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Baghya Nisha Radhakrishnan
Assistant Professor, Excel Engineering College, Namakkal–637303, Tamil Nadu, India

Rita Narayanan
Professor and Head, Department of Food Processing Technology, College of Food and Dairy Technology, Tamil Nadu-600051, Chennai, Tamil Nadu, India

Baskaran Dharmar
Faculty of Department of Livestock Products Technology (Dairy Science), Madras Veterinary College, Chennai-600007, Tamil Nadu, India

Palani Dorai R
Faculty of Department of Livestock Products Technology (Dairy Science), Madras Veterinary College, Chennai-600007, Tamil Nadu, India

Valli C
Faculty of Institute of Animal Nutrition, Kattupakkam, Tamil Nadu Veterinary and Animal Sciences University, Chennai–600007, Tamil Nadu, India

Correspondence:
Dr. Baghya Nisha Radhakrishnan
Assistant Professor, Excel Engineering College, Namakkal–637303, Tamil Nādu, India
Email: ritanarayanan@yahoo.com

Optimization of extraction conditions for anthocyanin from *Hibiscus rosasinensis* and its characterisation by Chromatography techniques

Baghya Nisha Radhakrishnan[1], Rita Narayanan[2], Baskaran Dharmar[3], Palani Dorai R[4], Valli C[5]

ABSTRACT

The present study was conducted to identify the better extraction conditions for anthocyanin from fresh and dry petals of hibiscus with potent antioxidant activity and polyphenol content. The variables used in this study are solvents (aqueous, ethanol, methanol and each added with acidified agents (1% citric acid, 0.1% HCl and 1% acetic acid), temperatures (40 ºC, 50 ºC, 60 ºC, 70 ºC and 80 ºC), steeping time (interval viz., 60min, 90min, 120 min, 150min, 180min and 210min and pH (1-9). The study revealed that the anthocyanin content was higher for fresh petals of hibiscus extracted with test solvent methanol acidified with 0.1% HCl with the mean and standard error values of 167.69±0.41 mg CAG/100g. From the results, the optimized condition for anthocyanin extraction chosen was at the temperature of 60ºC for 120min at pH of 3-3.5. However, for the food uses, fresh petals extracted with ethanol (acidified with 1% citric acid) was recommended. Their yield at optimized extraction condition was found to be 156.25 ± 0.13 mg CAG/100g with the total phenolic content of 4965.83±1.19 (mg./100g) and 75.33±0.33% antioxidant activity. The F-values for each response implied high significance of the fittest models. Thin Layer Chromatography (TLC) and Paper Chromatography (PC) chromatograms predicted that the major types of anthocyanin present in hibiscus petals were cyanidin and delphinidin.

Keywords: Anthocyanins, Antioxidants, *Hibiscus Rosasinensis*, Polyphenol, PC, TLC.

INTRODUCTION

Hibiscus rosasinensis is a traditional herb that belongs to the family Malvaceous and it is native to China. Originally, the red variety flowers of hibiscus were known for decorative addition in the home garden and cosmetic uses. In today's scenario, their petals constituents are exclusively identified for their medicinal properties because of their beneficial action against anti-tumor, hypoglycaemic, anti-inflammatory, antimicrobial, and hypertensive activity [1]. Hydroxyl citric acid compound present in red petals was found to aid in weight loss [2]. An increase in health and environmental concern over the use of synthetic colour has led to the production of natural colourants. Anthocyanin is one of the plant source colourants that are deliberately interested by food industry. Anthocyanin is formed of glycosides and anthocyanidins. Otherwise stated to made of hydroxy or polymethyl derivatives of poly (2-phenylbenzopyrylium) or flavylium salts. It is predominantly known for its antioxidant activity. They are responsible for the attractive colours of some fruits, flowers, vegetables and their products [3]. Anthocyanin has better ability to scavenge free radicals by donating their hydrogen atoms [4]. In the recent days, anthocyanin gains attention because of their health benefits owing by the antioxidants.

