OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF NATURAL COLORANT FROM HUYET DU LEAVES USING ETHANOL SOLVENT

TỐI ƯU HÓA QUÁ TRÌNH CHIẾT TÁCH CHẤT MÀU TỪ LÁ CÂY HUYẾT DỤ BẰNG DUNG MÔI ETANOL CÓ SỰ TRỢ GIÚP CỦA SÓNG SIÊU ÂM

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ABSTRACT

In this paper, the natural colorant from Vietnam Huyet du leaves were extracted by ultrasound-assisted extraction (UAE) technology using ethanol solutions. Three independent variables including extraction temperature (30-70 °C), ethanol concentration (5-95%) and sonication time (5-45 min) were investigated. The Box-Behnken design (BBD) with the help of Design Expert 10 was used to investigate the effect of process variables on the Huyet du colorant extraction process. Second-order mathematical regression model was developed and matched well with observed data. Response surface methodology (RSM) was performed to find out the optimal condition for total anthocyanin content as follow: extraction temperature of 69.85°C, ethanol concentration of 94.15% and sonication time of 30.49 min. In this optimal condition, the total anthocyanin content was determined as 46.43 mg/l extract. The experimental value under optimal condition was in good consistent with the predicted value. In addition, the UV/Vis and FTIR analyses were confirmed that anthocyanin compounds are the major colouring components of Huyet du extract. A rotary evaporator was employed for concentrating Huyet du anthocyanin solution and recovering ethanol solvent.

Keywords: *Huyet du leaves, Anthocyanin, ultrasound-assisted, box-Behnken design, optimisation.*

TÓM TẮT

Trong nghiên cứu này, chất màu anthocyanin trong lá Huyết dụ được chiết tách bằng dung môi hữu cơ là etanol với sự hỗ trợ của sóng siêu âm. Quá trình chiết tách được khảo sát thông qua các yếu tố độc lập bao gồm nhiệt độ (30 - 70°C), nồng độ etanol (5 - 95%) và thời gian (5 - 45 phút). Để lập kế hoạch thực nghiệm, phân tích và xử lý kết quả, các tác giả đã sử dụng mô hình Box-Behnken và phần mềm Design Expert 10. Kết quả thu được điều kiện tối ưu cho hàm lượng chất màu anthocyanin tại nhiệt độ 69,85°C, nồng độ etanol 94,15% và thời gian 30,49 phút và với hàm lượng anthocyanin cực đại đạt 46,43mg/l dịch chiết. Lặp lại thí nghiệm ở điều kiện chiết tối ưu thu được giá trị hàm lượng chất màu anthocyanin trong dịch chiết lá Huyết dụ tương hợp với giá trị thu được từ phần mềm quy hoạch thực nghiệm. Các phân tích UV-Vis và FTIR cho thấy thành phần chính trong dịch chiết lá Huyết dụ là các hợp chất anthocyanin. Sử dụng phương pháp cắt quay chân không để cô đặc dung dịch chất màu và thu hồi dung môi.

Từ khóa: *Lá Huyết dụ, Anthocyanin, sóng siêu âm, Box-Behnken, tối ưu hóa.*

1. INTRODUCTION

Recently, researchers are working in the field of natural colorants extensively as they are non-toxic, non-allergic, non-carcinogenic and eco-friendly [1-4]. Natural colorants are being used in beverages, desserts, jams, jellies, sauces, cosmetics, toothpaste, medicines, etc. There are various organic compounds responsible for natural colorants such as flavonoids, flavonols, anthocyanins, chlorophyll, carotene, quinines, imines, polymethines, napthaquinones, anthraquinonoids, indigoids, dihydropyrans, etc. Among them, anthocyanins are the most abundant class.

