

Microencapsulation of Kabocha Pumpkin Carotenoids

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Abstract—Kabocha pumpkin (*Curcubita maxima* [Duchesne ex Lamb.]) is a potential source of carotenoids. However, the usage of carotenoids is limited due to their instability and also their susceptible degradation against harmful conditions such as base and acidic conditions, oxidation, and illumination. In this study, kabocha carotenoids were incorporated into microencapsulation containing chitosan, sodium alginate and sodium tripolyphosphate. The objective of this study is to determine the formulation of coating agents, carotenoid stability in acidic conditions for mimicking the microencapsulation process, and to characterize the microencapsulated carotenoids including the determination of the efficiency of carotenoid incorporation into microencapsulates. A mixture of sodium alginate, chitosan and sodium tripolyphosphate (0.19 g : 1.92 g : 0.24 g, w/w/w) was the best of coating agents according to the physical characteristics and also its moisture content. Microcapsules obtained with and without addition of carotenoids were determined to be a microparticle size by SEM analysis. The products of microencapsulated carotenoids have the water content of around 5.4% to 7.1%. The highest efficiency of microencapsulation obtained was 91% at the carotenoid concentration of $117.98 \mu\text{g} \cdot \text{g}^{-1}$ (0.5 %, w/v), although the efficiency was decreased with increasing carotenoids added to the microcapsules probably due to over loading of carotenoids used. The pattern of this efficiency was in line with L^* and $^{\circ}\text{hue}$ values, whereas not only a^* , b^* , and chroma values, but total carotenoids, and total provitamin A also increased.

Index Terms—Carotenoids, emulsion, kabocha (*Curcubita maxima* [Duchesne ex Lamb.]), microencapsulation, pumpkin.

I. INTRODUCTION

Carotenoids have several functional benefits for human body. Carotenoids have roles in epithelisation process, influencing cell progression of the fibroblast, antioxidant, in protecting agent of UV radiation and decreasing the skin cancer risk. In addition, some types of carotenoids have a role as provitamin A [1]. However carotenoids are susceptibly degraded by harmful conditions, i.e. light radiation, high

temperature, and the presence of acid or oxygen. These conditions may degrade their quality on disappearance of color, rancidity and decrease in bioactivity and food functional roles [2].

Some protecting techniques have been explored to protect pigments from the degradation. Encapsulation is a common way that protects bioactive molecules by entrapping them into other substances and also that changes the size of particles into nano- or microparticles. Microencapsulation is encapsulation which produces micro particles (1 μm to 1000 μm) [3]. Microencapsulation has been applied in some kind of food products and usually uses spray-drying (high temperature) as a drying method while microencapsulation conducted by freeze dryer is still rare. The Freeze drying method produces the best quality for final product and does not change the bioactive composition in food because it uses low temperature. Suitable coating agents are needed in the encapsulation process, because the coating agents give protecting barrier to the bioactive compounds. Chitosan and sodium alginate are two kinds of coating agents that have been used for microencapsulation in pharmacy. These two agents give a good synergy in forming transparent, flexible, and strong film, and have high tensile strength [4], [5]. Preparation of microcapsule via emulsification with biopolymer combined with freeze drying technology is known to produce microspheres, having a particle size ranging between 20 μm and 5 000 μm [5].

Kabocha pumpkin (*Cucurbita maxima*) Duchesne is a potential carotenoids source. Kabocha has higher carotenoids content ($285.91 \text{ mg} \cdot 100 \text{ g}^{-1}$) than local pumpkins ($26.62 \text{ mg} \cdot 100 \text{ g}^{-1}$) [6]. In present study, the microencapsulation process of carotenoids from kabocha was conducted by freeze dryer. The best proportion of chitosan-sodium alginate-sodium tripolyphosphate (STPP) as coating agents of microencapsulated carotenoids would be chosen. The addition of different carotenoid concentrations was evaluated to determine microcapsules characteristics, such as color properties and encapsulation efficiency. The final product of microencapsulated kabocha carotenoids can be utilized as natural carotenoid powder with high stability.