Petals/calyxes of traditional red variety *Hibiscus rosasinensis* are a potential source of anthocyanins that exhibits exceptional antioxidant activity which might attracts many researchers to find it as a natural colorant that can be employed in foods, pharmaceuticals, and cosmetics. Several parameters could affect the extraction of anthocyanin compound from the complex plant matrix includes solvent composition, solvent to solid ratio, temperature, time and pH [5]. Anthocyanin is traditionally extracted by aqueous or mixture of aqueous with some organic solvents such as ethanol, methanol, and acetone [6]. Besides, anthocyanin is a sensitive pigment that may degrade or alter from its original state when subjected to a high temperature, pH and solvents. Hence it is very important to offer precise extraction conditions for maximum recovery of anthocyanin. The present investigation is an attempt to investigate the effect of different extraction conditions on anthocyanin content from fresh & dry petals of hibiscus. Henceforth the obtained crude extract can be used as natural colourant for food industry.

MATERIALS AND METHODS

Hibiscus flowers were procured from the local market, Chennai were used for the study. Flowers were stored in the freezing condition until analysis. All the glass wares and reagents used in the study were of

analytical grade. For extraction, fresh petals separated from the flower stalk was subjected to immediate soaking in solvents. For the dried analysis, petals were shade dried for 15 hours at room temperature until the moisture content reaches below 10%. To optimize the well-suited condition for anthocyanin extraction, various parameters were considered. It includes solvents, temperature, time and ph.

Red petals were extracted using aqueous (water), ethanol and methanol solvents. The experiment was also conducted by added the solvents with acidifying agents such as 0.1 % HCl, 1% citric acid and 1% acetic acid. For food purpose, fifty percent of solvent was considered to be safe. Hence, fifty percent of each solvent (50% water + 50% alcohol/aqueous) was taken for the study as the test solvents. The sample was mixed with 50% of test solvents in the ratio of 1:10 as per the modified procedure of Rashad *et al*. (2016) [7]. Solvents with extract mixture was shaken in the rotary shaker at 100rpm for 2 hrs under room temperature to evaporate the solvent. The resulting extracts were filtered using Whatman No.1 filter paper. The supernatant was filtered and stored in amber bottles at 4 ℃ in refrigerated condition until analysis.

Effect of various temperature such as 40 ℃, 50 ℃, 60 ℃, 70 ℃ and 80 ℃ on yield of anthocyanin content using the selected food grade solvent was carried out as per the modified procedure of Nayal and Babar [8]. Optimization of steeping time was done by repeating same procedure with the selected solvent at different time interval viz., 60min, 90min, 120 min, 150min, 180min and 210min at optimized temperature as per the modified procedure of Nayal and Babar [8].

Effect of pH Buffer solutions (1 to 9) on anthocyanin yield was carried out using buffer solutions (1-9) as per the procedure of Wahyuningsih *et al*. (2017) [9]. Accurate pH was measured with a pH electrode and temperature probe.

Total monomeric anthocyanin content in the crude extracts was determined using pH differential method spectrophotometrically. Briefly, buffer solution used are 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). Samples were diluted with buffer solution (1:10) and the absorbance was measured at the wavelength of 520nm and 700nm. The content of total anthocyanins was expressed as cyanidin-3-O-glucoside (mg) equivalent per 100g of the mass of the sample according to the following equation [10].

$$\text{Anthocyanin pigment (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Were,

A	=	(A520nm – A 700nm) $pH_{1.0}$ – (A520nm – A700nm) $pH_{4.5}$
MW	=	449.2 g/mol for cyanidin-3-glucoside (molecular weight)
DF	=	Dilution factor
ε	=	26,900 (molar absorptivity of cyanidin-3-glucoside)
1	=	cell path length in (1cm)
1000	=	factor for conversion from g to mg.

Estimation of Total phenolic content (TPC)

Total phenolic content (TPC) of crude hibiscus anthocyanin extracts was determined using Folin-Ciocalteu assay. Sample extract (200µL) was allowed to react with 0.25 ml of Folin-Ciocalteu reagent (1:9 with water) and 750µL of sodium carbonate (7%, w/v). The tubes were incubated for 30 min in the dark at room temperature and absorbance was measured at 765 nm using a UV/Vis spectrophotometer against the blank sample. A standard calibration plot was generated using known concentrations of gallic acid (10 µm/mL to 500 µm/mL). Total phenolic content was calculated from the calibration plot and expressed as mg gallic acid equivalents (mg GAE) of phenol/g of extract [11].

