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Development of lipid nanoparticle (LNP) system for encapsulation of flavonoid molecules

플라보노이드계 물질 포집을 위한 지질나노입자의 개발

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석사학위논문

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ABSTRACT

Quercetin, naringenin, and hesperetin as flavonoids show biological activities including anti-oxidation, anti-inflammatory, and anti-cancer effects. However, their application in functional food is greatly restricted mainly due to poor water-solubility. Lipid nanoparticle (LNP) has been proposed as a new delivery system in order to improve the bioavailability of lipophilic flavonoid molecules. Here, the stable LNP system incorporating the flavonoids was developed. In response surface methodology concerning the hydrodynamic particle size (Z-average) and the contents of stable LNPs (yield), the content of fully hydrogenated canola oil, squalene, Tween 20, and soybean lecithin for preparing optimum blank LNPs were determined as 3.5, 1.5, 0.6, and 1.1 wt %, respectively. Therefore, LNP systems encapsulating the flavonoids (0–0.5 wt% of lipid matrix) were fabricated with the determined ratios, respectively, and then their physicochemical characteristics (yield, Z-average, PDI value, and ζ-potential) were characterized (>94%, ~150 nm, 0.14–0.18, and <-40 mV). Additionally, the highest entrapment efficiencies of quercetin, naringenin and quercetin LNPs were observed at the concentration of 0.3 wt% in lipid phase (82.8, 89.0, and 90.0%, respectively), which was contributed to their solubility in the lipid phase. These optimum LNP systems loading flavonoids were digested by

simulated intestinal juice within 60 min, and released the molecules from the matrices to the aqueous medium (37°C) in 50% (v/v) ethanol within 12 h. These results could be used as the basis of further study to develop beverages with flavonoids.

Keywords: quercetin, naringenin, hesperetin, lipid nanoparticle (LNP), fully hydrogenated canola oil (FHCO), squalene, soybean lecithin.

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I. INTRODUCTION

The public demand in functional food product incorporating bioactive compounds has been growing with desire for the health promotion and the disease prevention (Aditya, et al., 2014, Fathi, et al., 2012). A number of studies for biological activities of functional materials are being conducted. Flavonoids molecules are one of the most common bioactive compounds in phenolic plant constituents studied for a long time. Due to their numerous biological activity (Peterson and Johanna, 1998), flavonoids have generally used in herbal medicines and food supplements worldwide (Manthey and Guthrie, 2002).

Quercetin, naringenin, and hesperetin are natural flavonoid group of polyphenols widely present in various fruits and vegetables (Erlund, 2004). These molecules were well known for a variety of biological activities, such as anti-oxidant, anti-inflammatory, and anti-cancer effect. However, they have critical limitations in food application such as the low water-solubility and the instability in physiological medium (Yen, et al., 2009). Some research showed that the bioavailable amount of the intake was < 17% in rats and even <1% in men (Li, et al., 2009). As a result, their oral application in food is greatly restricted.

Various colloidal delivery systems have been proposed to overcome these limitations. Liposomes, nanoemulsions, and lipid nanoparticle (LNP) systems, including solid lipid nanoparticle(SLN) and nanostructure lipid carrier (NLC), are the most common lipid based delivery systems (Joye, et al., 2014). Such systems are used as vehicles to deliver poorly water-soluble bioactive ingredients into body.

Among various delivery systems, LNP is an emerging carrier system for lipophilic bioactive molecules due to various advantages over other colloidal carriers (Salminen, et al., 2014). Distinct advantages of LNP system are high bioavailability, non-toxicity, easily adoptability for large scale production, and controlled release properties (Chen, et al., 2010). Besides, they are prepared using biodegradable and biocompatible lipid, which make these carrier system appropriate for food applications (Fathi, et al., 2012). Because bioactive ingredients are incorporated into solidified lipid matrix of LNPs, bioactive molecules could be protected from environment and delivered safely into body. Solid lipid nanoparticle (SLN) is a first generation LNP of novel delivery system, which is a nanoemulsion in solid state at room temperature and body temperature. Nanostructured lipid carrier (NLC) is a second generation LNP that is replaced the certain portion of solid lipid with liquid lipid. Recently, NLC has attracted more attention because they

could improve the drawbacks of SLN such as the low bioactive molecules loading and the bioactive molecules expulsion caused by their highly ordered lipid matrix (Tamjidi, et al., 2013). The addition of liquid lipid in solid lipid could improve the colloidal stability of LNP by interfering perfect formation of lipid crystals and increase the space for accommodating bioactive ingredients. Therefore, appropriate selection of liquid lipid type and contents is crucial for the successful production of stable LNP system.

Squalene, one of the main lipids found in the surface of skin, has been widely used as the liquid lipid phase for the LNP system. And it has several biological activities like skin hydration, anti-oxidation, and anti-tumor activities (Li, Zhao, Ma, Zhai, Li and Lou, 2009, Huang, et al., 2009). Fang at al. (Fang, et al., 2008) found that LNP containing squalene showed the smaller particle size and the more controlled release pattern from LNP than other liquid lipid based LNPs.

Type of emulsifiers composing LNPs mainly affect physical properties and the colloidal stability of the system. In other words, emulsifiers could not only characterize the particle size and the ζ-potential in direct, but also indirectly influence the crystallization and the polymorphic behavior of the lipids (Bunjes, et al., 2003). Therefore, careful selection of suitable emulsifiers is the most important step to prepare stable LNP system.

Tween 20 as a non-ionic surfactant is commonly used in food system because of their non-toxicity and high emulsifying capability (Choi, et al., 2014). However, the amount of emulsifier such as Tween 20 in food system is strictly limited according to regulations. Meanwhile, soybean lecithin, a food-grade emulsifier, is also widely used in pharmaceutics, cosmetics and food (Rydhag and Wilton, 1981). Previous studies have shown that phospholipids in lecithin can be used to impact the physical stability of SLN. However, phospholipids alone are not sufficient to stabilize nanoemulsion (Salminen, Helgason, Aulbach, Kristinsson, Kristbergsson and Weiss, 2014). In this regard, combination of more than one emulsifier for producing LNP could improve the stability of LNP system.

The aims of the study were: (1) to develop a stable LNP system for encapsulating lipophilic bioactive molecules, and (2) to evaluate the potentials of LNPs for food fortification by entrapping flavonoids such as quercetin, naringenin, and hesperetin. Therefore, for the first purpose, the effects of the liquid lipid type and contents in the lipid matrix, and emulsifier composition on a stable LNP formation were investigated. For the latter purpose, the effects of the flavonoid type and content on encapsulation efficiency, particle size distribution, ζ-potential, yield, thermal properties, and release pattern were investigated.

II. MATERIALS AND METHODS

2.1. Chemicals and reagents

Fully hydrogenated canola oil (FHCO) was gifted by Lotte Samkang Co. Ltd (Seoul, Korea). Canola oil (LCO) and soybean oil (LSO) were purchased from CJ Cheiljedang Co. (Seoul, Korea). Squalene (>98%) was supplied from Alfa Aesar (Heysham, England). Tween 20 and soybean lecithin were obtained from Ilshinwells Co., Ltd (Seoul, Korea) and Fisher Chemical (USA), respectively. Quercetin and naringenin were purchased from MP Biomedicals, LLC (Illkirch, France), and hesperetin was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Gas chromatography (GC)

Fatty acid methyl esters preparation from FHCO, LCO, and LSO were conducted for preprocess of samples, and gas chromatography was performed with Agilent 7890 gas chromatography (Agilent Technologies Inc., USA) (Garces and Mancha, 1993).

2.3. Power X-ray diffraction (XRD) analysis

The XRD patterns for FHCO stored overnight at room temperature were collected using an X-ray diffractometer (Bruker D8 Advance, Karlsruhe, Germany) with Cu Kα radiation at λ=1.54 Å (30 kV, 30 mA). Both results of small-angle X-ray scattering (2θ = 0–9.1°, 0.005°/s) and wide-angle X-ray scattering (2θ = 0–40°, 0.2°/s) were obtained with a general area detector diffraction system.

2.4. Lipid nanoparticle (LNP) preparation

2.4.1. Blank LNP preparation

FHCO was fully melted at 85ºC and three different liquid lipid were also maintained at 85ºC in a water bath. Lipid phases were formed by blending different weight ratios of FHCO and liquid lipid (0, 10, 20, and 30 wt%). Aqueous phase was prepared by adding various weight ratios of Tween 20 and soybean lecithin (4 : 0, 3 : 1, 2 : 2, 1 : 3, and 0 : 4) in double-distilled water containing 0.02 wt% sodium azide. The concentration of emulsifiers in the aqueous phase was 1.67 wt%. Course oil-in-water emulsions were prepared by homogenizing 5 wt% lipid phase with 95 wt%

aqueous phase using a high-speed blender (Ultra Turrax T25D, Ika Werke GmbH & Co., Staufen, Germany) at 8,000 rpm for 1 min and 11,000 rpm for 1 min (Table 1). Then droplet size was further reduced using a sonicator (VCX 750, Sonics & Materials Inc., Newtown, CT, USA) at an amplitude of 60% and a duty cycle of 1 s for 4 min at 95ºC. After the size reduction, post sonication was applied to the samples for 6 min at the same sonication condition mentioned above during cooling the emulsions using a jacketed beaker at 25ºC. Then the samples were kept at 25 ºC in the jacket beaker for 10 min with constant stirring. After preparation, the samples were stored at room temperature (25ºC).

2.4.2. Flavonoids loaded LNPs preparation

Flavonoids loaded LNPs were developed based on the optimal composition. LNPs incorporating three different core materials were processed with the previous method in the blank LNPs preparation. Before the preparation of LNPs, flavonoids molecules (quercetin, naringenin and hesperetin) were added to the molten lipid phase. The concentration of the molecules in the lipid phase was selected to be under flavonoid molecules' solubility limits (0.1–0.5 wt% in lipid phase), and then flavonoids loaded LNP systems were fabricated using the lipid phase loading the flavonoids.

Table 1

Composition of blank LNPs with different liquid lipid contents (0–30 wt%) and emulsifier compositions of Tween 20 and soybean lecithin

Sample	FHCO (wt%)	Liquid lipid (wt%)	Tween 20 (wt%)	Soybean lecithin (wt%)	Water (wt%)
0 BLK LNP [1)]	5	0	1.67	0	93.33
			1.25	0.42	93.33
			0.84	0.84	93.33
			0.42	1.25	93.33
			0	1.67	93.33
10 BLK LNP [2)]	4.5	0.5	1.67	0	93.33
			1.25	0.42	93.33
			0.84	0.84	93.33
			0.42	1.25	93.33
			0	1.67	93.33
20 BLK LNP [3)]	4	1	1.67	0	93.33
			1.25	0.42	93.33
			0.84	0.84	93.33
			0.42	1.25	93.33
			0	1.67	93.33
30 BLK LNP [4)]	3.5	1.5	1.67	0	93.33
			1.25	0.42	93.33
			0.84	0.84	93.33
			0.42	1.25	93.33
			0	1.67	93.33

1) 0 BLK LNP: blank LNP composed of 0 wt% liquid lipid and 100 wt% FHCO

2) 10 BLK LNP: blank LNP composed of 10 wt% liquid lipid and 90 wt% FHCO

3) 20 BLK LNP: blank LNP composed of 20 wt% liquid lipid and 80 wt% FHCO

4) 30 BLK LNP: blank LNP composed of 30 wt% liquid lipid and 70 wt% FHCO.

2.5. Contents of non-aggregated LNPs, yield (%)

The prepared LNP systems were diluted with double-distilled water in a ratio of 1 : 9 and the diluted samples were filtered through a glass microfiber filter with a 1 μm-pore size (GF/B, Whatman Ltd., Fisons, Loughborough, UK). The aggregated and unstable LNPs were left on the filters, and then stable LNPs sized < 1 μm were obtained. The contents of non-aggregated LNPs (yield %) was determined by calculating the difference of a filter weight between before and after a filtration.

$$yield\ (\%) = \left(1 - \frac{weight\ of\ aggregated\ lipid\ nanoparticles\ (g)}{weight\ of\ total\ lipid\ nanoparticles\ (g)}\right) \times 100$$

2.6. Differential scanning calorimetry (DSC)

The melting and crystallization behaviors of the bulk lipids and LNPs were observed by differential scanning calorimetry (Diamond DSC, Perkin-Elmer, Waltham, MA, USA). Bulk lipids and LNPs (25±1 mg) were placed in aluminum pans and hermetically sealed with sealer. An empty pan was also sealed and used as a reference. Each sample was heated from 25°C to 95°C by 5°C /min and then cooled to 10°C by 5°C/min. By analyzing DSC thermogram, melting temperature (T_m), crystallization temperature (T_c),

enthalpies of melting and crystallization were determined. Based on DSC thermograms, the crystallinity index (CI) was calculated to determine the degree of crystallinity of LNPs and the equation was given as follow (Schubert and Muller-Goymann, 2005):

$$\text{Crystallinity Index, CI (\%)} = \frac{\Delta H_{m\ LNP}\ (J \cdot g^{-1})}{\Delta H_{m\ bulk\ lipid}\ (J \cdot g^{-1})} \times 100$$

where $\Delta H_{m\ LNP}$ is the melting enthalpy of LNPs, $\Delta H_{m\ bulk\ lipid}$ is the melting enthalpy of bulk lipid.

