NUTRITION AND FOOD SCIENCE JOURNAL Open Access

SCIENTIFIC LITERATURE

RESEARCH ARTICLE

Antioxidant Activity and Optimisation of Ultrasonic-Assisted Extraction by Response Surface Methodology of Anthocyanins from Aronia Melanocarpa Berry

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ARTICLE INFO

Article history:
Received: 16 May 2017
Accepted: 10 October 2017
Published: 16 October 2017

Keywords:
Aronia melanocarpa;
Response surface methodology;
Ultrasound-assisted extraction;
Anthocyanin;
Antioxidant activity

Citation this article: Chen S, Meng X, Wang Y. Antioxidant Activity and Optimisation of Ultrasonic-Assisted Extraction by Response Surface Methodology of Anthocyanins from Aronia Melanocarpa Berry. Nutri Food Sci J. 2017; 1(1):112.

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ABSTRACT

The Based on one-factor-at-a-time experiments, response surface methodology was employed to optimize ultrasound-assisted extraction of Aronia melanocarpa berries (chokeberry; AMP). The optimum extraction conditions were determined as follows: ethanol concentration 62%, ultrasonic power 198 W, liquid-solid ratio 19 (mL/g) and ultrasonic time 44 min. Under this condition, AMP anthocyanin yield was 4.31893 mg/g with a 1.67 % error compared with the actual value, which showed that the modeland the optimization process was feasible. Additionally, superoxide anion radical (O^{2-}•) radical scavenging activities of AMP extracts and VC was comparatively analyzed and the result revealed that the antioxidant activity of AMP extracts was superior to VC.

Introduction

AMP that can be used in food, beverage, jam, canned food, preserved fruit, fruit juice, fruit wine and so on, which is also named the wild cherry berry, belongs to the Rosaceae and is native to Canada and North America [1,2]. The fruits of AMP, round in shape and black-purple in color, are a kind of new berry with extremely high nutritional and economic value [3]. “Berry” is used for describing any small edible fruit, usually tender and juicy, fruity, seeds in the flesh, sweet and sour flavor and have different color intensity, which is rich in polyphenols, including anthocyanins, flavonoids and proanthocyanidins [4,5]. Anthocyanin is a kind of natural pigment widely exists in plants and have been reported to possess a multiple biological activities, including antioxidant, anti-cancer, anti-thrombosis, improving immunity and promoting erythrolabe synthesis and other biological activities, particularly antioxidant activity that is 4.4 times higher than ascorbic acid [6,7]. Anthocyanins have been widely used in food and medicine and other fields due to their unique functional properties.

Ultrasonic-assisted extraction can improve the extraction rate of heat-sensitive biological active ingredients under rather low processing temperatures which is more effective than the conventional techniques of extraction [8,9]. The mechanical effect of ultrasound causes more solvents to penetrate into the tissue cells, then improve the mass transfer, and the breakage of the cell wall is favorable for the release of the tissue cell contents, which can reduces the

processing time and the usage of solvents compared with the traditional extraction methods. Additionally, $O^{2-\cdot}$ radical scavenging activity of AMP extracts is analyzed and the antioxidant activity of AMP extracts is evaluated through in vitro antioxidant activity test [10,11].

Materials and Methods

1. Plant Material

AMP, in the fully mature stage, were harvested in an orchard located in Liaoning province, China, in 2015 and was transported to the department of food science, Shenyang Agricultural University, China. The berries were stored in the refrigerator at -80°C until required.

2. Chemical Reagent

All chemical reagents used in this study were analytical grade. Hydrochloric acid, anhydrous ethanol, sodium acetate anhydrous, potassium chloride, pyrogallol and all the other reagents were obtained from the reagent management office in Shenyang Agricultural University (Liaoning, Shenyang, China).

