}} \times 100\%$$

2.6. Screening of the Forward Extraction Conditions Using a Plackett-Burman Design

Plackett-Burman design, an efficient technique for forward extraction conditions optimization [24], was used to pick factors that significantly influenced extraction yield and insignificant ones were eliminated in order to obtain a smaller, more manageable set of factors. The extraction conditions were screened by Plackett-Burman design for seven variables at two levels. The main effect of each

variable was simply calculated as the difference between the average of measurements made at the high setting and the average of measurements observed at the low setting of that factor.

2.7. Steepest Ascent

After selecting the most important factor affecting the forward extraction yield in the screening study, the steepest ascent method was used to construct a line through the center of the design [25], due to the contribution obtained by Plackett-Buman design. Consequently, some experiments were implemented along this line with intervals, and the response at each point was measured. If a maximum value is found, that point could be employed as the center point for the following optimization experimental design.

2.8. Optimization of the Forward Extraction Conditions Using a Box-Behnken Design

Once critical factors were identified via screening, a Box-Behnken design for the most important independent variables (CTAB concentration, pH, NaCl concentration, crude protein concentration). Each at three levels with three replicates at the centre points was employed to fit a polynomial model:

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y_i is the predicted response, X_iX_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the iith quadratic coefficient and β_{ij} is the ijth interaction coefficient. Design expert package (version 7.0, Stat-Ease, Inc., Minneapolis, MN, USA) was used for the experimental design and regression analysis of the data obtained.

3. Results and Discussion

3.1. Screening of the Forward Extraction Conditions Using a Plackett-Burman Design

Based on the earlier studies, a total of seven variable conditions were analyzed for their effect on forward extraction using a Plackett-Burman design. The variables chosen for the present study were CTAB concentration, pH, extraction temperature, alkyl alcohol than, extraction time, NaCl concentration and crude protein concentration. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as −1 (low level) and +1 (high level). The yield of forward-extraction, determined for each experimental design was shown in **Table 1**. The analysis of variance (ANOVA) for the experimental designs was calculated, and the significant levels of each extraction condition were determined by p-value (**Table 2**). The analysis showed that CTAB concentration (X_1), pH (X_2), NaCl concentration (X_6), crude protein concentration (X_7) had p-value below the significance level (0.05). Therefore, they were estimated to be significant (**Table 2**). The final equation is as follows:

$$Y = 49.6633 + 14.715X_1 - 9.66167X_2 - 10.0383X_6 + 8.98X_7 \quad (2)$$

Table 1. The experimental design using the Plackett-Burman method for screening of forward extraction conditions.

Run	X_1^*	X_2^*	X_3^*	X_4^*	X_5^*	X_6^*	X_7^*	The forward extraction yield (%)
1	40	8	20	4:1	20	0.04	1	39.36
2	20	8	40	2:1	20	0.04	2	32.21
3	40	4	40	4:1	10	0.04	2	72.5
4	20	8	20	4:1	20	0.02	2	35.58
5	20	4	40	2:1	20	0.04	1	19.36
6	20	4	20	4:1	10	0.04	2	45.02
7	40	4	20	2:1	20	0.02	2	82.3
8	40	8	20	2:1	10	0.04	1	29.3
9	40	8	40	2:1	10	0.02	2	84.25
10	20	8	40	4:1	10	0.02	1	19.31
11	40	4	40	4:1	20	0.02	1	78.56
12	20	4	20	2:1	10	0.02	1	58.21

*X_1: CTAB concentration (mmol/L); X_2: pH; X_3: Extraction temperature (˚C); X_4: alkyl alcohol than (V:V); X_5: Extraction time (min); X_6: NaCl concentration (mol/L); X_7: Crude protein concentration (mg/mL).

Table 2. Statistical analysis of Plackett-Burman design.

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	Prob > F
Model	5895.451	4	1473.863	15.16151	0.0015
X_1	2598.375	1	2598.375	26.72928	0.0013
X_2	1120.174	1	1120.174	11.52314	0.0115
X_6	1209.218	1	1209.218	12.43913	0.0096
X_7	967.6848	1	967.6848	9.9545	0.0160
Residual	680.4755	7	97.21079		
Cor Total	6575.926	11			

$R^2 = 0.896$.

The multiple correlation coefficient (R^2) of this first-order model is 0.896, which means that 89.6% of the data variation can be evaluated by the model. However, the difference between the adjusted R^2 value (83.7%) and the predicted R^2 value (69.6%) revealed that a first-order model is not an adequate mathematical equation for demonstrating the relationship between the significant independent variables and the response. Therefore, a second-order model should be employed for further investigation.

