CHAPTER 4

OBJECTIVE 2

CHAPTER 4 SECTION I

OPTIMIZATION OF BIOACTIVE COMPOUNDS FROM YELLOW AND RED TAMARILLO USING ULTRASOUND ASSISTED EXTRACTION

4.1.1. Introduction

Tamarillo is loaded with essential nutrients like proteins, vitamins, and minerals like potassium, phosphorus, etc. It is also rich in bioactive compounds like anthocyanins, carotenoids, flavonoids and phenolic compounds [6]. The colour of the fruit depends upon the maturity; yellow tamarillo is rich in carotenoids, red tamarillo have both anthocyanins and carotenoids, and purple tamarillo is majorly rich in anthocyanins [35]. The fruit is acidic, has slightly bitter and astringent taste and is consumed in raw form as well as processed to make juices, jellies and desserts [4, 35]. The phenolic composition in the yellow and red varieties of tamarillo were reported to be similar; anthocyanins are present in the red fruit and are absent in the yellow fruit [18]. Recovery of phenolic compounds can be done using the conventional Soxhlet extraction method as well as using non-conventional methods like pulsed-electric field, microwave and pressurized water, ultrasonic-assisted extraction, and supercritical fluid assisted extraction [24].

Response Surface Methodology (RSM) is an important statistical and mathematical tool used widely to optimize the process conditions by analyzing the data collected from suitable design. It helps to evaluate the relationship between processing variables on the responses critical for the process [16]. Optimization of extraction conditions is important to accurately determine the phenolic composition [3]. Box-Behnken design (BBD) has been used for evaluating the extraction efficiency of ultrasonic assisted extraction (UAE) [29, 33]. Ultrasonication is an effective and efficient technique that enhances the quality and yield of extraction in a short operating time [31]. UAE helps in extracting the bioactive compounds using shear force generated by the acoustic cavitation bubbles during the propagation of ultrasonic waves. The implosion of cavitation bubbles cause disruptions like surface peeling, particle size reduction and erosion on sample matrix, enabling fast mass transfer with high solvent penetration power in the sample, which ultimately favors extraction [8, 20]. In recent

years, UAE has been used by researchers for the extraction of the bioactive compounds from different food samples [21]. In comparison to the conventional extraction process, ultrasound comes under green extraction technique known for providing high extraction yield by minimizing the other extraction parameters like time, temperature and solvent consumption [2, 23]. UAE technique has been reported for the extraction of phenolic compounds from mulberry [12], sour cherry pomace [15], and dragon fruit peel [25].

Espin et al. [13] extracted phenolic compounds from the tamarillo fruit using ultrasound water bath but did not determine the optimized conditions for their extraction. Therefore, the main aim of this study was to optimize the ultrasound-assisted extraction conditions using ultrasound for maximum extraction of phenolic compounds from the pulp of yellow and red tamarillos available in north-east India and to see whether optimized conditions are similar for both varieties.

4.1.2. Materials and methods

4.1.2.1. Materials

Yellow and red colored tamarillos were purchased from the local market of Kohima, Nagaland. The yellow and red coloured tamarillos were selected based on their colour visualized in daylight. The red tamarillos were uniformly red in colour and the yellow ones had predominant yellow colour with orange specks towards both ends. Tamarillos were washed and cut into halves. Pulp and seeds were separated and freeze-dried. Freeze-dried pulp was ground into a powder using mixer grinder (Philips HL Model No. 1632, India) to pass through 0.3 mm sieve size, packed into zipper-lock polyethene bags, and kept at -20 °C until further analysis.

4.1.2.2. Ultrasonic assisted extraction

Extraction of the phenolic compounds from the pulp of tamarillo varieties was done using the method followed by Tabaraki et al. [32]. Briefly, 1 g of the freeze-dried sample was mixed with 40 mL of the distilled water, ethanol, acetone, and methanol at 100% concentration level. The extraction was done using probe ultrasonicator equipped with 6 mm probe (Takashi Ultrasonic Homogenizer Model No. U500, Japan), maximum power of 500 W, amplitude ranging from 0-100%, and frequency mode of 2 s active followed by 2 s rest.

4.1.2.3. Selection of variables

The selection of variables play an important role in the phytochemical extraction, and selection of the variables was done individually. The variables that affect the ultrasonication are extraction time, solvent type and concentration, temperature, sample to solvent ratio, the shape of vessel, and diameter of vessel [11]. In this study, yellow freeze-dried pulp was used to find out the optimization parameters and their range. Best solvent for extraction was selected from ethanol, methanol, acetone, and distilled water. For the selection of the best solvent, 1 g of sample was mixed with 20 mL of the solvent and subjected to ultrasonication, keeping cut off temperature of 40 ºC, treatment time of 10 min and ultrasound amplitude of 50%. For determination of best solvent concentration, the best solvent was used at different concentrations ranging from 20-100%, keeping all other experimental parameters constant. Solid to sample ratio of 1:30 was taken for determination of ultrasonication time keeping all other parameters of ultrasonication same. For solid to solvent ratio determination, the sample amount was variable keeping all other factors constant. For amplitude range determination, solvent concentration of 60% was taken keeping all other parameters constant. Selection of the best condition was done on the basis of maximum phenolic content in the extract [23].

4.1.2.4. Experimental Design

Optimization of UAE of polyphenols from freeze-dried tamarillo pulp was done using Response Surface Methodology (RSM). The experimental design followed was the Box-Behnken design that was prepared using Design Expert Version 7 software (State-Ease Inc., Minneapolis, MN, USA). The Box-Behnken design consisted of 17 experiments and comprised of three levels and three factors. The three independent variables that were taken were time (A: 5-15 min), ultrasonic amplitude (B: 20-60%) and solvent concentration (C: 50-80% in distilled water). Total phenolic content, total flavonoid content, and DPPH radical scavenging activity were taken as the dependent variables. The design was executed to maximize the dependent variables. Validation of the model was done by comparing experimental values with the predicted values.

4.1.2.5. Total phenolic content (TPC)

TPC in tamarillo pulp extract was determined according to Sablania et al. [26] with slight modifications. An aliquot of 0.5 sample (1:10 of extract: distilled water) was taken in a test tube followed by addition of 2.5 mL (1:10 diluted with distilled water) of Folin–Ciocalteu reagent. After 5 min, 2mL of sodium carbonate anhydrous (7.5%) was added and vortexed for proper mixing. The test tubes were kept in dark for 2 h and absorbance was read spectrophotometrically (CECIL Aquaris 7400) against a reagent blank at 725 nm. Gallic acid was used as standard, and amount of total phenolic content was expressed as mg GAE (gallic acid equivalents)/g of tamarillo pulp powder.

4.1.2.5. Total flavonoid content (TFC)

The flavonoid content of the samples was determined using the aluminum trichloride method [7]. Aliquots of 0.5 mL of the samples were taken in test tubes and mixed with 1.5 mL of 95% ethanol followed by 0.1 mL of 10% aluminum trichloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. The test tubes were vortexed for proper mixing. The test tubes were kept for incubation in dark at room temperature for 40 min. The absorbance of the samples was read at 415 nm against deionized water in UV-Vis spectrophotometer (Cecil, Aquarius 7400). Quercetin was used as standard and amount of TFC was expressed as mg QE (quercetin equivalent)/g of tamarillo pulp powder.

4.1.2.6. DPPH radical scavenging activity

Radical scavenging activity of the sample was determined according to Saikia et al. [27] with slight modifications. Briefly, 200 µL of sample extract was taken in a test tube followed by the addition of 2.8 mL of DPPH reagent (10^{-4} M). Distilled water served as control and the result was expressed in terms of radical scavenging activity (Eq. 1).

$$Radical\ Scavenging\ Activity\ \ (\%) = \frac{(Ac - As)}{Ac} \times 100 \quad (1)$$

Here, A_c denoted absorbance of control, A_s denoted absorbance of sample extract.

4.1.2.7. Statistical analysis

The results are expressed as the mean ± standard deviation of the triplicate readings. Data were assessed by ANOVA (analysis of variance) and statistical significance ($p< 0.05$) of the results were analyzed using Duncan multiple range test using the software (SPSS 24.0, IBM Corporation, Armonk, NY). In ANOVA, the total sum of squares helps express the total variation that can be attributed to various factors. The lack of fit test, fitted R^2, predicted R^2 and adequacy precision were considered for the adequacy of model. The model was considered adequate for predicting when a non-significant lack of fit ($p > 0.05$) is found, predicted R^2 is comparable to fitted R^2 and adequacy precision is higher than 4 [14].

4.1.3. Results and discussion

4.1.3.1. Selection of independent variables

For the extraction of phenolic compounds from tamarillo, four different solvents were used. As shown in Fig. 4.1.1a, acetone extract showed the highest amount of phenolic content among the solvents. For the determination of optimum solid to solvent ratio for the extraction of maximum bioactive compounds, four different ratios (1:20, 1:30, 1:40 and 1:50) were studied for 10 min of extraction with 60% acetone and 50% amplitude. In Fig 4.1.1b, we found that as the solid to the solvent ratio for extraction was increased, the phenolic content also increased. However, the extent of increase had narrowed down at higher ratios. Increase in the phenolic content with an increase in the solid to solvent ratio is related to mass transfer principle. Similar results were reported in the extraction of antioxidants from pomegranate peel [32] and black chokeberry [9] using ultrasonication as the extraction technique. Therefore, for the optimization of the extraction of bioactive compounds from tamarillo peel, 1:40 solid to solvent ratio was taken. Higher solid to solvent ratio means better extraction of the bioactive compounds but requires more solvent. There was not much difference in the phenolic content between 1:40 and 1:50 ratios.

In Fig 4.1.1c, among the different concentrations, the highest amount of phenolic content was found in 60% concentration acetone. Therefore, acetone was chosen as the extraction solvent and the 50- 80% acetone concentration was taken as the range for optimization of extraction of phenolic content. Due to the differences in the polarity of the

solvents, acetone with 0.355 polarity showed maximum phenolic content whereas ethanol and methanol with polarity of 0.654 and 0.762, respectively, showed lower extraction [34]. Similarly, 60% acetone concentration showed maximum extraction yield of phenolic compounds from star fruit [30] and peach [19].

For determination of the optimum range for time of extraction, different time periods (1, 5, 10, 15, 20, 25 and 30 min) of extraction were studied with acetone concentration of 60%, solid to solvent ratio of 1:30 and ultrasound amplitude of 50% with a cut off at 40°C temperature. As seen in Fig 4.1.1d, extraction of the phenolic compounds was found to increase as the extraction time was increased from 1 to10 min and decreased above 10 min of extraction. The decrease in the phenolic content after 10 min of extraction can be attributed to the degradation of phenolic compounds as reported by Espada-Bellido et al.[12] after ultrasonic extraction of mulberry. However, Porto et al. [10] reported maximum ultrasonic assisted extraction of phenolic content from grapes after 15 min of treatment. Therefore, 5-15 min time range was taken for optimization in this study. However, contrary to these findings, Carrera et al. [5] observed no significant differences in the quantities of phenolic compounds and condensed tannins extracted by UAE beyond extraction time of 6 min.

Amplitude plays an important role in the extraction of the bioactive compounds using ultrasound. In this study, different amplitudes (10, 20, 30, 40, 50, 60, 70 and 80%) for the extraction of phenolic compounds were studied with 10 min extraction time, 60% acetone concentration and 1:30 solid to solvent ratio. As seen in Fig 4.1.5, with increase in the extraction amplitude from 10 to 50%, phenolic content increased from 15.18 to 21.17 mg GAE/g. Increase in phenolic content by increasing the ultrasound amplitude was directly related to the greater impact of the cavitation phenomenon that occurs during ultrasonication at higher amplitude [17, 21]. However, extraction of phenolic content decreased as the amplitude of the ultrasound was increased from 50 to 70%. This may be due to the degradation of phenolic compounds because an increase in amplitude beyond an optimum level increases the severity of cavitation, which causes degradation of the already extracted phenolic compounds [28].

