Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol. 4, Suppl 1, 2011 ISSN - 0974-2441

Research Article

DEVELOPMENT AND STABILITY EVALUATION OF ASTAXANTHIN NANOEMULSION

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ABSTRACT

Astaxanthin is a naturally occurring carotenoid with strong antioxidant properties which shows low oral bioavailability due to its lipophilicity. The use of nanoemulsion-based formulations is one of several approaches found to be efficient in improving the bioavailability of lipophilic entities such as astaxanthin. This study explores the effects of various emulsifying conditions on the physicochemical properties and stability evaluation of astaxanthin nanoemulsion in order to optimize its production method. Astaxanthin nanoemulsion prepared in various emulsifying conditions was evaluated for its effects on the physicochemical characteristics and stability for a period of 3 months. The optimal formulation obtained required 5 minutes and 9000 rpm pre-homogenizing speed, high pressure (800 bar) and 5 cycles with composition of 4% surfactant, 16% oil and 80% water. Astaxanthin loaded in the optimal formulation were stable after the manufacturing process and storage at 5±3°C, 25±2°C/60±5% Relative humidity (RH) and 40°C±2°C/75%±5% RH for 3 months. The findings of this study will lead to the possibility of this formulation to be used as one of the method to improve astaxanthin bioavailability.

Keywords: Astaxanthin; Nanoemulsion; Physicochemical properties; Stability.

INTRODUCTION

Astaxanthin (3, 3'-dihydroxy-β-carotene-4, 4'-dione) is a carotenoid with red pigmenting properties, naturally occurring in yeast, algae, crustaceans, salmon and the asteroidean. It is closely related to other well-known carotenoids, such as β-carotene, zeaxanthin and lutein, thus they share many of the metabolic and physiological functions attributed to carotenoids. The presence of the hydroxyl and keto endings (Figure 1)[1] on each ionone ring confers some unique features, such as the ability to be esterified, a higher anti-oxidant activity and a more polar configuration than other carotenoids. Unlike some carotenoids such as canthaxanthin, there is no data on possible toxic or harmful effects of astaxanthin[2]. In fact scientific reports indicate that astaxanthin, because of its antioxidative properties which are 10 times more capable than other carotenoids[3], have anti tumor[4] and anti-inflammatory[5] activities, positive effects on blood pressure[6] as well as a cardioprotective effect[7].

Astaxanthin as other carotenoids cannot be synthesized by animals and must be acquired from the diet. Mammals lack the ability to synthesize astaxanthin or to convert dietary astaxanthin into vitamin A. Unlike β-carotene, astaxanthin has no pro-vitamin A activity in mammals[8]. Commercially astaxanthin is produced from both synthetic and natural sources. The primary natural source of astaxanthin for commercial uses is *Haematococus pluvialis*. This green microalgae synthesizes astaxanthin from the carotenoids, lycopene or phytoene. When it is exposed to extreme environmental conditions and ultraviolet light, it accumulates the highest level of astaxanthin and in this process the algae become red in colour.

Similar to other lipophilic compounds, astaxanthin shows very low bioavailability. One of the approaches that can be used to improve the bioavailability of lipophilic entities such as astaxanthin is to incorporate them in the fine particles of oil-in water (o/w) emulsion[9], liposome[10] and cyclodextrin[11]. In carotenoid family, most of the studies are focusing on improving β-carotene bioavailability by formulating oil-in-water emulsion[9], nanodispersion[12] and oil-in-water nanoemulsion[13]. Yuan *et al.*[13] successfully formulated stable oil-in-water β-carotene nanoemulsion and studied the influence of various emulsifying conditions on the physicochemical properties of the nanoemulsion. As far as we are concerned, there has been no publication on the astaxanthin nanoemulsion formulation. Therefore this study aims to look at the effects of various emulsifying conditions on the physical characteristics of astaxanthin nanoemulsion prepared by high pressure homogenizer and asses the stability of the optimal formulation obtained through size and drug content evaluation for a period of 3 months. The finding of this study will contribute to the possibility of this formulation to be used as one of the method to improve astaxanthin bioavailability.

MATERIALS AND METHODS

Materials

Astareal 10FC grade (an oil extract containing 10% w/w of standardized astaxanthin) was purchased from Fuji Chemical Industry (Nakaniikawa, Toyama, Japan), lecithin (L-α-phosphatidylcholine, Type IV-S ≥ 30% TLC (Thin layer Chromatography) - P3644) was puchased from Sigma (St Louis, Missouri, USA) while Tween 80 (polyoxyethylene (20) sorbitan monooleate) was purchased from Zulat Pharmacy (Selangor, Malaysia). Pure palm olein was purchased from FFM Berhad (Selangor, Malaysia). The water used was obtained from Elga PureLab (Marlow Buck, United Kingdom) water system. All other chemicals used were of analytical grade unless otherwise stated.

