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INTRODUCTION

Thailand has a great number of natural resources such as plants, animals, minerals, etc. These resources can produce active substances which have ability for cosmeceutical development. Astaxanthin (ASX) is the main carotenoid pigment found in various kinds of seafood including salmon, shrimp, lobster and especially green alga (*Haematococcus pluvialis*). It is shown to possess many biological activities beneficial to medical and cosmeceutical applications. This research focuses on the development of ASX from shrimp shell because it is the waste product of shrimp farming industry, one of the most important economic marine livestock of Thailand. ASX can be harvested from dried shrimp shell by extraction with organic solvents, followed by hydrolysis to convert the ester form into free form and subsequent separation of free ASX by column chromatography. ASX has a higher anti-oxidant activity[1)] and more polar configuration than other carotenoids, namely, zeaxanthin, lutein, canthaxanthin and beta-carotene[2)]. It is often claimed by manufacturers/suppliers as "super vitamin-E". ASX has important cosmetic application due to its ability to suppress hyper-pigmentation, inhibit melanin synthesis and photo-aging. However, the highly unsaturated structure of ASX renders it is very sensitive to heat, light and oxidative conditions[3)]. Solid lipid nanoparticles (SLN) have an important application in the personal care, cosmetics and health science fields because small droplet size can help the delivery of active ingredient into the skin and improve stability of skin-active material. Therefore, the main objective of this study was to extract free ASX from shrimp shell using organic solvent (isopropanol) and various hydrolysis conditions as well as to enhance the skin delivery of the obtained ASX by incorporating into SLN. The effects of extraction conditions on the yield and ASX purity as well as those of formulation parameters on the obtained SLN were investigated.

MATERIALS AND METHODS

Preparation of crude extract from shrimp shell: The fresh shrimp shell was removed from the shrimp and heated by hot air oven at temperature 70°C for 24 hours. The dried shrimp shell was grinded into powder by food processor. A crude extract was obtained by passive extraction using isopropanol as an organic solvent in 1:2 dried weight of shrimp shell per volume ratio for 30 minutes under mechanical stirring. The crude extract was filtered and evaporated using a rotary evaporator[4, 5)].

Hydrolysis reaction: Sodium hydroxide in methanol solution at different concentrations (0.01 N, 0.03N, 0.05N) were used for the saponification of crude extract under nitrogen in darkness with continuous mixing at 40°C for 5 hours[6)]. ASX free form was separated from the reaction by column chromatography and evaluated for the percentage yield by HPLC to find the most suitable hydrolysis condition.

Screening the type of solid lipids, surfactants, and co-surfactants: Types of solid lipids selected for the study were glyceryl behenate (Compritol®ATO 888), glyceryl monostearate (GMS), tristearin (Dynasan®118), cetyl palmitate (CP). The non-ionic surfactants with high HLB values were used which included polyoxyethylene 20 sorbitan monooleate (Tween®80), polyethylene-polypropylene glycol (Poloxamer®188), polyoxyethylene fatty alcohol ether (Brij®S721), PEG 40 hydrogenated castor oil (Cremophor®RH 40), and polyethylene glycol monostearate (Myrj®52). On the other hand, the co-surfactants initially chosen were in the alcohol group and included isopropanol, ethanol, benzyl alcohol, and propylene glycol. Miscibility between ASX and SLN components was observed at temperature 70°C by mixing the individual solid lipid with ASX at a ratio of 1:1, whereas the surfactant and co-surfactant were pre-mixed at a ratio of 1:1 before blending with ASX at the same ratio. The components that were found to be miscible with ASX without separation and precipitation would be chosen for subsequent SLN formulation.

Preparation of blank-SLN by microemulsion technique: Observing the type and quantity of solid lipid, surfactant and co-surfactant by using microemulsion technique as a preparation method that affects the SLN formation. Pseudoternary phase diagrams consisting of 3 components (solid lipid, surfactant:co-surfactant mix (1:1), and water) at temperature 70°C were constructed and the ratios in the isotropic zone able to form o/w microemulsion were selected in order to prepare SLN. Briefly, the aqueous phase (surfactant, co-surfactant and water) and the solid lipid phase were separately heated at 70°C and then the

aqueous phase was added to the solid lipid phase at constant temperature under agitation which could make a clear o/w microemulsion. SLN was prepared by dispersing the warm o/w microemulsion in the excess cold water (4°C) under mild mechanical stirring at a ratio of 1:20 v/v to obtain blank-SLN[7]. Physical characterization of SLN was evaluated by visual observation (no separation of solid lipid) whereas the particle size and polydispersity index were determined by photon correlation spectroscopy at 0, 2, and 4 weeks. SLN formulations which had an average particle size of less than 500 nm with uniform size distribution (measured by polydispersity index) would be selected for subsequent loading of ASX.

