"SUN PHARMA SCIENCE FOUNDATION AWARD"

1. Title: Carotenoids from carrot bio–waste: its extraction and encapsulation in emulsion based delivery system for food and pharmaceutical applications

2. Introduction

Carotenoids are lipid-soluble tetraterpenoids containing forty carbon atom chain. This is the backbone of the molecule from which other carotenoids are derived. They are characterized by a long aliphatic polyene chain composed of eight isoprene units. The end chain may be cyclic or complemented with oxygen containing functional group. Carotenoids are red, orange and yellow non-polar pigments found embedded in the membranes of chloroplasts and chromoplasts. These different shades of carotenoids depend on the presence of conjugated double bonds and functional groups (Rodriguez – Amaya *et al*., 2004). The carotenoids are classified into carotenes and xanthophylls based on the chemical structure. Carotenes are hydrocarbon carotenoids which contain only carbon and hydrogen in its polyene chain e.g. α-carotene, β-carotene, ɤ-carotene and lycopene, while xanthophylls are oxygenated carotenoids which contain excess oxygen in comparison to carotenes e.g. astaxanthin, lutein, violaxanthin, zeaxanthin, neoxanthin etc. (Sigurdson *et al*., 2017). Carotenoids are synthesized by plants and microorganisms, but not by the animals. Hence, animals derive carotenoids from plants itself (Bartley *et al*., 1995). There are more than 700 carotenoids pigments occurring in nature. The carotenoids have very essential role in biological functions of plants and animals. These carotenoids are accessory factors and are one of the important phytonutrients. Carotenoids are important group of pigments which are precursor of Vitamin A (Boeing *et al*., 2012), besides possessing very good antioxidant activity. It has been proposed that carotenoids are very good chemo preventive agents and lowers the risk of cancer due to its ability to quench singlet oxygen (Colditz *et al*., 1985). The major source of carotenoids mainly includes fruits and vegetables. Papaya, melons, peaches, squash, sweet potato, yam are the sources which contain carotenoids in moderately bioavailable form, whereas in carrots, tomato, spinach, pepper these are least bioavailable. Green micro-algae is also a very good source of carotenoids.

Carrot is one of the important root vegetables reported to be a good source of carbohydrates and minerals like calcium, phosphorus, iron and magnesium (Gopalan *et al*., 1980). It is rich in bioactive compounds like carotenoids and dietary fibers and possess appreciable levels of several other functional components having significant health-promoting properties. According to National Horticultural Database (NIH, 2015), China is the major

carrot producing country in the world and India ranks at 13th position. There are different varieties of carrots available in the market, differentiated on the basis of their colour and β–carotene content, i.e. yellow, orange, red and black carrots. Among these, orange carrots are the richest source of β–carotene. In fact, β–carotene is one of the important nutrients responsible for orange color of carrots. Carrot roots have numerous food applications such as they are traditionally used in salads, preparation of curries and *halwa* in India. Apart from these, it is also converted into processed products like juice, concentrate, dried powder, preserve, candy and canned carrots, etc. During the manufacturing of juices, higher amounts of pomace portion is produced which is generally disposed off as animal feed or manure. However, besides carrot, carrot by-product i.e. carrot pomace can be utilized for the extraction of valuable components from carrot. However, carrot pomace is reported to contain about 50% of β-carotene. Thus, making carrot pomace a potential source for extraction of β-carotene which possess high vitamin A activity and natural colorant properties.

Over the past twenty years, consumers have become increasingly aware of the ingredients used in the manufacturing of food and as such they require foods to be as 'natural' as possible. Thus, the demand of natural food colorants is increasing with the increase in awareness of ingredients used in manufacture of food products. Moreover, in order to make foodstuffs attractive vis-à-vis to increase the organoleptic acceptability of foodstuffs, synthetic colours are being added for decorative purposes and also for disguising low quality foods. These food colorants are used due to their simplicity in application and their stability under various processing and storage conditions. But, the acceptability by consumers to utilize the foods containing synthetic colorants is showing a downfall day-by-day. This is so because the man-made toxic colorants have hazardous impact on human health if their consumption exceeds Acceptable Daily Intake. The concern with the use of synthetic colors is regarded to their petrochemical origin. The health implications due to the consumption of synthetic colors include carcinogenicity, mutagenicity, genotoxicity, neurotoxicity, etc. Moreover, they can also induce certain types of hyperactivity and hypersensitivity reactions in the body (Amchova *et al*., 2015). On the other side, natural food colorants apart from providing colouring effects, helps in preventing various diseases and possess antioxidant and other pharmacological properties. The principle function of antioxidants is the inhibition of the initiation or propagation of oxidizing chain reactions by free radicals (which lead to oxidative damage to the human body) (Namiki, 1990). The antioxidant activity helps in the prevention or reduction in the risk of various types of degenerative diseases such as cardio vascular diseases,

osteoporosis, cancer and cataract formation by preventing the macular degeneration of retina cells (Dutta *et al*., 2005, Voutilainen *et al*., 2006).

The approaches adopted so far for extraction of natural colorants are less safe and thus inappropriate, like conventional solvent extraction methods. These methods involve the use of hazardous solvents. The commonly used organic solvents for extraction of worthy natural colors are acetone, methanol, ethanol, ethyl acetate, isopropyl alcohol, petroleum ether, and hexane etc., used individually or in combination (Sachindra *et al.*, 2005). Most organic solvents are flammable, volatile and often toxic and are responsible for environmental pollution and greenhouse effect. Additionally, there is a concern about the residual solvents which may cause potential health safety hazards. According to a report, the principal failure reason in marketplace for the natural colorants extracted from various fruits, vegetables and spices sources was mainly due to the problem of residual solvents in them (Simon *et al*., 2017). Thus, solvent removal is important due to health risks associated with their consumption or exposure (United States Pharmacopeia, 2013). On the other hand, green bio-refinery based concept is a new trend and need of hour that focuses on using green solvents that have the potential to protect from adverse health effects of solvents of petrochemical origin. As per the definition, green bio refinery concept is based on discovery and design of extraction processes which reduce energy consumption, allow use of alternative solvents and renewable natural products and ensure a safe and high quality extract/ product (Chemat *et al.*, 2012).

Carotenoids are required to be delivered through some means of delivery system. Various delivery systems like microemulsions, nanoemulsions, encapsulation, liposomes, niosomes, etc. may be useful. Thus, the present study was envisaged with the dual purpose of enriching flavoured milk with carotenoids from carrot bio-waste and also to utilize the extracted carotenoids in emulsion based delivery system as a colouring agent. However, both carotenoids as well as the powder developed can be taken as health supplements in the form of oil capsules as well as health sachet mix. The objectives of the study are:

3. Objectives:

3.1 Extraction of carotenoids from carrot bio-waste in flaxseed oil and its characterization

3.2 Optimization of emulsion-based delivery system of extracted carotenoids and its stability assessment

3.3 Application of dried delivery system (rich in carotenoids and omega-3 fatty acids) in flavoured milk as model food system

4 Materials and Methods:

4.1. Materials: Carrots were procured from the local market of Gharonda, Haryana. Crude flaxseed oil was obtained from the local market of Tilak Bazar Chowk, Delhi. WPC was procured from Milk Specialities Global which also contained soy lecithin. Lactose monohydrate was purchased from Sigma-Aldrich, India. Gum Arabic was purchased from Sigma-Aldrich, India. Cellulase from *Aspergillus niger* and Pectinases from *Aspergillus niger* were procured from Sigma Aldrich Chemicals Pvt. Ltd. Link road Bangalore, India. Reference standard (β-carotene) and all other chemicals were from sigma Aldrich Pvt. Ltd. or Hi-media.

4.2. Methods:

4.2.1 Objection 1: Extraction of carotenoids from carrot bio-waste in flaxseed oil and its characterization

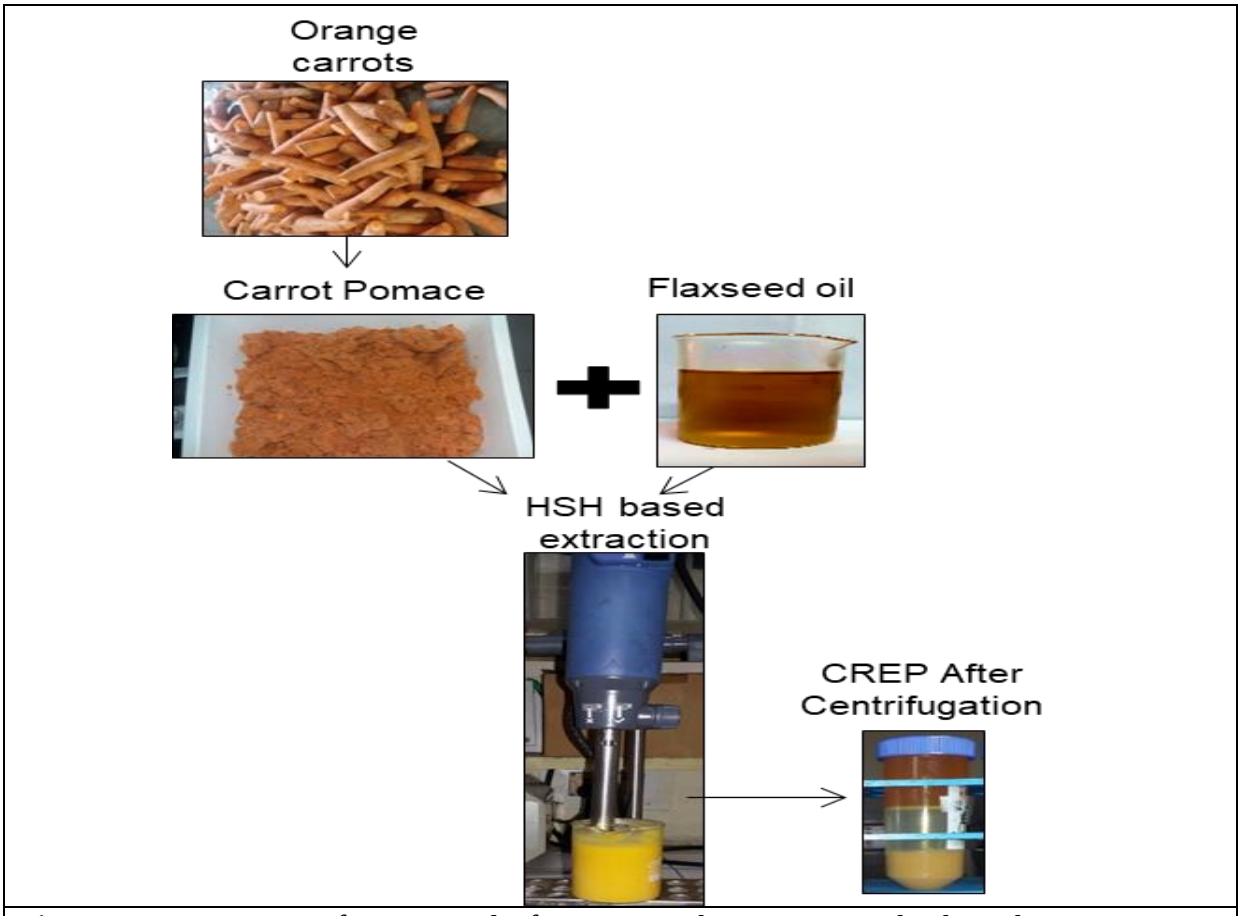


Figure: 1 Extraction of carotenoids from carrot bio-waste: High shear homogenization (HSH); Carotenoid rich extract from carrot pomace (CREP)

4.2.1.1 Processing of carrots

Carrots were washed with potable water, peeled and juice was extracted using Usha Lexus juicer grinder (2663 600 Watt). The bio-waste from carrots i.e. pomace was then blanched at different time/temperature combinations to check the blanching efficiency in order to inactivate the peroxidase enzyme. Blanched carrot pomace were portioned and packed in a food grade laminates of 150 g each and subsequently sealed and stored under frozen conditions at -23^{o}C ±2^{o}C.

