



Optimal Enzyme-Assisted Ethanol Extraction of Flavonoids from *Broccoli* by RSM and Research on Antioxidant Effect

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Abstract: Enzyme-assisted ethanol extraction of total flavonoids from *broccoli* and research on its antioxidant effect, were studied in this paper. Enzyme concentration, solid-liquid ratio, extraction temperature and time were determined as four single-factor in the experiment. According to the single-factor experimental results, the extract process was further optimized by RSM and Box-Behnken design. The optimum extract conditions were enzyme concentration of 0.15%, solid-liquid ratio of 5:30 (g: mL), extraction temperature of 70°C and time of 2 h, the experimental responding extraction ratio was 0.935% corresponding to the theoretical predictions of 0.953%. The experimental extraction ratio matched well with the theoretical value by solving the multiple regression equation. RSM has been proved to be an effective technique for optimization of extraction process and the fitted quadratic model has a predictive effect on target extracts. The scavenging effect of *broccoli* extracts on hydroxyl radicals displayed a significant dose-effect relationship.

Keywords: Enzyme-Assisted Ethanol Extraction, Response Surface Methodology, Box-Behnken Design, *Broccoli*, Antioxidant Effect

1. Introduction

Flavonoids are natural polyphenolic antioxidants with significant effects on anti-peroxidation and free radical scavenging. Flavonoids have significant antioxidant, anti-cancer, anti-inflammatory, bactericidal, anti-virus and regulating body immunity, etc [1-4]. The Extraction technology of flavonoids reported in the literature includes organic solvent extraction, ultrasonic extraction, microwave extraction, supercritical fluid extraction and Enzyme-assisted extraction [5-6].

Response Surface Methodology (RSM) is a statistical method in order to solve problems containing multiple variables. Multiple quadratic function was obtained by reasonable experimental design to fit the factors and response value, and the optimal process parameters were determined by analyzing regression equation. RSM has advantages of shorten experimental cycle, high accuracy of the regression equation, Interactions between multiple factors, etc [7]. RSM was demonstrated an effective statistic technique for optimizing complex processes, which has been successfully

used to optimize the extraction of total flavonoids from many medicine plants [8-9]. The total flavonoids extraction ratio was greatly influenced by extraction conditions, Box-Behnken Design was performed to predict the optimal extraction conditions [10].

There were related reports on extraction of total flavones from *broccoli* in the literature [11-13]. X. Zhou research group from Tianshui normal university reported the extraction of total flavones from *Broccoli* by RSM. the reported optimum extract conditions were ethanol concentration of 90%, solid-liquid ratio of 1.0:30 (g:mL), extraction temperature of 85.28°C and time of 1.82 h with responding extraction ratio of 0.853% [11]. J. Chen from Zhangzhou institute of technology reported Ultrasonic and cellulase assisted extraction of of total flavonoids from *broccoli* [12-13]. The former extraction ratio was 1.128% under ethanol concentration of 51% solid-liquid ratio of 1.0:20 (g:mL), extraction temperature of 53°C and ultrasonic power of 201w. The latter extraction ratio was reached up to 1.251% with the enzyme dosage of 0.84%, enzymolysis pH of 5.0 and enzymolysis time of 57 min.

Here, optimization of Enzyme-assisted ethanol extraction flavonoids from *broccoli* by RSM and determination of

flavonoids content by spectrophotometry were reported. Additional, cellulase and pectinase enzyme were assisted the extract process by destroying the cell walls. The hydroxyl radicals scavenging effect of *broccoli* extracts was also studied. This research was helpful in providing valuable flavonoids content data for development and utilization of *broccoli*.

2. Materials and Methods

2.1. Materials

Broccoli (purchased in chuxiong's agricultural market) dry crush Spare.