Astaxanthin nanoemulsions preparation

In order to prevent light sensitivity effect of astaxanthin, the nanoemulsion was prepared with minimal exposure to light. The oil-in-water red colour astaxanthin emulsion was prepared using ultrapure water as the continuous phase and palm olein containing astaxanthin as the disperse phase. Astaxanthin extract was first mixed with pure palm olein (15% w/w). Next, this solution was mixed with purified water (82.5% w/w) containing Tween 80 and lecithin as the emulsifier (2.5% w/w). The premix was homogenised using HSH (Silverson Homogeniser, UK) at 3000 rpm for 5 minutes followed by HPH (model APV 1000, APV Systems, Albertsland, Denmark).

Effects of Homogenization Condition on the Properties of Astaxanthin Emulsion

During the preparation of nanoemulsion, the effects of various homogenizing conditions were investigated. Several batches of emulsion according to the method explained above were prepared at various speeds and homogenizing duration. The speeds of homogenization were 3000 rpm, 6000 rpm and 9000 rpm with duration of 5 minutes, 10 minutes, 15 minutes and 20 minutes. Once optimal coarse emulsion is obtained, the effect of homogenization condition in terms of homogenizing pressure and cycle were evaluated. The pressures tested were 400 bar, 600 bar and 800 bar with 1, 3, 5, 6, and 7 cycles.

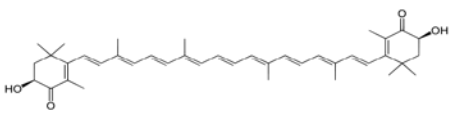


Fig. 1: Chemical structure of astaxanthin (3, 3'-dihydroxy-β-carotene-4, 4'-dione)

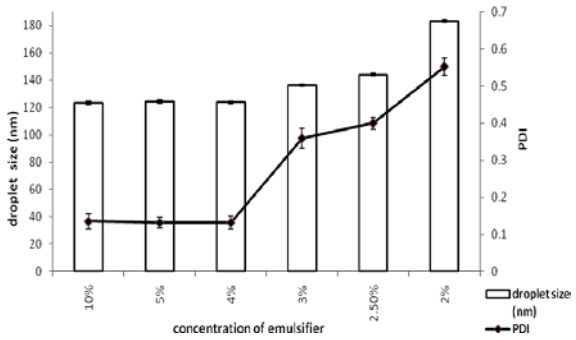


Fig. 2: Influence of different concentrations of surfactant on droplet size (nm) and size distribution (PDI) of astaxanthin nanoemulsions (mean±S.D., n = 3)

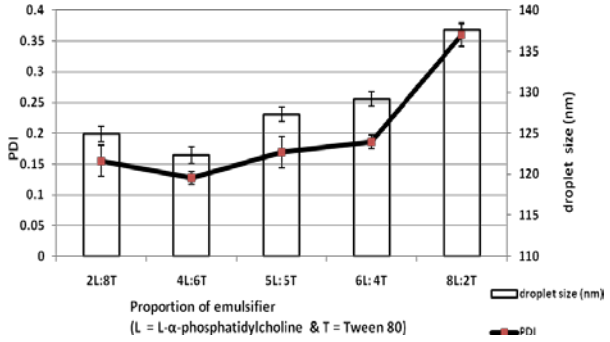


Fig. 3: Influence of different proportions of surfactants (L-α-phosphatidylcholine and Tween 80) on droplet size and size distribution of astaxanthin nanoemulsions (mean±S.D., n = 3)

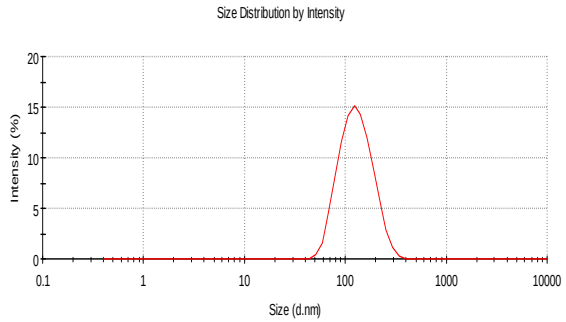


Fig. 4A: A typical profile of droplet size distribution in the astaxanthin nanoemulsion prepared with the following conditions: homogenization speed: 9000rpm, duration: 5 minutes, astaxanthin extract:oil:water:emulsifying agent=2:14:80:4, pressure: 800 bar, cycle: 5

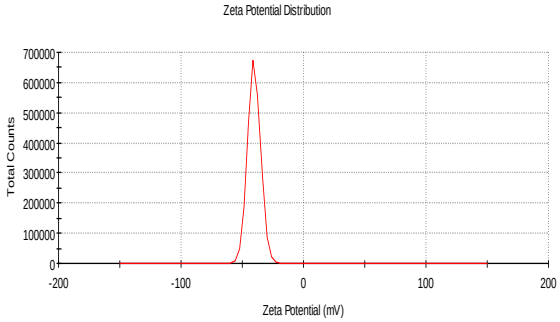


Fig. 4B: A typical profile of zeta potential in the astaxanthin nanoemulsion prepared with the following conditions: homogenization speed: 9000rpm, duration: 5 minutes, astaxanthin extract:oil:water:emulsifying agent=2:14:80:4, pressure: 800 bar, cycle: 5

Effects of Type and Concentration of Emulsifiers on the Properties of Astaxanthin Nanoemulsion

The nanoemulsion formed at optimal conditions was further investigated for the effects of type and concentration of emulsifiers. The types of emulsifiers used were Tween 80 and lecithin. Eleven batches were prepared by varying the emulsifier concentration and the ratio of Tween 80 (T) and lecithin (L). The emulsifier concentrations investigated were 2%, 2.5%, 3%, 4%, 5% and 10% with ratios of 2L:8T, 4L:6T, 5L:5T, 6L:4T and 8L:2T.

