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Kinetic Modelling of Anthocyanin Extraction from Grape *(Vitis vinifera)* using Response Surface Methodology

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Abstract: ***Anthocyanins are natural food colorants and functional compound, those substitute synthetic colours. One of the major source of Anthocyanin is Grapes, and by implementing suitable extraction process, it is possible to obtain high yield extract. In our study, we have optimized anthocyanin extraction from grape pomace (Vitis vinifera) by using response surface methodology. Owing to its simple, economical and efficient alternative to other extraction process, this method of extraction was found to be appropriate. Various parameter combinations were performed to determine the anthocyanin concentration such as extraction temperature, extraction time and pH and the process was optimized and validated. Various characterization of grapes and anthocyanin such as moisture, ash, total phenolic content, quantification of anthocyanin, HPLC and FTIR were also performed.***

Keywords*: Anthocyanins, response surface methods, Ultrasound-assisted extraction, Vitis vinifera*

I. INTRODUCTION

Colorants, especially natural ones are extremely necessary as it is considered safer than synthetic colours. Anthocyanins are water soluble natural pigments or colorants, which belong to phenolic compounds group seen in red, purple and blue colours of various fruits and flowers [4]. Red colour in Anthocyanin is due to the presence of cyanidin-3-glucoside [8]. When there are variations in pH, structural changes occur in phenolic substances present in this pigment such as cyanidin, delphinidin and pelargonidin [12]. It is important to select a good source of pigment that gives the best yield and is cost effective, to determine the appropriate method of extracting anthocyanin [11]. Grape and grape skin are found to be good sources of anthocyanins, and is easily obtainable. Kinetic modelling is an appropriate method in order to design and optimize extraction method to recover high quality and yield [2], [19]. The key phase is to select an optimum model. Therefore, appropriate selection of anthocyanin extraction kinetics is crucial, thereby improving the procedure accuracy, diminishing processing errors and good quality product [1], [13]. The most extensively used process for estimation of anthocyanin extraction is Response surface methodology (RSM) [9], [7], [15].

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In this paper, the anthocyanin extraction from grape pomace (***Vitis vinifera***) was optimized by applying response surface methodology, in order to define effect of ultrasonic assisted parameters such as extraction temperature, time and pH to determine the anthocyanin concentration using the optimum combination of these parameters. Finally, the optimized parameters were validated.

II. MATERIALS AND METHODS

A. Materials required for Anthocyanin extraction

Black grape pomace was obtained from Grapes Association, Mathampatti, Coimbatore. For anthocyanin extraction, chemicals such as HCL and ethanol were bought from M/s Precision Scientific Company, Coimbatore. For removal of mucilage, centrifuge was used, manufactured by Eltek. For concentrating the anthocyanin extract, Rotary vacuum evaporator was used, manufactured by CYBERLAB, model number RE 10C S84. Determination of total phenolic content was done by using Perkin Elmer Lamda 25 UV spectrophotometer.

B. Extraction of Anthocyanin

The grape pomace washed with hot water in order to eliminate undesirable particles were taken. The washed grapes were pulped using mixer and the extracted pulp was dried in cross flow drier at 45^0C for 7 hours and further grinded using a mixer-grinder. A known quantity of dried black grape pomace powder was taken for anthocyanin extraction was soaked in ethanolic HCl of 1.5N and kept it in dark for 2 hours without shaking or stirring. The residue was clarified and washed with ethanolic HCl solution till a clean solution was achieved. Anthocyanin extract was allowed to centrifuge at 3600rpm for 20minutes for mucilage separation. Then, anthocyanin was concentrated in the ratio of 10:1 by using rotary vacuum evaporator which has specified as rotation speed of RBF is 100rpm, vacuum pressure is about 337mbar and temperature of water bath is about 60°C and then stored in amber bottle in refrigerator.

C. Characterization of *Vitis vinifera* and Anthocyanin

- *Moisture content of dried grape pomace:*

The moisture content was analysed using gravimetric method described by [10]. Using hot air oven, the sample was dried at 110°C for 2 hours. The sample weight was measured before and after drying to determine the water mass loss. The difference between the weights of the wet and dry samples is the water mass.

$$\%moisture\ content = \frac{Wetweight - Dry\ weight}{Dry\ weight} \times 100$$

- *Ash:*

The ash value was determined by placing the sample in a crucible inside a heated muffle furnace for 4 hrs at 550°C. The sample was then cooled to less than 200°C in the furnace and further placed in a desiccator. Ash value was determined in order to find the inorganic matters present in a compound after removal of water and other organic matter by heating process [3].

$$\% \text{ ash} = \frac{\text{weight of residue (g)}}{\text{Sample weight(g)}} \times 100$$

- *Total Phenolic Content:*

The phenolic compound was determined by Folin-Ciocalteu's method spectrophotometrically. The standard was prepared as 100mg of tannic acid dissolved in 100ml of water and diluted 10 times for working standard. 0.5 ml of sample (Anthocyanin) and different aliquots (0.2 to 2 ml standard) were pipetted into different 10ml standard volumetric flask. The flask contains 0.5 ml Folin-Ciocalteu's reagent, 5ml of distilled water and 1.5 ml Na2CO3 solution and volume was made up with methanol and after 2 hours of oxidation of phenolic compounds, the absorbance was measured at 760nm. Tannic acid of (100µg per 100ml) was the standard used and samples were expressed in milligram tannic acid equivalent per 100 ml.

