Preparation and physical properties of carotenoids encapsulated in chitosan cross-linked tripolyphosphate nanoparticles

Phathanee Thamaket[1] **and Patcharin Raviyan**[1,*]

Abstract

Encapsulation of carotenoids in the chitosan cross-linked tripolyphosphate (TPP) was formulated at different ratios of chitosan: TPP to prepare the nanoparticles with superior aqueous solubility, efficient loading and release. The freeze dried sample prepared under the optimal formula of 3.00% (w/v) carotenoids, 1.50% (w/v) chitosan and 2.00% (w/v) TPP provided the particle with the average size of 317 nm and the encapsulation efficiency of 89.09%. Release of carotenoids in oil was relatively superior to ethanol and phosphate buffer, however, the encapsulated carotenoids could be effectively applied as a natural colorant in both oil-based salad cream and water-based drink. This study illustrated a potential approach to fabricate the carotenoids nanoparticles with competent aqueous solubility and releasing property.

Key words: carotenoids, chitosan, encapsulation, nanoparticles, tripolyphosphate

1. Introduction

Carotenoids are natural colorants with potent antioxidant property (Albanes, 1999; Erhardt *et al*., 2003; Mortensen, 2006). Nevertheless, carotenoids can be easily degraded by several factors including high temperature, solvent, acid, light, free radicals, iron and iodine, electron transfer, hydrogen abstraction, adduct formation and oxygen (Boon *et al.,* 2010). Prevention of carotenoids degradation by encapsulation in various complex matrixes have been reports (Elizalde *et al.*, 2002; Loksuwan, 2007; Nunes and Mercadante, 2007; Rascon *et al.*, 2011). However, increasing solubility of these lipophilic compounds in the water system that are common used in several food products is still required to be investigated.

Chitosan and its derivatives are the natural polycationic polysaccharides containing glucosamine and N-acetyl-glucosamine. They have been widely studied for applications in food, agriculture, biochemistry, cosmetic, pharmaceutical industries and waste water treatment because they are promptly available via cationic polyelectrolyte in acid solution. Furthermore, they have multipurpose properties and environmentally friendly raw material, non-toxic, biocompatible and biodegradable. The forms of the chitosan biopolymer could be liquid

[1] Division of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand, 50100

* Corresponding author : Tel: +6689-956-1078, E-mail: patraviyan@gmail.com

crystals, membranes and microcapsules (Harish-Prashanthand and Tharanathan, 2007; Rinaudo, 2006).

Despite several advantages, chitosan has some important drawbacks including poor releasing capability and poor solubility in water. These drawbacks could be improved by decreasing the particle size. Consequently, the recent studies have been focused on preparing the chitosan of nano-size (Hosseini *et al.,* 2013; Hu *et al.,* 2008; Kafshgari *et al.,* 2011; Lin *et al.,* 2007; Qi *et al.,* 2004). The chitosan nanoparticles exhibit a better activity as a result of their small size. Among many approaches, the cross-linking process with chemical agents is of particular interest to prepare the chitosan nanoparticles as it is a simple method (Tsai *et al.,* 2008).

Solubility of carotenoids could be achieved by modifying the structure of the carotenoids with encapsulated chitosan via crosslinking agent such as tripolyphosphate (TPP). Chitosan has a high density of amine groups in its backbone which are protonized to form NH_3^+ in acidic solution. These positively charged groups in chitosan can be chemically cross-linked with TPP.

TPP is a non-toxic compound that has been recognized as an acceptable food additive by the US Food and Drug Administration (Lin *et al.,* 2008). The quick gelling ability of TPP is the significant property that make it a potential cross-linker for an ionic gelation of chitosan (Gan and Wang, 2007; Mi *et al*., 2003). The chitosan–TPP matrix has been previously studies as the chelating resin of copper (II) ion (Lee *et al*., 2001) and the carrier of tea catechins (Hu *et al*., 2008).

According to the polar properties of the chitosan–TPP matrixes, carotenoids encapsulated in the chitosan–TPP nanoparticles have increasing water solubility. These modified properties could benefit several food industrial applications. Significantly, the process of ionic gelation of chitosan with TPP is feasible for scaling up in a particle processing operation (Stulzer *et al.,* 2009).

The purposes of this work were to determine the effects of TPP on encapsulation efficiency, releasing property and solubility of carotenoids in chitosan at various ratios of carotenoids to TPP. The selected encapsulated sample was tested as a natural colorant for a salad cream and a commercial drink.