Characterisation of Nanoemulsion Droplets

The average droplet size and droplet size distribution of the nanoemulsion were determined by dynamic light scattering using zetasizer (Malvern Instruments, Worcestershire, UK) which measures the Brownian motion of the droplet and its relation to the droplet size based on the principle that larger droplets have a slower motion.

The droplet size distribution is calculated according to the Mie Theory. The droplet surface charge (zeta potential) and polydispersity index (PDI) were determined by zetasizer. The polydispersity index (PDI) is an index that describes the variation in size.

The higher the PDI the wider is the droplet size distributed. Zeta potential values were determined from the electrophoretic mobility of the oil droplets via in-built software which used the Helmholtz-Smoluchowski equation. The measurements were carried out on diluted emulsion formulations. The refractive index was kept at 1.33 and viscosity at 1.0 cps.

Stability Evaluation of Astaxanthin Nanoemulsion

Stability of Astaxanthin in Production of Nanoemulsions by using HPH

The concentration of esterified astaxanthin in nanoemulsion was evaluated after nanoemulsion processing through high pressure homogenizer. This evaluation would indicate the effect of mechanical energy and heat during the nanoemulsion process. The amount of esterified astaxanthin that remained after processing was determined following the method developed by Britton, Liaaen-Jensen, and Pfander[14]. This method was chosen based on the fact that the esterified astaxanthin used in the formulation could not be determined through HPLC method. The nanoemulsion was diluted with acetone prior to analysis, and then the absorbance was measured with a Double Beam UV Spectrophotometer (Model 251-003, Hitachi High Technologies Corporation, Tokyo, Japan.) at wavelength of 474 nm. The concentration of esterified astaxanthin was obtained by referring to absorbances of a series of esterified astaxanthin standard solutions prepared under the same conditions.

Droplet Size Growth of Nanoemulsion during Storage (Ostwald Ripening Effect)

Ostwald ripening effect on the nanoemulsion stability was performed by evaluating the oil droplet characteristic of the optimum formulation of astaxanthin nanoemulsion for 12 consecutive days. The nanoemulsion was filled into an amber glass bottle and flushed with nitrogen gas prior to air-tight closure with a plastic cap. The nanoemulsion was then stored at 25°C and oil droplet properties characterization such as particle size and polydispersity Index (PDI) were determined at 0, 2, 4, 6, 8, 10 and 12 days.

Stability of Nanoemulsion during Storage

The effect of temperature and humidity on the optimum formulation of astaxanthin nanoemulsion was evaluated for 3 months under different storage conditions. The nanoemulsion prepared was filled into an amber glass bottle and flushed with nitrogen gas prior to air-tight closure with a plastic cap. Nanoemulsions were then stored at 5°C±3°C and 25°C±2°C/60%±5% RH and 40°C±2°C /75%±5% prior to droplet properties characterisation on the 7th, 30th, 60th and 90th days. The droplet properties of nanoemulsion evaluated in this

study were size, zeta potential value and PDI. The esterified astaxanthin content in the nanoemulsion was determined by the spectrophotometric analysis method developed by Britton *et al*[14].

Field Emission Scanning Electron Microscope (FESEM)

The astaxanthin nanoemulsions were also analyzed by field emission scanning electron microscope (FESEM). A small amount of emulsion sample was first fixed with 1% osmium tetraoxide in distilled water. After stabilising the fixed sample for 2 hours, it was then centrifuged at 10,000rpm for 15 minutes followed by distilled water rinsing. This process will be repeated 3 to 4 times in order to remove top layer particles. The washed sample was stabilised for 10 to 15 minutes prior to FESEM imaging. (FEI QUANTA 250 Field Emmission Scanning Electron Microscope (FEICO, USA) at high tension range 200 to 30,000 volts.)

Statistical Analysis

The data were analysed by one way analysis of variance (ANOVA) using the SPSS version 17. Significant differences of means were determined by the Duncan's multiple range tests. The whole data in drug formulation experiment were done at least in triplicate.

RESULTS AND DISCUSSION

Effect of Homogenization Conditions on the Properties of Astaxanthin Nanoemulsions

The production of small droplets in nanometer size range requires the application of high energy. HPH is one of the most frequently used techniques in nanoemulsion formation. Formation of coarse emulsion through homogenization by HSH followed by HPH is very crucial in order to form a uniform and stable nanoemulsion. The droplet size of an emulsion produced by homogenisation process depends on the speed which represents the energy of homogenisation. The higher the energy applied in the homogenisation of emulsion, the smaller is the droplet size produced. However, uniformity of droplet size distribution depended on the completion of droplet size reduction which required more energy. This can be observed from the high PDI values after homogenisation of emulsion by using the high speed homogenizer.

