OPTIMIZATION OF EXTRACTION OF BETALAIN FROM RED BEETROOT (*Beta vulgaris* var. *rubra* (L.) Moq)

Hoang Thi Ngoc Nhon*, Nguyen Thi Thanh Hang

Ho Chi Minh City University of Food Industry

*Email: *hoangthingocnhon1002@gmail.com*

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ABSTRACT

Betalain is a pigment found in plants, can be red-violet (betacyanin) or yellow (betaxanthin), soluble in water, a compound widely used in chemistry, medicine and pharmacy with the chemical stability in a wide pH range. Betalain is an antioxidant with antiviral, anti-inflammatory properties. They are found in high levels in red beetroot and are a food coloring additive. The aim of this work was to study factors affecting the extraction of betalain from red beetroots such as solvent type, solvent concentration, material-solvent ratio, temperature and time. The results showed that 2.955 mg/g of dry matter was gained in the suitable extraction conditions such as ethanol 20%, material-to-solvent ratio 1/25 (w/v) at 40 °C for 180 minutes. Optimization of extraction of betalains from red beetroot using the response surface method (RSM) with three-factor central composite rotatable design (CCRD) showed the optimized parameters including material-to-solvent ratio 1:22.96 (w/v), the temperature at 47.71 °C, the period of 183.65 minutes gave the highest content of betalain (3.467 mg/g dry matter).

Keywords: Betalain, *Beta vulgaris*, red beetroot, response surface method.

1. INTRODUCTION

Red beetroot (*Beta vulgaris* var. *rubra* (L.) Moq) is one of the many species of *Beta vulgaris*. It is the most widely cultivated root in North America, Central America and the United Kingdom [1]. Beetroots are considered the top 10 powerful root vegetables with a phenolic content of 50-60 μmol/g [2]. Betalains derived from red beetroots are water-soluble, nitrogen-containing plant pigments whose colors range from red-violet betacyanins to yellow betaxanthins, which are known with significant antioxidant and biological activities thanks to the combination with free radicals to help prevent oxidative-mediated oxidation and free radicals of biological molecules [3]. Natural pigments in general and betalain pigments, in particular, have good potential to replace synthetic pigments in food, cosmetics, pharmaceuticals and nutrition [4]. Colour is one of the most important attributes of foods, being considered as a quality indicator and determining frequently their acceptance. Although natural pigments have many technological disadvantages when compared to synthetic ones, including higher cost-in-use and lower color stability, consumers have preferred them over their synthetic counterparts because they are non-toxic to the human body and highly resistant to oxidation [5]. Betalain compounds commonly used in chemistry, medicine and pharmacology due to the chemical stability in a wide pH range, is a strong oxidant, has antiviral and anti-inflammatory properties... [6]. Betalains are water-soluble pigments with the stability from pH 3 to 7. They could use like colorants in foods with low acid content [7]. Because of those properties, betalains could be used as a food additive to avoid food discoloration or diverse foods. Betalain coloring was approved by the European Union and labeled as E162 [8]. This study found the optimized conditions for betalain extraction of the red beetroot.

2. MATERIALS AND METHODS

2.1. Materials

Fresh red beetroots were cultivated in Da Lat area (Vietnam) with GAP standards. The impurities (rubbish, peels...) were removed with tap water. The samples were then rinsed carefully with fresh water, then cut into thin slices and dried at 60 °C until less than 10% moisture content. It was ground into powder, sieved to an inhomogeneous size of 1 mm and stored in ziplock bags for all experiments.

2.2. Chemicals

Ethanol 99.5%, acid citric 99%, $Na_2HPO_4.12H_2O$ 98%, NaOH 99%, HCl 36.5%, methanol 99.5%, distilled water.

2.3. Methods

The parameters of experiments were investigated according to the research of Herbach *et al.* (2006) [9]. The betalain content (mg/g dry matter) is determined by UV/VIS spectrophotometry. Each experiment was repeated 3 times.

2.3.1. The effects of extraction solvent

The effects of solvents, namely water, ethanol, methanol on the extraction of betalain from beetroot were investigated. The dried samples of beetroot power (1 g) was added with the above solvents (distilled water, ethanol, methanol) in different experiments with the solvent concentration of 40% (ethanol, methanol), sample-to-solvent ratio 1/20. Batch extractions were carried out at 30 °C, 120 minutes and protected from light, then removed the residue and collected the supernatant.

