CHAPTER 7

OBJECTIVE 5

CHAPTER 7 SECTION I

EXTRACTION OF CAROTENOIDS FROM TAMARILLO PEEL USING HIGH SHEAR DISPERSER AND ULTRASOUND USING GREEN EXTRACTION SOLVENT

7.1.1 Introduction

Tamarillos are available in the three prominent varieties, namely purple, red and yellow tamarillos. Each variety has its own significance in terms of phytochemical properties. The yellow variety is known for its yellowish colour and is very rich in carotenoid compounds [24]. The predominant carotenoids reported in tamarillo fruit are β-cryptoxanthin and β-carotene, though other carotenoids like zeaxanthin and lutein are also reported to be present [23].

Carotenoids are known to possess various health beneficial properties like prevention of cancer, cardiovascular diseases, macular degeneration, possessing antioxidant, and anti-inflammatory activities and maintaining good health of the consumers [10]. Carotenoids are lipophilic tetraterpenes containing a series of conjugated double bonds and are very sensitive to heat, light, pH, oxygen, and other processing factors which affect their bioactivities [32]. Among the carotenoids, β-carotene is used to prevent vitamin A deficiency in children and pregnant women, therefore administration of this carotenoid is very important. However, their bioavailability is influenced by the ability of the compounds to reach the absorption site in the small intestine without degradation, thus, it is a crucial and challenging step for researchers to develop food products with enhanced bioaccessibility [2]. Even though these lipophilic compounds are readily soluble in organic solvents like hexane, acetone, methanol, cyclohexane, etc., these solvents can cause some health related problems in food systems [32]. Recently, consumers as well as researchers have shifted towards green extraction techniques where green solvents (mostly edible oils) are used in the place of organic solvents. Edible oils retain the bioactivity of the lipophilic carotenoids [6]. Olive oil is considered to be one of the best oils in terms of oxidative stability, sensorial, and health beneficial properties [22]. Due to its fatty acid composition,

especially monounsaturated fatty acid (oleic acid) contributes to 70-80 % of the total oil, olive oil is unique in itself and has attracted the attention of researchers and consumers alike [27]. More and more researchers are utilizing olive oil for the development of various new food products with health beneficial properties [22].

Nowadays, many novel extraction techniques are available for the extraction of carotenoids but researchers are more focused on those extraction techniques which maintains the naturalness of the product without altering its bioactivity with effective extraction and low energy consumption [6, 32]. There are several studies published on the extraction of carotenoids from a fruit sample using green solvents and high shear disperser [2] and ultrasound as the extraction means [6]. Green extraction means use of renewable plant resources and low energy intensive process that provides the contamination-free end product [27]. As carotenoids are fat soluble compounds therefore using oil as an extraction medium will promote the dissolution of the carotenoid compounds and ease the extraction. Ultrasound uses the cavitation phenomena for the extraction of bioactive compounds from the sample matrix. These cavitation bubbles that are produced will collapse after exceeding certain energy and generate micro turbulence in the extraction solvents due to which bioactive compounds get released in the extraction solvent [29]. The extraction of bioactive compounds by high shear disperser uses the mechanical forces to extract the bioactive compounds from the sample matrix, the mechanical force breaks the rigid matrix of the sample and it enables solubilization of the entrapped bioactive compounds in the sample matrix to the solvent medium [2]. Nowadays, researchers are more focused on using natural antioxidants because synthetic antioxidants are reported to have harmful effect on the human health [21]. Carotenoids have been added to the foods with high fat content to increase the shelf life of the food products. Such carotenoids-enriched foods are beneficial for human health because these pigments help in the inhibition of peroxyl radicals generated during lipid oxidation [5].

While processing of yellow tamarillo into various food products, the peel of this fruit that is rich in carotenoids is discarded as waste. Thus, the main objective of this research is to utilize the waste produced during processing of tamarillos and developed the carotenoids loaded oil based food product which possess better bioaccessibility. In this study, our work focused on determining the best optimum conditions for the extraction of the carotenoids from tamarillo peel in EVOO using HPH and UAE

techniques. Another aspect of the work was to develop the carotenoids-loaded functional food and determine the effects on carotenoids during *in-vitro* digestion.

7.1.2. Materials and methods

7.1.2.1. Plant materials and sample preparation

Yellow tamarillo was bought to Tezpur University, Assam from a local market in Kohima, Nagaland. Ripe fruits were thoroughly cleaned and washed and the peel of the fruits was manually separated and dried at 50°C in a tray drier (Labotech, BDI-51, B. D. Instrumentation, India). Dried peel was processed into powder form using mixer grinder (Philips HL Model No. 1632, India) and kept at -20 °C in zip lock pouch of LDPE (low density polyethylene) until further use. Extra virgin olive oil (Delmonte, India) (EVOO) was purchased from a local market in Tezpur.

7.1.2.2. High shear disperser (HSD)

The extraction of the carotenoids was done in extra virgin olive oil (EVOO) according to the method adopted by Baria et. al. [2] with some modification. A high shear disperser T25 Ultra-Turrax (IKA Works, Inc., NC, USA) was used for the extraction of the carotenoids from the tamarillo peel.

7.1.2.3. Ultrasound assisted extraction (UAE)

The carotenoids were extracted according to the method adopted by Chutia et al. [6] with some modification. The extraction was done using a probe sonicator (U500, Takashi, Japan) and EVOO was the extraction solvent used.

7.1.2.4. Experimental design

Optimization of the extraction conditions of carotenoids from the tamarillo peel was done using Response Surface Methodology (RSM). The experimental design was prepared using the three factor Face Cantered Composite design (FCCD) that comprised of three independent variables of time, temperature, and shear speed taking the help of Design Expert Version 7 software (State-Ease Inc., Minneapolis, MN, USA). In Table 7.1.1, the limits for the independent variables are reported for HSD. In Table 7.1.2, three independent variables are time, temperature and amplitude were selected the limits of the independent variables are reported for UAE. The dependent variable for the optimization process in both the extraction techniques was total carotenoids content (TCC) in the EVOO. Pre-heated oil was used for the extraction of the carotenoids and special attention

was given to maintain the temperature during experiments. The solid to solvent ratio was kept constant at 1:10 throughout all the experiments for HSD and UAE techniques. Design was executed to maximize the yield of carotenoids and validation of the model was done on the basis of comparing experimental and predicted values.

After extracting the carotenoids by HSD and UAE, every sample with the extracted carotenoids were centrifuged at 3000 x g at 25 °C (Eppendorf 5430R), stored under refrigeration temperature until further analysis.

Table 7.1.1. Limits for independent extraction of total carotenoids using HSD

Independent variables	-α	-1	0	+1	+α
Time (min)	3	5	7.5	10	12
Temperature (°C)	33	40	50	60	67
Speed (rpm)	3900	8000	14000	20000	2400

Table 7.1.2. Limits for independent extraction of total carotenoids using UAE

Independent variables	-α	-1	0	+1	+α
Time (min)	3	5	7.5	10	12
Temperature (°C)	33	40	50	60	67
Amplitude (%)	27	40	80	60	93

7.1.2.5. Extraction of the TCC

The extraction of the TCC was done according to the method adopted by Costamagna et al. (2013). Briefly, 1 mL EVOO oil was taken and added with 10 mL of solvent mixture (hexane:acetone:ethanol in the ratio of 2:1:1). The mixture was centrifuged at 5000 x g for 20 min at 4 °C (Eppendorf 5430R), and the top layer was separated and adjusted with hexane to 10 mL. Absorbance of the oil sample was read at 450 nm and results were expressed as mg of β-carotene equivalents (mg β-CE) per 100 g.

7.1.2.6. Determination of oil quality parameters

7.1.2.6.1. Acid value and peroxide value

The acid value and peroxide value of the optimized carotenoids enriched oil was calculated according to the standard method of AOAC [16].

7.1.2.6.2.Total phenolic content

The total phenolic content (TPC) in tamarillo was determined according to Saikia et al [31]. For the analysis, an aliquot of 0.5 mL of diluted sample extracts was taken in test tube and mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10). For blank, sample extract was replaced with distilled water. After 5 min of incubation, 2 mL of sodium carbonate (7.5%) was added into each test tube, vortexed and kept for 2 h in a dark place at room temperature. Absorbance was read by UV-Vis spectrophotometer (Thermo-Fischer Evolution A600) after incubation time against the reagent blank mixture. Gallic acid was used as standard, and results were expressed in mg GAE/100g.

7.1.2.6.3. DPPH radical scavenging activity

DPPH radical scavenging activity of tamarillos was calculated according to Saikia et al [31] with some modification. In a test tube, 200µl of sample extract was taken followed by the addition of 2.8 mL of DPPH radical prepared in methanol, vortexed and kept for 30 min in a dark place for incubation. The absorbance of sample was read at 517 nm using UV-Vis spectrophotometer (Thermo-Fischer Evolution A600) against blank (Eq. 1).

$$DPPH\ activity\ (\%) = \frac{A_o - A_s}{A_o} \times 100 \quad (1)$$

here A_o is absorbance of control blank, and A_s is sample absorbance

7.1.2.6.4. Colour values

The colour values of the HSD and UAE optimized extract were read in a Hunter colour spectrophotometer (Hunter Colour Lab Ultrascan Vis, USA). The instrument was standardized using the standards before measurement of the samples. Scale parameters for colour analysis were L* (lightness, 0=black to 100=white), a*(negative values indicate greenness and positive value indicate redness) and b* (negative values indicate blueness and positive values indicate yellowness) of the samples.

7.1.2.7. HPLC of phenolic acids of optimized extracts

The phenolic acids present in the EVOO extracts using HSD and UAE were identified according to the method adopted by Espin et al. [12] with some modifications. The phenolic acids samples were prepared according to the method adopted by Kıralan et al. (2009). The phenolic acids present in the HSD and UAE treated oil was identified and

quantified using reverse phase UHPLC (Utimate 3000, Thermo Scientific, USA) consisting of C-18 column with diode array detector. The flow rate was 0.5 mL/min, column temperature of 35 °C and wavelength was fixed to 330 nm. The program for the gradient flow 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.

7.1.2.8. HPLC of TCC of optimized extract

The TCC in the HSD and UAE treated samples were quantified using an UHPLC (Ultimate 3000, Thermo Scientific, USA). The reverse phase HPLC was equipped with C-30 column and diode array detector and identification of the carotenoid was done against standards. A gradient mode consisting of a mixture of solvent A (methanol/acetonitrile/water 84:14:4 v/v/v) and solvent B (100 % dichloromethane) was used. The flow rate was 1 mL/min, temperature was 25 °C and wavelength was fixed to 450 nm. The sample extract was filtered using a 0.22 µm syringe filter prior to injecting in HPLC. The program for the gradient flow was 100% A and 0% B initially, raised to 10% B at 4 min, 18% B at 12 min, 21% B at 17 min, 30% B at 20 min and maintained until 25 min, increased further to 39% B at 28 min, and finally to 60% B at 40 min [15].

7.1.2.9. Preparation of mayonnaise

The extracted carotenoids in the EVOO extract was used for the preparation of the mayonnaise according to the procedure followed by Kishk and Elsheshetawy [7] with some modification. The composition of mayonnaise (w/w) was oil 70, whole egg 19.1, salt 1.0, sugar 0.6, vinegar 5.6, mustard 1.6, white pepper 0.3, and lemon juice 1.6. At first, mustard and egg yolk were blended together in a mixer blender (Philips HL Model No. 1632, India), followed by addition of oil in small quantities until completely blended and mass of the (mayonnaise) puree was thickened. The product stored in airtight container in 4 °C until further analysis.

7.1.2.10. Sensory analysis

The sensory analysis of the carotenoids enriched mayonnaise was done according to the method adopted by de Souza Mesquita et al. [8] with some modification. The analysis was carried out by semi trained panellists who were free from allergies or intolerance. A nine point hedonic scale was used to score carotenoids-enriched

mayonnaise for colour, aroma, taste, texture, and overall acceptance. The score scale ranged from 1 (dislike extremely) to 9 (like extremely).