3. Determination of Total Anthocyanins

According to the structural characteristics of anthocyanins, the total anthocyanin content in the solution was calculated by the pH-differential method [12,13]. 5 g of completely broken samples were extracting with ethanol solution under certain conditions. 1 mL extract through the method of vacuum infiltration was taken and respectively added to 24 ml of potassium chloride buffer (pH=1.0) and sodium acetate buffer (pH=4.5). The mixed liquid was placed at room temperature for 20 min to make the mixed liquid equilibrium, and the absorbance of A was measured at 520 nm and 700 nm with a UV-Vis spectrophotometer (TU-1810, PuXi, China), respectively, with distilled water as blank. The absorbance value (ΔA) of the diluted sample was computed according to the following **Formula:** $\Delta A=(A\lambda max-A700)pH1.0-(A\lambda max-A700)pH4.5$

The total anthocyanins content expressed as mg cyanidin-3-glucoside equivalents per litre of extract, was computed according to the formula (1).

$$\text{total anthocyanins content}(mg/g)=\frac{\Delta A\times M\times V\times n}{\varepsilon\times m}$$

Where ΔA is absorbance, M (449.2) is cyanidin-3-glucoside molecular weight, V is total volume of extract, n is dilution ratio of extract, ε (26900) is the molar absorbtivity, m is sample mass.

4. Ultrasonic-assisted Extraction (UAE)

The UAE was performed in an ultrasonic device (SB25-12DTN, Nibo Scientz biological technology co., LTD, China). Sample (5 g) was extracted with absolute ethanol under these various conditions: ethanol concentration ranging from 0 to 100%, ultrasonic power from 0 to 200 W, liquid-solid ratio from 5 to 30 (mL/g), ultrasonic temperature from 0 to 60 ℃ and ultrasonic time from 10 to 60 min. The total anthocyanin extracts were computed according to the formula (1).

5. Experimental Design

Based on the basis of single-factor experiment, according to the Box- Behnken center design method of Design - Expert software, the response surface analysis method of the four factors and three levels was designed to optimize the extraction of AMP anthocyanins in ultrasonic process, and the levels and the factors of experimental design were shown in Table 1 [14].

6. Prepurification of Anthocyanins

After the liquid was filtered by vacuum, the filtrate was concentrated by rotary evaporator at 40 ℃ (RE-5203A, Shanghai Bilon Instruments Co., Ltd., China). Then, the concentrated samples were filtered under vacuum condition, and separated by glass column with macroporous resin HP-20 [15]. Deionized water was used to remove water soluble substances, then 75% ethanol elution was used to elute anthocyanins. The eluent was completely removed ethanol by rotary evaporation and then freeze-dried into powder by vacuum freeze dryer (LGO.2, Shenyang Aerospace Xinyang Quick Freezing Equip. Manuf. Co., Ltd., China). Purified anthocyanin powder was stored at 4 ℃. The purity was increased from 1.75% to about 30.81%.

7. $O^{2-\cdot}$ Scavenging Capacity Measurement

The determination procedure of $O^{2-\cdot}$ scavenging capacity was adapted from the method of Lu Zhu et al. 17 4.5mL Tris-HCl (50 mM, pH = 8.2) was added to a

tub and heated up in a water bath for 25 min at 25 °C. 2 mL different concentrations of the sample solution was added to the tub and then 0.4 mL pyrogallol solution (25 mM) using HCl (10 mM) as solvent. The mixed solution was shaken well successively, followed by reaction for 4 min, 2 drops of HCl was then added into the mixture to terminate the reaction. The ethanol solution with volume fraction of 80% was used as blank control and the absorbance was measured at 320 nm. $O^{2-\bullet}$ scavenging capacity was computed according to the formula (2).

$O^{2-\bullet}$ scavenging capacity (%) = [1-(A1-A2)/A]×100% (2)

Where A1 is the absorbance value of the system with sample solution and pyrogallol solution, A2 is the absorbance value of the system without pyrogallol solution and A is the absorbance value of the system without sample solution.

8. Statistical Analysis

Each experiment was done in 3 groups of parallel and the data was expressed as the mean ± standard deviation. The data of single factor and antioxidant experiment was analyzed by SPSS [16]. (SPSS Inc., Chicago, IL, USA) and the response surface was analyzed by Design-expert.v8.0.5b.