Preparation of astaxanthin-loaded SLN (ASX-SLN): The amount of 0.5%w/v ASX[8] was added to melted solid lipid as internal phase at 70°C. A warm surfactant, co-surfactant and water as continuous phase were heated at the same temperature. ASX dispersions were obtained by dispersing the warm microemulsion into cold water at a ratio of 1:20 as mentioned above. The ASX-SLN was measured for the physical stability in terms of particle size, polydispersity index (PDI), lack of ASX precipitation, entrapment efficiency (measured by ultrafiltration). The content of ASX was determined by HPLC which was modified from Navideh et al. (2010)[9]. The chromatographic analyses were performed using 4.6 cm×15 cm reverse phase column (C_{18}) at room temperature and a mobile phase consisting of methanol:dichloromethane:acetonitrile:water mixture (85:5:5:5 v/v). The column effluent (flow rate 0.8 ml/min) was continuously monitored at 474 nm to detect ASX.

RESULTS

Hydrolysis reaction of crude extract from shrimp shell: The percentage yields of free ASX after saponification and separation by column chromatography at different NaOH concentrations were 21.12, 52.34 and 32.14% w/w when using 0.01N, 0.03N and 0.05N NaOH respectively. The peak of free ASX was analyzed by HPLC as shown in Figure 1. The 0.03 N NaOH gave the highest amount of free ASX with least amount of degradation whereas the untreated crude extract gave very low peak of free ASX with the presence of two more lipophilic peaks at 10-20 and 35-75 min, attributed to be mono- and di-esters form of ASX, respectively (Figure 1-A).

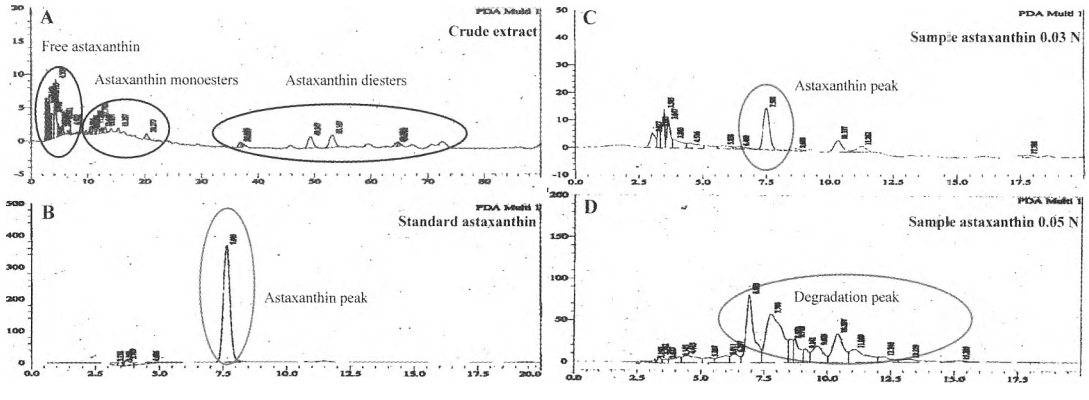

Figure 1. HPLC Chromatograms of ASX from various samples by using 2 mobile phase systems:
A: Methanol:dichloromethane:acetonitrile:water (28:46:22:4) for separation free ASX and esters form
B-D: Methanol:dichloromethane:acetonitrile:water (85:5:5:5) for separation free ASX and degradation products

