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Optimization parameters for the extraction of anthocyanins from lipote (*Syzygium curranii* (C.B. Robinson Merr.)) using acidified ethanol solvent system

Ilano, M.C.R., Tamayo, J.P. and *Rivadeneira, J.P.

Institute of Food Science and Technology, College of Agriculture and Food Science, University of the Philippines Los Baños, 4031, College, Laguna, Philippines

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Abstract

Lipote is one of the underutilized fruits in the Philippines that is a potential source of anthocyanins. In this study, various solvent mixing ratios were screened. Upon obtaining the best solvent system, the parameters (temperature, solid-liquid ratio (SLR), and extraction time) for extracting anthocyanins from lipote were optimized using Response Surface Methodology (RSM). The response considered for both the screening and optimization studies was the mean total anthocyanins content (TAC). Results showed that the best solvent ratio is 50:50:1 (v/v/v) of ethanol, water, and acetic acid, while the optimum extraction parameters were 8.4°C, 29.0% SLR, and 172.0 mins with a predicted TAC of 932.9 mg cyanidin-3-glucoside/L. Upon validation by actual extraction, 750.5 mg cyanidin-3-glucoside/L, equivalent to 80.4% of the expected yield, was obtained.

1. Introduction

Colour is one of the most noticeable attributes of food. It directly affects what consumers prefer to eat (Delgado-Vargas *et al*., 2003; Shim *et al*., 2011; Martins *et al.*, 2016). The colour of food, however, can be affected by processing and storage conditions. To prevent the adverse effects on food quality, food colourants are used. Colourants also standardize the colour of food products (Martins *et al*., 2016). Synthetic colourants are derived from chemical synthesis and are not commonly found in nature (Moss, 2002). However, there have been increasing concerns about the toxicity (long-term and short-term) and carcinogenicity of these colourants. Hence, the interest in using natural colourants for food applications grew. Natural colourants include those produced, stored, and extracted from living cells (Moss, 2002). One of these natural colourants is anthocyanins (Martins *et al*., 2016).

The global market for anthocyanins was USD 318 million and is expected to increase at a compound annual rate of 4.6%. By 2026, the projected market value of anthocyanins is USD 388 million (Market Data Forecast, 2021). In the Philippines, the anthocyanins market from local sources is still unavailable since most research was exploratory. However, with the continuous generation of data from different studies, the value of local raw materials as a source of anthocyanins increases.

Anthocyanins belong to a subgroup of phenolic compounds called flavonoids. It is one of the most widespread pigments found in plants (Damodaran *et al*., 2007). Anthocyanins give the salmon pink to red colour and violet to dark blue colour of most fruits, vegetables, and flowering plants. They are also found in some seed-bearing non-flowering plants, ferns, and bryophytes (Andersen and Jordheim, 2014). As a source of food colourants, anthocyanins' advantages include their safety, bright colour (especially in the red region), and water-solubility, leading to their ease in aqueous food systems (Markakis, 2012).

Anthocyanin extraction is carried out using solvents such as acidified ethanol, methanol, and acetone (Andersen and Jordheim, 2014). Acidified solvents are used since anthocyanins are not stable in neutral and alkaline solvents. These solvents help break down the cell wall and, at the same time, dissolve the water-soluble pigments. Although methanol is a more effective extractant and has a lower boiling point, ethanol is still preferred in food applications due to its lower toxicity (Jackman and Smith, 1996). In addition, weak organic acids are chosen over mineral acids because the latter can induce the hydrolysis of the ester linkage of potential aliphatic acyl groups in the glycosyl part of the compound (Andersen and Jordheim, 2014).

One promising source of anthocyanins is lipote (Figure 1). Lipote is *Syzygium curranii* (C. B. Robinson) Merr., a Philippine endemic tree. Summertime is its

*Corresponding author.
Email: *jprivadeneira@up.edu.ph*

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fruiting season, and it grows in some regions of the Philippines, specifically Isabela, Palawan, and Quezon Province. The fruits of lipote can be eaten fresh or processed into wine, jams, and jellies (Guiam, 2019). This paper aimed to optimize the parameters (temperature, SLR, and extraction time) for extracting anthocyanins from lipote using acidified ethanol solvent system through Response Surface Methodology (RSM).