2. Materials and methods

2.1 Materials

Carotenoids (oil, food grade) were purchased from Swanson, U.S.A. Chitosans powder (oligomer type, degree of deacytelated 94.50%) was purchased from Union Science, Thailand. Sodium tripolyphosphate (food grade) was purchased from Thai Polyphosphate and Chemicals, Thailand. Tween 80 (analytical grade) was purchased from Srichand United Dispensary, Thailand. Commercial carotenoids; Control (powder, food grade, 2.5% Mixed Carotenoids from *Dunaliella salina*) were purchased from E.I.D. Parry Nutraceuticals Ltd, India. Salad creams were purchased from Unilever Thai Holding Co. Ltd, Thailand. Commercial drink (10% concentrates grape juice with collagen 1000 ml) was purchased from Sapanan General Food Co., Ltd, Thailand.

2.2 Encapsulation method

Carotenoid was encapsulated by entrapping 2.0, 3.0, 4.0 and 5.0% (w/v) carotenoids in the Chitosan-TPP matrix. Firstly, 40 ml of each carotenoids solution was dropped by syringe into 200 ml of 1.5% (w/v) chitosan solution while stirring at 750 rpm for 2 h. Then, 1 ml of Tween 80 was gradually added and the mixture and was continuously stirred for another 2 h. Next, 20 ml of 0.5, 1.0 or 2.0% (w/v) TPP solution was dropped into the mixture and continuously stirred for 2 h. Finally, the mixture was homogenized at 5,000 rpm for 30 min. The obtained mixture was freeze dried at -40ºC, $133x10^{-3}$ mbar. The dried encapsulated carotenoids were crushed into the powder with a blender at 60 Hz, 37ºC for 3 min and stored at -20ºC for the further analysis (Yangchao *et al.,* 2011 with modification).

2.3 Encapsulation efficiencies

Total carotenoids in the encapsulated particles were determined by dissolving 0.1 g of the encapsulated powder in 100 ml of ethanol and measured for the absorbance by using a spectrophotometer (UV WINLAB version 2.85.04, PerkinElmer™, UK) at 450 nm. For the surface determination of carotenoids, 0.1 g of encapsulated carotenoids was dissolving in 100 ml of ethanol. The suspension was agitated in a vortex mixer at room temperature for 1 min and then filtered. The supernatant after filtered was measured for the absorbance using a spectrophotometer at 450 nm. The encapsulation efficiencies (EE) were calculated as the percentage of the ratio following equation (Saenz *et al*., 2009).

$$EE\ (\%) = [(A- B)/ C] \times 100 \quad (1)$$

A = total carotenoids in encapsulated particles

B = surface carotenoids

C = total carotenoids input

2.4 Measuring of the particles size by Scanning Electron Microscopy (SEM)

The particles size was observed under a scanning electron microscopy (SEM, JEOL Ltd, JSM-5910lv, Japan). Firstly, the samples were cast-dried on an aluminum pan before cutting into an appropriate size, and then adhered on the conductive carbon tapes. Subsequently, the samples were mounted on the specimen stubs and coated with a thin (<20 nm) conductive gold and platinum layer using a sputter coater. All images from SEM were codified using the Image J program (Image Processing and Analysis in Java: version 1.46r).

2.5 Solubility

Solubility of the encapsulated carotenoids powder was evaluated using deionized water and ethanol. One gram of the powder was dissolved in 10 ml of ethanol or distilled water. The suspension was centrifuged at 3,000 rpm for 10 min and dried in the hot air oven at 105ºC for 24 h (Shittu and Lawal, 2007). Weight of the solids recovered after drying was used to calculate the percentage of solubility. The percentage solubility was calculated as described by Sirirad (2009) as following:

$$Solubility\ (\%) = (A/B) \times 100 \quad (2)$$

A = Weight of dried sample that dissolved in the supernatant

B = Total weight of sample

2.6 Releasing property

The 0.1 g of encapsulated carotenoids was dissolved in 250 ml coconut oil or ethanol or phosphate buffer (PBS) pH 7.4. The mixture was shaken in a shaking water bath at 37ºC for 10 min. The absorbance values of the supernatant kept at 37ºC were recorded at holding times of 5, 10, 15, 30, 60, 120, 180, 240, 300, 360 and 420 min using the spectrophotometers at the wavelength of 450 nm. The releasing property of the encapsulated carotenoids was determined by plotting graph between the quantities of total carotenoids and holding time (Praphairaksit and Ngamsiri, 2007 with modification).