II. MATERIALS AND METHODS

A. Materials and Reagents

Kabocha and sunflower oil (Golden Bridge, Malaysia) were purchased from Lai-Lai Fruit Market (Malang, Indonesia). Chitosan and Sodium Tripolyphosphate (STPP) (Changzhou Kamadi Trading Co., Ltd, Changzhou, China), sodium alginate (Qingdao Hyzlin Biology Development, China), tween 80 (Sigma Aldrich, St. Louis, United States), demineralized water with resistivity $100 \times 104 \Omega \text{ cm}$, N_2 gas (UHP grade, PT Samator, Surabaya, Indonesia) were used directly. Glacial

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acetic acid, acetone, *n*-hexane are an analytical grade from Merck (Darmstadt, Germany).

B. Sample Preparation

The fruit of Kabocha pumpkin was separated from the peel and then cut into small pieces. The pumpkin fruit was dried by a vacuum oven (VO-200, Memmert, Schwabach, Germany) at 50 °C and 2 mbar for 24 h. The dried fruit was ground using a grinder (M20, IKA-Werke, Selangor, Malaysia). The pumpkin powders were kept inside a desiccator in dark condition for future analysis.

C. Carotenoids Extraction, Formulation of Coating Agents, and Stability Test in Emulsion System

Carotenoids were extracted from 20 g of dried kabocha powders with 30 mL of *n*-hexane by stirring for 40 min and then filtered through a filter paper. The extraction was repeated 2 times under a red light. The carotenoid extracts were dried by evaporation (Eyela SB-1100 rotary evaporator, Tokyo Rikakikai Co. LTD, Japan) and continued by the stream of nitrogen gas. The dried carotenoid extracts were kept at -30 °C.

Microencapsulation agents were comprised of sodium alginate, chitosan and STPP mixture (ACT) in 5 formulations (Table I). The microparticles with the ACT1 formulation were prepared by adding 96 mL of chitosan (2%, w/v) into 19.2 mL of sodium alginate solution (1%, w/v) and followed by addition of 4.8 mL of STPP solution (0.5%, w/v) and 0.96 mL of acetic acid. This mixture was homogenized three times by a UltraTurax Homogenizer T-18 (IKA) at 15 000 rpm for 5 min (1 rpm = 1/60 Hz). After storage at -15 °C for 24 h, the mixture was then lyophilized at -47 °C for 24 h by a freeze dryer (Labconco, Kansas City, USA). Other formulations of coating agents were prepared with the same manner as described above.

TABLE I: FORMULATIONS OF COATING AGENTS IN 120 G OF DEMINERALIZED WATER

Formula	Sodium alginate (1)		Chitosan (2)		STPP (3)		Mass Ratio of 1:2:3 (w/w/w)
	%	V	%	V	%	V	
ACT1	1.0	19.2	2.0	96.0	5.0	4.8	0.19:1.92:0.24
ACT2	2.0	37.5	4.0	75.0	2.0	7.5	0.75:3.00:0.15
ACT3	5.0	40.0	5.0	80.0	0.0	0.0	2.00:4.00:0.00
ACT4	2.0	40.0	2.0	80.0	0.0	0.0	0.80:1.60:0.00
ACT5	1.0	60.0	1.0	60.0	0.0	0.0	0.60:0.60:0.00

% = percentage (w/v); V = volume in mL; w = mass in g

Approximately 0.02 g of the dried carotenoid was dissolved in 0.2 mL of acetone and then titrated with 9.8 mL of acetic acid solution in different pH (pH 3 and pH 4) containing a Tween 80 solution (0.1%, w/v). The stability of the carotenoids was observed by recording the absorption spectrum in this emulsion at 0 min, 45 min, 100 min, and 150 min using 1700 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). The dried carotenoids in 10 mL of acetone were used as the control sample.