Figure 1. Fully-riped lipote.

2. Materials and methods

2.1 Sample preparation

Fully-riped lipote fruits were obtained from Infanta, Quezon, Philippines. The seeds were separated from the flesh and skin of the fruit. The skin and the flesh were dried at 50.0°C until constant weight. Dried samples were ground into powder form and then stored at room temperature.

2.2 Screening of extraction solvent

The extraction of anthocyanins from lipote was based on the method described by Kopjar *et al.* (2014) with some modifications. Extraction solvents of different ratios of ethanol, water, and acetic acid were prepared. The ethanol, water and 1.0 M acetic acid ratios (v/v/v) were 70:30:0, 70:30:1, 70:30:2, 50:50:0, 50:50:1 and 50:50:2. The prepared solvents were placed in glass bottles and stored under ambient conditions.

A total of 5 mL of the extraction solvent were added to 0.5 g of powdered sample in a centrifuge tube. The tube was covered with carbon paper, vortexed at high speed, and left undisturbed for 1 hr. The sample was then centrifuged at 3000 rpm for 20 mins. The extract was decanted and measured for its TAC using the pH differential method (Lee *et al*., 2005). All treatments were done in triplicates. The TAC were computed as mg cyanidin-3-glucoside per litre of the sample using equation 1.

$$TAC, \frac{mg}{L} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1} \quad (1)$$

Where:

$$A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$$

ε = 26 900 L $\cdot mol^{-1} \cdot cm^{-1}$, molar extinction coefficient for cyanidin-3-glucoside

Analysis of Variance (ANOVA) was done to determine a significant difference among the different ethanol, water, and acetic acid extraction solvents. Tukey's Honest Significant Difference test was used as the post-hoc test.

2.3 Optimization of extraction

Response Surface Methodology was used to optimize the temperature, SLR, and time of the extraction process. In addition, a face-centred Central Composite Design (CCD) was generated using Design Expert 10. The high and low levels of the process factors are shown in Table 1.

Table 1. High and low levels of the factors in optimization of extraction parameters.

Factor	Low Level (-1)	High Level (+1)
A – Time, mins	30.0 mins	180.0 mins
B – solid-liquid ratio (SLR), %	10.00%	30.00%
C – Temperature, °C	4.0°C	60.0°C

The extraction of anthocyanins was done the same way as that of the solvent system screening but with various times, temperatures, and SLR based on the CCD. Quantification of anthocyanins was done using the pH differential method. Results were analyzed using the Design Expert 10 software.

2.4 Validation of the optimum extraction parameters

Validation of optimized results generated by Design Expert 10 was done by performing actual runs in triplicate.

3. Results and discussion

3.1 Solvent system for anthocyanins extraction

Preliminary tests were done to determine which ethanol-water-acetic acid ratio was most suitable for anthocyanins extraction from lipote. Table 2 shows that the varied ratio of the extracting solvent significantly ($p<0.05$) affected the TAC of the extract. Solvent mixtures with acetic acid resulted in higher TAC than the non-acidified counterparts. Anthocyanins are stable in acidic conditions (Andersen and Jordheim, 2014), hence, the positive influence of acetic acid. Of the acidified solvent systems, the most appropriate ratio for extracting anthocyanins from lipote was 50:50:1 (v/v/v) of ethanol, water, and acetic acid.

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Table 2. Total anthocyanins contents of lipote extracts obtained from using different solvent ratios.