Results and Discussion

1. Single-factor Experimental Analysis

The effect of ethanol concentration on AMP anthocyanin yield was shown in Figure 1A. When the ethanol concentration was less than 60%, AMP anthocyanin yield increased as the ethanol concentration increased. The reason was that the ethanol concentration increased, which led to the reduction of water-soluble pigment, such as polysaccharide and pectin, which inhibited the dissolution of anthocyanins. When the ethanol concentration was greater than 60%, the polarity difference between anthocyanin and solvent was increased, and the dissolution of anthocyanins was inhibited. Therefore, the optimum ethanol concentration was 60%. The effect of ultrasonic power on AMP anthocyanin yield was shown in Figure 1B. When the ultrasonic power is less than 160 W, AMP anthocyanin yield increased as the ultrasonic power increased. Probably because the sample absorbed a large amount of solvent and expanded under the ultrasonic cavitation and mechanical action, so that the cell wall porosity was increased and the cell wall and cell membrane were destroyed and the anthocyanins were more easily dissolved. When the power was too high, the free anthocyanins were degraded under the mechanical action of ultrasound, at the same time not only the number of bubbles in the solvent was increased and the cavitation effect was reduced, but also AMP anthocyanin yield was affected. Therefore, the optimal ultrasonic power was 160 W. The effect of liquid-solid ratio on AMP anthocyanin yield was shown in Figure 1C. When the liquid-solid ratio was less than 20 (mL/g), AMP anthocyanin yield increased as the liquid-solid ratio increased. It was possible that the solvent was so little that too easy to reach saturation and then anthocyanins were difficult to extract completely. When the liquid-solid ratio was more than 20 (mL/g), AMP anthocyanin yield began to decrease as the liquid-solid ratio increased. The mass transfer process was accelerated because of the increase of the concentration difference. When the solvent was too much, the ultrasonic absorption of the samples was reduced and the impurities in the samples were dissolved, so that AMP anthocyanin yield was reduced, but also the burden of concentration was increased. Therefore, the best liquid-solid ratio was 20 (mL/g). The effect of temperature on AMP anthocyanin yield was shown in Figure 1D. When the temperature was greater than 40 °C, AMP anthocyanin yield decreased gradually. Because the heat resistance of anthocyanins was relatively poor, the increase of tempering temperature would cause the structure of anthocyanins change and decreased AMP anthocyanin yield. Therefore, the best ultrasonic temperature was 40 °C. The effect of extraction time on AMP anthocyanin yield was shown in Figure 1E. When the ultrasonic time was greater than 50 min, AMP anthocyanin yield decreased slowly. It was possible that the cell wall and cell membrane of the sample were completely broken under the action of ultrasound and the extension of

ultrasonic time would lead to the degradation of some anthocyanins. Therefore, the optimal ultrasonic time was 50 min [17].