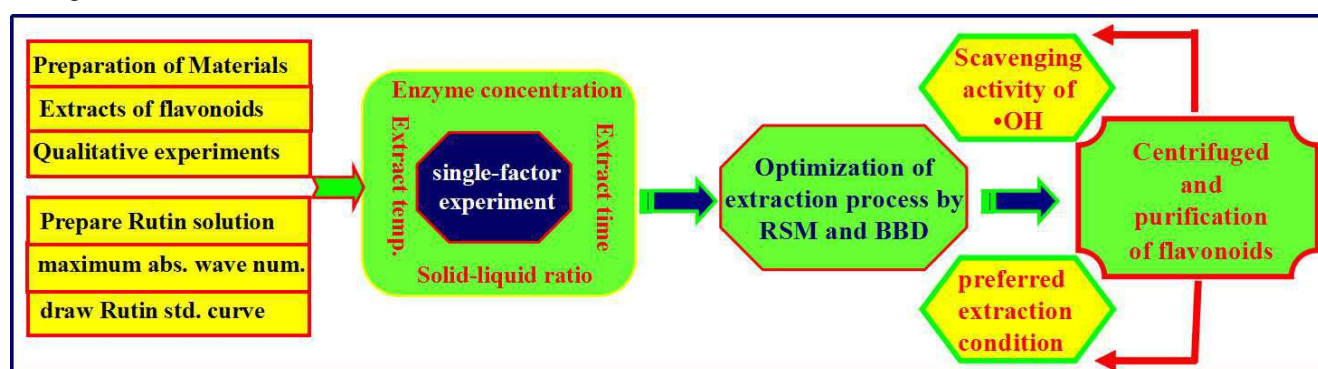


Figure 1. The experimental illustration of RSM optimization of broccoli flavonoids extraction and radical scavenging effects.

2.3. Optimization of the Extraction Process by RSM

Optimization of flavonoids extraction by RSM was operated as references[16]. Box-Benhnken Design combining with quadratic response model of four factors and three levels were performed to optimize extraction. First, the independent variables of four single-factors were determined, the level of

variables were coded by -1, 0, 1 based on the results of single-factor experiment (as shown in Table 1). A total of 25 points were designed, including points 16 factorial, 8 star points and 1 central points to ensure the precision of experiment.

Table 1. Factors and levels of Response Surface Methodology.

Code levels of independent variables	Independent variables			
	A: Enzyme concentration (%)	B: Solid-liquid ratio (mL·g ⁻¹)	C: Extraction temperature (°C)	D: Extraction time (h)
-1	0.05	3:30	60	2.0
0	0.10	5:30	70	3.0
+1	0.15	7:30	80	4.0

2.4. Study on Hydroxyl Radical Inhibition Activity

Total flavonoids was extracted from *broccoli* under the preferred conditions by RSM. The extracts was centrifuged, purification by macroporous resin, ethanol elution (ethanol volume fraction 80%), solvent evaporation, freeze-dried to obtain the total flavonoids powder. *Broccoli* total flavonoids solutions with different concentrations were prepared. Hydroxyl radical scavenging effect was operated as reference[16-18], *broccoli* total flavonoids and BHT solutions with different concentrations were added, the absorbance was measured under 510nm, the average of absorbance was collected by parallel experiments. The scavenging ratio was calculated as:

$$\text{The scavenging ratio of hydroxyl radical (\%)} = \frac{[A_0 - (A_x - A_{x_0})]}{A_0} \times 100$$

A_0 is the absorbance of control solution, A_x was the absorbance of *Broccoli* extract; A_{x_0} was background absorbance of the extract without H_2O_2 .

3. Results and Discussion

Chromogenic reaction of *broccoli* extracts was consistent with Rutin, which confirms that *broccoli* contained total flavonoids. The absorption spectra of Rutin and *broccoli* extracts determined the maximum absorption wavelength of 510nm. The linear regression equation was formulated as $A=3.283C-0.009$, $R^2=0.999$ by the standard curve (Figure 2).

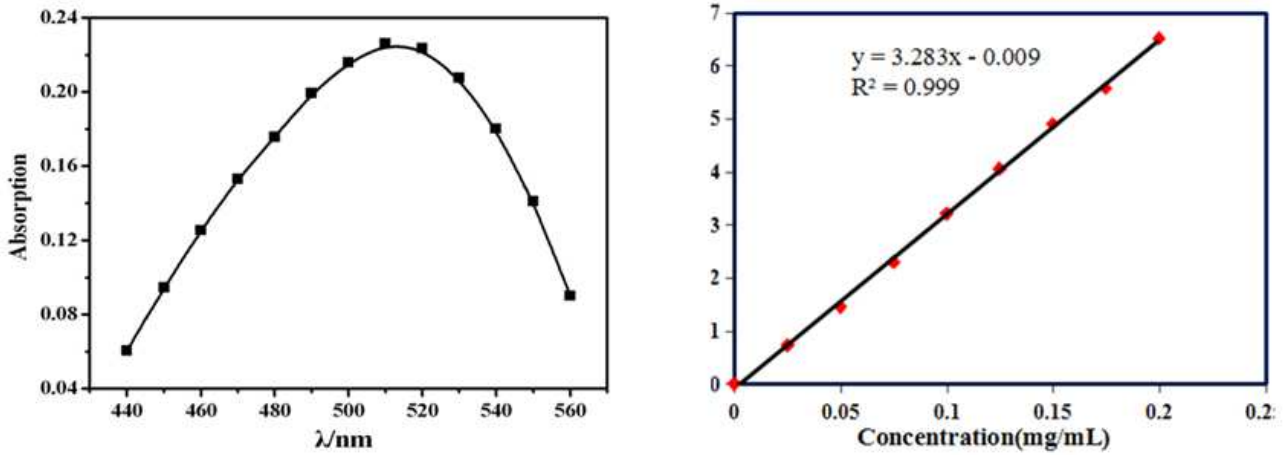


Figure 2. Absorption spectra and standard curve of broccoli extract.