D. Microencapsulation of Carotenoids

Preparation of carotenoids in emulsion system was adapted from the method of [7], while the microencapsulation of carotenoids was performed according to the method published elsewhere [8] with the slight modifications. Initially, the dried

carotenoids in the different masses were dissolved in trace amount of acetone (0.5 mL) and sunflower oil (1.5 mL) (the range of carotenoid concentration was 0.5 – 3.0%, w/v) and then added with Tween 80 (0.5 g) and sodium alginate (1%) in 19.2 mL of demineralized water. This emulsion was then homogenized at 1 500 rpm for 5 min. The carotenoid emulsion was mixed with chitosan (2%) in 96 mL of demineralized water and homogenized at 15 000 rpm for 1 min. Afterward STPP (5%, 4.8 mL) and acetic acid (2 mL) were added to this solution. The homogenization was continued for 5 min. To separate microencapsulated carotenoids from acidified water, the homogenized solution was centrifuged (Kubota, Tokyo, Japan) at 11 500 rpm and at 4° C for 20 min. The pellet of microencapsulated carotenoids was collected and stored at -15 °C for 24 h. The lyophilized process of microencapsulated carotenoids was performed by using a Freeze dryer (Labconco) at -47 °C for 24 h. The freeze-dried product was crushed in a mortar and sieved by a standard sieve (80 mesh).

E. Characterization of Microcapsules

Microcapsules (0.001 g) were extracted with a mixture of acetone and water solution (8:2, v/v) and then centrifuged at 10 000 rpm for 15 min. The supernatant was then partitioned with *n*-hexane and the hexane layer was dried under the stream of nitrogen. The dried carotenoid extract was then re-dissolved in *n*-hexane and an absorption spectrum was recorded in the range of 250 – 600 nm. The efficiency of microencapsulation was then calculated based on the ratio of the absorption at the position of absorption maximum between the microencapsulated carotenoids and initially added carotenoids.

The L^* , a^* , and b^* values of the microcapsules were recorded by a Colorflex EZ (Hunter Associates Laboratory, Inc., Reston, USA). The hue and chroma values were also calculated. Total carotenoids and pro-vitamin A were estimated based on Gross formula [9] and NAS-NRC [10], respectively.

F. Data Analysis

A SPSS 21 program with One-Way ANOVA (95%) and Duncan's Multiple Range Test was used for the statistical analysis.

III. RESULTS AND DISCUSSION

A. Determination of the Best Formula for Coating Agents

The composition of the coating agents is important for the final product. A high amount of sodium alginate produces a spongy structure due to the property of sodium alginate as a gel. Alginate contains gulunorat (G) and manuronat chains which the G chain will affect in the formation of gel [11]. The physical characteristics of formulations without the addition of STPP (ACT3, ACT4, and ACT5) revealed the sponge structure and formation of aggregates in the powders (Figure not shown). In addition, the moisture contents of these powders were higher than 10%. On the other hand, formulations with STPP (ACT1 and ACT2) produced dry and soft powders with lower moisture contents (less than 8%). However, the powders from the formulation of ACT2 were formed some aggregates with a sponge-like structure. Among 5 formulations tested, therefore, the best formulation of

coating agents was ACT1 in the following composition: sodium alginate: chitosan: STPP: demineralized water = 0.19 g:1.94 g:0.20 g:120 g. Besides the formulation, the process of microencapsulation is determined by the speed and duration of homogenization process, and other continuous procedures, i.e. centrifugation. Addition of ionic agent, such as STPP, can create cross interaction and improve the characteristic of the powder. STPP also facilitates chitosan to build crosslink with itself and addition of sodium alginate acts as a filler to strengthen the structure [8].

B. Stability Test of Carotenoids

The solution used for the microencapsulation was found to have low pH with a range of pH 3.6 to pH 4.0, therefore, a stability test on carotenoids was needed to ensure the formulation. The stability of kabocha carotenoids in the emulsion system with different pH was evaluated from their absorption spectra (Fig. 1). The absorption of carotenoids in acetone as a control (Fig. 1 a) showed a slight increase in the intensity due to evaporation of acetone during measurements.