7.1.2.11. In-vitro digestion of mayonnaise

The method of de Souza Mesquita et al. [8] was followed with some modification to determine the *in-vitro* digestion of the prepared mayonnaise. The digestion of the mayonnaise was done in oral, gastric and intestinal phases. Briefly, 10g of mayonnaise was taken in the conical flask and 10mL of the oral solution (7 mg of α-amylase and 25 µL of 0.3 M $CaCl_2$) was added, the pH was adjusted to 7.0 and incubated at 37 ℃ for 10 min. The oral phase was followed by the gastric phase, where sample was mixed with 20 mL of the salivary gastric fluid (SGF) (20mg of pepsin and 2.5 µl of $CaCl_2H_2O$), pH was adjusted to 3.0 using HCl followed by incubation for 2 h at 37 ℃ with continuous stirring. A known amount of sample was taken out from the gastric digesta and placed in ice bath for 10 min to stop the enzyme activities. In intestinal phase, 20mL of the simulated intestinal fluid (SIF) (37.5 mg of pancreatin and 40 mg of bile salts) was added into gastric phase mixture and pH of the solution was adjusted to 7.0 using NaOH. The crude digesta was placed in the ice bath to stop the enzyme action. The extraction of TCC present in initial, gastric and intestinal phase digesta was done The identification and quantification of individual carotenoids were done.

7.1.2.11. Calculation of bioaccessibility

The bioaccessibility of the carotenoids was calculated using Eq. 1 and reported in percentage (%).

$$\%\ Bioaccessability\ (BC) = \frac{Bioaccessabile\ content}{Total\ initial\ content} \times 100 \qquad (1)$$

7.1.2.12. Statistical analysis

All experiments were performed in triplicate and the experimental results are presented as mean ± standard deviation. Data were analyzed by ANOVA (analysis of variance) and Duncan multiple range test using statistical tool SPSS version 24.0 (SPSS 24.0, IBM Corporation, USA) [31].

7.1.3. Results and discussion

7.1.3.1. Effect of the independent variables on TCC using high shear disperser

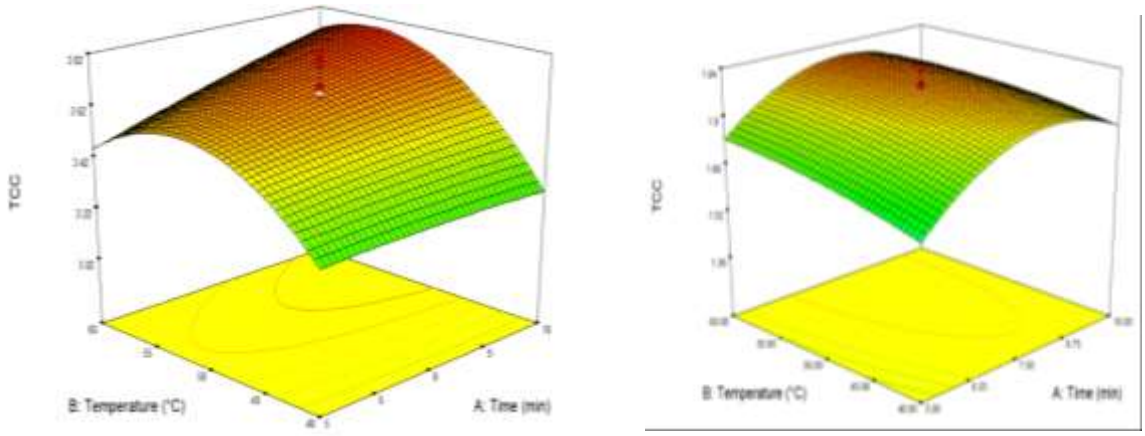
In HSD, three independent variables were used for the optimization of TCC extraction from the tamarillo peel in EVOO. The TCC values with their respective experimental conditions are reported in Table 7.1.3. In Fig. 7.1.1 (X1-X3), the response graphs for the TCC are given. It was found that all the individual factors showed positive influence on the carotenoids extraction but speed and temperature played important role during the extraction. It was noticed that the increase in temperature led to an increase in the carotenoids content but after exceeding the temperature above 50 °C, a negative impact on the carotenoids content was found. Baria et al. (2019) studied the extraction of carotenoids from mango peel and found that increase in extraction time has no significant effect on the carotenoids content, however, it was reported that increase in the speed showed positive relation with the carotenoids content.

$$\boldsymbol{TCC}\ (\boldsymbol{HSD}) = 3.67 + 0.067 \times A + 0.15 \times B + 0.26 \times C + 0.069 \times A \times B - 0.074 \times A \times C - 0.12 \times B \times C - 0.011 \times A^2 - 0.22 \times B^2 - 0.14 \times C^2$$

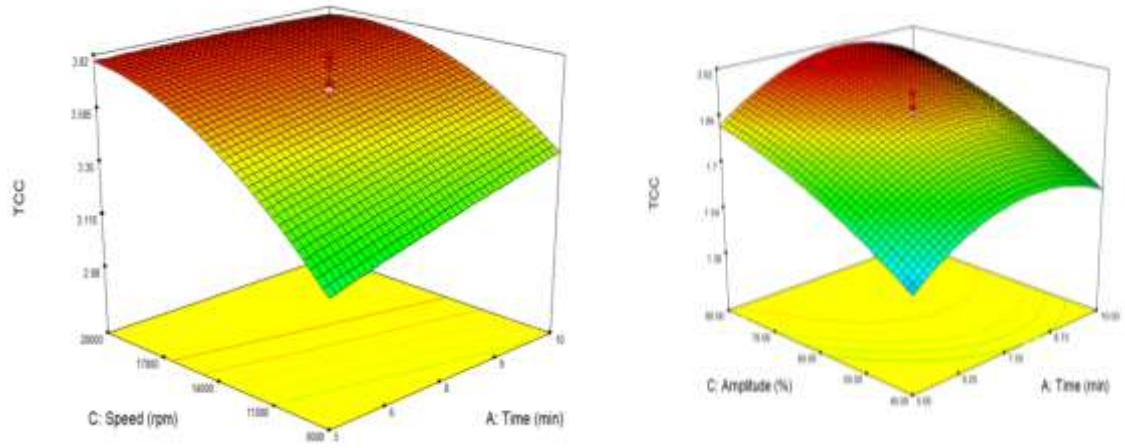
Our results on increase in speed and extraction time were found to correlate with this study. There was an increase in carotenoids extraction up to a certain time followed by a decrease [2]. Tiwari and his co-workers studied the extraction of carotenoids from carrot using flaxseed oil as the solvent by HSD technique and found that after a certain extraction time, no significant affect was found on the extraction of carotenoids [33]. The R^2 value was found to be 0.92 (Table 7.1.4). The model was significant and lack of fit (error) was found to be non-significant, as both the values play major role in evaluating the significance of model fitting [30].

Table 7.1.3. Experimental design for extraction of total carotenoids content using high shear disperser and ultrasound assisted extraction.

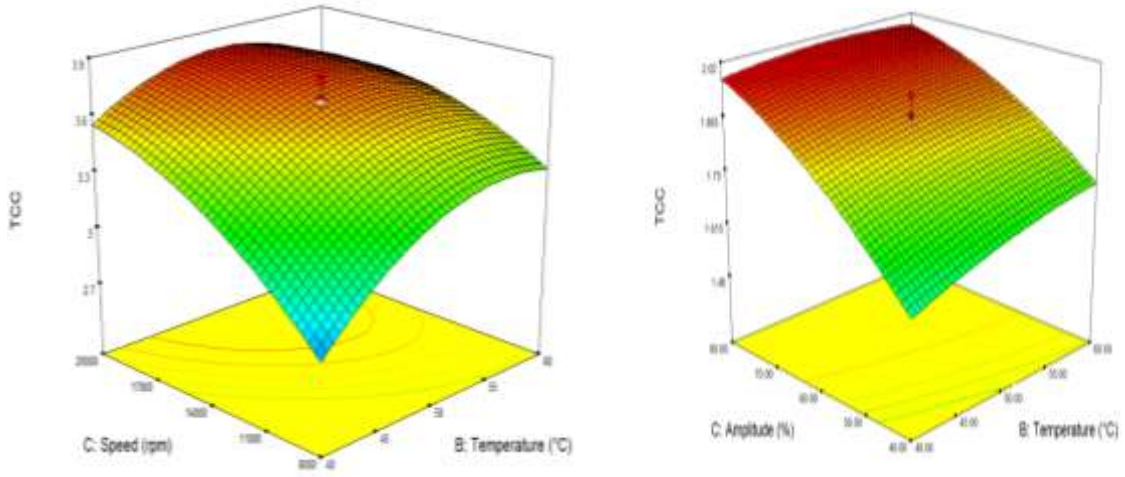
	High shear disperser				Ultrasound assisted extraction			
Run	**Time (min)**	**Temperature (°C)**	**C: Speed (rpm)**	**TCC (mg β CE/100 g)**	**Time (min)**	**Temperature (°C)**	**Amplitude (%)**	**TCC (mg β CE/100 g)**
1	5	40	8000	2.58	5	40	40	1.41
2	10	40	8000	2.76	10	40	40	1.53
3	5	60	8000	3.18	5	60	40	1.59
4	10	60	8000	3.55	10	60	40	1.65
5	5	40	20000	3.55	5	40	80	1.83
6	10	40	20000	3.35	10	40	80	1.94
7	5	60	20000	3.58	5	60	80	1.89
8	10	60	20000	3.74	10	60	80	1.94
9	3	50	14000	3.55	3	50	60	1.38
10	12	50	14000	3.79	12	50	60	1.62
11	7.5	33	14000	3.02	7.5	33	60	1.76
12	7.5	67	14000	3.16	7.5	67	60	1.83
13	7.5	50	3909	2.88	7.5	50	27	1.48
14	7.5	50	24091	3.75	7.5	50	94	1.97
15	7.5	50	14000	3.82	7.5	50	60	1.94
16	7.5	50	14000	3.69	7.5	50	60	1.87
17	7.5	50	14000	3.78	7.5	50	60	1.89
18	7.5	50	14000	3.47	7.5	50	60	1.87
19	7.5	50	14000	3.67	7.5	50	60	1.94
20	7.5	50	14000	3.58	7.5	50	60	1.79



(X1) (Y1)



(X2) (Y2)



(X3) (Y3)

Fig. 7.1.1. 3D response surface graphs of the extraction by the total carotenoids using (X1-X3) high shear disperser and (Y1-Y3) ultrasound assisted extraction.