3.1. Result of Single-Factor Experiment

Influences of each single factor on extraction ratio were shown in Figure 3. The optimum enzyme concentration was 0.10% with the responding extraction ratio of 1.984% for *broccoli*. Extraction ratio was significantly increased with the increasing of solid-liquid ratio, when the solid-liquid ratio was higher than 5:30 (g:mL), extraction ratio began to decrease. Higher than 5:30 (g:mL), resulted in a waste of materials; lower than 5:30 (g:mL), the concentration gradient of solid-liquid phase was too small, which was not conducive to the dissolution of total flavonoids. The preferred solid-liquid ratio was 5:30 (g:mL) with the responding extraction ratio of 4.44% for *broccoli*. With the temperature increasing,

extraction ratio increased significantly, the maximum of extraction ratios of 1.967% was achieved at 70 °C. The higher temperature would lead to ethanol evaporation and oxidative degeneration of flavonoids. The lower temperature decreases the rate of flavonoids dissolution. So the optimum extraction temperature was 70°C for extraction total flavonoids from *broccoli*. The optimum extraction time was 3.0 h with the responding extraction ratio of 3.107% from *broccoli*. Lower than 2.0h, the dissolution balances were not achieved, higher than 2.0h, the dissolution of other fat-soluble impurities complicated the post-separation and purification of total flavonoids.

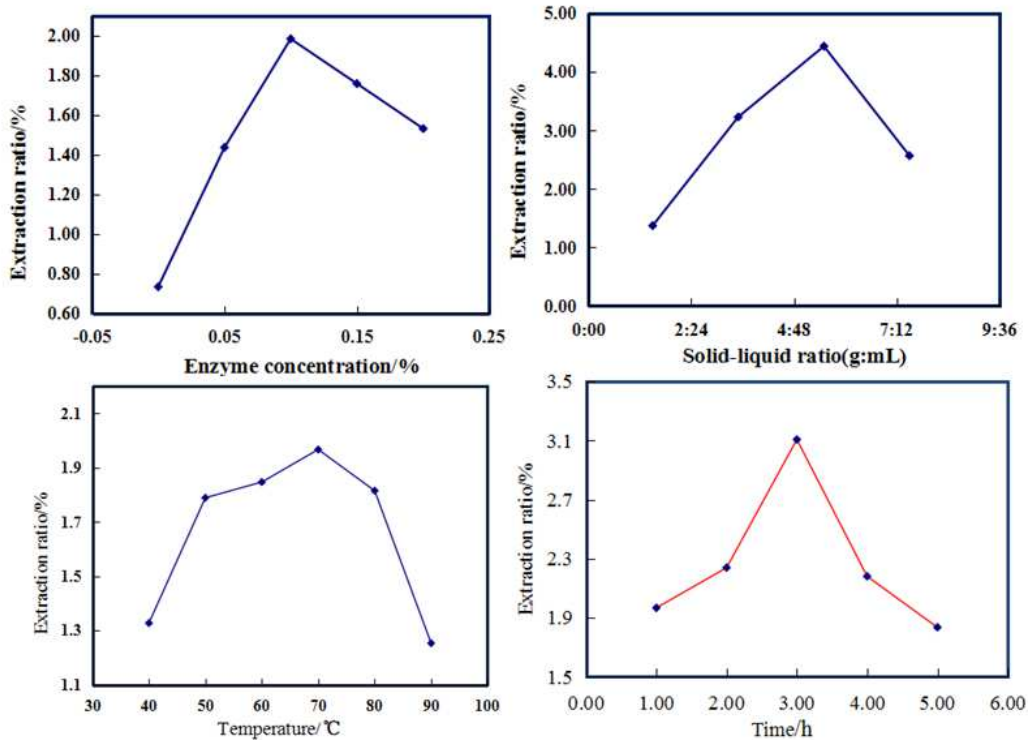


Figure 3. Effect of enzyme concentration, solid-liquid ratio, temperature and time on the extraction ratio of total flavonoids.

According to the results of single factor experiments, enzyme concentration of 0.05~0.15%; solid-liquid ratio of 3.0:30~7.0:30 (g:mL), extraction temperature of 60~80°C and extraction time of 2.0 ~ 4.0 h were determined as the factors and scope for response surface analysis.

