# CHAPTER IV
# RESULTS AND DISCUSSION

## 1. Preparation of lipid emulsions

The method of preparation is one of the important factors that influence the stability of emulsion. There are four steps of lipid emulsion preparation (Cuéllar *et al.*, 2005). In the first step, the oil and aqueous phases are conditioned by addition to both phases of components such emulsifier, glycerol and other components that are oil or water soluble. The emulsion is prepared in the second step that involves making oil-in-water coarse emulsion by combining the pre-heated aqueous and oil phase. Then the mixture is stirred by means of a high-speed mechanical mixer in order to achieve a fine emulsion with droplet size distribution of 1-5 μm. In the third step, the fine emulsion is homogenized by using a high pressure homogenizer. In the final step, the emulsion, after pH adjustment, is bottled, sterilized and stored at the recommended temperature.

### 1.1 Preparation of coarse emulsions

The coarse emulsions were prepared using egg phospholipid as main emulsifier. Generally, phospholipid used in the commercial lipid emulsion is either obtained from animal (egg) or vegetable (soybean) but the most frequently used emulsifier in commercial lipid emulsion is egg phospholipids because it is less toxic upon administration compared with synthetic emulsifiers (Hansrani, Davis and Groves, 1983; Nagasaka and Ishii, 2001; Nielloud and Marti-Mestres, 2000;). In this part, coarse emulsions were prepared using 10% purified soybean oil, 1.2% egg

phospholipid (EPC, Lipoid E80), which contain at least 80% of phosphatidylcholine, and glycerol for tonicity adjustment. The effect of cosurfactant on emulsion formation was also studied by adding sodium oleate, polyoxyethylene-sorbitan monooleate (Tween® 80) and *d*-α-tocopheryl polyethyleneglycol-1000-succinate (Vitamin E-TPGS). The details of compositions of each formulation are in Table 3. The coarse emulsions were prepared by using a high speed homogenizer in the range of 10,000 to 18,000 rpm for 5 and 10 minutes. The sample was then visually observed for the emulsion formation and the stability after storage for 24 hours at room temperature. The physical appearance of coarse emulsions was shown in Table 5.

**Table 5.** Physical appearance of coarse emulsions after 24 hours at room temperature.

| Homogenization | | Physical appearance of lipid emulsions | | | |
|---|---|---|---|---|---|
| Speed (rpm) | Time (min) | Rx1<br>1.2% Lipoid E80 | Rx5<br>1.2% Lipoid E80<br>0.9% Tween® 80 | Rx8<br>1.2% Lipoid E80<br>1.2% Vitamin E-TPGS | Rx13<br>1.2% Lipoid E80<br>0.03% Sodium oleate |
| 10,000 | 5 | 7.5% oil phase separation<br>3.2% water phase separation | 2.1% water phase separation | 4% water phase separation | 8.9% oil phase separation |
| | 10 | 1.1% oil phase separation<br>2.1% water phase separation | 2.2% water phase separation | 4% water phase separation | 10.6% creaming |
| 12,000 | 5 | 3.2% oil phase separation | 5.4% creaming | 3.3% creaming | 7.8% creaming |
| | 10 | 1% oil phase separation | 4.3% creaming | 2.2% creaming | 8.0% creaming |
| 15,000 | 5 | 2.1% oil phase separation | 3.3% creaming | 3.3% creaming | 6.4% creaming |
| | 10 | 13.0% creaming | 3.2% creaming | 3.3% creaming | 6.4% creaming |
| 18,000 | 5 | 1.1% oil phase separation<br>8.5% creaming | 2.2% creaming | 2.2%creaming | 1.1% oil phase separation<br>6.4% creaming |
| | 10 | 1.1% oil phase separation<br>8.5% creaming | 2.2% creaming | 2.2% creaming | 1.1% oil phase separation<br>6.4% creaming |

The results revealed that all formulations were unstable after 24 hours. At the homogenization speed of 10,000 rpm at either 5 or 10 minutes, the phase separation occurred in all formulations with the exception of Rx13, which showed creaming when the homogenization time was 10 minutes. When the speed of homogenization ranging from 12,000 to 18,000 rpm was used, all formulations showed only creaming or a very thin oil layer on the top of the surface, which became emulsion again by shaking or stirring. The instability of coarse emulsion might be due to the insufficient input energy necessary for emulsion formation. The smaller emulsion droplet could be achieved by passing the coarse emulsion through a high pressure homogenizer. The homogenization speed of 12,000 rpm for 5 minutes was selected for further preparation of coarse emulsion because of lower energy requirement.

### 1.2 Preparation of lipid emulsions by high pressure homogenization

Lipid emulsion was obtained by passing the coarse emulsion through a high pressure homogenizer. Many workers (Jumaa and Müller, 1998a; Leidtke *et al.*, 2000) studied the effect of homogenization pressure and number of recycling that influenced on the physicochemical properties of emulsions. In this study, the effect of homogenization cycle and pressure was also considered. Moreover, the different model of homogenizer was studied.

#### 1.2.1 Effect of homogenization cycle

The coarse emulsion was passed through the two models of a high pressure homogenizer, Emulsiflex C-50 and Emulsiflex C-5, at the pressure of 15,000 psi for 3, 5, 7 and 10 homogenization cycles. The measurement of particle size was investigated immediately after preparation. The size distribution of parenteral lipid

emulsion is a critical factor for patient safety because larger particles may cause embolism (Jeppsson *et al.*, 1976; Laval-Jeantet, Laval-Jeantet and Bergot, 1982). The volume weighted mean droplet size (D[4,3]) was mainly used for interpretation of particle size measurement in this study because it provides the mean value in volume which is the proportion of the oil droplets with respect to the whole internal phase volume. One more value, d (0.5), is the median diameter which means that 50% of the particles are smaller than the given size. The significant difference between particle size of emulsion passing various cycles of homogenizer was calculated using one-way ANOVA ($p<0.05$).

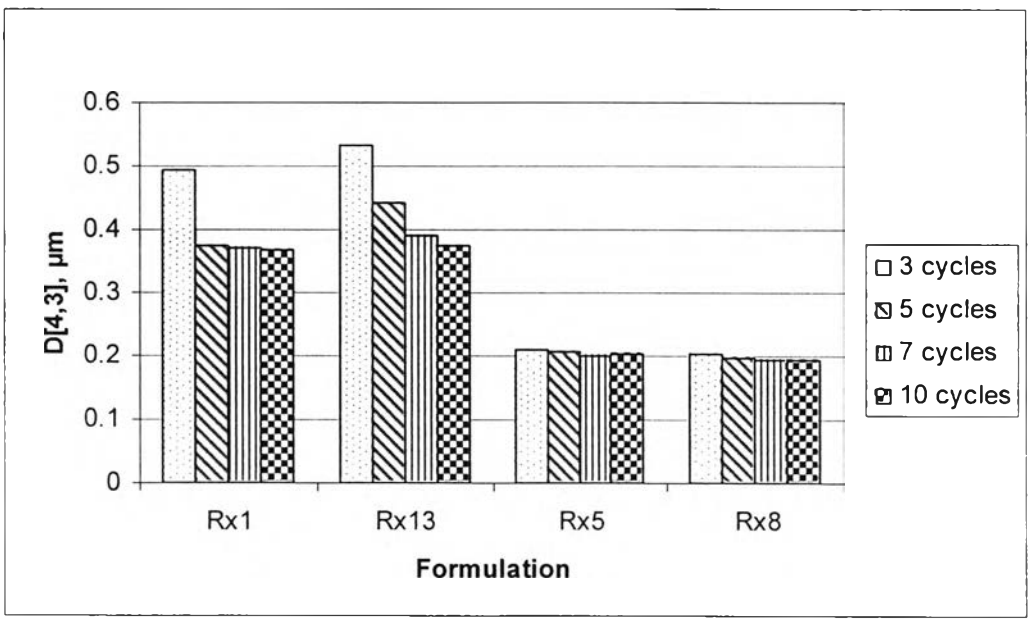


**Figure 9.** Volume weighted mean droplet size, D[4,3], of emulsions produced by Emulsiflex C-50 after 3, 5, 7 and 10 cycles at 15,000 psi (n = 3, S.D.< 0.01).