2.7 Application as a colorant in food products

Application in salad cream

The commercial salad cream without carotenoids or any other colorants was used in this experiment. The highest efficiency of encapsulated carotenoids was selected for this application. According to the FDA regulation of salad cream, carotenoids have been allowed to add in the maximum of 155.80 ppm. In this study, the sample of salad cream with 133.34 ppm of total carotenoids were prepared by mixing 30 g of the commercial salad cream with 0.6624 g of the encapsulated carotenoids. The control sample was the commercial salad cream with the same amount of the total carotenoids where it mixed with 0.5756 g of the commercial carotenoids. The L*, a* and b* values (Minolta Chroma meter CR-300, Japan), visual color and viscosity of all tested samples were measured at the beginning time, minimum release time (refer to the releasing properties section) and continuously measured every hour until the constant value was obtained at room temperature.

Application in commercial drink

The commercial drink without adding carotenoids or any other colorants was used in this experiment. The selected formula of carotenoids encapsulated chitosan-TPP of 3.0% (w/v) carotenoids with 2.0% (w/v) TPP was prepared according to method 3.3. According to the FDA regulation of commercial drink, carotenoids have been allowed to add in the maximum of 15.58 ppm. In this study, the sample of commercial drink with 10 ppm carotenoids were prepared by mixing 100 ml of commercial drink with 0.1670 g of the encapsulated carotenoids. The control sample was commercial drink with the same amount of total carotenoids, which the commercial drink was mixed with 0.1482 g of the commercial carotenoids.

The L*, a* and b* values, visual color and viscosity of all tested samples were measured at beginning time, minimum release time, sediment disappear time and continuous measured 1 hr until the value constant at room temperature.

2.8 Statistical analysis

The data was analyzed by SPSS (Statistical Package for the Social Sciences version 17) in Duncan's new multiple range tests. The different results between the experimental groups was considered at a 95% confidence interval ($p \leq 0.05$).

3. Results and discussion

3.1 Encapsulation efficiencies

The encapsulation efficiencies of the chitosan matrixes prepared with different percentage ratio of the encapsulated carotenoids were increased with the concentration of the TTP (Figure 1). The highest encapsulation efficiency was 89.09% which obtained from loading 3.0% (w/v) carotenoids into chitosan cross-linked with 2.0% (w/v) TPP. The higher concentration of the loading carotenoids did not provide higher encapsulation efficiency. This phenomenon was also observed in other polymeric matrices that over loading of encapsulated material caused a decrease of encapsulation efficiency (Liu and Park, 2009; Luo *et al.,* 2011; Shah *et al.,* 2009). The higher encapsulation efficiency resulted from an appropriate ratio of the cross-linked interaction of hydrogen bond between charged of NH_3^+ group in chitosan and carotenoids. When the concentration of carotenoids become restricted the amount of un-neutralized NH_3^+ groups presented, no further increased in the encapsulation efficiency (Konecsni *et al.,* 2012; Zhang and Kosaraju, 2007).