4.2.1.2 Enzymatic hydrolysis of carrot pomace and its scanning electron microscopic analysis

The frozen carrots were thawed in water at room temperature. Cellulase and pectinase suspension was prepared by dissolving these enzymes @ 0.35% and 0.5% in water equal to the quantity of carrot pomace, making a uniform suspension of carrot pomace and water (containing enzymes) in the ratio of 1:1 (Jori e*t al*., 2015). The pH was adjusted to 4 by using 12% HCl (v/v) and corrected using 10% NaOH (w/w) (Stoll *et al.*, 2003). The suspension was kept in the water bath for 3 hours at 45°C ± 1°C. Finally, the reaction was terminated by heating the suspension at 90°C for 5 min followed by cooling to 20°C.

A small quantity of carrot pomace (with and without enzyme treatment) was taken to check the effect of hydrolysis on tissues. The samples were finally examined by SEM (Hithachi, 3400N) at an acceleration voltage of 15kV under low vacuum of 0.00009 Torr and micrographs were recorded.

4.2.1.3 Extraction of carotenoid from carrot pomace in flaxseed oil

Extraction of carotenoid from carrot pomace was carried out by using high shear disperser (IKA T18) at 20 000 rpm for different time and Ultrasonication at 45% duty cycle, 750 Watt power with probe radius of 13 mm in flaxseed oil. The extraction was aimed at employing the green bio refinery concept, therefore, flaxseed oil was used as extracting medium in the ratio of 1:1 (carrot pomace to flaxseed oil). The mixture after each treatment was subjected to centrifuge @ 4000 rpm for 12 min to obtain supernatant layer of flaxseed oil rich in carotenoids.

The supernatant layer (pigmented oil rich in carotenoids) was analyzed for total carotenoids content (TCC), antioxidant activity (ABTS, DPPH and FRAP given by Awika *et al*. (2003), Cuendet *et al*. (1997), (Benzie and Strain, 1996), respectively. The color value (L*, a* and b*), peroxide value, conjugated acids and β-carotene through HPLC analysis.

High shear homogenizer **Ultrasonicator (probe type)**

Figure 2: Equipments used for extraction of carotenoids

4.2.2 Objective 2: Optimization of emulsion-based delivery system of extracted carotenoids and its stability assessment (may be taken in the form of **health sachet powder mix** with aqueous systems like milk).

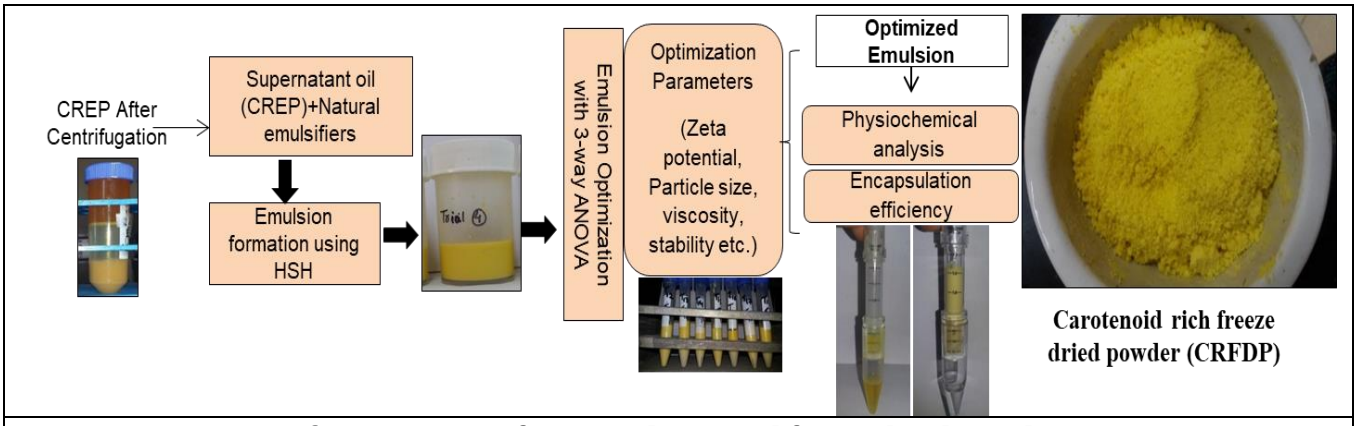


Figure: 3 Overview of Preparation of o/w emulsion and freeze dried powder preparation

4.2.2.1 Preparation of o/w emulsion

The o/w emulsion was prepared by following the method given in Figure 4 and table 1 using 3 factorial design. WPC-80, lactose and water were mixed on magnetic stirrer for half an hour to form a coarse emulsion. It was followed by addition of carotenoid rich extract in the coarse emulsion. This was followed by providing shear by using HSD at 22000 rpm for 20 min so as to get fine emulsion.

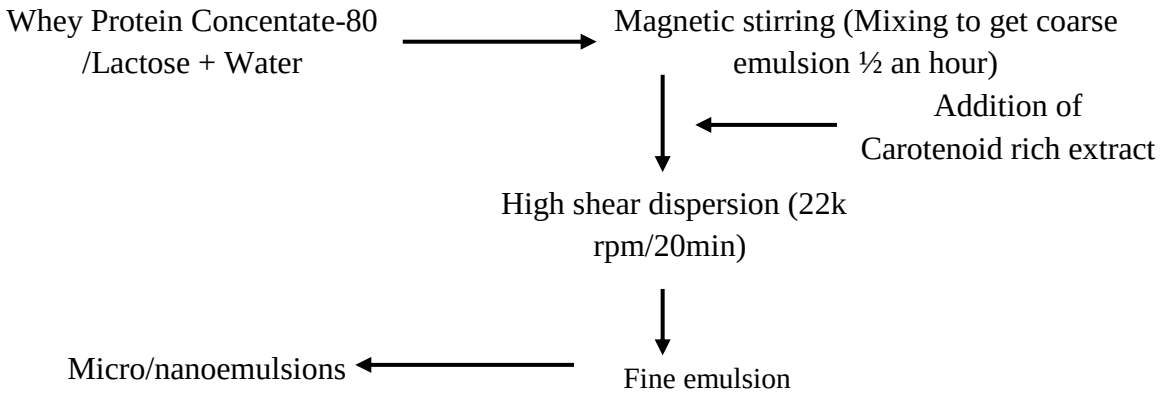


Figure 4: O/W Emulsion preparation

Table 1: 3^3 factorial design applied to optimize the preparation of emulsion based delivery system

Treatments	Carotenoid Rich Extract	WPC	Lactose
T1	30	5.0	5.0
T2	30	7.5	5.0
T3	30	10.0	5.0
T4	30	5.0	7.5
T5	30	7.5	7.5
T6	30	10.0	7.5
T7	30	5.0	10.0
T8	30	7.5	10.0
T9	30	10.0	10.0
T10	35	5.0	5.0
T11	35	7.5	5.0
T12	35	10.0	5.0
T13	35	5.0	7.5
T14	35	7.5	7.5
T15	35	10.0	7.5
T16	35	5.0	10.0

T17	35	7.5	10.0
T18	35	10.0	10.0
T19	40	5.0	5.0
T20	40	7.5	5.0
T21	40	10.0	5.0
T22	40	5.0	7.5
T23	40	7.5	7.5
T24	40	10.0	7.5
T25	40	5.0	10.0
T26	40	7.5	10.0
T27	40	10.0	10.0

4.2.2.2 Analysis of optimized emulsion:

The prepared emulsion was analysed for centrifugal stability, gravitation stability, total carotenoid content, morphology, encapsulation efficiency and antioxidant activity.

4.2.2.2.3 Freeze drying

The emulsion samples were first subjected to freezing at -23°C for 12-16 hours followed by drying under vacuum of 0.14 mTorr at -43°C in a freeze dryer.

4.2.3 **Objective 3:** Application of dried delivery system (rich in carotenoids and omega-3 fatty acids) in flavoured milk as model food system.

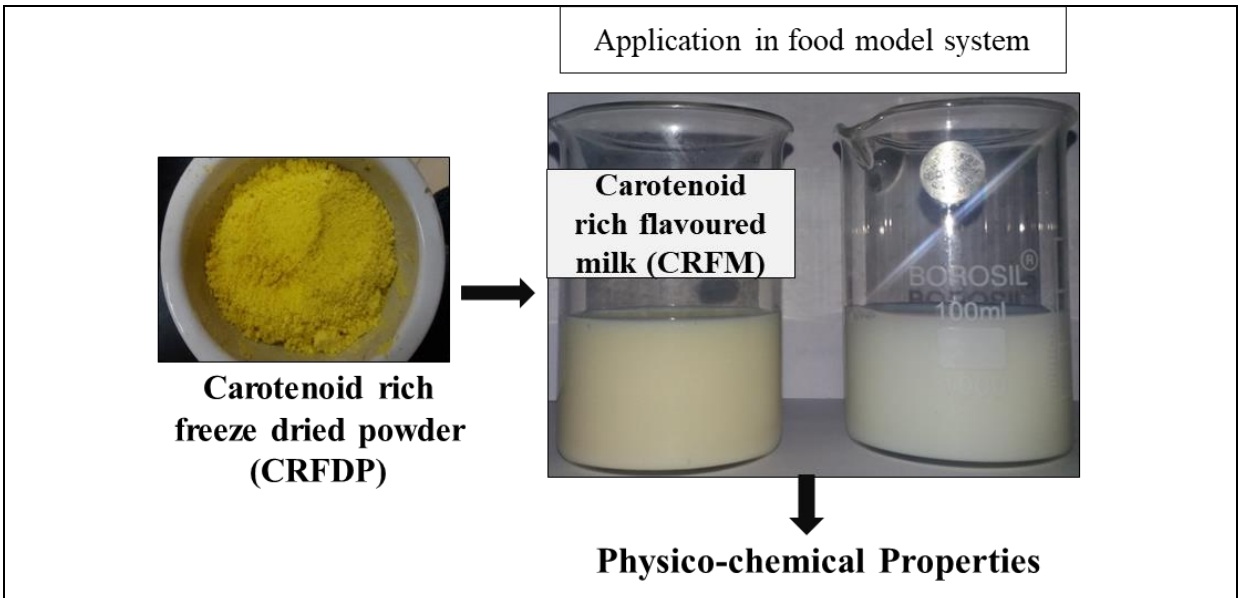


Figure 5: Preparation of flavored milk using carotenoid rich freeze dried powder

4.2.3.1 Preparation of flavored milk using carotenoid rich freeze dried powder

The flavoured milk was prepared using carotenoid rich freeze dried powder as per the method given in Figure 6.