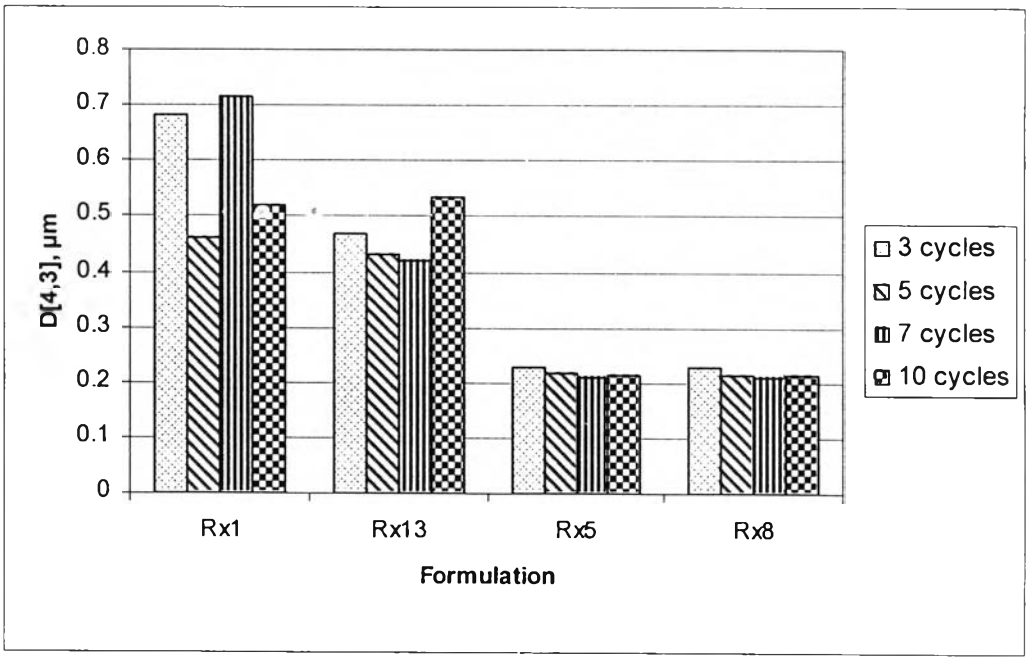


**Figure 10.** Volume weighted mean droplet size, D[4,3], of emulsion produced by Emulsiflex C-5 after 3, 5, 7 and 10 cycles at 15,000 psi (n = 3, S.D. < 0.01).

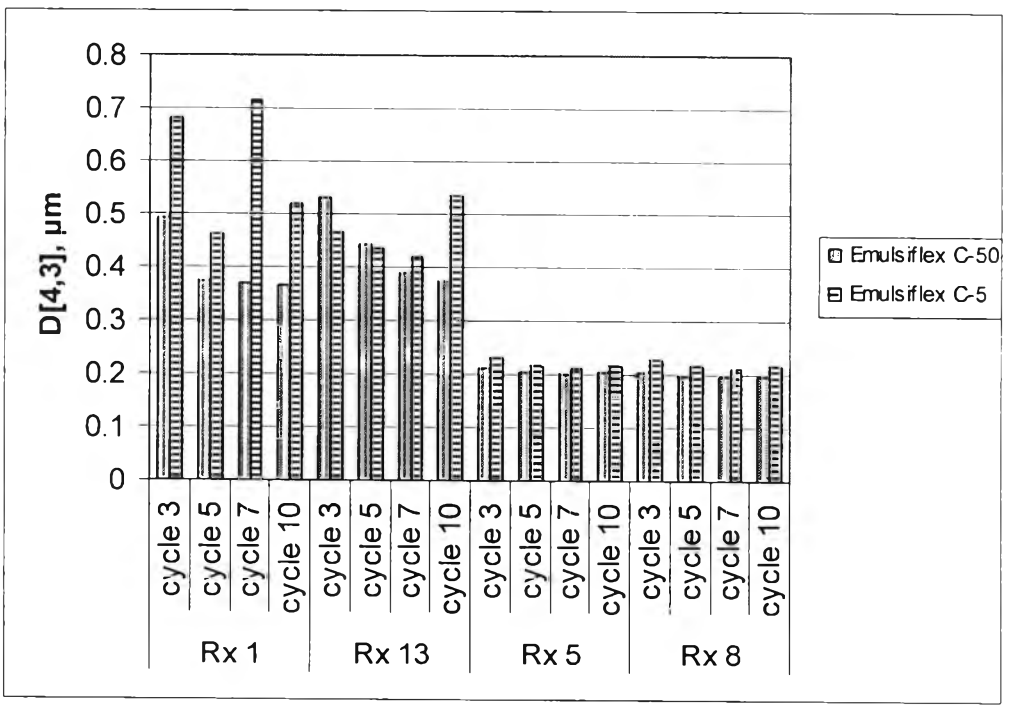


**Figure 11.** Comparison of particle size, D [4,3], of lipid emulsions produced by Emulsiflex C-50 and Emulsiflex C-5 at 15,000 psi and different homogenization cycles (n = 3, S.D. < 0.01).

The mean particle sizes, D[4,3], of Rx1 and Rx13 clearly showed the effect of recycling time (Figure 9). After being passed through the Emulsiflex C-50 for 3 cycles, the mean particle sizes of Rx1 and Rx13 were 0.493 ± 0.003 and 0.531 ± 0.005 μm, respectively. The mean particle sizes of both formulations were decreased to 0.373 ± 0.002 and 0.443 ± 0.002 μm, respectively when increased homogenization cycles from 3 to 5 cycles. The result illustrated that there were slightly decreased in the particle size of all formulations if the homogenization cycle was greater than 3. The same trend of particle size was occurred in all formulations that produced from Emulsiflex C-5 (Figure 10). Hence, the cycle number of 5 was chosen for further study. The size reduction after recycling the emulsion through the homogenizer was similar to that obtained from the study of Trotta, Pattarino and Ignoni (2002) which demonstrated that the higher cycle number was necessary in order to achieve fine particles. In addition, the particle sizes of lipid emulsion that produced from Emulsiflex C-50 and Emulsiflex C-5 were significantly different (Figure 11). The emulsions produced by Emulsiflex C-50 were smaller than those produced by Emulsiflex C-5 also with narrow particle size distribution (see appendix A). Normally, the batch size production of Emulsifilex C-50 claimed by manufacturer is larger than that of Emulsiflex C-5 with a capacity of emulsion production of 15-50 L/hr and 1-5 L/hr for Emulsiflex C-50 and Emulsiflex C-5, respectively. It was noticed that in this study Emulsiflex C-50 gave a stable pressure through the whole process, in contrary to Emulsiflex C-5 which its pressure rapidly drops when the small sample left in the sample tank These followed the review of Alison (1999) that the equipment changes during scale-up could affect the physical and chemical stability of the emulsion. Furthermore, Liedtke *et al.* (2000) illustrated the difference

in particle size of emulsion produced by different homogenization machine Emulsiflex C-3 (High pressure homogenizer of Avestin Co.) and Micron Lab 40 (Microfluidizer of Microfluidics Co.). Figures 12-14 showed the same trend of particle size, d(0.5), of emulsions effected by the recycling times.