Estimation of Antioxidant Activity (AA)

DPPH (2, 2-diphenyl -1-picrylhydrazyl) scavenging capacity of hibiscus extract was measured based on the method described by Blois [12]. with modifications described by Brand Williams [13]. This method expresses the colour change after radical scavenging by antioxidants from the reduction of purple to yellow colour. The remaining DPPH radicals that show maximum absorption at 517 nm will be measured. Briefly, 50 mL. of the sample extract was allowed to react with 1 ml of the DPPH solution (100 µM DPPH in methanol). The mixture was shaken vigorously, allowed to stand for 20 min in dark at room temperature and then absorbance was measured. The results were expressed as % of activity.

Thin layer chromatography (TLC)

For Thin later chromatography (TLC), silica gel plates (20cm X 20cm) were used. The sample was spotted on the plate and allowed to run in the solvent mixture containing butanol: acetic and water in the ratio of 4:1:2. The pigment spots were visually observed [14].

Paper chromatography (PC)

For Paper chromatography (PC), ethanolic extract of hibiscus was spotted on the Whatman No.1 filter and allowed to run in the solvent mixture containing petroleum ether and acetone in the ratio of 9:1. The chromatography paper was undisturbed for 30 minutes and the pigments spots were observed visually as per the procedure adopted by Muchuweti and Chikwambi [15]. The pigments spots were marked to determine the R_f values using the formula given below:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

All experiments were conducted in six trials. Analysis of variance and t – test was performed using VETSTAT software. The results were expressed as mean ± SE and the least significant difference at $P<0.05$ was calculated using Duncan's multiple range test to determine the significant differences on effect of solvents, time, temperature and pH on anthocyanin extraction. To show the significant difference between the treatments 3D surface graphs were used.

RESULTS AND DISCUSSION

Effect of varying solvents on total anthocyanin content of *Hibiscus rosasinensis* evidently showed that methanol and their acidified solvents represented high significant difference among other solvents followed by ethanol with their acidified solvents (Table 1). The findings shows that there is a significant difference ($P \leq 0.01$) on

varying solvents between fresh and dry petals. Better extraction yield was obtained when extracted using methanol acidified solvents followed by ethanol acidified solvents. Acidified methanol gave higher yield because methanol has high dielectric constant which enables extraction of more polar polyphenolic compounds compared to ethanol [16]. Fresh petals of hibiscus characterised to have maximum yield. Khoo *et al*. (2017) [17]. reported that ethanol is a safer extraction solvent and the use of weak acid like citric acid is advisable for extraction of anthocyanins. In that regard, ethanol acidified with citric acid with anthocyanin content of 114.02±0.38 (mg CGE/100g) was chosen as desirable solvent for the present study.

Table 1: Effect of varying solvents on anthocyanin# yield

S. No	Solvents	Anthocyanin content (mg CGE/100 g)		t - test
		FP	DP	
S1	Aqueous (A)	53.49^{a}±0.60	36.24^{a}±0.26	26.37**
S2	Ethanol (E)	104.28^{e}±0.43	78.38^{e}±0.43	42.31**
S3	Methanol (M)	132.48^{i}±0.47	115.4^{i}±0.49	25.17**
S4	A. HCl	96.59^{d}±0.62	62.17^{d} ±0.38	47.18**
S5	A. Citric	80.65^{c}±0.53	54.62^{c}±0.40	39.23**
S6	A. Acetic	76.46^{b}±0.35	48.78^{b}±0.26	63.33**
S7	E. HCl	125.65^{h}±0.58	106.43^{h}±0.19	31.74**
S8	E. Citric	114.02^{g}±0.38	91.23^{g} ±0.60	32.06**
S9	E. Acetic	111.48^{f}±0.56	86.98^{f}±0.43	34.69**
S10	M. HCl	167.69^{l}±0.41	151.40^{l}±0.60	22.56**
S11	M. Citric	154.69^{k}±0.81	140.31^{k}±0.14	17.54**
S12	M. Acetic	150.70^{j}±0.43	135.15^{j}±0.96	14.78**

Average of six trials, # Total anthocyanin content. ** represents statistically highly significant (P≤0.01) Small case superscripts represent significant differences between treatments.