2.7. Particle size and ζ-potential determination

The physical properties of the stable LNPs in nano-scale obtained after filtration were analyzed. Mean particle diameters (hydrodynamic diameter, z-average), size distribution (poly diversity index, PDI), and ζ - potential of the samples were measured by a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) using a helium-neon laser (λ = 633 nm).

2.8. Response surface methodology (RSM)

To optimize the formulation of main components (liquid lipid contents and ratio of emulsifiers), the response surface methodology (RSM) was applied. The statistical software SAS program (9.3 version, SAS Institute, Cary, NC., U.S.A.) was utilized for the RSM. Based on the experimental data, squalene contents in the lipid phase (X_1, 10–30%) and soybean lecithin contents in the emulsifier mixture (X_2, 50–100%) were selected as the independent variables. The yield value (Y_1), z-average (Y_2) and crystallinity index, CI (Y_3) were designated as the response variables. Variable levels were coded as -1 (low), 0 (central point) and 1 (high). To fit the experimental data, quadratic polynomial equation was given as follow:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 \quad (1)$$

where Y is the response; X_1 and X_2 are variables; β_0 is the model intercept coefficient; β_1 and β_2 are linear coefficients; β_{12} is interaction coefficients between the two factors; and β_{11}, and β_{22} are quadric coefficients. The range and levels of experimental design was listed in Table 2. The statistical analysis was conducted by ANOVA. The quality of fit of the polynomial model was determined by the coefficient of determination R2, and its statistical analysis was examined by the *F*-test in the SAS.

Table 2

Central composite design (CCD) for optimization of composition of stable LNPs system

Run	Variable levels (coded)		Experimental data		
	X_1	X_2	Y_1	Y_2	Y_3
	Liquid lipid contents (%)	Soybean lecithin contents (%)	Yield (%)	Z-average (nm)	CI (%)
1	10 (-1)	50 (-1)	56.3	165.0	72.9
2	10 (-1)	75 (0)	62.9	164.5	82.8
3	10 (-1)	100 (1)	30.7	176.8	98.1
4	20 (0)	50 (-1)	63.2	155.0	72.2
5	20 (0)	75 (0)	88.6	148.5	72.7
6	20 (0)	100 (1)	34.4	155.0	86.1
7	30 (1)	50 (-1)	92.9	163.4	32.9
8	30 (1)	75 (0)	94.8	154.5	31.6
9	30 (1)	100 (1)	53.7	188.3	60.5

2.9. Flavonoids entrapment efficiency, EE %

Entrapment efficiency, EE % was calculated in order to evaluate the capability of LNPs incorporating bioactive ingredients. The amount of encapsulated flavonoids was estimated by UV-spectrophotometry at each flavonoid molecules' absorbance wavelength. Flavonoids loaded LNPs were immersed in the same volume of *n*-butanol and centrifuged at 10,000 rpm for 10 min. The supernatant, *n*-butanol dissolving non-encapsulated flavonoids, was collected to determine the amount of flavonoid entrapped in the lipid matrix.

$$\text{EE \%} = \left(1 - \frac{amount\ of\ unentrapped\ drug}{amount\ of\ total\ drug\ added}\right) \times 100$$

2.10. Simulated small intestinal *in vitro* digestion test

To investigate the digestion patterns of the flavonoids loaded LNPs, simulated small intestinal *in vitro* digestion test was conducted. Composition and concentration of simulated small intestinal juice were listed in Table 3. Intestinal juice was composed of 60% (v/v) of duodenal juice, 30% (v/v) of bile juice, and 10% (v/v) of bicarbonate buffer. Twenty milliliter of LNPs were blended with the same volume of intestinal juice and digested by

intestinal juice at 37°C with moderate stirring. As enzymatic reaction progress, free fatty acids were produced from LNPs so that pH of the sample was lowered. Amount of fatty acid hydrolyzed from LNPs was calculated by determining the volume of 0.1M NaOH to neutralize the fatty acid for maintaining initial pH level (pH 8.3).

Table 3

Composition and concentration of *in vitro* simulated small intestinal juice

	Duodenal juice	Bile juice
Inorganic solution	40 mL NaCl (175.3 g/L)	30 mL NaCl (175.3 g/L)
	40 mL $NaHCO_3$ (84.7g/L)	68.3 mL $NaHCO_3$ (84.7g/L)
	10 mL KH_2PO_4 (8 g/L)	4.2 mL KCl (89.6 g/L)
	6.3 mL KCl (89.6 g/L)	150 μL HCl (35% g/g)
	10 mL $MgCl_2$ (5 g/L)	-
	180 μL HCl (35% g/g)	-
Organic solution	4 mL Urea (25 g/L)	10 mL Urea (25 g/L)
	9 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L)	10 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L)
	1 g BSA	1.8 g BSA
	9 g Pancreatin	30 g Bile
	1.5 g Lipase	-
pH	8.1±0.2	8.2±0.2

2.11. Flavonoids release experiment

Flavonoids released from LNPs were estimated by using dialysis bag method. Dialysis bags (MWCO 12 kDa, Sigma-Aldrich, St. Louis, MO, USA) were soaked in double-distilled water for 12 h before use. One milliliter of flavonoids loaded LNPs were put into a dialysis bag and then the sealed bag was suspended in 49 mL of 50% (v/v) ethanol solution on a water bath at 37ºC and, 100 rpm. One milliliter of aliquot was withdrawn at each time interval and replaced the same volume of fresh 50% (v/v) ethanol to maintain the sink condition. The amount of released flavonoids was determined by UV spectrometer.

$$\text{Flavonoids released (\%)} = \frac{Amount\ of\ flavonoids\ in\ media}{Amount\ of\ flavonoids\ encapsulated\ in\ LNPs} \times 100$$

2.12. Statistical analysis

All results were analyzed using a Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). Data represent the averages of at least three independent experiments or measurements.

III. RESULTS AND DISCUSSION

Part I. Preparation of stable blank LNPs

3.1. Characteristics of FHCO and the blended lipids

FHCO as solid lipid and, canola oil, soybean oil, and squalene as liquid lipids were selected for the matrices of LNP system. Because fatty acid compositions of each lipid were critical factor affecting properties of LNPs, characteristics of FHCO, liquid lipids, and the blended lipids with FHCO and liquid lipids in various ratios were characterized by GC, DSC and XRD. Fatty acid composition of FHCO, canola oil and soybean oil were seen in Table 4. According to the results, while the most abundant fatty acid in FHCO was stearic acid, oleic acid and linoleic acid were the most abundant in both canola oil and soybean oil. Thus, because FHCO could be composed with a lot of tristearin (TS) and other triacylglycerides, thermal and physical properties of FHCO were expected to be similar to those of TS.

Table 4

Fatty acid composition (%) of FHCO, canola oil, and soybean oil

Fatty acid	Oil sample		
	FHCO	Canola oil	Soybean oil
Lauric ($C_{12:0}$)	1.8	-	0.2
Myristic ($C_{14:0}$)	0.9	-	0.1
Palmitic ($C_{16:0}$)	8.8	4.4	10.2
Stearic ($C_{18:0}$)	79.5	1.8	3.8
Oleic ($C_{18:1}$)	4.2	58.3	23.2
Linoleic ($C_{18:2}$)	1.4	20.0	49.3
Linolenic ($C_{18:3}$)	-	8.3	4.9
Arachidic ($C_{20:0}$)	1.6	0.6	0.3
Erucic ($C_{20:1}$)	-	1.3	0.2
etc	1.8	5.3	7.7

Melting temperatures (T_m) of α and β polymorphic form of TS were known as 55.6°C and 72.1°C, respectively, and a crystallization temperature (T_c) of a α polymorphic form of TS was known as 51°C (Elisabettini, et al., 1996). In a DSC thermogram of FHCO (Figure 2a), the T_m of α and β form of FHCO and the T_c of α and β form of FHCO were similar to those of TS, respectively. Besides, β form of pure TS was characterized by the three specific diffraction lines of the β form at 0.46, 0.39, and 0.37 nm with WAXS (Elisabettini, Desmedt and Durant, 1996); and β form of commercial TS (Dynasan 118) was distinguished by a specific X-ray long spacing at 4.48 nm with SAXS (Bonnaire, et al., 2008). These values were also similar to results acquired form SAXS (4.49 nm) and WAXS (0.46, 0.39, and 0.37 nm) measurements of FHCO (Figure 1).

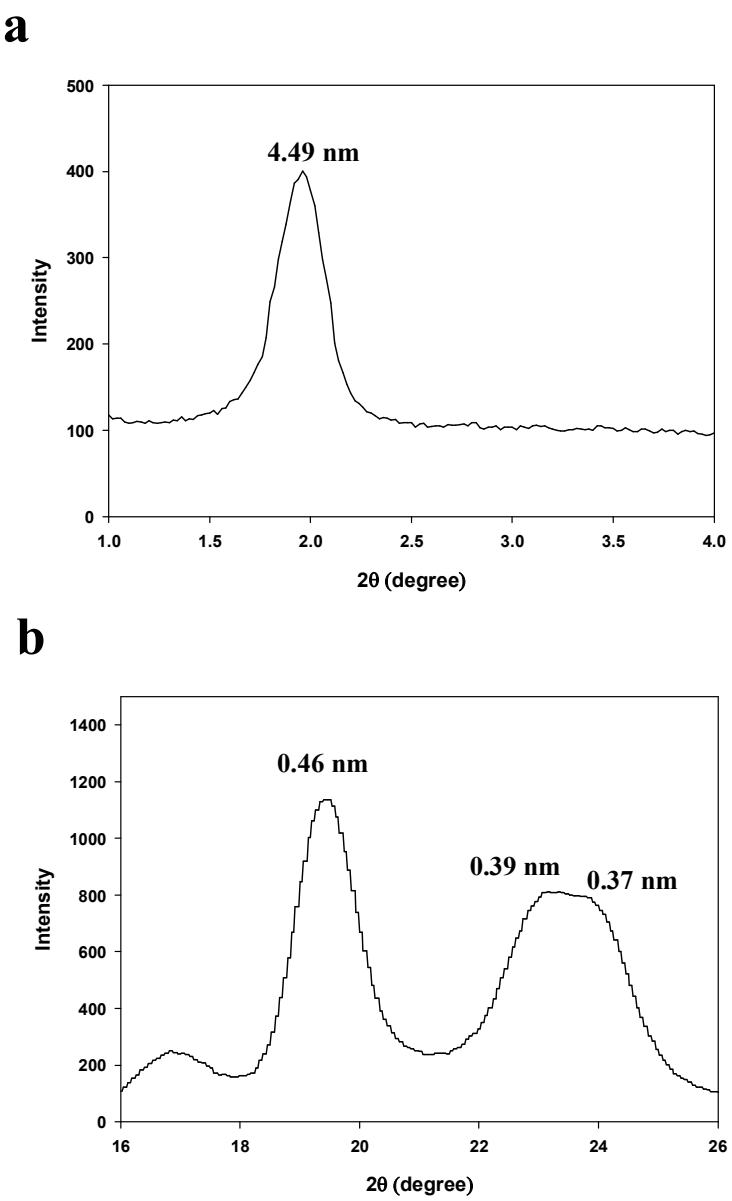

Figure 1. X-ray diffraction patterns of the β form of fully hydrogenated canola oil: (a) small angle X-ray scattering and (b) wide angle X-ray scattering.

DSC thermograms of FHCO and blended with each liquid lipid in various ratios (10–30 wt%) was shown in Figure 2. The thermograms of the bulk lipids mixed with the liquid lipid showed similar patterns of FHCO, which have a melting peak of β form and a crystallization peak of α form. However, the T_m and the T_c of blended lipids slightly differed from those of FHCO. FHCO samples blended with 10 and 20 wt% of soybean oil showed similar values of the T_m as 71.4 and 70.0°C to those of FHCO, respectively, whereas the T_m of 30 wt% of soybean oil was lower (67.4°C) than the T_m of other bulk lipids (Figure 2a, b, c, and d). In case of bulk lipids mixed with different weight ratios of squalene, T_m decreased as the contents of squalene increased. The lipids with 10, 20 and 30 wt% squalene have T_m of 68.1, 67.8 and 66.9°C, respectively (Figure 2e, f, and g). T_m (69.1°C) of the lipid blended with 10 wt% canola oil has similar to the T_m of FHCO whereas lipids with 20–30 wt% canola oil had lower value of T_m (both 67.3°C) (Figure 2h, i, and j).