2.3.2. The effects of solvent concentration

The effects of solvents' concentration (20%, 40%, 60%, 80%, 99.5%) and the control on the extraction of betalain from beetroot were investigated. The dried samples (1 g) in the above different solvents' concentration with the chosen solvent (experiment 2.3.1), sample-to-solvent ratio 1/20. Batch extractions were carried out at 30 °C, 120 minutes and protected from light, then removed the residue and collected the supernatant.

2.3.3. The effects of sample-to-solvent ratio (g/mL)

The effects of the sample-to-solvent ratio (1/10, 1/15, 1/20, 1/25, 1/30) were investigated. The dried samples (1 g) in the chosen solvents (experiment 2.3.1) with the concentration (experiment 2.3.2). Batch extractions were carried out at 30 °C, 120 minutes and protected from light, then removed the residue and collected the supernatant.

2.3.4. The effects of extraction temperature

The effects of extraction temperatures (30, 40, 50, 60 and 70 °C) were investigated. The dried samples (1 g) in the chosen solvents (experiment 2.3.1), the concentrations (experiment 2.3.2) and the sample-to-solvent ratios (experiment 2.3.3). Batch extractions were carried out at 120 minutes and protected from light, then removed the residue and collected the supernatant.

2.3.5. The effects of extraction time

The effects of extraction time (60, 120, 180, 240 and 300 minutes) were investigated. The dried samples (1 g) in the chosen solvents (experiment 2.3.1), the concentrations (experiment 2.3.2), the sample-to-solvent ratios (experiment 2.3.3) and extraction temperatures (experiment 2.3.4). Batch extractions were carried out and protected from light, then removed the residue and collected the supernatant.

2.4. Optimized extraction conditions

Optimization of the extraction of betalains from beetroot was carried out using the Response Surface Method (RSM). A central composite rotatable design (CCRD), five levels (±α, 0, ±1) consisting of 20 experimental runs were employed including six replicates at the center point. All the runs were carried out in duplicate. The design variables were the material-to-solvent ratio (X1, %), extraction temperature (X2, °C) and the extraction time (X3, mins) while the response variable was betalain content.

2.5. Analysis of betalain content

Betalain content in the extracts was determined spectrophotometrically, using a UV/VIS Thermo Scientific_Genesys_10S (USA). Betalain has an absorption maximum at 535-540 nm, but absorbs also at 476-478 nm. The quantity of betalain extracted from 1 g of vegetable material was calculated using the following equation:

$$m = \frac{A.DF.MW.1000}{\varepsilon\, x\, L} \quad (1)$$

The significance of the terms used is as follows:

m - the amount of extracted betalain per g of raw material used;

A - sample absorption;

MW - average molecular mass, in g/mol, (550 g/mol)

ε - molar extinction coefficient (60000 L/mol - betalain)

L - cuvet length (1 cm)

DF - dilution factor.

2.6. Statistical analysis

Statistical analysis of the experimental data was conducted using Microsoft Excel 2013. Results were expressed as means ± SD and statistical differences among experiments were compared by IBM SPSS Statistics 20 and JMP 10. Differences between the experiments were considered significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. The effects of solvents on betalain extraction

Solvent plays a key role in pigment extraction protocol [11]. The aim of the experiment is to find the appropriate extraction solvents with the parameters shown in section 2.3.1. The results were shown in Table 1.

Table 1. The effects of solvents on betalain extraction

No.	Solvents	Betalain content (mg/g)
1	Ethanol	$(1.985 \pm 0.005)^{c}$
2	Water	$(1.005 \pm 0.0229)^{b}$
3	Methanol	$(0.935 \pm 0.0132)^{a}$

Reported data are average values ± standard deviations. Data in the same columns with different superscripts were significantly different ($p < 0.05$).

The solvent has a significant effect on the amount of betalain extracted. Ethanol resulted in a higher betalain amount than methanol and water because methanol is volatile easily. According to Delgado-Vargas *et al.* (2000), methanol or ethanol with water is often required to extract entire pigments [12]. Ethanol reacts with hydrolysis to decompose betalain colorants, the nucleotide reaction to occur on carbon atoms in the double bond N=C. The presence of ethanol in a high water activity environment would increase the reaction rate of betalain decomposition [13].