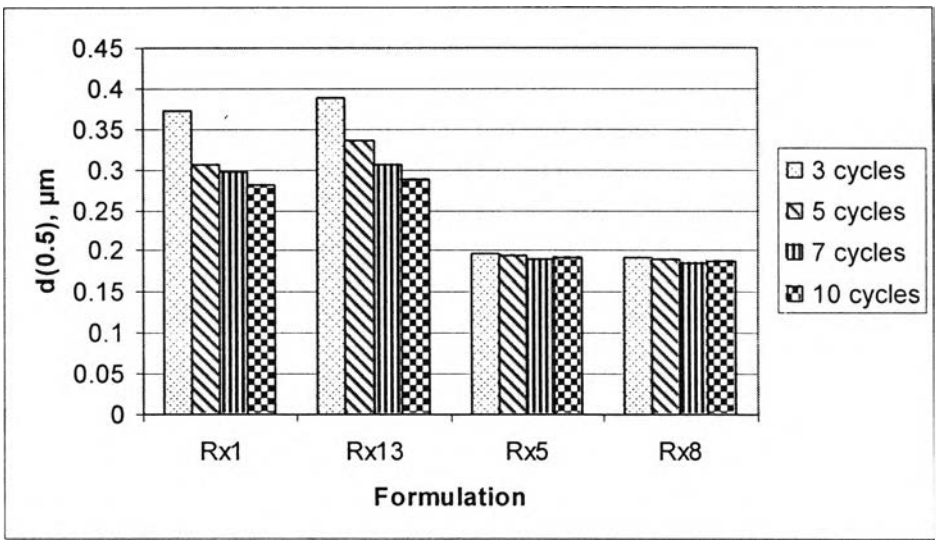


**Figure 12.** The mean diameter, d (0.5), of lipid emulsions produced by Emulsiflex C-50 after 3, 5, 7 and 10 cycles at 15,000 psi (n = 3, S.D. < 0.01).

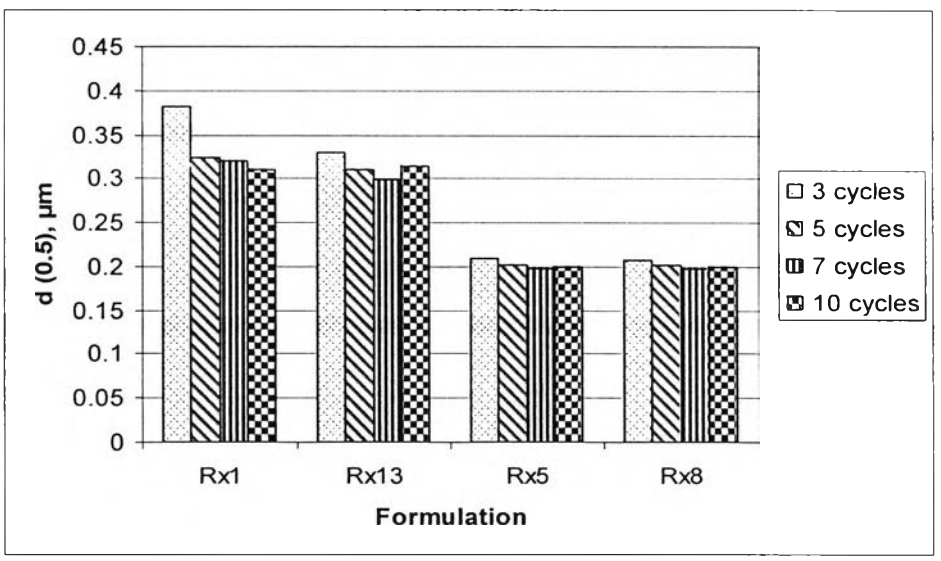


**Figure 13.** The mean diameter, d (0.5), of lipid emulsion produced by Emulsiflex C-5 after 3, 5, 7 and 10 cycles at 15,000 psi (n = 3, S.D. < 0.01).

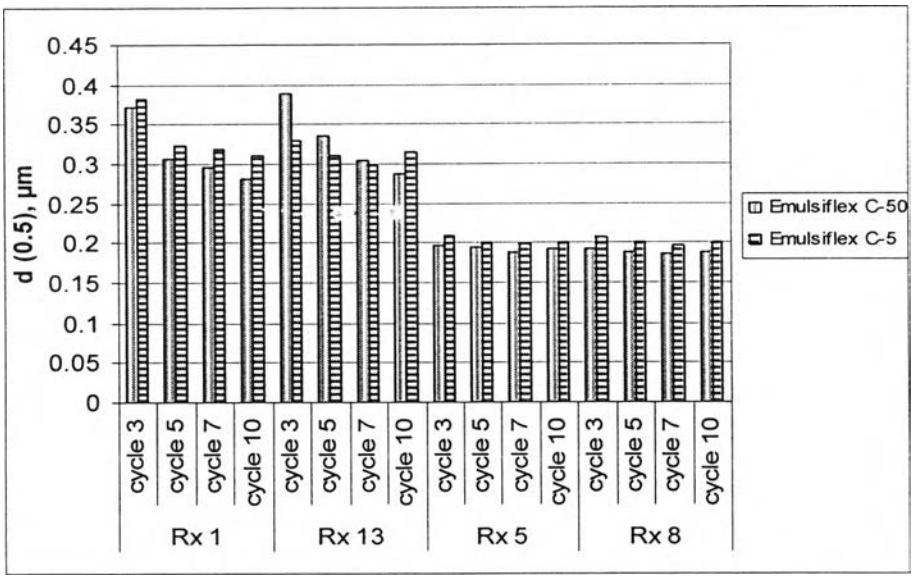


**Figure 14.** Comparison of the mean diameter, d (0.5), of lipid emulsions produced by Emulsiflex C-50 and Emulsiflex C-5 at 15,000 psi and different homogenization cycles (n = 3, S.D. < 0.01).

### 1.2.2 Effect of homogenization pressure

The coarse emulsion was passed through a homogenizer at the pressures of 10,000, 15,000 and 20,000 psi and at the number of 5 cycles. The measurement of particle size was investigated immediately after preparation. The significant difference between particle size of emulsion passing various pressure of homogenizer was calculated using one-way ANOVA ($p<0.05$).

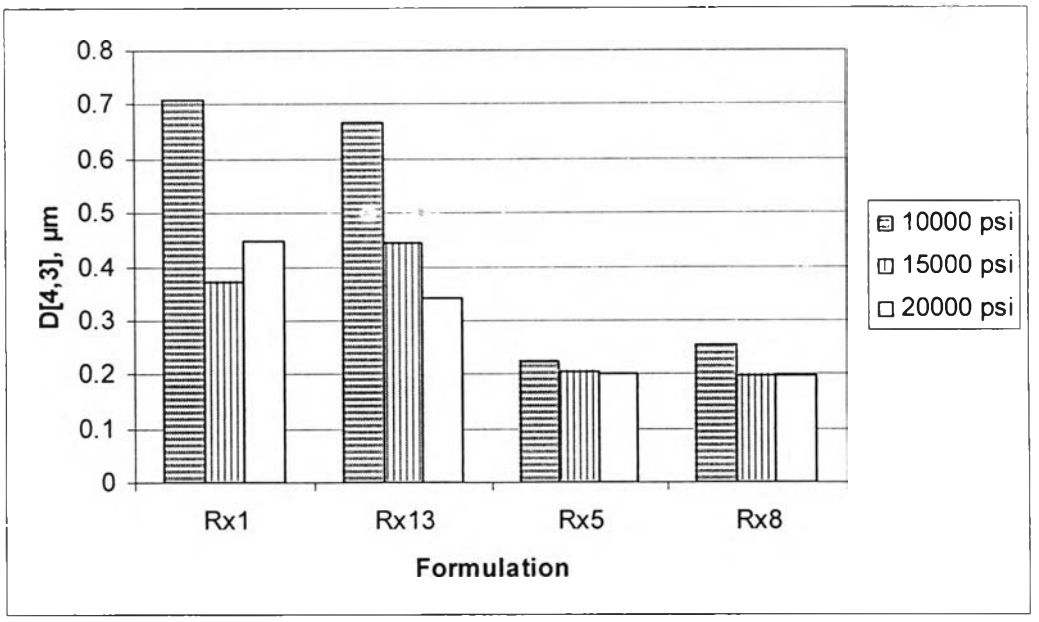


**Figure 15.** Volume weighted mean droplet size, D[4,3], of emulsions produced by Emulsiflex C-50 after 5 cycles at different homogenization pressures (n = 3, S.D.<0.01).