4.1.3.2. Fitting the model in the experimental design

Box-Behnken design was used for analyzing the effect of three independent variables (extraction time, amplitude, and solvent concentration) on three dependent variables for the optimization of UAE extraction of bioactive compounds present in tamarillo. In Table 4.1.1, experimental data with their responses for yellow and red tamarillo are presented. The quadratic model was used to analyze the interaction between the independent and dependent variables. For perfect model fitting, the *p-value* of the model should be significant, and lack of fit should be insignificant. The R^2 (coefficient of determination), adjusted R^2, predicted R^2, sum of squares are important parameters in the ANOVA table and are helpful to determine the adequacy and suitability of the respective model [3]. The quadratic model was found to be significant with the confidence level of $p<0.05$ and lack of fit and residual error were insignificant, which are essential criterion for fitting the mathematical model. The R^2 (coefficient of determination) of the responses viz. TPC, TFC and DPPH radical scavenging activity were 0.95, 0.94 and 0.95, respectively for yellow tamarillo pulp, and 0.97, 0.96 and 0.96, respectively for red tamarillo pulp. The coefficient of variation (%) for TPC, TFC and DPPH radical scavenging activity was 2.66, 3.21 and 0.46, respectively in the case of yellow tamarillo pulp, and 1.94, 3.34 and 0.94, respectively for red tamarillo pulp. The coefficient of variation value shows a positive impact on the experimental runs and good reliability over the outcome.

4.1.3.3. Effect of independent variables on the extraction of total phenolic content

The effect of the independent variables on the TPC content and antioxidant activity of the extract from yellow tamarillo pulp (A1, A2 and A3) and from red tamarillo pulp (X1, X2 and X3) are presented in Fig. 4.1.2. Extraction time, amplitude and solvent concentration showed significant effects on the extraction of phenolics in both the varieties of tamarillo. While all the independent variables showed positive effect on the extraction of phenolics, their interactions among themselves were mixed (Table 4.1.1) for both yellow and red tamarillos. In yellow tamarillo, acetone concentration showed greater effect on the phenolic content among the three independent variables. In Fig. 4.1.2 (A1), increase in time and amplitude during extraction showed an increase in the phenolic content to a certain extent and thereafter extraction yield of phenolics was found to decrease. Overall interaction

showed negative effect on the response. While extraction time and solvent concentration had positive interaction on phenolic content, a decrease in the phenolic content was observed when the amplitude and solvent concentration increased gradually. Increase in the extraction time caused an increase in the phenolic content of the tamarillo and had a coefficient value of 1.57 in the quadratic second order polynomial equation in its coded form for total phenolic content (Table 4.1.2). The highest phenolic content in yellow tamarillo pulp found experimentally was 24.74 mg GAE/g at extraction time 10 min, amplitude 40%, and acetone concentration 65%. Fig. 4.1.2 (X1, X2 and X3) shows the effect of the independent variables on the extraction of phenolic compounds from red tamarillo pulp. Amplitude and solvent concentration showed positive interaction between themselves whereas extraction time with amplitude and extraction time with acetone concentration showed to have negative effect. Highest phenolic content of 15.23 mg GAE/g at extraction time of 15 min, 40% amplitude and 50% solvent concentration were extracted experimentally. At the optimized extraction conditions for yellow tamarillo pulp, phenolic content was 15.16 mg GAE/g. Increase in the concentration of solvent leads to lesser extraction of phenolic compounds because of an increase in solvent polarity [26].

4.1.3.4 Effect of independent variables on the extraction of total flavonoids content

The response surface 3D contour plots with the independent variables of extraction time, ultrasound amplitude and acetone concentration on the extraction of total flavonoids from the pulp of yellow tamarillo and red tamarillo are shown in Fig. 4.1.3 (B1, B2 and B3) and (Y1, Y2 and Y3) respectively. Highest flavonoid content of 5.64 mg QE/g and 4.98 mg QE/g in yellow tamarillo and red tamarillo, respectively was extracted. In yellow tamarillo, ultrasound amplitude and acetone concentration played an important role in extracting the flavonoids from the pulp. Increase in the extraction time and amplitude helped to release more flavonoids and had a positive impact on the extraction.

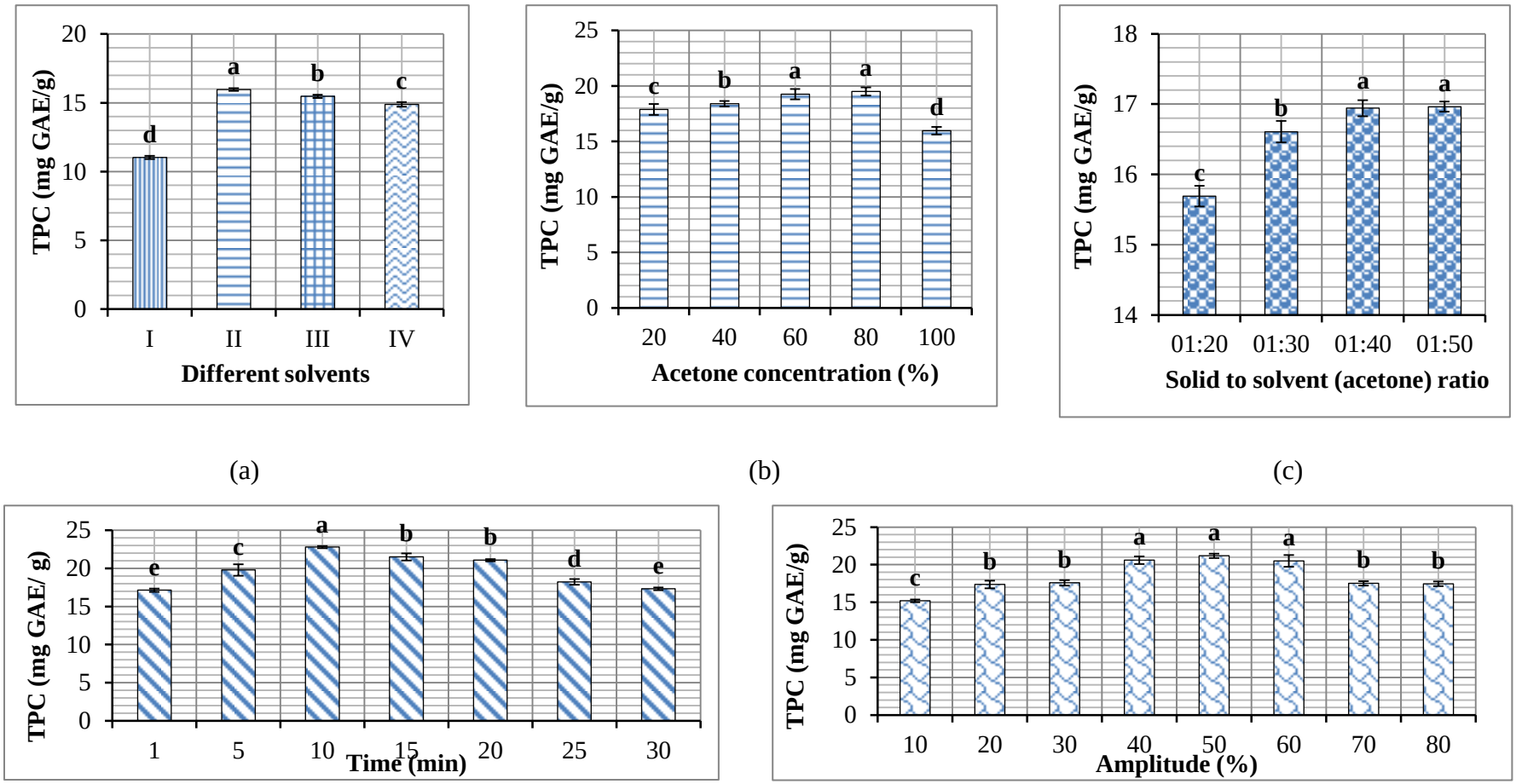
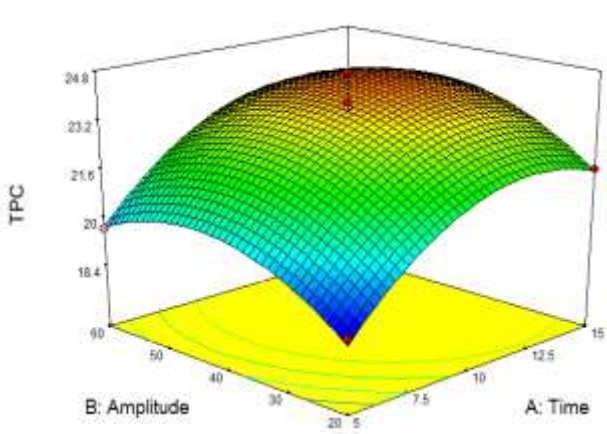
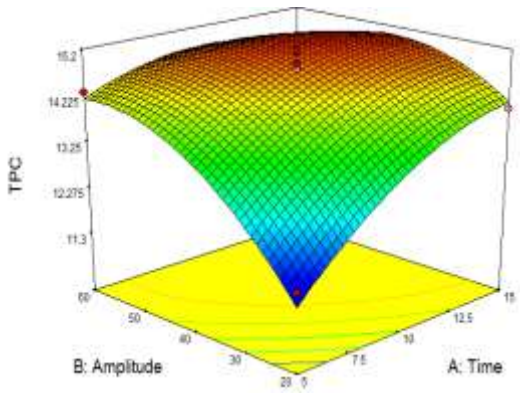


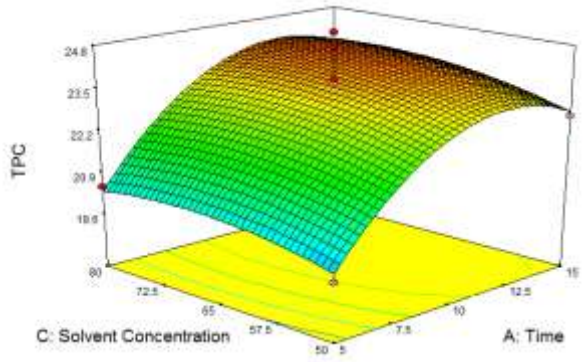
Fig. 4.1(a-e). Effect of (a) solvents (I-Distilled water, II: Acetone, III: Ethanol, IV: Methanol); (b) Acetone concentration; (c) solid to solvent (acetone) ratio; (d) Time; (e) Amplitude on extraction of total phenolic content (TPC) using UAE. Values are means of three determinations ± SD. $^{a\text{-}e}$ represents the difference between the data, same letter means no difference, different letter means significant difference ($P < 0.05$).



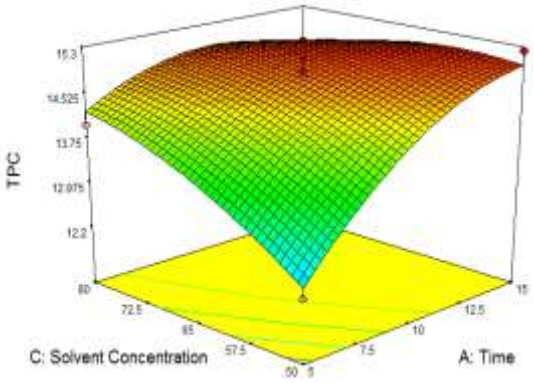
(A1)



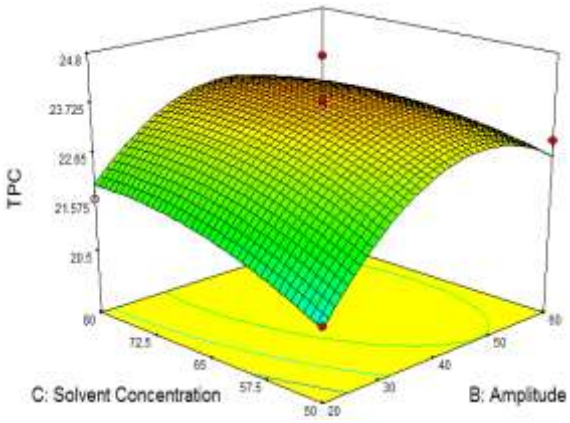
(X1)



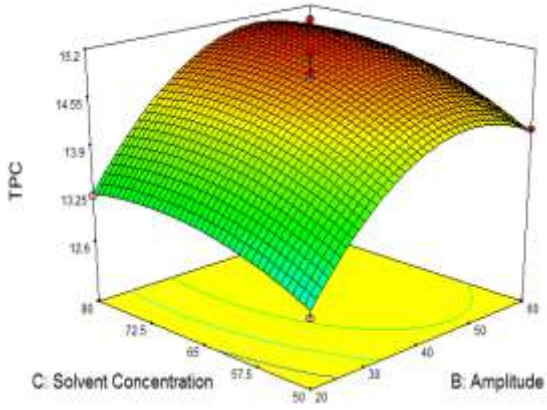
(A2)



(X2)



(A3)



(X3)

Fig. 4.1.2. Response surface plots showing effect of UAE parameters on total phenolic content extraction from yellow tamarillo pulp (A1, A2 and A3) and red tamarillo pulp (X1,X2 and X3).