Ethanol, water and 1M acetic acid ratio of solvent (v/v/v)	Total Anthocyanins Content (MG cyanidin-3-glucoside-L^{-1})
50:50:0	297.7±9.1^{bc}
50:50:1	373.2±17.3^{a}
50:50:2	308.1±5.8^{b}
70:30:0	273.2±10.4^{c}
70:30:1	295.1±3.9^{bc}
70:30:2	283.9±1.2^{bc}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($p<0.05$)

3.2 Optimization of parameters for anthocyanins extraction

Table 3 shows the TAC generated from different extraction conditions. The range of TAC obtained was from 134.1 to 928.7 mg cyanidin-3-glucoside per liter. Statistical analysis showed that both the two-factor interaction model and the quadratic model were suggested. However, the latter model was considered due to its higher significance of "lack of fit" and higher predicted and actual R-squared values. This also implies that a quadratic model would fit the data better than the two-factor interaction model. Based on the results of ANOVA, only SLR and its interaction with extraction time were significant ($p<0.05$). Therefore, these two terms had a significant effect on the TAC. The quadratic polynomial equation (in terms of coded factors) relating TAC to the extraction parameters (temperature, SLR, and extraction time) is shown in Equation 2.

$$TAC = 614.41 + 199.50B + 152.58BC \quad (2)$$

Where B = SLR and C = Time

Of the three individual factors, SLR had the highest effect on TAC. The significance of SLR is in agreement with other studies like that of Cacace *et al*. (2003), where SLR is the most critical variable (as compared to temperature and solvent composition in the extraction of anthocyanins from milled berry (Takeuchi *et al.*, 2009). Cissé *et al*. (2010) also reported that SLR is one of the significant factors in extracting anthocyanins from *Hibiscus sabdariffa*.

The interactions of parameters were plotted to visualize their effects on TAC. Figures 2a and 2b show that an increase in temperature will slightly increase the TAC of the extract. This can be due to the rise in anthocyanins solubility in the solvent and increased diffusion rate at higher temperatures (Cissé *et al*., 2012). This trend continues until a certain point where further temperature rise results in a decrease in TAC — possibly due to the degradation of anthocyanins. In Figures 2a and 2c, increasing the SLR resulted in increased TAC. This is because the high SLR increased the availability of the source of anthocyanins. In terms of extraction time,

Table 3. Total anthocyanins contents of lipote extracts derived using different temperature, solid-liquid ratio, and time of extraction.

Std. No.	Run no.	Temperature (°C)	Solid-liquid ratio (%)	Time (mins)	Total Anthocyanins Content (mg cyanidin-3-glucoside-L^{-1})
1	2	4.0	10.0	30.0	193.3
2	9	60.0	10.0	30.0	574.2
3	7	4.0	30.0	30.0	423.8
4	14	60.0	30.0	30.0	412.9
5	18	4.0	10.0	180.0	134.1
6	6	60.0	10.0	180.0	230.1
7	19	4.0	30.0	180.0	928.7
8	11	60.0	30.0	180.0	725.4
9	4	4.0	20.0	105.0	561.9
10	5	60.0	20.0	105.0	323.4
11	10	32.0	10.0	105.0	203.3
12	3	32.0	30.0	105.0	839.1
13	15	32.0	20.0	30.0	590.3
14	16	32.0	20.0	180.0	457.2
15	1	32.0	20.0	105.0	812.8
16	20	32.0	20.0	105.0	528.5
17	8	32.0	20.0	105.0	484.7
18	12	32.0	20.0	105.0	625.4
19	13	32.0	20.0	105.0	686.3
20	17	32.0	20.0	105.0	787.5