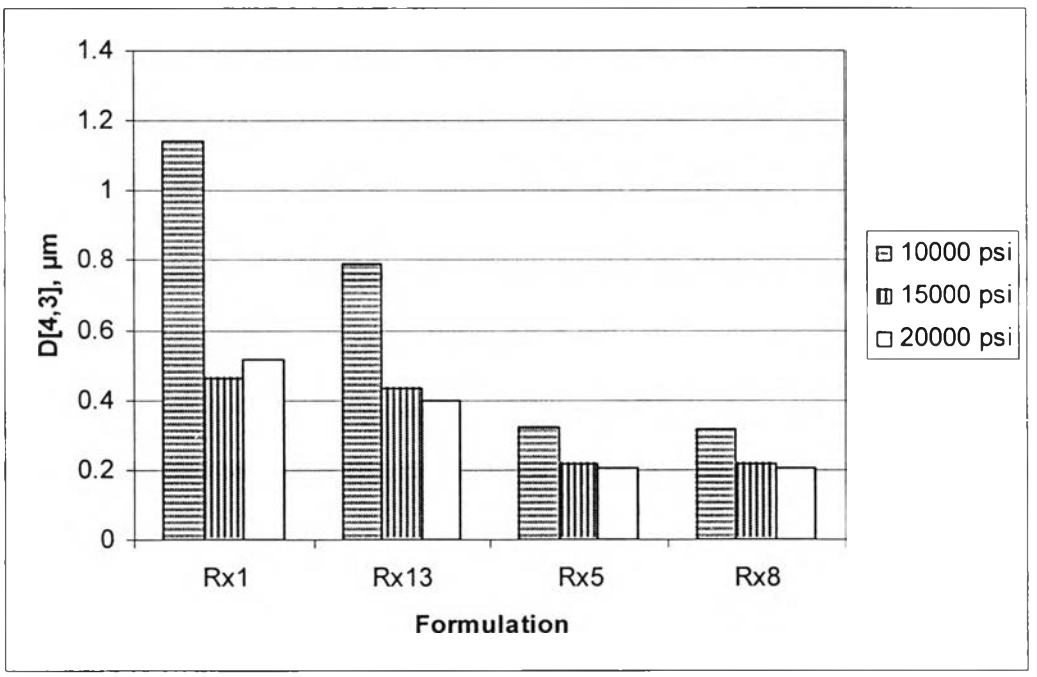


**Figure 16.** Volume weighted mean droplet size, D[4,3], of emulsions produced by Emulsiflex C-5 after 5 cycles at different homogenization pressures (n = 3, S.D.<0.01).

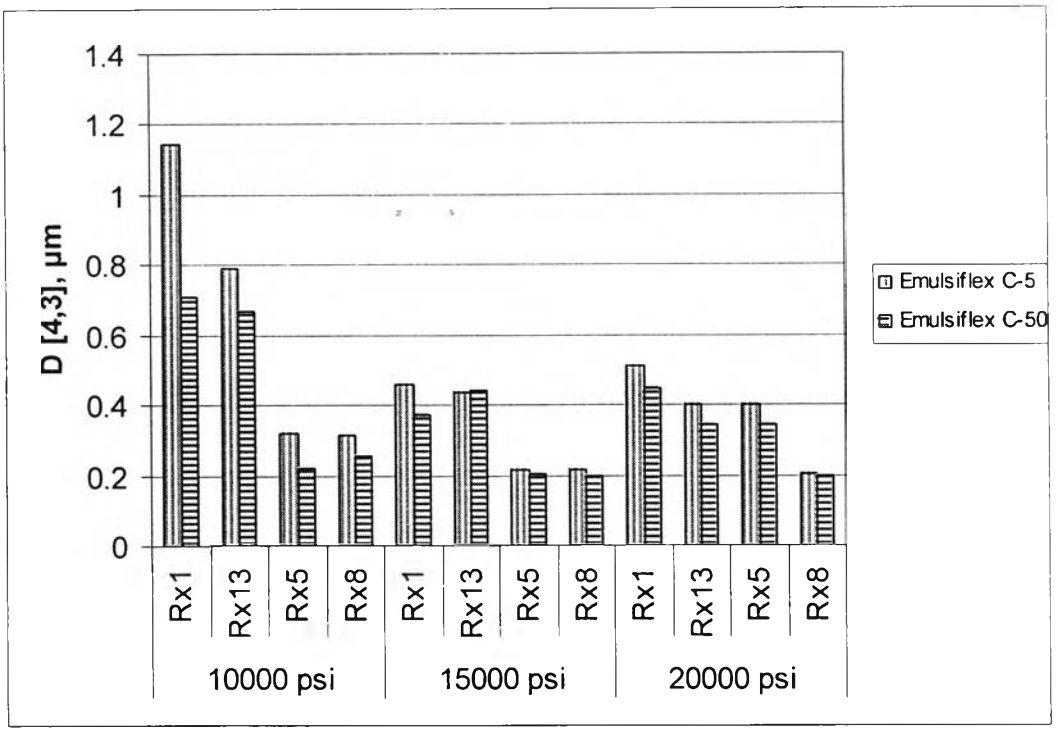


**Figure 17.** Comparison of particle size D[4,3] of emulsions produced from Emulsiflex C-50 and Emulsiflex C-5 after 5 cycles at different homogenization pressures (n = 3, S.D.<0.01).

The influence of homogenization pressure on the particle size was similar to the number of homogenization cycles. The higher homogenization pressure exhibited the small particle size of emulsions produced by either Emulsiflex C-50 or Emulsiflex C-5 (Figures 15-17). Furthermore, the particles produced by Emulsiflex C-50 were smaller than those produced by Emulsiflex C-5. There was slight difference in particle size between the pressure of 15,000 and 20,000 psi, so the pressure of 15,000 psi was selected for further experiments. The optimum homogenization pressure of 15,000 psi and 5 homogenization cycles led to a significant decrease in particle size, especially in Rx1 and Rx13, regardless of different models of high pressure homogenizer. The mean diameter, d (0.5), of the particles were followed the same trends of D[4,3]. (Figures 18-20).

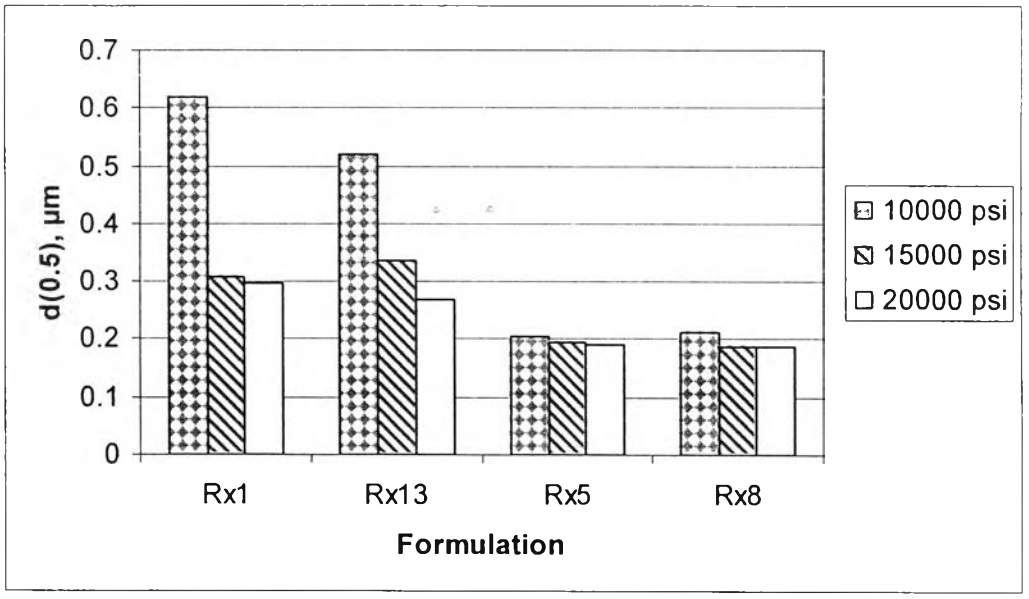


**Figure 18.** The mean diameter, d (0.5), of lipid emulsions produced by Emulsiflex C-50 after 5 cycles at different homogenization pressures (n = 3, S.D.<0.01).