Study on varying temperature on anthocyanin yield from fresh petals and dry petals are given in Table 2. The results of the study revealed that there is a significant difference (P≤0.01) between fresh and dry petals anthocyanin content. Among varying temperatures, 60 ℃ showed high significant difference with greater anthocyanin content with the mean and SE values of 134.62±0.61 (mg CGE/100g) in the fresh petals and 106.95±0.21 (mg CGE/100g) in the dry petals. Comparatively, fresh petals gave the maximum yield than the dry petals. The results also showed that increase in temperature from 40 to 60 ℃ gradually increased anthocyanin yield whereas at above 60 ℃ anthocyanin tend to decrease. The findings were correlated with the findings of Nayal and Babar [8]. who also suggested that optimum extraction temperature for anthocyanin is 60 ℃? The reason behind the lesser stability of anthocyanin may be due to the reason that, at high temperature phenolic compounds like anthocyanins present in the crude extract are getting degraded enzymatically by polyphenol oxidase enzyme.

Table 2: Effect of varying temperature on anthocyanin# yield using solvent ethanol acidified with 1% citric acid

Treatments	Temperature (ºC)	Anthocyanin content (mg/100g)		t - test
		FP	DP	
T1	40	114.41^{a} ±0.24	99.74^{a}±0.38	31.94**
T2	50	124.41^{c} ±0.21	104.03^{c}±0.23	61.86**
T3	60	134.62^{e} ±0.61	106.95^{d}±0.21	43.08**
T4	70	126.98^{d} ±0.10	104.15^{c}±0.10	146.58**
T5	80	121.84^{b} ±0.10	101.42^{b}±0.20^{c}	89.72**

Average of six trials, # Total anthocyanin content. Small case superscript represents significant differences between treatments. ** represents statistically highly significant (P≤0.01)

Anthocyanin content during varying steeping time showed a high significant difference (P≤0.01) between fresh petals and dry petals (Table 3). The present study was investigated with optimum extraction temperature of 60 ℃ using selected solvent (ethanol acidified with 1% citric acid). Comparatively, the results of treatments show that longer drying time 210 min reduced the total anthocyanin yield with mean and SE values of 132.42±0.23 (mg CGE/100g) whereas at 120 min anthocyanin yield was higher with the mean and SE values of 155.78±0.15 (mg CGE/100g). The results were in accordance to Nayal and Babar [8]. who indicated that two hours of extraction at 60 ℃ showed better retention of anthocyanins? The anthocyanin yield was found to be maximum in fresh petals compared to dry petals.

Table 3: Effect of steeping time on anthocyanin# yield using solvent ethanol acidified with 1% citric acid at optimized temperature 60 ℃

Treatments	Time (min)	Anthocyanin content (mg/100 g)		t - test
		FP	DP	
t1	60	138.42^{b} ±0.23	109.70^{b}±0.22^{a}	96.91**
t2	90	151.52^{e} ±0.26	111.49^{c}±0.18^{d}	149.80**
t3	120	155.78^{f} ±0.15	116.49^{e}±0.17^{e}	165.20**
t4	150	146.57^{d} ±0.14	113.54^{d}±0.15^{f}	122.34**
t5	180	144.34^{c} ±0.21	108.56^{b}±0.14^{c}	139.43**
t6	210	132.42^{a}±0.23	103.34^{a}±0.94^{b}	29.96**

Average of six trials, # Total anthocyanin content. Small case superscript represents significant differences between treatments. ** represents statistically highly significant (P≤0.01)

From table 1, 2 & 3, treatments shown with high anthocyanin content was chosen for the current study. The findings showed that there is a significant difference (P≤0.01) upon varying pH between fresh and dry petals (Table 4). It was found that pH had the great influenced the anthocyanin yield. At acidic pH (1, 3) red colour of anthocyanin was bright showed higher absorbance. But when the pH is increased the colour changed slightly to light colour resulted with less absorbance. The results of the study were in accordance to Wahyuningsih *et al.* (2017) [9]. who accounted that anthocyanin is more stable in acidic pH and degrades at alkaline ph.? This study declares that the stability of flavylium ion responsible for redness is stable at acidic condition and colour degrades at increasing pH condition.