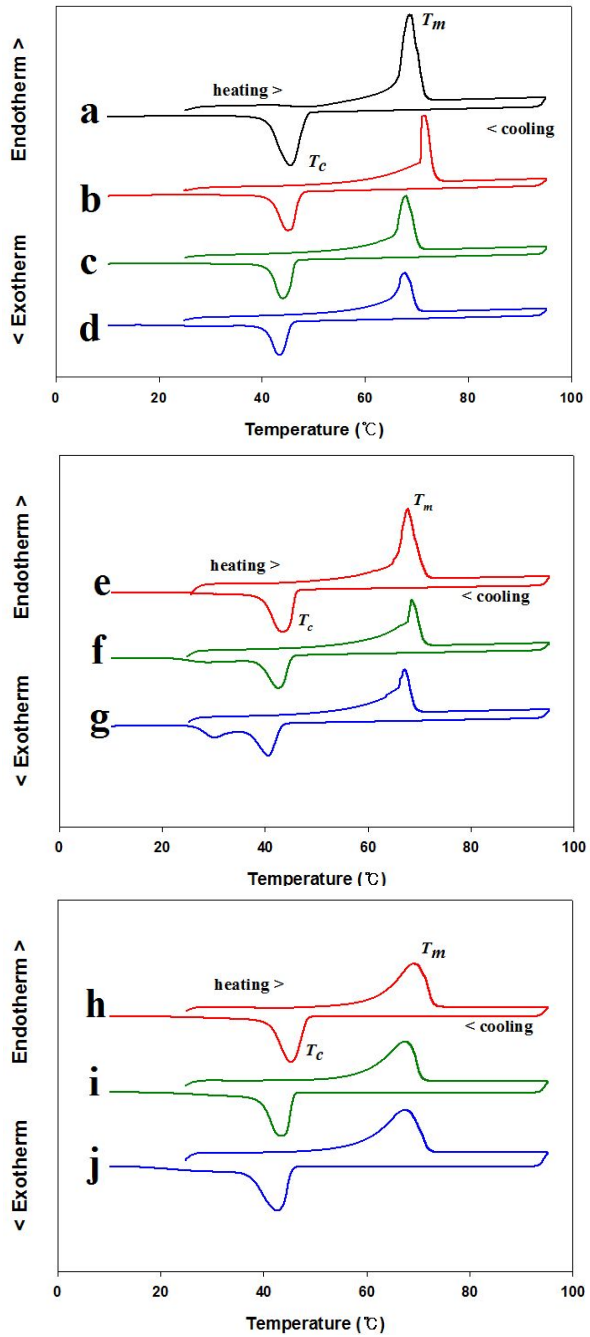

Figure 2. DSC thermograms, melting temperature (T_m) and crystallization temperature (T_c) of bulk FHCO lipids containing (a) 0, (b) 10, (c) 20, (d) 30 wt% soybean oil, (e) 10, (f) 20, (g) 30 wt% squalene, and (h) 10, (i) 20, (j) 30 wt% canola oil.

3.2. Visual stability of LNPs

LNP samples were prepared using the various types and the contents of liquid lipid and the ratio of two emulsifiers (Tween 20 and soybean lecithin). Unstable LNP system visually showed the destabilized phenomenon such as creaming. Because it was difficult to observe the creaming of freshly prepared samples, all samples were diluted tenfold. The diluted LNPs were separated into two layers (Figure 3); creaming layer at the top region and well-dispersed layer below the creaming region. The length of creaming layer was decreased as liquid lipid contents increased from 0 to 30 wt% at every emulsifier compositions. All LNP samples based on three liquid lipids showed similar tendency. These results suggested that increases of liquid lipid contents in the lipid phase could stabilize LNPs. Furthermore, LNP samples stabilized with single emulsifier, both Tween 20 and soybean lecithin alone, have showed unstable phenomenon (creaming). However, mixture of two emulsifiers in different weight ratios could stabilize particles synergistically. Moreover, LNPs prepared with weight ratio of Tween 20 and soybean lecithin as 1 : 3 were the most stable composition among the formulations.

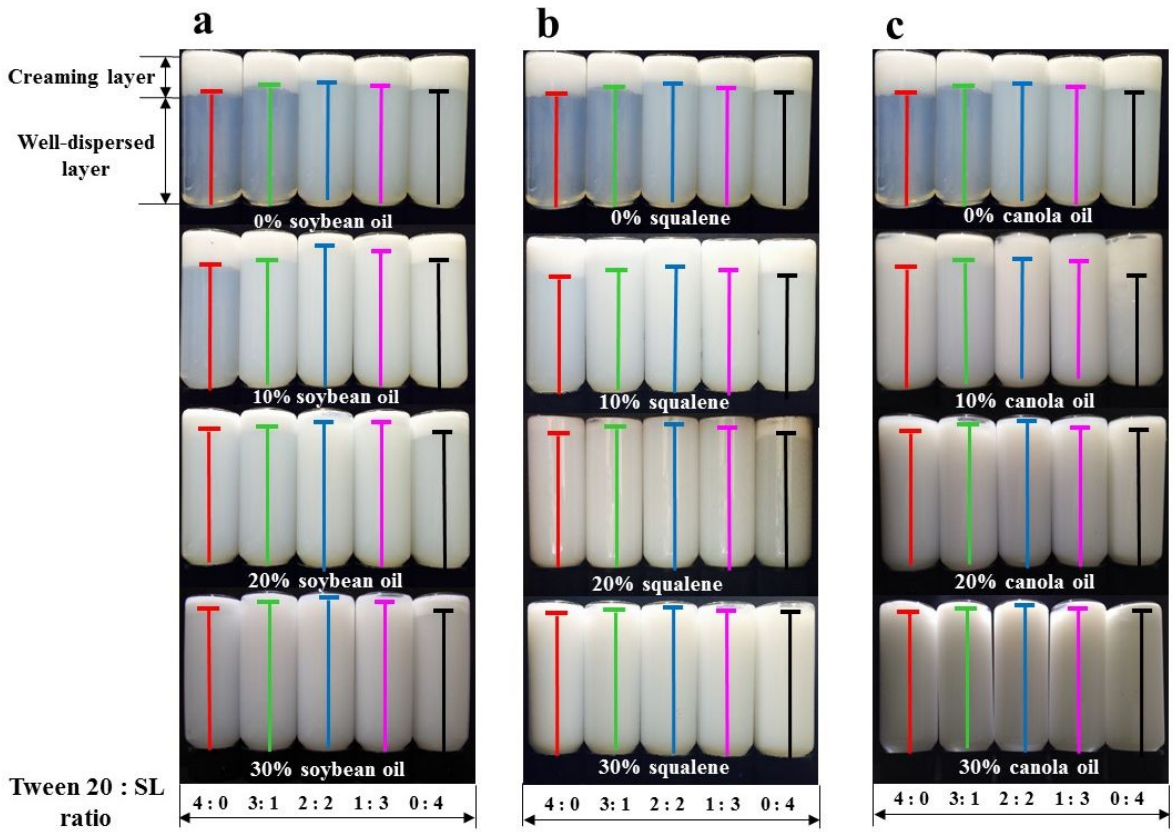

Figure 3. Visual stability of LNPs diluted tenfold. (a) LNPs with 0–30 wt% soybean oil, (b) LNPs with 0–30 wt% squalene, and (c) LNPs with 0–30wt% canola oil (Each bar means the length of well-dispersed layer; and SL, soybean lecithin).

3.3. Contents of non-aggregated LNPs, yield (%)

LNPs were diluted with double-distilled water in a ratio of 1 : 9 and the diluted samples were filtered through a glass microfiber filter with a 1 μm-pore. The aggregated LNPs in micron scale remain on the filter. The contents of non-aggregated LNPs were determined by calculating the filter weight difference before and after the filtration. Following the Stokes' law, large particles could float faster than small particles in the nano scale (<1 μm). Therefore, the yield could indicate the contents of LNPs <1 μm, in other words, the contents of stable (non-aggregated) LNPs.

LNPs with hydrophobic area exposed to aqueous phase could interact other LNPs at the similar state and aggregate (Silverstein, 1998). In other words, LNPs insufficiently covered with emulsifiers strongly tend to interact each other, and form aggregates of micron sized particles. Consequently, the aggregated unstable particles readily creamed upwards because of density difference caused by particle size. Therefore, the LNP system prepared with materials at the best formulation could show the high yield value. As shown in Table 5, with higher liquid lipid contents (from 0 to 30 wt%), yield value increased at every emulsifier composition and oil type. LNPs made by only one emulsifier (Tween 20 : soybean lecithin = 4 : 0, 0 : 4) had lower yield values <60%. Especially, LNPs fabricated using canola oil

showed the lowest yield value over other liquid oil LNPs. Among different weight ratio of liquid oil, 30wt% liquid in lipid matrix showed higher yield values (Table 5). At this liquid lipid contents (Figure 4), LNPs composed of emulsifier the ratio of Tween 20 and soybean lecithin at 1 : 3 show the highest yield value (soybean oil based LNPs: 92.7±0.52%, squalene based LNPs: 94.8±0.40%, and canola oil based LNPs: 86.5±0.81%). It was evidence that the use of two surfactants (Tween 20 and soybean lecithin) could stabilize LNPs better than using only one surfactant.

Table 5

Yield (%) of three different liquid lipid based LNP (0–30 wt%) with different Tween 20 (T20) : soybean lecithin (SL) ratios

Sample		Yield[1)] (%)		
T20 : SL	Oil (wt%)	Soybean oil	Squalene	Canola oil
4 : 0	0	38.5 ± 3.0 ij	38.5 ± 3.0 hij	38.5 ± 3.0 def
	10	52.8 ± 1.1 efgh	43.6 ± 1.7 gh	12.6 ± 3.0 hi
	20	50.4 ± 2.3 efgh	49.8 ± 0.7 efg	8.3 ± 2.7 i
	30	45.4 ± 4.4 ghij	57.5 ± 7.4 def	38.4 ± 2.2 def
3 : 1	0	41.7 ± 3.4 hij	41.7 ± 3.4 ghi	41.7 ± 3.4 de
	10	52.0 ± 4.4 efgh	44.2 ± 3.8 gh	13.4 ± 1.6 hi
	20	56.2 ± 3.5 efg	58.5 ± 4.8 cd	29.3 ± 2.1 fg
	30	69.8 ± 6.2 cd	79.1 ± 2.6 b	25.0 ± 2.3 g
2 : 2	0	55.5 ± 1.3 efg	55.5 ± 1.3 cdef	55.5 ± 1.3 bc
	10	57.8 ± 0.8 ef	56.3 ± 1.8 cde	9.00 ± 1.6 i
	20	80.7 ± 4.1 abc	63.2 ± 1.2 c	79.9 ± 2.1 a
	30	86.2 ± 2.2 ab	93.0 ± 0.5 a	79.6 ± 2.5 a
1 : 3	0	47.6 ± 0.6 fghi	47.6 ± 0.6 fg	47.6 ± 0.6 cd
	10	61.2 ± 1.1 de	62.9 ± 3.5 c	63.3 ± 2.6 b
	20	75.6 ± 3.3 bc	88.6 ± 0.7 a	84.1 ± 1.9 a
	30	92.7 ± 0.5 a	94.8 ± 0.4 a	86.5 ± 0.8 a
0 : 4	0	35.2 ± 2.6 j	35.2 ± 2.6 ij	35.2 ± 2.6 ef
	10	37.8 ± 2.8 ij	30.7 ± 1.8 j	21.9 ± 4.0 gh
	20	47.8 ± 1.9 fghi	34.4 ± 3.4 ij	7.3 ± 5.4 i
	30	48.9±2.38 fghi	53.7 ± 3.7 def	23.8 ± 0.1 g

[1)] Different letters from a to j in a column are significantly different (p <0.05).

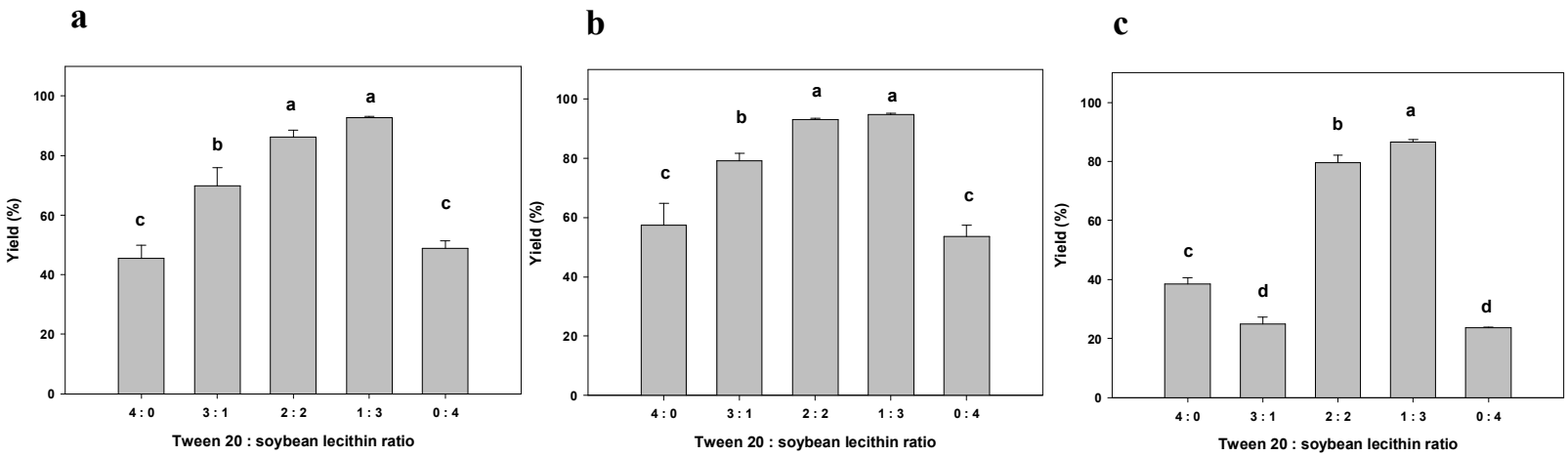

Figure 4. Yield (%) of (a) 30 wt% soybean oil LNPs, (b) 30 wt% squalene LNPs, and (c) 30 wt% canola oil LNPs with different emulsifier compositions; and different letters (a–c) are significantly different (p <0.05).