It can be seen from Equation (2) that all the significant factors, except pH (X_2), NaCl concentration (X_6) had a positive sign. Therefore, increasing their value would result in an increase in the level of forward-extraction efficiency. Further statistical analysis revealed that the difference between the means of the center point and factorial trials in this design was significant ($P < 0.05$). This indicated that the optimum levels for forward-extraction efficiency would be

beyond the experimental ranges chosen for the Plackett-Burman design. Therefore, the steepest ascent method should be used. All the other insignificant variables were neglected and optimum combinations of these four were further analyzed by a steepest ascent design.

3.2. Steepest Ascent

The steepest ascent method was used to construct a line through the center of the design, due to the contribution obtained by Plackett-Burman first-order equation. Consequently, some experiments were implemented along this line with defined intervals, and the response at each point was measured. If a maximum value is found, that point could be employed as the center point for the following optimization experimental design. These results are summarized in Table 3.

3.3. Further Optimization of the Extraction Conditions Using a Box-Behnken Design

3.3.1. Statistical Analysis and Model Fitting

Experiments were carried out in duplicates to arrive at an optimum combination of the four conditions above using Box-Behnken design. Based on the results of steepest ascent experiments, Table 4 gave the design and results of experiments carried out by the Box-Behnken design. The results obtained were submitted to ANOVA on Design-expert 7.0 package and the regression model was given as

$$\begin{aligned} Y = {} & 82.244 + 0.1917A + 3.9B - 0.895C - 1.3483D - 1.7225AB - 1.285AC \\ & + 2.2575AD + 1.45BC + 5.3575BD + 1.015CD \\ & - 6.6749A^2 - 4.9474B^2 - 4.019C^2 - 5.145D^2 \end{aligned} \quad (3)$$

1) Analysis of variance (ANOVA) for the extraction yield of grape seeds protein

The analysis of variance (ANOVA) was conducted to test the significance of the fit of the second-order polynomial equation for the experimental data as shown in Table 5. The Model F-value of 10.37 implies the model is significant. There is only a 0.01% chance that a "Model F-value" could occur due to noise. The *P*-values are used as a tool to check significance of each variable, which also indicate the interaction strength between each independent variable. The smaller *P*-values, the bigger the significance of the corresponding variable. *P*-values in

Table 3. Design and data from the steepest ascent experiment.

Run	CTAB concentration (mmol/L)	pH	NaCl concentration (mol/L)	crude protein concentration (mg/mL)	The forward extraction yield (%)
1	30	6	0.03	1.5	55.49
2	33	5.8	0.025	1.7	64.02
3	36	5.6	0.02	1.9	73.24
4	39	5.4	0.015	2.1	78.68
5	42	5.2	0.01	2.3	71.45
6	45	5	0.005	2.5	68.47

Table 4. Box-Behnken experimental design and results for protein extraction yield.

Trial No.	Coded variables[a]				Uncoded variables				The forward extraction yield (%)	
	A	B	C	D	A	B	C	D	Experimental	Predicted
1	−1	−1	0	0	33	5	0.015	2.1	65.23	64.81
2	1	−1	0	0	45	5	0.015	2.1	69.2	68.64
3	−1	1	0	0	33	5.8	0.015	2.1	75.5	76.05
4	1	1	0	0	45	5.8	0.015	2.1	72.58	72.99
5	0	0	−1	−1	39	5.4	0.01	1.9	76.1	76.34
6	0	0	1	−1	39	5.4	0.02	1.9	73.24	72.52
7	0	0	−1	1	39	5.4	0.01	2.3	70.9	71.61
8	0	0	1	1	39	5.4	0.02	2.3	72.1	71.85
9	−1	0	0	−1	33	5.4	0.015	1.9	72.28	73.84
10	1	0	0	−1	45	5.4	0.015	1.9	66.23	69.71
11	−1	0	0	1	33	5.4	0.015	2.3	70.23	66.63
12	1	0	0	1	45	5.4	0.015	2.3	73.21	71.53
13	0	−1	−1	0	39	5	0.01	2.1	71.22	71.72
14	0	1	−1	0	39	5.8	0.01	2.1	79.23	76.62
15	0	−1	1	0	39	5	0.02	2.1	64.55	67.03
16	0	1	1	0	39	5.8	0.02	2.1	78.36	77.73
17	−1	0	−1	0	33	5.4	0.01	2.1	69.5	70.97
18	1	0	−1	0	45	5.4	0.01	2.1	74.23	73.92
19	−1	0	1	0	33	5.4	0.02	2.1	71.3	71.75
20	1	0	1	0	45	5.4	0.02	2.1	70.89	69.56
21	0	−1	0	−1	39	5	0.015	1.9	78.3	74.96
22	0	1	0	−1	39	5.8	0.015	1.9	73.25	72.04
23	0	−1	0	1	39	5	0.015	2.3	60.2	61.55
24	0	1	0	1	39	5.8	0.015	2.3	76.58	80.06
25	0	0	0	0	39	5.4	0.015	2.1	80.2	82.24
26	0	0	0	0	39	5.4	0.015	2.1	82.56	82.24
27	0	0	0	0	39	5.4	0.015	2.1	83.26	82.24
28	0	0	0	0	39	5.4	0.015	2.1	82.9	82.24
29	0	0	0	0	39	5.4	0.015	2.1	82.3	82.24