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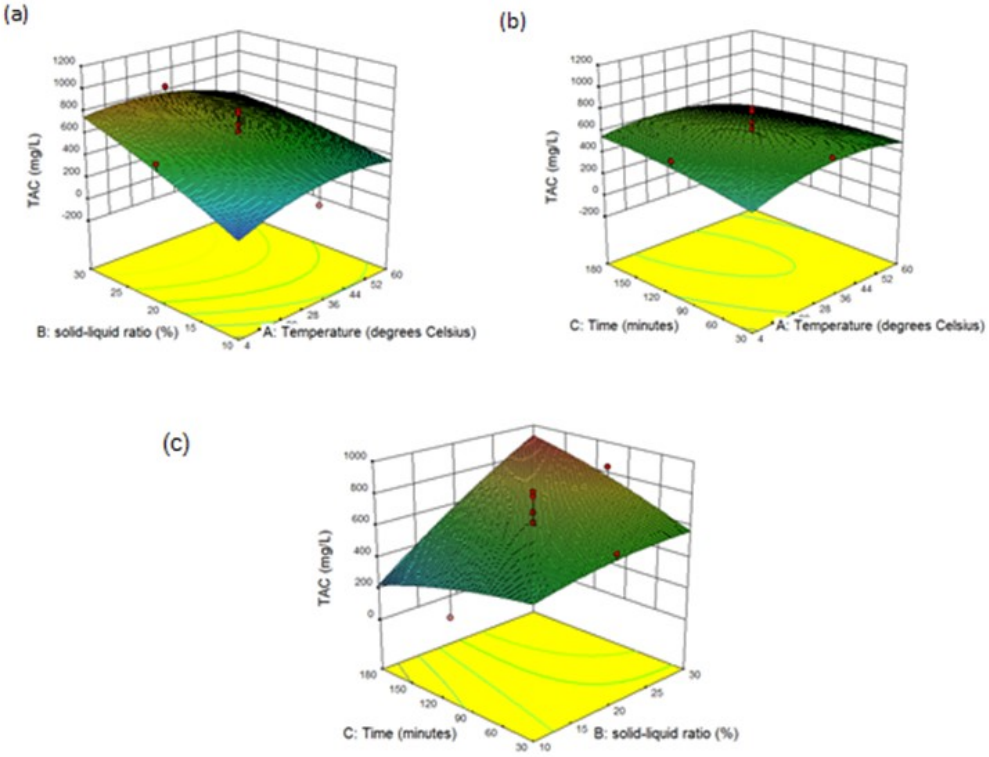

Figure 2. Response surface model showing (a) the effect of temperature and solid-liquid ratio (SLR) on total anthocyanins content (TAC) at a constant time of 105 mins; (b) the effect of time and temperature on TAC at a constant 20% SLR, and (c) the effect of time and SLR on TAC at a constant temperature of 32°C.

Figure 2b shows that extraction time has no effect on the TAC at all temperatures. On the other hand, Figure 2c showed that increasing the extraction time increased TAC for samples with high SLR and had no effect on those with low SLR. The quadratic model predicted that the optimum condition was extracting 29.4% SLR of lipote at 8.4°C for 172.0 mins. This combination of parameters predicts 932.9 mg cyanidin-3-glucoside/L.

3.3 Validation of optimized conditions

The predicted optimum extraction condition was verified by performing an actual run of the optimized parameters. After three trials, the TAC was 750.5 mg cyanidin-3-glucoside/L or 80.4% of the predicted yield. The actual TAC was within the intervals required for model verification, from 622.5 to 1,243.3 mg cyanidin-3-glucoside/L. Hence, the model can be considered valid for the prediction of optimum conditions. The model can also predict future extractions of anthocyanins from lipote using an ethanol-acetic acid mixture as a solvent.

4. Conclusion

The solvent systems were screened to extract anthocyanins from lipote using ethanol, water, and acetic acid mixture, followed by the optimization of extraction parameters (temperature, SLR, and time). It was found that the best solvent system was 50:50:1 (v/v/v). The presence of acetic acid increased the TAC of the extract due to the greater stability of anthocyanins at lower pH. The optimized conditions for the extraction of anthocyanins were 8.4°C, 29.4% SLR, and 172.0 mins. The predicted and actual TAC after extraction were 932.9 and 750.5 mg cyanidin-3-glucoside/L, respectively. The actual TAC was within the intervals required for model verification; hence, the model generated was valid.

Conflict of interest

The authors declare no conflict of interest.

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