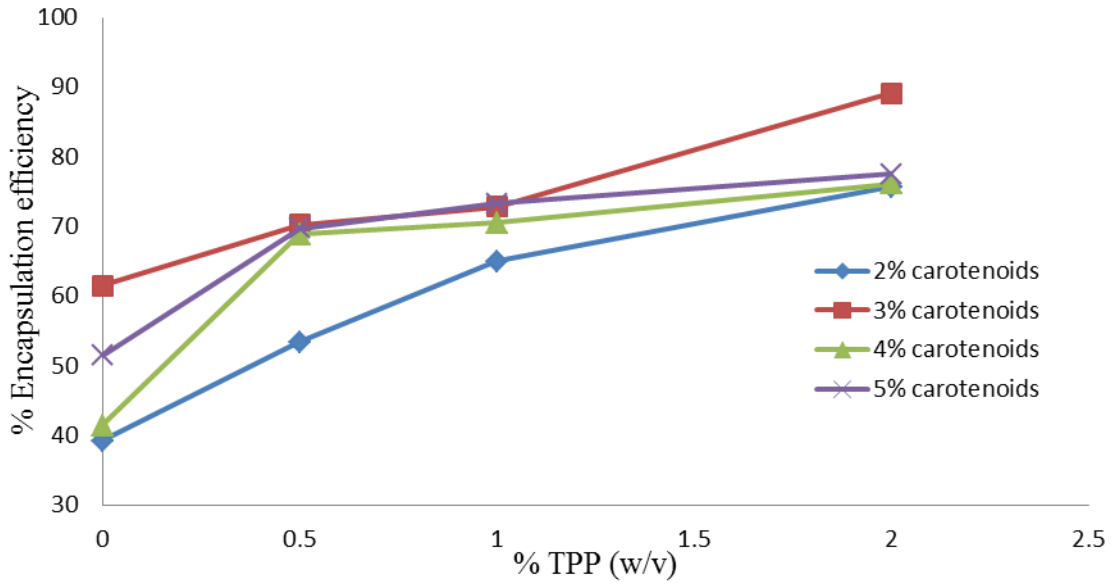

Figure 1 Encapsulation efficiencies of carotenoids encapsulated in chitosan-TPP

3.2 Particle size

The encapsulated carotenoids prepared under the optimal formula appeared in regular elongated shape and rough surface (Figure 2). The average diameter of the carotenoids encapsulated in chitosan-TPP was 317 nm. The previous studies also reported the nano-size of chitosan-TPP particles (Hu *et al*., 2008; Luangtana-anan *et al*., 2005; Tsai *et al*., 2008; Rodrigues *et al.,* 2012; Chattopadhyay and Inamdar, 2012).

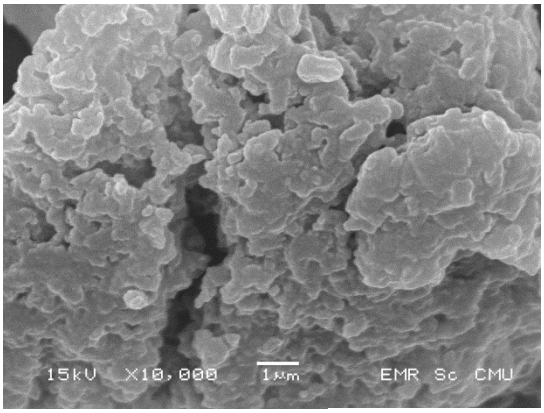

Figure 2 SEM image of 3.0% (w/v) carotenoids encapsulated in 1.5% (w/v) chitosan and 2.0% (w/v) TPP

3.3 Solubility

Ethanol and water solubilities of carotenoids encapsulated in the chitosan cross-linked TPP were increased with increasing ratio of TPP (Figure 3). These results indicated that solubility was affected by the particle size. The carotenoids in nature are lipophilic compounds which cannot dissolve in aqueous media. Advantageously, this study indicated the chitosan cross-linked with TPP could improve the solubility of carotenoids in aqueous solution effectively. The improving solubility caused by the cross-linking between group I and NH_4^+ (cations) of chitosan with PO_4^{3-} (anions) of TPP (Harold and Charles, 2001; Ralph *et al.,* 2011).

Solubility of carotenoids could be achieved by modifying the structure of carotenoids by encapsulation in chitosan cross-linking with TPP. According to the polar properties of the chitosan–TPP matrixes, encapsulated carotenoids had the desired properties of increasing water solubility. These modified properties would benefit several food industrial applications especially the process of ionic gelation of chitosan with TPP is feasible for the scaling up in a particle processing operation.

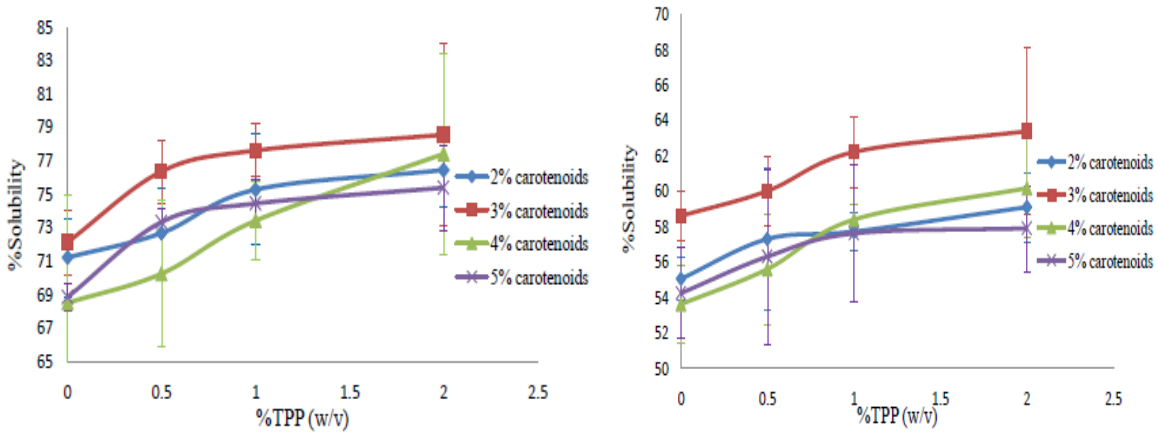

Figure3 Solubility of the carotenoids encapsulated in chitosan-TPP in ethanol (a), and water (b)