- *Quantification of Anthocyanin:*

After the extraction, anthocyanin was allowed to check the concentration of anthocyanin by using UV spectrophotometer at 540nm. The anthocyanin concentration [6] was expressed as Cyanidin-3-glucoside equivalents:

$$\text{Concentration of anthocyanin (mg/litre)} = \frac{A \times Mw \times DF}{\varepsilon \times 1} \times 1000$$

where A- absorbance at 540nm

MW - molecular weight (g/mole) 449.2 g/mole for Cyanidin-3-glucoside,

DF- dilution factor

ε - extinction coefficient (34300) for Cyanidin-3-glucoside

$$\text{Dilution factor} = \frac{sample + solvent\ volume}{sample\ volume}$$

- *HPLC Analysis:*

The technique was done to analyse anthocyanin compounds. It is primarily used in identification of compounds. HPLC is a specific type liquid chromatography that uses the computer system with sampler, mobile phase reservoirs, pumps, column and a detector. The sampler injects the small amount of sample onto the column and compounds separate based on their polarity. An UV-visible detector records the presence of compound. A flow rate of 1.0 ml/min and about 5µL injection volume were used for sample. The column temperature was 35±5°C. UV-Visible detection was at 520nm.

- *FT-IR SPECTROSCOPY INTERPRETATION:*

The analysis was recorded to determine the functional groups present in the sample. About 0.10g of potassium bromide was blended with mortar and pestle for approximately 10 minutes and made as a pellet and then sample (anthocyanin) was dropped on pellet. The disc was conditioned in a desiccators placed in an oven at 80°C for 16 hours before analysis. The spectra of Anthocyanin sample were obtained using IR PRESTIGE-21, manufactured by M/s SHIMADZU-8400, Japan.

D. Experimental Design for RSM

The method was employed to determine the effect of the ultrasonic assisted extraction parameters temperature, time and pH to determine anthocyanin concentration using optimum combination of these parameters. Dependent and independent variables refer to values the change in correlation to each other. Dependent variables are those intentionally employed to invoke a change in dependent variables. Precisely, "if A is given, then B occurs", where A denotes independent variables and B denotes the dependent variables. Considering the review of the results obtained from the trails undertaken, the process parameters which from the independent variables were chosen for each extraction condition chosen. Levels of independent and dependent variables used for study are: Independent variables: pH (3,4,6), Time (20min, 40min, 60min), Temperature (30^0C, 50^0C, 70^0C). Dependent variables: Concentration Response(B) Concentration of anthocyanin from dried waste black grapes at 700nm was studied using a spectrophotometer.

Factorial design for RSM: The developed experimental design was a factorial 3^3, where the factors were pH, time and temperature at the levels 3,4,5 and 20min, 40min, 60min and 30^0C,50^0C,70^0C for each factor respectively.

Table 1: Experimental Design Matrix for complete factorial for variables

Test Set	pH	TIME (minutes)	TEMPERATURE (^{0}C)
1	4	`40	30
2	3	60	70
3	3	20	30
4	6	20	30
5	6	60	30
6	4	60	50
7	4	40	50
8	3	40	70
9	4	40	50
10	3	20	50
11	3	40	50
12	3	60	30
13	4	40	50
14	6	60	70
15	6	40	50
16	4	60	50
17	4	20	70
18	4	40	30
19	6	40	50
20	6	20	70

III. RESULTS AND DISCUSSION

- **Characterization of *Vitis vinifera* and Anthocyanin**

The moisture content of the grape pomace was found to be 2.3%. This could be because of components such as salts, sugars or fat, increasing viscosity and thus slowing drying process. Ash value was determined in order to find the inorganic matter present in the grape pomace after removal of moisture and organic matter by the application of heat.

The ash content was found to be 0.23%. Total phenolic content was estimated from standard curve of tannic acid and found to be about 37.2mg/dl as per Folin's method. The concentration of anthocyanin in grape pomace was found to be about 844.7mg/L.

- **FTIR Interpretation of Anthocyanin**

When there is absorption of infrared radiation, the amplitude of vibrational frequencies increases. This is directly proportional to concentration of molecule, states Beer lambert law. Various forms of vibrations such as stretching, bending, twisting and rotating are unique depending on wavelength, frequency of vibration and the bond itself.

