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Tri Nhut Pham[1,2], Thi Ha Xuan Huynh[1,2], Thien Hien Tran[1], Dai-Viet N. Vo[3], Tran Quoc Toan[4], Tri Duc Lam[1,5], Xuan Tien Le[6], Hieu Vu-Quang[1,*]

[1]NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[2]Faculty of Chemical Engineering and Food Technology, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[3]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Malaysia
[4]Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, Ha Noi, Vietnam
[5]Graduate School of Biotechnology, Kyung Hee University, South Korea
[6]Department of Chemical Engineering, HCMC University of Technology, VNU-HCM, Ho Chi Minh City, Vietnam
Email: vqhieu@ntt.edu.vn, labams2013@gmail.com

Abstract. Anthocyanins are naturally occurring compounds that are responsible for a wide variety of colors in many plants, fruits and vegetables. In this study, the extraction of natural anthocyanins from Vietnamese *Carissa carandas* L. beverage was optimized using response surface methodology (RSM). We applied a Box−Behnken design consisting of three levels and three factors. Examined factors are extraction temperature (ranging from 40 to 60 ℃), liquid to solid ratio (ranging from 2:1 to 4:1), extraction time (ranging from 30 to 60 min). Using 60% ethanol as solvent for the process, we determined the maximum yields of anthocyanin was 273.786 mg/L. This yield corresponds to extraction conditions of 3:1 (v/w) liquid to solid ratio, temperature of 48.10 ℃ with a 44.08 min extraction time. The experimental results also fit well with the proposed response model of anthocyanin yield (R^2 = 0.9992). Therefore, this study suggested optimization of different extraction methods for the defatted fruit parts.

1. Introduction

Carissa carandas L., categorized as herb in Apocynaceae family, is an underutilized fruit plant which thrives well throughout tropical and sub-tropical climate [1-3]. They are also edible and rich in minerals such as iron, calcium, magnesium and phosphorus. After being dried, the fruit is shrunk and changed to dark brown colour. Naturally, fruits owe their ability to dye fabrics and threads to the pigment that they contain. Such pigment could be anthocyanins, which are a very large group of red-blue plant pigments, present in the *Carissa carandas* L. fruit [4-7]. The uses of natural bio-pigments, such as anthocyanins from fruit and food products, are an excellent alternative to synthetic colors. Anthocyanins are also non-toxic and the source from which they are extracted are renewable.

Extraction of anthocyanin was conventionally carried out with solvents, such as ethanol, methanol, water, acetone, or mixtures, and often with of small acid addition to prevent nonacylated compounds

from deteriorating [8,9]. To optimize such parameters and produce an efficient experimental procedure, Response surface methodology (RSM) is commonly used. The method whose core is a nonlinear model and is widely applied in chemistry, biology, and agriculture for condition optimization of the systems. RSM is superior to "one to one factor" method, owing to its ability to allow for simultaneous and mutual interaction of process variables [10-20].

Considering the aforementioned background, this study aimed to optimize the parameters for anthocyanin extraction and maximize the anthocyanin yield from Vietnamese *Carissa carandas* L. Beverage by employing RSM with three-variable, three-level Box-Behnken design (BBD). The results were used to evaluate the influence of temperature, liquid-solid ratio, and time factor on the extracted yields. The extract obtained from this extraction method could be further isolated and purified for a certain anthocyanin or can be used as food colorants or in parapharmaceutical products.

2. Experimental Section

Sample Preparation and Chemicals

Fresh *Carissa carandas* L. fruit were obtained in mature stage in July 2018 from Tien Giang province, Viet Nam. The harvested *Carissa carandas* L. fruit were washed and kept in a non-hygroscopic bag. Lastly, 10g *Carissa carandas* L. fruit was cut into 1-2 mm portions. The extraction of anthocyanins was carried out with ethanol (C_2H_5OH), purchased from Sigma Aldrich (US).

Extraction of Anthocyanins

Various amounts of 60% ethanol containing 0.1 (v/v) hydrochloric acid (pH~1.0) were mixed with Freeze-dried powder samples (1.0 g) [21] to produce mixtures with liquid-solid ratios ranging from 2:1 to 4:1 (v/w). The mixtures were then placed in thermostatic water bath with temperatures and durations ranging from 40-60 (°C) and 30–60 (min), respectively, followed by centrifugation at 4000 rpm for 10 min at 25 °C. The resulted supernatant was filtered through filter paper for total anthocyanin evaluation. The pH of supernatant was scanned from 400 nm to 700 nm. Spectrophotometric pH-differential method was used to determine the total anthocyanin content [22].