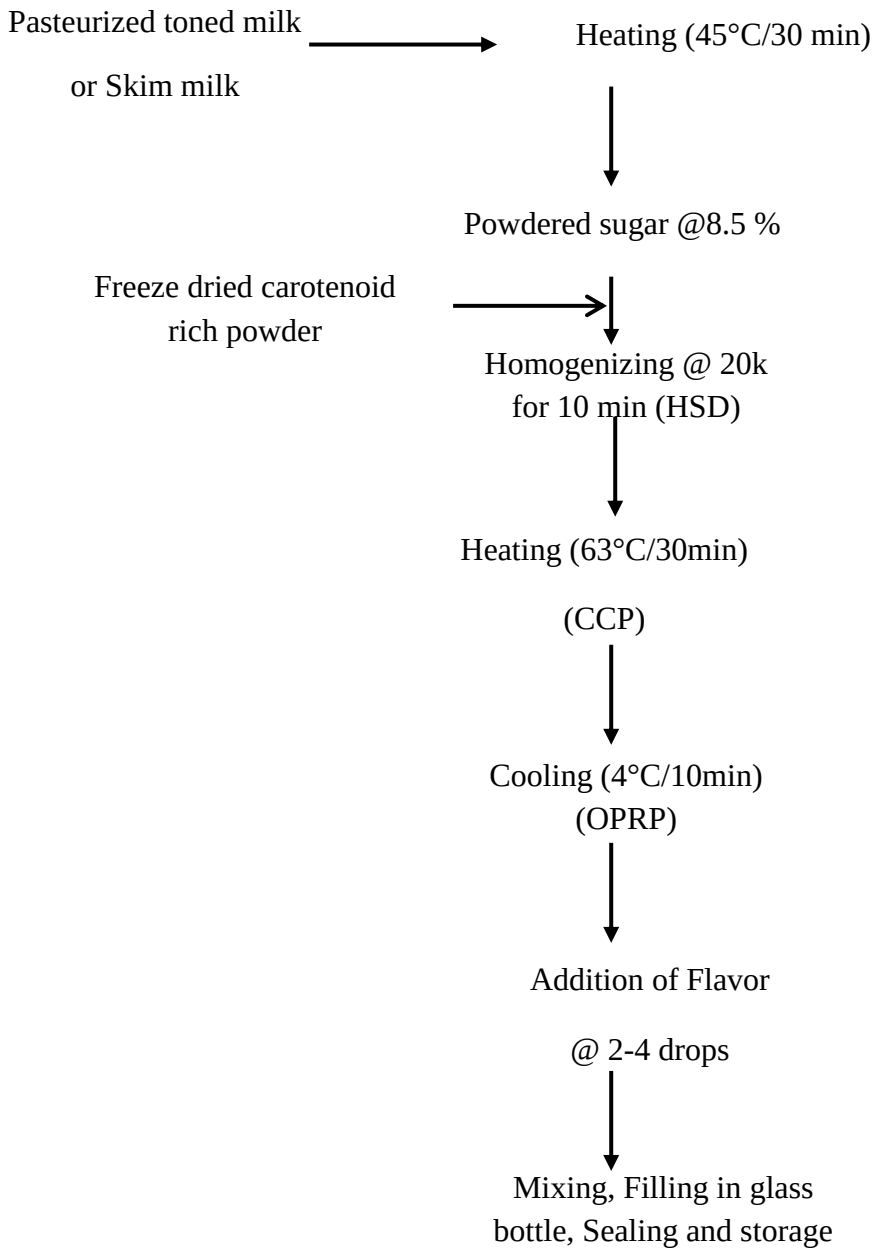


Figure 6: Preparation of carotenoid rich flavored milk using carotenoid rich powder

4.2.3.2 Physicochemical analysis of carotenoid rich flavored milk (CRFM)

The physico-chemical analysis of carotenoid rich flavoured milk include compositional analysis, pH, acidity and functional analysis

5. Results

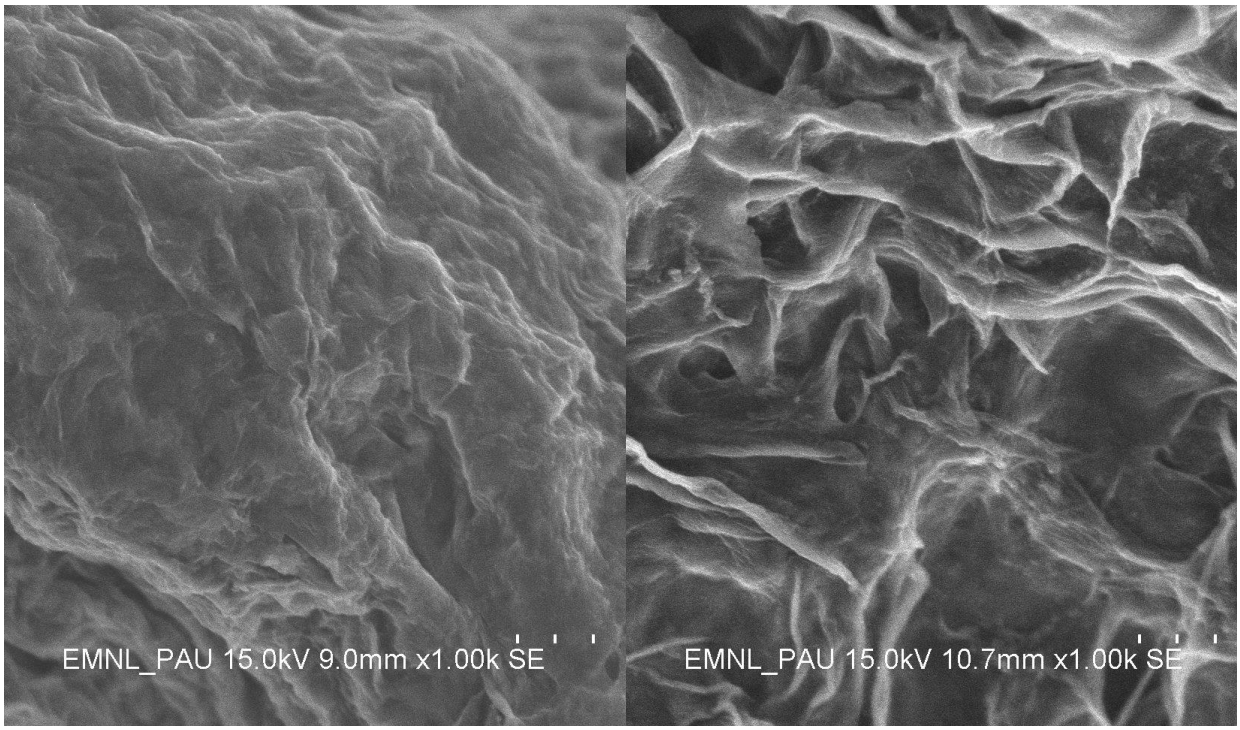
5.1 Objective 1: Extraction of carotenoids from carrot bio-waste in flaxseed oil and its characterization

5.1.1 Enzymatic treatment of carrot pomace

The total carotenoid content of carrot pomace without enzymatic treatment and extraction was 53.86±0.084 µg/g (approx.), while after enzymatic treatment it was 73.03±1.182 µg/g (approx.).

Pomace without enzymatic treatment **Pomace with enzymatic treatment**

Figure 7: Carrot Pomace before and after enzymatic hydrolysis



Without Enzymatic Treatment **With Enzymatic Treatment**

Figure 8: SEM images of carrot Pomace with and without enzymatic treatment

5.1.2 Effect of enzymatic hydrolysis on morphology of carrot pomace

Scanning electron microscopy of the carrot pomace was done to study the difference in the structure of carrot pomace before and after enzymatic treatment at microscopic level. Figure 8 shows scanning electron micrograph of the freeze-dried carrots with and without pre-treatment. Untreated carrot tissue had a smaller cavities or no cavities and uniformity in the structure indicating the intactness, while the enzyme treated samples showed an opened up structure.

5.1.3 Extraction of Total carotenoids content by employing different techniques

The preliminary trials were conducted for selection of the ratio of carrot pomace to flaxseed oil to be used in the further study for extraction of carotenoids. For this, different carrot pomace to flaxseed oil ratios were selected and extraction of carotenoids was done using ultrasonication assisted (UAE) and high shear disperser (HSD) technique at a constant time (Table 2). UAE was done at 45% duty cycle for 4 min, while HSD was carried out at 20 000 rpm for 4 min. The extracted samples were analyzed for total carotenoid content (TCC). The TCC content was significantly higher for both the techniques when the ratio of carrot pomace to flaxseed oil was kept at 1:1. In case of carrot pomace to flaxseed oil ratio of 1:0.5, the recovery was lower which might be due to the saturation of the oil with carotenoids and this might have caused some of the carotenoids to be left out in the pellet after centrifugation. On the other hand, in the case when ratio of carrot pomace to flaxseed oil was 1:2, there was significant decrease in the TCC which may be due to dilution of carotenoids with the larger quantity of flaxseed oil used for the extraction purpose. Thus, the results of the study revealed that carrot pomace to flaxseed oil ratio of 1:1 yielded highest carotenoid content by using both the techniques and hence this ratio was selected for further study.