[a]A: CTAB concentration (mmol/L), B: pH, C: NaCl concentration (mol/L), D: Crude protein concentration (mg/mL).

this study less than 0.01 indicate model terms are very significant. Among model terms, B, BD, A^2, B^2, C^2, D^2 are significant with a probability of 99%. P-values greater than 0.05 indicate the model terms are not significant. Here the R^2 value was 91.21%, which could explain 91.21% variability of the response. It indicates a good agreement between experimental and predicted values and implies that the mathematical model is very reliable for protein extraction field in the present study. At the same time, a very low value 3.3 of coefficient of the variation (CV) clearly indicated a very degree of precision and a good deal of reliability of the

Table 5. ANOVA for response surface quadratic model for protein extraction field.

Factors	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	856.6741	14	61.19101	10.37369	<0.0001
A-A	0.4408	1	0.440833	0.074734	0.7886
B-B	182.52	1	182.52	30.94255	<0.0001
C-C	9.6123	1	9.6123	1.62957	0.2225
D-D	21.8160	1	21.81603	3.698464	0.0750
AB	11.8680	1	11.86803	2.011982	0.1779
AC	6.6049	1	6.6049	1.119726	0.3079
AD	20.3852	1	20.38523	3.4559	0.0842
BC	8.41	1	8.41	1.425744	0.2523
BD	114.8112	1	114.8112	19.4639	0.0006
CD	4.1209	1	4.1209	0.698615	0.4173
A^2	289.0022	1	289.0022	48.99444	<0.0001
B^2	158.7693	1	158.7693	26.9161	0.0001
C^2	104.8199	1	104.8199	17.77007	0.0009
D^2	171.6984	1	171.6984	29.10796	<0.0001
Residual	82.58144	14	5.898674		
Lack of Fit	76.83792	10	7.683792	5.351277	0.0601
Pure Error	5.74352	4	1.43588		
Cor Total	939.2555	28			

experimental values.

3.3.2. Analysis of Response Surface

Response surface plots are shown in **Figure 1**, which depict the interactions between two variables by keeping the other variables at their zero levels for forward extraction yield. The effect of CTAB concentration and pH on the yield of extracted grape seeds protein is shown in **Figure 1(a)**.

Figure 1(b) represents the interaction between CTAB and NaCl concentration. Lower and higher levels of both CTAB and NaCl concentration did not result in higher forward extraction yields. The shape of the response surface curves showed a moderate interaction between these tested variables.

Figure 1(c) depicts the interaction of CTAB concentration and crude protein concentration where the shape of the response surface indicated no positive interaction between these two factors.

Figure 1(d) shows the effects of pH and concentration of NaCl on the forward extraction yield of grape seeds protein. This result showed that pH changes were more effective than NaCl concentration changes for yield extraction.

The graph shown in **Figure 1(e)** & **Figure 1(f)** indicates that pH, NaCl concentration and crude protein concentration had a quadratic effect on protein extraction.