Experimental Design for Optimization

RSM, in conjunction with a Box-Behnken design, was employed using Design-Expert software (Version 11.0) to optimize the parameters of anthocyanin extraction from the fruit. The independent variables were liquid to solid ratio, extraction time, and extraction temperature and were termed as X_1, X_2 and X_3, respectively. The response variable (Y) was the anthocyanin yield. Details of the independent factors and their corresponding ranges are described in the table 1. Experimental data were fitted to the following second-order model (Equation 2):

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

where Y, β_0, and β_n are predicted response, intercept and coefficients of model terms, respectively. The statistical significance of coefficient estimates and statistical indicators of the model was calculated by analysis of variance (ANOVA).

Table 1. Levels and Independent factors of the natural anthocyanin extraction process from Vietnam *Carissa carandas* L. fruit.

Levels	Independent factors		
	X_1 (v/w)	X_2 (Min)	X_3 (°C)
-α	1.32:1	33.18	19.77
-1	2:1	30	40
0	3:1	45	50
+1	4:1	60	60
+α	4.68:1	66.82	70.23

Statistical Analysis

Actual experiments results, derived from triplicate trials, and were summarized as means ± SD. Accrued data were analyzed with the SPSS 14.0 package (SPSS Inc., Chicago, IL, USA, 2005). Significance of a term is recognized if its p-value is lower than 0.05

3. Results and Discussion

The 20-experiment Box-Behnken design was detailed in the Table 2. The yields of anthocyanins were described in both experimental and predicted values. The following equation showed fitted model in terms of response variable and actual factors: $Y = 273.65 - 0.3116X_1 - 0.1712X_2 - 1.36X_3 + 0.1420X_1X_2 - 1.67X_1X_3 + 0.8953X_2X_3 - 3.15X_1^2 - 2.79X_2^2 - 3.72X_3^2$ (3)

Table 2. Box-Behnken design arrangement and response.

| No	Independent factors			Y (mg/L)		No	Independent factors			Y (mg/L)	
	X_1	X_2	X_3	Actual	Predicted		X_1	X_2	X_3	Actual	Predicted
1	2:1	30	40	265.32	265.20	11	5:1	19.77	50	265.91	266.04
2	4:1	30	40	267.68	267.64	12	5:1	70.23	50	265.39	265.46
3	2:1	60	40	262.82	262.78	13	5:1	45	33.18	265.31	265.42
4	4:1	60	40	265.87	265.79	14	5:1	45	66.82	260.73	260.83
5	2:1	30	60	264.09	264.03	15	5:1	45	50	273.93	273.65
6	4:1	30	60	259.87	259.77	16	5:1	45	50	273.83	273.65
7	2:1	60	60	265.29	265.19	17	5:1	45	50	273.73	273.65
8	4:1	60	60	261.52	261.50	18	5:1	45	50	273.56	273.65
9	1.32:1	45	50	265.14	265.26	19	5:1	45	50	273.33	273.65
10	4.68:1	45	50	264.14	264.22	20	5:1	45	50	273.57	273.65

Analysis of variance (ANOVA) for the resulted equation was shown in the Table 3. The model F-value of 1397.98 implied that the signal to noise ratio was adequately measured. Low p-value of F-value also suggested that "model F-Value" of this large could occur due to noise with the chance of lower than 0.01% [23]. In addition, the coefficient of determination (R^2) was higher than 0.99 and *Coefficient of Variation* % was lower than 6.98%, showing reasonable fitness and reproducibility of the model. As reported by the ANOVA results, X_1, X_2, X_3, X_1X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant model terms. The F-value of lack-of-fit statistic of 0.49 rejected the null hypothesis that the data was unfitted to the proposed model, and subsequently, affirmed the validity of the produced quadratic model. Regarding R^2, Le Man et al. and Chauhan and Gupta proposed that R^2 of any model is acceptable when it is greater than 0.75 [24, 25], which is also in line with the ANOVA results of the model.. Therefore, except for X_2X_3, remaining linear and non-linear terms of the model are showed to be significant predictors of anthocyanin yield. Hence, the relevance of independent variables on the TAC is ranked as follows: $X_1 > X_2 > X_3$.