As mentioned above, high energy input during premix by using high speed homogenizer reduced the droplets size with an increase in polydispersity index (PDI). The increase in PDI after homogenisation with high speed homogenizer was caused by the droplets of emulsion produced that were not uniform with multi peaks and broad droplet size distribution. However, when the emulsion passed through HPH for a few cycles, a very fine nanoemulsion with low value of PDI was produced.

As for the time interval at the particular speed, although there is a significant difference ($P<0.05$) between sizes, (Table 1) a five-minute interval was chosen in order to reduce the heat generated which might have decreased the stability of the dispersed astaxanthin. Therefore, 9000rpm and 5 minutes were chosen for the pre-homogenization speed and time respectively for the astaxanthin nanoemulsion formulation. It is widely known that homogenization pressure can significantly influence the characteristics of the emulsion as the turbulence and shear force formed during homogenization process can affect particle size and size distribution[15]. In this study, the influence of homogenization pressure and number of cycles on the properties of astaxanthin nanoemulsion was studied by varying the pressure from 400 to 800 bar.

As illustrated in Table 2, increasing the homogenization pressure resulted in a significant ($P<0.05$) decrease in the particle size and PDI of the nanoemulsion. This is due to the fact that by increasing the homogenizing pressure, the shear and turbulence force formed during homogenization increased and this resulted in the formation of smaller and more uniform particle size. The effect of homogenization cycle on the astaxanthin nanoemulsion properties is also presented in Table 2. As predicted, increasing the homogenizing cycle resulted in significant decrease ($P<0.05$) in both particle size and PDI. However, after passing through the emulsion in the homogenizer for more than five times, subsequent passes had no significant difference on the particle size and PDI (Table 2). This is due to the fact that after five cycles the formation of nanosize particles was already maximised, therefore any subsequent cycles would not significantly reduce the particle size further. This result agreed with the findings of Yuan *et al.*[13] on the formation of β-carotene nanoemulsion.

Effects of Type and Concentration of Emulsifiers on the Properties of Astaxanthin Nanoemulsions

Safety is an important criterion in choosing a surfactant as a large amount of emulsifier may cause GIT irritation. Nonionic surfactants are less toxic than their ionic counterparts and provide oil-in-water nanoemulsion stability which makes them more favourable among formulators[16]. The study on an ideal concentration of the emulsifier used in the formulation is very important in order to ensure that the amount of emulsifier used is just enough to stabilize the droplet in an o/w nanoemulsion more efficiently. In this study we used the combination of two emulsifiers namely Tween 80 (polyoxyethylene (20) sorbitan monooleate) and lecithin (L-α-phosphatidylcholine) fixed at 40%: 60%. Both are less toxic and less irritant to the GIT. As shown in Figure 2, increasing the emulsifier concentration from 2% to 5% resulted in a significant decrease ($P<0.05$) in the droplet size. It is due to the fact that a smaller droplet size means greater surface area, which requires more emulsifiers to cover. However, the effect of emulsifier concentration on the droplet size reached a maximum value at 4%. It is likely that at 4% concentration, all the droplets in the emulsion were fully covered by the emulsifier and excessive emulsifiers (more than 4%) in the system would not be utilized unless the droplet size could further be reduced. Although the 4% and 5% concentrations resulted in insignificant difference in droplet size and PDI, the former concentration was chosen as the amount of surfactant was minimal and just enough to produce the desired average droplet size (123.8±1.03 nm).

It is widely known that hydrophilic-lipophilic balance (HLB) value of the non-ionic surfactant plays an important role in predicting the emulsion stability and controlling the emulsion rheological quality. The right blend of low and high HLB value can provide a synergistic effect in enhancing the stability of the emulsion. A suitable combination of emulsifiers will lead to a greatly enhanced stability as compared to the individual emulsifier. In this study the influence of different combinations of emulsifiers was investigated. The combinations used in the study were 2L:8T, 4L:6T, 5L:5T, 6L:4T, 8L:2T (L for lecithin and T for Tween 80). As shown in Figure 3, 4L: 6T resulted in the smallest droplet size and PDI value. As reported by Hatanaka, Kimura, Lai-Fu, Onoue, and Yamada[17] emulsifier or blend HLB values of 10 and above are required to produce stable o/w emulsion. From this study only 2L:8T and 4L:6T with HLB values of 12.8 and 10.6 respectively, fall within this category. As reported by Griffin[18], the required HLB value for lipophilic ingredient in W/O emulsion plays an important role in determining emulsion stability. The required HLB for oil is the HLB value of the surfactant that will provide the lowest interfacial tension between oil phase and water phase. When the lowest interfacial tension between these two phases is achieved, the lowest amount of surfactant is needed to produce a stable emulsion. This study used palm olein as the disperse phase. Palm olein (long chain triglycerides) was chosen due to the fact that vegetable oils containing carotenoids can provide a lipophilic surrounding and stimulate pancreatic secretions required to transport astaxanthin from the food matrix to the enterocytes[19]. With the required HLB value of 10 for palm oil, 4L:6T with a HLB value of 10.6 is the right combination for both emulsifiers. As shown in Figure 3, 4L:6T resulted in the smallest particle size and PDI value compared to other combinations.