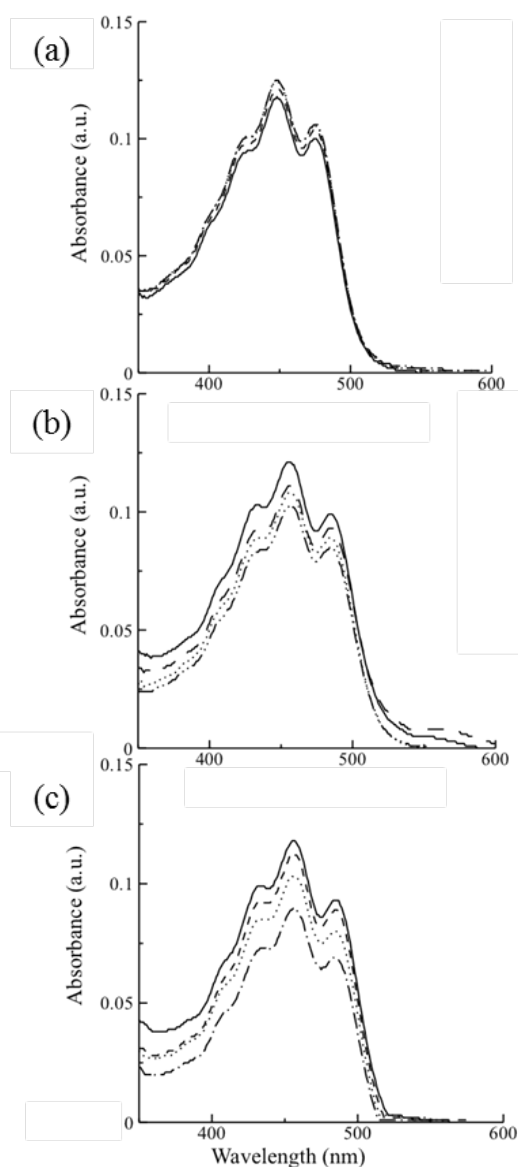


Fig. 1. Absorption spectra of kabocha carotenoids in acetone (a), emulsion systems at pH 4 (b) and pH 3 (c) incubated at room temperature for 0 min (—), 45 min (---), 100 min (.....) and 150 min (-.-.-).

In the emulsion with a low pH, it was shown that the

carotenoid spectra decreased in the intensity. After 150 min the absorbance of carotenoids has decreased to 18.8% and 15.2% in the emulsion with pH of 3 and 4, respectively. Based on the stability test, the maximum processing time for microencapsulation of carotenoids was set to be 60 min which least affects the carotenoid stability of 7.8% for both pH.

In Fig. 1, the spectra of carotenoids dissolved in acetone solution have 3 bands with absorption maxima at 424 nm, 448 nm, and 474 nm. In the emulsion system, the bands were red-shifted to about 6 nm to 8 nm. This shift is due to the aggregation process of carotenoids with their environment and it is usually called as a J-aggregation. Previously it was reported that a bathochromic shift of carotenoids occurred because of the interaction of carotenoids with the surfactants that produces an aggregate [12]. In the case of fucoxanthin, the presence of water caused the aggregation of fucoxanthin in acetone solution which shows the bathochromic shift up to 7 nm [13]. It was suggested that the presence of water in carotenoid solution might cause aggregation of carotenoids because there is a strong hydrophobic tensile strength [14].

C. Characterization of Microencapsulated Carotenoids

The microencapsulated carotenoid powders have low moisture contents in the range of 5.41% to 7.08%. The moisture contents of the microencapsulated kabocha carotenoids were in line with the results reported before [15]. Moisture contents of microencapsulated powders dried by different methods of spray-freeze drying, freeze-drying, and spray-drying were 4.15% to 6.63%, 6.7% to 6.99% and 3.56% to 6.40%, respectively [15].