Preparation of blank-SLN by microemulsion technique: Preliminary test with solid lipids showed that both Dynasan®118 and GMS were able to mix with other ingredients. The surfactants used were Poloxamer®188, Brij®S721, Myrj®52, Tween®80 and Cremophor®RH 40. The latter four surfactants produced good miscibility results for several formulations whereas Poloxamer®188 could form only w/o microemulsion and it was thus excluded from further study. Isopropanol, ethanol, benzyl alcohol, and propylene glycol were selected as co-surfactant. Propylene glycol with chosen solid lipid and surfactant could not form microemulsion at all whereas benzyl alcohol could form only w/o microemulsion. Hence, only isopropanol and ethanol were used in subsequent experiments. The pseudoternary phase diagrams were constructed using two different types of lipids, i.e., Dynasan®118 and GMS, by water titration method at 70°C. Water was gradually added to the mixtures of lipid and mixed surfactants (1:1 combination of surfactant and co-surfactant) at various ratios under continuous stirring until the mixture became turbid liquid or gel formation. To find the boundary line of microemulsion region, the ratios of lipid, mixed surfactants and water were calculated. The data were plotted and the phase diagrams were constructed (a representative diagram is shown in Figure 2). 10% of solid lipid, and various percentages of mixed surfactants (not more than 50%) and water within the area of o/w microemulsion formation of

each phase diagram were chosen to prepare SLN. The particle size of both SLN dispersions (Dynasan-SLN and GMS-SLN) with various surfactant:co-surfactant ratios are shown in Figure 3.

Preparation of astaxanthin-loaded SLN (ASX-SLN): To study the effects of ASX loading and other ingredients in the formulation on particle size and entrapment efficiency, seven blank-SLN formulations were selected to entrap ASX. All SLN formulations (3 batches each) were prepared to the final ASX concentration of 0.50% w/v. The photos of freshly prepared SLN are shown in Figure 4 whereas Table 1 shows the data of particle size, polydispersity index, entrapment efficiency of the blank-SLN and ASX-SLN.

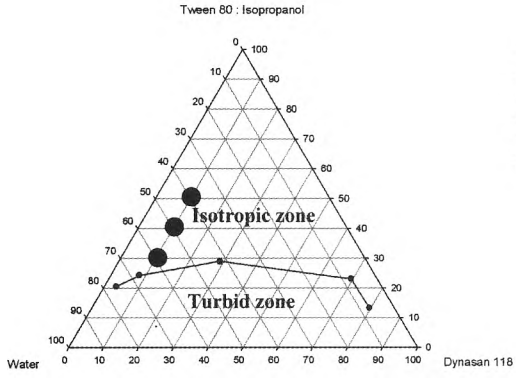

Figure 2. The pseudoternary phase diagram of Dynasan®118, mixed surfactants (Tween® 80:Isopropanol) and water at 70°C. The three black dots in isotropic zone were selected to form SLN.

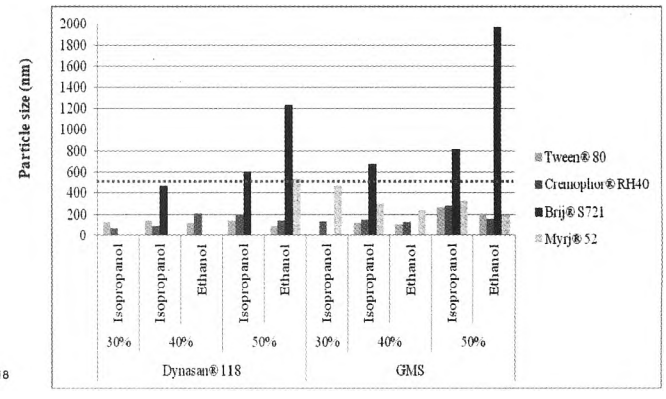

Figure 3. The particle size of freshly prepared blank-SLN dispersions containing 10% Dynasan®118 or GMS as solid lipid with various surfactants:co-surfactants (1:1).

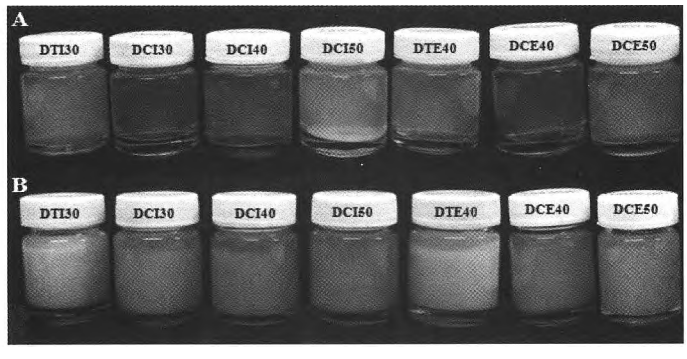

Figure 4. Freshly prepared SLN (A: Blank-SLN, B: ASX-SLN)