Table 7.1.4. ANOVA table for HSD

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.49	9	0.28	11.39	0.0004	significant
A-Time (min)	0.081	1	0.081	3.35	0.0971	
B-Temperature (°C)	0.35	1	0.35	14.46	0.0035	
C-Speed (rpm)	1.05	1	1.05	43.1	< 0.0001	
AB	0.059	1	0.059	2.42	0.1509	
AC	0.025	1	0.025	1.02	0.3366	
BC	0.086	1	0.086	3.55	0.0889	
A^2	5.443E-004	1	5.443E-004	0.022	0.8841	
B^2	0.65	1	0.65	26.73	0.0004	
C^2	0.25	1	0.25	10.47	0.0089	
Residual	0.24	10	0.024			
Lack of Fit	0.16	5	0.032	1.89	0.2516	not significant
Pure Error	0.084	5	0.017			
Cor Total	2.74	19				
R^2	0.91					
Adj. R^2	0.83					
C.V. %	4.55					

7.1.3.2 Effect of the independent variables on TCC using UAE

During extraction of carotenoids using UAE, three independent variables were used for optimizing the extraction conditions. The TCC values with the respective experimental conditions are shown in Table 7.1.3. In Fig. 7.1.1 (Y1-Y3), the response 3D graphs are reported. The response curves show that all the individual factors have significant role on the TCC value but highest effect was shown by amplitude of the ultrasound. It was found that with an increase in the amplitude of the ultrasound, increase in the TCC value in the extraction medium occurred. Li et al. [20] studied the extraction of carotenoids using sunflower oil and reported that increase in the ultrasound intensity showed an increase in the extraction yield of carotenoids. Higher ultrasound amplitude indicates that more power is given into the extraction system to disrupt the cell structure which is helpful to extract the extractable compounds in the solvent mixture [26]. However, initially there is an increase in the TCC with time but after exceeding a certain period of time, no further increasing in the extraction yield was observed.

$$\boldsymbol{TCC\ (UAE)} = 1.88 + 0.054 \times A + 0.035 \times B + 0.16 \times C - 0.015 \times A \times B - 2.500E - 003 \times A \times C - 0.030 \times B \times C - 0.012 \times A^2 - 0.018 \times B^2 - 0.043 \times C^2$$

During extraction, temperature has an important role because rise in the temperature above 50 °C enhances the fluidity and favours the rapid extraction of carotenoids compounds due to decrease in the viscosity of the oil which helps in the extraction process [14]. Our results indicates that increase in the temperature up to 50 °C showed an increase in the carotenoids content, but further increase in temperature led to loss of carotenoids. Our results on the effect of temperature on TCC was found to be in agreement with the reported study [6], where an increase in the temperature above 50 °C exhibited negative effect on the extraction yield of carotenoids from passion fruit peel. The R^2 value of the quadratic model was found to be 0.91 and model was significant with non-significant lack of fit (Table 7.1.5).

7.1.3.3. Optimization of response surface methodology and comparison of the two extraction methods

The optimization of the extraction conditions using two different extraction techniques for the extraction of TCC from tamarillo peel in EVOO taken as solvent was done. Highest extraction yield was found using HSD in comparison UAE. HSD was able to extract about 0.90 times more TCC in comparison to UAE sample. As high shear disperser uses mechanical force to extract the bioactive compounds from the sample matrix, therefore maximum extraction yield was found in the high shear disperser in comparison to ultrasound assisted extraction. The optimized conditions for HSD were time of 5.50 min, temperature of 49 °C and speed of 15000 rpm and at these conditions, the predicted and experimental values of TCC were 3.79 and 3.81 mg βCE/100g, respectively, closer to each other with the desirability value 0.96 (Table 7.1.6). Similar results of high yield of carotenoids content from mango peel was found in HSD in contrast to UAE [2].

Table 7.1.5. ANOVA table for UAE

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.854422	9	0.094936	12.36815	0.0003	significant
A-Time (min)	0.065767	1	0.065767	8.568095	0.0151	
B-Temperature (°C)	0.069089	1	0.069089	9.000899	0.0133	
C-Amplitude (%)	0.508926	1	0.508926	66.30244	< 0.0001	
AB	0.0002	1	0.0002	0.026056	0.8750	
AC	5E-05	1	5E-05	0.006514	0.9373	
BC	5E-05	1	5E-05	0.006514	0.9373	
A^2	0.203097	1	0.203097	26.45933	0.0004	
B^2	0.002305	1	0.002305	0.300326	0.5957	
C^2	0.001539	1	0.001539	0.200476	0.6639	
Residual	0.076758	10	0.007676			
Lack of Fit	0.050025	5	0.010005	1.871256	0.2541	not significant
Pure Error	0.026733	5	0.005347			
Cor Total	0.93118	19				
R^2	0.91					
Adj. R^2	0.84					
C.V. %	5.88					

Table 7.1.6. Predicted and experimental values of extracted total carotenoids using high shear disperser and ultrasound assisted extraction.

High shear disperser					
Time (min)	Temperature (°C)	Speed (rpm)	Predicted values	Experimental values	Desirability
5.50	49	15000	3.79	3.81	0.96
Ultrasound assisted extraction					
Time (min)	Temperature (°C)	Amplitude (%)	Predicted values	Experimental values	Desirability
8	50	76	1.99	2.01	1

In UAE, the optimized condition for TCC extraction was time of 8 min, temperature 50 °C and amplitude of 76%, giving the predicted and experimental values of 1.99 and 2.01 mg βCE/100g TCC, respectively, which were closer to each other with the

desirability value of 1.00. Researchers have studied the effect of high pressure homogenization and ultrasound on lycopene present in tomato juice and reported that ultrasound was better and a promising technique for enhancing the bioaccessibility of carotenoids [34]. The validation of the model was done by analysing the relative deviation (Eq. 4). The relative deviation for HSD and UAE was found to be 0.52% and 0.99%, respectively. Kadiri et al. [18] reported that relative deviation of less than 10% signifies that model was valid and appropriate for further analysing. Coefficient of variations (C.V.) value expresses the deviation from the mean values and shows the reliability of the experiment. The C.V. value for HSD and UAE was 4.55 and 5.88%, respectively and recommended C.V. value of less than 10% specifies the better reliability on the results [17].

Researchers have widely reported that ultrasound is an emerging and promising technique for extraction of bioactive compounds [3] but our results revealed that application of high shear disperser is better for the extraction of the carotenoids from tamarillo peel in relation to ultrasound. Tiwari et al. [33] also observed that high shear is better for extraction of carotenoids over ultrasonication.

7.1.3.4. Effect of HPH and UAE on acid value, peroxide value, total phenolic content, in vitro antioxidant activity, and colour properties of oil

Acid value and peroxide value (PV) are considered as important parameters that determine the quality of oil. In Table 7.1.7, the acid and peroxide values of the control, HPH and UAE treated oil are reported. Applying HSD and UAE to the EVOO can cause mechanical disruption which surely affects the flavour, aroma of the EVOO and ultimately leads to oxidation to some extent. Acid value corresponds to the hydrolytic products formed during processing and peroxide value indicates the primary products from from oxidation reaction [19] . Acid value of the fresh EVOO was found to be 0.39 mg KOH/g oil. Increase in the acid value was found after HSD and UAE treatments but no significant difference was observed between the two techniques ($p < 0.05$). The PV of the fresh EVOO was found to be 8.2 (meq O_2/kg), but after applying extraction treatments, PV values increased to 9.2 meq O_2/kg in HSD treatment and 8.66 meq O_2/kg in UAE treatment. The high acid and peroxide value of HSD sample was related to higher mechanical sheer in comparison to UAE. The increase in the acid and peroxide values of the HSD and UAE treated oil may be due to the splitting of ester linkage of triglyceride

molecules during treatment [11]. Similar increases in acid and peroxide values were found in cotton seed oil after application of ultrasound [13]. According to the Codex Alimentarius standard, the acceptable limits of acid and peroxide values in the vegetable oil are below 4.0 mg KOH/g oil and 15 meq O_2/kg oil. Our results revealed that though HSD and UAE treatments change the oil quality parameters by increasing the acid value and peroxide value, the values however were within the acceptable limits and suitable for consumption [6].

The TPC present in both the extract is reported in the Table 7.1.7. Significant difference in the TPC was found. The TPC in HSD extracted oil was found to be 10.25 mg GAE/mL and in ultrasound the TPC was found to be 8.47 mg GAE/mL. An increase of 17% was found in HSD treated samples which clearly shows that greater extraction occurred in HSD treatment. Chanioti et al. [4], applied different types of extraction methods for extraction of phenolic compounds from olive pomace and reported that high shear disperser gives better result in extraction. Theoretically, HSD and UAE facilitate the extraction of bioactive compounds from the tamarillo peel, but more extraction was found in HSD samples. HSD leads to more disruption in the cell of tamarillo peel which favours more release of the bioactive compounds in the solvent. The DPPH activity in the HSD and UAE treated samples was found to be 19.63% and 16.15%. Higher phenolic content was reported to improve the *in-vitro* antioxidant activity in the tamarillo samples [31]. Carotenoids are known for possessing antioxidant activity because of their unique structure that comprises of conjugated double bonds which enables the quenching of the free radicals or singlet oxygen during metabolic processes [28]. HSD treated sample shown higher carotenoids content in comparison to UAE treated sample which directly correlated with our results of better antioxidant activity in HSD sample.

Colour is the most important property in assessing the quality of the food products. Significant difference in L*, a*, b* values of carotenoids extracted oil using HSD and UAE was found; highest value was shown by the control sample (Table 7.1.7). HSD treated samples registered higher L*, a*, b* values than UAE and control sample (Table 7.1.7). A higher value of redness and yellowness is a clear indicator of more carotenoids release during extraction in oil. Similar results of lower darkness and higher redness and yellowness in the carotenoids from carrots extracted in flaxseed seed oil using microwave as an extraction medium were reported [11]

Table 7.1.7. Total phenolic content, *in-vitro* antioxidant activity, and colour values of olive oil extract after high shear treatment and ultrasound assisted extraction.

Parameters	Untreated olive oil	Treated olive oil	
		HSD	UAE
Acid Value (mg KOH/g oil)	00.39 ± 0.01[b]	00.46 ± 0.02[a]	00.44 ± 0.01[a]
Peroxide value (meq O_2/kg)	08.24 ± 0.02[b]	09.20 ± 0.05[a]	08.66 ± 0.04[a]
TPC (mg GAE/mL oil)	00.71 ± 0.11[c]	10.25 ± 0.69[a]	08.47 ± 0.36[b]
DPPH radical scavenging activity (%)	3.32 ± 0.11[c]	19.63 ± 0.39[a]	16.15 ± 0.26[b]
L*	89.36 ± 1.23[a]	64.95 ± 0.84[c]	69.51 ± 0.51[b]
a*	2.87 ± 0.08[c]	16.55 ± 0.51[a]	12.29 ± 0.22[b]
b*	36.23 ± 0.02[c]	83.31 ± 0.21[a]	62.68 ± 0.51[b]

Values expressed as mean ± SD. Values in the superscript within a row are significantly different by ANOVA test ($p < 0.05$).

7.1.3.5. HPLC of phenolic acids

The phenolic acids present in the optimized conditions of HSD and UAE treated samples was analysed using HPLC. As seen in Fig 7.1.2, four phenolic acids were identified in HSD and UAE treated samples. The identified phenolic acids present in HSD and UAE treated sample were gallic acid, chlorogenic acid, caffeic acid and p-coumaric acid. Increase in the phenolic acids was found in the HSD treated samples except for chlorogenic acid. Probably chlorogenic acid hydrolysed to caffeic acid and quininc acid [25]. The concentration of individual phenolic acids is reported in Table 7.1.8. Espin et al. [12] reported that chlorogenic, caffeic and p-coumaric acids are found in the tamarillo from Ecuador region, while gallic acid was only found in the tamarillo from New Zealand [9]. The concentration of gallic acid in HSD and UAE treated samples was found to be 27.59 and 24.56 μg/g, respectively. In four phenolic acids, chlorogenic acid was found to be dominant in concentration in HSD and UAE treated sample. The total phenolic acid concentration in HSD and UAE was 242 and 239 μg/g (Table 7.1.8), this shows that there was insignificant difference in the concentration of phenolic acids, even though significant difference in the total phenolic content in the two extracts were observed (Table 7.1.7).