3.4 Releasing property

The releasing properties of carotenoids encapsulated in chitosan-TPP were evaluated using coconut oil, ethanol and phosphate buffer solution (PBS) at pH 7.4. The sampling procedure was carried out continuously until the concentrations of carotenoids in the tested solvents were constant, which were taken about 420 min. As shown in Figure 4, releasing patterns of control and encapsulated carotenoids in all solvents were relatively similar. The maximum releasing of the control carotenoids and the encapsulated carotenoids were 6.57 and 6.55 ppm in coconut oil at 180 min respectively. In ethanol, the release of control carotenoids was 5.42 ppm, while that of the encapsulated carotenoids was 5.24 ppm at 240 min. In PBS, the release of control carotenoids was 2.08 ppm, and that of the encapsulated carotenoids was1.95 ppm at 300 min.

This result demonstrated that the releasing efficiency in oil was better to ethanol and PBS. However, the releasing properties of encapsulated carotenoids and control were rather similar. The discrepancy could be due to the effect of the encapsulation matrix. Matalanis *et al.,* (2011) reported that the primary factor that controlled the releasing characteristic was the encapsulation matrix.

The previous study on releasing of carotenoids from the chitosan-TPP has not been reported in the literature yet. However, releasing rate of any bioactive core material should be generally affected by many factors including the properties of coating materials such as molecular weight/molar ratio of chitosan, quantity of TPP and the assay employed. Recently, McConnell *et al*. (2008) reported that chitosan films prepared without TPP were completely degraded when incubated at 37ºC with pancreatin enzymes and the degree of degradation depended upon the level of TPP added. Moreover, Lin *et al*. (2008) reported that chitosan-TPP particles become unstable and start breaking down at pH > 7.2 due to the deprotonation of the amino group of chitosan.