FTIR spectra is obtained between 500- 3500 cm-1. The broad band spectra of 3500-3300cm^{-1} is associated with Phenol OH groups and sugar vibration. This shows changes in shape and intensity, and once complexes form shift to low frequency. Vibration band of C═O group at 1638.60 cm^{-1}, indicated slight change to small frequency. The M ─ O band at the range 1050-1000 cm^{-1} linked to metal ion interaction. Due to heavy mass of selected atom, this band link to low frequency region.

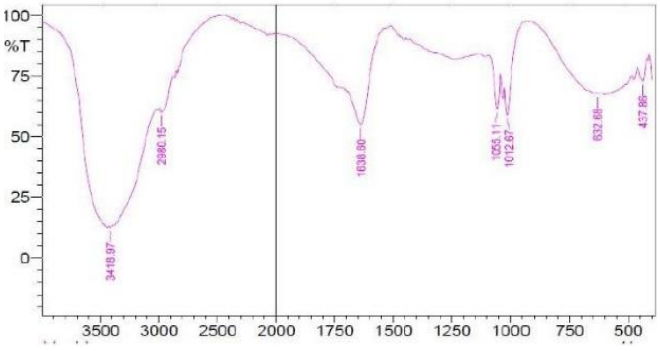

Fig 1: FTIR Spectra of anthocyanin

Table 2: FTIR Profile of Anthocyanin

S.No	Wave Number(cm-1)	FUCNTIONAL GROUP
1.	3418.97	Phenol OH group
2.	2980.15	Aliphatic hydrogen (CH2 group)
3.	1638.60	C=O group
4.	1055.11, 1012.67	M-O (metal ion with active group of pigment)

- **HPLC ANALYSIS OF ANTHOCYANIN:**

The resulting first peak, the retention time of 8.83 minutes showed a molecular ion 449.2, signifying the presence of cyanidin-3- glucoside and the second peak, the retention time of 9.24 minutes, showed a molecular ion 595, signifying the presence of cynidin-3-rutinoside. This result was confirmed according to [5].

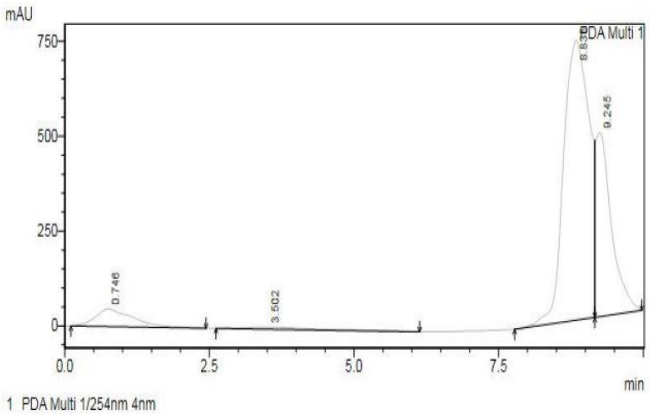

Fig 2: HPLC Profile of Anthocyanin

- **Response Surface Methodology**

To optimize three independent variables selected for the study, namely pH, time and temperature, a three levels Central composite design (CCD) was applied as represented in Table 3. Comparison of actual and predicted values of Anthocyanin extract is represented in Fig.3 are calculated using regression model. This model was valued using determination coefficient (R2) of 0.97, which indicates a reasonable fit of the model to the actual data (Table 4). The values of adjusted and predicted coefficient of determination are 0.94 and 0.92, which indicates the values are appropriate as the difference is less than 0.2 A higher value than 4 is preferred.

Table 3: Experimental design of Central composite design and total anthocyanins of ultrasonic-assisted grape pomace extract

Test set	pH	Time (min)	Temperature (^{0}c)	Concentration of anthocyanin (mg)
1	4	40	30	0.76
2	3	60	70	0.91
3	3	20	30	0.73
4	6	20	30	o.47
5	6	60	30	0.49
6	4	60	50	0.89
7	4	40	50	0.84
8	3	40	70	0.86
9	4	40	50	0.85
10	3	20	50	0.81
11	3	40	50	0.90
12	3	60	30	0.87
13	4	40	50	0.88
14	6	60	70	0.48
15	6	40	50	0.51
16	4	60	50	0.87
17	4	20	70	0.83

18	4	40	30	0.82
19	6	40	50	0.64
20	6	20	70	0.52

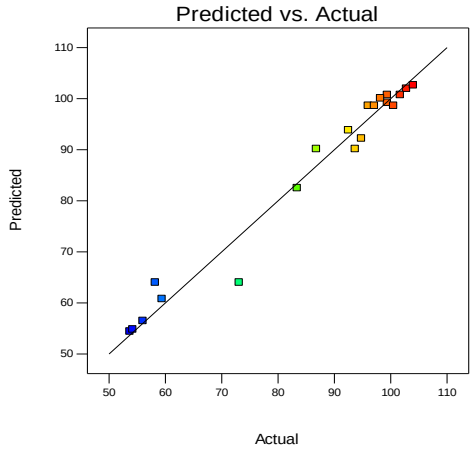

Fig 3: Comparison of actual and predicted values of Anthocyanin extract.