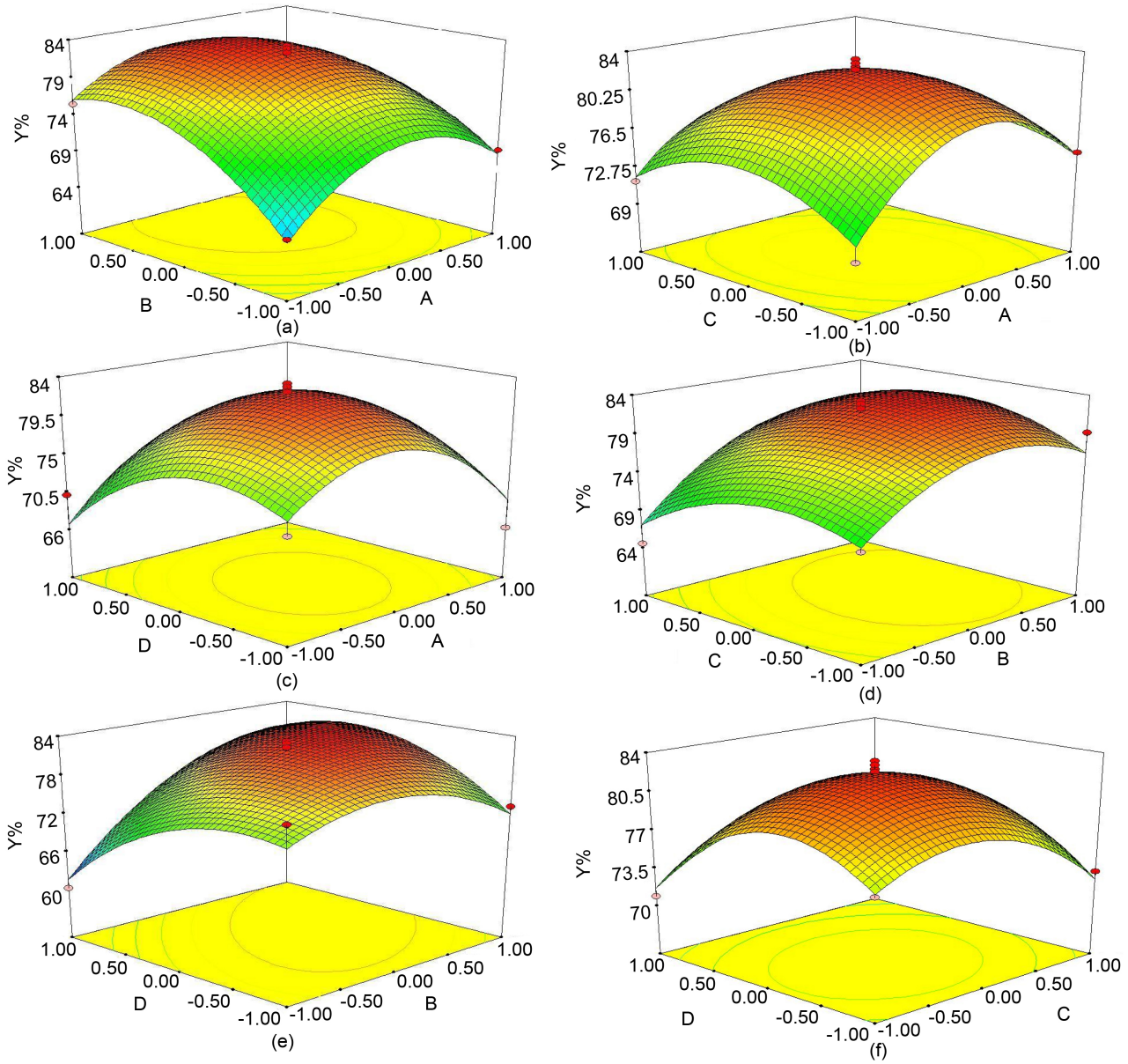

Figure 1. The response surface plot showing the effects of the forward extraction parameters on grape seeds protein yield. (a) at varying CTAB concentration (A) and pH (B), (b) at varying CTAB concentration (A) and NaCl concentration (C), (c) at varying CTAB concentration (A) and crude protein concentration (D), (d) at varying pH (B) and NaCl concentration (C), (e) at varying pH (B) and crude protein concentration (D), (f) at varying NaCl concentration (C) and crude protein concentration (D).

3.3.3. Optimum Conditions and Model Verification

In order to optimize processing conditions of grape seeds protein extraction , the first partial derivatives of the regression model were equated to zero according to A, B, C and D. From the model, optimum conditions for grape seeds protein extraction were prepared as follows: CTAB concentration 38.84 mmol/L, NaCl concentration 0.01 mol/L, crude protein concentration 2.12 mg/mL. The pH of the aqueous phase was 5.58. Under such conditions, the yield of forward extraction process was predicted to be 83.06%.

To ensure the predicted result was not biased toward the practical value, experiment rechecking was performed by using these modified optimal conditions:

CTAB concentration 39 mmol/L, NaCl concentration 0.01 mol/L, crude protein concentration 2.1 mg/mL. The pH of the aqueous phase was 5.6. A mean value of 82.3% (N = 3) was obtained from real experiment. The results of analysis confirmed that the response model was adequate for reflecting the expected optimization, and the model of Equation (3) was satisfactory and accurate.

4. Conclusion

The data presented in this article demonstrate the feasibility of the forward extraction of protein from grape seeds by reverse micelles. Based on the Plackett-Burman design and steepest ascent, response surface methodology (RSM) was used to estimate and optimize the experimental variables: CTAB concen- tration, pH, NaCl concentration and crude protein concentration. The optimal forward extraction conditions for grape seeds protein by reverse micelles were determined as follows: CTAB concentration 39 mmol/L, NaCl concentration 0.01 mol/L, crude protein concentration 2.1 mg/mL; the pH of the aqueous phase was 5.6. Under these conditions, the forward extraction yield of grape seeds protein was 82.3%, which was closed with the predicted yield value. The data presented in this article demonstrate the feasibility of the forward extraction of protein from grape seeds by reverse micelles. Reverse micelles extraction was an efficient method compared to conventional solvent extraction. These results demonstrated the successful extraction of protein with Reverse micelles extraction, providing potential benefits for industrial extraction of protein from grape seeds.

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Conflict of Interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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