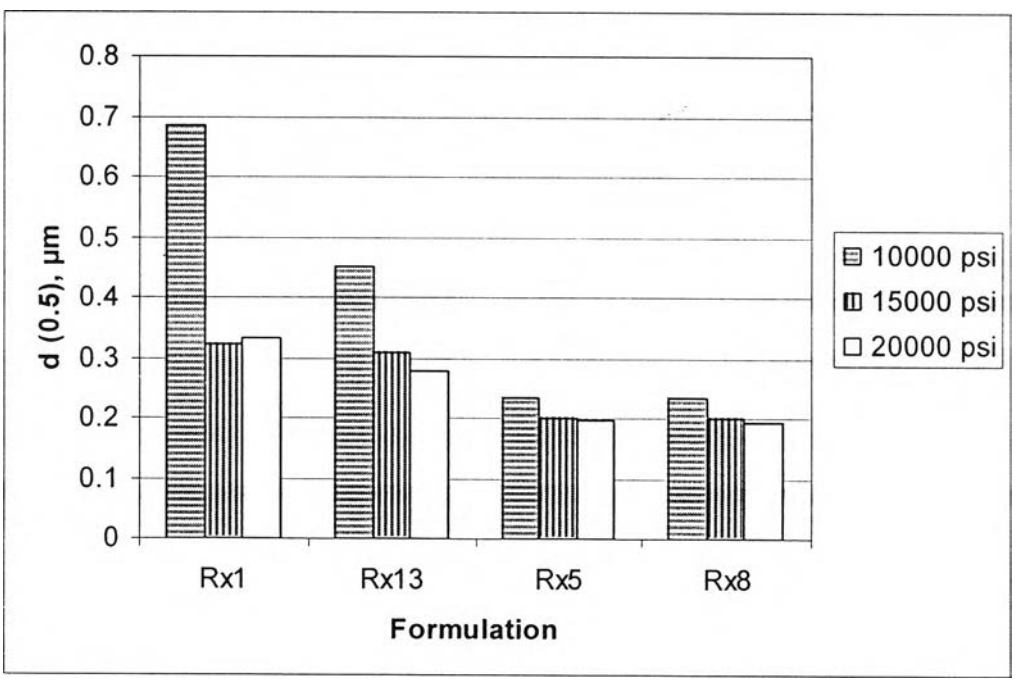


**Figure 19.** The mean diameter, d (0.5), of lipid emulsions produced by Emulsiflex C-5 after 5 cycles at different homogenization pressure (n = 3, S.D.<0.01).

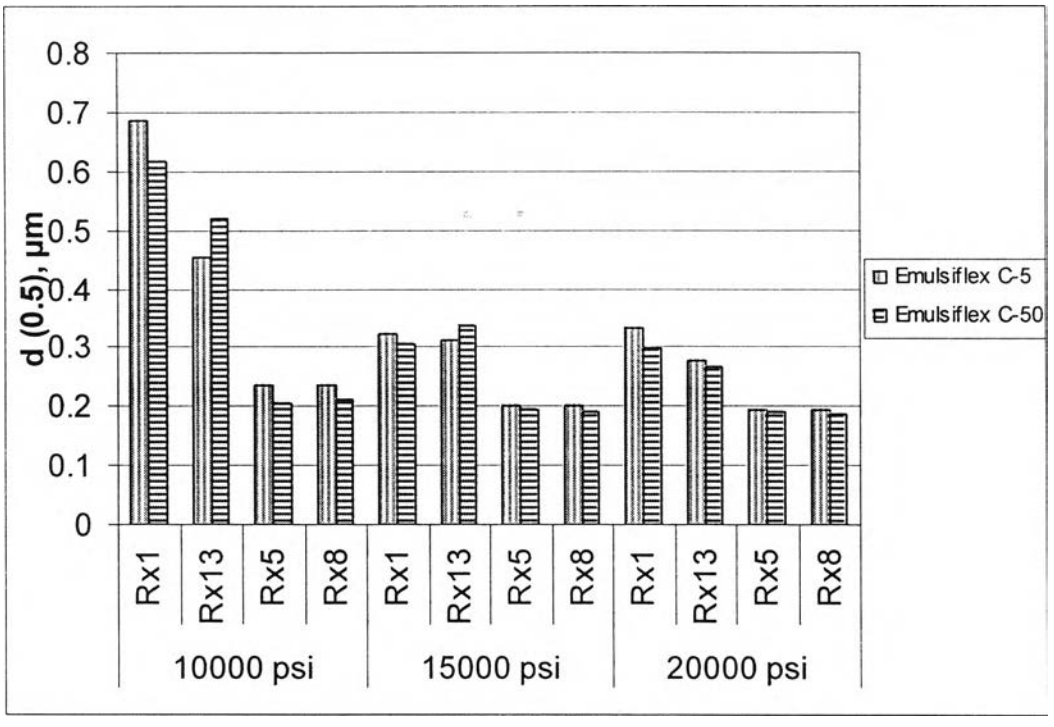


**Figure 20.** Comparison of the mean diameter, d (0.5) of lipid emulsions produced from Emulsiflex C-50 and Emulsiflex C-5 after 5 cycles at different homogenization pressures (n = 3, S.D.<0.01).

Many researchers studied the effect of homogenization pressure and cycle on the particle size of emulsions. For example, Jumaa and Müller (1998a) revealed that the particle size of lipid emulsion could be reduced by using the pressure between 30 and 35 Mpa at eight cycles which leads to a decrease in D99 values from 4.5 to 1.35 µm. The D99 value is the volume diameter 99% obtained from LD, that means 99% of the particles are below the given size.

**2. Study on the emulsion compositions**

The emulsion was prepared by using the high speed homogenizer at 12,000 rpm for 5 minutes then it was passed through Emulsiflex C-50 at the pressure of 15,000 psi for 5 cycles, the condition obtained from 1.1 and 1.2. The effect of

autoclaving and types of cosurfactant on the physicochemical properties and stability was investigated. The appropriate amounts of three types of cosurfactant, sodium oleate, Tween®80 and Vitamin E-TPGS were studied.

### 2.1 Effect of autoclaving

Autoclaving is generally a necessary process to sterilize lipid emulsions, as sterile filtration cannot be used due to the large particle size of lipid emulsions (Jumaa and Müller, 1999). Lipid emulsions, therefore, should display sufficient stability against this stress process if a suitable formulation is to be achieved (Hansrani, Davis and Groves, 1983).

A series of emulsions (Rx 1-22) containing different types of cosurfactant were prepared using different amounts of oil. The emulsions were examined immediately before and after autoclaving at 121° C for 15 minutes.