3.2. Response Surface Optimization of Extraction process

3.2.1. Multiple Regression Model and Analysis of Variance (ANOVA)

The extraction processes of total flavonoids from *broccoli*

was further optimized by RSM. According to the single-factor experimental results of 3.1, enzyme concentration of 0.05~0.15%; solid-liquid ratio of 3.0:30~7.0:30 (g:mL), extraction temperature of 60~80°C and extraction time of 2.0 ~ 4.0 h were selected as the actual levels of factors to maximize the extraction ratio of total flavonoids by Box-Behnken Design, as listed in Table 1. A total of 25 experiments were designed, including 16 factorial experiments, 8 star experiments and 1 central experiments to estimate the absolute error.

Table 2. Experimental design and results for extraction ratio by using Box-Behnken Design and RSM.

Std	Run	Independent variables				Response 1 extraction ratio (%)
		A: Enzyme concentration (%)	B: Solid-liquid ratio (g:mL)	C: Extraction temperature (°C)	D: Extraction time (h)	
14	1	0.10	7:30	60	3	1.9818
23	2	0.10	3:30	70	4	2.2080
7	3	0.10	5:30	60	4	1.7664
12	4	0.15	5:30	70	4	2.1972
21	5	0.10	3:30	70	2	2.3550
16	6	0.10	7:30	80	3	1.7838
25	7	0.10	5:30	70	3	1.9932
15	8	0.10	3:30	80	3	2.4090
20	9	0.15	5:30	80	3	1.7088
4	10	0.15	7:30	70	3	1.9080
9	11	0.05	5:30	70	2	1.9200
6	12	0.10	5:30	80	2	2.1024
10	13	0.15	5:30	70	2	2.0736
5	14	0.10	5:30	60	2	2.1024
3	15	0.05	7:30	70	3	1.8249
18	16	0.15	5:30	60	3	2.0220
17	17	0.05	5:30	60	3	1.7664
2	18	0.15	3:30	70	3	2.1540
24	19	0.10	7:30	70	4	1.9662
19	20	0.05	5:30	80	3	1.9644
8	21	0.10	5:30	80	4	2.1900
13	22	0.10	3:30	60	3	2.0640
11	23	0.05	5:30	70	4	1.9119
1	24	0.05	3:30	70	3	1.7880
22	25	0.10	7:30	70	2	2.0340

The RSM experimental design and results of extraction ratio for *broccoli* was shown in Table 2. Extraction ratio ranged from 1.708% to 2.409%. The maximum of extraction ratio was recorded under the experimental conditions of enzyme concentration of 0.10%; solid-liquid ratio of 3:30 (g:mL), extraction temperature of 80°C and extraction time of 3.0h with the responding extraction ratio of 2.409% . The experimental data was analyzed by using Design-Expert8.0, the response variable of extract ratio and the four independent variables were related by the following multiple regression

equation:

$$\text{Extract ratio} = +1.99+0.074A-0.12 B+0.038 C-0.029D-0.071 AB-0.13AC+0.033AD-0.14 B C+0.020 BD+0.11 CD-0.10 A^2+0.055B^2-0.022C^2+0.098 D^2$$

$$\text{Extract ratio} = -0.24321+28.94275* \text{enzyme concentration} + 0.31811* \text{solid-liquid ratio}+0.062812* \text{extraction temperature} -1.47580* \text{extraction time} -0.70725* \text{enzyme concentration} * \text{solid-liquid ratio}-0.25560* \text{enzyme concentration} * \text{extraction temperature}+0.65850 * \text{enzyme concentration} * \text{extraction time}-6.78750E-003* \text{solid-liquid ratio} * \text{extraction temperature}+9.90000E-003 * \text{solid-liquid}$$

ratio *extraction time+0.010590* extraction temperature * extraction time-40.05000* enzyme concentration² + 0.013641* solid-liquid ratio² - 2.23500 E-004* extraction temperature²+ 0.098363 * extraction time².

Table 3 showed the analysis of variance (ANOVA) for the multiple regression equation, The Model F-value of 3.45 implies the model is significant. There is only a 2.75% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, The linear term of solid-liquid ratio and the interaction terms of solid-liquid ratio and extraction temperature were were significant for response variables. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The analysis result of ANVOA indicated the response variable and the four independent variables were not a simply

linear relationship. The value of R² (0.8248) and R_{Adj}² (0.5883) for the multiple regression equation was not approaching, indicated that the model need to be further optimized. The adequate precision value of 7.584 was much higher than the desirable value of 4.00, which presented a higher "signal (response) to noise (deviation)" and indicated that the model were significant for the extraction process, and the P value for the model were less than 0.05. This model can be used to navigate the design space. The lower value of coefficient of the variance (C.V. =5.86%) also indicated that the model have a predictable effect for the extraction process of total flavonoids.