3.4. Crystallinity index (CI) of LNPs

Because a degree of crystallization could greatly influence the capability to encapsulate guest molecules such as bioactive molecules, the crystallinity index (CI) is a crucial parameter determining usefulness of LNP. High CI value could mean that the lipid matrix is highly ordered crystallized in the highly ordered state, which leads to little space to incorporate bioactive molecules into lipid. The CI value of the LNPs decreased with increasing liquid lipid contents regardless of types of liquid lipid (Table 6). This may be attributed to interfering liquid lipid molecules in perfect and ordered formation of FHCO β crystal (Lacatusu, et al., 2014). Crystals in the LNP prepared using solid lipid only are arranged more dense and tighter than those composed from solid and liquid lipid mixture. Under the certain liquid lipid type and contents, CI values were significantly influenced by the composition of two emulsifiers. In case of the soybean oil LNP, the CI value was higher at single emulsifier conditions and lower at Tween 20 and soybean lecithin ratio of 2 : 2 and 1 : 3 (except 0 wt% soybean oil and 100 wt% FHCO LNPs). Among the soybean oil based LNPs formulations, 30 wt% soybean oil contents and the same content of Tween 20 and soybean lecithin (1 : 1) showed the lowest CI value of 54.5%. Squalene based LNPs had similar trend with soybean oil LNPs whereas 30 wt% squalene content and

ratio of emulsifiers at Tween 20 : soybean lecithin = 1 : 3 showed the lowest CI value of 26.7%. Canola oil LNP samples had similar trend with other oil based LNPs, and showed the lowest CI value of 68.1% at 30 wt% canola oil content and Tween 20 : soybean lecithin ratio of 3 : 1. Therefore, due to the low crystallinity properties, squalene as liquid lipid in LNPs showed the potential to form stable carrier systems for the incorporation of bioactive molecules.

Table 6

Crystallinity index of three different liquid lipids based LNPs (0–30 wt%) with different Tween 20 (T20) : soybean lecithin (SL) ratios

Sample		Crystallinity index[1)]		
T 20 : SL	Oil (wt%)	Soybean oil	Squalene	Canola oil
4 : 0	0	95.6 ± 2.0 ab	95.6 ± 2.0 ghi	95.6 ± 2.0 ab
	10	83.3 ± 0.7 bcde	83.9 ± 2.0 bcdef	93.6 ± 1.2 abc
	20	79.9 ± 5.3 defg	80.8 ± 2.7 defg	88.0 ± 0.8 bcde
	30	75.3 ± 2.5 efgh	67.6 ± 1.1 ghi	72.5 ± 3.6 hi
3 : 1	0	92.5 ± 2.3 abcd	92.5 ± 2.3 abcd	92.5 ± 2.3 abcd
	10	82.9 ± 0.8 bcde	78.1 ± 3.2 efg	86.2 ± 3.1 cdef
	20	78.4 ± 9.2 efg	72.4 ± 0.5 efghi	78.5 ± 2.8 efg
	30	67.4 ± 2.6 gh	58.9 ± 1.1 i	76.7 ± 1.4 ghi
2 : 2	0	93.2 ± 2.1 abc	93.2 ± 2.1 abcd	93.2 ± 2.1 abc
	10	77.8 ± 1.6 efg	72.9 ± 6.8 efgh	83.4 ± 1.4 efg
	20	67.1 ± 2.3 ghi	71.9 ± 3.6 fghi	72.4 ± 0.9 hi
	30	54.5 ± 3.1 i	28.7 ± 0.3 j	69.8 ± 2.1 hi
1 : 3	0	96.7 ± 6.2 a	96.7 ± 6.2 ab	96.7 ± 6.2 ab
	10	80.8 ± 10.0 cdef	82.8 ± 1.3 cdef	83.7 ± 6.1 defg
	20	69.9 ± 3.3 fgh	72.7 ± 3.4 efgh	70.1 ± 3.0 hi
	30	72.3 ± 2.9 hi	26.7 ± 0.3 j	68.1 ± 2.1 i
0 : 4	0	95.0 ± 3.7 ab	95.0 ± 3.7 abc	95.0 ± 3.7 abc
	10	92.4 ± 1.4 abcd	98.1 ± 0.1 a	97.2 ± 2.1 a
	20	85.2 ± 3.3 abcde	86.1 ± 12.4 abcde	84.2 ± 1.0 defg
	30	77.3 ± 2.9 efg	60.5 ± 7.6 hi	90.2 ± 0.6 abcde

[1)] Different letters from a to j in a column are significantly different (p <0.05).

3.5. Particle size distribution and ζ -potential

Particle size of LNPs is a significant factor of the gastrointestinal uptake and clearance (Das and Chaudhury, 2011). Furthermore, the small size could lead to increasing stability against gravitational separation because of Browning motion of LNPs (Fathi, et al., 2012). In general, the particle size < 300 nm is desirable for the intestinal transport. In all formulations, mean particle size values (z-average) of the stable particles were in the range of 123.2 to 188.3 nm and had a unimodal size distribution (Table 7, 8, and 9). In addition, PDI values of the stable LNPs were in the range of 0.15 to 0.33 and these values were within the acceptable range (<0.5) (Aditya, et al., 2012). Z-average of LNPs increased with of liquid lipid content increased except 30 wt% liquid lipid content at every liquid lipid type. LNPs stabilized with one emulsifier alone showed relatively larger particle size and broad size distribution (PDI) whereas LNPs stabilized with two surfactants had smaller particle size and narrow size distribution.

ζ -potential means the overall surface charge of particles and the degree of repulsion between similarly charged particles in colloidal dispersion. In some research, the stability of the dispersion after preparation and during storage was predicted by ζ -potential (Das and Chaudhury, 2011). Upon different ratios of Tween 20 and soybean lecithin, in the present

research, the absolute value of ζ -potential differed from each formulation. Because of the negative charged portion in soybean lecithin, the absolute value of ζ -potential was greatly influenced by the amount of soybean lecithin in the aqueous phase. As a result, in the whole prepared LNP systems, the absolute value of ζ -potential increased from -20.4 to -51.9 mV with increment of the soybean lecithin content in total emulsifier mixture from 0 to 100 wt%. Generally, the particles could be recognized as electrostatically stable when the absolute value of ζ -potential is over 30 mV (Muller, et al., 2001). At the high ζ -potential, the repulsion force between LNPs is high enough to prevent the aggregation, and ultimately leads higher emulsion stability (Fathi, Varshosaz, Mohebbi and Shahidi, 2012). Therefore, LNPs containing soybean lecithin > 25 wt% in the emulsifier mixture could be recognized as electrostatically stable formulations.

Table 7

Size, PDI value, and ζ -potential of 0–30 wt% liquid soybean oil (LSO) LNPs with different Tween 20 (T20) : soybean lecithin (SL) ratios

Sample		Parameter[1)]		
T20 : SL	LSO (wt%)	Z-average (nm)	PDI value	ζ-potential (mV)
4 : 0	0	123.2 ± 1.2 i	0.24 ± 0.01 cd	-20.6 ± 1.2 ab
	10	146.9 ± 2.9 g	0.29 ± 0.01 a	-23.6 ± 0.1 b
	20	180.7 ± 1.8 a	0.28 ± 0.03 ab	-20.4 ± 0.2 a
	30	184.9 ± 3.0 a	0.26 ± 0.01 abc	-20.4 ± 0.2 a
3 : 1	0	135.5 ± 1.2 h	0.22 ± 0.01 cdef	-35.2 ± 1.3 c
	10	157.5 ± 2.1 f	0.29 ± 0.01 cdefg	-38.7 ± 3.0 def
	20	170.6 ± 0.7 b	0.23 ± 0.01 cde	-37.2 ± 0.7 cd
	30	163.8 ± 0.9 cde	0.19 ± 0.02 efg	-38.2 ± 0.4 cde
2 : 2	0	145.6 ± 2.6 g	0.20 ± 0.01 defg	-39.1 ± 0.2 defg
	10	162.5 ± 0.8 def	0.21 ± 0.01 cdef	-39.5 ± 0.7 defg
	20	168.8 ± 0.4 bc	0.21 ± 0.02 cdefg	-41.9 ± 0.7 ghij
	30	159.0 ± 2.5 ef	0.18 ± 0.02 efgh	-41.5 ± 1.2 fghi
1 : 3	0	144.9 ± 1.3 g	0.23 ± 0.01 cde	-40.9 ± 0.1 efgh
	10	157.7 ± 1.5 f	0.22 ± 0.01 efgh	-43.4 ± 0.2 hij
	20	167.3 ± 1.3 bcd	0.20 ± 0.03 defg	-44.6 ± 0.6 ijk
	30	158.9 ± 1.6 ef	0.17 ± 0.01 gh	-44.9 ± 0.7 gh
0 : 4	0	165.1 ± 1.8 cd	0.20 ± 0.02 defg	-47.5 ± 1.1 kl
	10	182.8 ± 1.4 a	0.19 ± 0.01 efgh	-41.5 ± 1.0 efgh
	20	166.0 ± 1.6 bcd	0.15 ± 0.01 h	-48.1 ± 1.3 l
	30	168.4 ± 0.9 bc	0.18 ± 0.00 fgh	-48.3 ± 0.7 l

[1)] Different letters from a to l in the each column are significantly different (p <0.05).

Table 8

Size, PDI value, and ζ -potential of 0–30 wt% squalene (SQ) LNPs with different Tween 20 (T20) : soybean lecithin (SL) ratios

Sample		Parameter[1)]		
T 20 : SL	SQ (wt%)	Z-average (nm)	PDI value	ζ-potential (mV)
4 : 0	0	123.2 ± 1.2 i	0.24 ± 0.01 bc	-20.6 ± 1.2 a
	10	175.6 ± 2.3 b	0.33 ± 0.03 a	-23.2 ± 1.0 b
	20	183.7 ± 1.9 a	0.26 ± 0.01 b	-22.8 ± 0.1 b
	30	171.6 ± 1.9 bc	0.21 ± 0.01 cde	-22.4 ± 0.8 ab
3 : 1	0	135.5 ± 1.2 h	0.22 ± 0.01 bcd	-35.2 ± 1.3 c
	10	167.7 ± 2.7 cd	0.23 ± 0.02 bcd	-35.7 ± 0.4 cd
	20	166.0 ± 2.1 cd	0.20 ± 0.01 def	-37.7 ± 0.9 de
	30	164.9 ± 2.4 d	0.19 ± 0.01 defg	-40.2 ± 0.4 f
2 : 2	0	145.6 ± 2.6 g	0.20 ± 0.01 cde	-39.1 ± 0.2 ef
	10	165.0 ± 1.0 d	0.22 ± 0.01 cd	-40.1 ± 0.6 f
	20	154.8 ± 0.5 e	0.19 ± 0.01 def	-39.8 ± 1.0 ef
	30	163.4 ± 3.8 d	0.17 ± 0.01 efgh	-43.0 ± 0.2 g
1 : 3	0	144.9 ± 1.3 g	0.23 ± 0.01 bcd	-40.9 ± 0.1 fg
	10	164.5 ± 1.4 d	0.20 ± 0.01 def	-46.3 ± 0.9 hi
	20	148.4 ± 1.3 fg	0.19 ± 0.01 defg	-46.0 ± 0.5 h
	30	154.5 ± 2.5 ef	0.14 ± 0.01 h	-45.8 ± 0.6 h
0 : 4	0	165.1 ± 1.8 d	0.20 ± 0.02 def	-47.5 ± 1.1 hi
	10	176.8 ± 0.8 b	0.15 ± 0.01 h	-48.4 ± 0.7 ij
	20	155.0 ± 2.1 e	0.15 ± 0.01 gh	-50.1 ± 0.6 jk
	30	188.3 ± 1.6 a	0.16 ± 0.01 fgh	-51.9 ± 0.4 k

[1)] Different letters from a to k in the each column are significantly different (p <0.05).

Table 9

Size, PDI value, and ζ -potential of 0–30 wt% liquid canola oil (LCO) LNPs with different Tween 20 (T20) : soybean lecithin (SL) ratios

Sample		Parameter[1)]		
T 20 : SL	LCO (wt%)	Z-average (nm)	PDI value	ζ-potential (mV)
4 : 0	0	123.2 ± 1.2 k	0.24 ± 0.01 cdef	-20.6 ± 1.2 a
	10	173.0 ± 0.9 fg	0.33 ± 0.01 a	-26.3 ± 0.5 b
	20	183.8 ± 1.0 abc	0.29 ± 0.02 ab	-27.0 ± 0.8 b
	30	185.8 ± 2.0 ab	0.25 ± 0.01 cde	-26.2 ± 0.9 b
3 : 1	0	135.5 ± 1.2 j	0.22 ± 0.01 defgh	-35.2 ± 1.3 c
	10	180.5 ± 1.1 cde	0.26 ± 0.01 bcd	-37.0 ± 0.4 cd
	20	185.7 ± 3.4 ab	0.23 ± 0.01 cdefg	-42.5 ± 1.5 fg
	30	181.4 ± 1.1 bcd	0.23 ± 0.01 cdefg	-37.4 ± 0.9 cd
2 : 2	0	145.6 ± 2.6 i	0.20 ± 0.01 fghi	-39.1 ± 0.2 de
	10	176.7 ± 0.5 def	0.27 ±0.01 bc	-35.3 ± 1.1 c
	20	184.5 ± 0.6 abc	0.23 ± 0.01 cdefgh	-44.9 ± 0.9 ghi
	30	176.2 ± 0.9 ef	0.20 ± 0.02 ghi	-42.6 ± 0.8 fgh
1 : 3	0	144.9 ± 1.3 i	0.23 ± 0.01 cdefgh	-40.9 ± 0.1 ef
	10	169.2 ± 0.3 gh	0.20 ± 0.01 fghi	-44.0 ± 0.5 gh
	20	171.9 ± 1.0 fg	0.20 ± 0.02 efghi	-45.1 ± 0.6 hij
	30	178.7 ± 1.3 de	0.19 ± 0.03 hi	-44.6 ± 1.0 gh
0 : 4	0	165.1 ± 1.8 h	0.20 ± 0.02 fghi	-47.5 ± 1.1 j
	10	183.9 ± 3.2 abc	0.23 ± 0.01cdefg	-47.4 ± 0.4 ij
	20	173.2 ± 1.0 fg	0.19 ± 0.01 hi	-44.6 ± 0.5 gh
	30	187.9 ± 2.1 a	0.17 ± 0.03 i	-47.2 ± 0.4 ij

[1)] Different letters from a to j in the each column are significantly different (p <0.05).