Table 4.1.1. Experimental data and their response in Box-Behnken design for yellow and red tamarillo pulp.

Run	Factor 1	Factor 2	Factor 3	Yellow tamarillo			Red tamarillo		
	A: Time (min)	B: Amplitude (%)	C: Solvent Concentration (%)	Response 1	Response 2	Response 3	Response 1	Response 2	Response 3
				TPC (mg/g)	TFC (mg/g)	DPPH (%)	TPC (mg/g)	TFC (mg/g)	DPPH (%)
1	10	40	65	24.74	4.98	96.32	14.65	4.54	94.32
2	10	20	50	20.64	4.65	92.32	12.65	3.64	87.65
3	10	60	80	22.43	5.64	95.65	14.98	4.32	92.23
4	15	40	50	22.68	4.64	94.65	15.23	4.98	91.64
5	5	60	65	19.64	4.54	94.65	14.32	3.65	87.68
6	10	40	65	23.67	5.42	96.32	14.78	4.97	95.32
7	15	20	65	21.64	4.32	93.65	13.98	4.14	90.46
8	10	40	65	22.79	5.21	95.64	15.16	4.88	95.65
9	10	40	65	23.64	5.32	95.64	14.82	4.68	94.36
10	5	20	65	18.65	4.21	92.32	11.62	3.55	87.32
11	10	40	65	22.79	5.21	95.64	14.88	4.64	94.36
12	10	20	80	21.64	5.26	93.64	13.23	4.36	92.21
13	15	60	65	22.64	4.94	95.32	13.98	4.64	89.64
14	10	60	50	22.94	5.14	93.65	14.14	4.58	91.32
15	5	40	50	19.64	4.12	93.98	12.24	3.79	88.32
16	5	40	80	20.45	4.88	94.12	13.98	4.65	93.32
17	15	40	80	23.94	5.42	95.68	14.65	4.86	95.32

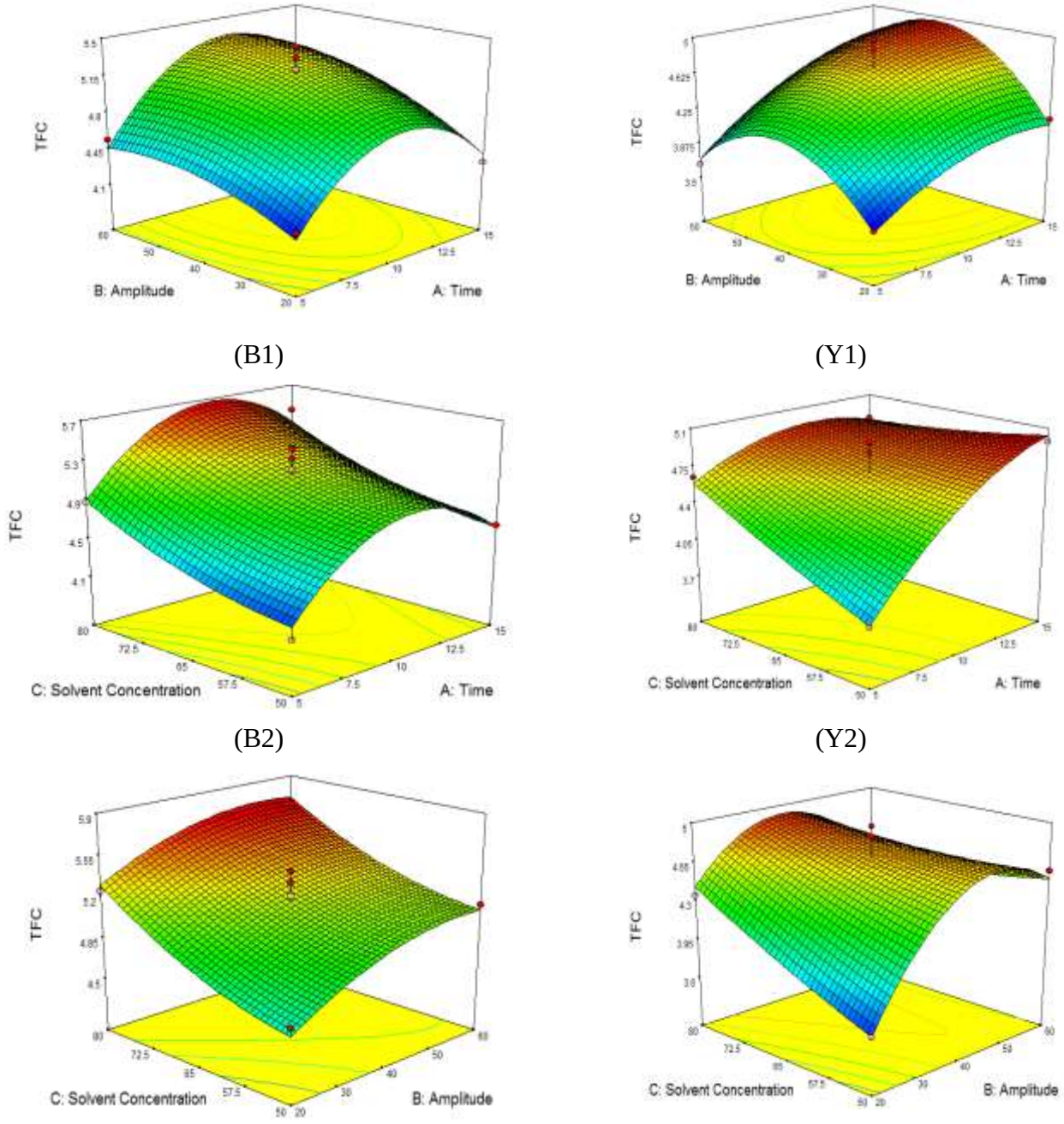


Fig 4.1.3. Response surface plots showing effect of UAE parameters on total flavonoids content extraction from yellow tamarillo pulp (B1,B2 and B3) and red tamarillo pulp (Y1,Y2 and Y3).

Table 4.1.2. Optimized response polynomial equations fitted in quadratic model for yellow and red tamarillo pulp extract**.**

Output variables	2nd order polynomial equations (Quadratic model)	R^2	*p-value*	Adjusted R^2	Lack of fit
	Yellow tamarillo				
TPC (mg GAE/g)	$TPC\ Yellow = +23.72 + 1.5A + 0.63B + 0.32C + 2.500E - 003AB + 0.11AC - 0.38BC - 1.66A^2 - 1.42B^2 - 0.39C^2$	0.9509	0.0009	0.8877	0.7942
TFC (mg QE/g)	$TFC\ Yellow = +5.21 + 0.20A + 0.23B + 0.33C + 0.072AB + 5.00E - 003AC - 0.027BC - 0.56A^2 - 0.15B^2 + 0.11C^2$	0.9464	0.0011	0.8775	0.5890
DPPH (%)	$DPPH\ Yellow = +96.11 + 0.53A + 0.92B + 0.56C - 0.17AB + 0.22AC + 0.17BC - 0.67A^2 - 1.46B^2 - 0.84C^2$	0.9492	0.0010	0.8839	0.4620
	Red tamarillo				
TPC (mg GAE/g)	$TPC\ Red = +14.86 + 0.71A + 0.74B \times +0.32C - 0.68AB - 0.58AC + 0.065BC - 0.55A^2 - 0.83B^2 - 0.28C^2$	0.9708	0.0001	0.9332	0.1255
TFC (mg QE/g)	$TFC\ Red = +4.74 + 0.37A + 0.19B + 0.15C + 0.100AB - 0.25AC - 0.25BC - 0.20A^2 - 0.55B^2 + 0.029C^2$	0.9594	0.0004	0.9071	0.8477
DPPH (%)	$DPPH\ red = +94.80 + 1.30A + 0.40B + 1.77C - 0.30AB - 0.33AC - 0.91BC - 2.36A^2 - 3.66B^2 - 0.29C^2$	0.9625	0.0003	0.9142	0.1686

Table 4.1.3. Predicted and experimental values using optimized conditions for maximum TPC, TFC, and antioxidant activity.

	Time (min)	Amplitude (%)	Solvent Conc. (%)	TPC (mg GAE/g)		TFC (mg QE/g)		DPPH (%)		Desirability
				Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	
Yellow	12	46	78	24.06	23.96	5.64	5.61	95.78	96.31	0.94
Red	12	43	73	15.07	14.97	4.89	4.86	95.65	95.22	0.97

Solvent Conc. is solvent concentration, Pred. is Predicted value and Exp. is Experimental value

From the quadratic equation (Table 4.1.2) for TFC in yellow fruit extract, it was seen that the ultrasound amplitude with solvent concentration had a negative effect on the extraction process. In Table 4.1.2, time, amplitude and solvent concentrations had significant effect on total flavonoids ($p<0.1$). For the red tamarillo pulp (Table 4.1.2), only extraction time with ultrasound amplitude had positive effect, otherwise time with acetone concentration and amplitude with acetone concentration had a negative impact. Thus, the independent variables that affect extraction of flavonoids concentration from yellow tamarillo are different from red tamarillo (Table 4.1.3).

4.1.3.5. Effect of independent variables on DPPH radical scavenging activity of the extract

The 3D contour plots of response surface on the DPPH radical scavenging activity on yellow and red tamarillo fruits are given in Fig 4.1.4(C1, C2 and C3) and (Z1, Z2 and Z3), respectively. Maximum DPPH radical scavenging activity of 96.64% in yellow tamarillo and 95.65 % in red tamarillo were recorded. Highest radical scavenging activity in yellow tamarillo is the due to the higher phenolic and flavonoids content in yellow tamarillo than red tamarillo. Phenolic compounds and flavonoids are responsible for the high antioxidant property [26].

In red tamarillo pulp, extraction time and solvent concentration had significant effect on the DPPH radical scavenging activity. As the extraction time and amplitude were increased, the DPPH radical scavenging activities also increased but after attaining the maximum value, radical scavenging activity decreased with increase in the value of independent variables. This may be related to degradation of the extracted bioactive compounds by longer extraction time and higher amplitude. From Fig 4.1.4(Z2 and Z3), it was observed that solvent concentration had a positive effect on DPPH radical activity with high coefficient value of 1.77 (Table 4.1.2). It can be stated that an increase in solvent concentration enhanced the radical scavenging activity. Extraction time did not have significant effect on the antioxidant activity, as was also reported by Carrera et al. [5].