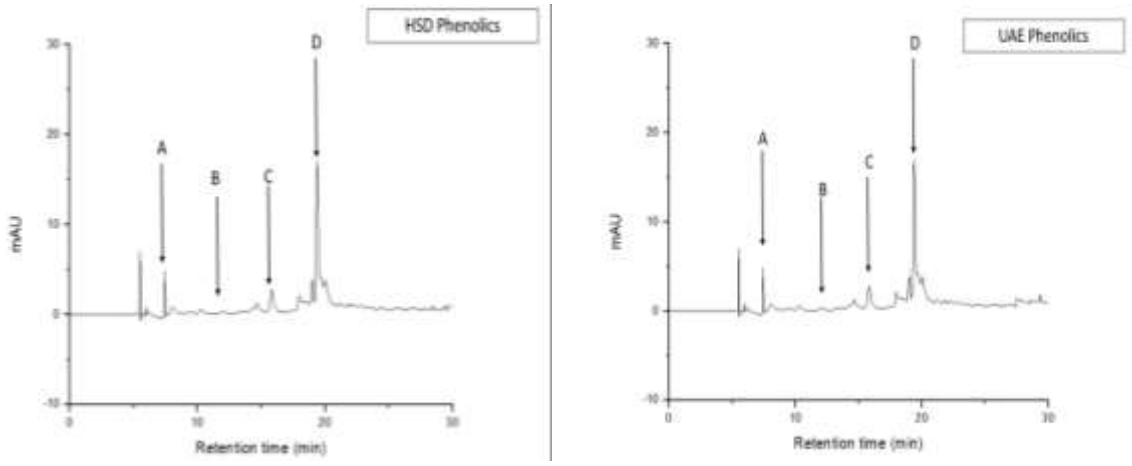


Fig. 7.1.2. HPLC chromatogram for phenolic acids present in optimized HSD and UAE sample, A: gallic acid, B: chlorogenic acid, C: caffeic acid and D: p-coumaric acid

7.1.3.6. HPLC of carotenoids

The carotenoids present in the HSD and UAE extracts using optimized conditions were analysed using HPLC. Fig. 7.1.3 shows that three carotenoids were identified in HSD and UAE treated samples. The identified carotenoids present in the sample were zeaxanthin, β-cryptoxanthin and β-carotene. An increase of 26, 14 and 25% was found for zeaxanthin, β-cryptoxanthin and β-carotene, respectively in the HSD treated sample in comparison to UAE sample (Table 7.1.8). Mertz et al. [24] studied the carotenoids composition of the tamarillo from the Ecuador region and reported that β-cryptoxanthin and β-carotene are the predominant carotenoids present in the tamarillo. The greater release of bioactive compounds in HSD also helped in the increased release of carotenoids in HSD extract than UAE extract.

Table 7.1.8. Quantification of identified phenolic acids and carotenoids in olive oil extract.

	Phenolic acids (µg/g)				**Carotenoids (µg/100 g)**		
	Gallic acid	**Chlorogenic acid**	**Caffeic acid**	**p-coumaric acid**	**Zeaxanthin**	**β-cryptoxanthin**	**β-carotene**
HSD	27.59	135.49	62.47	16.50	53.65	194.32	593.35
UAE	24.56	137.05	61.93	15.83	42.36	169.36	472.36

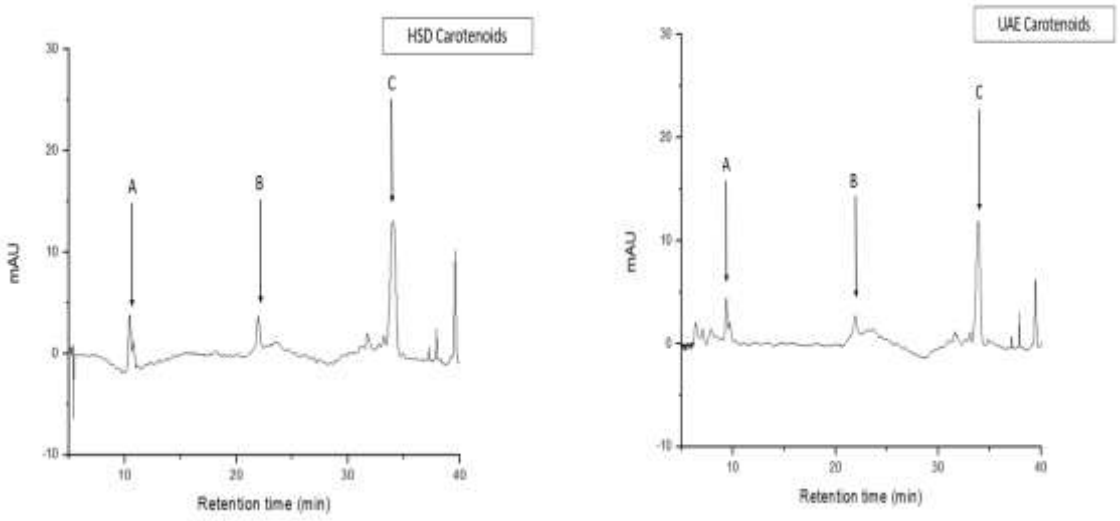


Fig. 7.1.3. HPLC chromatograms for carotenoids present in optimized HSD and UAE sample, A: zeaxanthin, B: β-cryptoxanthin, C: β-carotene.

7.1.3.7. Sensory characteristics of mayonnaise

EVOO enriched with carotenoids using optimized HSD conditions was used for the preparation of the mayonnaise. In Table 7.1.9, the sensory attributes of the carotenoids-enriched mayonnaise and control mayonnaise (without carotenoids) are given. The data showed that was no significant difference in the sensory parameters ($p < 0.05$) between the two mayonnaise samples. However, mayonnaise prepared without carotenoids incorporation got higher scores in comparison to the carotenoids-enriched mayonnaise. The overall acceptability of the two mayonnaise samples was same.

Table 7.1.9. Sensory evaluation of carotenoids enriched and without enriched mayonnaise

	Sensory parameters				
Samples	Taste	Colour	Aroma	Texture	Overall acceptability
Without Carotenoids	7.98 ± 0.85^{a}	7.83 ± 0.74^{a}	8.03 ± 0.71^{a}	8.23 ± 0.50^{a}	8.40 ± 0.77^{a}
Carotenoids enriched	7.63 ± 0.85^{a}	7.68 ± 0.96^{a}	7.81 ± 0.93^{a}	7.56 ± 0.86^{a}	7.33 ± 1.06^{a}

Values are expressed as Mean ± SD. Values in the same row with different letters are significantly different by ANOVA test ($p < 0.05$).

7.1.3.8. In-vitro digestion of mayonnaise

The *in-vitro* digestion of carotenoids-incorporated mayonnaise was done to determine the bioaccessibility of TCC and individual carotenoids. In Fig. 7.1.4, the *in-vitro* digestion of prepared mayonnaise in different phases of digestion is reported and the effect on individual carotenoids during digestion was studied. The concentration of TCC present in the undigested, gastric and intestinal phases were 1.78 ± 0.13, 0.45± 0.03 and 0.76± 0.04 mg βCE/g of sample, and the values were significantly different. The bioaccessibility of the carotenoids in gastric phase was 25%, which increased in the intestinal phase to 43%. However, initially the carotenoids content of HSD extracted oil was 3.81 mg βCE/100 g. The EVOO oil used in mayonnaise had 2.66 mg βCE/100g of carotenoids, but the extracted oil from mayonnaise was reduced to 1.78 mg βCE/100g, which indicated that there was a loss of 33% 33% during preparation of mayonnaise. The reduction in the carotenoids during processing of mayonnaise is related to many factors, like carotenoids being very sensitive to light, air, and heat, degradation can occur during the whipping step of mayonnaise preparation. In the gastric phase, the liberation of carotenoids was found to be low, which can be explained to be the effect of low pH of the digestion solution that causes a delay in the release from the sample matrix and decreases the stability of carotenoids [1]. However, in intestinal phase pH is adjusted to 7.0 and enzymes action enables for better release of the carotenoids from the sample matrix. Similar results of increase in carotenoids content from gastric to intestinal phase were reported in pumpkin by products extracted in corn oil as an extraction solvent (Lyu et al., 2021).

Cryptoxanthin and β-carotene were the most abundant carotenoids present in the mayonnaise samples in the intestinal phase. However, while analysing the individual carotenoids, zeaxanthin which was present in the HSD extracted oil was absent in the mayonnaise sample during the gastric phase of digestion. β-In Table 7.1.10, the concentration of the carotenoids in the oral, gastric and intestinal phases is reported. It was observed that more release of the carotenoids occurred in the intestinal phase than in the oral and gastric phases.

Table 7.1.10. *In- vitro* digestion of identified carotenoids in prepared mayonnaise.

Carotenoids identified	Digestion phase		
(µg/100g)	Initial	Gastric	Intestinal
β-cryptoxanthin	180.29	N.D	170.95
β-carotene	465.73	88.22	255.23

*N.D stands for not detected

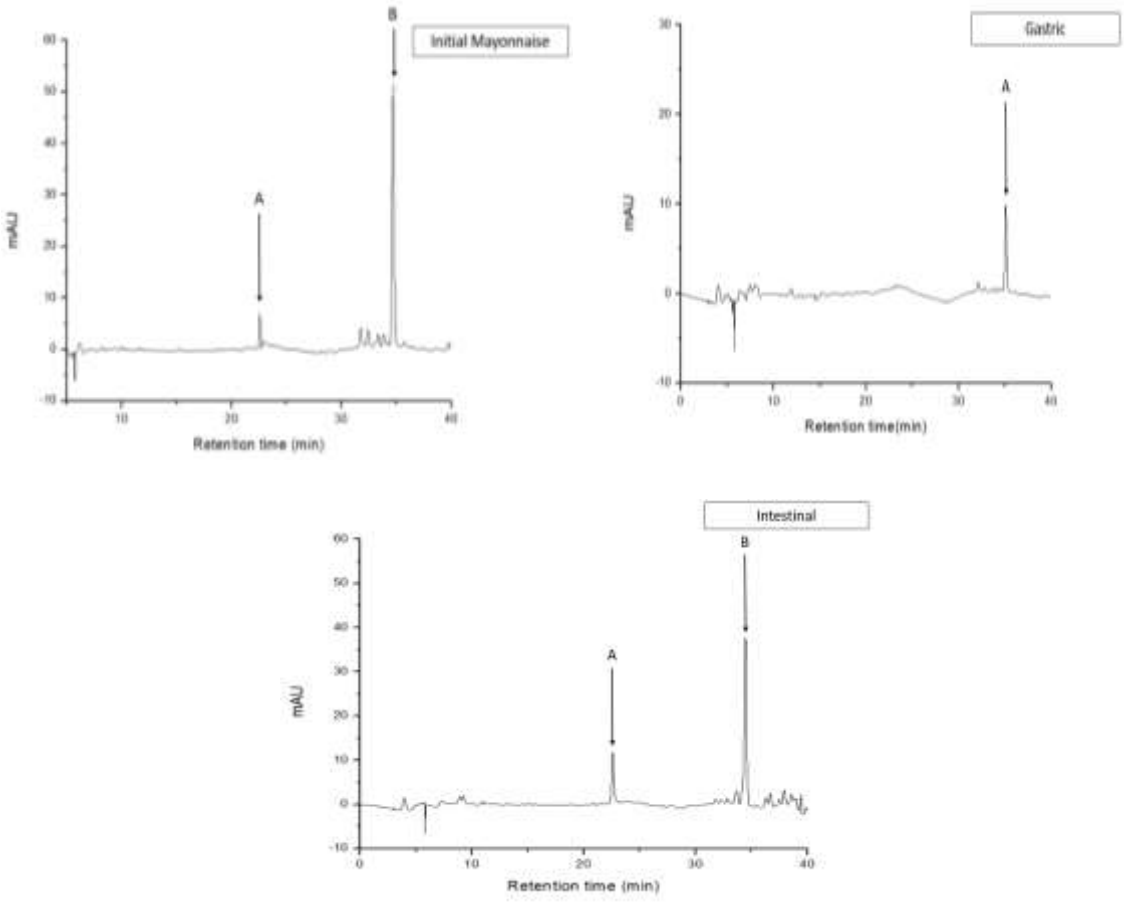


Fig. 7.1.4. HPLC of in-vitro digestion of carotenoids present in mayonnaise

7.1.4. Conclusion

Yellow tamarillo peel is a great source of bioactive compounds, especially carotenoids that provides many health beneficial properties. Optimization of extraction parameters of both HSD and UAE was done and extraction of total phenolic content and carotenoids was higher after HSD treatment than UAE treatment, The extraction of

carotenoids using the two extraction techniques caused changes in the acid and peroxide values but found to be in the acceptable range for consumption. Better extraction yield of carotenoids was found in HSD treated samples with the value of 3.81 mg βCE/100 g. Our results indicated that carotenoids extracted from tamarillo peel using EVOO as the solvent and high shear disperser as the extraction technique can be used to prepare carotenoids-enriched mayonnaise. Carotenoids-enriched mayonnaise showed acceptability for the evaluated sensory attributes. The in vitro digestibility of carotenoids was observed to be high in the intestinal phase. This is the first report on utilising yellow tamarillo peel in the development of a functional product like mayonnaise and in the process also obtain value-added product from tamarillo peel that is discarded as a waste.