The result of analysis of variance (ANOVA) showed that significant levels of the four factors were sorted by solid liquid ratio > enzyme concentration > extraction temperature > extraction time. The linear term of solid liquid ratio and the interaction term of solid liquid ratio and extraction temperature were significant for the response variables.

Table 3. ANOVA for Response Surface Quadratic Model for broccoli.

Source of deviation	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	significant
Model	0.669027	14	0.047788	3.449420	0.0275	
A	0.065712	1	0.065712	4.743238	0.0544	
B	0.182361	1	0.182361	13.16320	0.0046	s
C	0.017282	1	0.017282	1.247484	0.2902	
D	0.010075	1	0.010075	0.727207	0.4137	
AB	0.020008	1	0.020008	1.444229	0.2571	
AC	0.065331	1	0.065331	4.715762	0.0550	
AD	0.004336	1	0.004336	0.312998	0.5882	
BC	0.073712	1	0.073712	5.320713	0.0438	s
BD	0.001568	1	0.001568	0.113193	0.7435	
CD	0.044859	1	0.044859	3.238039	0.1021	
A ²	0.028306	1	0.028306	2.043184	0.1834	
B ²	0.008406	1	0.008406	0.606752	0.4541	
C ²	0.001410	1	0.00141	0.101807	0.7562	
D ²	0.027318	1	0.027318	1.971885	0.1905	
Residual	0.138538	10	0.013854			
Cor Total	0.807566	24				
R-Squared	0.8284		Adj R-Squared	0.5883		
Adeq Precision	7.584		C.V. %	5.86		

3.2.2. RSM Analysis and Research on the Optimum Extract Process

The multiple regression models could be vividly reflected by the 3D response surface and Contour lines plots, as shown in Figures 4-9. The 3D response surface plots reflected the effects of multiple independent variables on the response value, the sensitivity of response value to different factors could also be analyzed. The corresponding surfaces were more steeper, indicated its extremely significant impact on response value. The closer the curve to the center in the Contour lines plots, the greater of the value corresponding response variable; contour lines with circular indicated weak interactions between independent variables, contour lines

with oval indicated strong interaction. An increase of each independent variable resulted in an initial increase and then decrease of response variables.

The interaction effect of enzyme concentration and solid-liquid ratio on the extraction ratio was illustrated in the 3D response surface plots of Figure 4, the corresponding surface of solid-liquid ratio was more steeper than that of enzyme concentration, indicated its significant impact on responding value. cricle-like Contour lines indicated the weak interaction effect of the two terms on responding values with the p value of 0.2571. the maximum extraction ratios of 2.409% was achieved as enzyme concentration of 0.13% and solid-liquid ratio of 3.0: 30 (g:mL).

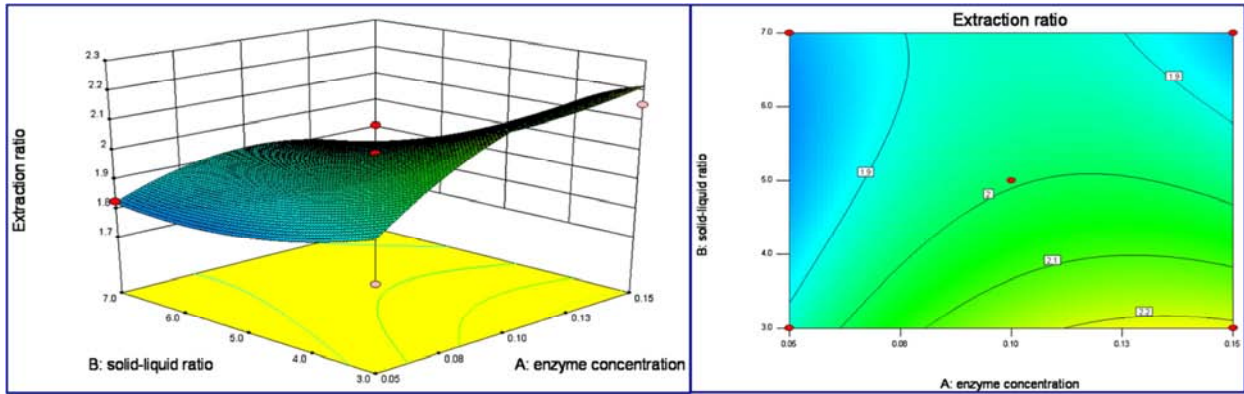


Figure 4. RSM analyses for interactive effects of enzyme concentration and solid-liquid ratio.