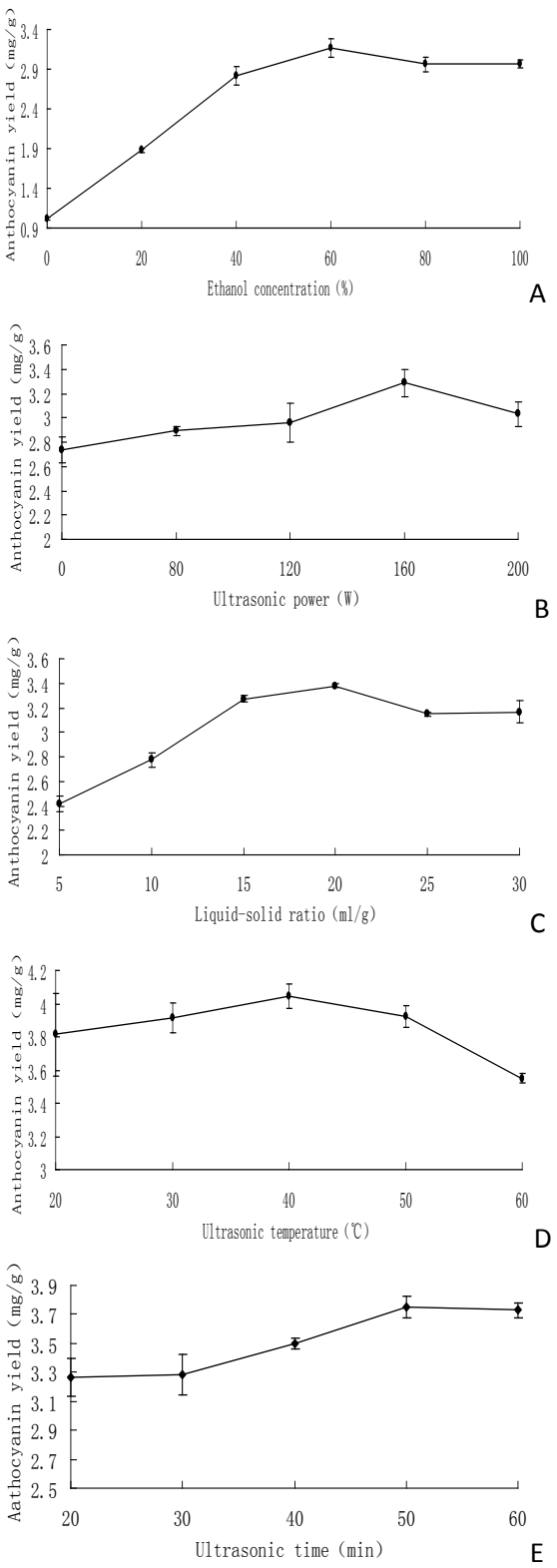

Figure 1: Effects of different extraction parameters on anthocyanin yield: (A) ethanol concentration, (%); (B) ultrasonic power, (W); (C) liquid-solid ratio, (mL/g); (D) ultrasonic temperature,(℃); (E) extraction time. (min).

2. Response Surface Experimental Results and Analysis

2.1. Design and results of response surface experiment of box –behnken: According to the design theory of the response surface experiment of Box - Behnken, on the basis of single-factor experiment, 4 factors were selected at 40 ℃ that were ethanol concentration (A), ultrasonic power (B), liquid-solid ratio (C) and ultrasonic time (D). The response surface experiment of four factors and three levels was carried out with AMP anthocyanin yield as the response value [18]. There were 29 experimental points, including 24 factorial points and 5 null points and the experimental design and results were shown in Table 2.

2.2. Model establishment and significance test: Design-expert.v8.0.5b was used to analyze the experimental datas and the two - order polynomial model of the four factors and of AMP anthocyanin yield was obtained by regression fitting. The model was as follows: $Y=4.28+0.044A+0.023B-0.071C-0.068D+0.028AB+0.10AC-0.050AD-0.033BC-0.079BD+0.053CD-0.29A^2-0.043B^2-0.20C^2-0.14D^2$. Where Y was the predictive value of AMP anthocyanin yield. From Table 3, the regression model was extremely significant ($p<0.0001$) and the lack of fit was not significant ($p= 0.1210> 0.05$), which shown that the experimental error was small. The results that R^2 was 0.9804, R^2_{adj} was 0.9607 and R^2_{pred} was 0.8955 shown that the model had a good fitting degree, which could explain the change of the 96.07% of response value and the independent variable have a significant linear relationship with the response value and be used to predict the response under different variables. Variance analysis showed that the factor of A and C and D had extremely significant effect ($p<0.01$). In this model, the effects of various factors on AMP anthocyanin yield were as follows: liquid-solid ratio (C) > eultrasonic time (D) > thanol concentration (A) > ultrasonic power (B).

2.3. Optimization and verification of response surface conditions: From the results, the interactive effects of

AC, AD, BD and CD were extremely significant, the response surface plots were shown in figure 2.