Anthocyanins are water-soluble flavonoid colorants, and are largely responsible for attractive colors of flowers, leaves, and fruits [5-6]. Chemically, anthocyanins are glycosides and their aglycones, i.e. the sugar free colorants are known as the anthocyanidins. The various anthocyanins are shown to possess the same carbon skeleton and differed only in the nature of substituent groups. The fundamental nucleus is 2-phenyl chromenylium chloride (or flavylium chloride), Fig. 1a. All the anthocyanin has been considered to be derivatives of 3,5,7-trihydroxy flavylium chloride. Various anthocyanins differ in the number and position of other hydroxyl groups, methoxy groups and sugar residue. They are well-known pharmacological properties and strong biological functions such as antiinflammatory and antioxidant activities. In addition, they exhibit color changes with variation of pH, thus anthocyanins have been utilized as a natural indicator for different types of acid-base titrations [7].

Huyet du plant (Cordyline fruticosa L.) comprises more than 480 species distributed in tropical and subtropical regions of the world [8-10]. In Vietnam, Huyet du is often found in gardens as an ornamental plant, because it is an evergreen flowering plant, and its leaves are very conspicuous and attractive. Huyet du leaves may be reddish purplish, glossy green, or marked with various combinations of purplish, red, yellow and white [11]. According to literature, Huyet du leaves affluent in natural colorant of anthocyanin flavonoids. These phenolic compounds could be isolated using a suitable solvent polarity such as water, methanol, ethanol, etc. To extract anthocyanins, it is recommended using acidic media because the acid can denature function of plant cell membrane, then, dissolve the colorants, as well as preventing the oxidation of the phenolic compounds.

In our earlier study, the anthocyanin compound from red cabbage had been extracted by ultrasound-assisted extraction (UAE) technology using acid-ethanol solvent [12]. In this work, we investigate the effects of processing factors including extraction temperature, ethanol concentration and sonication time of UAE on anthocyanins from Huyet du leaves, and determine the optimum extraction conditions by response surface methodology (RSM).

2. EXPERIMENTAL SECTION

2.1. Material

Huyet du leaves were collected in Bach khoa university campus in December 2016, and were washed with distilled water, then dried at room temperature before use. Analytical grade ethanol, formic acid, hydrochloric acid, potassium hydrogen phthalate and potassium and potassium chloride were supplied by Xilong chemical industry incorporated Co. Ltd., China. All the experiments were carried out in triplicate and average values are reported.
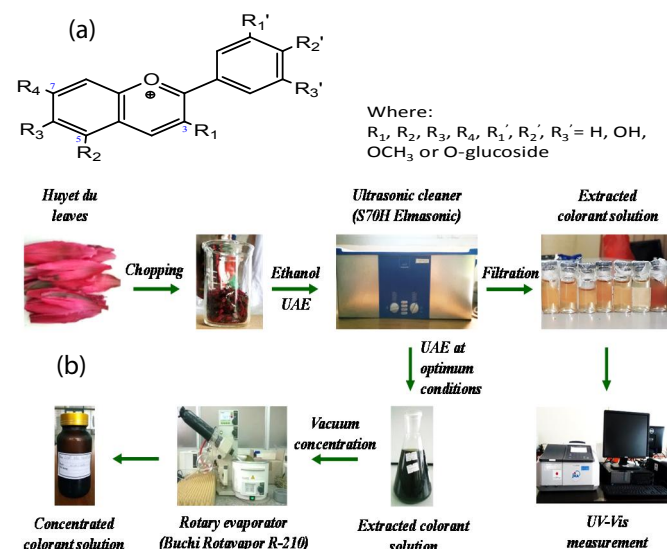
2.2. Ultrasound-assisted extraction (UAE)

Fig. 1. (a) The molecular structure of anthocyanin; (b) Schematic diagram of UAE of natural colorant from Huyet du leaves

Huyet du leaves were chopped by use of a blender (Philips HR2100). The extraction process was carried out by use of solid-liquid extraction method with ultrasound support. In a typical procedure, 4.0 g samples were immersed in 20 ml aqueous ethanol solutions, kept in capped glass tubes, and then immersed in a water bath. UAE process was performed in Elma S70H Elmasonic (Germany) with the power and working frequency of 750 W and 37 kHz, respectively. The extraction temperature, sonication time and ethanol concentration were controlled according to the experimental design conditions, Table 1. After extraction, the tubes were immediately cooled to room temperature using chilled water. The extracts were filtered through filter papers, and the filtrates were collected in a flask. The schematic diagram of UAE of natural colorant from Huyet du leaves in aqueous ethanol solution was presented in Fig. 1b.