Among various weight ratios of liquid lipid in the lipid phase, LNPs composed of 30 wt% liquid lipid showed the most stable in the visual stability (Figure 3), higher yield values and lower CI than other liquid lipid contents. At these formulations, particle size and PDI values were compared within liquid lipid type. LNPs with 30 wt% soybean oil had the particle size in the range of 158.9 to 184.9 nm and the PDI values from 0.17 to 0.26 (Figure 5a). The LNP stabilized by Tween 20 or soybean lecithin alone was significantly larger whereas the LNP with ratio of Tween 20 and soybean lecithin at 1 : 3 showed the smallest in the particle size and the PDI. Absolute value of ζ -potential increased from -20.4 to -48.3 mV with the amount of soybean lecithin increased (Figure 6b). The LNP composed of 30 wt% squalene had particle sizes ranging from 154.5 to 188.3 nm and PDI value from 0.14 to 0.21 (Figure 6a). Similar to LNPs with soybean oil, LNPs stabilized by Tween 20 or soybean lecithin alone resulted in significantly larger particle size and PDI value. Absolute value of ζ -potential increased from -22.4 to -51.9 mV, which was slightly larger than LNP composed of 30 wt% soybean oil (Figure 6b). As shown in Figure 7a, LNPs composed of 30 wt% canola oil had larger particle size between 176.2 and 185.8 nm in comparison to those of LNPs composed of soybean oil or squalene. However, ζ -potential showed similar value with those of other liquid lipid based LNPs (Figure 7b).

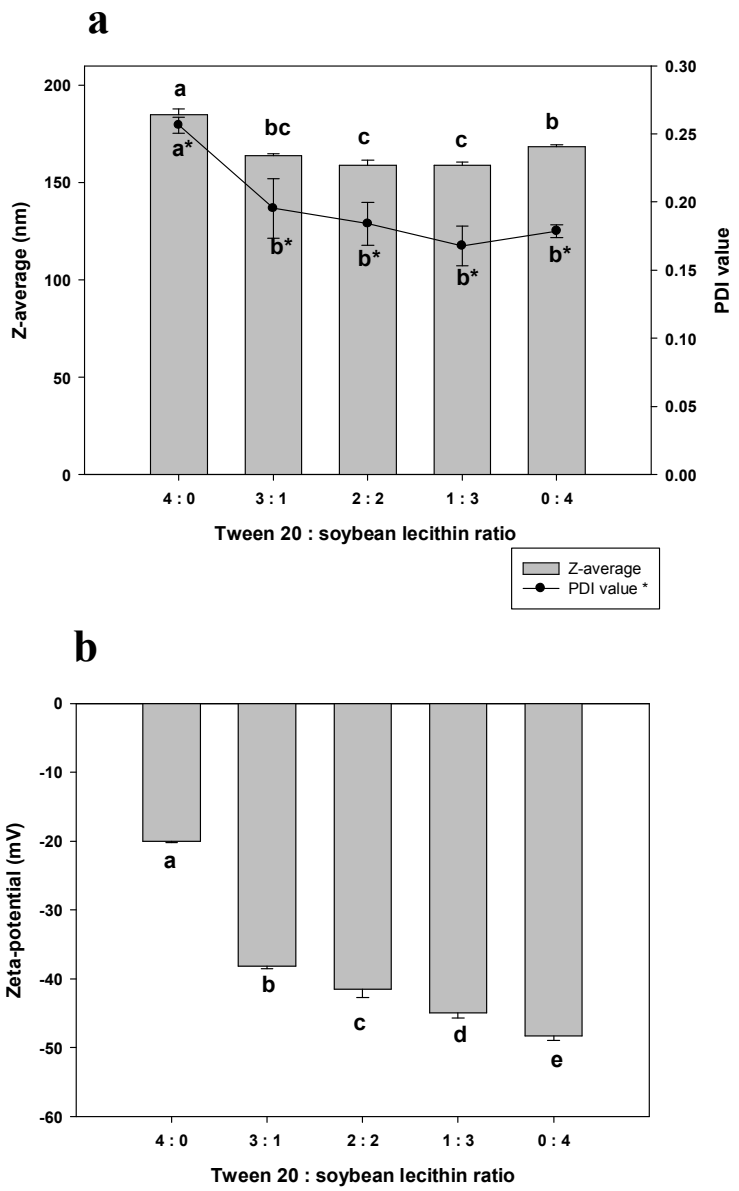

Figure 5. (a) Particle size (Z-average) and (b) ζ -potential of 30 wt% soybean oil LNPs with different Tween 20 : soybean lecithin ratios; and different letters (a–e) are significantly different (p <0.05).

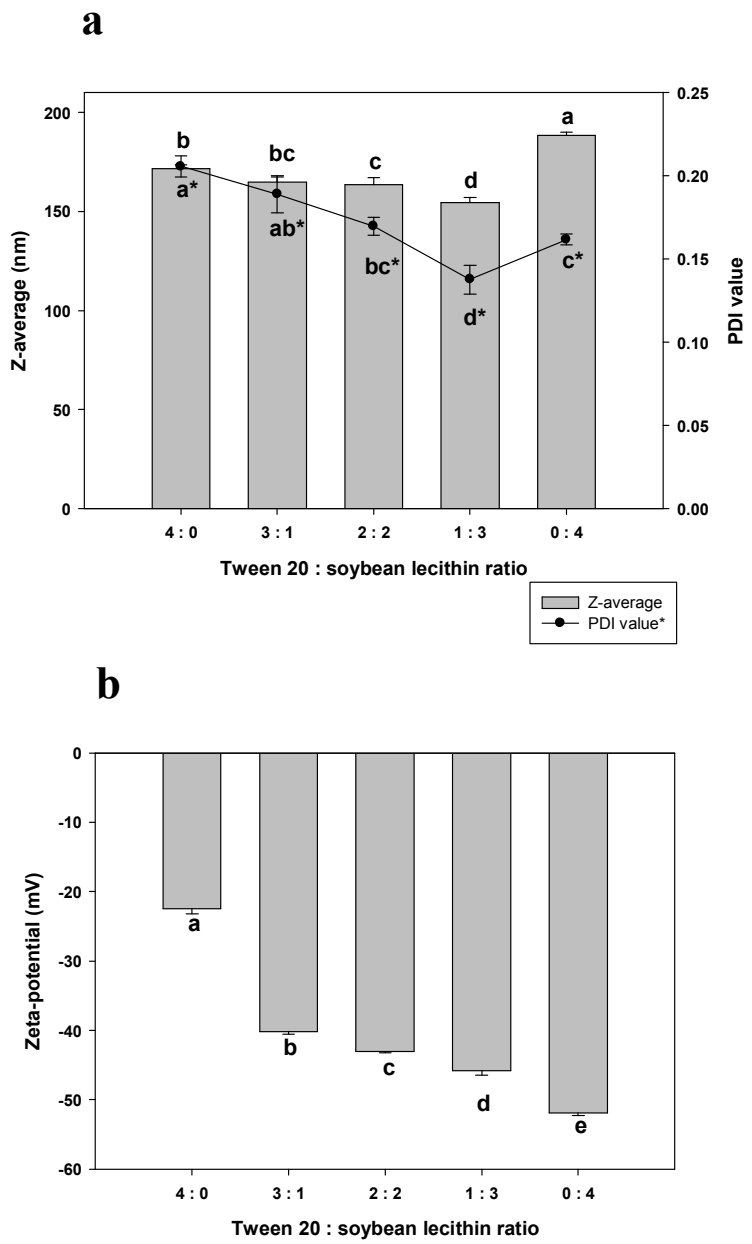

Figure 6. (a) Particle size (Z-average) and (b) ζ -potential of 30 wt% squalene LNPs with different Tween 20 : soybean lecithin ratios; and different letters (a–e) are significantly different (p <0.05).

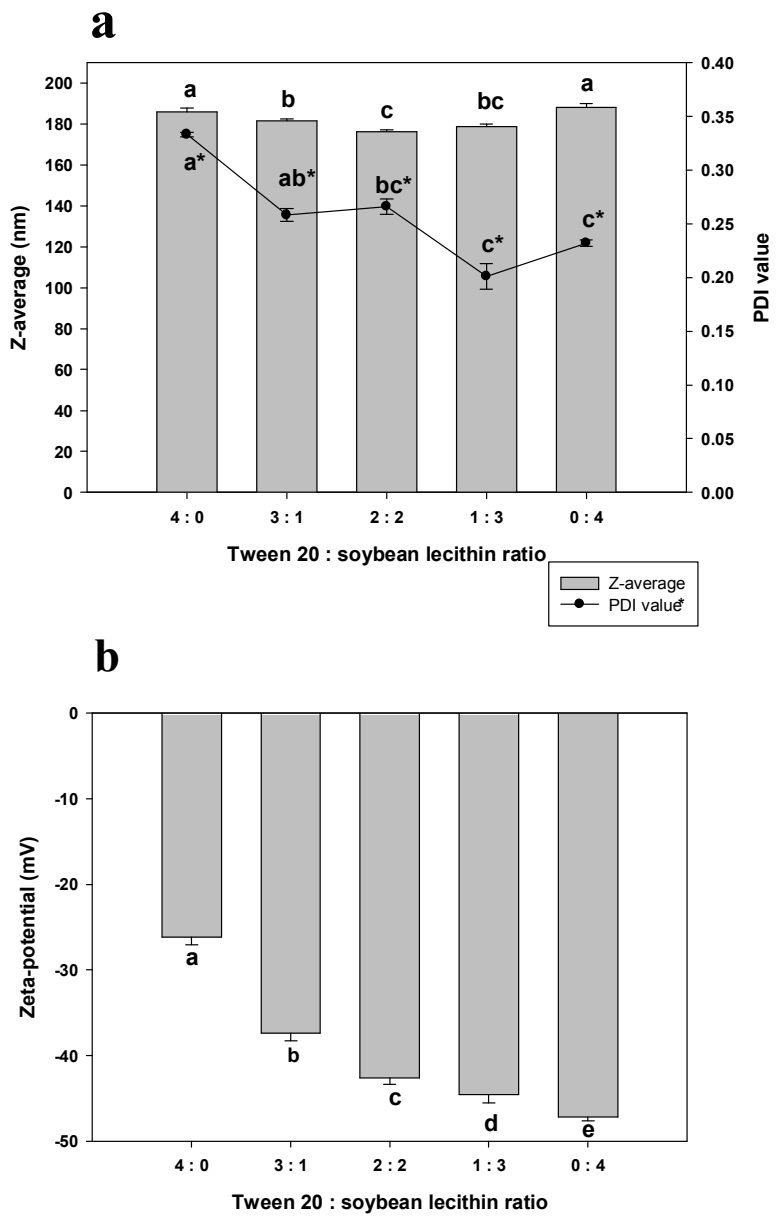

Figure 7. (a) Particle size (Z-average) and (b) ζ -potential of 30 wt% canola oil LNPs with different Tween 20 : soybean lecithin ratios; and different letters (a–e) are significantly different (p <0.05).

3.6. Response surface methodology (RSM)

In the previous section, squalene showed high potential to produce stable LNPs. Squalene based LNPs had the largest yield value and the smallest crystallinity index among all samples. Therefore, response surface methodology (RSM) was conducted to determine the optimum formulations of the squalene content (X_1) and the emulsifier composition (X_2) for suitable LNP system preparation. Independent and response variables were analyzed to get regression equation for prediction of the response under the given range of the conditions. The polynomial regression equation obtained for yield (Y_1), particle size (Y_2), and CI (Y_3) was as follows:

$$Y_1 = 53.78333 + 1.296167X_1 + 6.106267X_2 - 0.01374X_1X_2 + 0.0315{X_1}^2 - 0.04304{X_2}^2 \quad (2)$$

$$Y_2 = 314.232778 - 7.36558X_1 - 2.7914X_2 + 0.00248X_1X_2 + 0.15928{X_1}^2 + 0.012896{X_2}^2 \quad (3)$$

$$\mathrm{Y_3} = 01.88333 + 3.224667\mathrm{X_1} - 1.539067\mathrm{X_2} - 0.00248\mathrm{X_1X_2} - 0.1389{\mathrm{X_1}}^2 + 0.012896{\mathrm{X_2}}^2 \quad (4)$$

Based on this polynomial equation, three-dimensional (3D) graph for predicted responses was drawn (Figure 8). As shown in Figure 8a, the yield values were increased with increasing squalene content in the lipid, and

the composition of emulsifiers reached the optimum condition between ratio of 2 : 2 and 1 : 3.