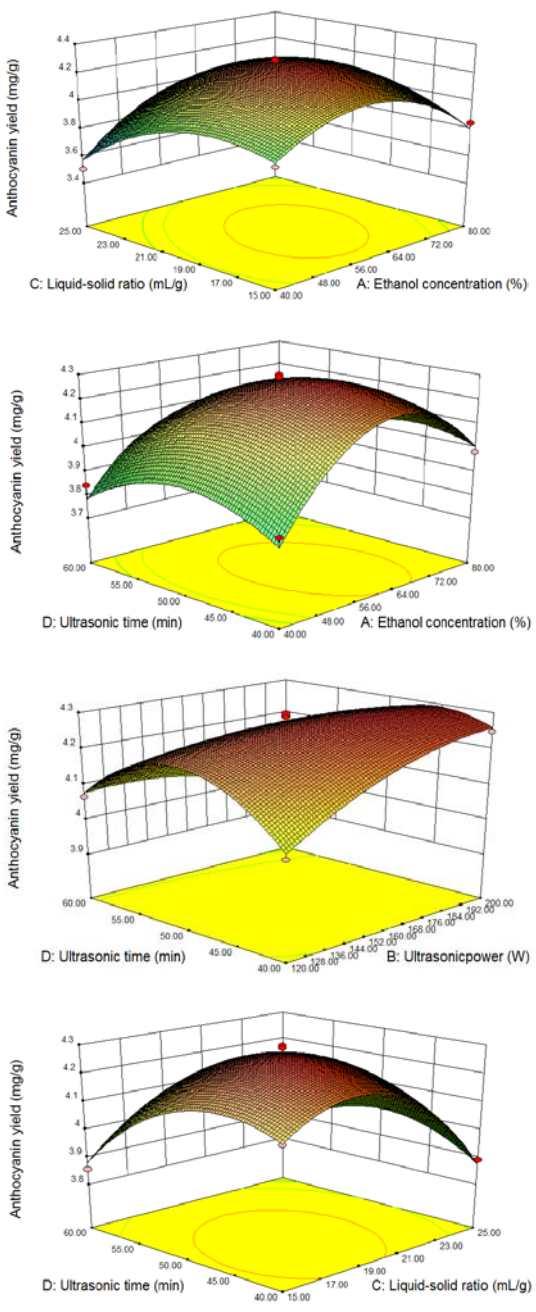

Figure 2: Response surface and contour plots showing the effects of different extraction parameters on anthocyanin yield.

The figure 2 had shown the interactive effects of the factors that were ethanol concentration, ultrasonic power, liquid-solid ratio and ultrasonic time on the amount of anthocyanins extraction from Aronia melanocarpa berries. Through analyzing the equation, the optimum extraction conditions were as follows: ethanol concentration was 62.41%, ultrasonic power was 198.04 W, liquid-solid ratio was 18.50 (mL/g) and ultrasonic time was 44.25 min [19]. At this time, the most theoretical value of anthocyanins was 4.31893 (mg/g). Taking into account the feasibility of the actual operation, the process parameters were adjusted as follows: ethanol concentration was 62%, ultrasonic power was 198 W, liquid-solid ratio was 19 (mL/g) and ultrasonic time was 44 min [20]. Under the optimal conditions, 3 parallel confirmatory experiments were performed and AMP anthocyanin yield was 4.248 (mg/g). The relative error between the actual value and the predicted value is about 1.67%, which proves the validity of the model and has practical value [21].

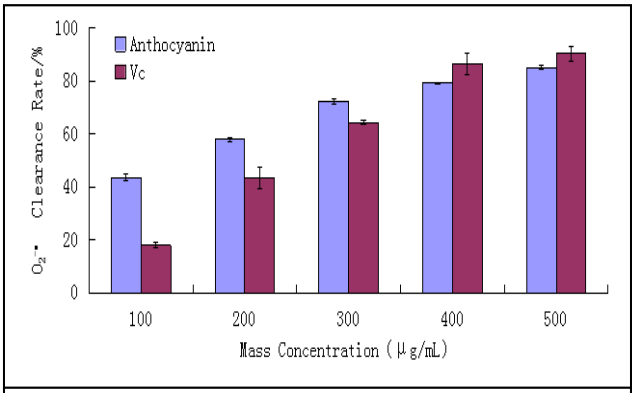

Figure 3: $O^{2-\cdot}$ scavenging activity of AMP extracts and VC.

Table 1: Factors and levels of response surface design.

Level	Factor			
	A:Ethanol concentration (%)	B:Ultrasonic power (W)	C:Liquid-solid ratio (ml/g)	Dextraction time (min)
-1	40	120	15	40
0	50	160	20	60
1	60	200	25	80

2. $O^{2-\cdot}$ Scavenging Capacity

$O^{2-\cdot}$ that was produced in the metabolism of organisms could cause mutation and necrosis of protein, polysaccharide and nucleic acid and even led to organismic oxygen poisoning [22]. The pyrogallol caused auto-oxidation in the alkaline environment and produced $O^{2-\cdot}$. The intermediate product had strong absorption in the UV region and AMP extracts had inhibitory effect on auto-oxidation of the pyrogallol that reflected the strong $O^{2-\cdot}$ scavenging capacity [23]. It can be seen from Figure 3 and Table 3 that $O^{2-\cdot}$ scavenging percentages of AMP extracts was on the rise with the

Table 2: Design and results of Box-Behnken experiments.