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DEVELOPMENT OF CAROTENOIDS LOADED NANOEMULSION USING ULTRASONICATION

7.2.1. Introduction

An emulsion is a homogenous mixture consisting of two immiscible liquids (water and oil), which is stabilized with the help of a surfactant [7]. The emulsions are divided into two categories, viz. conventional and nanoemulsion, based on the mean particle size (PS) of the droplets [30]. If the PS is below 500 nm, it is referred to as a nanoemulsion, whereas for a size above 500 nm, it is referred to as a conventional emulsion [30]. Nanoemulsion (NE) is preferable to conventional emulsion because of its better physicochemical properties, optical transparency, and bioavailability [13]. NE possesses various advantages over conventional emulsion, like enhanced bioavailability and stability under different conditions [12]. However, the mixing of two immiscible liquids into a stable NE is a tedious process and properties like rheology, appearance, texture, stability, and flavor of the food products are affected [14]. There are many techniques for the preparation of NE, like high-pressure homogenization, ultrasonication, microfluidization, spontaneous emulsification, etc. and each technique has its own merits and demerits [12]. Of all the techniques, ultrasound is the most widely employed technique used for the development of NE. Ultrasound is a very effective, efficient, and economical lab-scale technology that is used for the formation of NE [22]. In ultrasound, the high energy input is known to produce the cavitation effects, which in turns breaks down the large droplet of emulsion into smaller droplets and ultimately NE is formed, but the other factors like ultrasonication time, emulsifier, oil, temperature etc. need to be optimized for better and stable NEs [13]. The main ingredient for NE is the stabilizer. There are many emulsifiers used for the fabrication of NEs. Among them, Tween 20 (T20, chemically called polyoxyethylene sorbitan monolaurate) is a food-grade, non-ionic surface-active agent widely employed in food industries for the development of NEs [28]. Tween 20 was used to stabilize β-carotene compounds in NE to prevent the degradation of bioactive compounds [24].

The main objective of this study was to optimize the fabrication of NE using tamarillo seed oil and the carotenoids extracted from tamarillo peel using ultrasonication as a fabrication technique and to evaluate the effect of ultrasonication on the PS and carotenoids retention under different environmental conditions.

7.2.2. Materials and methods

7.2.2.1. Plant materials

Yellow tamarillo was purchased from the local market of Kohima, Nagaland and directly brought to Tezpur University (Assam, India). Peel and seeds of the fruits were collected and stored under -20 °C until further analysis. The peel and seeds were dried at 50 °C in a tray drier (Labotech, BDI-51, B. D. Instrumentation, India). Peel and seeds were separately processed into powder form in a mixer grinder (Philips HL Model No. 1632, India) and kept at -20 °C in zip lock pouches of LDPE (low density polyethylene) until further use.

7.2.2.2. Extraction of tamarillo seed oil

Powdered tamarillo seed sample was used for the extraction of oil using hexane as the extraction solvent. The oil was extracted in a Soxhlet apparatus (SOCS PLUS, India) for 4 h. After extraction, the solvent was evaporated, and fat was stored in an amber coloured glass vial until further analysis. The yield of tamarillo seed oil was 23.15 ± 3.25 g/100g of dry weight.

7.2.2.3. GC-MS analysis of the tamarillo seed oil

The sample preparation for fatty acid determination of the tamarillo seed oil was done according to the method adopted by Durante et al. [10] with some modification. Briefly, 40 µL of tamarillo seed oil was mixed with 2 mL boron trifluoride in methanol (14%) solution in a 15 mL falcon tube. The falcon tube was placed in a water bath at 55 °C for 1.5 h with continuous stirring. After incubation, 2 mL of saturated sodium bicarbonate was added in the mixture followed by the addition of 3 mL of n-hexane. The above extract was separated and GC analysis was done.

The fatty acid composition of the tamarillo seed oil was determined using Agilent 7890 gas chromatograph equipped with MS (Jeol, Accu TOF GCV) and flame ionization detector. The initial temperature was kept at 200 °C for 1 min and subsequently increased to 230 °C at 1.5°C mL/min and then held for 1 min. The flow rate for nitrogen as the

carrier gas was kept at 1 mL/min. The compounds were identified using online NIST-library spectra and published MS data literature.

7.2.2.4. Extraction of carotenoids

Dried tamarillo peel powder was used for the extraction of carotenoids. The extraction of carotenoids was done according to Baria et al. [5], with some modification. The carotenoids were extracted in tamarillo seed oil using high shear disperser (T25 Ultra-Turrax IKA Works, Inc., NC, USA) at 15000 rpm, for 5 min with a cut-off temperature of 50 °C. The carotenoids-enriched oil was stored in an amber coloured glass vial until further analysis. This oil was used for the fabrication of the NE for the experiments. The total carotenoids content in the tamarillo sample was calculated according to the method adopted by Orqueda et al. [23] with some modifications. Briefly, 1mL of sample was mixed into 10 mL of hexane:acetone:ethanol (2:1:1 v/v/v) and centrifuged at 7000 x g for 10 min. The upper layer of mixture was separated carefully and adjusted to 10 mL using hexane. The absorbance of hexane containing carotenoids was read at 450 nm (Thermo-Fischer Evolution A600) and values were reported as mg of β-carotene equivalents (mg β-CE)/ g of sample.

7.2.2.5. Preparation of carotenoids-loaded NE

7.2.2.5.1. Experimental design

A three factor Box-Behnken design (BBD) was used for the fabrication of the carotenoids-loaded NE. Three independent variables were taken for the optimization of the study: oil concentration (1-10%), surfactant (1-3%), and treatment time (10-30 min), and the dependent variables used for the optimization were particle size (PS), polydispersity index (PDI), and encapsulation efficiency (EE). The design was executed to minimize the PS and PDI and maximize the EE. Validation of the model was done based on comparing experimental and predicted values.

7.2.2.5.2. Preparation of NE

The oil in water NEs were prepared by mixing the carotenoids extracted from tamarillo seed oil, surfactant, and double distilled water. The aqueous phase was prepared by mixing double distilled water and surfactant, whereas tamarillo seed oil with carotenoids extract was the dispersed phase. The concentration of dispersed phase was mentioned in the experimental deign (Table 7.2.1). A probe type ultrasonicator (Rivotek,

Model no. 1700, India) equipped with 12 mm probe, maximum power (250 W), amplitude (80%) and frequency mode (5 s active, rest 5 s) was used for the fabrication of NE. The cut-off temperature in ultrasound was set at 40 °C and the fabricated NEs were transferred to 50 mL centrifuge tubes and stored in dark under refrigeration temperature until further analysis.

7.2.6. Characterization of NE

7.2.6.1. Determination of PS and PDI

The determination of PS and PDI of the fabricated carotenoid-loaded NEs was done according to the method adopted by Sotomayor-Gerding et al. [29] with some modification. Before measurement, the NEs were diluted in MilliQ water (1:100) and then analysed by dynamic light scattering (DLS) (Anton Paar, Litesizer 500) at 25 °C. Same instrument was used to determine zeta potential of the NEs.

7.2.6.2. Encapsulation efficiency

The encapsulation efficiency of the fabricated NE was determined according to the method adopted by Orqueda et al. [23] with some modifications. The sample was mixed with the extraction solvent (acetonitrile:methanol 70:30) for release of carotenoids from NE. After release, the sample was filtered through 0.2 µm pore size. The sample was read at 450 nm in UV-Vis spectrophotometer (Cary 60, UV-Vis spectrophotometer, Agilent Technologies, Germany). The total carotenoids of the NE were expressed in mg β-carotene/100 mL of NE (Eq. 1).

$$EE\ (\%) = \frac{Initial\ carotenoids\ - Carotenoids\ in\ aqueous\ phase}{Initial\ carotenoids} \times 100 \qquad (1)$$

7.2.2.7. Stability of carotenoids-loaded NE against pH and ionic strength

The stability of the carotenoids-loaded NE was evaluated against pH and ionic strength according to method adopted by Zhu et al. [34] with some modifications. The freshly prepared carotenoids-loaded NE was adjusted to pH values of 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 using disodium hydrogen phosphate-citrate buffer. For determining the ionic strength of the NE, freshly prepared NE was added with an aqueous 3 M NaCl to give the concentration of 0.01, 0.02, 0.05, 0.10 and 0.20 M and left for incubation at 25 °C for 4 h. After incubation, the PS of the NE was determined.

7.2.2.8. Physical stability of carotenoids-loaded NE at stimulated gastrointestinal conditions

The physical stability of carotenoids-loaded NE was calculated according to the method adopted by Zhu et al. [34] with some modification. Freshly prepared carotenoids-loaded NE at optimized condition was diluted 10 times with simulated gastric fluid (pH 2) and kept for incubation in a shaking incubator at 37 °C for 2 h. After gastric phase, the sample was again diluted 10 times with the simulated intestinal phase (pH 7) and kept for incubation for 2 h at 37 °C. The TCC and PS were determined before and after each digestion process.

7.2.2.9. Effect of storage temperature on stability of the carotenoids-loaded NE

The effect of storage temperature on the physical and chemical stability of the carotenoids-loaded NE was analyzed according to Luo et al. [18] with some modification. The sample was stored at 4, 25 and 55 °C for up to 14 days. The PS of the NE was measured on 0, 3, 7, 10, and 14 days of storage (as described in Sec 7.2.6.1).

7.2.2.10. Transmission electron microscopy

The morphology of the carotenoids-loaded NE was analyzed by transmission electron microscopy (TECNAI G2 20 S-TWIN, USA). Briefly, the NE was dropped on the copper mesh, dried and covered with the carbon film and stained with phosphotungstic acid and the excess water was removed very carefully from the mesh using a filter paper. The mesh was kept for drying at 50 °C and the image of the NE was observed using the software (TIA, FEI imaging software) and analysed [32].

7.2.2.11. Statistical analysis

All experiments were performed in triplicate and the experimental results are presented as mean ± standard deviation. Data were analysed by ANOVA (analysis of variance) and Duncan multiple range test with statistical significance ($p < 0.05$) using statistical tool SPSS version 24.0 (SPSS 24.0, IBM Corporation, USA) [26]. The other statistical parameters, including coefficient of determination (R^2), root mean squared error (RMSE), relative deviation (R_d) and coefficient of variation (C.V. %) were calculated using Eq. 2-5, respectively.

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

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(predicted - experimental)}{n}} \quad (3)$$

$$R_d = \frac{100}{n}\sum_{i=1}^{n}\frac{|experimental - predicted|}{experimental} \quad (4)$$

$$C.V.(\%) = \frac{Standard\ deviation}{Mean} \times 100 \quad (5)$$

7.2.3. Results and discussion

7.2.3.1. GC-MS analysis of the tamarillo seed oil

The identification of the fatty acid methyl esters present in tamarillo seed oil was done using GC-MS. In Fig 7.2.1, the fatty acids in the chromatogram were identified using NIST Mass Spectral Library as linoleic acid, oleic acid, palmitic acid, and stearic acid. Researchers studied the extraction of tamarillo seed oil using supercritical CO_2 and reported the presence of linoleic acid, oleic acid, palmitic acid, stearic acid, linolenic acid and palmitoleic acid [9]. Yilmaz et al. [31] reported that the major fatty acids in tamarillo seed oil were palmitic (14.43%), stearic (5.93%), oleic (22.26%), and linoleic (53.63%) acids.