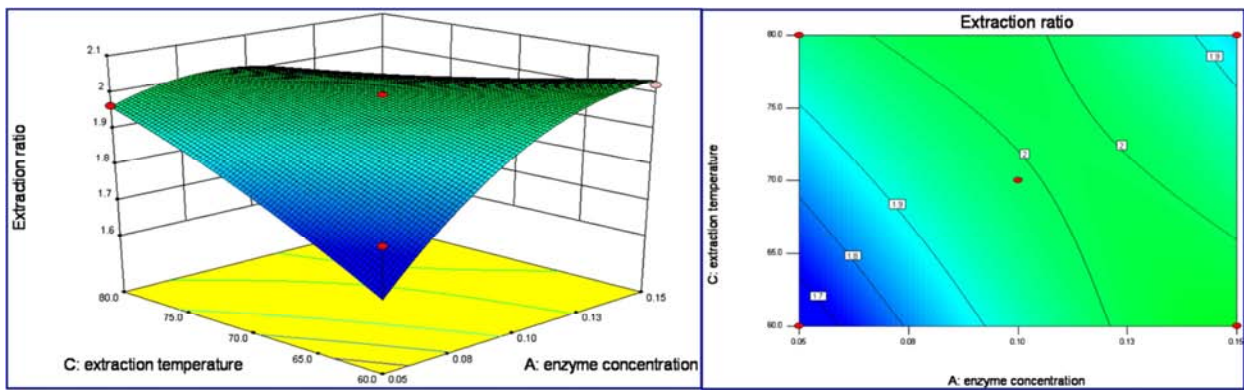


Figure 5. RSM analyses for interactive effects of enzyme concentration and extraction temperature.

The interaction terms of enzyme concentration and extraction temperature on the responding values were displayed in the 3D response surface plots of Figure 5, the impact of the former term on extraction ratio was more significant than that of the latter. Contour lines plots was approaching to Oval, indicated the interaction effects of the above two terms on the responding value began to significant with $P=0.0550$. the maximum extraction ratios of 2.409% was obtained as the enzyme concentration of 0.13% and extraction temperature of 79.7°C.

The impact of the enzyme concentration on the responding value was more significance than that of extraction time, as

shown in Figure 6. Contour lines plots also indicated the weak interaction effects of the above two terms on the response values $P = 0.5882$. The maximum extraction ratios of 2.409% was obtained as enzyme concentration of 0.13% and extraction time of 3.8 h. From the 3D response surface plots of Figure 7, the impact of solid-liquid ratio and extraction temperature terms on the extraction ratios was significant for the responding value with $p=0.0438$. Oval-like Contour lines plots indicated significance interaction effect of above two terms on the response values. the maximum extraction ratios of 2.409% was achieved as the solid-liquid ratio of 3:30· (g:mL) and extraction temperature of 79.7°C.

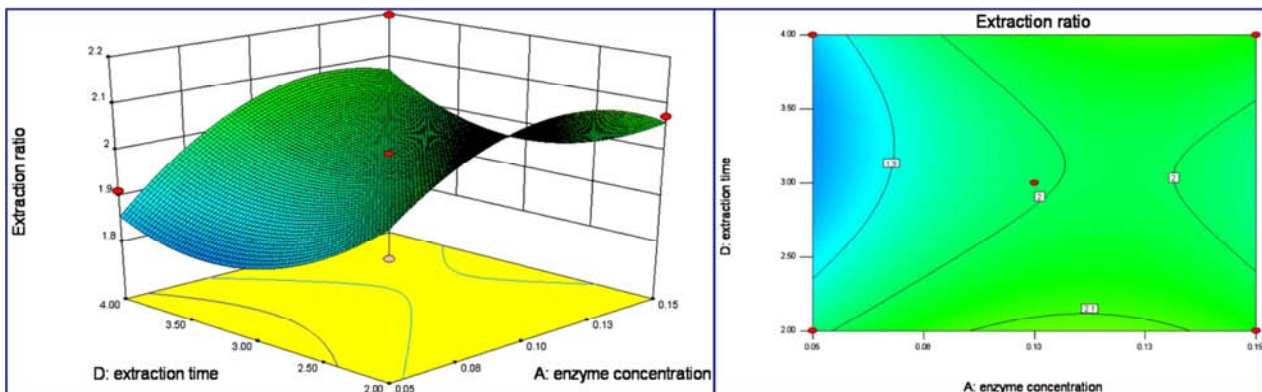


Figure 6. RSM analyses for interactive effects of enzyme concentration and extraction time.

