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RESEARCH PAPER

Optimization of various solvents for the extraction of anthocyanins from 'sapinit' (Philippine wild raspberry) using response surface methodology

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Abstract

'Sapinit,' officially known as the Philippine wild raspberry, is a promising source of natural colourants such as anthocyanins. In this study, extraction of anthocyanins from 'sapinit' using three solvent systems, namely, acidified acetone, acidified ethanol and acidified methanol were evaluated and optimized. The optimized extraction conditions were: 4°C, 150 mins, and 50% solid-to-liquid ratio (SLR) for the acidified acetone solvent system; 110°C, 10 mins, and 60% SLR for the acidified ethanol solvent system; and 26°C, 90 min, and 24% SLR for the acidified methanol system. These optimum conditions resulted in extracts with total monomeric anthocyanin content (TMA) of 540 mg cyanidin-3-glucoside/L, 622 mg cyanidin-3-glucoside/L, and 89 mg cyanidin-3-glucoside/L, respectively. Validation procedures showed the generated models were able to predict the optimum TMA, with at most a 7% difference from the actual values. Moreover, acidified ethanol was found to be the most appropriate solvent for the extraction of anthocyanins from 'sapinit.'

1. Introduction

Colour is one of the critical factors in food quality assessment as it influences the consumers' preferences and choices of food (Pathare *et al*., 2013). Raw and manufactured food products have a range of acceptable colour appearances depending on the different factors associated with the consumer, as well as the condition of the surroundings at the time of observation. During food processing, the inherent colour of a food may change due to the associated chemical, physical and biological reactions. Hence, there is a need for a stable colourant to preserve or maintain the desired colour. Depending on the objective, a colourant may be added prior to, during, or after the processing for as long as it is in a stable form.

Natural pigments as a replacement for synthetic pigments have gained attention due to some reported health hazards of the latter. Studies have shown that synthetic pigments, specifically artificial food colours, affect children's intelligence and attention causing hyperactivity, and allergy when consumed more than 50 milligrams (Kanarek, 2011). Natural pigments are a good alternative to synthetic colourants because of their beneficial health effects. Natural colourants have been used in confectionery, dairy products, sauces and beverages (Carocho *et al*., 2015). Among the various types of natural pigment is anthocyanin, a water-soluble compound that is reported to possess antioxidant and antimicrobial properties, improve visual and neurological health and protect against some non-communicable diseases (Khoo *et al*., 2017).

Anthocyanins are the red, blue, or purple pigments found in flowers, fruits and some other plant parts. It is a flavonoid with a positive charge at the oxygen atom of the C-ring of the basic flavonoid structure. The colour and stability of anthocyanins are influenced by pH, light, temperature and structure (Mattioli *et al*., 2020). Hence, conditions during its extraction from plant sources must be established and optimized to maximize the quantity of the colourant.

In the Philippines, a promising source of anthocyanins is the Philippine wild raspberry, locally known as 'sapinit' (*Rubus rosifolius* L.). It is an endemic raspberry that can be found in certain areas of Laguna, Palawan and Quezon provinces. Studies performed on the extract showed that phenolic compounds, specifically anthocyanins, are its major component. In addition, the extract has shown antimicrobial properties against *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella enterica* serovar Typhi*, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Candida*

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albicans and *Aspergillus oryzae* (Alvarez *et al.*, 2013). The anthocyanins extracted from 'sapinit' have a wide range of potential applications, especially as a natural colourant in the food industry.

A lot of methods for the extraction of anthocyanin from different plant sources have been explored and reported. The polar characteristic of anthocyanins allows for its solubility in polar solvents such as alcohols, acetone and water. The traditional way of extracting anthocyanins from fruit samples involves the use of acidified ethanol, methanol, acetone, water, or a mixture of two or more of these solvents. The reaction of a sample to different solvents could vary; hence, protocols vary from one sample to another. For instance, ethanol and methanol acidified with hydrochloric acid have been used to extract anthocyanins from blueberries (Singh *et al*., 2022), Lapins cherries (Blackhall, 2018), and mashua (Chirinos *et al.*, 2007). Meanwhile, acetone acidified with hydrochloric acid was used in hulless pigmented barley (Lee *et al*., 2013). Ethanol, methanol, and acetone were among the most accepted solvents for anthocyanin extraction.