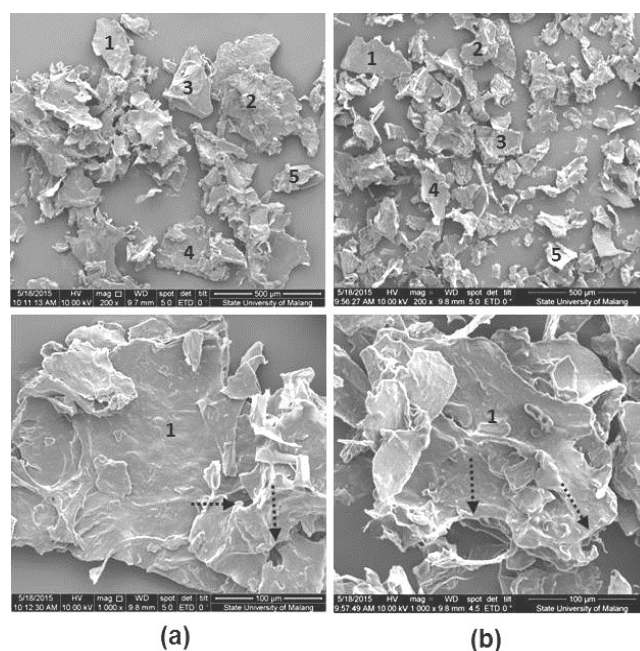


Fig. 2. SEM images of the empty microcapsules of ACT1 formulation (a) and the carotenoid-filled microcapsules with the addition of 3% carotenoids (b) observed at magnifications of 200 times (above) and 1000 times (below). Five specimens indicated with the numbers were used for calculating the average of diameter of microcapsules and the arrow indicates a porous structure of microcapsules.

SEM images of microcapsules depict the morphological characteristics of microparticles. The microencapsulated carotenoid powders have smaller diameter than that of empty one (Fig. 2, 200 times magnification). The large diameter of the empty microcapsules might be affected by the overlapped

powders during the SEM analysis and also the microcapsules have not been sieved. The average diameters of the empty and carotenoid-filled microcapsules were 337.88 μm and 214.26 μm , respectively, indicating that microencapsulation process has produced the micro-scale particles. Reference [5] revealed that microencapsulation by a freeze drying method produces a varied particle size of around 20 - 5000 μm .

At the magnification of 1000 times (Fig. 2, below), carotenoid-filled microcapsules appeared a rougher surface than the empty microcapsules. It is suggested that the pigment has been entrapped to the coating agents. Other characteristics of microcapsules were irregular shape with porous and slab features and a variation in particle size probably due to the sample preparation during freeze drying and crushing steps. These SEM results were in agreement with other study by [16]. The morphology of freeze dried microcapsules was irregular shape having a slab-like structure. These morphological features came from the freeze dried matrix subjected to crush by mortar.

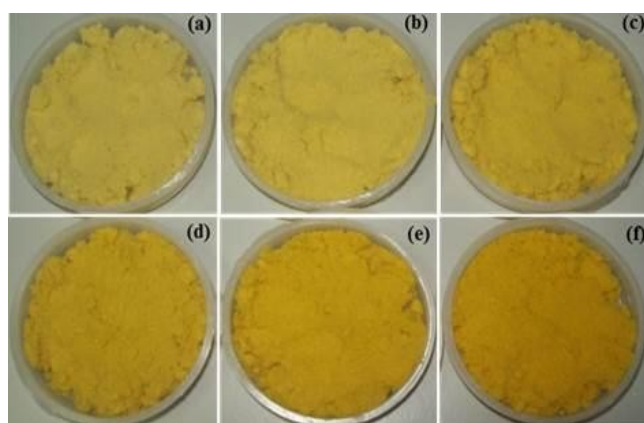


Fig. 3. Microencapsulated carotenoids powder with the ACT1 formulation using different addition of carotenoid concentrations, i.e. 0.5 % (a), 1.0 % (b), 1.5 % (c), 2.0 % (d), 2.5 % (e) and 3.0 % (f).