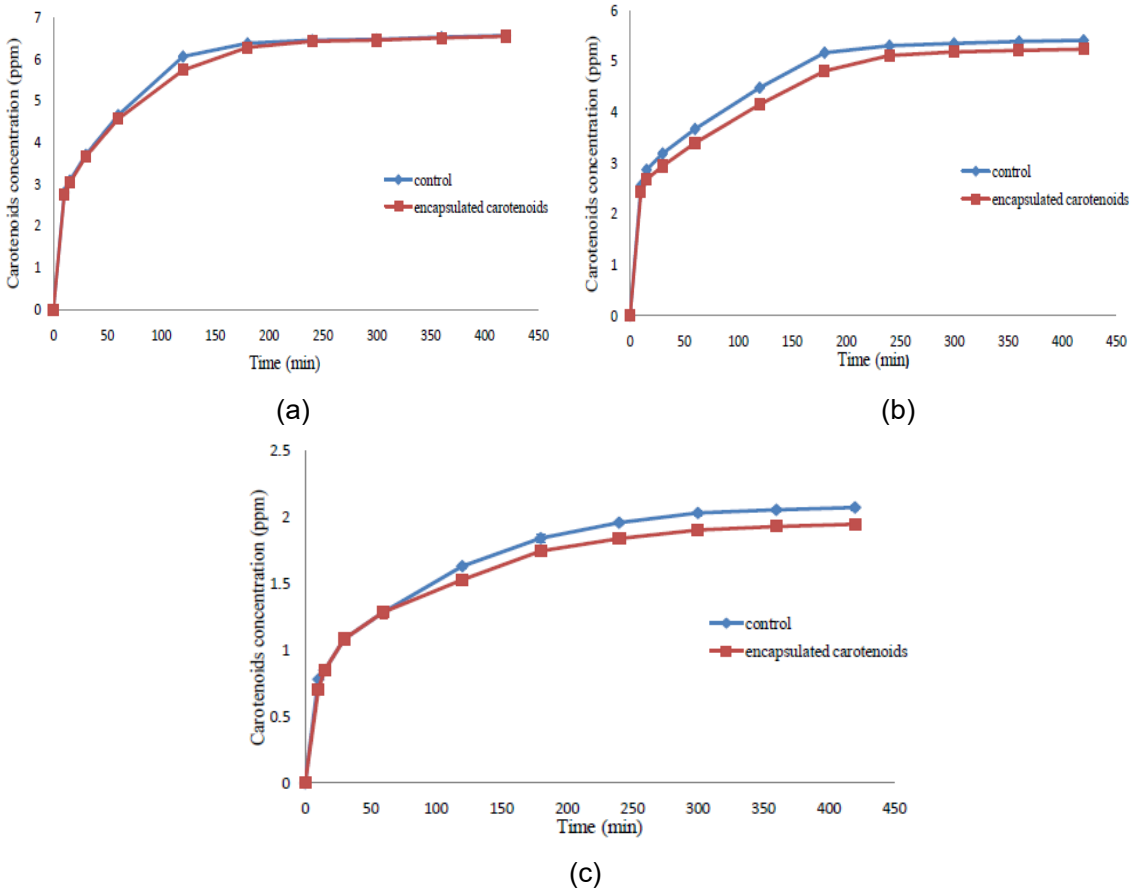

Figure 4 Releases of carotenoids encapsulated in chitosan-TPP in oil (a), ethanol (b), and phosphate buffer (PBS) at pH 7.4 (c)

3.5 Application as a food colorant

For food colorant study, the 3.0% (w/v) carotenoids encapsulated in 2.0% (w/v) chitosan-TPP was selected as colorant in the white commercial salad cream and the commercial drink made from the clear concentrated grape juice. The salad cream was selected as the lipid based model food, while the commercial drink was used as the aqueous based model food.

Salad cream

The 133.34 ppm of carotenoids was adequate to provide the color of salad cream. The visual colors of the commercial salad cream, salad cream mixed with the encapsulated carotenoids and salad cream mixed with the control carotenoids were white, orange and yellow as presented in Figure 5. Therefore, the encapsulated carotenoids achieved higher a* and less b* as compared to those values of the control sample.

Color of the salad cream mixed with the encapsulated carotenoids slightly increased with time. Then, the observed color was constant at the storage time of 3 h (Figurer 6). This minor increase suggested the fast releasing of carotenoids from the chitosan-TPP matrix and

the result agreed with the releasing property in coconut oil that the carotenoids would take 3 h to be completely released from the particle. In addition, separation of salad cream mixed with carotenoids color was not appeared after keeping at room temperature for 7 h. Moreover, addition of control or encapsulated carotenoids did not affect viscosity of the salad cream (Table 1). The prepared carotenoids could be used as colorants in food system satisfactory.

Figure 5 Appearances of the original salad cream (left),
salad cream mixed with carotenoids encapsulated in chitosan-TPP (middle),
salad cream mixed with the control (right)