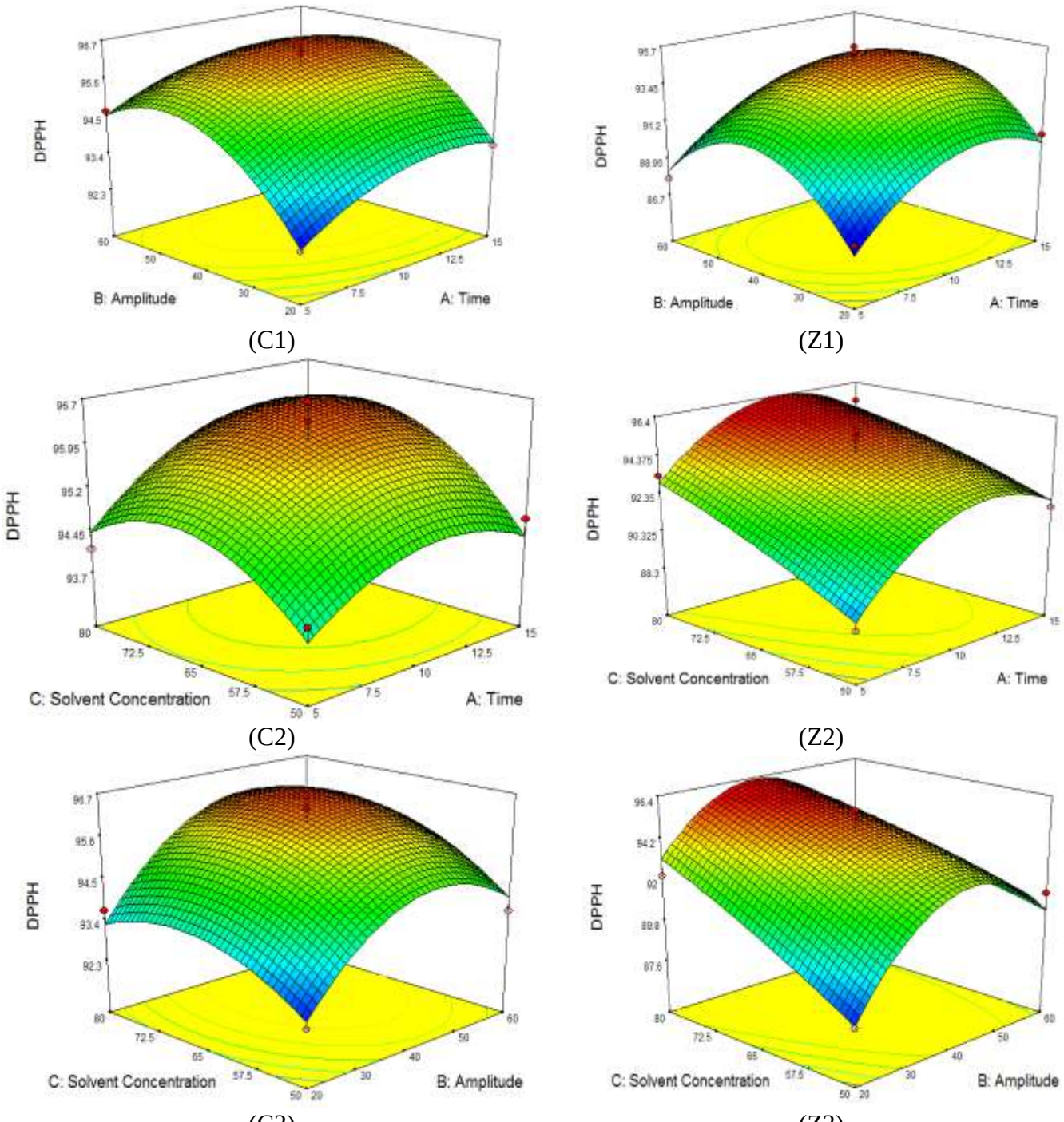


Fig 4.1.4. Response surface plots showing effect of UAE parameters on DPPH radical scavenging activity of yellow tamarillo pulp (C1,C2 and C3) and red tamarillo pulp (Z1,Z2 and Z3).

4.1.3.6 Optimization

In Table 4.1.3, the predicted and optimized conditions along with desirability are given. For the optimization, all the independent variables were chosen to be in range and bioactive compounds were maximized for finding out the predicted values. The total phenolic content was 23.96 mg GAE/g and 15.07 mg GAE/g, total flavonoid content was 5.61 mg QE/g and 4.86 mg QE/g and DPPH radical scavenging activity was 96.31% and 95.22% for yellow tamarillo and red tamarillo pulp, respectively. All the experimental values were found to be very close to predicted values for all the responses (Table 4.1.3). The desirability for yellow tamarillo and red tamarillo was 0.94 and 0.97, respectively, which satisfies the results and confirmed the reliability of the model applied.

Orqueda et al. [22] studied the chemical properties of yellow orange tamarillo from Argentina and reported the total phenolic content and flavonoid content to be 415.2 mg and 223.80 mg per100 g of dry pulp powder. Acosta-Quezada et al. [1] studied 23 varieties of tamarillo and found that total phenolic content ranged from 2.4-6.2 g/100g of dry powder. Espin et al. [13] studied the phenolic profile of 4 varieties of tamarillo using HPLC and reported that total hydroxycinnamoyl derivatives were in the range of 60.3−421.6 mg/100 g dry weight. It therefore appears that there is wide variation in the phenolic and flavonoids content in tamarillos available in different parts of the world.

4.1.4. Conclusion

The optimization of the extraction of bioactive compounds from yellow and red tamarillos using UAE technique taking extraction time, ultrasound amplitude and solvent concentration as independent variables was done by response surface methodology. The study showed that solvent concentration played an important role in the extraction of the bioactive compounds and acetone was found to be the best solvent for extraction. For yellow tamarillo, ultrasound amplitude of 46% and solvent concentration of 78% gave maximum extraction. For red tamarillo, ultrasound amplitude of 43% and solvent concentration of 73% gave maximum extraction of bioactive compounds. Extraction time of 12 min gave better results for the extraction of the bioactive compounds from yellow and red tamarillo samples. Optimum conditions of the extraction variables differed for the two tamarillo varieties, probably due to the nature of the bioactive compounds present. Further, UAE as an

extraction technique was an efficient green technique that was able to extract maximum phenolic compounds with high antioxidant activity. Study also revealed the very potent antioxidant property of both yellow and red tamarillos.

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OPTIMZATION OF CONDITIONS OF SUPERCRITICAL FLUID EXTRACTION AND ULTRASOUND ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS AND ANTHOCYANINS FROM PURPLE TAMARILLO

4.2.1. Introduction

Tamarillo fruit are available in different varieties, these varieties are distinguished according to fruit colour as yellow, purple and red [22]. The skin of purple tamarillo is purple and pulp and mucilage covering the seeds are purple or red in colour [6]. The purple tamarillo fruit's distinctive colour and flavour are due to the presence of anthocyanins and other beneficial bioactive compounds. Besides giving an appealing colour, anthocyanins are known for their anti-oxidant, anti-tumour, and anti-inflammatory properties [8]. Extraction techniques with their defined extraction parameters are very important for extracting the maximum yield of bioactive compounds from sample matrix. Yield and concentration of bioactive compounds vary with the type of extraction technique and process conditions used [22]. Researchers are presently focusing on achieving maximum yield with minimum effect on bioactivity using minimum quantity of solvent, therefore on the basis of these requirements, the most useful and widely employed new techniques that have been adapted nowadays for the extraction of phytochemicals are Supercritical fluid extraction (SCFE) and Ultrasound assisted extraction (UAE) [19].

SCFE is a clean, fast, efficient, and very effective method for the extraction of bioactives from fruits and vegetables. The technique uses eco-friendly CO_2 gas as an extraction medium, which acts like a liquid solvent that helps in enhancing yield with low chances of contamination in a short time when compared to the conventional method. The conversion of CO_2 gas into liquid solvent is achieved after exceeding the critical pressure of 7.4 MPa and critical temperature of 31ºC [19]. UAE helps in effective extraction [17] with high yield in a short time [21]. Factors that play important role in UAE extraction are solvent pH and concentration, temperature, ultrasound amplitude, solid to solvent ratio, and time [22]. In UAE, acoustic waves are generated in the solvent medium, creating regions of

compression and rarefaction producing cavitation bubbles. When these bubbles burst in solvent, extensive shock wave-induced damage to the cell wall of sample matrix occurs, resulting in release of extractable compounds from the sample into the solvent [20]. Response surface methodology (RSM) is employed for the optimization of extraction parameters using Box-Behnken design (BBD). BBD design consists of having experiments at central points and middle points and overall fewer experiments are required than other designs [22]. In reported works, only one type of extraction process is mainly focused upon or SCFE followed by UAE techniques are used for improved extraction efficiency [27]. Castro-Vargas et al. [2] made a comparative study of SCFE and conventional solvent extraction of antioxidants from red tamarillo and observed greater antioxidant activity in SCFE extract. The aim of this research was to optimize the phenolic content and anthocyanin content of tamarillo extract in acidified co-solvent as a solvent medium using SCFE and UAE and make a comparative analysis of the phenolic compounds and anthocyanins present using HPLC. This is the first report in which two extraction techniques viz. SCFE and UAE was used for the comparison for phenolic content and anthocyanins from purple tamarillo peel.

4.2.2. Materials and methods

4.2.2.1. Plant materials and sample preparation

Tamarillo was procured from the local market of Gangtok, Sikkim, India and transported to Tezpur University. Fruits were sealed in low-density polyethene zip pouch and stored in a plastic container in a deep freezer (-20 ºC). Freeze-dried powder was prepared just before extraction. For freeze-dried powder preparation, frozen fruits were allowed to thaw at RT (room temperature) and rinsed with clean water. Peel with the pulp of tamarillo was manually separated from the seed jelly with a sharp stainless-steel knife in a low-lit room to ensure less damage and then freeze dried (Lyo Lab, Lyophilisation Systems, Inc.) for 12 h. The freeze-dried peel-pulp portion was powdered using household mixer grinder (Crompton Greeves), passed through a standard sieve mesh (425μm) and stored in an air-tight container at -20 °C until further analyses.

4.2.2.2. Supercritical fluid extraction (SCFE)

SCFE was carried out in a Waters (SCF 100) system. Acidified co-solvent mixture of ethanol and distilled water in the ratio of 1:1 was used as the modifier that was brought to pH 2.0 with citric acid. Sample weight taken was 2 g. The flow rate of modifier was 1 mL/min and flow rate of CO_2 was 5 mL/min.

In SCFE, major parameters that play important roles in extracting the extractable compounds from the sample matrix are time, temperature, pressure, and the flow rate of supercritical CO_2 and modifier. For determining the time for SCFE, 2 g sample was taken, and temperature and pressure were kept at 50 °C and 165 bar, respectively. For determination of temperature, time and pressure were kept at 45 min and 165 bar, respectively. For pressure, the time and temperature were kept at 45 min and 50 °C, respectively. The limits of all the parameters were selected based on maximum total phenolic content obtained in the extract. The extraction parameters and their ranges are mentioned in Table 4.2.1.

Table 4.2.1. Extraction limits selected for independent variables to extract total phenolic content and total monomeric anthocyanins from purple tamarillo peel using SCFE and UAE.

Independent variables of SCFE	**Symbol**	**Level**		
		-1	0	+1
Time (min)	A	30	45	60
Temperature (ºC)	B	40	50	60
Pressure (bar)	C	150	165	180
Independent variables of UAE	**Symbol**	**Level**		
		-1	0	+1
Time (min)	A	10	20	30
Temperature (ºC)	B	40	50	60
Amplitude (%)	C	20	40	60

4.2.2.3. Ultrasound-assisted extraction (UAE)

UAE was carried out in a probe type ultrasonicator (TAKASHI Model No U-500, JAPAN) with power of 500 W and amplitude range from 0-100 % at a constant frequency of 20.5 KHz. For extraction, 2 g of freeze-dried powder sample was added to 50 mL of acidified co-solvent mixture (pH 2.0).

Time, temperature, and amplitude were the independent variables selected for optimization. For determining the time, 1g sample was mixed in 25 mL of extraction solvent and the mixture was treated from 5-40 min and temperature and amplitude were maintained at 50 °C and 50%, respectively. For determining the temperature, sample was mixed in the extraction solvent (1:25), the temperature varied from 35-65 °C, and time and amplitude were 15 min and 50%, respectively. For amplitude, extraction was done for 15 min at 50 °C and the amplitude was varied from 10-80%. The limits of all the three independent variables were decided based on total phenolic content. All the extraction parameters are given in Table 4.2.1 with their limits.

4.2.2.4. Extraction of bioactive compounds

A three level Box-Behnken design with three independent variables was used for the optimization of parameters for extraction of anthocyanins and phenolic compounds by SCFE and UAE methods (Table 4.2.1). Total phenolic content and total monomeric anthocyanins content were taken as the responses for optimization of extraction of bioactive compounds using SCFE and UAE.