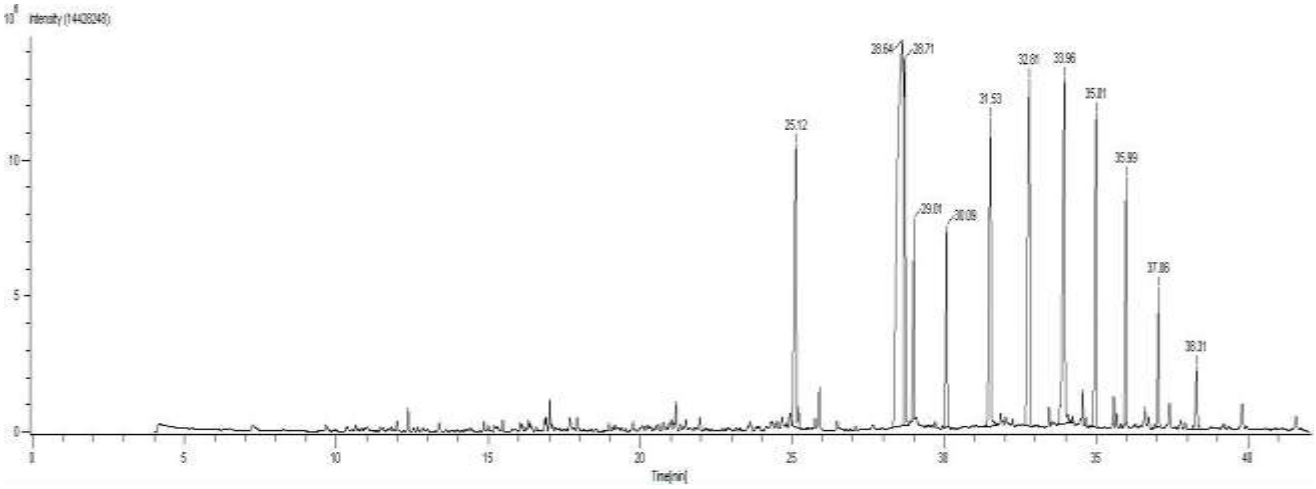


Fig. 7.2.1. GC-MS chromatogram of the tamarillo seed oil

7.2.3.2. Fitting the model in experimental design

Box-Behnken design was used for the optimization of carotenoids-loaded NE using ultrasonication as the fabrication technique. In this experimental design, three independent variables were taken (oil concentration, surfactant concentration, and ultrasonication time) for predicting the experimental set (Table 7.2.1). The quadratic model was found to give a significant fit with $p< 0.05$ for optimization process and the residual error was found to be non-significant. The R^2 (coefficient of determination) and adjusted R^2 were used to check the adequacy of the model and p-values were used to analyse the effect of independent variables on the dependent variables. Analysis of variance was done at a confidence level of 95%. The R^2 of the response, viz. PS, PDI, and EE was found to be 0.97, 0.94 and 0.92, respectively (Table 7.2.2). All the responses were found to be best fitted in the quadratic model.

Table 7.2.1. Experimental data with their resposne in Box-Behnken design of carotenoids loaded NE

Run	A: Oil Concentration (%)	B:Surfactant Concentration (%)	C:Time (min)	PSA (nm)	PDI	EE (%)
1	1	1	20	306	0.57	85
2	10	1	20	555	0.24	62
3	1	3	20	305	0.44	85
4	10	3	20	264	0.29	80
5	1	2	10	246	0.46	72
6	10	2	10	291	0.36	52
7	1	2	30	217	0.46	71
8	10	2	30	296	0.24	52
9	5.5	1	10	405	0.31	55
10	5.5	3	10	253	0.28	60
11	5.5	1	30	299	0.21	71
12	5.5	3	30	205	0.25	67
13	5.5	2	20	216	0.28	85
14	5.5	2	20	184	0.31	79
15	5.5	2	20	202	0.24	82
16	5.5	2	20	163	0.29	83
17	5.5	2	20	228	0.27	75

7.2.3.3. Effect of independent variables on PS

The effect of independent variables on the PS of carotenoids-loaded NE can be assessed from Fig. 7.2.2 (X1, X2 and X3). The PS size varied from 163-555 nm (Table 7.2.1), which was affected by the three independent variables, viz. oil concentration, surfactant concentration, and ultrasonication time. PS analysis is the most basic and convenient method for determining the stability of NE [33] The mathematical relationship of the interaction of the independent variables on PS is reported in Table 7.2.2. It was observed that oil concentration had positive impact on PS, whereas surfactant and ultrasonication time had negative impact on PSI. However, combined impact of surfactant and ultrasonication had positive impact on PS. It was found that increase in oil content promoted an increase in the PS of the emulsion. Researchers reported that smaller the size of the NE possesses better stability [20]. It was observed that increasing the surfactant concentration and ultrasonication time helped to reduce the PS of the NE. An increase in the concentration of surfactant decreased the interfacial tension between the dispersed and continuous phase that helped in the reduction of the PS [20]. Low concentration of surfactant and higher oil content was observed to lead to an increase in the PS, as seen in Fig. 7.2.2 (X1). The low concentration of surfactant probably did not allow the proper mixing of the dispersed phase into continuous phase. However, it is also reported that increasing the surfactant concentration beyond required amount causes the PS to increase and make the NE unstable [2]. An increase in ultrasonication time enhanced the kinetic energy of the system which in turn led to particle-particle collision and helped to get the desired PS of NE [1]. Our results of decrease in PS with increase in the surfactant concentration and ultrasonication time agreed with the results reported by Mehmood et al. [21].

7.2.3.4. Effect of independent variables on PDI

The effect of independent variables on PDI are reported in Fig. 7.2.2 (Y1, Y2 and Y3). All the independent variables individually showed significant but negative impact on the PDI. PDI gives an indication of the heterogeneity of the PS [17] and was found to range from 0.21-0.57 (Table 7.2.1). It was found that increase in oil and surfactant concentration had positive impact on the PDI. The increased value of the PDI indicates that more particles are present in the solution but of non-uniform size, which is an undesirable property for NE. Our results showed that the negative impact of oil

concentration and ultrasonication time led to the decrease in the PDI values. Similar results of decrease in the PDI values while increasing the oil and ultrasonication time was reported by Ahmad et al. [1]. The respective effect of oil concentration and ultrasonication is shown in Fig. 2 (Y3). The uniformity of the particles present in the NE is reflected by the low PDI value [25]. An emulsion having PDI more than 0.3 is termed as polydisperse and less than 0.3 is classified as monodisperse; monodisperse NE are more stable [11].

Table 7.2.2. Optimized response polynomial equations fitted in quadratic model of carotenoids loaded NE

Response	Equation	R^2	Adjusted R^2
Particle size	$PS = 198.60 + 41.50 \times A - 67.25 \times B - 22.50 \times C - 72.50 \times A \times B + 8.50 \times A \times C + 14.50 \times B \times C + 65.45 \times A^2 + 93.45 \times B^2 - 1.55 \times C^2$	0.97	0.93
PDI	$PDI = 0.28 - 0.10 \times A - 8.750E - 003 \times B - 0.031 \times C + 0.045 \times A \times B - 0.030 \times A \times C + 0.018 \times B \times C + 0.11 \times A^2 - 5.250E - 003 \times B^2 - 0.010 \times C^2$	0.94	0.86
Encapsulation efficiency	$EE = 80.80 - 8.38 \times A + 2.37 \times B + 2.75 \times C + 4.50 \times A \times B + 0.25 \times A \times C - 2.25 \times B \times C - 2.15 \times A^2 + 0.65 \times B^2 - 16.90 \times C^2$	0.92	0.82

7.2.3.5. Effect of independent variables on EE

The effect of independent variables is reported in Fig. 2 (Z1, Z2 and Z3). All the independent variables showed positive and significant impact on EE. The EE ranged from 52-85%. It is clear that oil concentration had significant impact on the encapsulation efficiency and increase in the oil concentration led to enhanced encapsulation of carotenoids in NE (Table 7.2.2). It was found that increasing the surfactant concentration and ultrasonication time had positive impact on the EE of carotenoids-loaded NE. Response surface 3D graphs showed positive relation between oil and surfactant concentration (Fig. 7.2.2Z1), and between surfactant concentration and ultrasonication time (Fig. 7.2.2Z2). Surfactant helped in the entrapment of the carotenoids that were present in the oil and increase in ultrasonication time favoured the formation of NE. Researchers reported that increase in sonication time enhances the kinetic energy of the

system, which leads to particle-particle collision, whereas increase in surfactant concentration helps in the stabilization of NE by preventing particle coagulation [1].

7.2.3.6. Optimization of process conditions

In Table 7.2.3, the predicted and experimental values of the carotenoids-loaded NE using ultrasonication as extraction technique are given. For optimization, all the independent variables were kept within the range, while among the responses, PS and PSI were minimized and EE was maximized. The PS, PDI, and EE were found to be 199 nm, 0.27, and 81.95%, respectively, and their predicted values were 198 nm, 0.26, and 81.45%, respectively. Our results of predicted and experimental values were close for each response. The EE of 81.45% and PS of 198 nm suggested that carotenoids were effectively encapsulated in the NE with good stability. The desirability of the optimization was found to be 0.89 (Table 7.2.3). The relative deviation (R_d) of the all the dependent variables i.e., PS, PDI, and EE was found to be 0.50, 3.70, and 1.48, respectively, calculated using Eq. 2. Relative deviation value less than 10% signified that model was valid and appropriate for further analysis [15].

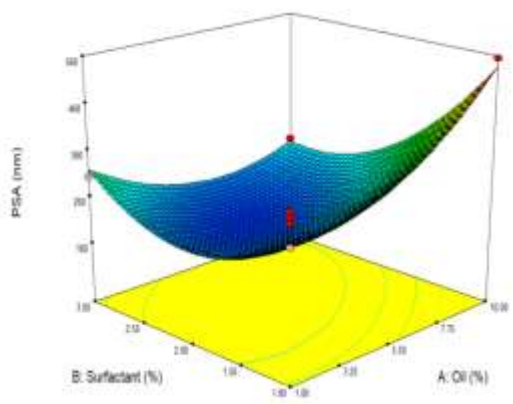
Table 7.2.3. Predicted and experimental values using optimized conditions for PSA, PDI and EE.

Oil (%)	**Surfactant (%)**	**Time (min)**	**PSA (nm)**		**PDI**		**EE (%)**		**Desirability**
			Pred.	Exp.	Pred.	Exp.	Pred.	Exp.	
6.17	2.74	21	198	199	0.26	0.27	92.90	91.54	0.88

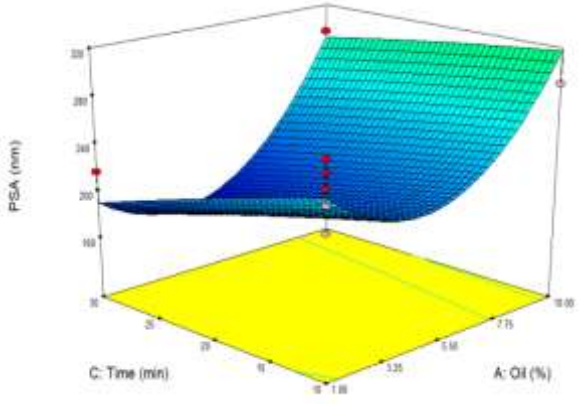
Pred. stands for predicted value, Exp. Stands for experimental value

7.2.3.7. Zeta potential of optimized NE

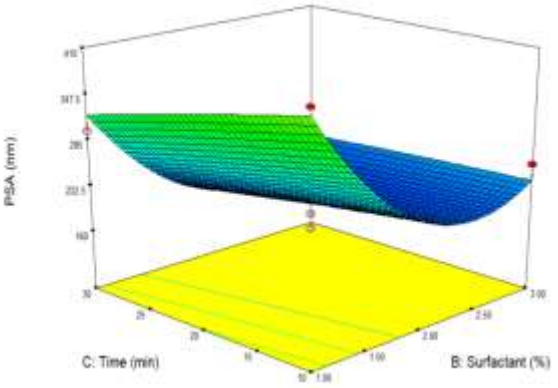
Zeta potential determines the surface charge present on the droplet surface and it is an important measurement that determines the stability of the NE [11]. The zeta potential value of the optimized carotenoids-loaded NE was found to be -32.61. Banasaz et al. [4] reported that zeta potential value less than -30mV and more than 30mV is desirable for a stable NE while preventing the agglomeration of particles.