The RSM 3D graph showed that the liquid lipid content (X_1) and the soybean lecithin content (X_2) significantly affected the yield value (Y_1), the particle size (Y_2), and the CI (Y_3). Because the formulation of >30 wt% squalene in the lipid phase would produce liquid droplets, the squalene content in the lipid were fixed at the 30 wt%. Therefore, the optimal values of yield, particle size, and CI value were calculated by polynomial equation (2), (3), and (4) at the content of 30 wt% squalene.

The yield value was expected to be the maximum at the composition of the 30 wt% squalene content in the lipid and the 65 wt% soybean lecithin in the emulsifier mixture. The particle size was expected to be minimum (158.9 nm) at the composition of the 30 wt% squalene content in the lipid and the 64.8 wt% soybean lecithin in the emulsifier mixture. And the CI value was calculated to be minimum (32.03%) at the composition of the 30 wt% squalene content in the lipid and the 56.8% of soybean lecithin in the emulsifier mixture. Among the three response factors, the yield value and the particle size were considered as more important parameters of stable LNP than CI value. Therefore, the optimal formulation was as follows; Squalene as liquid lipid, 30 wt% squalene with 70 wt% FHCO, and the ratio of Tween 20 and soybean lecithin was 0.35 : 0.65, respectively. All LNP systems

incorporating quercetin, naringenin or hesperetin were prepared at this optimal composition.

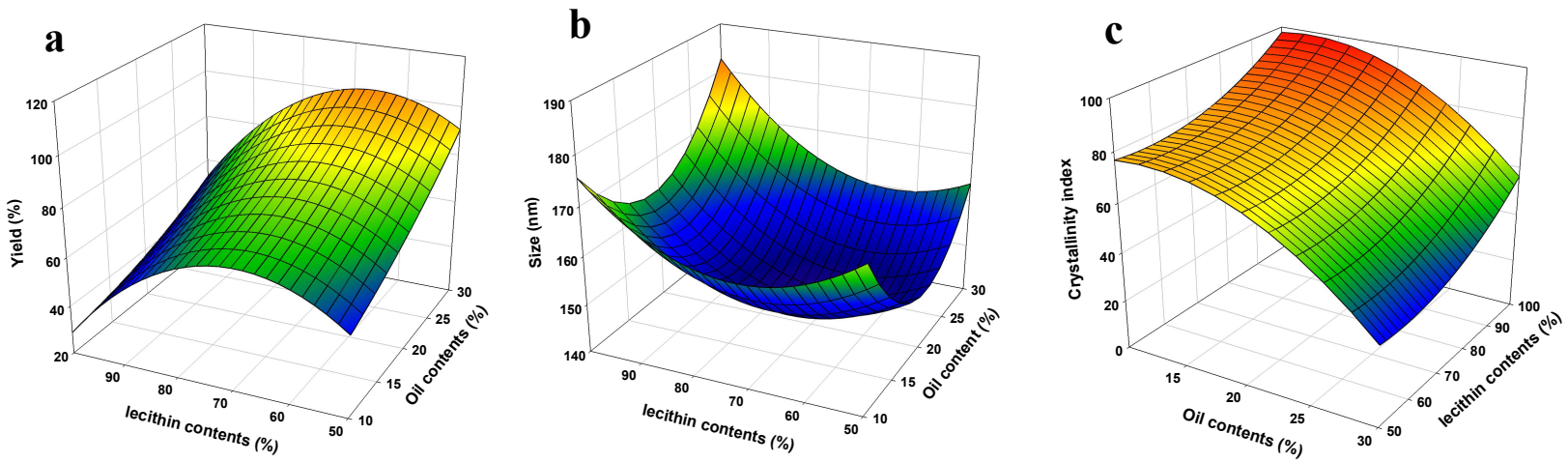

Figure 8. Response surface 3D graphs showing the effect of soybean lecithin contents and squalene contents on (a) yield (%), (b) size (nm), and (c) crystallinity index.

Part II. Application of the stable LNP system to encapsulate flavonoid molecules

3.7. Preparation of LNPs loading flavonoid molecules

I developed and optimized a formulation in aspects of the colloidal stability and the efficiency for encapsulating lipophilic bioactive ingredients. To apply this carrier system directly into foods, I chose flavonoid molecules; quercetin, naringenin and hesperetin. The capability of LNP incorporating biological ingredients was evaluated by analyzing of particle size, ζ-potential, encapsulation efficiency, thermal properties, release profiles, and digestion patterns. I also studied effects of the difference of flavonoid molecule properties on physicochemical characteristics and release profiles of core materials from the lipid matrix. The solubility of each flavonoid molecules in the lipid phase (30 wt% squalene, 70 wt% FHCO) was determined before the fabrication of flavonoids loaded LNPs. The concentration of the bioactive materials in the lipid phase was selected to be under flavonoid molecules' solubility limits at > T_m of the lipid. The solubility of bioactive molecules in the lipid matrix is an important factor, prior to incorporate them into the LNP system, because solubility could greatly influence the flavonoids loading capacity, the entrapment efficiency

and the subsequently utility of LNP system (Jaspart, et al., 2005) (Kasongo, et al., 2011). In this study, the concentrations of flavonoid molecules in the lipid were fixed up to 0.5 wt% in the lipid phase according to their solubility (Figure 9). At > T_m of the lipid, all flavonoids molecules well solubilized in the lipid phase up to 0.3 wt% in lipid phase whereas the concentration of flavonoids above 0.4 wt% of the lipid phase showed some insoluble sediment in the vials.

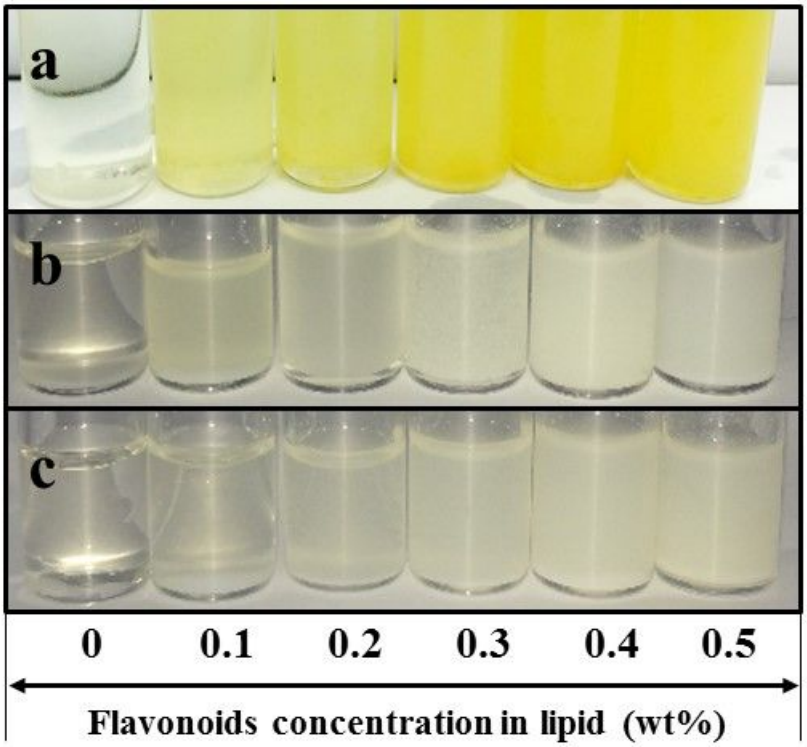

Figure 9. Solubility of (a) 0–0.5 wt% quercetin, (b) 0–0.5 wt% naringenin, and (c) 0–0.5 wt% hesperetin in lipid phase at > T_m.

3.8. Yield (%) of flavonoids loaded LNPs

After ×1/10 dilution of the fresh LNP systems, the aggregated and micron-size LNPs were left on the filter (pore size = 1μm), and then the yield of samples were recorded as Figure 10. Yield values of 0.1–0.5 wt% quercetin loaded LNPs were around 94% and both naringenin and hesperetin loaded LNPs showed similar values to those of quercetin loaded LNPs. These results were also similar to the yield of blank LNPs. Therefore, it explained that the incorporation of 0.1–0.5 wt% flavonoid molecules in the lipid matrix could have no effect on yield values of LNPs.

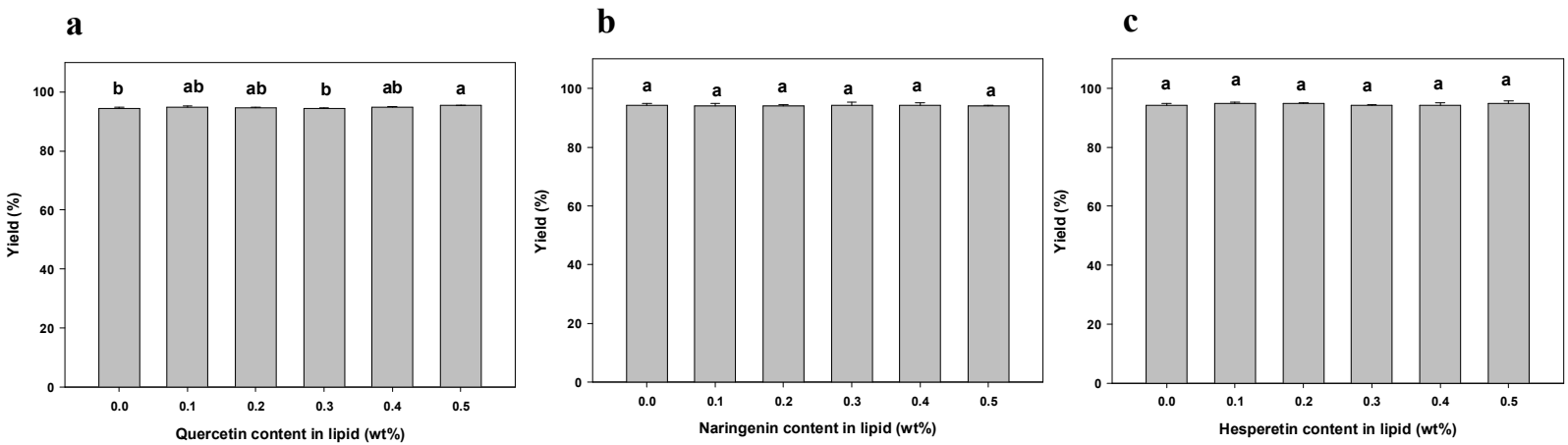

Figure 10. Yield (%) of (a) 0–0.5 wt% quercetin loaded LNPs, (b) 0–0.5 wt% naringenin loaded LNPs, and (c) 0–0.5 wt% hesperetin loaded LNPs; and different letters (a–b) are significantly different (p <0.05).

3.9. Particle size distribution and ζ -potential of flavonoid molecules loaded LNPs

Particle size distribution and ζ -potential of flavonoids loaded LNPs were shown in figure 11, 12, and 13. Mean particle size (Z-average) of the blank and the quercetin loaded LNPs were in the range of 149.1 to 156.2 nm and had PDI value from 0.14 to 0.17 (Figure 11a). Naringenin loaded LNPs showed mean particle size of LNPs from 148.8 to 156.2 nm and PDI values from 0.14 to 0.17 (Figure 12a). Hesperetin loaded LNPs had mean particle size from 149.9 to 155.7 nm and their PDI values from 0.14 to 0.18 (Figure 13a). The results showed that all flavonoids loaded LNPs had mean particle size around 150 nm and these values were not significantly different from the values of blank LNPs. In addition, all the prepared LNPs loading flavonoids had small PDI values (<0.17) indicating their narrow size distribution. Thus, it is an evidence that the incorporation of flavonoid molecules in these concentration range (0–0.5 wt%) in LNPs would have no significant effect on their size distribution. All of the prepared LNPs composing the flavonoids had similar ζ -potential values from -40 to -45 mV (Figure 11b, 12b, and 13b). It was suggested that the ζ -potential of flavonoids loaded LNPs in our formulations would not be mainly influenced by the flavonoids concentration in lipid phase but by the amount of soybean lecithin.

