CHAPTER 3

Methodology

3.1 Apparatus

1. Syringe: 10 ml (Nipro Co. Ltd., Thailand)
2. Needle: 26G (Nipro Co. Ltd., Thailand)
3. Spectrophotometer: UV WINLAB version 2.85.04 (PerkinElmer^{TM}, UK)
4. Chroma meter: Model D25-2 (Minolta Chroma meter CR-300, Japan)
5. Water activity meter: Serie 3 (Aqualab, USA)
6. Micropipette: L-100 (Mettler-Toledo InternationalInc, Thailand)
7. Homogenizer: ULTRA-TURRAX® T50 basic (IKA Werke, Germany)
8. Freeze dryer: FreeZone 4.5 (Labconco, USA)
9. Scanning electron microscope: JSM-5910lv (JEOL Ltd., Japan)
10. Hot air oven: UNE 400 model (Memmert, Germany)
11. Hot plate stirrer: IKA®C-MAG HS7 (IKA Werke, Germany)
12. Magnetic stirring bar: IKAFLON® 50 (IKA Werke, Germany)
13. Shaking water bath: WNB29 (Memmert, Germany)
14. Blender: HR2011/70 (Philips, Netherlands)
15. Brookfield digital viscometer: DV-II+ (Brookfield Engineering Laboratories, Germany)
16. Particle size Analyzer Laser: Mastersizer S (Malvern Instruments Limited, UK)

3.2 Materials and reagents

1. Carotenoids: full spectrum carotenoid complex, food grade (Swanson, U.S.A)
2. Chitosans powder: King crab chitosan oligomer type, degree of deacytelated 94.5% (Union Science, Thailand)
3. Sodium tripolyphosphate: $NH_5P_3O_{10}$, food grade (Thai Polyphosphate and Chemicals, Thailand)
4. Tween 80: Polyoxyethylene 20 sorbitan monooleate, analytical grade (Srichand United Dispensary, Thailand)
5. Deionized water: H_2O, analytical grade (RCI Labscan, Thailand)
6. Acetic acid: CH_3COOH, MW 60.05, 99.8%, analytical grade (Merck, Germany)
7. Ethanol: Ethyl alcohol, C_2H_5OH, MW 46.07, analytical grade (Merck, Germany)
8. Ethanol: Ethyl alcohol, C_2H_5OH, MW 46.07, HPLC grade (Merck, Germany)
9. Standard β-carotene: $C_{40}H_{56}$, MW 536.87, Type I, synthetic, ≥93% (Fluka, U.S.A)
10. Methanol: Methyl alcohol, CH_3OH, MW 32.04, analytical grade (Merck, Germany)
11. ABTS: 2, 29-azinobis (3- ethylbenzothiazoline-6- sulfonic acid) diammonium salt, $C_{18}H_{18}N_4O_6S_4 \cdot (NH_3)_2$, MW 548.7 (Merck, Germany)
12. Potassium persulfate: di-potassium peroxdisulfate, $K_2S_2O_8$, MW 270.32, analytical grade (Sigma-Aldrich, UK)
13. Monobasic sodium phosphate: NaH_2PO_4, MW 119.98, analytical grade (Sigma-Aldrich, UK)
14. Dibasic sodium phosphate: Na_2HPO_4, MW 141.96, analytical grade (Sigma-Aldrich, UK)
15. Salad cream (Best Food: Unilever Thai Holding Co. Ltd, Thailand)
16. Commercial drink: 10% concentrates grape juice with collagen, 1000 ml, Sappe Beauti Drink (Pink), (Sapanan General Food Co., Ltd, Thailand)
17. Commercial carotenoids: 2.5% Mixed Carotenoids from Dunaliella salina, Parry Nutraceuticals (A Division of E.I.D. Parry Ltd, India)

3.3 Preparation of carotenoids encapsulated in chitosan-TPP

3.3.1 Preparation of stock solution

The carotenoids solutions of 2.0, 3.0, 4.0 and 5.0% (w/v) were prepared by thoroughly dissolving2.0, 3.0, 4.0 and 5.0 g carotenoids in 100 ml ethanol, respectively. The 1.5% (w/v) chitosan was prepared by dissolving 15 g chitosan powder in 1000 ml of 1% acetic acid (Appendix A1). The 0.5, 1.0 and 2.0% (w/v) tripolyphosphate (TPP) solution were prepared by dissolving 0.5, 1.0 and 2.0 g sodium tripolyphosphate in 100 ml deionized water.

3.3.2 Encapsulation method

Carotenoid was encapsulated by entrapping 2.0, 3.0, 4.0 and 5.0% (w/v) carotenoids in the Chitosan-TPP matrix. First, 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 hr. Then, 1 ml of Tween 80 was gradually added and continuously stirred the mixture for another 2hr. Next, 20 ml of0.5, 1.0 or 2.0% (w/v) TPP solution was dropped into the mixture and continuously stirred for 2hr. Finally, the mixture was homogenized at 5,000 rpm for 30 min. The obtained mixture was freeze dried at -40°C, $133x10^{-3}$ mbar. The dried encapsulated carotenoids was crushed into the powder with a blender at 60 Hz, 37°C for 3 min and stored at -20°C for the further assay (Yangchao *et al.*, 2011 with modification).

3.4 Analysis of the physical characteristics of carotenoids encapsulated in chitosan-TPP

3.4.1 Particle characterization, particle size and interstitial pore fraction observed under the Scanning Electron Microscopy (SEM)

The particles were observed under a scanning electron microscopy (SEM, JEOL Ltd, JSM-5910lv, Japan). Samples were first 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. Representative SEM images were reported. All images from SEM were codified using the Image J program (Appendix D) (Image Processing and Analysis in Java: version 1.46r).