2.3. Characterization of the colorant extracts

In order to determine anthocyanin contents of the colorant extracts, aliquots of the filtrates were diluted in ethanol to the concentrations appropriate for analysis. The absorbances of the diluted samples were measured at 533nm and 700nm with Unico 4802 Double Beam UV/Vis Spectrophotometer. The total anthocyanin content of the samples (TAC, mg cyanidin-3-glucoside (C3G)/l of Huyet du extract) were determined by pH differential method using two buffer systems: pH 1.0 (KCl + HCl) and pH 4.5 ($KHC_8H_4O_4$ + NaOH). The TAC of samples was calculated according to the Lambert-Beer law, using molar absorptivity of C3G ($E^{1\%}_{1cm}$ = 26900) [12, 13].

$$TAC\ (mg/l) = A \times M \times DF \times 1000/(\varepsilon \times L) \qquad (1)$$

where A (absorbance) = $(A_{\lambda max} - A_{700nm})_{pH=1.0}$ - $(A_{\lambda max} - A_{700nm})_{pH=4.5}$;

M (molecular weight) = 449.2 g/mol for C3G;

DF (dilution factor) = 8.25;

1000 = conversion from g to mg;

ε (molar absorptivity coefficient in l/mol/cm for C3G) = 26,900;

L (path length) = 1 cm.

Chemical charaterization of anthocyanin compound was obtained via Fourier-transform infrared (FTIR) spectra, which was recorded uisng a Thermo Scientific Nicolet 6700 FTIR in transmittance mode within the wavenumber range of 4000 to 500 cm^{-1}.

2.4. Experimental design and data analysis

The Box-Behnken design (BBD) with three levels and three variables was used in experimental design. The independent variables and their levels were presented in Table 1. The BBD consisted of 17 combinations (including 5 replicates of the centre point) and was performed in triplicate. Design-Expert® version 10.0 software (Stat-Ease, Inc., USA) was employed for constructing the design matrix, graph plotting, and data analysis. The design matrix and experimental results were showed in Table 2. Results using response surface regression were fitted to a second-degree polynomial equation, as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{33} X_3 X_3$$
$$+ \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \qquad (2)$$

where Y is the predicted response; β_0, β_1, β_2, β_3, β_{11}, β_{22}, β_{33}, and β_{12}, β_{13}, β_{23} are regression coefficients for intercept, linear, quadratic and interactions terms, respectively; X_1, X_2 and X_3 are the coded independent variables.

Table 1. Range of coded and actual values for Box-Behnken design

Independent variables	Unit	Codes	Coded levels		
			-1	0	+1
Temperature (A)	ºC	X_1	30	50	70
Ethanol concentration (B)	%	X_2	5	50	95
Time (C)	min	X_3	5	25	45

Table 2. Box-Behnken design matrix and experimental results

Run[a]	Sample	Extraction temperature (A, °C)	Ethanol concentration (B, %)	Sonication time (C, min)	TAC[b] (mgC3G/l)
1	T2C2M2	50	50	25	16.256±2.893
2	T1C2M3	30	50	45	15.43±2.755
3	T3C3M2	70	95	25	46.427±0.138
4	T2C3M1	50	95	5	33.753±0.138
5	T1C3M2	30	95	25	45.325±0.689
6	T2C1M1	50	5	5	16.945±0.138
7	T2C2M2	50	50	25	16.256±0.964
8	T3C2M1	70	50	5	17.221±3.031
9	T1C1M2	30	5	25	24.109±4.409
10	T2C2M2	50	50	25	16.256±1.102
11	T2C3M3	50	95	45	36.232±0.138
12	T1C2M1	30	50	5	15.843±3.031
13	T2C1M3	50	5	45	16.67±3.444
14	T2C2M2	50	50	25	16.256±1.929
15	T3C2M3	70	50	45	20.803±1.515
16	T2C2M2	50	50	25	16.256±2.893
17	T3C1M2	70	5	25	26.864±3.031

[a] Run number was used for identification purposes only and does not indicate the order in which the experimental runs were conducted.
[b] All results are the means ± SD (n = 3).