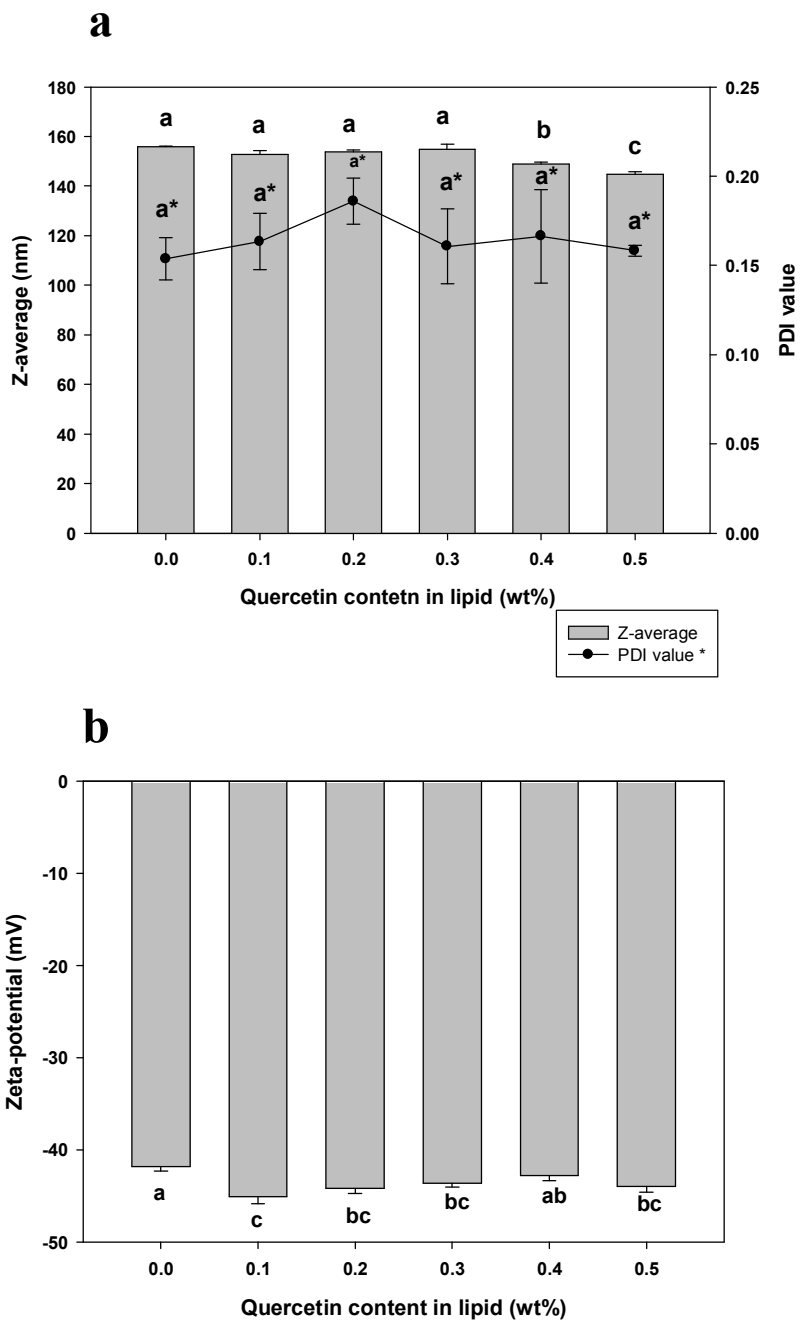

Figure 11. (a) Particle size (Z-average) and PDI value, and (b) ζ -potential of 0–0.5 wt% quercetin in lipid LNPs; and different letters (a–c) are significantly different (p <0.05).

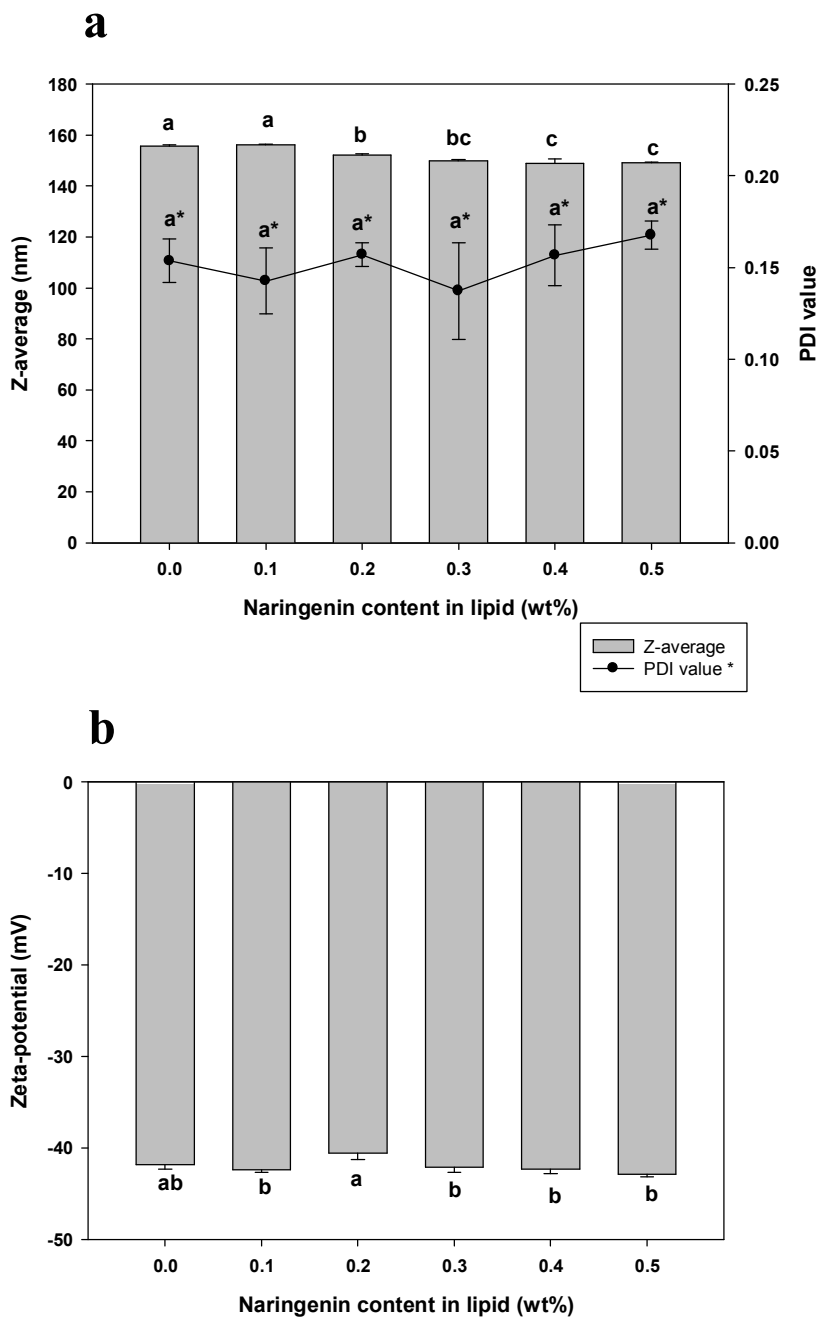

Figure 12. (a) Particle size (Z-average) and PDI value, and (b) ζ -potential of 0–0.5 wt% naringenin in lipid LNPs; and different letters (a–c) are significantly different (p <0.05).

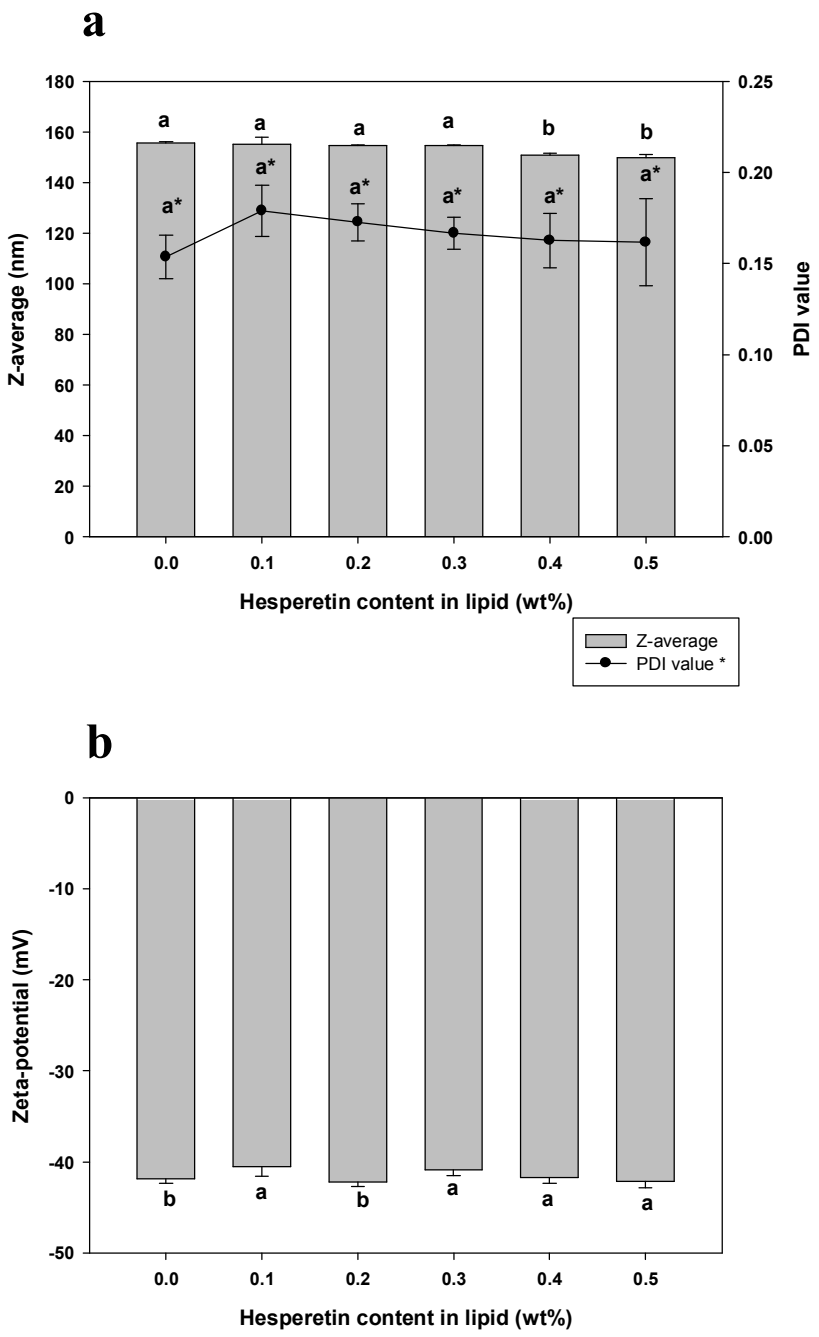

Figure 13. (a) Particle size (Z-average) and PDI value, and (b) ζ -potential of 0–0.5 wt% hesperetin in lipid LNPs; and different letters (a–b) are significantly different (p <0.05).

3.10. Flavonoids entrapment efficiency, EE %

Many researchers had wanted to encapsulate amount of bioactive molecules in carrier systems (Fathi, Varshosaz, Mohebbi and Shahidi, 2012). That is, obtaining higher entrapment efficiency (EE %) is the most desirable goal of developing drug-loaded LNPs. To investigate the effect of the flavonoids concentration on the EE %, LNPs at various flavonoids concentrations in the lipid (0–0.5 wt%) matrix were prepared. As shown in the data for quercetin-loaded LNPs (Figure 14), EE % increased from 69.5 to 81.1% as the quercetin concentration in the lipid increased from 0.1 to 0.3 wt%. However, EE % decreased from 81.1 to 64.25% as the quercetin concentration in the lipid decrease from 0.3 to 0.5 wt%. Meanwhile, naringenin loaded LNPs had high EE % over 90% at 0.3wt% naringenin in the lipid, which indicated that naringenin could be well encapsulated into the lipid matrix. Additionally, Hesperetin loaded LNPs showed EE % ranging from 72.5 to 89.0% at 0.1 wt% and 0.3wt% hesperetin contents in the lipid, respectively. In all flavonoids loaded LNPs, EE was the highest at the 0.3wt% content. In other word, at the level of 0.3 wt% concentration, quercetin, naringenin and hesperetin were well incorporated in the lipid matrix. Consequently, the difference of EE % among each flavonoids loaded LNP system might be caused by the difference of solubility in lipid phase.

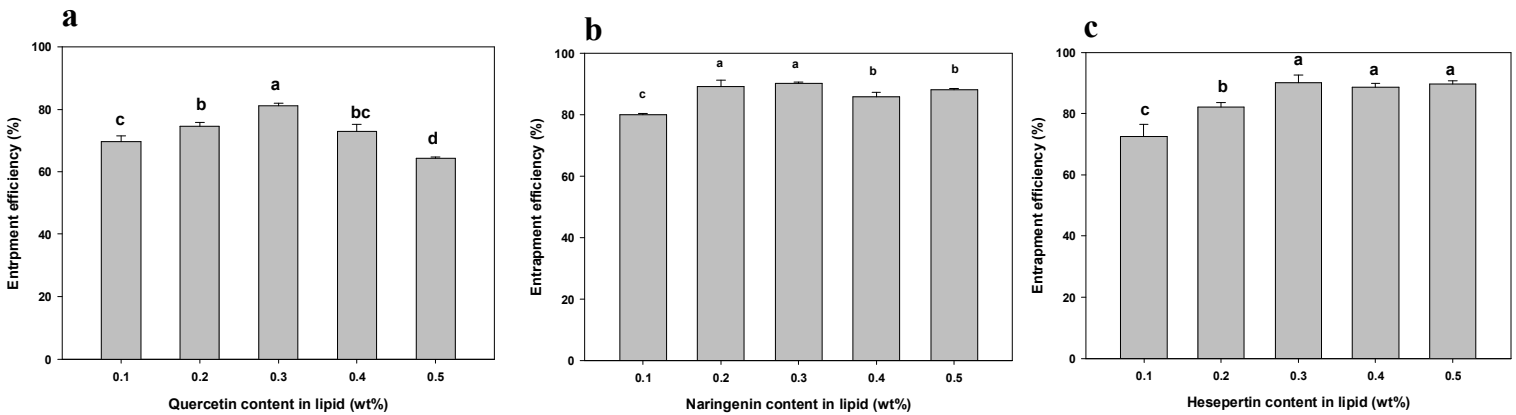

Figure 14. Entrapment efficiency (%) of (a) 0.1–0.5 wt% quercetin loaded LNPs, (b) 0.1–0.5 wt% naringenin loaded LNPs, and (c) 0.1–0.5 wt% hesperetin loaded LNPs; and different letters (a–d) are significantly different (p <0.05).