4.2.2.5. Total phenolic content (TPC)

TPC of fruit extract was analyzed according to Sablania et al. [26]. An aliquot of 0.5 mL of sample was taken in a test tube followed by addition of 2.5 mL of Folin Ciocalteu reagent (1:10 diluted with distilled water) and allowed to stand for 5 min, then 2 mL of sodium carbonate anhydrous (7.5%) was added to each of the test tubes and vortexed them. The absorbance was noted down after 2 h of incubation in the dark (test tubes were covered by aluminum foil to avoid contact with air) using UV-Visible spectrophotometer (CECIL Aquaris 7400) against a reagent blank at 725 nm. Gallic acid was used as standard and the amount of total phenolic content was expressed as mg GAE (Gallic acid equivalents) /g of tamarillo fruit.

4.2.2.6. Total monomeric anthocyanin content (TMAC)

Total monomeric anthocyanin content was measured by the pH differential method [7]. One reagent of pH 1.0 using potassium chloride (1.86 g/L di-ionized water) and another reagent of sodium acetate buffer of pH 4.5 (32.8 g/L of di-ionized water) were prepared. The sample extract was added to the above-prepared reagents; dilution factor was noted while adding the extract. The absorbance of each sample mixture was taken at wavelengths 520 and 700 nm in triplicate. Anthocyanins content was calculated as mg cyanidin-3-glucoside (C3G)/g of dry material using Eq. 1 & 2.

$$Anet = Abs510 - Abs700 \quad (1)$$

$$Anthocyanin\ \left(\frac{mg}{g}\right) = \frac{Anet}{26900}.MW.DF.\frac{V}{Wt} \quad (2)$$

Where, *Anet* is net absorbance, *MW* (449.2) is molecular weight, *DF* is dilution factor, *V* is volume of sample, Wt is weight of sample used for extraction and 26900 is molar absorptivity of cyanidin-3-glucoside (C3G).

4.2.2.7. HPLC analysis of phenolic acids

The phenolic acids in the sample extract were identified and quantified using reverse phase UHPLC (Utimate 3000, Thermo Scientific, USA) with the help of standards in C18 (4.6 × 250 mm) column and diode array detector. The sample extract was filtered through 0.45µm syringe filter prior to injecting. A gradient mode consisting of two solvents, A (0.1% formic acid) and B (acetonitrile) at 35ºC was used [6]. The flow rate of the solvent was kept at 0.5 mL/min and wavelength was kept at 330 nm and the gradient flow pattern was 15% B for 5 min, 20–35% B for 10 min, 35–50% B for 10 min, 50–60% B for 5 min, and 60% B for 5 min.

4.2.2.8 HPLC analysis of anthocyanins

The anthocyanins in the sample extract were identified and quantified using UHPLC (Utimate 3000, Thermo Scientific, USA) with the help of anthocyanin standards in reverse phase in C18 column using diode array detector and a calibration curve was developed. The sample extract was filtered through 0.45µm syringe filter prior to injecting. A gradient mode

consisting of two solvents, solvent A (0.1% triflouroacetic acid) and B (acetonitrile) at 35ºC was used [6]. The flowrate of the solvent was kept at 0.5 mL/min at a wavelength of 520nm using gradient flow with 10% B for 3 min, 10–15% B over 12 min, 15% B for 5 min, 15–18% B over 5 min, 18–30% B over 20 min, 30–35% B over 5 min, and re-equilibration to initial solvent parameters.

4.2.2.9. Stability of the anthocyanins

The thermal stability of the optimized extracts from UAE and SCFE were tested according to the method adopted by Ferreira et al. [7]. The optimized extract was kept in a falcon tube (50 mL) and heated in a water bath at 50, 70 and 90 °C, and sample was collected at 0.5,1, 2, 3, 4, 5 and 6 h intervals and placed in ice cooled bath to stop further degradation. The analysis was done in triplicate and the amount of anthocyanins present was determined.

4.2.2.10. Scanning electron microscopy

The residue remaining after SCFE and UAE extraction were collected and dried at 40ºC in a hot air oven. The sample was placed on the SEM stubs using double-sided tape and coated with a thin layer of gold. The SEM images were captured using JSM -6390LV scanning electron microscope (174 JEOL, Japan) at 15kV at x500 and x5500 magnifications [14].

4.2.2.11. Statistical analysis

The effect of extraction conditions was optimized using software (Design Expert 7.0, State-Ease Inc., Minneapolis, MN, USA). ANOVA was performed to identify the effect and interaction on independent variables and responses. A p-value of less than 0.05 was statistically significant for a model. The other statistical parameters, including coefficient of determination (R^2), root mean squared error (RMSE) and relative deviation (R_d) were calculated using Eq.3-5, respectively.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(predicetd - experimental)}{\sum_{i=1}^{n}(avergae\ value - experimental)} \quad (3)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(predicted - experimental)}{n}} \quad (4)$$

$$R_d = \frac{100}{n}\sum_{i=1}^{n}\frac{|experimental - predicted|}{experimental} \quad (5)$$

4.2.3. Results and discussion

4.2.3.1. Determining the limits of independent variables of SCFE

The range of the independent variables was selected based on the experiments trials on TPC (Fig. 4.2.1). Highest extraction of phenolics that was significantly different was found at 50 min of extraction followed by 55 and 60 min (Fig. 4.2.1a). Decrease in the TPC occurred at 65 min, probably due to the degradation of phenolic compounds. Highest phenolic content was reported at 55 °C and lowest at 70 °C (Fig. 4.2.1b). There was significant difference in the phenolic content as the temperature increased from 35 °C to 55 °C, which may be due to release and solubility of phenolic compounds in the extracting solvent. Phenolic content increased as pressure was raised to 165 bar and decreased beyond it (Fig. 4.2.1c). Increase in pressure during extraction causes disintegration of the sample matrix that helps to release the bioactive compounds; however, excessive pressure causes degradation [9]. Therefore, for optimization of bioactive compounds using SCFE, the selected range for time was 30-60 min, temperature was 40-60 °C, and pressure was 150-180 bar (Table 4.2.1).

4.2.3.1.1. Effect of independent variables on responses using SCFE

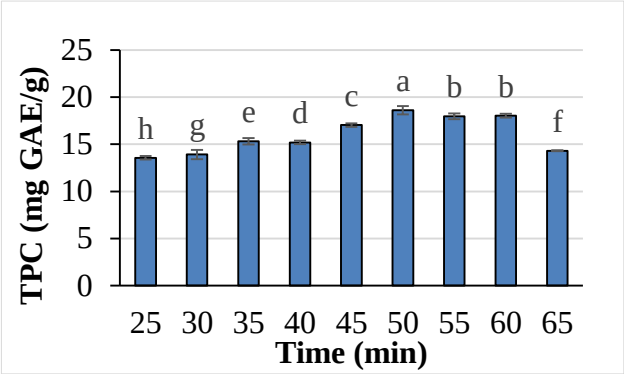
The Box-Behnken design was used to optimize TPC and TMAC using supercritical extraction technique. Seventeen experimental conditions and their response are given in Table 4.2.4. The phenolic content ranged from 11.65 -16.05 mg GAE/g of dry powder sample. The interaction between the independent variables during TPC extraction is presented in Fig. 4.2.2 (X1, X2 and X3). It was observed that increase in time from 30 to 45 min showed a positive impact on TPC as also increase in pressure up to a certain extent had a positive impact [13]. Besides rupturing of the sample matrix [4], increase in density of supercritical CO_2 at higher solvent pressure facilitates the extraction of bioactive material

directly into the solvent. TPC increased as the temperature increased from 40 °C to 50 °C and thereafter decreased. Garcia-Mendoza et. al. [8], reported that an increase in temperature tends to increase the vapor pressure and enhances the diffusivity of CO_2 that ultimately increases the extraction. In Eq. 3, the interaction of time, temperature, and pressure on TPC are given. In SCFE, modifier plays an important role in extracting the extractable compounds from the sample because it increases the solvating power which ultimately increases the extraction efficiency [23]. Linear effect of all independent variables had a positive impact on extraction of phenolic compounds. The interaction term of time and pressure only had positive effect, whereas interaction term of time with temperature and temperature with pressure showed negative effects on TPC.

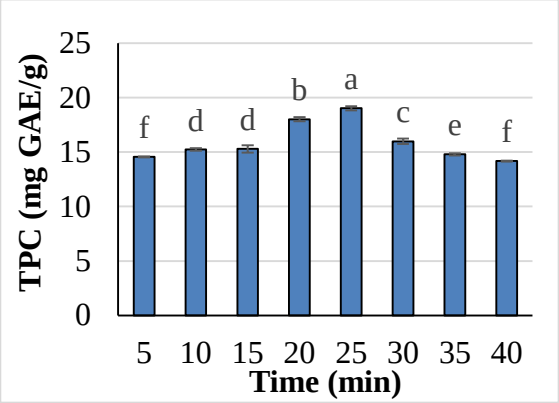
$$TPC_{SCFE} = +15.73 + 0.44 \times A + 0.42 \times B + 0.32 \times C - 0.12 \times A \times B + 0.46 \times A \times C - 0.72 \times B \times C - 0.95 \times A^2 - 1.87 \times B^2 - 0.093 \times 6C^2 \quad (6)$$

$$TMAC_{SCFE} = +.0.62 + 0.018 \times A + 02.500\text{E} - 003 \times B + 0.030 \times C + 5.000\text{E} - 003 \times A \times B - 5.000\text{E} - 003 \times A \times C - 0.015 \times \text{B} \times \text{C} - 0.012 \times A^2 - 0.052B^2 + 0.016 \times C^2 \quad (7)$$

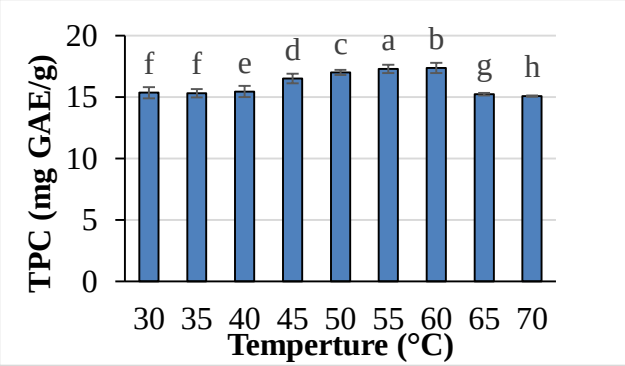
TMAC varied from 0.50-0.63 mg C3G/g dry sample. As seen in Eq. 7, during SCFE, pressure and time had positive effects on anthocyanins extraction and temperature showed negative impact. Fig. 4.2.2 (Y1, Y2 and Y3) illustrates the effect of the independent variables on the response. As temperature and time increase, a gradual increase in anthocyanins occurred but after achieving maximum, it gradually decreased [18]. However, in Fig. 4.2.2 (Y2 and Y3), increase in pressure with time and temperature increased anthocyanins content. SCFE at higher pressure and temperature not only increases concentration but also the stability of anthocyanins [12]. Anthocyanins extraction decreased at 60 °C, supporting Ryu and Koh [24] findings on anthocyanins from black soybean. High pressure increases the solvating power due to increase in density which leads to an increase in extracted anthocyanins. While CO_2 and modifier help in extraction of phenolic compounds, acidified solvent (modifier) is reported to be beneficial for the rupture of the cell wall of sample matrix for anthocyanins extraction [23].