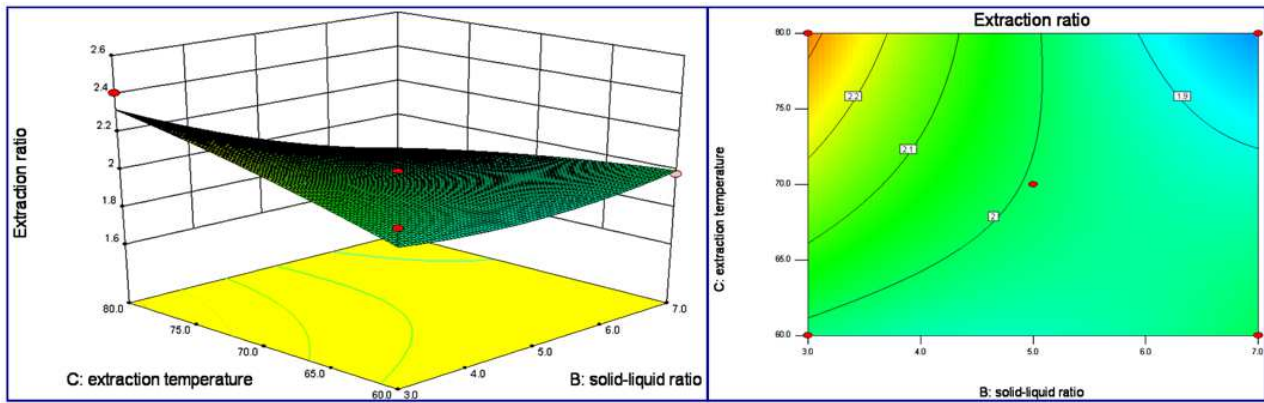


Figure 7. RSM analyses for interactive effects of solid-liquid ratio and extraction temperature.

The 3D response surface and Contour lines plots of the interaction effect of solid-liquid ratio and extraction time on extraction ratio was shown in Figure 8, the interaction effect of the above two terms was all not significant. While the linear of solid-liquid ratio has a significance effect on the extraction ratio from the more steeper surface. The maximum extraction

ratio was achieved as 2.409%. Both the linear and interaction effects of extraction temperature and time on the extraction ratio were not significant as in the 3D response surface and Contour lines plots of Figure 9. The maximum extraction ratio of 2.409% was achieved under extraction temperature of 79.7°C and time of 3.8 h.

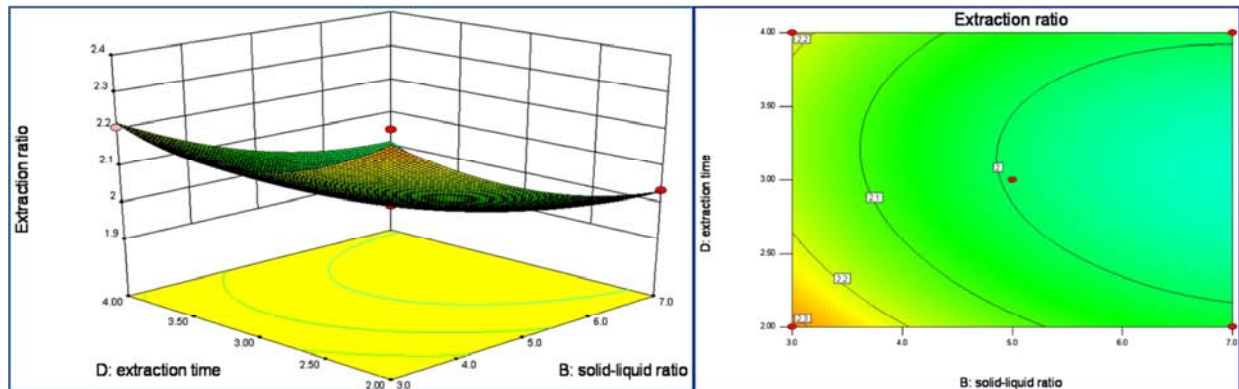


Figure 8. RSM analyses for interactive effects of solid-liquid ratio and extraction time.