3.11. Thermal properties of flavonoids loaded LNPs

All prepared LNPs were analyzed by DSC in order to investigate the effects of flavonoids incorporation on the melting and the crystallization behavior of LNPs. As shown in Figure 15, 0.3 wt% quercetin loaded LNP system had a similar thermogram pattern to that of blank LNPs, especially in a melting peak and crystallization peaks. Blank LNP have a melting peak at 61.9°C and crystallization peak at 47.2 and 18.9°C. A melting and crystallization peaks of quercetin loaded LNPs were not significantly different from those of blank LNPs. DSC thermogram of both 0.3 wt% naringenin and hesperetin loaded LNPs also showed similar patterns with those of blank and quercetin loaded LNPs. These results indicated that the incorporation of flavonoids molecules at the concentration level of 0.3 wt% in the lipid matrix could have no effect on thermal properties of LNP system.

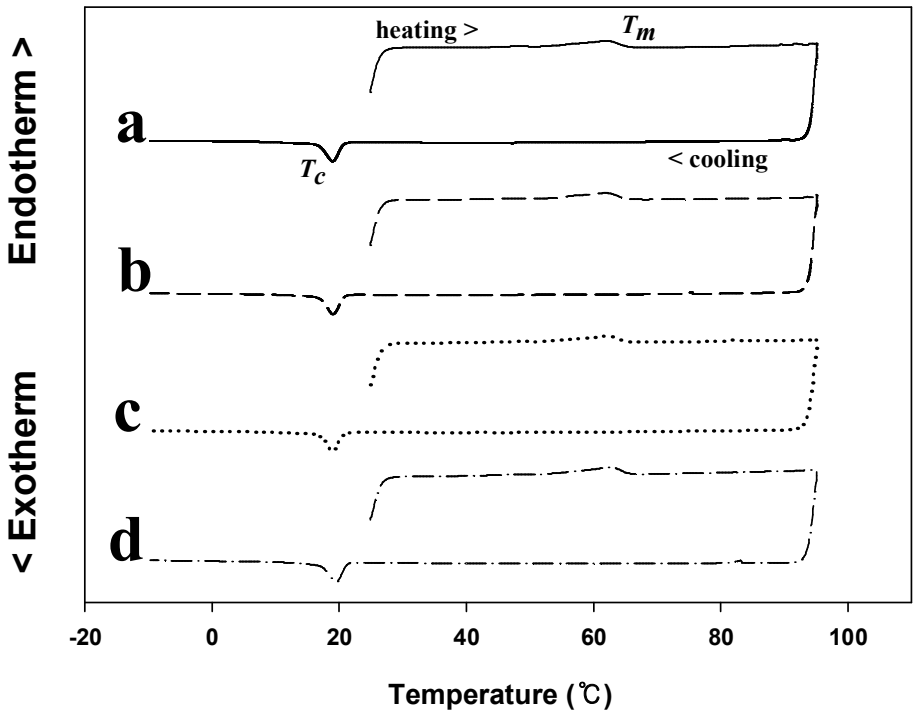

Figure 15. DSC thermograms, melting temperature (T_m) and crystallization temperature (T_c) of LNPs containing (a) blank, (b) 0.3 wt% quercetin, (c) 0.3 wt% naringenin, and (d) 0.3 wt% hesperetin in the lipid phase.

3.12. Release pattern of flavonoids loaded LNPs

Cumulative release profiles from the matrix of LNPs loading 0.3 wt% flavonoids were shown in Figure 16. For all the prepared LNPs containing 0.1–0.5 wt% flavonoids, release profiles were observed with similar pattern. At the initial stage of release, a burst release were occurred for 2 h, which means flavonoids were release rapidly from LNPs (80% of flavonoids were release from LNPs). After rapid release, the rate of release from LNPs became slower and the remaining of flavonoids inside the LNPs (20% of flavonoids) were released within 12 h.

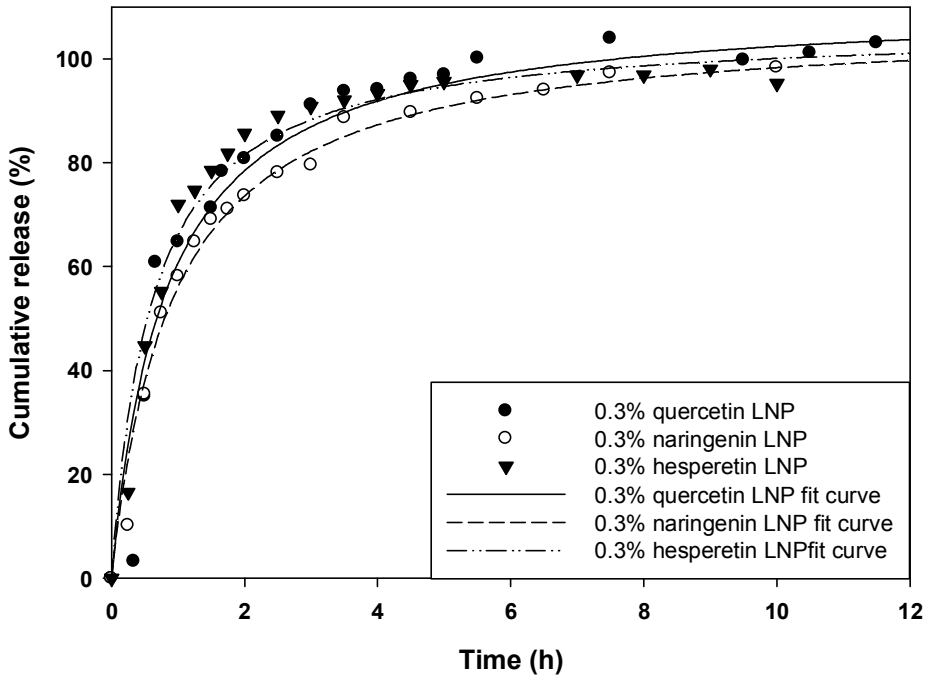

Figure 16. Cumulative release of (●) 0.3 wt% quercetin loaded LNP, (○) 0.3 wt% naringenin loaded LNP, and (▼) 0.3 wt% hesperetin loaded LNP in 50% (v/v) ethanol 37°C medium with 100 rpm shaking.

3.13. Simulated small intestinal *in vitro* digestion test

Simulated small intestinal *in vitro* digestion test of flavonoid-loaded LNPs was conducted in order to figure out hydrolysis patterns of the lipid (triacylglycerols) in the intestinal circumstance. Free fatty acid were produced from LNPs and pH of the sample was lowered as enzymatic reaction progress due to the hydrolysis of triacylglycerols. The amount of hydrolyzing fatty acid molecules from LNPs was depicted by the volume of 0.1 M NaOH to neutralize the fatty acid and maintain initial pH 8.3. Digestion patterns were shown in Figure 17. Consequently, flavonoid-loaded LNPs were digested by simulated small intestinal medium within 60 min.

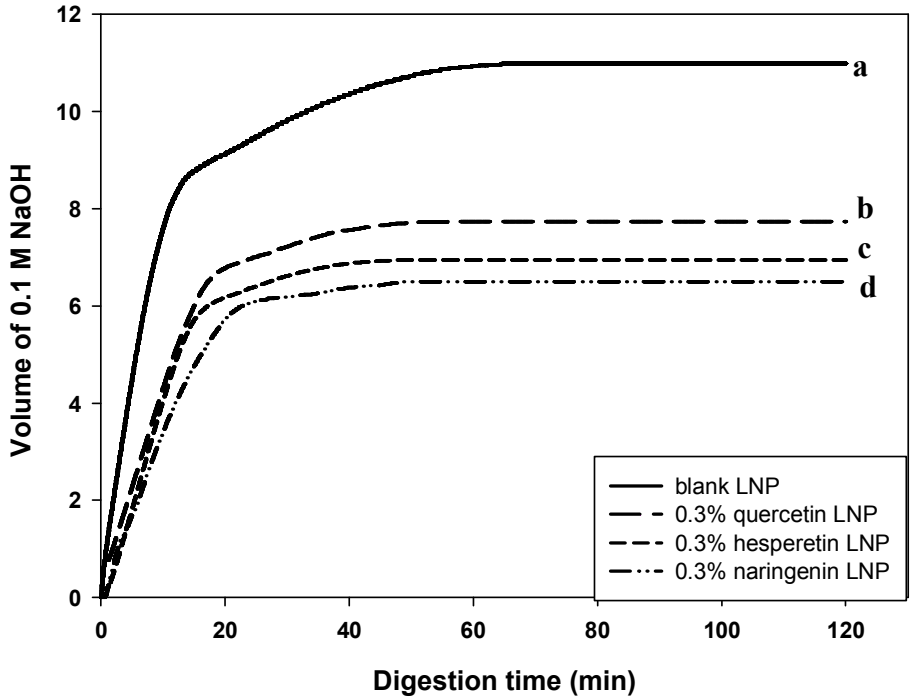

Figure 17. Titration curves of (a) blank LNP, (b) 0.3 wt% quercetin LNP, (c) 0.3 wt% hesperetin LNP, and (d) 0.3 wt% naringenin LNP by 0.1 M NaOH solution.

IV. CONCLUSIONS

In this study, the stable LNP system for incorporating quercetin, naringenin, and hesperetin was developed. RSM was used to optimize the components of the liquid lipid type, the liquid lipid content in lipid phase, and ratio of Tween 20 and soybean lecithin. The best stable LNP was obtained at the condition of the blended lipid phase (30 wt% squalene + 70 wt% FHCO) and the emulsifier mixture (Tween 20 : soybean lecithin =7 : 13). Flavonoids loaded LNPs were prepared at the optimum formulation. Physicochemical characteristics (yield, particle size, PDI value, and ζ-potential) of 0–0.5 wt% flavonoid loaded LNP systems prepared with the optimum formulation suggested that all systems were stable. The highest EE % values were 82.8, 89.0, and 90.0% at 0.3 wt% quercetin, naringenin, and hesperetin concentration in the lipid phase, respectively, which were mainly due to their solubility in the lipid phase. For all flavonoid loaded LNPs, flavonoid molecules released from the lipid matrix to the aqueous phase medium within 12 h. In addition, these LNP systems loading flavonoids were digested by simulated intestinal juice within 60 min. In conclusion, this research could be used as the basis of further study to the functional beverages for the delivery of lipophilic bioactive molecules.

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VI. 국문초록

Quercetin, naringenin, hesperetin은 자연계에 널리 존재하는 천연 플라보노이드계 물질로 항산화, 항암, 항염효과와 같은 다양한 생리활성을 나타낸다. 그러나 이러한 물질들은 낮은 수용성으로 인해 기능성식품에 적용하는데 한계를 가진다. 이러한 단점을 극복하기 위해 다양한 전달 시스템이 주목 받고 있다. 이 중 지질나노입자는 지용성 활성물질의 생리학적 이용률을 높이기 위해 제안된 새로운 전달체계이다. 본 연구에서는 플라보노이드 물질의 가용성을 증가시키기 위하여 안정한 지질나노입자 시스템을 개발하였다. 반응표면분석법을 이용하여 지질나노입자를 구성하는 액체 기름의 종류와 함량, 유화제의 비율에 따른 지질나노입자의 물리적 특성과 안정성을 비교하는 실험을 수행하였다. 입자 크기, ζ-potential, 수율, 결정화도 등의 지표를 비교하여 지질나노입자 생산의 최적 조건을 도출하였다. 최적 조건의 지질나노입자는 스쿠알렌 30% 와 경화카놀라유 70%로 이루어진 기름으로, 35% Tween 20과 65%의 대두 레시틴을 포함한 수용상으로 만들어 진다. 최적 지질나노입자 조건을 이용하여 위의 세가지 플라보노이드 물질을 포집하는 연구를 진행하였다. 0.1–0.5%의 플라보노이드 물질을 포함한 지질나노입자는 94%

이상의 높은 수율과 평균 150 nm의 작은 입자 크기를 가지며 -40 mV 이하의 안정적인 ζ-potential을 나타냈다. 또한 각각의 플라보노이드 물질을 포집한 지질나노입자는 0.3% 농도에서 가장 높은 포집능(EE %)을 나타내었고, 이 농도에서 quercetin 함유 지질나노입자는 82.8%, naringenin 함유 지질나노입자는 89.0%, hesperetin 함유 지질나노입자는 90.0%의 포집능을 나타냈다. 이러한 포집능의 차이는 각 물질의 지질에 대한 용해도 차이에서 기인한다. 또한 플라보노이드 물질을 함유한 지질나노입자는 인공장액에 의해 60분 안에 소화되었으며, 지질 내에 있는 플라보노이드 물질은 37ºC의 50%(v/v)의 에탄올 환경에서 12시간 이내에 외부환경으로 빠져 나왔다. 본 연구를 통해 개발된 지질나노입자는 지용성생리활성 물질을 포집하고 전달할 수 있는 유용한 시스템이라는 것을 증명하였고, 이를 바탕으로 플라보노이드 및 다양한 지용성 활성 성분을 포집하는 시스템 연구의 기초가 될 것으로 기대된다.

주요어: quercetin, naringenin, hesperetin, 지질나노입자, 경화카놀라유(FHCO), 스쿠알렌, 대두 레시틴.