In this study, three different solvent systems (acidified ethanol, acidified methanol, and acidified acetone) for the extraction of anthocyanins from 'sapinit' were evaluated and optimized using response surface methodology.

2. Materials and methods

2.1 Materials

Fresh 'sapinit' samples were obtained from Mount Banahaw in Dolores, Quezon, Philippines. The samples were oven-dried (TV-30u Oven, Memmert, Strasse, Schwabach) at 50°C for 72 hrs, then pulverized using a countertop grinder (DV-525 Molinillo de Café, DVTech®, Madrid, Spain), and stored in an airtight sealed container at 25°C. Common chemicals and reagents, unless otherwise stated, were analytical reagent grade purchased from Sigma-Aldrich Pte Ltd., Singapore.

2.2 Optimization of parameters for the extraction of anthocyanins

The three solvent systems were evaluated and optimized using Response Surface Methodology (RSM). Extraction solvent systems were composed of a mixture of solvent (acetone/ethanol/methanol), distilled water and 1 M hydrochloric acid at a 70:30:1 (v/v/v) ratio. For each solvent system, the parameters evaluated were temperature, time and solid-liquid ratio (SLR).

The combinations of parameters for each solvent system were based on a Central Composite Design (CCD) generated using Design Expert 10.0 (StatEase, Inc). Table 1 shows the range of parameters used for each experimental design, and a total of twenty combinations were generated per solvent system. Depending on the SLR, a 10 mL mixture of ground 'sapinit' and the assigned solvent system was placed in a 15-mL centrifuge tube covered with carbon paper. The mixture was vortexed (Vortex V-1 Plus, BOECO, Hamburg, Germany) at high speed for 2 mins and then incubated at a specified temperature for a specific length of time. The mixture was then centrifuged (Centrifuge Z 326 K, Hermle Labortechnik GmbH, Germany) at 3,000 rpm for 15 mins.

The supernatant was separated by filtration and analyzed for total monomeric anthocyanin (TMA) content using the pH differential method as described by Lee *et al*. (2005). In this method, the supernatant was diluted in buffers of different pH (1 and 4.5), without exceeding a 1:4 sample-to-supernatant ratio. The dilution was also adjusted such that the absorbance of the sample (in pH 1 buffer) at 520 nm is within the linear range of the spectrophotometer, or between 0.2 to 1.4. After the necessary dilutions, the absorbance was measured at 520 and 700 nm using a UV/VIS spectrophotometer (Spectrophotometer CT-2600, E-Chrom Tech Co., Ltd., Taipei, Taiwan). Distilled water was used as a blank. The TMA concentration was calculated using Equation 1.

$$\text{Total Monomeric Anthocyanins (TMA)} = \frac{A \times MW \times DF \times 1000}{\varepsilon} \quad (1)$$

Where A = Absorbance of the sample = $(A_{520nm} - A_{700nm})_{pH1} - (A_{520nm} - A_{700nm})_{pH4.5}$, MW (molecular weight) = 449.2 g/mol for cyanidine-3-glucoside (cyd-3-glu), DF = dilution factor determined, ε = 26,900, molar extinction coefficient of cyd-3-glu in $L \cdot mol^{-1} \cdot cm^{-1}$ and 1000 = conversion factor from g to mg

The Design Expert 10.0 was used to run statistical

Table 1. Range of parameters used for the optimization of extraction of anthocyanins from 'sapinit'.

Solvent system	Extraction temperature, °C		Extraction time, mins		Solid-liquid ratio, %	
	-	+	-	+	-	+
Acidified acetone	4	70	30	150	10	50
Acidified ethanol	10	110	10	180	10	60
Acidified methanol	10	40	60	120	10	30

-: is the minimum, +: is the maximum

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tests for the optimization experiment. The software analyzed the responses using multiple regressions, and the resulting significance coefficients were evaluated using F-Test. A model was developed, and Analysis of Variance (ANOVA) determined its acceptability. Upon generating a valid model, the optimum combinations of extraction parameters were determined by numerical optimization (n = 3).