Color of the microencapsulated carotenoid powders was visually seen in the range from yellow to orange (Fig. 3). The addition of carotenoids significantly affected to the color change of the microencapsulated carotenoid powders. Table II summarizes color values, i.e. L^* , a^* , b^* , C^* and $^{\circ}\text{Hue}$, from the samples. The positive correlations of a^* , b^* and C^* with the amount of added carotenoids were seen because of increment of carotenoid concentration, while L^* and $^{\circ}\text{Hue}$ gave a negative correlation. The results of color measurement were in agreement with the literatures [17], [18]. A study has reported that L^* value had a negative correlation to the addition of pigment, because higher pigment content will increase the darkness of sample as a consequence on decrease in L^* value. An L^* value had a strong correlation with the total carotenoids, whereas b^* and chroma values had a moderate correlation with total carotenoids and had a strong correlation with the increase in lutein. The degree of hue value had a negative correlation with total carotenoids [17]. Other report showed that a^* , b^* , and C^* values also have a positive correlation with β -carotene content and total carotenoids [18].

Total carotenoids and pro-vitamin A of microencapsulated carotenoids powder increased with the increase in the carotenoid concentrations (Table III). Reference [9] reported the daily need for vitamin A in a man is 1 000 RE 8. It means that by consuming about 100 g of microencapsulated

carotenoids (the addition 2.5 % and 3.0 % carotenoids), it may fulfil the daily need for vitamin A. Based on SNI 7709–2012, maximum fortification of vitamin A to the vegetable oil is 45 $\text{IU}\cdot\text{g}^{-1}$. The addition of 1.05 g to 4.20 g of the microencapsulated carotenoids to 1 g of vegetable oil is enough to achieve the standardization of food fortification. The increase in carotenoid concentration added in microencapsulation process caused the decrease in efficiency. Table III summarizes the percentage of encapsulation efficiency with the addition of several portions of carotenoids. This result showed the same trend with other reports. Reference [19] concluded that the loading carotenoids with high concentration did not provide better encapsulation efficiency. This phenomenon was also observed in other polymeric matrices that over loading of encapsulated materials caused some decrease in the encapsulation efficiency [20].

TABLE II: CHROMATIC VALUES OF THE MICROENCAPSULATED CAROTENOIDS FROM KABOCHA POWDERS WITH ACT1 FORMULATION

% Carotenoids (w/v)	L^*	a^*	b^*	$^{\circ}\text{Hue}$	C^*
0.5	76.21 $\pm 2.14^a$	3.64 $\pm 0.62^a$	48.91 $\pm 2.26^a$	85.76 $\pm 0.62^a$	49.05 $\pm 2.28^a$
1.0	76.61 $\pm 0.46^a$	7.85 $\pm 0.92^b$	60.33 $\pm 3.57^b$	82.59 $\pm 0.78^b$	60.84 $\pm 3.60^b$
1.5	74.74 $\pm 0.86^{ab}$	9.25 $\pm 0.35^c$	65.74 $\pm 3.39^c$	81.97 $\pm 0.66^b$	66.39 $\pm 3.31^c$
2.0	72.77 $\pm 0.60^b$	10.19 $\pm 0.10^c$	69.99 $\pm 3.33^d$	81.70 $\pm 0.36^{bc}$	70.73 $\pm 3.30^d$
2.5	70.34 $\pm 2.57^c$	11.66 $\pm 0.43^d$	72.34 $\pm 1.14^e$	80.85 $\pm 0.23^c$	73.28 $\pm 1.18^{de}$
3.0	70.34 $\pm 1.21^c$	14.02 $\pm 1.17^e$	74.69 $\pm 1.20^f$	79.37 $\pm 0.87^d$	76.00 $\pm 1.21^e$
Y	-1.49x + 78.72	1.84x + 3.00	4.83x + 48.42	-1.07x + 85.78	5.04x + 48.41
R^2	0.96	0.94	0.90	0.88	0.91

*Means \pm SD. The difference notation shows that the data are significantly different at $p \geq 0.05$.