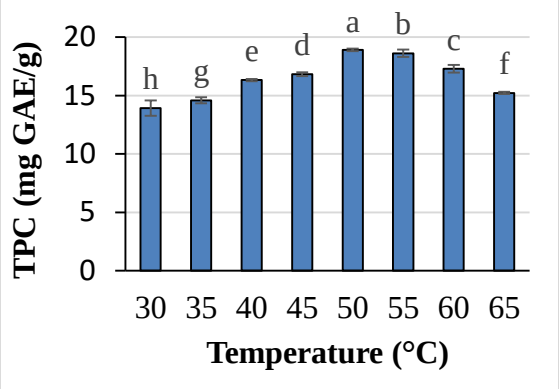
(a)



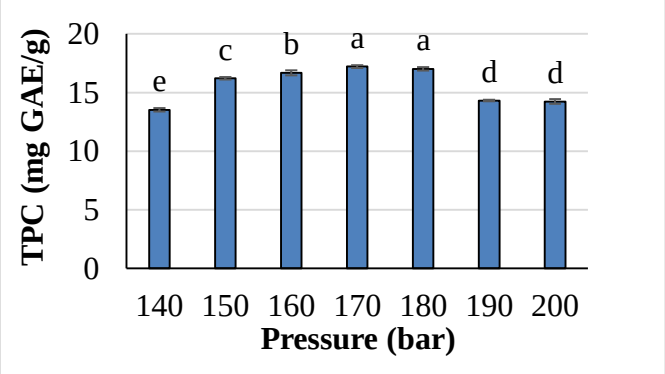
(x)



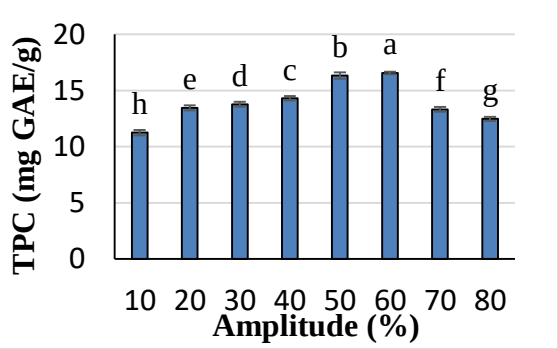
(b)



(y)



(c)



(z)

Fig. 4.2.1. Effect of supercritical fluid extraction parameters (a) extraction time, (b) temperature, (c) pressure on total phenolic content; effect of ultrasound assisted extraction parameters (x) extraction time, (y) temperature, (z) amplitude on total phenolic content. Different letters on the bars (a-i) show significant difference ($p < 0.05$) between means according to the Duncan's multiple range test.

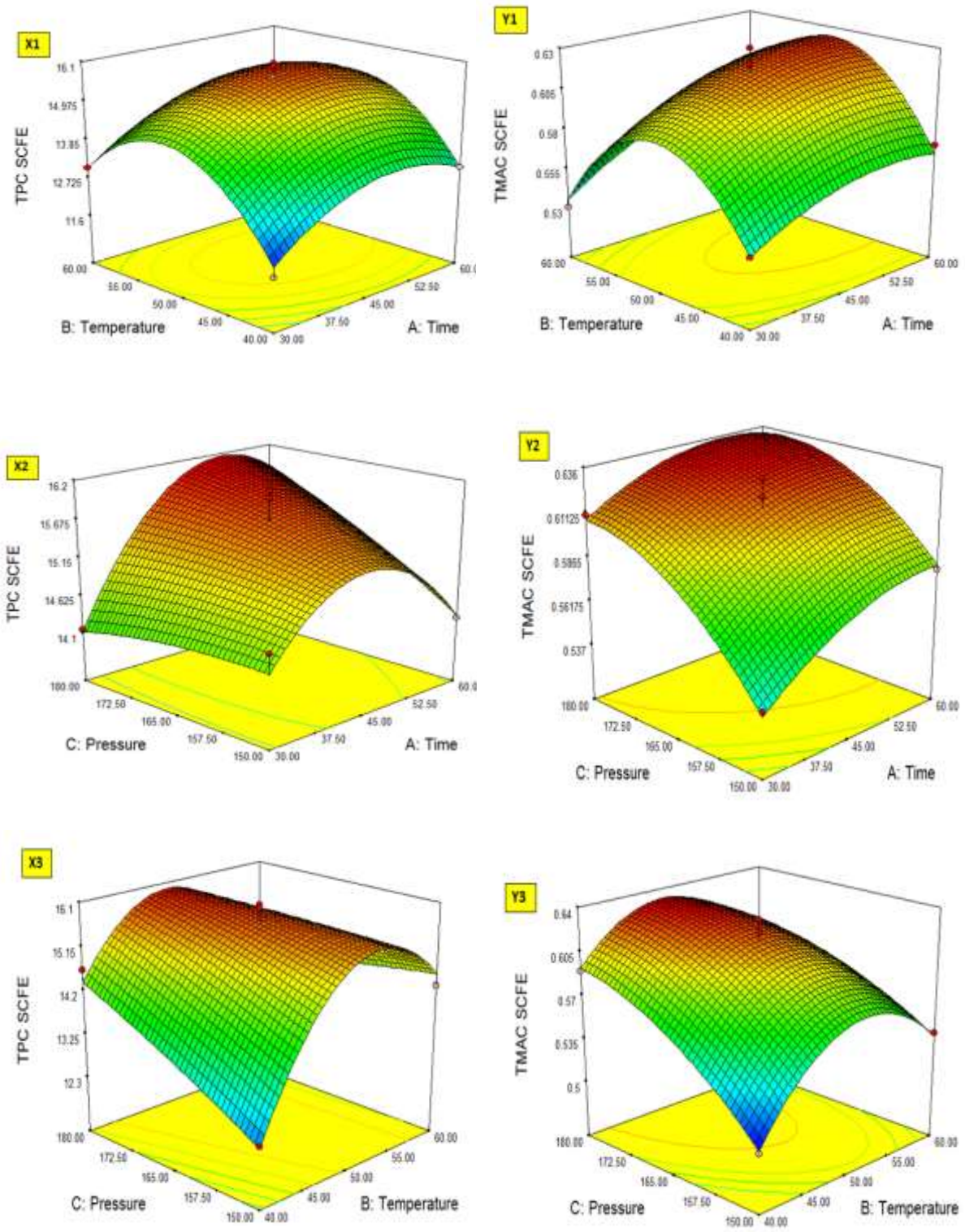


Fig. 4.2.2. Response surface (3D graphs) for supercritical fluid extraction.

4.2.3.1.2. Verification of model for SCFE

Four models namely, Linear, 2 Fi, Quadratic, and Cubic were applied for the optimization of the TPC and TMAC extraction from purple tamarillo using SCFE. Table 4.2.2 presents coefficients of regression R^2, Adequate R^2, Predicted R^2 and *p* value of all the models. The adequacy of the individual model was tested using Design Expert 7.0 software. Among the models, Quadratic model was found best for the optimization of extraction of phenolic compounds and anthocyanins content with R^2 value of 0.97 and 0.98, respectively and *p* values less than 0.0001. A high R^2 value with low *p* value is required for fitting of the model [24]. Even though cubic model showed highest R^2 value, its F value was high and, therefore, quadratic model was selected for optimization. The interactions and effect of independent variables on the response with quadratic model are given in Table 4.2.3. The coefficient of variance (CV) values of the model for TPC and TMAC was 2.29 and 1.27 %, respectively in SCFE; CV values being less than 10% indicates that the model satisfies the fitting parameters.

Table 4.2.2. Adequacy of model tested using SCFE for optimization of response.

Source	Std.Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	Prob > F	Remarks
TPC $_{SCFE}$							
Linear	1.337731	0.138185	-0.06069	-0.45853	39.37161	0.5715	
2FI	1.425709	0.247	-0.2048	-1.43262	65.66611	0.7023	
Quadratic	0.32883	0.97196	0.935909	0.715389	7.682788	< 0.0001	Suggested
Cubic	0.276893	0.988639	0.954556		+	0.2624	Aliased
TMAC$_{SCFE}$							
Linear	0.034187	0.38965	0.2488	0.004326	0.024786	0.0840	
2FI	0.037542	0.433837	0.09414	-0.73676	0.043235	0.8525	
Quadratic	0.007368	0.984735	0.965109	0.918153	0.002038	< 0.0001	Suggested
Cubic	0.008367	0.988752	0.955009	-	+	0.7158	Aliased

Table 4.2.3. Anova table of extraction of total phenolic content and total monomeric anthocyanin content by SCFE.

	Total phenolic content		Total monomeric anthocyanin content	
	Sum of Squares	p-value Prob > F	Sum of Squares	p-value Prob > F
Model	26.23708	0.0001	0.024514	< 0.0001
A-Time	1.53125	0.0070	0.00245	0.0003
B-Temperature	1.386113	0.0090	5E-05	0.3692
C-Pressure	0.812813	0.0288	0.0072	< 0.0001
AB	0.055225	0.4980	1E-04	0.2168
AC	0.837225	0.0272	1E-04	0.2168
BC	2.0449	0.0034	0.0009	0.0047
A^2	3.768067	0.0006	0.000557	0.0150
B^2	14.70018	< 0.0001	0.011167	< 0.0001
C^2	0.036809	0.5779	0.001146	0.0025
Lack of Fit	0.450225	0.2624	0.0001	0.7158
Mean	14.36353		0.580588	
C.V. %	2.289341		1.269038	
PRESS	7.682788		0.002038	
Std. Dev.	0.32883		0.007368	
Adeq Precision	15.70175		23.00515	

4.2.3.2. Determination of the limits of independent variables of UAE

For optimization using UAE, three independent variables were decided (time, temperature, and amplitude) and based on TPC limits, the range of independent variables were decided (Fig. 4.2.1x-z). For the determination of time range, time varied from 5-40 min while temperature and amplitude were kept constant at 50 °C and 50%, respectively. Significant difference ($p < 0.05$) was found in TPC and maximum extraction occurred at 25 min. The decrease in TPC was due to degradation of bioactive compounds on prolonged treatment [22]. In determining the limits of temperature, it was noticed that as the temperature was increased from 40 to 50 °C, increase in the extraction of phenolics occurred that however decreased at higher temperature due to degradation of phenolic compounds. This supported the findings of Albuquerquea et al. [1]. For amplitude, the highest phenolic content was observed at 60% followed by 50% due to the degradation of phenolic compounds.

4.2.3.2.1. Effect of independent variables on response using UAE technique

In Table 4.2.4, all the experimental trials and their specific extraction parameters with their respective response are given. The phenolic content from the sample extract ranged from 16.32-21.04 mg GAE/g of sample. The best response was recorded with processing parameters of 20 min time, 50 °C temperature and 50% amplitude. In Fig. 4.2.3 (A1-A3), the 3D response surface graphs are given with interactions of the independent variables in terms of responses. The responses for the experimental conditions were fitted in the second-order polynomial equations to understand the interactions between independent variables and response. In Eq. 5, individually time, temperature and amplitude showed positive effect on the phenolic content, however only interaction terms of time and temperature showed positive effect. As shown in Table 4.2.5, TPC was highly affected by ultrasound temperature and amplitude. The quadratic terms A^2, B^2 and C^2 were highly significant ($p < 0.01$). The interaction for AB, CA was non-significant at $p < 0.05$. Increase in time from 10 to 20 min and temperature from 40 to 50 °C led to an increase in TPC, and thereafter there was a gradual decrease, which may be due to excessive heat generation that degrades the bioactive compounds. Higher extraction temperature and time helps to extract the extractable compounds from the sample matrix and increase the solubility with enhanced mass transfer [10], but beyond limits, their concentration decreases due to degradation. Amplitude showed a positive effect on the extraction of the phenolic compounds because the increase in the amplitude is directly related to enhanced diffusion and increased mass transfer of bioactive compounds into the extracting solvent [3].

$$TPC_{UAE} = +20.57 + 0.028 \times A + 1.10 \times B + 0.66 \times C + 0.32 \times A \times B - 0.89 \times A \times C - 0.13 \times B \times C - 1.17 \times A^2 - 1.70 \times B^2 - 0.66 \times C^2 \quad (8)$$

$$TMAC_{UAE} = +0.71 + 1.250\text{E}-003 \times A + 7.500\text{E}-003 \times B + 1.250\text{E}-003 \times C - 0.013 \times A \times B - 0.015 \times A \times C - 2.500\text{E}-003 \times B \times C - 0.073 \times A^2 - 0.055 \times B^2 - 2.500\text{E}-003 \times C^2 \quad (9)$$

TMAC ranged between 0.54 and 0.74 C3G/g of dry weight of the sample. The second-order polynomial equation for response TMAC using UAE is mentioned in Eq. 9 that helps to determine the interactions between independent variables on responses. In Fig. 4.2.3 (B1-B3), the 3D response surface graphs are given with interactions of the independent

variables in terms of response. In Fig. 4.2.3 (B1), an increase in the temperature and time caused an increase in the TMAC and after reaching the maximum, it decreased gradually. However, an increase in the amplitude showed an increase in the anthocyanins content in the sample extract. Similar results were obtained in the case of extraction of anthocyanins using ultrasound from jabuticaba epicarp [1]. Solvent plays a major role in the extraction of polyphenols from sample matrix [25]. Acidified solvent enhances anthocyanins extraction and improves stability of anthocyanins at acidic pH (2.0-2.3) [11]. Extraction solvent with 50% ethanol in water acidified to pH 2.0 improved extraction of anthocyanins from elderberry using ultrasound [5].