The model adequacies were checked by R^2, adjusted R^2, predicted R^2 and F-value. After selecting the most accurate model, the analysis of variance (ANOVA) was conducted to determine the statistical significance of the regression coefficients by evaluating the P-value < 0.05. The optimal conditions for the extraction of annatto pigment were determined by RMS. Experiments were carried out in triplicate at the optimal conditions, and the results were compared with the predicted values to check the reliability of the predictive extraction model.

3. RESULTS AND DISCUSSION

3.1. Fitting the models

The Box-Behnken design with 17 experiments was employed to evaluate the effects of temperature, ethanol concentration and sonication time on the total anthocyanin content. The values of the response variable for UAE process are listed in Table 2.

Analysis of variance (ANOVA) showed the quadratic polynomial model was highly significant with the P-value is less than 0.0001 for TAC (Table 3). The F-value of 389,48 for TAC implied the model was significant. There was only a 0,01% chance that an F-value this large could occur due to noise. Moreover, the hight values of R^2 and R^2_{adj} for TAC confirmed that the quadratic models were the more adequate than other models. As shown in Table 3, the absence of values of lack of fit and pure error indicated well reproducibility of the experimental data.

Table 3. Analysis of variance for the fitted quadratic polynomial model for the total anthocyanin content

Source	Total anthocyanin content				
	SS	DF	MS	F-value	P-value
Model	1799.35	9	199.93	389.48	< 0.0001
A-Temp.	14.07	1	14.07	27.40	0.0012
B-Et. conc.	744.00	1	744.00	1449.39	< 0.0001
C-Time	3.61	1	3.61	7.03	0.0329
AB	0.68	1	0.68	1.33	*0.2865*
AC	3.99	1	3.99	7.77	0.0270
BC	1.90	1	1.90	3.69	*0.0961*
A^2	123.91	1	123.91	241.39	< 0.0001
B^2	825.32	1	825.32	1607.82	< 0.0001
C^2	79.91	1	79.91	155.68	< 0.0001
Residual	3.59	7	0.51		
LoF	3.59	3	1.20		
PE	0.000	4	0.000		
Total	1802.94	16			
$R^2 = 0.9980$ $R^2_{adj} = 0.9954$					

Temp.: Temperature; Et. conc.: Ethanol concentration;
SS: sum of squares; DF: degree of freedom; MS: mean square;
LoF: Lack of Fit; PE: Pure Error.

Table 4 showed each regression coefficient values of TAC with respective P-values. Factors presenting significant with P < 0.05 were included in the models. All of linear effects, interaction effects and the quadratic effects were significant for TAC, except the interaction effects of temperature-ethanol concentration (AB) and ethanol concentration-time (BC). Thus, using the regression coefficients from the adjusted models in Table 4, the following model equations were generated:

TAC (mg/l) $= 16.26 + 1.33X_1 + 9.64X_2 + 0.67X_3 +$
$$+ X_2 X_3 + 5.42 X_1 X_1 + 14 X_2 X_2 - 4.36 X_3 X_3 \qquad (3)$$

Table 4. Estimated regression coefficients for the total anthocyanin content

Factor	Coefficient estimate		P-value
	in terms of Coded factors	in terms of Actual factors	
Constant	+16.26	+48.69798	< 0.0001
A-Temp.	+1.33	-1.32935	0.0012