No.	A	B	C	D	Y:Anthocyanin yield(mg/g)
1	-1	-1	0	0	3.89
2	1	-1	0	0	3.917
3	-1	1	0	0	3.909
4	1	1	0	0	4.049
6	0	0	1	-1	3.89
7	0	0	-1	-1	3.856
8	0	0	1	-1	3.839
9	-1	0	0	-1	3.856
10	1	0	0	-1	3.982
11	-1	0	0	-1	3.839
12	0	0	0	-1	3.764
13	0	-1	-1	0	4.065
14	0	1	-1	0	4.144
15	0	-1	1	0	4.016
16	0	1	1	0	3.961
17	-1	0	-1	0	3.892
18	1	0	-1	0	3.843
19	-1	0	1	0	3.503
20	1	0	1	0	3.86
21	0	-1	0	-1	4.04
22	0	1	0	-1	4.247
23	0	-1	0	-1	4.065
24	0	1	0	-1	3.956
25	0	0	0	0	4.29
26	0	0	0	0	4.256
27	0	0	0	0	4.247
28	0	0	0	0	4.297
29	0	0	0	0	4.287

Table 3: Variance analysis for four elements quadratic regression model.

Source	Sum of Squares	df	Mean Square	F value	P-value Prob>F	significance
Model	0.97	14	0.069	49.96	<0.0001	**
A	0.023	1	0.023	16.71	0.0011	**
B	6.211E-003	1	6.211E-003	4.50	0.0522	**
C	0.060	1	0.060	43.63	<0.0001	*
D	0.055	1	0.055	40.11	<0.0001	**
AB	3.192E-003	1	3.192E-003	2.31	0.1505	**
AC	0.041	1	0.041	29.86	<0.0001	**
AD	0.010	1	0.010	7.32	0.0171	**
BC	4.489E-003	1	4.489E-003	3.25	0.0928	*
BD	0.025	1	0.025	18.09	0.0128	**
CD	0.011	1	0.011	8.14	0.0931	
A^2	0.54	1	0.54	387.94	<0.0001	**
B^2	0.012	1	0.012	8.65	0.0107	**
C^2	0.26	1	0.26	191.39	<0.0001	**
D^2	0.13	1	0.13	97.01	<0.0001	**
Residual	0.019	14	1.380E-003			
Lack of Fit	0.017	10	1.732E-003	3.47	0.1210	
Pure Error	1.997E-003	4	4.993E-004			
Cor Total	0.98	28				
R^2	0.9804					
R^2_{adj}	0.9607					
R^2_{pred}	0.8955					

increase of the mass concentration. When the concentration was in the range of 100-300 g/mL, $O^{2-\bullet}$ scavenging percentages of AMP extracts was higher than that of VC. When the concentration was more than 100-300 g/mL, the growth rate of $O^{2-\bullet}$ scavenging

percentages of AMP extracts slowed down, less than VC. The regression equation of $O^{2-\bullet}$ scavenging capacity of AMP extracts is: Y=36.364+0.104X (R^2=0.961), and the regression equation of $O^{2-\bullet}$ scavenging capacity of VC is: Y=4.282+0.187X (R^2=0.958). When the AMP extracts concentration was 131 g/mL, $O^{2-\bullet}$ scavenging percentages reached 50%. In conclusion, AMP extracts in this system showed good $O^{2-\bullet}$ scavenging capacity.

Conclusion

According to the design theory of the response surface experiment of Box -Behnken, on the basis of single-factor experiment, 4 factors were selected at 40 ℃ that were ethanol concentration (A), ultrasonic power (B), liquid-solid ratio (C) and ultrasonic time (D). The response surface experiment of four factors and three levels was carried out with AMP anthocyanin yield as the response value. Design-expert.v8.0.5b was used to analyze the experimental datas and the two - order polynomial model of the four factors and of AMP anthocyanin yield was obtained by regression fitting. Taking into account the feasibility of the actual operation, the process parameters were adjusted as follows: ethanol concentration was 62%, ultrasonic power was 198 W, liquid-solid ratio was 19 (mL/g) and ultrasonic time was 44 min. Under the optimal conditions, 3 parallel confirmatory experiments were performed and AMP anthocyanin yield was 4.248 (mg/g). The relative error between the actual value and the predicted value is about 1.67%, which proves the validity of the model and has practical value. In the experiment of $O^{2-\bullet}$ scavenging activity, in a certain range of concentration (100-300 g/mL), the antioxidant activity of AMP extracts scavenging activity was than VC.

Acknowledgement

The supports from the Ministry of Agriculture Special Public Welfare Industry (201303073-04)

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