From the experiments, it was found that a slight change in the white color of emulsion to soymilk-like emulsion after autoclaving was observed in Rx 1, 2, 3, 18 and 22. There was no sign of instability observed in all systems both before and after autoclaving except for Rx 7 and Rx 15 which were creaming after autoclaving. Both formulations contained 1.2% EPC as a major emulsifier blended with 0.4% Tween®80 (Rx 7) and 0.015% sodium oleate (Rx 15). This instability occurred may be due to the improper amount of surfactant. As the results shown in Table 6, the particle sizes of Rx 1, 2, 3, 18 and 22 were increased. This was similar to the study of Jumaa and Müller (1999) that showed the change in particle size of lipid emulsion after autoclaving. Rx 1-3 contained only EPC as an emulsifier which can be hydrolysed to free fatty acid and lysophospholipids (LPL) during the autoclaving

process (Rabinovich-Guilatt *et al.*, 2005). The two main chemical degradation pathways of the phospholipids are oxidation and hydrolysis (Grit and Crommelin, 1993). While the first degradation pathway can be minimized by the addition of antioxidants or the removal of oxygen, hydrolysis of the ester functionalities in presence of water is virtually unavoidable. As a result, the interfacial film of phospholipids was not strong enough to prevent droplet coalescence upon the thermic process. Furthermore, by increasing the amount of cosurfactant, the results illustrated no change in mean particle size with the exception of Rx 7, 15, 18 and 22. There was a creaming layer occurred in Rx 7 and Rx 15 while Rx 18 and Rx 22 had a significant change in mean particle size. This phenomenon occurred possibly due to the small amount of cosurfactant which is not enough to form the strong interfacial film. An increase in concentration of cosurfactant could lead to effectively form a strong and stable interfacial film between phospholipids and cosurfactant. Jumaa and Müller (1998b) suggested that nonionic surfactant, i.e., Cremophor® EL, Poloxamer® 188, Solutol® HS15 and Tween® 80 were usually combined with phospholipids to improve the stability of the surfactant layer. A close-packed film was obtained by combination of emulsifiers which conferred steric stability to the dispersed droplets.

Moreover, the ability of surfactant molecules to give the necessary curvature of the interfacial film required to form fine emulsions has been related to the packing geometry, which is the ratio between hydrocarbon volume, optimum head group area and tail length of the molecule at the interface. Phospholipids has a packing parameter around 0.8 and this value is further increased if the oil phase penetrates into the alkyl chains of the phospholipids molecule. In order to produce fine oil-in-water emulsions, it is necessary to reduce this parameter by using cosurfactants, thus

allowing the interfacial film sufficient flexibility to take up the curvature required to form fine emulsions (Trotta, Pattarino and Ignoni, 2002). The cosurfactants used in this experiments is more hydrophilic than phospholipids, incorporation of this cosurfactant at the oil-water interface could form a mixed monolayer with phospholipids. The flexibility of this mixed film is greater than that of phospholipids, because the different structures of the two molecules prevent close packing at the interface.

**Table 6.** Particle size of Rx1-22 before and after autoclaving. ( n = 3, S.D.<0.01).

| Formulation | Oil (%) | Emulsifier | Particle size (μm) Before autoclaving | | Particle size (μm) After autoclaving | |
|---|---|---|---|---|---|---|
| | | | D [4,3] | d (0.5) | D [4,3] | d (0.5) |
| Rx1 | 10 | 1.2% EPC | 0.357 | 0.286 | 0.388 | 0.323 |
| Rx2 | | 1.5% EPC | 0.330 | 0.276 | 0.400 | 0.345 |
| Rx3 | | 2.0% EPC | 0.372 | 0.299 | 0.486 | 0.401 |
| Rx4 | | 1.2% EPC + 1.2% Tween® 80 | 0.193 | 0.185 | 0.193 | 0.184 |
| Rx5 | | 1.2% EPC + 0.9% Tween® 80 | 0.195 | 0.186 | 0.193 | 0.185 |
| Rx6 | | 1.2% EPC + 0.6% Tween® 80 | 0.198 | 0.189 | 0.199 | 0.189 |
| Rx7 | | 1.2% EPC + 0.4% Tween® 80 | 0.201 | 0.191 | creaming | creaming |
| Rx8 | | 1.2% EPC + 1.2% Vit E-TPGS | 0.191 | 0.183 | 0.191 | 0.183 |
| Rx9 | | 1.2% EPC + 0.9% Vit E-TPGS | 0.194 | 0.186 | 0.194 | 0.186 |
| Rx10 | | 1.2% EPC + 0.6% Vit E-TPGS | 0.196 | 0.187 | 0.195 | 0.187 |
| Rx11 | | 1.2% EPC + 0.4% Vit E-TPGS | 0.203 | 0.192 | 0.222 | 0.207 |
| Rx12 | | 1.2% EPC + 0.06% sodium oleate | 0.344 | 0.278 | 0.330 | 0.271 |
| Rx13 | | 1.2% EPC + 0.03% sodium oleate | 0.319 | 0.276 | 0.314 | 0.279 |
| Rx14 | | 1.2% EPC + 0.02% sodium oleate | 0.505 | 0.306 | 0.387 | 0.302 |
| Rx15 | | 1.2% EPC + 0.015% sodium oleate | 0.376 | 0.306 | creaming | creaming |
| Rx16 | 20 | 1.2% EPC + 1.2% Tween® 80 | 0.204 | 0.194 | 0.205 | 0.194 |
| Rx17 | | 1.2% EPC + 0.9% Tween® 80 | 0.212 | 0.199 | 0.211 | 0.199 |
| Rx18 | | 1.2% EPC + 0.6% Tween® 80 | 0.224 | 0.207 | 0.305 | 0.291 |
| Rx19 | | 1.2% EPC + 1.2% Vit E-TPGS | 0.205 | 0.194 | 0.206 | 0.194 |
| Rx20 | | 1.2% EPC + 0.9% Vit E-TPGS | 0.213 | 0.200 | 0.214 | 0.200 |
| Rx21 | | 1.2% EPC + 0.6% Vit E-TPGS | 0.230 | 0.213 | 0.230 | 0.212 |
| Rx22 | | 1.2% EPC + 0.4% Vit E-TPGS | 0.251 | 0.231 | 0.358 | 0.332 |

The effect of autoclaving can be seen not only in particle size but in other physicochemical parameters such as pH and surface charge of the droplet.

**Table 7.** Zeta potential of Rx1-22 before and after autoclaving (mean ± S.D., n = 3).

| Formulation | Oil (%) | Emulsifier | Zeta potential (mV) | |
|---|---|---|---|---|
| | | | Before autoclaving | After autoclaving |
| Rx1 | 10 | 1.2% EPC | -37.33 ± 1.00 | -38.10 ± 0.87 |
| Rx2 | | 1.5% EPC | -38.47 ± 0.38 | -39.83 ± 1.20 |
| Rx3 | | 2.0% EPC | -38.60 ± 1.74 | -40.67 ± 0.84 |
| Rx4 | | 1.2% EPC + 1.2% Tween® 80 | -34.57 ± 0.99 | -38.23 ± 0.25 |
| Rx5 | | 1.2% EPC + 0.9% Tween® 80 | -33.93 ± 0.61 | -36.47 ± 0.06 |
| Rx6 | | 1.2% EPC + 0.6% Tween® 80 | -37.37 ± 0.87 | -40.27 ± 0.29 |
| Rx7 | | 1.2% EPC + 0.4% Tween® 80 | -37.77 ± 0.31 | creaming |
| Rx8 | | 1.2% EPC + 1.2% Vit E-TPGS | -36.00 ± 0.10 | -37.20 ± 0.40 |
| Rx9 | | 1.2% EPC + 0.9% Vit E-TPGS | -37.03 ± 0.55 | -38.77 ± 0.12 |
| Rx10 | | 1.2% EPC + 0.6% Vit E-TPGS | -35.43 ± 0.21 | -36.63 ± 0.50 |
| Rx11 | | 1.2% EPC + 0.4% Vit E-TPGS | -35.90 ± 0.10 | -38.07 ± 0.06 |
| Rx12 | | 1.2% EPC + 0.06% sodium oleate | -39.57 ± 0.35 | -39.90 ± 0.10 |
| Rx13 | | 1.2% EPC + 0.03% sodium oleate | -39.43 ± 0.25 | -39.50 ± 0.26 |
| Rx14 | | 1.2% EPC + 0.02% sodium oleate | -39.33 ± 0.12 | -39.50 ± 0.26 |
| Rx15 | | 1.2% EPC + 0.015% sodium oleate | -39.20 ± 0.36 | creaming |
| Rx16 | 20 | 1.2% EPC + 1.2% Tween® 80 | -40.30 ± 3.52 | -43.33 ± 0.12 |
| Rx17 | | 1.2% EPC + 0.9% Tween® 80 | -41.23 ± 1.39 | -44.60 ±0.10 |
| Rx18 | | 1.2% EPC + 0.6% Tween® 80 | -43.00 ± 0.10 | -44.80 ± 0.46 |
| Rx19 | | 1.2% EPC + 1.2% Vit E-TPGS | -36.47 ± 2.50 | -39.13 ± 0.76 |
| Rx20 | | 1.2% EPC + 0.9% Vit E-TPGS | -38.33 ± 1.60 | -39.87 ± 0.97 |
| Rx21 | | 1.2% EPC + 0.6% Vit E-TPGS | -38.33 ± 1.42 | -40.27 ± 1.12 |
| Rx22 | | 1.2% EPC + 0.4% Vit E-TPGS | -37.90 ± 1.25 | -39.30 ± 0.95 |