B-Et. conc.	+9.64	-0.47325	< 0.0001
C-Time	+0.67	+0.41505	0.0329
AB	-0.41	-4.59167E-4	*0.2865*
AC	+1.00	+2.49688E-3	0.0270
BC	+0.69	+7.65000E-4	*0.0961*
A²	+5.42	+0.013562	< 0.0001
B²	+14.00	+6.91383E-3	< 0.0001
C²	-4.36	-0.010891	< 0.0001
P<0.05 (significant); P>0.05 (non-significant)			

3.2. Effects of extraction conditions on the total anthocyanin content

Base on the regression coefficients shown in Table 4, all terms including temperature, ethanol concentration and sonication time had significant linear (p< 0.01) and quadratic (p<0.05) effects on the response. The negative quadratic coefficients of sonication time evidenced the existence of an optimal extraction condition leading to a maximal total anthocyanin content. Fig. 2 showed that TAC increased when the sonication time increased, and reached maximum value at certain time about 30 min. Above this value, stable region followed by decrease zone of anthocyanin content was observed, as demonstrated by the negative quadratic coefficient of this term in the regression equation. This effect of sonication time could be due to ultrasonic waves exposure along with prolonged extraction process leading to anthocyanin degradation [9, 12-15].
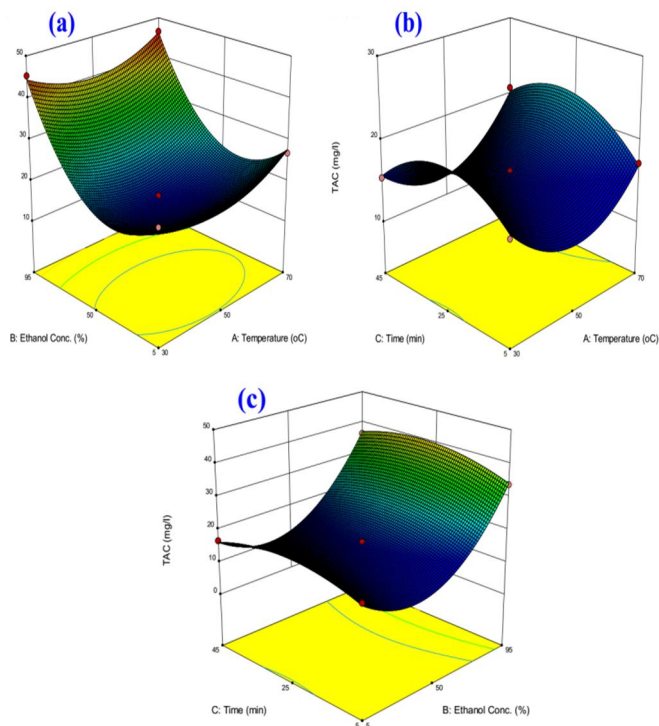

Fig. 2. Response surface plots showing the combined parameter effects of (a) temperature and ethanol concentration at fixed time of 25 min; (b) temperature and time at fixed ethanol concentration of 50%; (c) ethanol concentration and time at fixed temperature of 50°C on the total anthocyanin content of the Huyet du extracts

3.3. Optimisation of the extraction process and the model validation

The maximum predictive values of TAC were determined using the numerical optimization technique of DX10 software, in agreement with the plot analysis. The optimal condition for TAC was listed as follow: extraction temperature of 69.85°C, ethanol concentration of 94.15% and sonication time of 30.49 min. Under this condition, the predicted TAC was calculated as 46.43 mgC3G/l. Further experimental trials were performed with triplicates in the optimal region (70°C, 94.2% ethanol and 30.5 min sonication) to validate the model and compare the predicted response with the practical results. A mean value of TAC was 46.42 ± 0.27 mgC3G/l. This practical result is close to the predicted TAC confirming the predictability of the model. Thus, the second-order polynomial model could be effectively applied to predict the total anthocyanin content of extract from Huyet du leaves in the UAE process. After the optimal condition was established, the chemical composition of the optimized Huyet du extract was determined and will be described more detail in the following section.