4.2.3.2.2. Verification of model for UAE

Four different models (Linear, 2 Fi, Quadratic and Cubic) were applied for the optimization of UAE for extraction of TPC and TMAC from purple tamarillo fruit. Quadratic model was selected because of its high R^2, Adequate R^2 and less p-value, which are desirable conditions for model fitting (Table 4.2.6).

The R^2, Adequate R^2 and p-values for the quadratic model applied in TPC were 0.97, 0.94 and < 0.0001 and for TMAC 0.97, 0.94 and < 0.0001, respectively. The ANOVA table for TPC and TMAC is given in Table 4.2.5. The model was found to be significant at $p<0.05$ as lack of fitness was non-significant and residual values were low. The CV for TPC_{UAE} and $TMAC_{UAE}$ was 1.96 and 1.86, respectively.

Table 4.2.4. Experimental data of the responses for SCFE and UAE in BOX-Behnken design.

	Supercritical fluid extraction					Ultrasound assisted extraction				
Run	**A Time (min)**	**B Temperature (°C)**	**C Pressure (bar)**	**TPC (mg GAE/g)**	**TMAC (mg C3G/g)**	**A Time (min)**	**B Temperature (°C)**	**C Amplitude (%)**	**TPC (mg GAE/g)**	**TMAC (mg C3G/g)**
1	30 (-1)	40 (-1)	165 (0)	11.65	0.54	10 (-1)	40 (-1)	40 (0)	16.81	0.54
2	60 (+1)	40 (-1)	165 (0)	13.05	0.57	30 (+1)	40 (-1)	40 (0)	16.32	0.59
3	30 (-1)	60 (+1)	165 (0)	13.02	0.53	10 (-1)	60 (+1)	40 (0)	18.45	0.70
4	60 (+1)	60 (+1)	165 (0)	13.95	0.58	30 (+1)	60 (+1)	40 (0)	19.25	0.59
5	30 (-1)	50 (0)	150 (-1)	14.65	0.54	10 (-1)	50 (0)	20 (-1)	17.22	0.69
6	60 (+1)	50 (0)	150 (-1)	14.32	0.58	30 (+1)	50 (0)	20 (-1)	18.96	0.73
7	30 (-1)	50 (0)	180 (+1)	14.15	0.61	10 (-1)	50 (0)	60 (+1)	20.32	0.71
8	60 (+1)	50 (0)	180 (+1)	15.65	0.63	30 (+1)	50 (0)	60 (+1)	18.49	0.69
9	45 (0)	40 (-1)	150 (-1)	12.36	0.50	20 (0)	40 (-1)	20 (-1)	16.38	0.67
10	45 (0)	60 (+1)	150 (-1)	14.32	0.54	20 (0)	60(+1)	20 (-1)	20.73	0.72
11	45 (0)	40 (-1)	180 (+1)	14.65	0.59	20 (0)	40 (-1)	60 (+1)	18.96	0.63
12	45 (0)	60 (+1)	180 (+1)	13.75	0.57	20 (0)	60 (+1)	60 (+1)	20.38	0.74
13	45 (0)	50 (0)	165 (0)	15.65	0.62	20 (0)	50 (0)	40 (0)	20.87	0.69
14	45 (0)	50 (0)	165 (0)	15.36	0.61	20 (0)	50 (0)	40 (0)	20.34	0.68
15	45 (0)	50 (0)	165 (0)	16.05	0.63	20 (0)	50 (0)	40 (0)	20.77	0.68
16	45 (0)	50 (0)	165 (0)	15.96	0.61	20 (0)	50 (0)	40 (0)	21.04	0.69
17	45 (0)	50 (0)	165 (0)	15.64	0.62	20 (0)	50 (0)	40 (0)	19.84	0.71

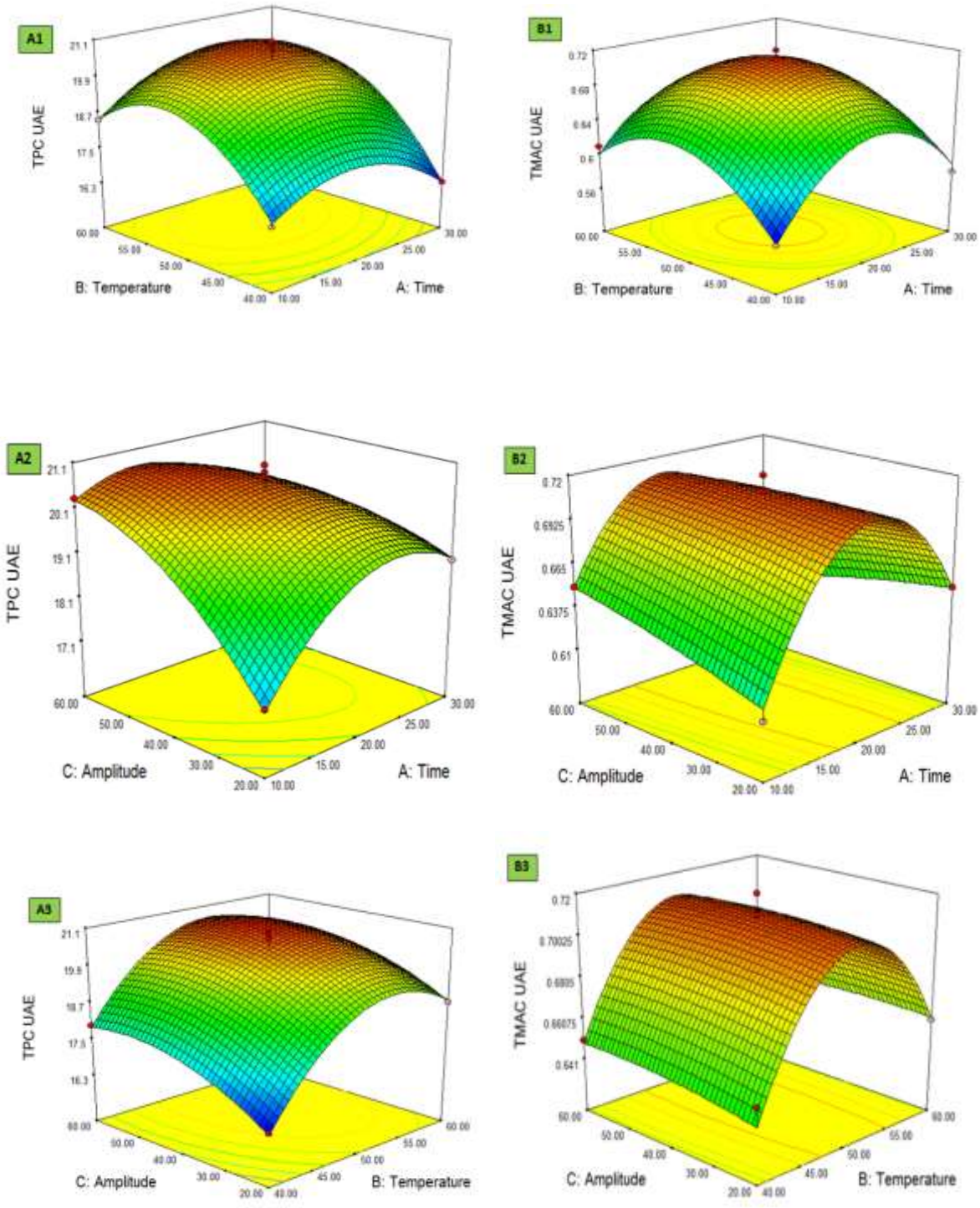


Fig. 4.2.3. Response surface (3D graphs) for ultrasound assisted extraction.

Table 4.2.5. Anova table of extraction by UAE.

	Total phenolic content		Total monomeric anthocyanins content	
	Sum of Squares	**p-value Prob > F**	**Sum of Squares**	**p-value Prob > F**
Model	38.36904	< 0.0001	0.039151	< 0.0001
A-Time	0.00605	0.8408	1.25E-05	0.7786
B-Temperature	9.5922	< 0.0001	0.00045	0.1230
C-Amplitude	3.4848	0.0016	1.25E-05	0.7786
AB	0.416025	0.1275	0.000625	0.0777
AC	3.186225	0.0020	0.0009	0.0423
BC	0.065025	0.5164	2.5E-05	0.6918
A^2	5.736727	0.0004	0.022132	< 0.0001
B^2	12.12908	< 0.0001	0.012737	< 0.0001
C^2	1.818853	0.0086	2.63E-05	0.6843
Lack of Fit	0.0381	0.9811	0.000425	0.4986
Mean	18.91471		0.648824	
C.V. %	1.972898		1.865032	
PRESS	2.073163		0.007738	
Std. Dev.	0.373168		0.012101	
Adeq Precision	14.86513		23.00515	

4.2.3.3. Optimization and comparison of techniques

In Table 4.2.7, the optimized conditions for SCFE and UAE are mentioned. In both the techniques, all the independent variables were kept in range and responses were maximized. The desirability for SCFE and UAE was 1.00 and 0.95, respectively, and experimental results were found to be near to the predicted model. In SCFE, the experimental values for TPC and TMAC were found to be 16.12 ± 0.05 mg GAE/g and 0.62 ± 0.02 mg C3G/g, respectively. In UAE, the experimental values for TPC and TMAC were found to be 21.07 ± 0.14 mg GAE/g and 0.71 ± 0.03 mg C3G/g, respectively. The validation of the experimental and predicted values was done using relative deviation. In SCFE, the relative deviation for TPC and TMAC was found to be 0.25 and 1.63%, respectively. In UAE, the relative deviation for TPC and TMAC was 0.90 and 1.63%, respectively.

Kadiri et al. [15] reported that relative deviation of less than 10% signifies validity of the model, which is appropriate for further use. Therefore, the model was inferred to be satisfactory for optimization of extraction conditions.

Table 4.2.6. Adequacy of model tested using UAE for optimization of responses.

Source	Std.Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	Prob > F	Remarks
TPC $_{UAE}$							
Linear	1.421288	0.332531	0.1785	-0.03761	40.82369	0.1420	
2FI	1.503113	0.425742	0.081187	-0.45566	57.27104	0.6650	
Quadratic	0.373168	0.975224	0.943369	0.947307	2.073163	< 0.0001	Suggested
Cubic	0.483911	0.976192	0.90477	-	+	0.9811	Aliased
TMAC $_{UAE}$							
Linear	0.055263	0.011823	-0.21622	-0.60494	0.064481	0.9837	
2FI	0.061767	0.050403	-0.51936	-1.95176	0.118591	0.9366	
Quadratic	0.012101	0.974488	0.941686	0.807412	0.007738	< 0.0001	Suggested
Cubic	0.012247	0.985066	0.940264	-	+	0.4986	Aliased

The TPC and TMAC values of the extract obtained from SCFE and UAE techniques using optimized conditions are shown in Table 4.2.7. UAE showed higher yield of TPC and TMAC than SCFE. In UAE technique, ultrasonic waves are powerful for generating microjets from bubbles that disintegrate sample matrix. This disruption enhances the extraction process and increases the concentration of bioactive compounds in the solvent [16]. Omar et al. [20] too reported higher polyphenol extraction from citrus peel using UAE as compared to SCFE. UAE takes less time and experimental cost is low. In SCFE, lower extraction yield may depend on factors like the solubility of polyphenols, temperature, extraction time, pressure, modifier concentration, flow rate, etc.