**Table 8.** pH of Rx1-Rx22 before and after autoclaving (Mean ± SD, n = 3)

| Formulation | Oil (%) | Emulsifier | pH | |
|---|---|---|---|---|
| | | | Before autoclaving | after autoclaving |
| Rx1 | 10 | 1.2% EPC | 8.05 ± 0.02 | 6.12 ± 0.17 |
| Rx2 | | 1.5% EPC | 8.04 ± 0.02 | 5.66 ± 0.07 |
| Rx3 | | 2.0% EPC | 8.04 ± 0.04 | 5.34 ± 0.07 |
| Rx4 | | 1.2% EPC + 1.2% Tween® 80 | 8.03 ± 0.01 | 7.07 ± 0.13 |
| Rx5 | | 1.2% EPC + 0.9% Tween® 80 | 8.03 ± 0.02 | 6.85 ± 0.07 |
| Rx6 | | 1.2% EPC + 0.6% Tween® 80 | 8.08 ± 0.02 | 7.24 ± 0.01 |
| Rx7 | | 1.2% EPC + 0.4% Tween® 80 | 8.01 ± 0.02 | creaming |
| Rx8 | | 1.2% EPC + 1.2% Vit E-TPGS | 8.08 ± 0.06 | 6.89 ± 0.10 |
| Rx9 | | 1.2% EPC + 0.9% Vit E-TPGS | 8.08 ± 0.05 | 6.74 ± 0.03 |
| Rx10 | | 1.2% EPC + 0.6% Vit E-TPGS | 8.02 ± 0.01 | 6.84 ± 0.07 |
| Rx11 | | 1.2% EPC + 0.4% Vit E-TPGS | 8.05 ± 0.01 | 6.67 ± 0.02 |
| Rx12 | | 1.2% EPC + 0.06% sodium oleate | 8.01 ± 0.02 | 7.91 ± 0.05 |
| Rx13 | | 1.2% EPC + 0.03% sodium oleate | 8.04 ± 0.01 | 7.37 ± 0.07 |
| Rx14 | | 1.2% EPC + 0.02% sodium oleate | 8.05 ± 0.02 | 7.40 ± 0.06 |
| Rx15 | | 1.2% EPC + 0.015% sodium oleate | 8.04 ± 0.02 | creaming |
| Rx16 | 20 | 1.2% EPC + 1.2% Tween® 80 | 8.03 ± 0.02 | 6.84 ± 0.09 |
| Rx17 | | 1.2% EPC + 0.9% Tween® 80 | 8.05 ± 0.02 | 6.73 ± 0.06 |
| Rx18 | | 1.2% EPC + 0.6% Tween® 80 | 8.05 ± 0.02 | 6.63 ± 0.11 |
| Rx19 | | 1.2% EPC + 1.2% Vit E-TPGS | 8.05 ± 0.01 | 6.67 ± 0.04 |
| Rx20 | | 1.2% EPC + 0.9% Vit E-TPGS | 8.06 ± 0.01 | 6.68 ± 0.01 |
| Rx21 | | 1.2% EPC + 0.6% Vit E-TPGS | 8.06 ± 0.01 | 6.97 ±0.10 |
| Rx22 | | 1.2% EPC + 0.4% Vit E-TPGS | 8.04 ± 0.03 | 6.76 ± 0.05 |

Tables 7 and 8 illustrated an increase in the negative charges of the emulsion and a decrease in pH, respectively in all formulations after autoclaving. This effect comes from the hydrolysis of phospholipids which is producing lysophospholipids and free fatty acids. It is believed that the release of lysophospholipids and free fatty acids may affect the physicochemical stability of the emulsions (Hansrani, Davis and

Groves, 1983). Rabinovich-Guilatt *et al.* (2005) also found that the zeta potential of the emulsion were -35 and -39 mV before and after autoclaving, respectively, determined by the presence of the overall negatively charged phospholipids in the EPC. The used egg phospholipids contains mainly phosphatidylcholine (PC), which is zwitterionic in form and neutral over a wide range of pH, and negative-charged phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidic acid (PA) and ionization is markedly pH dependent (Jeong, Oh and Kim, 2001).

It could be concluded that the physicochemical properties of the emulsion were changed after autoclaving. The use of optimal emulsifier could minimize the physicochemical changes leading to instability. Many investigations revealed that using a combination of emulsifiers, ionic lipids or nonionic emulsifiers, providing a synergistic effect on stability, which will be discussed in the next experiment.

### 2.2 Effect of cosurfactant

From previous study, three types of cosurfactant, Tween® 80, Vitamin E-TPGS and sodium oleate, were mixed with 1.2% w/w of EPC to improve the stability of emulsion. Tween® 80 and Vitamin E-TPGS were mixed with EPC at the weight ratios of 1:1 to 1:3, so the concentration of Tween® 80 and Vitamin E-TPGS used were ranging from 0.4% to 1.2% w/w. Sodium oleate was mixed at the weight ratios of 1:20 to 1:80. In the present study, the concentrations of sodium oleate were ranging from 0.015% to 0.06% w/w. It was mentioned that only small amount of sodium oleate was added due to its hemolytic effect (Jumaa and Müller, 2000). The

emulsions, Rx1 - Rx15, were stored at room temperature to observe their stability by determining the physicochemical properties.

### 2.2.1 Particle size

The particle sizes of stable emulsion both before and after autoclaving were determined by laser diffractometer (Mastersizer ® 2000). In order to select the suitable emulsifier system, the D[4,3] was used for comparing the droplet size in each preparation. The results are shown in Table 9.

**Table 9.** Particle size of lipid emulsions containing various emulsifiers at room temperature (Mean ± SD, n = 3).

| Rx | Emulsifier | Particle size (μm) | | | |
|---|---|---|---|---|---|
| | | unautoclaved | autoclaved | 1 week | 4 weeks |
| Rx1 | 1.2% EPC | 0.357 + 0.003 | 0.388 ± 0.006 | 0.382 ± 0.002 | creaming |
| Rx2 | 1.5% EPC | 0.330 + 0.000 | 0.400 ± 0.001 | 0.399 ± 0.001 | creaming |
| Rx3 | 2.0% EPC | 0.372 ± 0.003 | 0.486 ± 0.007 | 0.498 ± 0.017 | creaming |
| Rx4 | 1.2% EPC + 1.2% Tween® 80 | 0.193 ± 0.000 | 0.193 ± 0.001 | 0.193 ± 0.001 | 0.193 ± 0.000 |
| Rx5 | 1.2% EPC + 0.9% Tween® 80 | 0.195 ± 0.001 | 0.193 ± 0.000 | 0.193 ± 0.000 | 0.193 ± 0.000 |
| Rx6 | 1.2% EPC + 0.6% Tween® 80 | 0.198 ± 0.001 | 0.199 ± 0.001 | 0.199 ± 0.001 | 0.199 ± 0.001 |
| Rx7 | 1.2% EPC + 0.4% Tween® 80 | 0.201 ± 0.000 | creaming | creaming | creaming |
| Rx8 | 1.2% EPC + 1.2% Vit E-TPGS | 0.191 ± 0.000 | 0.191 ± 0.000 | 0.192 ± 0.000 | 0.192 ± 0.000 |
| Rx9 | 1.2% EPC + 0.9% Vit E-TPGS | 0.194 ± 0.000 | 0.194 ± 0.001 | 0.195 ± 0.000 | 0.195 ± 0.000 |
| Rx10 | 1.2% EPC + 0.6% Vit E-TPGS | 0.196 ± 0.001 | 0.195 ± 0.000 | 0.195 ± 0.001 | 0.195 ± 0.001 |
| Rx11 | 1.2% EPC + 0.4% Vit E-TPGS | 0.203 ± 0.001 | 0.222 ± 0.001 | 0.221 ± 0.003 | 0.221 ± 0.001 |
| Rx12 | 1.2% EPC + 0.06% sodium oleate | 0.344 ± 0.003 | 0.330 ± 0.011 | creaming | creaming |
| Rx13 | 1.2% EPC + 0.03% sodium oleate | 0.319 ± 0.009 | 0.314 ± 0.002 | creaming | creaming |
| Rx14 | 1.2% EPC + 0.02% sodium oleate | 0.505 ± 0.210 | 0.387 ± 0.010 | creaming | creaming |
| Rx15 | 1.2% EPC + 0.015% sodium oleate | 0.376 ± 0.001 | creaming | creaming | creaming |