Optimal Nanoemulsion Formulation Profile

Figure 4A shows a typical profile of droplet size distribution in the astaxanthin nanoemulsion prepared with the optimal formulation. The droplet size distribution was unimodal and typically covered the range between 30 to 300nm. The mean diameter (z-average) of the

nanoemulsion droplets ranged between 116nm and 127nm with polydispersity indices (PDI) in the range of 0.116 – 0.138.

Figure 4B shows a typical profile of the zeta potential of the astaxanthin nanoemulsion prepared with the optimal formulation. The zeta potential value was unimodal and typically covered the range between -30mV to -60mV. These results therefore proved that astaxanthin nanoemulsion prepared as follows: 2% astaxanthin, 4% surfactant, 14% oil and 80% water, with 5 minutes, 9000 rpm pre-homogenizing speed at 800 bar pressure and 5 cycles produced stable astaxanthin emulsions with the disperse droplets in the nanometer range.

Field Emmision Scanning Electron Microscopic (FESEM) Observation of Astaxanthin Nanoemulsion

The astaxanthin nanoemulsion was observed under the field emission scanning electron microscope (FESEM). Two samples were prepared for observation. The first sample was the freshly prepared emulsion and the second sample was analysed after storage for 3 months at 4°C. The results are illustrated in Figure 5. Most of the droplets are spherical in shape and the agglomeration of smaller oil droplet was clearly shown in Figure 5A.

In general, there were no significant changes in the oil droplet size and shape of both preparations. Both sample 1 and sample 2 recorded oil droplet size of below 200nm with insignificant changes on the oil droplets shape. This was supported by the result from FESEM image (Figure 5) and zetasizer (Figure 6). This result appeared to be consistent with the result produced by Yuan *et al*.[13] on β-carotene nanoemulsion where the oil droplet size was reported to be unchanged after being kept for 1 month at 4°C.

Stability of Astaxanthin Nanoemulsions

Stability of Astaxanthin in Nanoemulsions by Using High Pressure Homogenizer

Results of the astaxanthin content before and after manufacturing process for three concentrations of astaxanthin are presented in Figure 7. There are no statistically significant differences between the amount of astaxanthin in the emulsion before and after the manufacturing process. This might be due to the precaution taken during the manufacturing process such as minimal exposure of the formulation to oxygen and light. The results also implied that the heat generated during HPH process did not alter the astaxanthin content in the formulation.

Droplet Size Growth of Nanoemulsion during Storage (Ostwald Ripening Effect)

The physicochemical stability of an emulsion during storage depends on the emulsion formulation. A suitable formulation results in stable emulsion over a long period of time. Changes in particle size, PDI or zeta potential indicate that flocculation/coalescence and Oswald ripening is happening. In this study the optimal formulation of astaxanthin nanoemulsion was tested for any potential Oswald ripening occurrence by monitoring any changes on particle size and PDI for 10 days.

Table 3 shows the result of droplet size and size distribution of the optimal formulation monitored every 2 days for a 10 day's storage period. The nanoemulsion droplet size ranged from 121.5±1.16 nm to 122.9±2.14 nm with the PDI approximately in the range of 0.123±0.008 to 0.131±0.008. Results also show that there is no significant difference in the droplet size and PDI of astaxanthin nanoemulsion after 10 days, proving that the astaxanthin nanoemulsion prepared with the optimal formulation was stable and free from Oswald ripening.

Effect of Temperature and Humidity on Nanoemulsion Characteristics during Storage

In order to ensure that every formulation studied can be applied to the commercial product, it is crucial for the formulation to be stable in terms of the particle size, PDI and drug content for a longer period of time. Therefore, the stability of astaxanthin nanoemulsion was further studied by monitoring any changes in particle size, PDI and zeta potential after the emulsion was kept under International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use conditions (ICH guidelines): 5°C±3°C, 25°C±2°C/60%±5% relative humidity (RH) and 40°C±2°C/75%±5% RH for 3 months (Table 4).

Results show that there are no significant differences in droplet size and PDI of the nanoemulsion during the 3 month's storage at various temperatures. The zeta potential values for all samples kept for 3 months at 5°C±3°C, 25°C±2°C /60%±5% RH and 40°C±2°C /75%± 5% RH were within the recommended range which was less than -30 mV. Based on the results, a conclusion can be made that the astaxanthin nanoemulsion prepared with the optimal formulation was stable for up to three months regardless of the storage temperature.