Analysis of Variance (Table 5) shows model evaluation. The analysis revealed F-value of 40.82 indicates the model is significant. A "Lack of fit" having p value 0.9616 and F value of 0.17 indicates the "lack of fit" was not significant comparative to the pure error. Application of RSM enabled to optimize the Anthocyanin extraction parameters for obtaining optimum operating conditions from ***Vitis vinifera*** at an extraction concentration of 1:3, time 20min and temperature of 50^0C.

Table 4: Determination Coefficient values of adjusted and predicted

Std. Dev.	4.17	R- squared	0.9735
Mean	85.25	Adj R-squared	0.9496
C.V. %	4.90	Pred R-squared	0.9220
PRESS	512.56	Adeq Precision	16.343
-2 Log Likelihood	100.04	BIC	130.00
		AICc	144.48

Table 5: Analysis of Variance of the total anthocyanin contents for response surface quadratic model of the grape pomace model

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob>F**	
Model	6396.71	9	710.75	40.82	<0.0001	Signi - ficant
A-pH	218.96	1	218.96	12.57	0.0053	
B-Time	108.43	1	108.43	6.23	0.0317	
C-Temp	178.81	1	178.81	10.27	0.0094	
AB	106.83	1	106.83	6.14	0.0327	
AC	12.91	1	12.91	0.74	0.4093	
BC	30.46		30.46	1.75	0.2154	
		1				
A^2	334.67	1	334.67	19.22	0.0014	
B^2	12.51	1	12.51	0.72	0.4165	
C^2	121.87	1	121.87	7.00	0.0245	
Residual	174.13	10	17.41			
Lack of Fit	25.67	5	5.13	0.17	0.9616	Not significant
Pure Error	148.46	5	29.69			
Cor Total	6570.84	19				

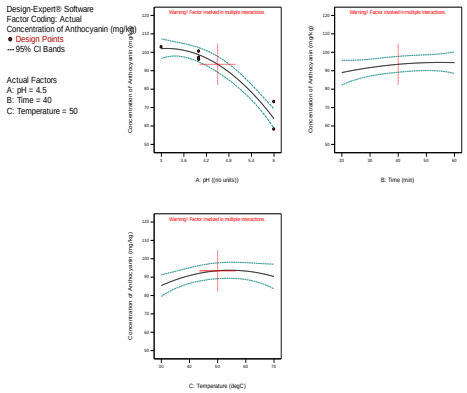

Fig 4: Concentration of Anthocyanin with pH, Time and Temperature with respect to actual factors

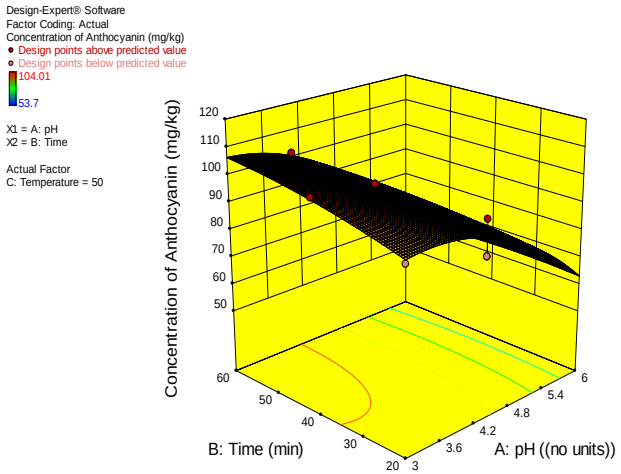

Fig 5: Response surface plots of the Concentration of Anthocyanin extract from Grape pomace as a function of extraction time, temperature and pH

IV. CONCLUSION

Anthocyanins from grape pomace (*Vitis vinifera*) extraction was performed based on ultrasonic assisted extraction technology and to optimize the process variables by response surface methodology.

The study was performed in different concentration, time and temperature in 1:3, 1:4, 1:5 and 20mins, 40mins, 60mins, and 30^0C, 50^0C, 70^0C. Characterization studies of grapes and anthocyanin such as moisture, ash, total phenolic content and quantification of anthocyanin. HPLC analysis was performed to identify of compounds and FTIR profile determined the functional groups present in the sample. An extraction concentration of 1:3, time 20min and temperature of 50^0C was found to be the optimum operating condition for Anthocyanin extraction applying response surface methodology.

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