1) Particle size measured by the Mastersizer

The particles were observed by Particle size Analyzer Laser (Mastersizer S, Malvern Instruments Limited). Powder sample were dispersed in the dispersing medium and using a pump speed of 2500 rpm, 2 min. The conditions tested were 300 RF mm range lens, λ: 633 nm He-Ne laser source, 2.40 mm beam length, 0.05-900 μm particle size ranges analysis, small sample dispersion unit (MS1) in a 600 ml beaker.

3.4.2 Encapsulation efficiencies

For the determination of surface carotenoids, 0.1 g of encapsulated carotenoids was dissolving in 100 ml of ethanol. These dispersions were agitated in a vortex at room temperature for 1 min and then filtered. The supernatant after filtered was measuring for the absorbance using a UV spectrophotometer at 450 nm. Total carotenoids in the encapsulated particles were determined by dissolving 0.1 g of the encapsulated powder in 100 ml of ethanol and measuring for the absorbance using a UV spectrophotometer at 450 nm. The absorbance values were calculated using the standard curve (Appendix B1, B3). The encapsulation efficiencies (EE) were calculated as the percentage of the ratio following equation (Saenz *et al*., 2009).

EE (%) = [(A- B)/ C] x 100

A = total carotenoids in encapsulated particles

B = surface carotenoids

C = total carotenoids input

3.4.3 Color

The values of the luminance or lightness (L*) and the two chromatic components of red-green colors (a*), and yellow-blue colors (b*) of the encapsulated carotenoids were measured using the chromameter. The L* value were ranged from 0 to 100. The a* component would be the positive and negative values for red and green

colors, respectively, The b* component would be the positive and negative values for yellow and blue colors, respectively.

3.4.4 Solubility

Solubilities of the encapsulated carotenoids powder were 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 before drying in the hot air oven at 105°C for 24 hr (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) x 100

A = Weight of dried sample that dissolved in the supernatant

B = Total weight of sample

3.5 Analysis of chemical characteristics of encapsulated carotenoids by Fourier Transform Infrared Spectrometer (FT-IR)

The carotenoids powders encapsulated in the chitosan with and without TTP were determined for the following chemical characteristics using the FT-IR (Appendix A2).

3.6 Release property

The 0.1 g of carotenoids encapsulated samples were dissolved in 250 ml coconut oil or ethanol or pH 7.4 phosphate buffer (PBS) prepared as described in Appendix A2. The mixture was shaken in a shaking waterbath at voltage of 230V/55H_z, 37°C for 10 min. The absorbance values of the supernatant kept at 37°C 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 using a graph of plotted points that show the

relationship between the quantities of total carotenoids and times (Praphairaksit and Ngamsiri, 2007 with modification).

3.7 Storage stability of encapsulated carotenoids

The samples were stored in the amber glass bottles and kept at 5, 25 or 40°C for 60 days. The remaining concentration of carotenoids, color and antioxidant activity were determined at 0, 3, 6, 9, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 storage days (Pu *et al.,* 2011 with modification). The values of R-square obtained from the trend lines of zero order, first order and second order plots of the carotenoids degradation during storage were compared. The degradation rate constant of carotenoids during storage was calculated using an appropriated degradation kinetic (Chiu *et al*., 2007 with modification).

3.7.1 Antioxidant property by ABTS radical cation assay ($ABTS^{\bullet+}$)

1) Preparation of the $ABTS^{\bullet+}$ solution

The $ABTS^{\bullet+}$ solution was prepared by mixing 2 mL of 7 mM ABTS solution (Appendix A3) with 35.5 µL of 140 mM potassium persulfate solution (Appendix A4). The mixture was kept in the dark at room temperature for 12-16 hr to allow the completion of radical generation before dilution with deionized water to adjusted the initial absorbance to 0.700±0.02 at 734 nm.

2) Measurement of inhibition activity

One ml of $ABTS^{\bullet+}$ solution was mixed with 10 µl of sample solution prepared by dissolving 0.1 g of encapsulated carotenoids in 10 ml HPLC grade ethanol. The mixture was kept in the dark at room temperature for 6 min before measuring the absorbance at 734 nm. The distilled water was used as the control (Roberta *et al.,* 1999). Percent inhibition of samples was calculated as following:

Inhibition (%) = [(A–B) / A] x 100

Where;

A= absorbance of control sample

B= absorbance of encapsulated carotenoids sample

3.8 Application of encapsulated carotenoids as colorant in food products.

3.8.1 Application in salad cream

The commercial salad cream without 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 salad cream, carotenoids have been allowed to add in the maximum of 155.8 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 (calculation as shown in Appendix F1.1). The control sample was the salad cream with the same amount of the total carotenoids, which the commercial salad cream was mixed with 0.5756g of the commercial carotenoids (calculation as shown in Appendix F1.2).

The L*, a* and b* values, visual color and viscosity of all tested samples were measured at beginning time, minimum release time (refer from section 3.6) and continuous measured 1 hr until the value constant at room temperature.

3.8.2 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 (calculation as shown in Appendix F2.1). 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 (calculation as shown in Appendix F2.2).

The L*, a* and b* values, visual color and viscosity of all tested samples were measured at beginning time, minimum release time (refer from section 3.6), sediment disappear time and continuous measured 1 hr until the value constant at room temperature.

3.9 Statistical analysis

The factorial in CRD was used in this experiment. The data was analyzed by Duncan's new multiple range tests. Statistical significance was determined for the differences between the experimental groups at a 95% confidence interval ($P \leq 0.05$).