The carrot pomace to flaxseed oil ratio of 1:1 was subjected to different extracting techniques i.e. ultrasonication (probe method) and high shear disperser for different times (min). UAE was done at 45% duty cycle, while HSD was carried out at 20 000 rpm for different time period. It is evident from Table 3 that as the time increased from 2 to 12 minutes, the extraction of carotenoids increased significantly ($p<0.05$) using both the techniques. This could probably be due to more shearing action which in turn facilitated in extraction of more carotenoids because of higher time of exposure by complete rupturing of carrot pomace matrix. Further, in case of high shear disperser technique, it was observed that there was non-significant increase in the total carotenoid content of the extract when time was increased from 12 to 14 min. Moreover, the total carotenoids recovered from HSD technique was significantly higher as compare to that of UAE. The TCC of extract treated with HSD at

12 min was 82.66±0.06 µg/ml with recovery of 94.8±0.08%, while from that of ultrasonication treated samples at same treatment time was 21.67±0.40 µg/ml with very less recovery of carotenoids in the extract i.e. 11.36±0.54% (Table 3).

Table 2: Effect of different ratio of carrot pomace to flaxseed oil (C:F) on extraction of carotenoid

Ratio (C:F)	Time	Extraction technique	
		UAE (Ultrasound assisted extraction) (Probe type) (TCC content µg/g)	High shear dispersion (HSD) (TCC content µg/g)
1:0.5	4 min	7.59 ± 0.06^{bx}	61.72 ± 0.131^{by}
1:1	4 min	12.304 ± 0.067^{cx}	66.49 ± 0.075^{cy}
1:2	4 min	4.13 ± 0.075^{ax}	35.65 ± 0.622^{ay}

Mean ± SD (n=3)
Different superscripts ($^{a-b}$) are significantly different with each other within a column ($p<0.05$),
Different superscripts ($^{x-y}$) are significantly different with each other within a row ($p<0.05$)

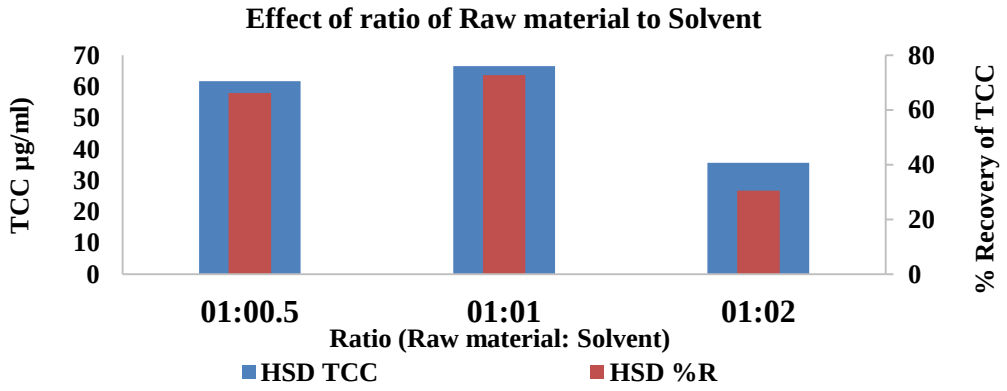


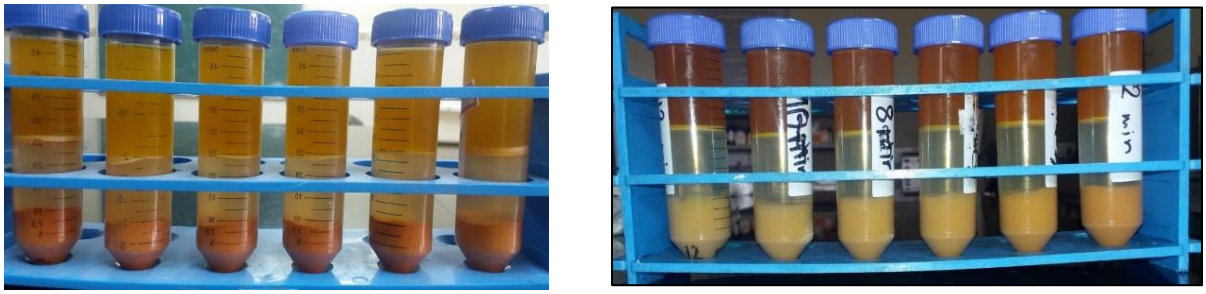
Figure 9: Effect of different ratio of carrot pomace to flaxseed oil (C:F) on extraction of carotenoid

Table 3: Effect of extraction time and technique on the total carotenoid content and its recovery

Time (min.)	Extraction technique (TCC content µg/g)			
	UAE (probe method)		HSD	
	TCC	% Recovery	TCC	% Recovery
2	14.02 ± 0.02^{ax}	1.32±0.02	62.16 ± 0.18^{ay}	66.55±0.24
4	15.28 ± 0.30^{ax}	2.62±0.41	67.64 ± 24^{by}	74.3±0.33
6	16.41 ± 0.61^{bx}	4.17±0.02	69.32 ± 1.12^{cy}	76.6±1.5
8	17.83 ± 0.20^{cx}	6.1±0.27	77.08 ± 1.12^{dy}	87.23±0.16

10	19.4 ± 0.46^{dx}	8.25 ± 0.63	79.12 ± 1.81^{ey}	90.02 ± 0.32
12	21.67 ± 0.40^{ex}	11.36 ± 0.54	82.66 ± 0.06^{fy}	94.8 ± 0.08
14	-	-	82.98 ± 1.10^{f}	94.19 ± 0.98

Mean ± SD (n=3),
Different superscripts ($^{a-f}$) are significantly different with each other within column ($p<0.05$),
Different superscripts ($^{x-y}$) are significantly different with each other within row ($p<0.05$)



Ultrasonication (pellet contains carotenoids) **High shear dispersion (pellet is colorless)**

Fig 10 : Comparison between High shear disperser and Ultrasonication

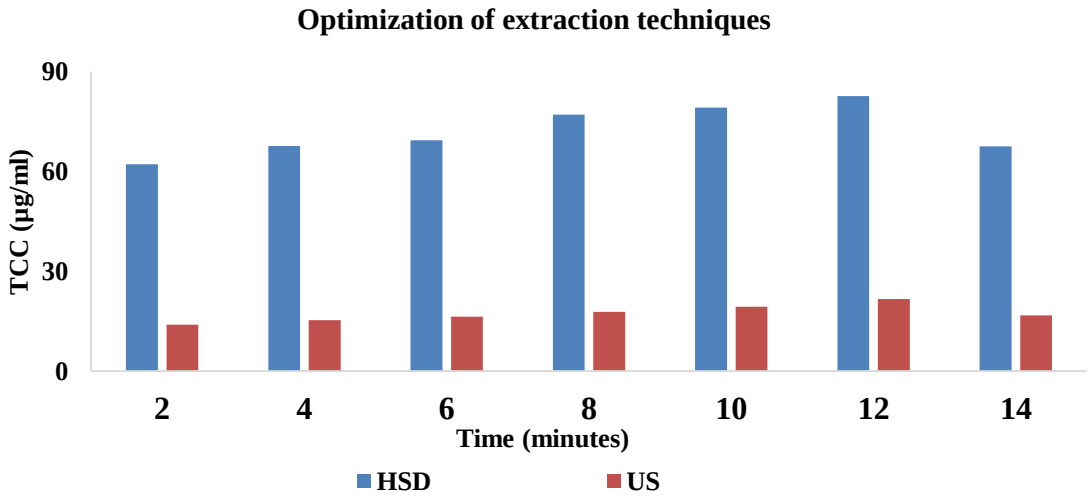


Fig. 11 : Effect of extraction time and technique on the total carotenoid content and its recovery

5.1.4 Comparison of the physico-chemical properties of carotenoid rich extract with extracting medium (control)

The extracting medium i.e. flaxseed oil and the carotenoid rich extract were analyzed for various parameters namely, total carotenoids content (µg/g), antioxidant activity (ABTS, µg Trolox eq./ ml; DPPH, µg Trolox eq./ml; FRAP, µM Trolox eq./ml), Color values (L*; a*; b*; Chroma; Hue, h*), peroxide value (m eq. / 1000 g of sample), conjugated acids in % (conjugated dienes, conjugated trienes, conjugated tetraenes and conjugated pentaenes) (Table 4). The green bio-refinery concept was used for the extraction of carotenoids in the

flaxseed oil (i.e. extracting medium). The green bio-refinery approach included extraction, centrifugation of treated pomace and phase separation carried out sequentially.

Table: 4 Physico-chemical analysis extracting medium and carotenoid rich extract

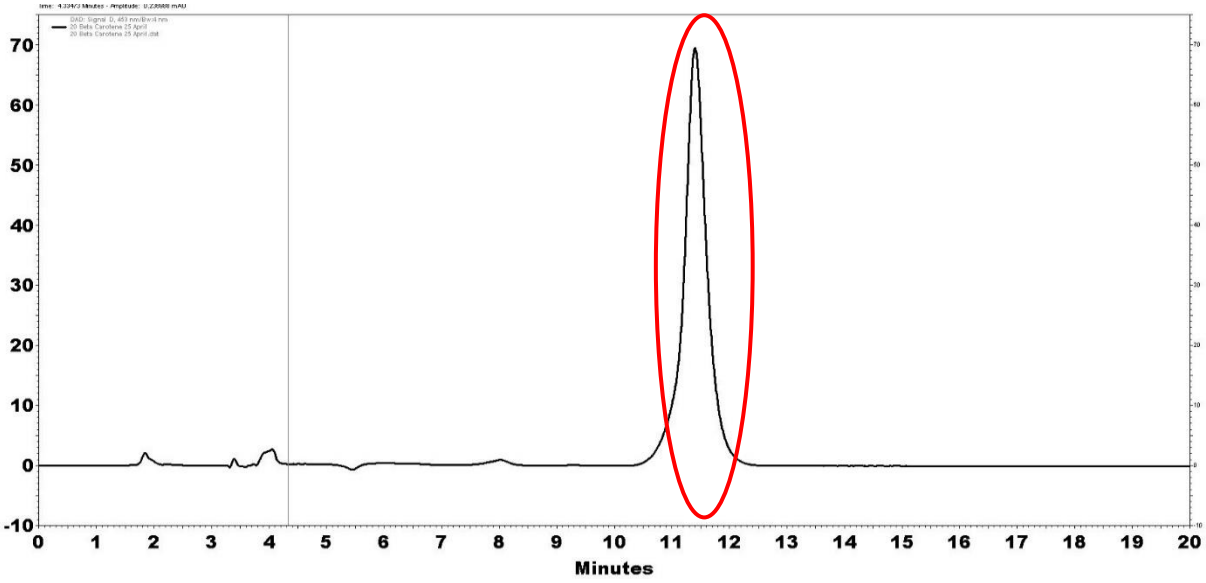
Parameter	Flaxseed oil	Carotenoids rich extract
Total carotenoids content (µg/g)	13.38±0.141[a]	82.66±0.06[b]
Antioxidant activity		
ABTS (µg Trolox eq./ml)	930.33±64.61[a]	1596.04±69.45[b]
DPPH (µg Trolox eq./ml)	255.32±9.05[a]	380.21±39.62[b]
FRAP (µM Trolox eq./ml)	521.91±12.65[a]	941.20±19.91[b]
Color values		
L*	4.085 ±0.12[a]	18.65 ±0.037[b]
a*	1.502 ±0.025[a]	19.42 ±0.206[b]
b*	2.163 ±0.106[a]	27.94 ±0.65[b]
Chroma	2.63±0.07[a]	34.03±0.41[b]
Hue	55.19±0.79[a]	55.19±0.92[a]
Peroxide value (meqO_2/1000 g of sample)	1.0736±0.104[a]	1.0493±0.08[a]
Conjugated acids (%)		
Conjugated Dienes	0.311±0.00009[a]	0.406±0.0005[b]
Conjugated Trienes	0.0423±0.0007[a]	0.055±0.0007[b]
Conjugated Tetraenes	0.0009±0.0006[a]	0.0046±0.0006[b]
Conjugated Pentaenes	0.0001±0.00002[a]	0.0004±0.00003[b]