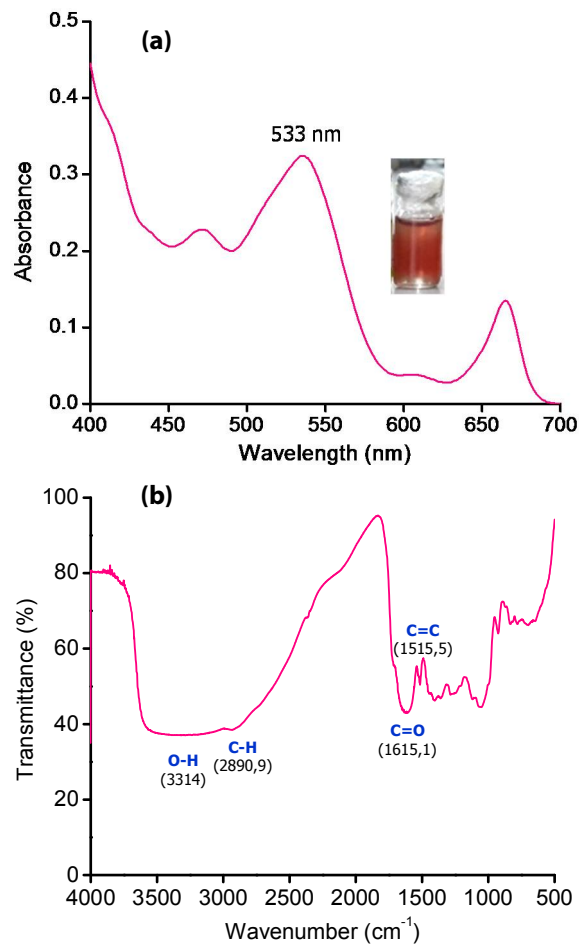### 3.4. Characterization of the Huyet du extract

Fig. 3. (a) UV/Vis spectrum of Huyet du extract in an aqueous ethanol solution, (b)FTIR spectrum of Huyet du colorant

In order to determine the profile of Huyet du extract, the spectrophotometric UV-Vis and FTIR analyses of the Huyet du extract were employed. As shown in Fig. 3a, the UV/Vis spectrum of Huyet du extract in an aqueous ethanol solution revealed clear absorption maxima at 665 nm, 533 nm and 472 nm, which are in a good agreement with the literature reports for Huyet du anthocyanins [9-10]. The FTIR spectrum obtained from the dried Huyet du colorant was shown in Fig. 3b. This spectrum presented a broad band at 3314 cm^{-1} which is ascribed to the stretching vibration of the OH groups and a peak at 2890.9 cm^{-1} associated with the stretching vibrations of the hydrocarbon skeleton, as well as with sharp bands at 1615.1 and 1515.5 cm^{-1} were ascribed to the stretching vibration of C=O (in ketone groups) and C=C (in aromatic skeletons), respectively. These absorption bands are typical of flavonoid compounds, in particular anthocyanin molecules. Based on these results, it could be concluded that the major colouring components of Huyet du extract are anthocyanin compounds.

4. CONCLUSION

This paper provided evidence that ultrasound-assisted extraction is an effective technique for the extraction of natural colorant from Huyet du leaves using ethanol solutions. The Box-Behnken design showed that extraction temperature, ethanol concentration and sonication time were statistically significant variables that affected on the UAE of Huyet du anthocyanin content, quantified by the UV/Vis analyses. The adequacy of the model obtained by RSM was demonstrated by the close agreement of the predicted responses (46.43 mgC3G/l) with the experimental result (46.42 ± 0.27 mgC3G/l) under optimal extraction conditions (69.85°C extraction temperature, 94.15% ethanol concentration and 30.49 min sonication time). The major colouring components of Huyet du extract are anthocyanin compounds, which were confirmed via UV/Vis and FTIR measurements. The concentrated Huyet du anthocyanin solution obtained by rotary evaporation under vacuum will be used for dyeing textile materials in ongoing research.

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