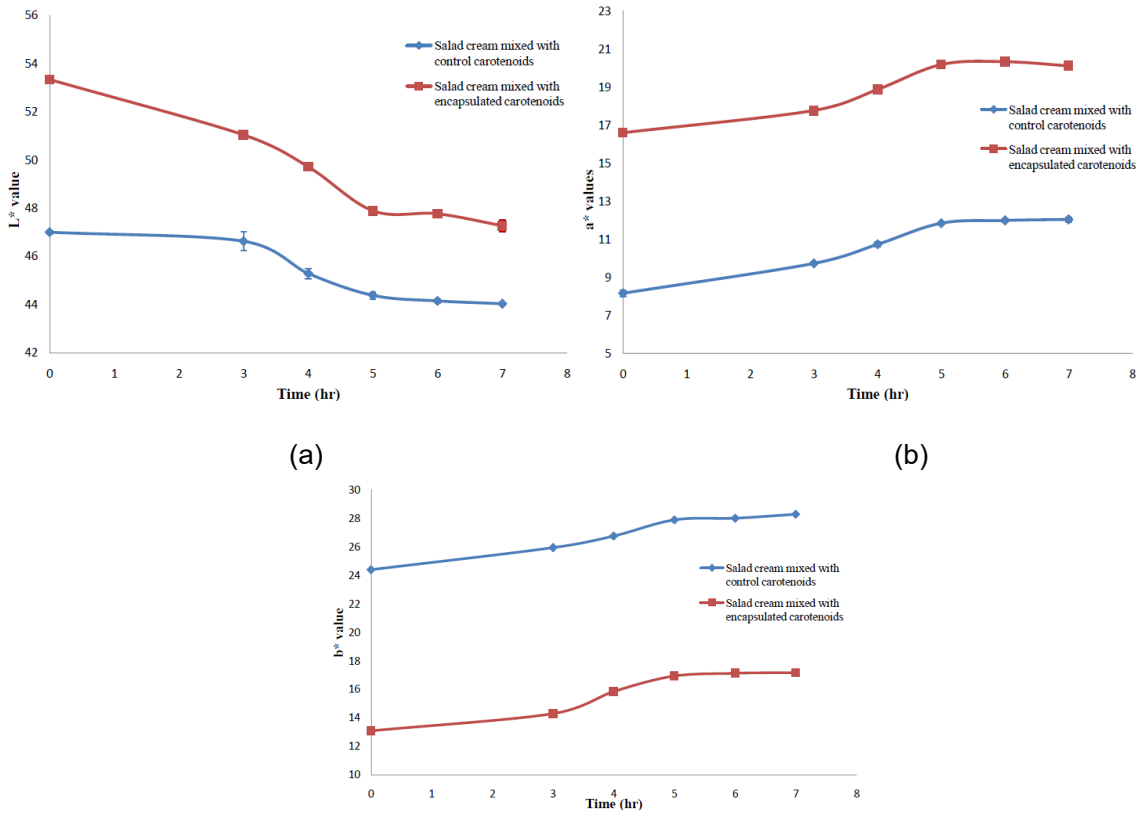

Figure 6 Color values of the salad cream samples (a) L* values (b) a* values (c) b* values

Table 1 Viscosity of salad cream

Hr	Viscosity (centipoises)		
	Salad cream	Salad cream with control carotenoids	Salad cream with encapsulated carotenoids
0	8933.73^{ns} ± 2.60	8936.88^{a} ± 2.83	8936.58^{a} ± 3.40
3	8933.96^{ns} ± 3.16	8937.78^{a} ± 0.39	8937.60^{a} ± 0.18
4	8934.16^{ns} ± 3.29	8938.20^{a} ± 0.18	8938.36^{a} ± 0.09
5	8934.00^{ns} ± 3.22	8939.25^{a} ± 0.82	8939.75^{ab} ± 0.41
6	8934.03^{ns} ± 3.18	8942.58^{b} ± 0.47	8942.81^{ab} ± 0.40
7	8934.16^{ns} ± 3.12	8945.11^{b} ± 0.17	8946.41^{c} ± 0.18

Note: Values were mean ± S.D. (n=5).

Different letter (a-c) in the same column indicated significant differences (p<0.05) between samples.

Commercial drink

The Food and Drug Administration (2010) has controlled the maximum quantity of carotenoids in drink at 15.58 ppm. In this experiment, 10 ppm of carotenoids was enough to provide color of the commercial drink. The commercial drink made from concentrated grape juice, Sappe Beauti Drink brand, was used for this study because this product was clear and had no color. Therefore, changing of color after adding this juice with carotenoids would be easy to observe. Figure 7 showed the original appearance of the commercial drink as well as the samples mixed with carotenoids. The sample with the carotenoids encapsulated in chitosan-TPP had the orange color with the positive a* and b*, while the sample with the control carotenoids contained a slightly deeper orange color. The discrepancy of color between carotenoids samples might be due to the difference sources of raw material. The a* and b* values of the tested samples were increased with time and the values were constant at the storage time of 14 h (Figure 8). The sample with the carotenoids encapsulated in chitosan-TPP had slightly sedimentation of the particles. However, the sedimentation disappeared at the storage time of 14 h. The rupture of particles should explain increasing a* and b* values after keeping the sample for 14 h. Addition of the control carotenoids did not change the viscosity of the drink. However, the use of encapsulated carotenoids resulted in slight increase of the viscosity (Table 2). The higher viscosity could be caused by the gel-like forming of chitosan-TPP in water (Berger *et al.,* 2004).

Figure 7 Appearances of the original drink (A), commercial drink mixed with carotenoids encapsulated chitosan-TPP (B), commercial drink mixed with control (C)

Table 2 Viscosity of the drink samples

H	Viscosity (centipoises)		
	Commercial drink	**Drink mixed with control carotenoids**	**Drink mixed with encapsulated carotenoids**
0	$2.01^{ns} \pm 0.01$	$2.32^{a} \pm 0.02$	$4.53^{a} \pm 0.02$
5	$2.02^{ns} \pm 0.01$	$2.34^{ab} \pm 0.01$	$4.56^{b} \pm 0.01$
14	$2.02^{ns} \pm 0.01$	$2.36^{b} \pm 0.01$	$4.58^{bc} \pm 0.01$
15	$2.03^{ns} \pm 0.02$	$2.37^{b} \pm 0.02$	$4.59^{cd} \pm 0.01$
16	$2.03^{ns} \pm 0.01$	$2.37^{b} \pm 0.01$	$4.60^{d} \pm 0.01$

Note: Values were mean ± S.D. (n=5).