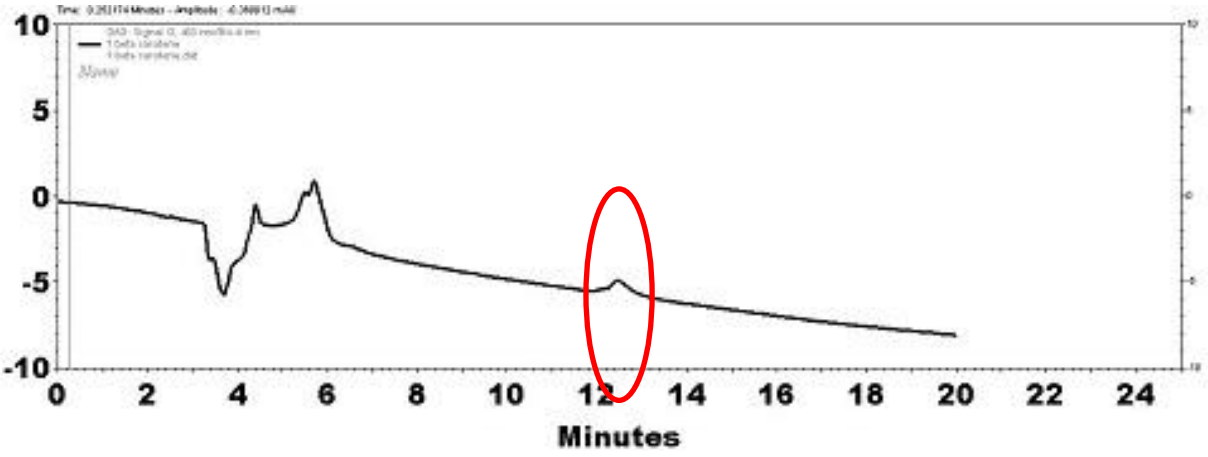
Mean ± SD (n=3),
Different superscripts ([a-b]) are significantly different with each other within a row ($p<0.05$)

5.1.5 Estimation of beta-carotene using High Performance Liquid Chromatography (HPLC)

Carotenoids are the major lipophilic antioxidants present in carrot performing many health promoting functions in human body. Among the different varieties of carrots, orange carrots mainly contain appreciable amount of β-carotene over other carotenoids. The standard curve was prepared between the concentration 0.7-30µg/g and according to the peak area its content was estimated. The typical chromatogram of the standard of β-carotene indicates that the retention time for beta-carotene was 11.68 minutes. The peak area of 40 95 734 was

obtained for a standard of 30 µg/g, while flaxseed oil and CRE showed peak area of 95 133 and 25 75 129, respectively. The amount of beta carotene in the both flaxseed and CRE was calculated by plotting the peak area of each sample in standard curve equation (at the retention time) followed by multiplying with dilution factor. Thus, the β-carotene content of flaxseed oil was approximately 6.75 µg/ g, while for CRE it was 78.37 µg/g.





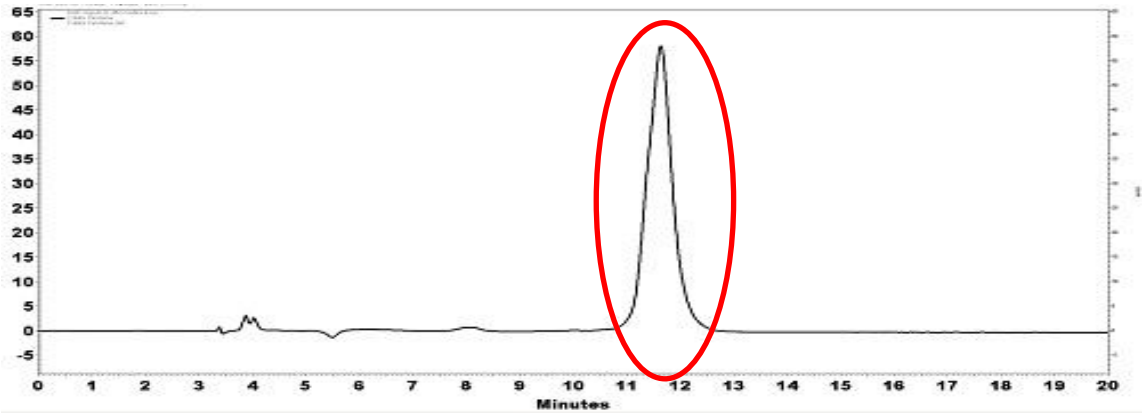


Fig 12 : Peak for standard β-carotene indicating the retention time of 11.68 min; Peak for β-carotene in flaxseed oil; Peak for β-carotene for carotenoid rich extract

5.2 Objective 2 Optimization of emulsion-based delivery system of extracted carotenoids and its stability assessment

5.2.1 Effect of different levels of carotenoid rich extract (CRE), whey protein concentrate and lactose on physico-chemical properties of emulsion

The effect of different concentrations of CRE, whey protein concentrate and lactose was studied on the physico-chemical properties, namely, particle size, zeta potential, viscosity, L*, a*, b* values of the emulsion (Table 5).

5.2.2 Particle size, zeta potential, viscosity and color values (L*, a*, b*)

The lowest average particle size was obtained with 35% of carotenoid rich extract (CRE) i.e. 298.94 nm followed by 40% (362.66 nm) and 30% (419.79 nm). In case of WPC, the lowest average particle size was obtained with WPC @ 10% level i.e. 189.02 nm and highest average particle size was obtained with 5% level of WPC that is 589.56 nm as given in Table 5. In case of lactose, mean particle size ranged between 341.84 nm and 379.02 nm which was due to 10 and 7.5% level of lactose, respectively in emulsion. The particle size due to 5% lactose was 360.53 nm.

In case of zeta potential, with increasing level of CRE in emulsion viz. 30%, 35% and 40%, the average value of zeta potential decreased significantly to -23.03 mV, -22.17mV, -20.5mV, respectively ($p<0.05$) (Table 5). According to zeta potential, 35% CRE, 5% WPC and 7.5% is the best combination.

In case of Viscosity, with increasing level of WPC, at 5%, 7.5% and 10%, the average apparent viscosity increased i.e. 0.027 Pa.s, 0.043 Pa.s and 0.136 Pa.s, respectively ($p<0.05$). In case of lactose also there was significant difference found in the average apparent viscosity in all the three levels as given in Table 5.

For color, different levels of CRE in emulsion have significant effect on average b* value ($p<0.05$). At different levels of CRE i.e. 30%, 35% and 40%, the b* value increased to 8.82, 9.86 and 10.3, respectively. In case of WPC, b* value was deceased from 5% (50.87) to 7.5% (50.15) of WPC, while at 10%, it increased significantly to 50.45. In case of lactose, with increasing concentration of lactose, the b* value significantly increased ($p<0.05$) as shown in Table 5. The highest b* value (54.05±0.07) was obtained in the combination possessing 40% CRE, 5% WPC and 10% Lactose.

The final emulsion was prepared using 35% CRE, 10% WPC, 5% Lactose and 1% Gum arabic (T12 combination).

Table 5: Physico-chemical properties of emulsions prepared using different concentrations of carotenoid rich extract (CRE), whey protein concentrate and lactose

Treatments	CRE (%)	WPC (%)	Lactose (%)	Particle Size	Zeta Potential	L*	a*	b*	Viscosity	Gravitational stability * (days)	Centrifugal stability**
T1	30	5.0	5.0	636.17± 69.86	-26.47± 2.13	76.62 ± 0.16	8.35± 0.17	44.96 ±0.17	0.012± 0.0014	1	0
T2	30	7.5	5.0	391.33± 32.70	-23.63± 0.50	76.78 ± 0.10	8.32± 0.08	44.73 ±0.35	0.029± 0.0059	5	0
T3	30	10.0	5.0	173.83± 23.19	-20.30± 1.17	76.46 ± 0.01	8.10± 0.01	43.06 ±0.08	0.072± 0.0011	15	0
T4	30	5.0	7.5	761.60± 111.7	-25.17± 0.94	75.79 ± 0.60	9.03± 0.01	47.82 ±0.18	0.017± 0.0006	1	0
T5	30	7.5	7.5	358.03± 80.30	-23.23± 3.30	76.21 ± 0.07	8.58± 0.35	45.60 ±0.31	0.028± 0.0002	2	0
T6	30	10.0	7.5	268.50± 60.73	-20.13± 1.26	76.00 ± 0.07	9.34± 0.23	48.09 ±0.18	0.194± 0.0357	15	1
T7	30	5.0	10.0	643.37± 128.7	-23.97± 0.86	75.98 ± 0.11	9.25± 0.35	47.89 ±0.30	0.024± 0.0034	1	0
T8	30	7.5	10.0	349.73± 47.04	-23.43± 1.15	75.96 ± 0.05	9.17± 0.09	47.90 ±0.30	0.036± 0.0016	1	1
T9	30	10.0	10.0	195.67± 35.13	-21.03± 0.86	75.99 ± 0.12	9.26± 0.26	48.34 ±0.68	0.092± 0.0036	15	0
T10	35	5.0	5.0	476.93± 47.4	-24.37± 1.36	75.04 ± 0.09	10.09 ± 0.06	52.36 ±0.13	0.027± 0.0010	2	0
T11	35	7.5	5.0	193.07± 71.9	-21.37± 0.28	75.83 ± 0.03	9.62± 0.08	49.59 ±0.12	0.051± 0.0004	7	1
T12	35	10.0	5.0	120.03± 8.20	-16.57± 0.49	75.11 ± 0.04	9.66± 0.32	50.29 ±0.62	0.124± 0.0115	15	1
T13	35	5.0	7.5	446.45± 17.8	-26.67± 0.80	75.13 ± 0.09	9.82± 0.11	51.41 ±0.53	0.033± 0.0033	3	0