The driving force of the extraction process is the difference in concentration inside and outside the environment. Solvent molecules would diffuse into the material via a capillary structure, which leads to a concentration gradient. Betalain molecules would transfer into the solvent. Thanks to the affinity reaction, betalain will be dissolved from the material into solvent-water based on the concentration gradient. Water is not efficient for extraction due to there is no affinity agent.

3.2. The effects of solvents concentration on betalain extraction

The investigation of ethanol concentration was conducted to gain a suitable concentration. The results were shown in Table 2.

Table 2. The effects of solvent concentration on betalain extraction

TN	Solvent concentration	Betalain content (mg/g)
1	Control	$(1.055 \pm 0.0087)^{a}$
2	Ethanol 20%	$(2.26 \pm 0.0626)^{e}$
3	Ethanol 40%	$(2.025 \pm 0.015)^{d}$
4	Ethanol 60%	$(1.835 \pm 0.0132)^{c}$
5	Ethanol 80%	$(1.705 \pm 0.0507)^{b}$
6	Ethanol 99.5%	$(1.105 \pm 0.02)^{a}$

Reported data are average values ± standard deviations. Data in the same columns with different superscripts were significantly different ($p < 0.05$).

The solvent concentration effects significantly on betalain content with the highest content (2.26±0.0626 mg/g (at a concentration of 20%) and tends to decrease with the increasing solvent concentration. Ethanol solvents of 20% - 50% were proposed by Delgado-Vargas *et al.* (2000) to exploit the entire betalain in beetroot [12]. The study of P. Simon *et al.* (1993) recommended that 20% ethanol was the best solvent to extract betalain. Betalain exhibits high stability at low water activity. In fact, the decomposition reaction of betalain involves water, the decomposition due to the reduction of water activity reduces, which leads to low reagent reactivity or oxygen solubility.

However, ethanol with a higher concentration affects the structure and stability of betalain. Moreover, if betalain reacts with pure ethanol, the intermediate compounds will form quickly. In this way, betalain will be significantly decomposed. The solvent with appropriate ethanol concentration and water activity would minimize the reaction of betalain decomposition [13]. Nguyen Quoc Duy *et al.* confirmed that 20% ethanol was highly effective in the extraction of betalain pigment from beetroot as well as being safe [11]. A higher ethanol concentration would inhibit betalain extraction. From another side, water lacks affinity agents resulting in lower betalain content. Nevertheless, with just a sufficient amount of ethanol affinity agent to create a solvent-ethanol system (20% ethanol), extraction efficiency increased significantly more than twice.

3.3. The effects of material-to-solvent ratios on betalain extraction

The high material-to-solvent ratio leads to higher extraction yield but betalain in the medium was lower [11]. Thus, the appropriate material-to-solvent ratio is to avoid wasting solvents as well as to obtain the highest betalain content. The results were shown in Table 3.

Table 3. The effects of material-to-solvent ratios (g/mL) on betalain extraction

No.	Material-to-solvent ratio	Betalain content (mg/g)
1	1/10	$(1.9 \pm 0.0492)^a$
2	1/15	$(2.2 \pm 0.01)^b$
3	1/20	$(2.265 \pm 0.0976)^b$
4	1/25	$(2.52 \pm 0.005)^c$
5	1/30	$(2.525 \pm 0.0095)^c$

Reported data are average values ± standard deviations. Data in the same columns with different superscripts were significantly different ($p < 0.05$).

The results indicated that material-to-solvent ratios affect the amount of obtained betalain. The lower the solvent concentration, the higher the extraction ability since bioactive compounds react well solvent, which results in extraction efficiency [11], the expanded tissue and material cells facilitate solvent penetration into the cell as well as help betalain penetrate the membrane and diffuse into the solvent. However, the yield recovery of the bioactive components obtained will not continue to increase at a balance situation [14]. In the experiments, the betalain content increased significantly with the increase of material-to-solvent ratio. Because the extraction process will stop as the balance set between inside material and outside solvent, the higher material-to-solvent ratio will result in a higher betalain amount diffused into the solvent to achieve balance status. Besides, the dilute solvent extraction needs a concentration stage to remove the solvent. This means that it is costly as well as more oxygen in the solvent makes the antioxidant activity of betalain weaken. So, the appropriate ratio between materials and solvents is required to gain the highest extraction efficiency and cost savings. In the experiment, material-to-solvent ratio 1/25 was chosen.