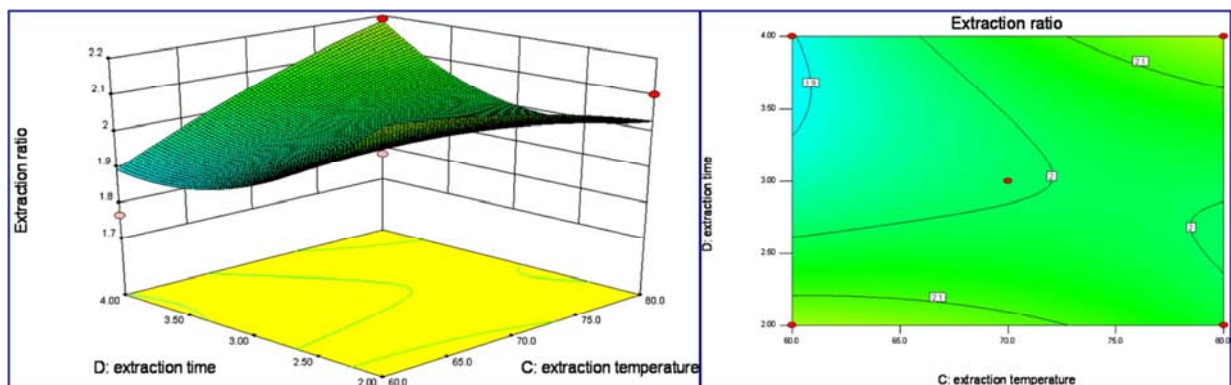


Figure 9. RSM analyses for interactive effects of extraction temperature and time.

The optimum values of the selected variables were obtained by solving the multiple regression equation. The values obtained were A=0.13%, B=3.0:30 (g:mL), C=79.7°C and D=3.8 h, with the corresponding extraction ratio of 2.409% for *broccoli*, calculated by Design-Expert 8.0 software. In the experiment, three triplicate experiments were performed under preferred extract conditions of enzyme concentration of 0.10%, solid liquid ratio of 3:30 (g:mL), extraction

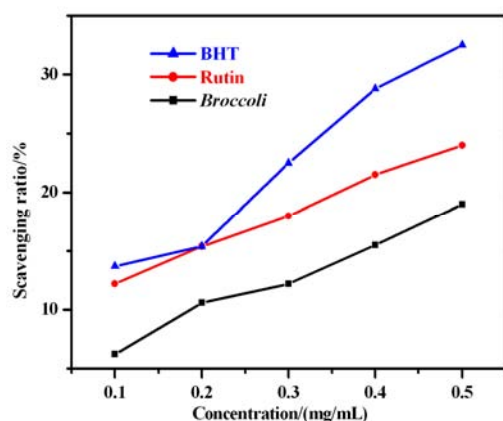
temperature of 80°C and time of 2h. the average maximum value of extraction ratio was 2.409% for *broccoli*. The obtained experimental data were listed in Table 4, the experimental extraction ratio and calculated values of response variable matched each other very well, which indicated that the model were reliable for the extraction process of *broccoli*.

Table 4. The obtained experimental data of extraction ratio under preffer experimental conditions.

Run	Enzyme concentration (%)	Solid-liquid ratio (g:mL)	Extraction temperature (°C)	Extraction time (h)	extraction ratio	Average value
1	0.10	3:30	80	3	2.409	
2	0.10	3:30	80	3	2.409	2.409
3	0.10	3:30	80	3	2.409	

3.3. Study on Hydroxyl Radical Scavenging Activity

The Flavonoids compounds had a scavenging effect on hydroxyl radical, superoxide radicals and •DPPH radicals as the o-dihydroxy from the structural benzene ring [14-16]. Hydroxyl radical scavenging effects of *broccoli* extracts, Rutin and BHT with different concentrations were measured and listed in Figure 10. With the concentration increasing, the scavenging ratio for hydroxyl radicals increased, which showed a significant degree of dose-effect relationship. Hydroxyl radical scavenging effects of the three antioxidant with the same concentration were sorted by BHT > Rutin > *broccoli* extract. The reasons were analyzed as follows: First, o-dihydroxy from benzene rings were partly methylated, leading to the reduction of scavenging activity on hydroxyl radical [16-18]. Second, the lack of necessary separation and identification for extracts, and the presence of impurities also affected their scavenging effects.

**Figure 10.** Scavenging activity comparison of broccoli extracts, Rutin and BHT.

4. Conclusions

The RSM and BBD were successfully employed to optimize the enzyme-assisted extraction of flavonoids from *broccoli*. The linear term of solid liquid ratio, interaction term of solid liquid ratio and extraction temperature, displayed significant effects on the responding value of extraction ratio. The experimental central points for Box-Behnken Design were selected. The extraction process was further optimized by RSM and Box-Behnken Design. The optimum extract conditions were enzyme concentration of 0.13%, solid-liquid ratio of 3.0:30 (g:mL), extraction temperature of 79.7°C and time of 3.8 h with maximum extraction ratio of 2.409 % by solving the multiple regression equation. The experimental

extraction ratio matched well with the calculated value, which indicated the predictive effect of the fitted quadratic model on target extracts. The scavenging activity of extracts on hydroxyl radicals displayed a significant dose-effect relationship. The isolation, purification and structure identification of *broccoli* extracts, relationship between antioxidant activity and structure of flavonoids, and related research works are underway.

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