Figure 8 shows the effect of various storage conditions on astaxanthin content. Results show that there are no statistically significant differences between astaxanthin content within the 90-days period of storage at 5°C±3°C and 25°C±2°C/60%±5% RH. These results disagreed with those of Yuan *et al*.[13] who found that β-carotene was degraded by about 14-25 % at the end of the 30 day's storage period at 4°C and 25°C. This might be due to the fact that the nanoemulsion prepared in this experiment managed to prevent the oxidation effect on the astaxanthin loaded in the disperse phase. Besides that precautions taken during the experiment such as the use of amber screw cap bottles to store the emulsion and nitrogen flushing after every sampling might contribute to these results. As predicted, the astaxanthin content was degraded by about 28–29% at 90 days for samples stored at 40°C±2°C/75%±5% RH. Astaxanthin which is sensitive to heat might have degraded after being exposed to the high temperature for a long period of time. The results of this experiment are very encouraging since the optimal formulation developed has the ability to maintain astaxanthin stability for up to 90 days if kept at 25±2 °C/60%±5% RH.

Table 1: Effect of premix speed and time interval on particle size and size distribution of astaxanthin emulsions (mean±SD, n =3)

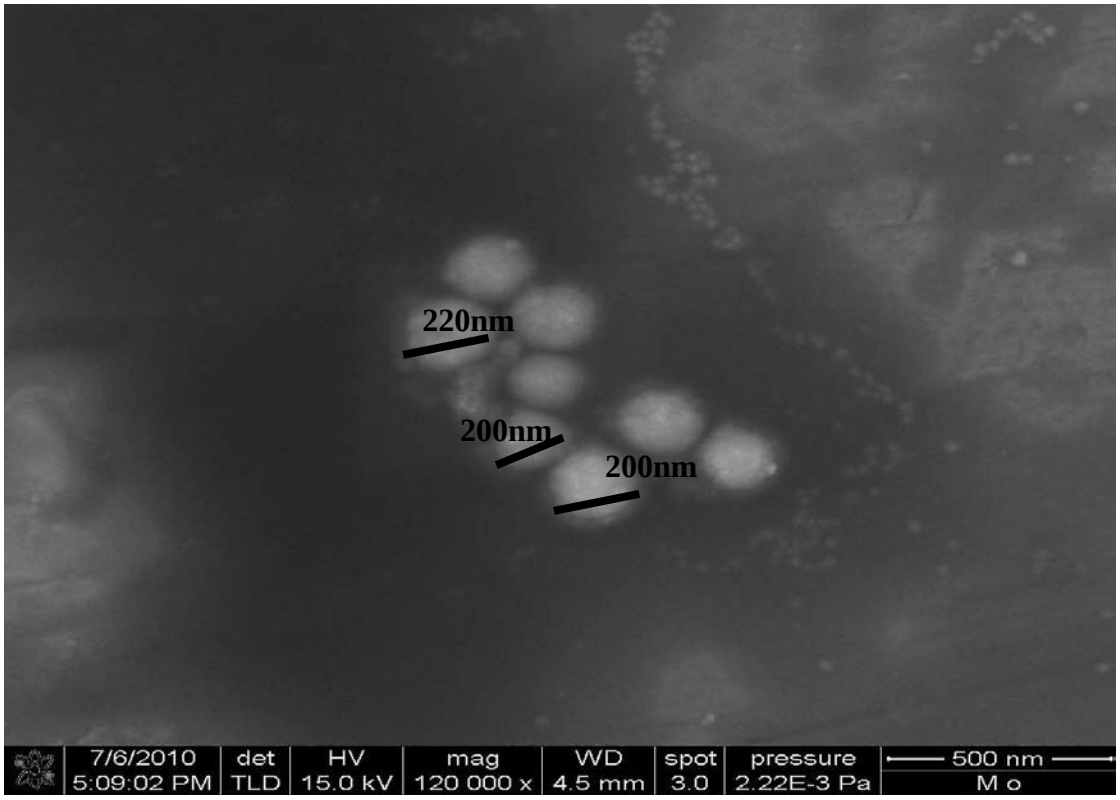
Speed (rpm)	Time (min)	D±S.D. (μm)	PDI±S.D.
3000	5	22.90±0.002^{a}	0.387±0.001^{A}
	10	20.04±0.003^{b}	0.391±0.003^{A}
	15	18.46±0.002^{c}	0.391±0.001^{A}
	20	17.33±0.004^{d}	0.394±0.012^{A}
6000	5	17.45±0.009^{e}	0.528±0.001^{B}
	10	15.50±0.010^{f}	0.535±0.007^{C}
	15	15.30±0.001^{g}	0.535±0.001^{C}
	20	15.00±0.001^{h}	0.554±0.001^{D}
9000	5	8.90±0.011^{i}	0.673±0.001^{E}
	10	8.70±0.005^{j}	0.637±0.009^{F}
	15	8.53±0.006^{k}	0.625±0.011^{FG}
	20	8.34±0.003^{l}	0.613±0.002^{G}

D = average diameter, PDI = polydispersity index, S.D. = standard deviation, rpm = rotation per minute, n = number of sample; $^{a-l}$ for the D values, mean followed by a different lower case superscript letter are significantly different (P<0.05); $^{A-G}$ for the PDI values, mean followed by a different capital superscript letter are significantly different (P<0.05)