Sample	% Astaxanthin loading (w/v)	Size (nm) ± SD	PDI ± SD	Entrapment efficiency (%) ± SD
DTI30	Blank	128.6 ± 1.38	0.421 ± 0.041	-
DTI30	0.50%	338.0 ± 17.91	0.462 ± 0.116	90.18 ± 1.19
DCI30	Blank	140.5 ± 24.91	0.372 ± 0.131	-
DCI30	0.50%	192.6 ± 6.16	0.371 ± 0.079	88.08 ± 0.37
DCI40	Blank	106.7 ± 18.31	0.625 ± 0.055	-
DCI40	0.50%	161.8 ± 5.44	0.350 ± 0.050	85.21 ± 1.26
DCI50	Blank	142.3 ± 1.15	0.423 ± 0.028	-
DCI50	0.50%	165.0 ± 9.98	0.292 ± 0.030	76.87 ± 0.71
DTE40	Blank	151.7 ± 17.03	0.405 ± 0.091	-
DTE40	0.50%	333.4 ± 17.03	0.323 ± 0.072	54.43 ± 3.51
DCE40	Blank	96.6 ± 7.80	0.497 ± 0.087	-
DCE40	0.50%	158.7 ± 6.66	0.462 ± 0.103	83.72 ± 2.16
DCE50	Blank	135.9 ± 3.08	0.440 ± 0.027	-
DCE50	0.50%	146.4 ± 2.77	0.433 ± 0.048	67.99 ± 2.82

Table 1. Particle size, polydispersity index and entrapment efficiency data of blank-SLN and ASX-SLN. [The first letter of the formula coding represents types of solid lipid (D = Dynasan®118). The second letter represents types of surfactant (T = Tween®80, C = Cremophor®RH40). The third letter represents types of co-surfactant (I = Isopropanol, E = Ethanol). The fourth number represents percentages of surfactant:co-surfactant in o/w microemulsion region.]

DISCUSSION

In the present experiment, 0.03 N NaOH resulted in a mild saponification of crude extract that gave the highest percentage yield with very little ASX degradation. Higher NaOH concentration should be avoided to minimize the ASX degradation. A total of 31 selected formulations within o/w microemulsion region composed of 10% solid lipid were able to form SLN. Size determination was generally used as a characterization tool. As the surfactant:co-surfactant in the formulation increased, the bigger particle size was obtained. The different type of co-surfactants (Isopropanol and Ethanol) in the same formulation apparently did not affect the particle size. The solid surfactants (Brij®S721 and Myrj®52) could generate larger SLN than the liquid surfactants (Tween®80, Cremophor®RH 40). For this reason, the liquid state of Tween®80 and Cremophor®RH 40 might be able to form a more flexible film at the o/w interface than those of solid surfactants. The appearance of Dynasan-SLN was clearer than that from GMS-SLN indicating the average larger particle size of the latter. This might be due to the long carbon chain length of GMS which results in higher melting point and at room temperature may promote rapid lipid recrystallization. Since a wide range of surfactant and co-surfactant could be used to obtain good physical stability, Dynasan-SLN was selected to further load the obtained ASX. All ASX-SLN formulations showed good physical appearances. No coalescence, gel formation and phase separation occurred. The mean diameters of all formulations were in nanometer range. It was found that the mean particle size of ASX-SLN by microemulsion technique method was around 100-350 nm. The polydispersity index indicated that the formulations were moderately polydisperse. Determination of the amount of entrapped ASX in SLN was carried out by ultrafiltration and analyzed by HPLC. The results in Table 1 clearly show that all the formulations gave high entrapment efficiency about 54-90% depending on the formulations.

CONCLUSION

The concentration of methanolic NaOH solution was found to be important in promoting the hydrolysis of ASX esters and minimizing the degradation of free ASX during saponification. Blank-SLN formulations made from GMS gave larger particle size than Dynasan®118. Increasing the percentage of surfactant:co-surfactant resulted in the augmentation of mean particle size. Seven ASX-SLN formulations were prepared from Dynasan®118. ASX-SLN prepared in this study had an average particle size of 100-350 nm and showed high entrapment efficiency. In conclusion, the preliminary results from this study show potential promise of SLN as a cosmetic delivery system for ASX as it offers an affordable method of making a high loading preparation with suitable particle size range. Further studies on their stability and permeation characteristics are now underway.

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