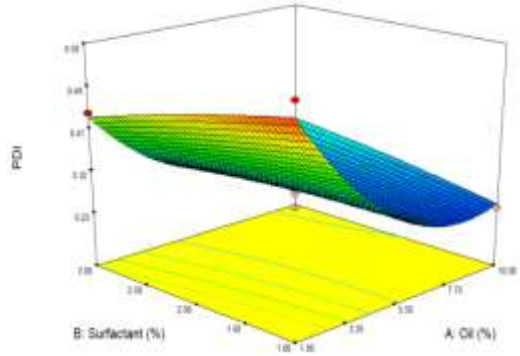
X1



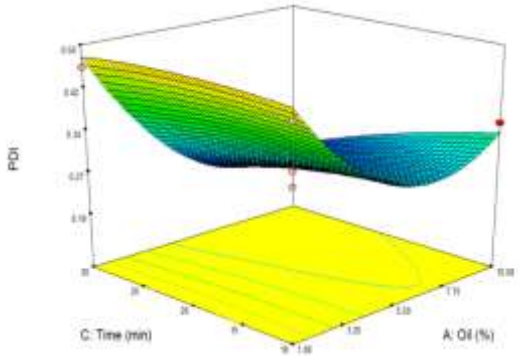
X2



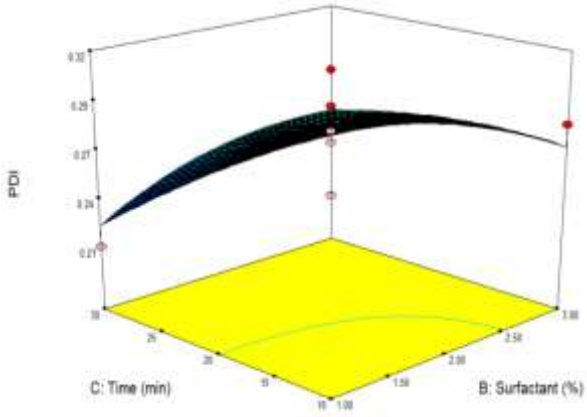
X3



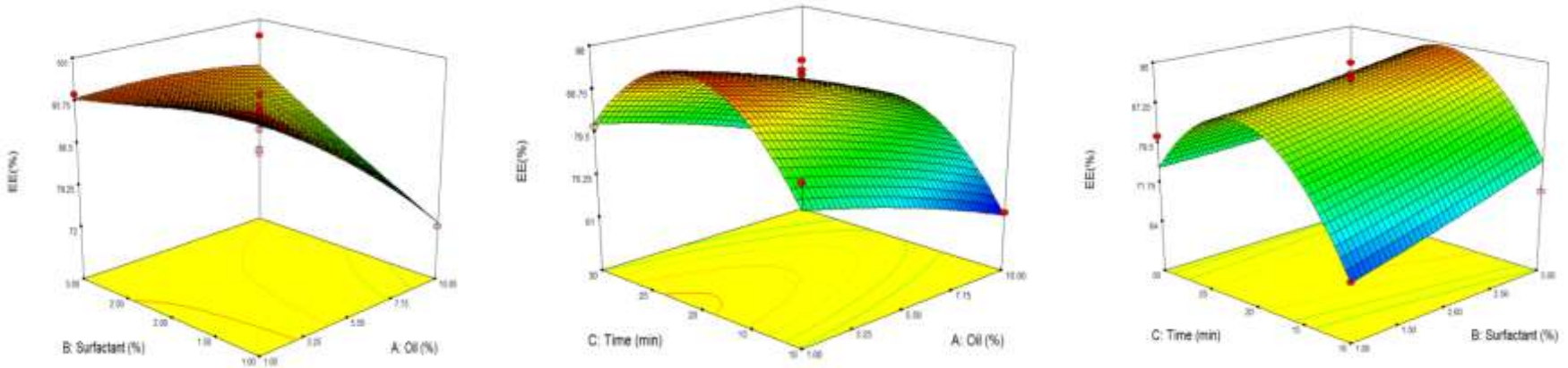
Y1



Y2



Y3



Z1 Z2 Z3

Fig. 7.2.2. Response surface graphs of carotenoids loaded NE for PS (X1, X2 and X3), PDI (Y1, Y2 and Y3), and EE (Z1, Z2 and Z3).

7.2.3.8. Effect of pH on PS and carotenoids stability

The change in PS of the optimized carotenoids-loaded NE in different pH solutions was evaluated. As seen in Fig. 7.2.3(A1), the PS of the NE changed at different pH. However, the PS remained below 300 nm in all the pH solutions, which showed the stability of the NE in different pH environments. Further, the NE remained most stable at neutral pH and maintained its PS. Similar results of change in the PS with change in pH were reported for linseed oil NE [29].

In Fig. 7.2.3 (A2), the effect of pH on the total carotenoids content can be understood. It was observed that at low pH values of 2 and 3, the release of the carotenoids was less than 60%. However, on increasing the pH values from 4 to 7, carotenoids retention in the NE was enhanced. Sotomayor-Gerding et al. [29] reported that particle coalescence and instability of astaxanthin NE occurred at pH 2.0. Another group of researchers reported that carotenoids-loaded NE attained better stability and exhibited good release at neutral pH [6] because in acidic environement the carotenoids are pronated and chances of undergoing cis-trans isomerization is increased drastically [24]. Thus, our result of high release of carotenoids from pH 4 onwards was found to be in agreement with the above reported study.

7.2.3.9. Effect of ionic strength on PS and carotenoids stability

In Fig 7.2.3 (B1), the PS and carotenoids stability of the optimized carotenoids-loaded NE at pH 7.0 and 25 °C were determined in solutions of different ionic strength ranging from 0.01 to 0.06 mol/L. It was observed that increasing the ionic strength led to a gradual increase in the PS of the NE. At lower ionic concentrations, the change was very minimal and PS was 221 nm but higher ionic concentration enlarged the PS to 290 nm because at higher ionic strength, agglomeration of particles increases and consequently the destabilization rate of NE increases gradually [24].

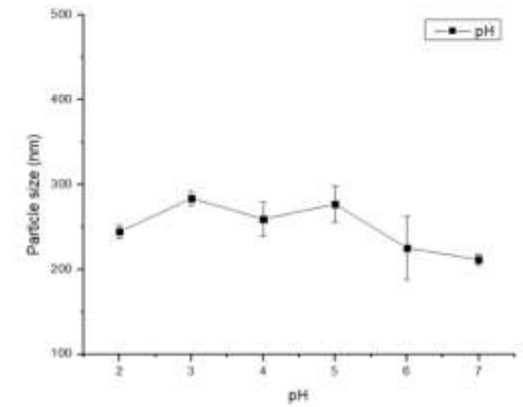
In Fig 7.2.3 (B2), the effect of ionic strength on carotenoids retention was determined. It was observed that with an increase in ionic strength, carotenoids retention in the NE was negatively impacted. At higher ionic concentration, carotenoids are not stable and degradation of occurs. Qian et al. [24] reported that at high ionic strength more fading of β-carotene-loaded NE was observed because at high salt concentration leads to destabilize the NE. At low salt concentration, the electrostatic repulsion between NE was

so strong but as the ionic strength increased the hydrophobic and Van der waals forces increase gradually cuase to break the NE.

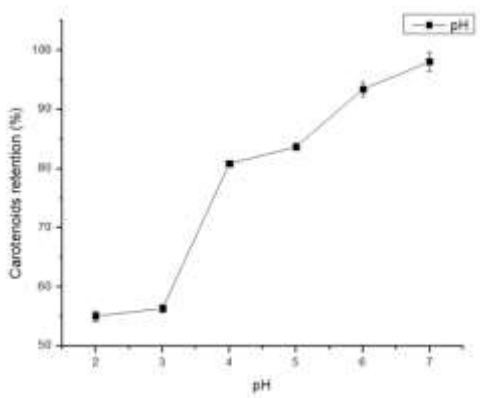
7.2.3.10. Effect of temperature on PS and carotenoids release during storage

Three different temperatures (4, 25, and 55°C) were used to determine the storage stability of the carotenoids-loaded NE for up to 14 days. The PS of carotenoids-loaded NE was noticed to increase at higher temperature, however, no visual changes and separation of phases were observed in the NE. The upsurge in PS at higher temperature might be due to the agglomeration of particles. Similar results of increase in the average PS with respect to temperature were reported for linseed microemulsion [3]. In Fig. 7.2.3 (C1), on 14^{th} day, the PS was found to be 290, 351, and 490 nm on storage at 4, 25, and 55°C, respectively. Chances of phase inversion as well as chances of coalescence in NE increase when non-ionic surfactant-coated NE is stored at higher temperature for a long period [24]. Qian et al. [24] therefore, recommended storing the NE at lower temperature. Contrary to it, our results indicated that carotenoids-loaded NE was stable at 55°C for 14 days.

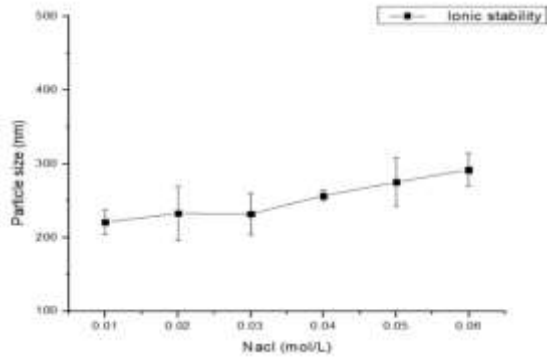
Our results show that the fall in carotenoids level in the NE occurred at all temperatures during the first 3 days and thereafter the fall was gradual, but highest fall was found at 55 °C, as seen in Fig. 7.2.3 (C2). Qian et al. [8] explained that, at higher temperature the chances of the breakdown of the NE are increased, which ultimately affects the carotenoids retention. Almost whole of β-carotene degradation was observed in carotenoids-loaded NE stored at 50 °C after 30 days [8]. Zhou et al. [32] reported that β-carotene-loaded NE was more stable in semisolid oil rather than liquid oil. Therefore, our results of GC-MS of tamarillo seed oil have shown the presence of palmitic acid (saturated fatty acid), which has shown to be a better dispersed phase to fabricate the carotenoids loaded NE.