T14	35	7.5	7.5	264.60± 6.50	-22.70± 1.08	75.09 ± 0.11	9.80± 0.20	51.11 ±0.29	0.039± 0.0007	5	0
T15	35	10.0	7.5	225.37± 14.04	-21.67± 0.72	74.78 ± 0.58	9.63± 0.10	51.37 ±0.33	0.222± 0.0005	15	1
T16	35	5.0	10.0	534.73± 85.4	-24.60± 3.16	75.10 ± 0.11	10.26 ± 0.48	52.10 ±0.03	0.035± 0.0018	2	0
T17	35	7.5	10.0	265.73± 2.67	-22.47± 0.61	75.07 ± 0.04	9.91± 0.01	51.18 ±0.22	0.050± 0.0033	7	0
T18	35	10.0	10.0	166.53± 16.06	-19.17± 2.23	75.14 ± 0.06	9.96± 0.05	52.06 ±0.05	0.111± 0.013	7	0
T19	40	5.0	5.0	658.97± 9.59	-24.63± 1.42	75.34 ± 0.06	10.32 ± 0.04	53.48 ±0.19	0.022± 0.0005	5	0
T20	40	7.5	5.0	308.67± 56.1	-20.70± 0.45	75.34 ± 0.12	10.35 ± 0.14	53.61 ±0.34	0.043± 0.0031	7	0
T21	40	10.0	5.0	285.83± 19.7	-19.60± 1.22	75.61 ± 0.13	10.36 ± 0.04	53.47 ±0.03	0.049± 0.0018	7	0
T22	40	5.0	7.5	607.23± 22.1	-24.03± 0.90	75.74 ± 0.21	10.48 ± 0.07	53.83 ±0.25	0.031± 0.0011	1	0
T23	40	7.5	7.5	304.43± 22.7	-20.43± 1.84	75.67 ± 0.24	10.26 ± 0.08	53.83 ±0.25	0.067± 0.0027	5	0
T24	40	10.0	7.5	177.97± 15.6	-18.10± 1.08	75.67 ± 0.19	10.24 ± 0.03	53.83 ±0.37	0.101± 0.0036	7	1
T25	40	5.0	10.0	542.33± 43.3	-22.77± 1.60	75.86 ± 0.14	10.23 ± 0.08	54.05 ±0.07	0.042± 0.0043	4	0
T26	40	7.5	10.0	291.03± 38.4	-19.63± 0.77	75.87 ± 0.04	10.21 ± 0.04	53.88 ±0.03	0.052± 0.0007	12	1
T27	40	10.0	10.0	87.53± 9.07	-14.63± 2.00	75.64 ± 0.11	10.33 ± 0.15	53.60 ±0.06	0.260± 0.0085	13	1

* indicates number of days till which the sample did not show phase separation

** 0 indicates no centrifugal stability, while 1 indicate sample was stable after centrifugation

5.2.3 Encapsulation Efficiency of emulsion

The encapsulation efficiency was checked and was observed to be 92.206±0.636%. It was calculated by evaluating the total carotenoid content as a marker. For determination of encapsulation efficiency, the encapsulated CRE and un-encapsulated CRE (after dissolving in ethyl acetate) were passed through 100kDa MWCO membrane.

CRE dissolved in ethyl acetate **Optimized emulsion containing CRE**

Fig: 13 : Encapsulation efficiency of oil in water emulsion

5.2.4 Physico-chemical analysis of the emulsion (Total Carotenoid Content, antioxidant, color, morphology, encapsulation efficiency)

A new lot of carrot was procured for formation of emulsion as the older lot got exhausted. The new lot had higher TCC content (102.24±0.169µg/g) in comparison to the older lot. The TCC of optimized emulsion was 34.6 µg/g and it was significantly lower than the CRE ($p<0.05$).

ABTS activity of the emulsion was lower than that of CRE which was 1133.28±25.67 µg Trolox eq./ml.

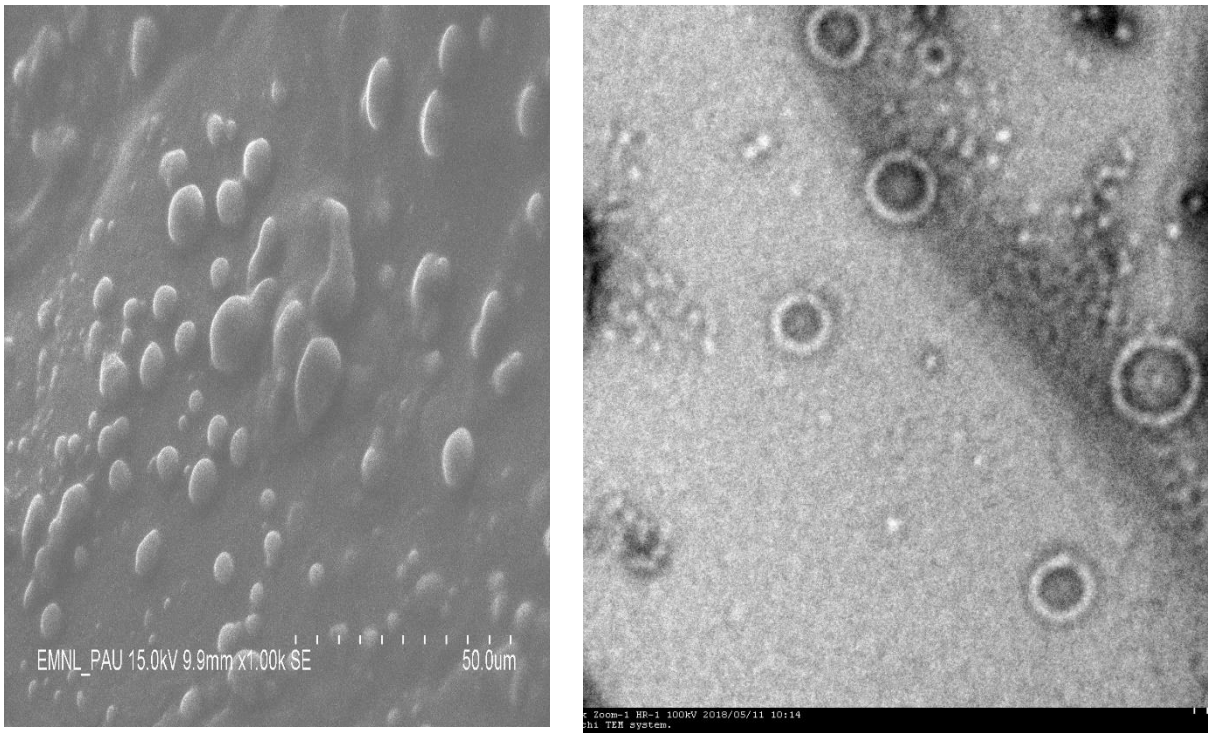
L* value increased significantly which might be due to increase in whiteness of emulsion caused by WPC and lactose. a* value of the emulsion was observed to decrease significantly ($p<0.05$) which indicates the decrease in redness, while b* value was increased showing that after incorporating CRE to emulsion, yellowness increased.

Scanning electron microscopy was used to provide information about changes in surface morphology and results obtained are shown in the.

Table 6: Physico-chemical analysis of the emulsion

Parameter	CRE	Emulsion
Total carotenoids content (µg/g)	**102.24±0.169[a]**	**34.6±0.137[b]**
Antioxidant Activity		
ABTS (µg Trolox eq./ml)	**1833.23±60.83[a]**	**1133.28±25.67[b]**
DPPH (µg Trolox eq./ml)	**445.83±17.29[a]**	**292.07±23.22[b]**
FRAP(µM Trolox eq./ml)	**1057.80±16.62[a]**	**622.96±18.91[b]**
Color values		
L*	**23.85±0.063[a]**	**74.21±0.32[b]**
a*	**21.77±0.421[a]**	**10.12±0.342[b]**
b*	**29.41±0.356[a]**	**51.94±0.450[b]**

Mean ± SD, (n=3) [a &b] Mean values with different superscripts in columns are significantly different with each other ($p<0.05$)



(a) Emulsion droplets (at 1k) **(b) Size of the emulsion droplets**

Figure 14: SEM images for optimized oil in water emulsion; Oil droplets coated with WPC and lactose to form emulsion

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Figure 15 : Freeze drying of emulsion to convert it into powder

5.3 Objective 3: Application of dried delivery system (rich in carotenoids and omega-3 fatty acids) in flavoured milk as model food system

5.3.1 Drying of emulsion

The optimized emulsion was dried in freeze dryer and the freeze dried powder was used for further incorporation in the product (Figure 15).

5.3.2 Optimization of carotenoid rich powder for the preparation of carotenoid rich flavoured milk

The optimization of carotenoid rich flavoured milk was done on the basis of sensory scores. For the preparation of flavoured milk, the level of sugar, speed of homogenization using HSD and time were kept constant @ 8.5 %, 20 000 rpm and 10 min, respectively, while the level of carotenoid rich powder (CRP) was varied at a level of 8%, 10% and 12% on the basis of preliminary trials. A non-significant difference was observed between control and carotenoid rich flavored milk (CRFM) containing 8%, 10% and 12% level of CRP for the scores obtained for color and appearance, odour, mouthfeel and overall acceptability. However, the scores for taste decreased significantly from 7.25±0.36 to 6.45±0.43 when 10 and 12% of CRP, respectively was added. This could be attributed to typical taste offered by flaxseed oil. Thus, on this basis of the scores obtained for taste, 10% level of CRP was selected for further study.

5.3.3 Proximate analysis of the CRFM

The proximate analysis of the optimized flavored drink was carried out and all the parameters were found to be significantly higher ($p<0.05$) in CRFM except ash and total sugar content which showed non-significant difference ($p>0.05$) between control and

CRFM. Fat content of CRFM was higher than control which can mainly be attributed to the CRP containing CRE. The significant difference in the protein content of control and CRFM could be attributed to the presence of WPC in the coat materials.