Figure 1 showed surface and contour plots demonstrating the effects of two variables on the response function (anthocyanin yield). In each plot, one variables is fixed while other variables vary, as reflected by two bottom horizontal axes. In figure 2A, given any value of X_1, anthocyanin yield (Y) mounted up proportionally with X_2 to the peak value, and started to diminish after that. Similar relationship could also be observed for X_1 when X_2 was fixed. Figure 2B illustrated relationship between yield, liquid-solid ratio and temperature. The plot indicated that, compared to that of temperature the variations of solid-liquid ratio were more influential on Y The effect of temperature (X_3) and time (X_2) is shown in Figure 1C. Visually, when X_3 exceeds 45 ℃, a slight increase of X_2 could lead to a considerable decline of yield. Therefore, from the slope of the surface, one may conclude that temperature and liquid-solid ratio are two key factors in achieving high extraction efficiency of anthocyanins. To obtain the optimal values of the factors, the resulted equation was evaluated by the software. It is revealed that the optimal conditions of the extracting process from *Carissa carandas* L. fruit were 3:1 (v/w) liquid to solid ratio, temperature 48.1 ℃ with 44.08 min of extraction time, corresponding to Y = 273.786 mg/L. A set of triplicate experiment was conducted following the calculated optimum conditions. These confirmation experiments showed the results of 273.784 ±0.45 mg/g. This concurs with the predicted optimal value, suggesting that the actual data is well fitted and the extraction process is optimized.

Table 3. Analysis of Variance (ANOVA) for the regression equation

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	Prob. > F	Comment
Model	437.60	9	48.62	1397.98	< 0.0001	significant

X_1	1.33	1	1.33	38.14	0.0001	
X_2	0.4001	1	0.4001	11.50	0.0069	
X_3	25.42	1	25.42	730.81	< 0.0001	
X_1X_2	0.1613	1	0.1613	4.64	0.0567	
X_1X_3	22.43	1	22.43	644.95	< 0.0001	
X_2X_3	6.41	1	6.41	184.35	< 0.0001	
X_1^2	143.04	1	143.04	4112.56	< 0.0001	
X_2^2	112.39	1	112.39	3231.46	< 0.0001	
X_3^2	199.68	1	199.68	5741.10	< 0.0001	
Residual	0.3478	10	0.0348			
Lack of Fit	0.1140	5	0.0228	0.4878	0.7752	not significant
Pure Error	0.2338	5	0.0468			
Std. Dev.= 0.1865		**Mean = 267.05**		**C.V. % = 0.0698**		**R^2 = 0.9992**
Adjusted R ²= 0.9985		**Predicted R ²= 0.9972**		**Adeq. Precision = 105.2679**		
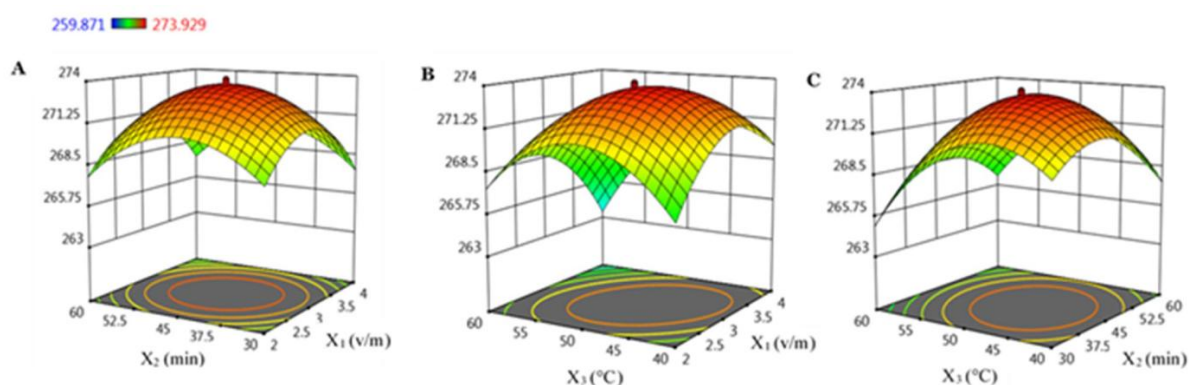
Figure 1. Response surface plots of the natural anthocyanin yielded (Y) from the Vietnamese *Carissa carandas* L. fruit extract, corresponding to pairwise comparisons of three factors.

Conclusions

We successfully attempted RSM with an experimental design to optimize the extraction process of natural anthocyanins from the Vietnamese *Carissa carandas* L. fruit. We showed that the highest anthocyanin yield was 273.786 mg/L, corresponding to optimal combination of independent variables of temperature at 48.1 °C, time at 44.08 min, and liquid-solid ratio at 3:1. Of the three examined factors, temperature was more dominant when it comes to influencing the efficiency of anthocyanin extraction. The resulted optimum condition could be applied in methanolic extraction of anthocyanin pigments, facilitating the production of natural colourants in textile, solar cell, and food industry.

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