Table 4: Effect of varying pH on anthocyanin# yield using selected solvent ethanol acidified with 1% citric acid at optimized temperature and time (60 ℃ for 120 min)

Treatments	pH	Anthocyanin content (mg CGE /100 g)		t - test
		FP	DP	
P1	1	167.78^{e} ±0.40	140.20^{e}±0.11	64.90**
P2	3	156.25^{d} ±0.13	116.58^{d}±0.14	201.97**
P3	5	129.78^{b} ±0.13	107.61^{b}±0.14	110.51**
P4	7	145.87^{c} ±0.21	115.68^{c}±0.09	129.30**
P5	9	96.24^{a} ±0.24	102.63^{a}±0.20	20.29**

Average of six trials, # Total anthocyanin content. Small case superscript represents significant differences between treatments. ** represents statistically highly significant (P≤0.01)

From the previous table results, optimised extraction condition for hibiscus anthocyanin extraction was ethanol acidified with 1% citric acid at 60 ℃ for 120min at pH 3. Significant difference was found between fresh and dry petals from the total phenolic content and antioxidant activity values (Table 5). TPC and AA were found to be maximum for hibiscus fresh petals with mean and SE values of 4965.83±1.19 (mg G/100g) and 75.33% respectively. The findings were comparable with the reports of Make *et al.* (2013) [18]. who acquired similar values TPC and AA to be 4598.16±106.8 (mg G/100g) and 83.01±0.1% respectively?

Table 5: Total anthocyanin content (TAC)#, Total phenolic content (TPC)# and Antioxidant activity (AA)# at Optimized extraction condition (Ethanol acidified with 1% citric acid - 60 ℃ for 120min at pH 3-3.5)

Sample	TAC (mg CGE / 100g)	TAC t-test	TPC (mg G/100g)	TPC t - test	AA (% of Inhibition)	AA t - test
FP	156.25^{b}±0.06	201.97**	4965.83^{b}±1.19	1012.33**	75.33^{b}±0.33	47.00**
DP	116.58^{a}±0.14		3255.17^{a}±1.93		44.00^{a}±0.5	

Average of six trials, # Total phenolic content and #Antioxidant activity small case superscripts represent significant differences between treatments. ** represents statistically highly significant (P≤0.01)

The chromatogram spots of TLC had purple (A) and magenta (B) shades. and PC were yellow (A) and purple (B) shades. From PC chromatogram, it was depicted that the purple colour may due to cyanidin and yellow pigment spot may correspond to the presence of carotenoids. From TLC chromatogram, it was predicted that the purple and magenta colour of hibiscus extracts corresponds to the presence of cyanidin and delphinidin as the major anthocyanin. The results were in par with Tonfack *et al* [19]. who represented that red colour of the hibiscus is due to the delphinidin 3-O-sambubioside and cyanidin 3-O-sambubioside?

Table 6: Characterization of type of anthocyanin in hibiscus extract

Parameters observed	Paper chromatography	Thin layer chromatography
Natural spot color in daylight	Yellow, Purple	Magenta, Purple
R_f values	0.78, 0.64	0.52, 0.62
Probable type of pigment	Carotenoid, Delphinidin	Delphinidin, Cyanidin,

CONCLUSION

The present study conducted to optimize various parameters (temperature, solvents, time and pH) for providing effective extraction condition for maximum yield of anthocyanins revealed that methanol with HCl was giving more yield compared to other test solvents. However, methanol is toxic solvent, their adverse effects on human health should be considered, when the final extract is recommended for pharmaceutical and food purposes. From the results, anthocyanins extracted using ethanol (acidified with 1% citric acid) at 60 ℃ for 2 hrs at the pH 3–3.5 was chosen as optimum condition. The results may be of interest for food manufacturers to use the hibiscus extract both as natural colorants and antioxidant. The results of PC and TLC of hibiscus extract indicated that the major type of anthocyanin responsible for the colour of anthocyanin is cyaniding and Delphinidin.

Conflict of Interest

None declared.

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None declared.

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