Table 7: Sensory scores for optimization of level of extract in carotenoid rich flavoured milk

Sample	Color & Appearance	Odor	Taste	Mouthfeel	Overall acceptability
C	7.66±0.47[a]	7.85±0.71[a]	7.80±0.61[a]	7.70±0.91[a]	7.55±0.82[a]
8%	6.94±0.51[a]	7.35±0.75[a]	7.20±1.10[a]	6.85±0.60[a]	7.15±0.36[a]
10%	7.22±0.58[a]	7.31±0.46[a]	7.25±0.36[a]	7.1±0.48[a]	7.2±0.61[a]
12%	7.00±0.70[a]	7.05±0.41[a]	6.45±0.43[b]	6.65±0.50[a]	7.25±0.25[a]

Data are presented as means ± SEM (n=10)

[a-b] Means with different superscripts in same column differ significantly ($p<0.05$)

6. Discussion

6.1 Objective 1 Extraction of carotenoids from carrot bio-waste in flaxseed oil and its characterization

6.1.1 Enzymatic treatment of carrot pomace

The total carotenoid content after giving enzymatic treatment was found to be increased significantly ($p<0.05$). The major advantage of enzymatic hydrolysis is that it decreases the activation energy of the chemical reaction and provide milder conditions for the process. These benefits attract researchers to use the enzyme aided extraction for carotenoids separation (Deenu *et al*., 2013). The similar results are obtained in the present investigation vis-à-vis increase in the extraction of carotenoids from carrot pomace after application of enzymes pectinase @ 0.5% and cellulase @ 0.35% level simultaneously.

6.1.2 Effect of enzymatic hydrolysis on morphology of carrot pomace

It is clear from the Figure 8 that enzymatic treatment has helped in extraction of carotenoids from carrot by opening up structure and enhancing the release of bound carotenoids present in cell wall and cellulosic material of the carrot.

6.1.3 Extraction of Total carotenoids content by employing different techniques

The results of the study revealed that carrot pomace to flaxseed oil ratio of 1:1 yielded highest carotenoid content by using both the techniques and hence this ratio was selected for further study. .

Cha *et al*. (2010) also reported that recovery of β-carotene using UAE was less when compared with pressurized liquid extraction. The results in the present study are in accordance with the literature i.e. HSD was found to be better approach as compared to UAE due to higher percent recovery of total carotenoid content in flaxseed oil. It is also clear from the Figure 10 as the pellet obtained in case of HSD treated extract was almost colorless, while that obtained by UAE sample showed the coloring effect of carotenoids. Thus, 12 min using HSD carried out at 20 000 rpm for 12 min was selected as maximum possible carotenoids could be recovered with at this combination.

6.1.4 Comparison of the physico-chemical properties of carotenoid rich extract with extracting medium (control)

Total carotenoid content (TCC), Antioxidant activity, color values

The pretreatment given to carrot pomace i.e. enzymatic treatment followed by high shear disperser technique were found to be a good combination for extracting the carotenoids from carrot pomace using green bio refinery concept. Carotenoids are the oil soluble pigments and thus could be extracted efficiently when flaxseed oil was used as the extracting medium.

However, there is also a possibility that the other oil soluble and oil insoluble pigments too could have been extracted with the extracting medium, but the chances for this are less as carrots contain high proportion of lipophilic carotenoids pigments (Surles *et al*., 2004). Previously, Sachindra *et al*., (2006) carried out the extraction of carotenoids from shrimp waste using oil (sunflower) as extracting medium. The carotenoids from carrot pomace are reported to be rich in β-carotene which possess very good pro-vitamin A activity. Therefore, this extract could also be used as dietary supplement along with providing coloring attributes to food products (Surles *et al*., 2004). Furthermore, ω-3 rich flaxseed oil is known for its health beneficiary properties like it has been suggested to prevent the chances of diabetes, cancer, arthritis, inflammatory diseases, depression, heart disease, hypertension, memory problems, weight gain and some allergies (Ruxton *et al*., 2004).

The ABTS antioxidant activity is based on the extent of reduction of the ABTS radical cation and is based upon single electron transfer mechanism. The ABTS free radical scavenging activity of carotenoids is mainly due to carboxyl or hydroxyl functional group at terminal ends as well as the number of conjugated double bonds in hydrocarbon chain. It is mainly based on the ionization potential of the carotenoids in non-polar solvents (Miller *et al*., 1996). Hence, increase in antioxidant activity is mainly contributed by extracted carotenoids with some minor lipid soluble antioxidant components in carotenoids rich extract. ABTS antioxidant activity permits the determination of both hydrophilic and lipophilic antioxidants. However, hydrophilic antioxidants are better reflected by ABTS assay than DPPH assay. The difference in the absorbance values before and after particular time period i.e. 10 min in our case can be used to estimate the hydrophilic and hydrophobic antioxidant activity. After sample addition the decrease in absorbance is directly proportional to amount of antioxidants in the sample (Arnao *et al*., 2001).

The principle of FRAP antioxidant activity is based on reduction of ferric ion to ferrous ion at low pH (3.6) via electron transfer mechanism with antioxidant resulting in colored ferrous-tripyridltriazine complex formation due to which it leads to increase in absorbance (Benzie and strain, 1996). The ferric reducing activity is mainly influenced by the size of the conjugated double bond (C=C) system. The electron-withdrawing characters of the carbonyl oxygen affects the decrease of FRAP value of carotenoids.

FRAP and ABTS are, however, based on single electron transfer mechanism, but the values for both the parameters were different. This could probably be due to different reaction conditions adopted to measure both the antioxidant activities, especially in terms of the pH value (pH for FRAP being 3.6, while pH of ABTS being 7.4) and the oxidizing

molecules (ferric di-TPTZ vs. ABTS) leading to different antioxidant activities. The variation in the results may be attributed to the varying reaction conditions and the effectiveness of the antioxidant components present in carotenoids extract towards ABTS and FRAP free radicals (Muller *et al*., 2011).

DPPH is a chemical compound that possesses a proton free radical with a characteristic absorption which decreases significantly on exposure to proton radical scavengers (Yamaguchi *et al.*, 1998). Muller *et al.* (2011) reported that carotenoids do not scavenge DPPH free radical, hence DPPH antioxidant activity was observed less in assay involving DPPH as compared to ABTS and FRAP antioxidant activities. The low antioxidant activity might be due to less contribution of carotenoids to scavenge DPPH antioxidant and whatever antioxidant activity is there that may be due to some other antioxidants present in the extract such as α-tocopherol (present in flaxseed oil) which may act as radical scavenger (Jiang *et al*., 2014).

This increase in L*, a*, b* values was mainly attributed to the extraction of lipophilic carotenoids in flaxseed oil as the extracting medium. The L* value were increased from 4.085 ±0.12 to 18.65 ±0.037 indicating more bright colour of the extract as compared to flaxseed oil. a* value increased significantly from 1.502 ±0.025 to 19.42 ±0.206 indicating that colour of extract shifted towards the red shade and b* value increased from 2.163 ±0.106 to 27.94 ±0.65 which reveals that there was increase in the yellow shade. The increase in red and yellow shade of extract can mainly be attributed to the carotenoids providing red and yellow colours.

There was no significant difference ($p>0.05$) between the peroxide value of flaxseed oil as well as carotenoid rich extract (Table 4.4) which was within limits as given by New Zealand Food Regulations (1984) for edible fats and oils i.e. 10 meq peroxides/ kg of oil and the Codex Alimentarius Commission (1999) i.e. 5 meq peroxides/kg of oil, which means that oil and carotenoid- rich extract are free from oxidation which indicate the freshness of flaxseed oil and it was free from oxidation. Burten & Ingold, (1984) reported that carotenoids can prevent the vegetable oils from being oxidized and can act as potent antioxidant for oils.

6.1.5 Estimation of beta-carotene using High Performance Liquid Chromatography (HPLC)

The amount of beta carotene in the both flaxseed and CRE was calculated by plotting the peak area of each sample in standard curve equation (at the retention time) followed by multiplying with dilution factor. Thus, the β-carotene content of flaxseed oil was approximately 6.75 µg/ g, while for CRE it was 78.37 µg/g. Koley and coworkers (2014)

reported that the amount of amount of β-carotene present in orange variety of carrots is approximately 45 µg/g, while our study shows a very high content of the beta-carotene which may be due to change in variety or cultivar of orange carrot. Tadesse *et al*. (2015), on the other hand, reported that fresh carrot contains approximately 71 ppm of β-carotene. The results of the present study are in accordance with the reported value. β-carotenes are reported to be highly unsaturated compounds with extensive conjugated double-bond systems that are susceptible to oxidation, isomerisation and other chemical changes during processing and storage (Shi and Maguer, 2000). However, in our study the higher retention of β-carotene could be attributed to proper storage and processing of carrot pomace.

6.2 Objective 2: Optimization of emulsion-based delivery system of extracted carotenoids and its stability assessment

Effect of different levels of carotenoid rich extract (CRE), whey protein concentrate and lactose and their interaction on physico-chemical properties of emulsion

The effect of different concentrations of CRE, whey protein concentrate and lactose was studied on the physico-chemical properties, namely, particle size, zeta potential, viscosity, L*, a*, b* values of the emulsion (Table 5).

6.2.1.1 Particle size, zeta potential, viscosity and color values (L*, a*, b*)

It has been reported in past that the particle size should be less for greater emulsion stability because when the particle size is smaller in that case the Brownian motion will exceed the Stoke's law and particles will remain suspended in the emulsion, while in case of larger particles, they get settled down in the bottom which will affect the stability of emulsion and same is evident with the results obtained for the present combination vis-à-vis highest stability with respect to gravity separation (i.e. 15 days) and centrifugal stability. Rosenberg and Young (1993) reported that when whey protein concentrates are used as a wall material, spherical capsules with smooth and wrinkle-free surfaces are formed. The encapsulation of milk fat was done successfully with particle size ranging between 50-600 nm. The core material was reported to be uniformly distributed throughout the interior of the wall matrices with no visible pores or cracks exposing the core material to the environment.

According to zeta potential relating to emulsion stability, whey protein concentrates being negatively charged at neutral pH, showed negative zeta-potential on emulsion droplets (Goyal *et al*., 2015). The pH near to isoelectric point of protein shows zero zeta potential resulting in no electrostatic repulsion. A further increase in anionic groups leads to increase in net negative charge and decrease in positive charge as is followed in case of Whey protein concentrates (Kulmyrzaev and Schubert, 2004). Taherian *et al*. (2011) reported that whey protein concentrates can lead to stronger electrostatic repulsion and superior emulsion stability. Our data of zeta potential shows the negative value which reveal that this hypothesis is true.

L* value indicates the increase in lightness (towards 100) and darkness (towards zero) of the emulsion. Thus, lower L* value indicates the extract was on lighter side which depends upon the amount of WPC and lactose used for the emulsion preparation. The highest L* value was obtained in case when the CRE was used at the rate of 40%, while WPC and lactose both at 7.5%. This could be due to the higher level of CRE and adequate level of WPC and lactose used to coat the CRE. Therefore, 7.5% level for both WPC and lactose was best suited to yield high L* value i.e. 75.04±0.09.

The increase in redness values are expressed as a* value of the emulsion. Thus, with increasing level of CRE, a* value would be higher. The highest a* value was obtained with 40% level of CRE as it contains highest levels of carotenoids, while in case of WPC and lactose, 7.5% WPC and 10% Lactose are sufficient to provide red shade to the emulsion. If WPC and lactose are used at higher levels, these may provide colour masking effect.