3.4. The effects of temperature on betalain extraction

Saguy *et al.* asserted that betalain was often unstable at high temperatures since the decomposition rate increased rapidly with the increase of temperature and heating time [15]. The effects of temperature extraction were shown in Table 4.

Table 4. The effects of extraction temperature on betalain content

No.	Temperature (ºC)	Betalain content (mg/g)
1	30	$(2.53 \pm 0.016)^{b}$
2	40	$(2.93 \pm 0.187)^{e}$
3	50	$(2.89 \pm 0.235)^{d}$
4	60	$(2.61 \pm 0.164)^{c}$
5	70	$(2.435 \pm 0.212)^{a}$

Reported data are average values ± standard deviations. Data in the same columns with different superscripts were significantly different ($p < 0.05$).

The temperature significantly affected betalain content. In the experiment, the highest betalain obtained was at 40 °C. The higher temperatures might lead to betalain decomposition due to its unstable activity. From 30 ºC to 40 ºC, temperature help to soften the plant tissue, increase the permeability of cell membranes and release biological compounds into the solvent. To a certain extent, temperature increasing facilitates the extraction by improving the pigment's solubility as well as the diffusion coefficient [15]. Betalain can be decomposed by the reaction of isomerization, decarboxylation, hydrogenation and hydrolysis under the influence of heat and acid. Roy *et al.* reported that the betalain extraction from beetroot was optimal at 40 °C [16]. The study of Elbe *et al.* confirmed that betalain is commonly known as thermally unstable pigments, the decomposition rate increases rapidly along with the increase of temperature and heating time [17]. Since the temperature increased from 40 °C to 50 °C, 60 °C, 70 °C, the betalain content decreased. This might come from two reasons. Firstly, betalain easily decomposes at high temperatures. Secondly, with moderate concentration, ethanol creates a suitable affinity to help the betalain extraction but higher temperatures lead to ethanol evaporation. This leads to decrease the concentration of ethanol, resulting in a change in the affinity, which affects betalain extraction.

3.5. The effects of time on betalain extraction

The betalain content and color in the extract increase significantly with a longer time of extraction. However, a longer extraction time leads to the balance between the material and the extract, which limits the diffusion of betalain into solution [11]. The effects of time on the betalain extraction were shown in Table 5.

Table 5. The effects of time on betalain extraction

No.	Time (minutes)	Betalain content (mg/g)
1	60	$(1.725 \pm 0.0304)^{a}$
2	120	$(2.925 \pm 0.0328)^{b}$
3	180	$(2.955 \pm 0.0132)^{c}$
4	240	$(3.005 \pm 0.0377)^{c}$
5	300	$(2.94 \pm 0.0173)^{b}$

Reported data are average values ± standard deviations. Data in the same columns with different superscripts were significantly different ($p < 0.05$).

From Table 5, betalain content gradually increased until 180 minutes and got the remain from 180 minutes to 240 minutes. The main reason is that the content of red powder extracted from red beetroot decreases with increasing temperature and time of extraction [18]. Therefore, the extraction time of 180 minutes was chosen in the experiment.

3.6. Optimizing betalain extraction

Table 6. Optimization of betalain extraction

No.	Factors			Betalain content (mg/g)
	X_1 (material-to-solvent ratio, g/mL)	X_2 (Temperature, °C)	X_3 (Time, mins)	
1	-1	-1	-1	2.134
2	-1	-1	1	2.123
3	-1	1	-1	3.134
4	-1	1	1	3.262
5	1	-1	-1	3.105
6	1	-1	1	2.574
7	1	1	-1	3.02
8	1	1	1	2.855
9	-1.68	0	0	3.026
10	1.68	0	0	2.891
11	0	-1.68	0	2.876
12	0	1.68	0	2.985
13	0	0	-1.68	2.891
14	0	0	1.68	2.729
15	0	0	0	3.412
16	0	0	0	3.398
17	0	0	0	3.402
18	0	0	0	3.421
19	0	0	0	3.391
20	0	0	0	3.387