4.2.3.4. HPLC profile of phenolic acids

The HPLC profile of the phenolic acids of the extracts from SCFE and UAE techniques extracted under optimized conditions are presented in Fig. 4.2.4a and Fig. 4.2.4b, respectively. The main phenolic acids identified in the extract of purple tamarillo extract were gallic acid, chlorogenic acid, caffeic acid and p-coumaric acid. The concentration of phenolic acids was higher in UAE extract than SCFE (Table 4.2.8). The highest concentration was seen for gallic acid with 2100.84 and 1572.63 µg/g in UAE and SCFE extracts, respectively, followed by chlorogenic acid, caffeic acid and p-coumaric acid. Espin et al. [6] observed that purple tamarillo is a good source of chlorogenic, caffeic and p-coumaric acids.

Table 4.2.7. Optimized conditions for SCFE and UAE with predicted and experimental values.

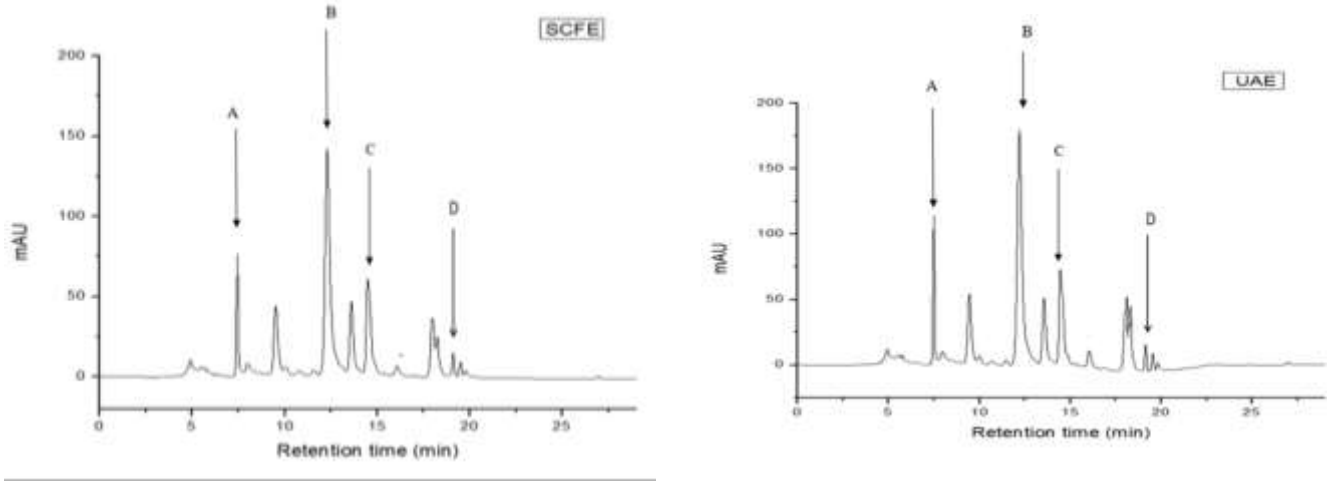
Supercritical fluid extraction						
			Predicted value		Experimental value	
Time (min)	Temperature (°C)	Pressure (bar)	TPC **(mg GAE/g)**	TMAC **(mg C3G/g)**	TPC **(mg GAE/g)**	TMAC **(mg C3G/g)**
49.42	49.28	176.63	16.08	0.63	16.12	0.62
Ultrasound assisted extraction						
			Predicted value		Experimental value	
Time (min)	Temperature (°C)	Amplitude (%)	TPC **(mg GAE/g)**	TMAC **(mg C3G/g)**	TPC **(mg GAE/g)**	TMAC **(mg C3G/g)**
19.14	51.53	50.53	20.87	0.70	21.06	0.71

4.2.3.5. HPLC profile of anthocyanins

Delphinidin-3-O-rutinoside, cyanidin-3-O-rutinoside and pelargonidin-3-O-rutinoside were the three anthocyanins identified in the extract of SCFE (Fig. 4.2.c) and UAE (Fig. 4.2.5d). Pelargonidin-3-O-rutinoside content was 308.44 µg/g and 213.78 µg/g in UAE and SCFE extracts, respectively (Table 4.2.8). However, Espin at al. [6] reported pelargonidin-3-O-rutinoside and delphinidin-3-O-rutinoside as the main anthocyanins present in purple tamarillo from New Zealand.

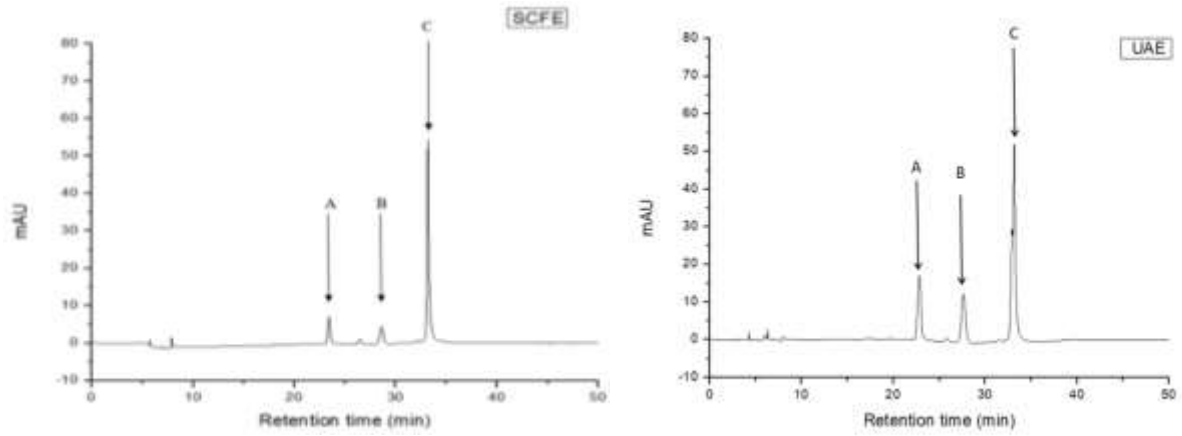
Table 4.2.8. Phenolic acids and anthocyanins content present in optimized extract of SCFE and UAE extracts of purple tamarillo.

Peak order	Compounds	SCFE (µg/g)	UAE (µg/g)
	Phenolic acids		
1	Gallic acid	1572.63	2100.84
2	Chlorogenic acid	1277.53	1481.43
3	Caffeic acid	136.92	140.35
4	p-Coumaric acid	57.07	106.01
	Anthocyanins		
1	Delphinidin 3-rutinoside	41.26	90.67
2	Cyanidin-3-*O*-rutinoside	38.27	58.87
3	Pelargonidin-3-O-rutinoside	213.78	308.44



(a) (b)

Fig. 4.2.4. HPLC profile of phenolic compounds in optimized extract of (a) SCFE and (b) UAE; A: gallic acid, B: chlorogenic acid, C: caffeic acid and D: p-coumaric acid.



(c) (d)

Fig. 4.2.5. HPLC profile of anthocyanins in optimized extract of (c) SCFE and (d) UAE. A: Delphinidin 3-rutinoside, B: Cyanidin-3-O-rutinoside and C: Pelargonidin 3-O-rutinoside.

4.2.3.6. Anthocyanins stability

The thermal degradation of anthocyanins was studied at three different temperatures for different intervals of time. In Fig. 4.2.6, the degradation of anthocyanins with respect to temperature and exposure time is indicated. It was found that highest degradation of anthocyanins was found at 90 °C followed by 70 °C and very less degradation of anthocyanin was found at 50 °C. No difference in the degradation of anthocyanins in UAE and SCFE extracts was observed. Our results agree with the degradation of anthocyanins extracted from blueberry bagasse in acidified solution [7].

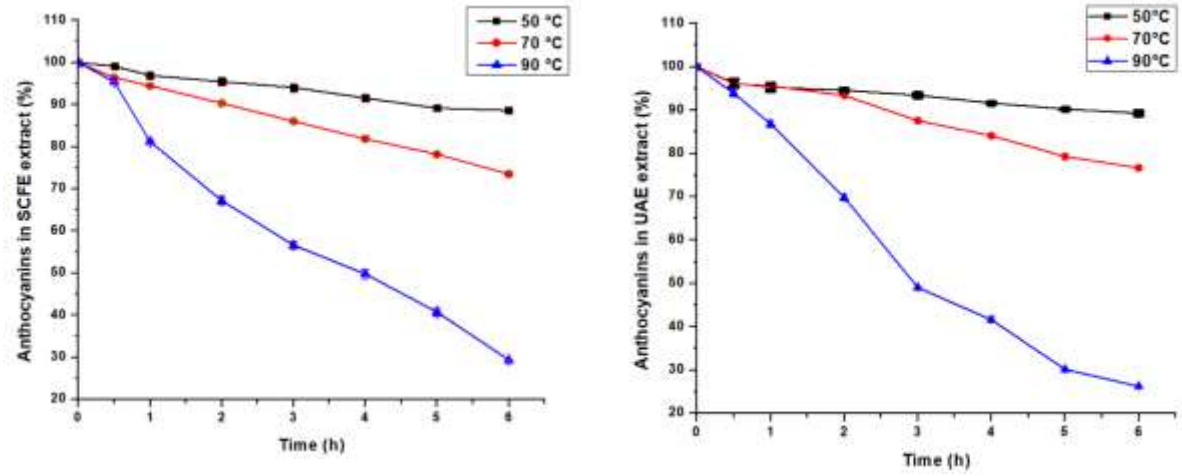


Fig. 4.2.6. Effect of temperature on extracted anthocyanins from purple tamarillo using SCFE and UAE techniques.

4.2.3.7. Scanning electron microscopy

The tamarillo residue left after SCFE and UAE were observed for morphological differences at ×500 and ×5500 magnifications (Fig. 4.2.7). Visible difference was found in the control and samples subjected to SCFE and UAE. UAE caused greater structural damage and crack formation than SCFE, which supports the higher release of TPC and TMAC from the sample matrix as reported above [14].

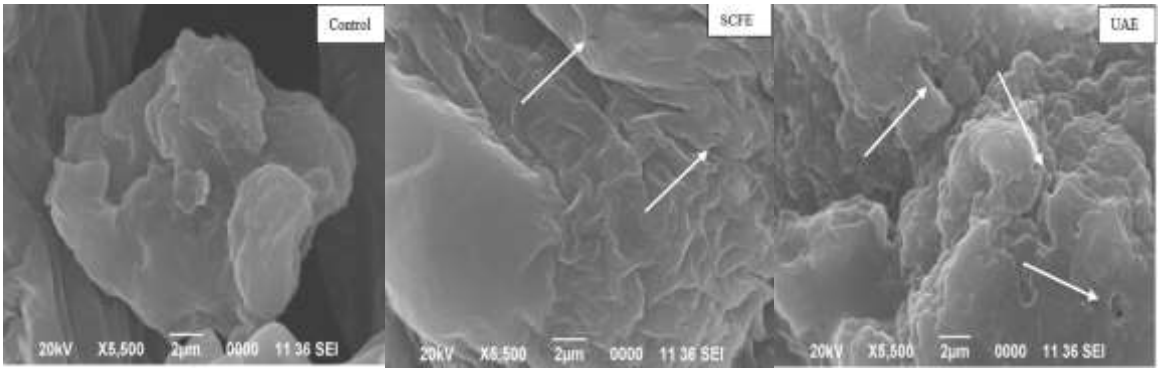


Fig. 4.2.7. SEM images of Control, SCFE and UAE treated samples at different magnifications**.**

4.2.4. Conclusion

Optimization of the extraction process for purple freeze-dried tamarillo was successfully done using response surface methodology for the extraction of total phenolics and anthocyanins content. Among the two techniques studied, UAE extracted higher amount of phenolics and anthocyanins than SCFE. Gallic acid was the major phenolic acid and pelargonidin-3-O-rutinoside was the major anthocyanin in purple tamarillo, as identified by HPLC. To the best of our knowledge, this is the first report on the comparison of UAE and SCFE for the extraction of phenolic compounds from the purple tamarillo and the results indicated that UAE technique allows for greater extraction of phenolic acids. Freeze-dried powder of UAE extract can be helpful for use as natural colorant in food systems.

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