3. Results and discussion

3.1 Optimization and validation of parameters for the extraction of anthocyanins

3.1.1 Acidified acetone solvent system

The model equation generated (based on coded factors) for anthocyanins extraction using the acidified acetone solvent system is

$$TMA = 320.82 - 62.38A + 11.94B + 155.52C$$

Where TMA = Total Monomeric Anthocyanins, mg/ L cyd-3-glu equivalents, A = extraction temperature, °C, B = extraction time, mins and C = solid-liquid ratio, %

Based on the Analysis of Variance (ANOVA), the generated model was significant. Moreover, the Lack of Fit was found to be non-significant compared to the pure error calculated. A non-significant value for the lack of fit is an indicator that the generated model fits with the data.

With regards to the model values, the linear effects of the SLR and extraction temperature were found to be significant while the linear effect of the incubation time was found to be insignificant. Moreover, the negative coefficient for the temperature implies that lower extraction temperatures generally yield higher anthocyanin contents. In addition, the positive coefficient for the SLR implies that a higher SLR generally yields higher anthocyanin contents in the extracts. These results are similar to that of Garcia-Viguera *et al*. (1998) where the use of acetone permitted lower temperature for sample preparation. Moreover, their study also resulted in an increased concentration of extracted anthocyanins as the SLR increased. The response surface model showing the effect of SLR and extraction temperature in anthocyanin extraction using acidified acetone is shown in Figure 1.

The linear model predicted that the optimum combination of extraction conditions was 4, 150 min, and 50% SLR. The predicted TMA was 551 mg cyanidin -3-glucoside/L while the validated TMA was 540 mg cyanidin-3-glucoside/L or 98% of the predicted yield. Thus, the model can be used to predict the yield of extraction of anthocyanins from ‘sapinit’ using acidified acetone.

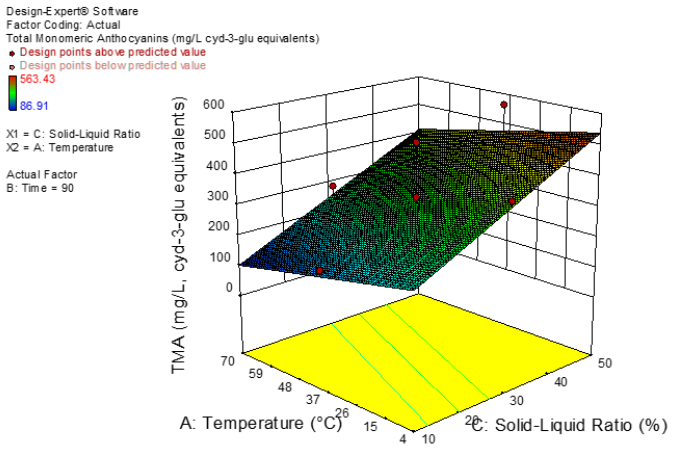

Figure 1. Response surface model for the extraction of anthocyanins from ‘sapinit’ using acidified acetone.

3.1.2 Acidified ethanol solvent system

The model equation (based on coded factors) for anthocyanins extraction using the acidified ethanol was:

$$TMA = = 378.80 + 45.29A - 9.88B + 238.43C$$

Where TMA = Total Monomeric Anthocyanins, mg/ L cyd-3-glu equivalents, A = extraction temperature, °C, B = extraction time, mins and C = solid-liquid ratio, %.

Based on ANOVA, the generated coded model was significant. In addition, there was a non-significant lack of fit, indicating that the model fits with the data.

In terms of the effects of the factors on the response, the extraction temperature and SLR positively affected TMA. Meanwhile, the incubation time negatively affected the response factor. The significance of temperature and SLR agree with the results of Fan *et al*. (2008), Yang *et al*. (2009) and Anuar *et al*. (2008), where the acidified ethanolic solvent was used to extract anthocyanins from purple sweet potato, purple corn and *Melastoma malabathricum* fruit, respectively. Extraction time, on the other hand, was only significant for anthocyanins extraction in purple corn samples and *Melastoma malabathricum* fruit. The response surface model showing the effect of temperature and SLR on the extraction of anthocyanins using acidified ethanol is shown in Figure 2.