Different letter (a-d) in the same column indicated significant differences ($p<0.05$) between samples

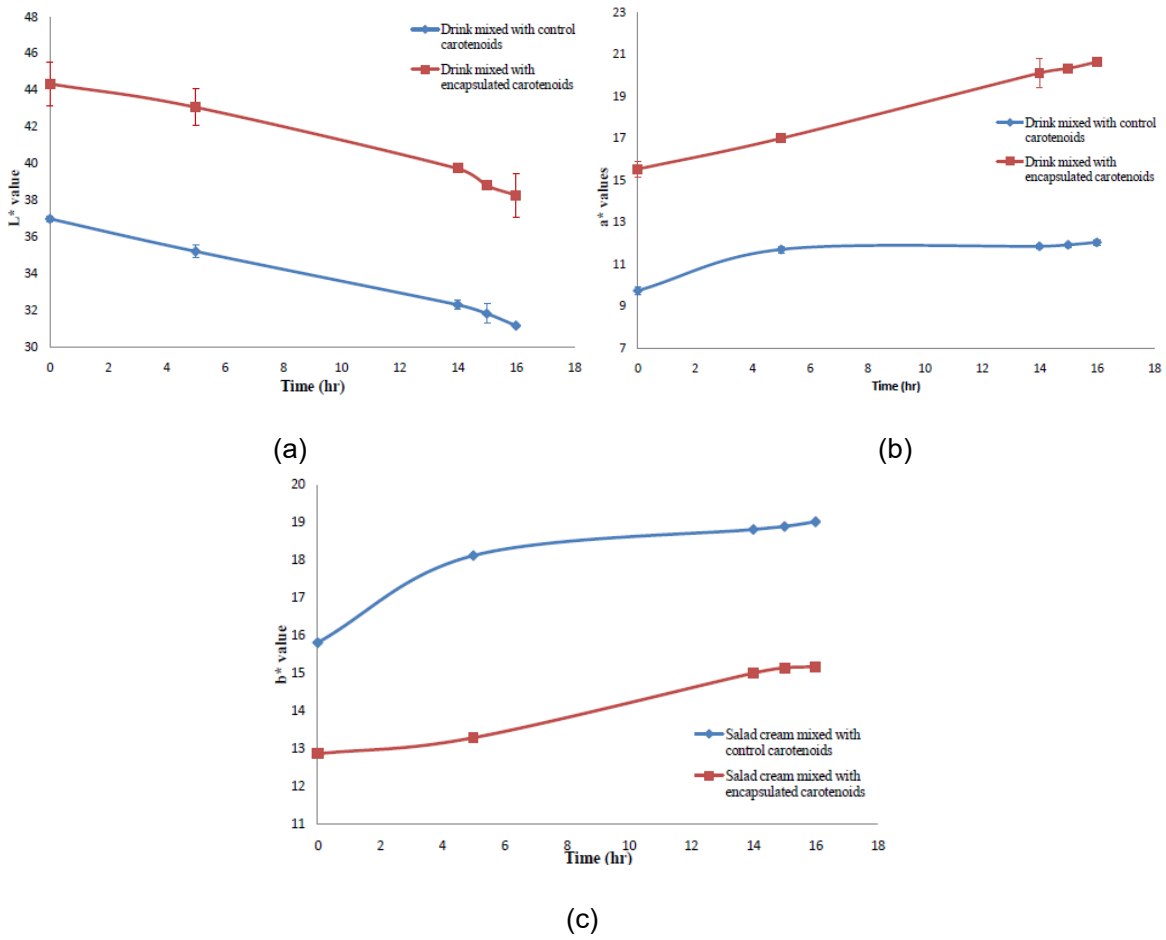

Figure 8 Color values of the salad drink samples (a) L* values (b) a* values (c) b* values

4. Conclusion

Encapsulation of 3.0% (w/v) carotenoids in 1.5% (w/v) chitosan and 2.0% (w/v) TPP was the optimal formula that could encapsulate carotenoids up to 89.09% within 36 h of encapsulation. The obtained nanoparticles has satisfactory releasing capability and solubility in both aqueous and lipid systems. Applications of the encapsulated samples in salad cream and commercial drink suggested that the developed carotenoids could be successfully used as the natural colorant for food products.

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