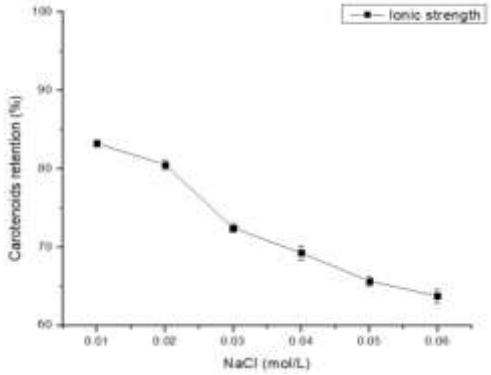
A1



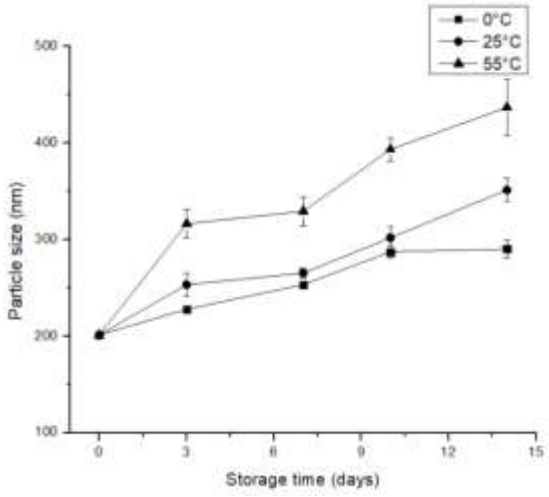
A2



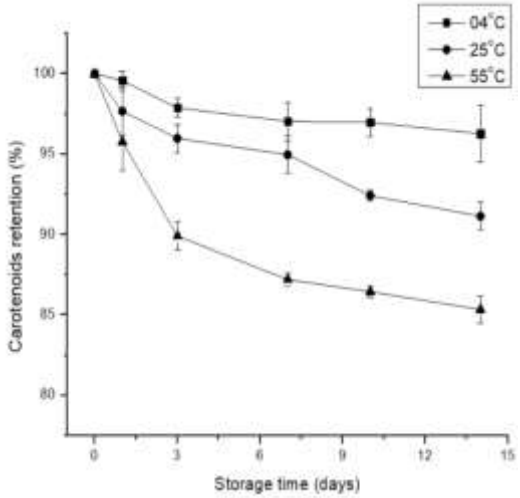
B1



B2



C1



C2

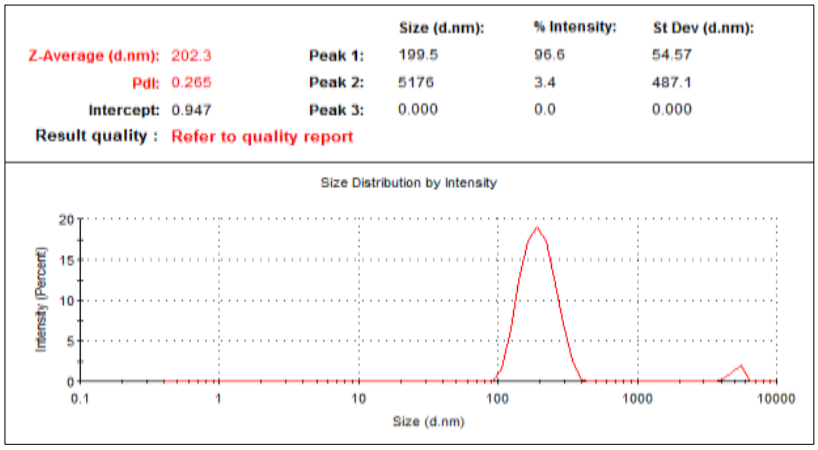
Fig. 7.2.3. Effect on PS (A1) in different pH, (B1) ionic strength, and (C1) storage temperature, and carotenoids retention (A2) in different pH, (B2) ionic strength, and (C2) storage temperature.

7.2.3.11. In-vitro digestion of NE

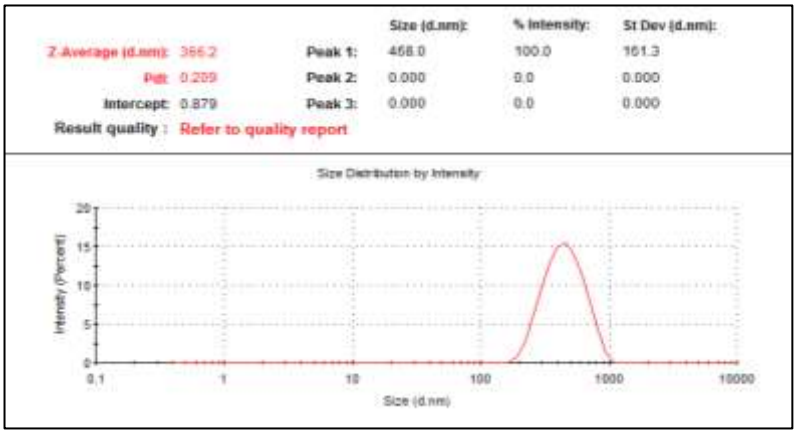
The particle size of the NE was evaluated at each phase of digestion. The PS of the initial, gastric, and intestine phase was 201, 468 and 579, respectively (Fig. 7.2.4). It was observed that an increase in PS occurred during digestion; this increase was due to the action of enzymes on the NE [27] and change in the pH of the digestive fluids. The PS was increased as the particles moved to gastric and intestinal phases. Sotomayor-Gerding et al. [29] reported that PS of the NE was distorted when treated with different pH and enzymes. The other main reason for increase in the PS after digestion is related to fatty acids; these fatty acids are released during and after the digestion process and causes an agglomeration of the droplets [19]. It was reported that after intestinal digestion the PS of the astaxanthin loaded NE increased from initial level of 178 nm to 879 nm [29]. The TCC of the NE was 0.78 mg βCE/100 mL, however during digestion the TCC of the gastric and intestinal phase was found to be 0.1 and 0.45 mg βCE/100mL, respectively. HPLC data revealed that β-cryptoxanthin and β-carotene were the major carotenoids present in the NE (Fig. 7.2.5). In the undigested NE, the concentration of β-cryptoxanthin and β-carotene was 26 and 125 µg/100 mL, respectively (Table 7.2.4), whereas in the intestinal phase the concentration was 13 and 61 µg/100 mL, respectively. Researchers reported that decrease in the release of TCC in gastric and intestinal phase was due to the action of enzymes and change in pH but the increase of the released TCC in the intestinal phase (pH 6-7) was related to the lipid digestion [16].

Table 7.2.4. Concentration of carotenoids in *in-vitro* digestion assessed by HPLC

Carotenoids identified (µg/100 mL)	Initial	Gastric	Intestinal
β-cryptoxanthin	26.62	0.00	13.32
β-carotene	125.56	20.51	61.11



Initial



Gastric

Intestine

Fig 7.2.4. Particle size of *in-vitro* digested sample

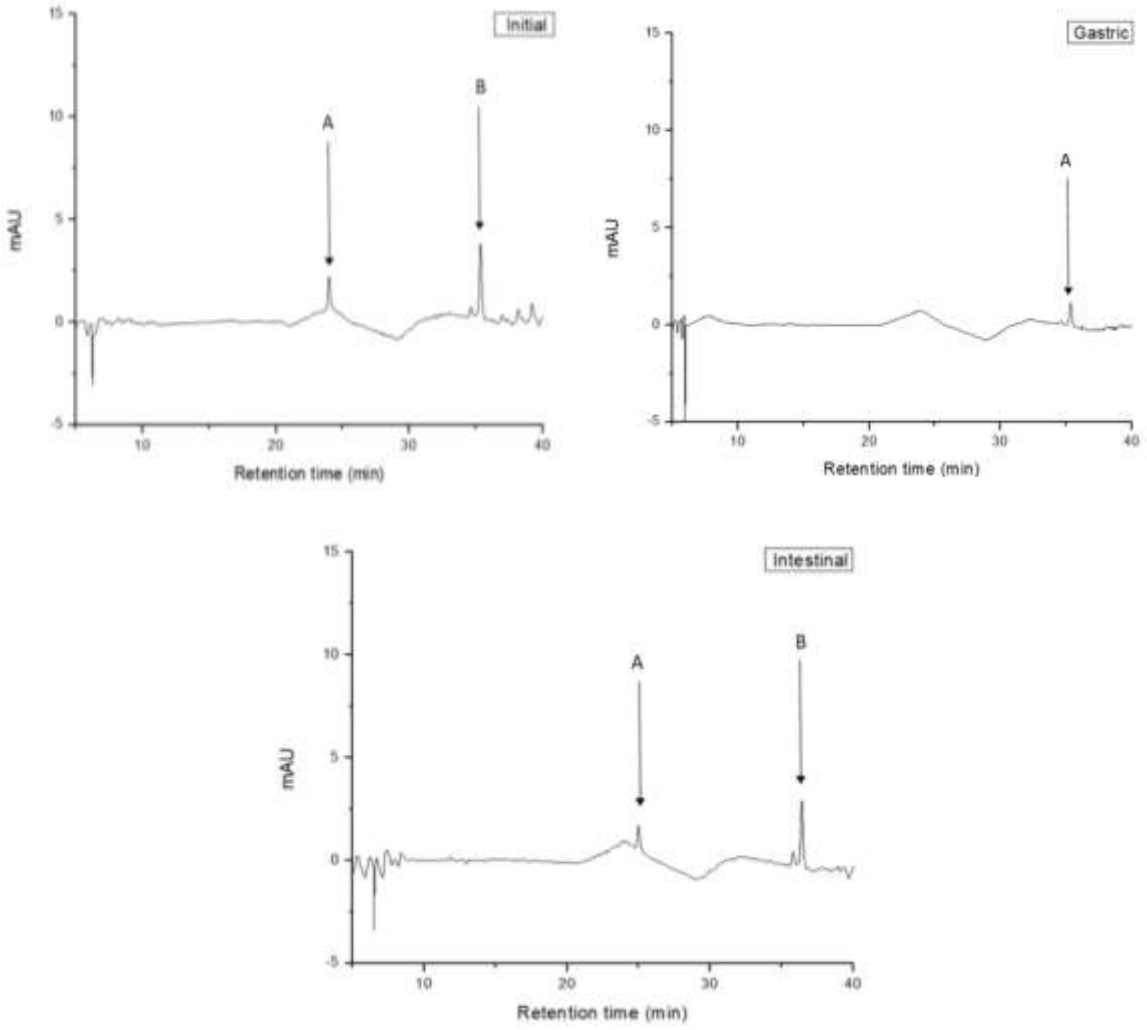


Fig. 7.2.5. HPLC chromatograms of in vitro digestion of carotenoid loaded NE

7.2.3.12. TEM

DLS is considered to be the most convenient and easy method to detect the size of the NE but, TEM is recommended for reconfirming the accuracy of the DLS results [19]. In Fig. 7.2.6, the TEM results showed that the size of the particle was between 218 and 220 nm, however, many particles smaller than 220 nm were also visible; these were uniform in shape and uniformly disbursed. The difference between the TEM and DLS results was because TEM discloses the local PS whereas; DLS reveals the average PS of the NE [19]. Thus, TEM results are in agreement with our DLS results of the NE obtained through the optimization process.

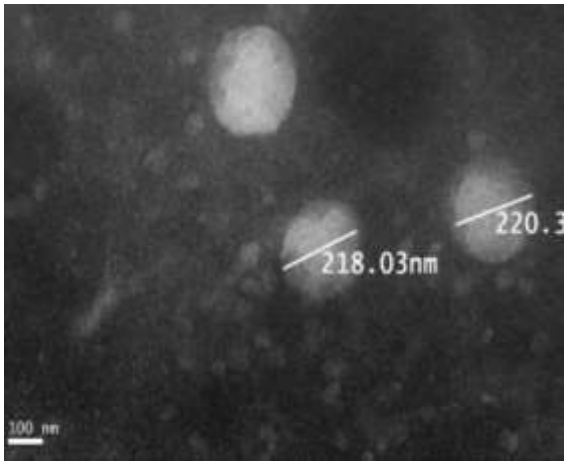


Fig. 7.2.6. TEM iamges of carotenoids loaded nanomeulsion

7.2.4. Conclusion

The optimized carotenoids-loaded NE fabricated by ultrasonication technique exhibited good encapsulation efficiency and emulsion stability. β-cryptoxanthin and β-carotene were the major carotenoids. Carotenoids retention in the NE was enhanced at higher pH even though PS increased. However, ionic strength, and storage temperature of 55 ℃ adversely affected carotenoids retention in the NE. The PS was increased during in vitro digestion and more carotenoids were released in intestinal phase in comparison to gastric phase. This study showed that the tamarillo seed is rich in oil and this oil can be used as a solvent to extract the carotenoids that are present in good quantity in the peel of yellow tamarillo. Seeds are a by-product in the tamarillo processing unit and there is scope for its judicious utilization. One such way can be taking the seed oil for the fabrication of carotenoids-loaded NE using the carotenoids extracted from the peel of yellow tamarillo, another by-product of processing.

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