The linear model predicted that the optimum conditions were 110°C, 10 mins and 60% SLR. The model predicted that these parameters would give a TMA of 672 mg cyanidin-3-glucoside/L. To validate the model, the sample was extracted using the suggested optimum conditions. The TMA was found to be 622 mg cyanidin-3-glucoside/L, which was 93% of the predicted yield. Thus, the model can be used to predict the result of anthocyanin extractions from ‘sapinit’ using acidified

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ethanol as a solvent.

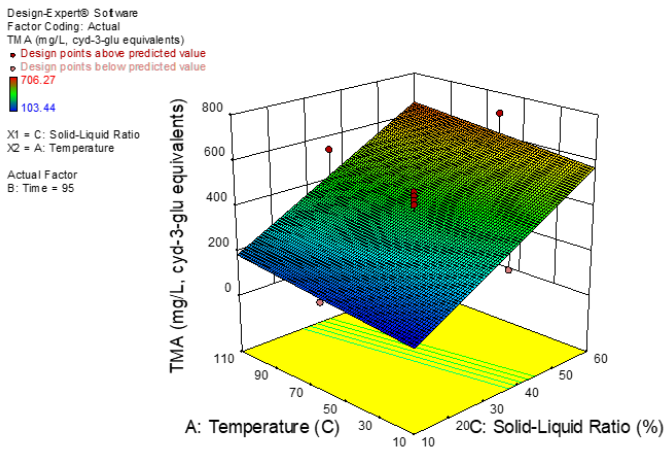

Figure 2. Response surface model for the extraction of anthocyanins from 'sapinit' using acidified ethanol.

3.1.3 Acidified methanol solvent system

The model equation (based on coded factors) for anthocyanin extraction using the methanol-HCl solvent system is as follows:

$$TMA = 83.72 + 1.51A - 7.72B + 14.76C - 3.16AB + 3.38AC + 0.76BC - 45.92A^2 - 0.49B^2 - 19.43C^2$$

Where TMA = Total Monomeric Anthocyanins, mg/L cyd-3-glu equivalents, A = extraction temperature, °C, B = extraction time, mins and C = solid-liquid ratio, %.

Based on ANOVA, the generated model was significant. Meanwhile, the Lack of Fit was non-significant, hence, the model fits with the data.

With regard to the model terms, only the quadratic effect of the temperature was found to be significant. Increasing the extraction temperature up to a certain point resulted in increased TMA while further temperature increases lowered the yield of anthocyanins. As explained by Escribano-Bailon and Santos-Buelga (2003), an appropriate temperature can improve the efficiency of extraction by increasing the compound solubility and diffusion characteristics and decreasing the viscosity of the extraction solvent. On the other hand, higher temperatures have been proposed to hasten anthocyanin degradation (Blackhall *et al.*, 2008; Silva *et al.*, 2017). The response surface model showing the effect of temperature for anthocyanin extraction using acidified methanol is shown in Figure 3.

The quadratic model predicted that the optimum conditions were 26°C, 90 mins and 24% SLR. The combination of these extraction parameters is also predicted to produce 87 mg cyanidin-3-glucoside/L. To validate the model, the sample was extracted using the suggested optimum conditions. The TMA was found to be 89 mg cyanidin-3-glucoside/L, which was 93% of the predicted yield. Thus, the model can be used to predict the result of anthocyanin extractions from 'sapinit' using acidified methanol as a solvent.