ANOVA analysis by JMP software gave the below regression equation (2), which illustrates the relationship between the betalain content (Y) and factors and unaffected factors are excluded from the equation ($p>0.05$).

$$Y = 3.404 + 0.184X_2 - 0.243X_1X_2 - 0.176X_1^2 - 0.186X_2^2 - 0.229X_3^2 \quad (2)$$

The factors, namely X_2 ($p<0.05$), X_1^2, X_2^2, X_3^2, X_1X_2 have effects on the betalain content. In detail, X_2 has a positive effect while X_1^2, X_2^2, X_3^2 and X_1X_2 have a negative effect on betalain content. Overall, these factors impact betalain content significantly.

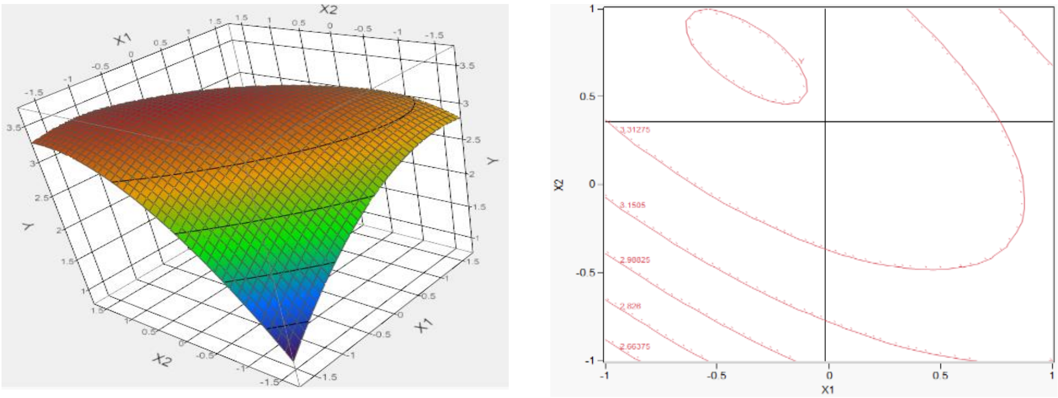

Figure 6. The response surface and contour plots for the effects of extraction temperature and the material-to-solvent ratio on the betalain content

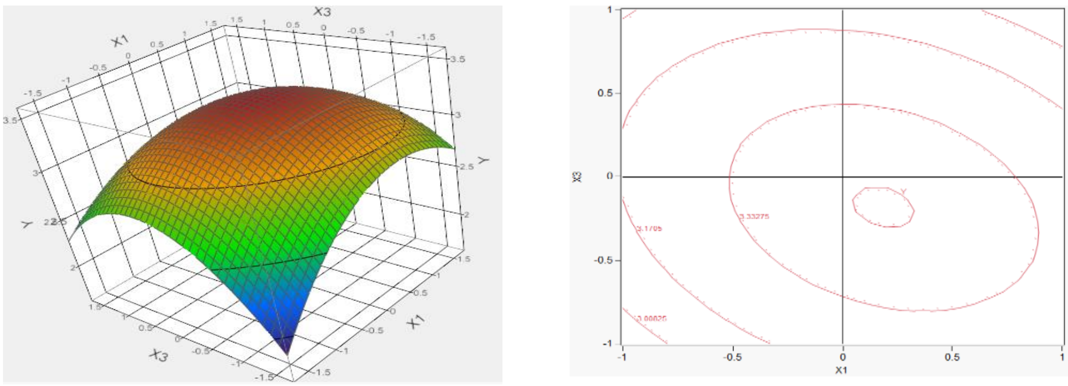

Figure 7. The response surface and contour plots for the effects of extraction time and the material-to-solvent ratio on the betalain content