The b* value indicates the increase in yellow shade of the emulsion. The increasing level of CRE resulted in higher b* value, while in case of WPC and lactose, the amount of WPC showing higher b* value was 5% and for lactose it was 7.5%. Lesser amount of both WPC and lactose are required to produce more of yellowness because if both of them are used at higher levels they may possess some color masking effects. The highest b* value (54.05±0.07) was obtained in the combination possessing 40% CRE, 5% WPC and 10% Lactose.

6.2.3 Encapsulation Efficiency of emulsion

The encapsulation efficiency of more than 90% indicates the proficient encapsulation of CRE, while in another case i.e. control the CRE was passed through the same filters but after dissolving in ethyl acetate. Ethyl acetate was taken because CRE was soluble in ethyl acetate and it was more compatible with the membranes of the filters used. However, initially the

trials were taken by dissolving CRE in other solvents like hexane. CRE was found to be dissolved in hexane, but it was observed that hexane was incompatible with these filters. Therefore, ethyl acetate was selected as the choice of solvent. Young *et al*. (1993) did the encapsulation of anhydrous milk fat using whey protein concentrates and lactose as encapsulating material in which they observed the effects of microencapsulating agent type and concentration (10 to 30% w/w) and of fat load (25 to 75% w/w) on the microencapsulation yield and encapsulation yields of more than 90% were obtained for all evaluated systems. Sari and coworkers (2015) also studied the encapsulation efficiency by using whey protein concentrates and Tween-20 as the coating material and found the encapsulation efficiency of approx. 90%.

Physico-chemical analysis of the emulsion

Total carotenoid content, antioxidant activity, color values, morphology,

The total carotenoid content is mainly because only 35% of CRE was used as core material. The other ingredients used for formulation of emulsion i.e. WPC and lactose do not have influence on the TCC of emulsion.

The antioxidant activity is mainly contributed by carotenoids present in the CRE. However, the antioxidant activity could have been contributed by whey proteins also. Since whey proteins are good source of some of the bio-active peptides (Power *et al*., 2013) possessing antioxidant activity and radical scavenging capacity.

In DPPH and FRAP, the antioxidant activity was predominantly due to carotenoids present in CRE and WPC. Gad *et al*. (2010) reported that the whey proteins possess very good radical scavenging activity. The researchers estimated the antioxidant activity in terms of DPPH. The authors also carried out the in vivo studies and reported that whey proteins in conjunction with some other antioxidant rich component can prevent the liver damage caused by carbon tetrachloride.

L* value increased significantly which might be due to increase in whiteness of emulsion caused by WPC and lactose. a* value of the emulsion was observed to decrease significantly ($p<0.05$) which indicates the decrease in redness, while b* value was increased showing that after incorporating CRE to emulsion, yellowness increased.

Scanning electron microscopy revealed that surface of the droplets showed no visible fissures and cracks. The varied size, while uniform shape was observed in all the droplets. Similar results were obtained in the SEM analysis carried out by Shen *et al*. (2013) who compared microencapsulation of astaxanthin by using whey protein isolates (WPI) and

sodium caseinate as natural emulsifiers and they found that by using WPI smoother texture can be obtained with very little dents on the surface.

TEM analysis of the carotenoid rich emulsion was done and resultant microphotographs indicates that most of the particles are spherical in shape showing the integrity of WPC and lactose as the coating material. who prepared β-carotene rich emulsions using artificial emulsifiers and revealed that the nano-emulsion droplets were non-aggregated and nearly spherical in shape in TEM analysis.

6.3 Objective 3: Application of dried delivery system (rich in carotenoids and omega-3 fatty acids) in flavoured milk as model food system.

6.3.1 Drying of emulsion

The optimized emulsion was dried in freeze dryer and the freeze dried powder was used for further incorporation in the product.

6.3.2 Optimization of carotenoid rich powder for the preparation of carotenoid rich flavored milk

The optimization of carotenoid rich flavoured milk was done on the basis of sensory scores. For the preparation of flavoured milk, the level of sugar, speed of homogenization using HSD and time were kept constant @ 8.5 %, 20 000 rpm and 10 min, respectively, while the level of carotenoid rich powder (CRP) was varied at a level of 8%, 10% and 12% on the basis of preliminary trials. A non-significant difference was observed between control and carotenoid rich flavored milk (CRFM) containing 8%, 10% and 12% level of CRP for the scores obtained for color and appearance, odour, mouthfeel and overall acceptability. However, the scores for taste decreased significantly from 7.25±0.36 to 6.45±0.43 when 10 and 12% of CRP, respectively was added. This could be attributed to typical taste offered by flaxseed oil. Thus, on this basis of the scores obtained for taste, 10% level of CRP was selected for further study.

6.3.3 Proximate analysis of the CRFM

The proximate analysis of the optimized flavored drink was carried out and all the parameters were found to be significantly higher ($p<0.05$) in CRFM except ash and total sugar content which showed non-significant difference ($p>0.05$) between control and CRFM. Fat content of CRFM was higher than control which can mainly be attributed to the CRP containing CRE. The significant difference in the protein content of control and CRFM could be attributed to the presence of WPC in the coat materials.

7. Statistical Analysis: All the experiments were carried out in triplicate. Results are expressed as mean ± standard deviation (SD). The data obtained during optimization were analyzed statistically using ANOVA method in SPSS tool (IBM SPSS Statistics Version 20) as function of Duncan Post Hock Test ($p<0.05$). The data for three factorial design was analyzed using Proc GLM method of SAS 9.3. The data obtained during storage were analyzed statistically using paired sample t-test along with ANOVA method in SPSS tool (IBM SPSS Statistics version 21) as function of Duncan Post Hock Test ($p<0.05$).

8. Impact of the research in the advancement of knowledge or benefit to mankind

Most importantly, the oil rich in carotenoids can be directed to food and pharmaceutical applications as natural colorant and health supplement.

Usability of research

- This research aid with finding the efficacy of carotenoids as natural colorant: Synthetic food colours though not banned in most of the countries but are threat to food safety. Therefore, pigments extracted from horticultural waste are the most suitable alternatives. Henceforth, Carotenoids extracted in this work can be effectively used as natural colorant in variety of food products.
- This work will provide links for novel formulations as a source of β-carotene: Owning to pro-vitamin A activity β-carotene, a sub-category of carotenoids have important place in the pigments extracted from horticultural commodities. Carrot contains 90% of carotenoids as β-carotene, therefore this type of research is highly suitable to extract these valuable neutraceutical.
- This work is based on environment friendly approch: Carrot bio-waste is the major solid residue produced form carrot juice processing industry which contains at least 50 % of the carotenoids. Thus, utilizing such valuable and underutilized carotenoid source can be highly beneficial to maintain the concept of novel circular economy and decreasing the environmental burden of waste disposal.
- This work is focused to emphasize the reduction of usage of organic solvent: Earlier more emphasis on the extraction of natural pigments was given using organic solvents. Such protocols leaves residual solvents after processing which again possess various adverse health effects including hindering in normal cellular metabolisms thereby cellular degeneration, CNS depressant, and chronic encephalopathy etc. Thus, in future also optimizing and working on techniques that can replace the organic solvents with vegetable oils for the extraction of natural pigment can be a breakthrough.

Outcome of research

Output from objective 1: Carotenoids rich oil extract can be potentially used as natural colorant in oil based foods like butter; spreads etc. in food industry and oils based capsules for pharma industry

Output from objective 2: Powder prepared from emulsion of carotenoids can be used directly as natural colorant for aqueous/water based foods and health mix sachets

Output from objective 3: Application in of freeze dried powder was indicated in flavoured milk

<table>
<tr><th colspan="3">Overall output from all the 3 objectives</th></tr>
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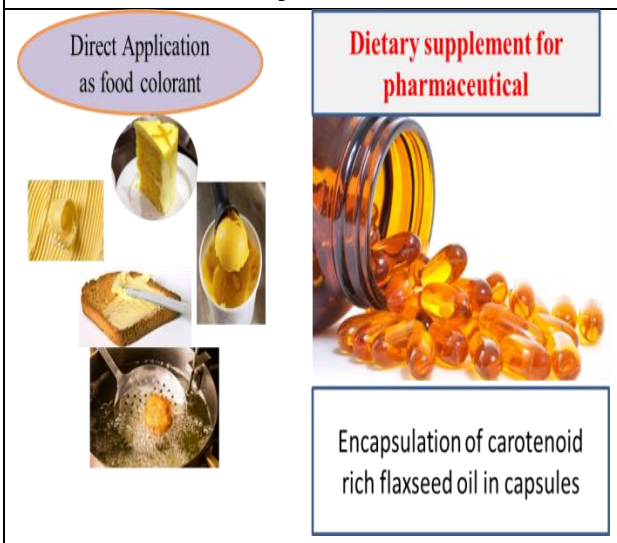
<td> 
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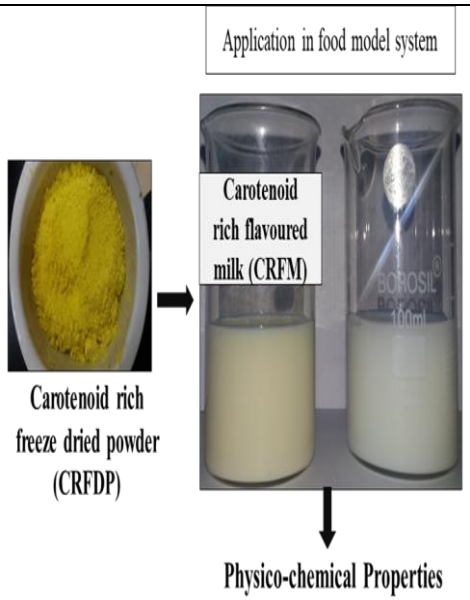
<td> 
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Overall output from sustainable development point of view: Utilization of bio-wastes from food processing industries is a promising approach for the extraction of nutraceutical and bioactive components. The innovation can facilitate the link between food, agro-waste and pharmaceutical industry thus creating jobs in renewable energy section of agro industries. This work may enhance the resource efficiency of horticultural bio-waste thereby regional and global collaborations in maintaining sustainable development goals. The adoption of green technology is always imperative to realise the sustainable development goals. Currently, the main challenge for the extraction of natural colors is extraction medium. There is unfair use of hazardous organic solvents for the extraction of natural colors which are highly toxic for environment as well. This innovation can be a potential contributor to improve the environment. Hence, it can go well with the aim of utilization of technology while at the same time maintaining circular economy which can stimulate the economy.

9. Litrature

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**This research was carried out at ICAR-National Dairy Research Institute, Karnal Haryana.*

Applicant's signature