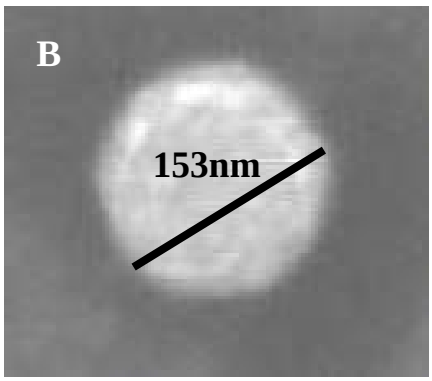
Table 2: Effect of homogenizing pressure and number of cycles on the particle size and size distribution of the astaxanthin nanoemulsion (mean±SD, n = 3)

HPH Pressure (Bar)	HPH Cycle	D±S.D. (nm)	PDI±S.D.
400	1	724.7±0.814[a]	1.348±0.023[A]
	3	636.8±1.400[b]	1.004±0.044[B]
	5	425.6±1.206[c]	0.998±0.023[B]
600	1	420.4±1.850[d]	0.737±0.027[C]
	3	327.40±1.800[e]	0.725±0.011[C]
	5	180.10±1.720[f]	0.837±0.020[D]
800	1	274.1±1.760[g]	0.716±0.019[E]
	3	184.2±1.590[h]	0.591±0.015[F]
	5	122.9±1.550[i]	0.128±0.010[G]
	6	122.6±0.670[i]	0.120±0.014[G]
	7	122.7±1.400[i]	0.120±0.017[G]

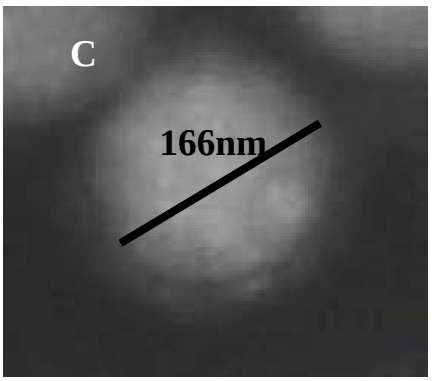
HPH = high pressure homogenizer, D = average diameter, PDI = polydispersity index, S.D. = standard deviation, n = number of sample .[a-i] for the D values, mean followed by a different lower case superscript letter are significantly different (P<0.05). [A-G] for the PDI values, mean followed by a different capital superscript letter are significantly different (P<0.05).



A) agglomeration of spherical shape astaxanthin oil globules



B) Freshly prepared emulsion



C) After 90 days of storage at 4° C.

Fig. 5: Environmental Scanning Electron Microscopy (ESEM) image of astaxanthin nanoemulsion prepared with optimal formulation.

Table 3: Oswald ripening studies on astaxanthin nanoemulsion prepared with the optimal formulation (mean±S.D., n = 3). There is no statistically significant changes in both size and PDI

Storage period (days)	D±S.D. (nm)	PDI±S.D.
0	122.90±1.11	0.127±0.011
2	122.90±2.14	0.131±0.008
4	121.50±1.16	0.125±0.010
6	122.20±0.38	0.123±0.008
8	122.10±0.78	0.124±0.010
10	122.20±0.62	0.129±0.016

D = mean diameter, PDI = polydispersity index

Table 4: Effect of temperature and duration of storage on particle size, PDI and zeta potential of the astaxanthin nanoemulsion (mean±S.D., n = 3)

Temp (°C/% RH)	Duration (days)	Size (nm)	PDI	Zeta Potential
5±3	0	122.4±1.22^{a}	0.116±0.015^{A}	-41.33±0.306
	7	122.8±1.44^{a}	0.119±0.021^{A}	-48.77±0.802
	30	123.2±2.25^{a}	0.116±0.020^{A}	-54.13±0.208
	60	124.0±1.25^{a}	0.130±0.037^{A}	-57.93±1.234
	90	122.7±1.33^{a}	0.116±0.018^{A}	-60.13±0.802
	7	123.5±1.16^{b}	0.129±0.015^{B}	-57.37±1.159
25±				
2/60±	30	123.8±0.87^{b}	0.138±0.022^{B}	-55.43±0.404
5	60	122.4±2.43^{b}	0.121±0.013^{B}	-50.03±0.231
	90	123.4±1.06^{b}	0.130±0.013^{B}	-52.13±2.801
	7	122.3±1.55^{c}	0.131±0.019^{C}	-57.33±0.651
40±				
2/75	30	123.6±1.11^{c}	0.140±0.011^{C}	-65.10±1.539
5	60	122.2±1.66^{c}	0.134±0.011^{C}	-56.53±0.321
	90	124.5±1.86^{c}	0.135±0.019^{C}	-58.77±0.473

D = Average diameter; PDI = polydispersity index; S.D. = standard deviation, n = number of sample, °C = temperature in degree Celcius, RH = relative humidity; $^{a-c}$ for the D values, mean value followed by a different lower case superscript letter are significantly different (P<0.05) $^{A-C}$ for the PDI values, mean value followed by a different capital superscript letter are significantly different (P<0.05)