TABLE III: TOTAL CAROTENOIDS, PROVITAMIN A, AND MICROENCAPSULATION EFFICIENCY OF THE MICROENCAPSULATED CAROTENOIDS FROM KABOCHA POWDERS WITH THE ACT1 FORMULATION

% Carotenoids (w/v)	Total Carotenoids ($\mu\text{g} \cdot \text{g}^{-1} \text{dw}^*$)	Total Provitamin A		% Efficiency*
		RE/100 g dw*	IU/100 g dw*	
0.5	117.98 $\pm 10.41^a$	321.90 $\pm 28.39^a$	1 073.00 $\pm 94.64^a$	90.86 $\pm 6.17^a$
1.0	150.00 $\pm 11.70^b$	474.14 $\pm 36.99^b$	1 580.47 $\pm 123.28^b$	83.37 $\pm 7.82^{ab}$
1.5	207.38 $\pm 18.00^c$	691.03 $\pm 59.97^c$	2 303.42 $\pm 199.92^c$	78.20 $\pm 2.93^b$
2.0	235.51 $\pm 24.06^d$	822.68 $\pm 84.03^d$	2 742.26 $\pm 280.10^d$	62.51 $\pm 4.68^c$
2.5	279.17 $\pm 13.47^e$	1 045.88 $\pm 50.47^e$	3 486.27 $\pm 168.23^e$	58.55 $\pm 5.68^c$
3.0	318.29 $\pm 24.64^f$	1 287.00 $\pm 99.64^f$	4 290.01 $\pm 332.15^f$	54.72 $\pm 3.36^c$
0.5	117.98 $\pm 10.41^a$	321.90 $\pm 28.39^a$	1 073.00 $\pm 94.64^a$	90.86 $\pm 6.17^a$
1.0	150.00 $\pm 11.70^b$	474.14 $\pm 36.99^b$	1 580.47 $\pm 123.28^b$	83.37 $\pm 7.82^{ab}$

*Means \pm SD. The difference notation shows that the data are significantly different at $p \geq 0.05$.

The centrifugation process also caused the decreasing in

microencapsulation efficiency. It was estimated that some pigments having high polarity were soluble in aqueous solution and dissolved into the supernatant. HPLC analysis was performed on microencapsulated carotenoid powders and the supernatant after centrifugation [21]. The results showed that the polar carotenoids, i.e., antheraxanthin, lutein, and zeaxanthin were present in higher amount in the supernatant than non-polar carotenoids such as α - and β -carotene (1.1 to 1.9 times), while in the microencapsulated carotenoid powders, the relative concentration of non-polar carotenoids was 1.5 to 4.4 times higher than that of polar carotenoids. This result proved the loss of polar carotenoids in the supernatant during the centrifugation process. Centrifugation process is commonly used to separate bioactive compounds which have been entrapped into coating agents from untrapped one [8].

IV. CONCLUSION

Kabocha carotenoids can be entrapped with 2% chitosan, 1% sodium alginate and 5% STPP (1.92 g : 0.19 g : 0.24 g, w/w/w) to have a better microencapsulated products. The empty and carotenoid-filled microcapsules have an irregular shape, stable and porous structures, microsphere size with the average diameters between 214.26 μm and 337.88 μm . The concentration of carotenoids added to the microencapsulation influences the micro encapsulation efficiency, color values, total carotenoids and also provitamin A.

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Ms. Indrawati is an author of Indonesian peer-reviewed papers as well as several international publications. She has been awarded for a double degree scholarship by Indonesian Government to pursuit her graduate study for two years and a half. She received the award as one of the best presenters in Natural Pigments Conference for South-East Asia in three consecutive years.



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