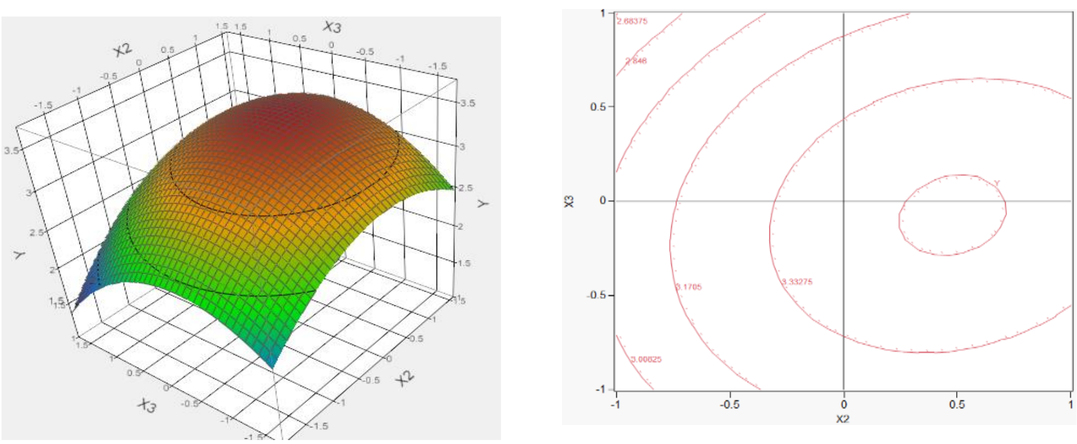

Figure 8. The response surface and contour plots for the effects of temperature and time on the betalain content

Table 7. The results of optimizing betalain extraction

Factors	Coding	Real values
X_1 (g/mL)	-0.4086	1/22.957
X_2 (^{0}C)	0.7714	47.714
X_3 (mins)	0.0609	183.654

To verify the results of the optimization, the triplicate experiments at the optimal conditions: material-to-solvent ratio around 1/23 (g/mL), temperature about 48 °C in approximately 184 minutes. The betalain content from verified experiments was $3.467^a \pm 0.0027$ mg/g in comparison with 3.470 mg/g total betalain content at theory optimal conditions. Thus, there is a compatible model between the theory and the experiment. In this way, the optimal model and its conditions were reliable.

4. CONCLUSION

The study results in betalain extraction conditions of ethanol 20% solvent, material-to-solvent ratio 1/25 (w/v), 40 °C and 180 minutes. The betalain content was 2.955 mg/g. At the optimal conditions material-to-solvent ratio 1/22.96 (w/v), 47.71 °C and 183.65 minutes), the betalain content was 3.467 mg/g. The study is a prominent base to create useful natural food additives as well as economic benefits.

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TÓM TẮT

TỐI ƯU ĐIỀU KIỆN TRÍCH LY THU NHẬN BETALAIN TỪ CỦ DỀN
Beta vulgaris var. *rubra* (L.) Moq

Hoàng Thị Ngọc Nhơn*, Nguyễn Thị Thanh Hằng
Trường Đại học Công nghiệp Thực phẩm TP.HCM
*Email: *hoangthingocnhon1002@gmail.com*

Betalain là sắc tố trong thực vật có màu từ đỏ-tím (betacyanin) đến màu vàng (betaxanthin), hòa tan trong nước, là hợp chất được sử dụng nhiều trong hóa học, y học và dược với tính ổn định về mặt hóa học trong khoảng pH rộng. Betalain có tính oxy hóa, có khả năng kháng virus, kháng viêm. Chúng được tìm thấy với hàm lượng cao trong củ dền và là một phụ gia thực phẩm tạo màu. Mục tiêu của nghiên cứu này là khảo sát các yếu tố ảnh hưởng dung môi: loại dung môi, nồng độ dung môi, tỷ lệ nguyên liệu/dung môi, nhiệt độ, thời gian đến khả năng trích ly betalain từ củ dền (*Beta vulgaris* var. *rubra* (L.) Moq). Kết quả thu được hàm lượng betalain 2,955 mg/g chất khô ở các điều kiện: dung môi ethanol 20%, tỷ lệ nguyên liệu/dung môi 1/25 (w/v) tại nhiệt độ 40 °C trong thời gian 180 phút. Tối ưu hóa quá trình trích ly betalain từ củ dền theo phương pháp đáp ứng bề mặt (RSM) với 3 yếu tố theo mô hình CCRD cho thấy, với tỷ lệ nguyên liệu/dung môi 1:22,96 (w/v), nhiệt độ 47,71 °C, thời gian 183,654 phút thu được hàm lượng betalain tối ưu là 3,467 mg/g chất khô.

Từ khóa: Betalain, *Beta vulgaris*, củ dền, mô hình bề mặt đáp ứng.