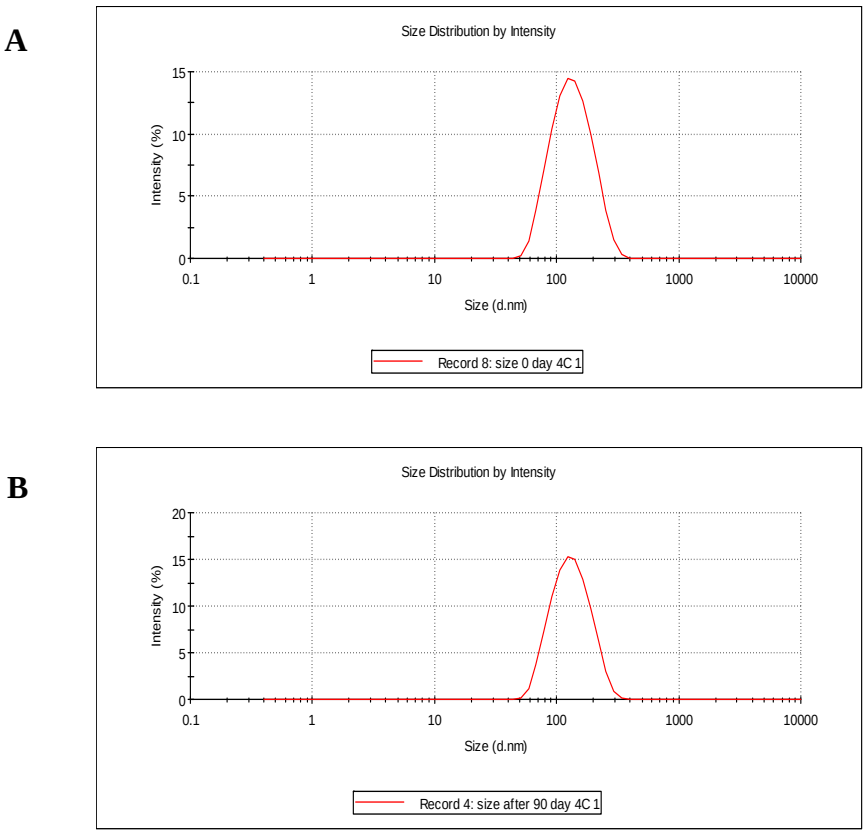


Fig. 6: Profile of particle size distribution of astaxanthin nanoemulsion prepared with optimal formulation; A) Freshly prepared emulsion B) After 90 days of storage at 4° C

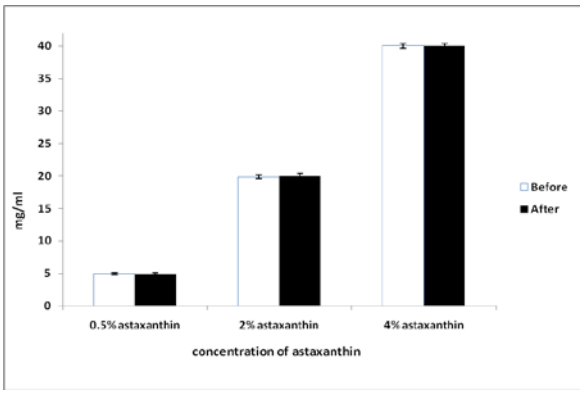


Fig. 7: Astaxanthin content in the formulation before and after manufacturing (mean±SD, n = 3)

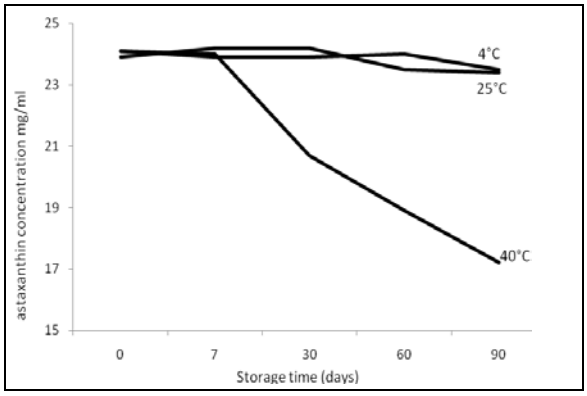


Fig. 8: Astaxanthin content in nanoemulsion during storage at 5°C±3°C, 25°C±2°C/60%±5% RH and 40°C±2°C/75%±5% RH

CONCLUSION

In conclusion this study confirmed that the droplet size and size distribution (PDI) of astaxanthin nanoemulsion were influenced by the homogenization pressure and number of cycles, as well as type and concentration of emulsifier blend. The optimal formulation obtained from this study needed 5 min and 9000 rpm pre-homogenizing speed, with 4% w/w surfactant, 2% w/w astaxanthin, 14% w/w oil and 80% w/w water. High pressure (800 bar) and 5 cycles were also needed to produce the optimal nanoemulsion. Astaxanthin loaded in the optimal formulation did not produce any statistically significant change in the particle size and drug content. Also, there was no statistically significant ($P<0.05$) change in the particle size and astaxanthin content during storage at 5°C±3°C, 25°C±2°C/60%±5% relative humidity (RH) and 40°C±2°C/75%±5% RH for 3 months.

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