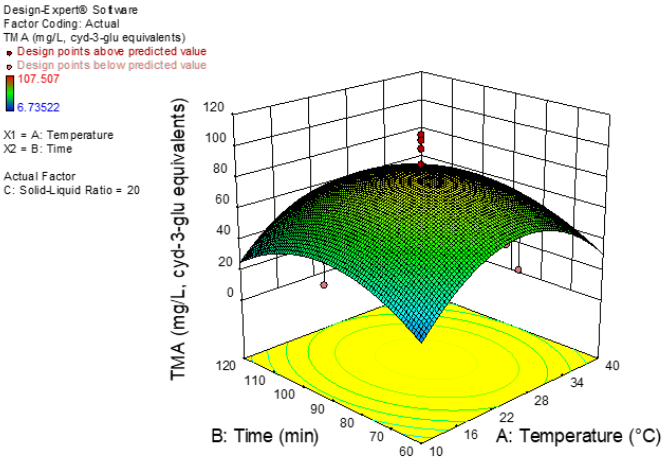

Figure 3. Response surface model for the extraction of anthocyanins from 'sapinit' using acidified methanol.

3.2 Comparison of optimized conditions from different solvent systems

Figure 4 shows the actual TMA content produced from the optimized extraction using acidified acetone, acidified ethanol and acidified methanol systems. Results showed that among the three solvent systems, optimized extraction using an acidified ethanol solvent system produced the highest TMA. This result concurs with the review of Hidalgo and Almajano (2017), who reported that ethanol was the most effective when compared with water, acetone, hexane, ethyl acetate and methanol in extracting antioxidant chemicals. The result also agreed with the study of Rockenbach *et al*. (2008) who reported that the TMA content of grape bagasse ethanolic extracts was higher than that of the grape bagasse acetone extracts. However, this result contradicts that of Singh *et al*. (2022), which showed that acidified methanol is more efficient than acidified ethanol in extracting blueberry anthocyanins. It also contradicts the result of Chirinos *et al*. (2007) which showed that for extracting anthocyanins

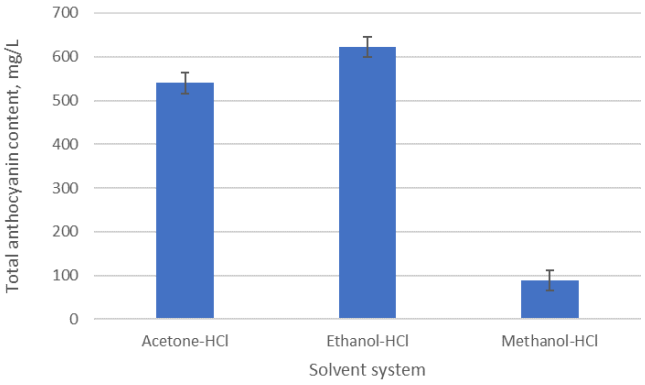

Figure 4. Actual total monomeric anthocyanin content of extracts from acidified acetone, acidified ethanol and acidified methanol solvent systems (n = 3).

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from mashua, methanol and acetone have more or less the same efficiency and ethanol has a lesser efficiency than both methanol and acetone. In a study by Wang *et al*. (2016), different extraction solvents were found to have different efficiencies when extracting anthocyanins from different plant sources. Thus, contradictions observed can be due to the inherent differences in the composition and structure of blueberries, mashua and 'sapinit.'

4. Conclusion

The optimum combination of time, temperature, and solid-liquid ratio (SLR) for the extraction of anthocyanins from 'sapinit' using three solvent systems: acidified acetone; acidified ethanol; and acidified methanol were evaluated and optimized. For the acidified acetone solvent system, optimum extraction conditions were found to be 4°C, 150 mins, and 50% SLR. For the acidified ethanol solvent system, optimum extraction conditions were 110°C, 10 mins, and 60% SLR. Lastly, for the acidified methanol solvent system, optimum extraction conditions were 26°C, 90 min, and 24% SLR. These extraction conditions can be used for future extractions of anthocyanins from 'sapinit.' A comparison of actual yields from the optimum conditions showed that the acidified ethanol solvent system was most suitable for extraction, followed by the acidified acetone solvent system, and finally the acidified methanol solvent system. Thus, the ethanol solvent system can be used to maximize the anthocyanin content from 'sapinit' extracts.

Conflict of interest

The authors declare no conflict of interest.

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