The results demonstrated that the particle size of the prepared lipid emulsion containing cosurfactant was smaller than those containing EPC alone (Rx 1). Even increasing the concentration of EPC (Rx2-3), the particle size of lipid emulsion containing a mixture of surfactant were lower. As can be seen from the results, Rx6 and Rx10 which contained 1.2% EPC and either 0.6% Tween® 80 or 0.6% Vitamin E-TPGS, respectively, their particle sizes after autoclaving were dramatically dropped from 0.388 μm (autoclaved Rx1) to below 0.2 μm and their particle size distribution was narrower than emulsion containing EPC alone (see appendix A).

It is known that the emulsifier with HLB value in between 10 and 15 is considered to be appropriate for o/w emulsification (Kan *et al.*, 1999) and higher HLB is prone to create o/w emulsion and micelles simultaneously. The HLB value of the cosurfactant used are 15 for Tween® 80; 13 for Vitamin E-TPGS, and 18 for sodium oleate. The HLB value of EPC is around 9, so the HLB values of the system used here (cosurfactant plus EPC) were estimated, based on the additive rule in term of weight fraction. The HLB value of the surfactant mixture is approximately to be from 12 to 10 for Tween® 80 and Vitamin E-TPGS, respectively. For sodium oleate, the HLB value of the system is about 9.43 to 9.11 which is not appropriate for o/w emulsification process. Thus, the larger particle size of the system containing sodium oleate and EPC can be seen.

Nonionic surfactants (i.e., Tween® 80 and Vitamin E-TPGS) stabilize an emulsion by the method called steric stabilization from two forces; osmotic forces and entropic effects (Lawrence, 2004).

(i) Osmotic (solvation) forces: nonionic surfactants usually contained the polyethylene chain or hydrophilic polymer chain as the hydrophilic portions. When

two droplets come in close contact, the polymer chain would overlap and the region became more concentrate. This led to the osmotic gradient resulting in the dilution of the overlap area by water molecules and the solution forces occurred which pushed the droplets apart.

(ii) Another forces or mechanism was called " The entropic effects ". When the polymer chain overlapped, the entropy of the system was lost. This resulted in thermodynamically unfavorable condition which forces the droplets to be separated.

For the effect of storage period on the particle size, it was found that some formulations (Rx1-3) were unstable after storage for 4 weeks while Rx12-15 (EPC + sodium oleate) were creaming after storage for 1 week. However, the emulsion containing Tween® 80 and Vitamin E-TPGS (Rx4-11) remained stable after 4 weeks, exception for Rx7 which was creaming after autoclaving. These might be due to the small amount of cosurfactant added that was not enough to form the strong film around the droplet as explained previously in 2.1.

### 2.2.2 Zeta potential

The zeta potential of emulsions are mainly due to the charges of surfactant coating droplets. If an anionic surfactant is used i.e., phosphatidylglycerol, zeta potential shows negative value. Positive zeta potential can be achieved by addition of cationic surfactant i.e.,stearylamine. The emulsion can be stabilized by electrostatic repulsive force by these charged molecules. When only nonionic surfactant stabilizes an emulsion, no electrostatic charges present to stabilize droplet. Thus nonionic surfactants stabilize emulsion by steric stabilization as described before.

In this experiment, all preparations had the negative zeta potential from negative charge of some phospholipids fraction. The high value of zeta potential of more than -30 mV is desirable in most of emulsion prepared in order to ensure a high energy barrier which caused repulsion of adjacent droplets resulting in the formation of stable emulsions (Klang and Benita, 1998). Emulsifiers can stabilize the emulsion droplet not only by the formation of a mechanical barrier, but also by producing an electrical repulsive of surface charges. The surface charges of the droplets were produced by the ionization of interfacial film-forming component which showed an enormous effect when the ionic surfactants were used. The zeta potential of an emulsion droplet was dependent upon the extent of ionization of the emulsifying agent.

The results shown in Table 10 indicated that zeta potential of emulsion was changed with the storage time. In all formulations with the exception of Rx12 – 15, it was found that the negative surface charge of oil droplet increased. The zeta potential of emulsions containing EPC and anionic sodium oleate were not changed after autoclaving and consequence to showed instability after storage for 1 week. This might be due to insufficient amount of sodium oleate to form a strong film around the oil droplets. In contrast to emulsions containing nonionic surfactants, Tween® 80 and Vitamin E-TPGS, which remained stable up to 4 weeks due to an increase in zeta potential. This follows the theory that the greater the zeta potential, the more likely the suspension is to be stable. Thus, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electrostatic repulsion (Heurtault *et al.*, 2003). However, after 4-week storage, the negative zeta potential of emulsions containing Tween® 80 was lower than those containing Vitamin E-TPGS.

**Table 10.** Zeta potential of lipid emulsions containing various emulsifiers at room temperature (Mean ± SD, n = 3).

| Rx | Emulsifier | Zeta potential (mV) | | | |
|---|---|---|---|---|---|
| | | unautoclaved | autoclaved | 1 week | 4 week |
| Rx1 | 1.2% EPC | -37.33 ± 1.00 | -38.10 ± 0.87 | -40.17 ± 0.06 | creaming |
| Rx2 | 1.5% EPC | -38.47 ± 0.38 | -39.83 ± 1.20 | -39.87 ± 0.67 | creaming |
| Rx3 | 2.0% EPC | -38.60 ± 1.74 | -40.67 ± 0.84 | -40.03 ± 0.60 | creaming |
| Rx4 | 1.2% EPC + 1.2% Tween® 80 | -34.57 ± 0.99 | -38.23 ± 0.25 | -36.47 ± 0.06 | -41.53 ± 0.15 |
| Rx5 | 1.2% EPC + 0.9% Tween® 80 | -33.93 ± 0.61 | -36.47 ± 0.06 | -37.73 ± 0.81 | -41.63 ± 0.29 |
| Rx6 | 1.2% EPC + 0.6% Tween® 80 | -37.37 ± 0.87 | -40.27 ± 0.29 | -39.00 ± 0.35 | -42.50 ± 0.26 |
| Rx7 | 1.2% EPC + 0.4% Tween® 80 | -37.77 ± 0.31 | creaming | creaming | creaming |
| Rx8 | 1.2% EPC + 1.2% Vit E-TPGS | -36.00 ± 0.10 | -37.20 ± 0.40 | -35.90 ± 0.53 | -43.37 ± 0.75 |
| Rx9 | 1.2% EPC + 0.9% Vit E-TPGS | -37.03 ± 0.55 | -38.77 ± 0.12 | -37.67 ± 0.95 | -47.63 ± 0.64 |
| Rx10 | 1.2% EPC + 0.6% Vit E-TPGS | -35.43 ± 0.21 | -36.63 ± 0.50 | -36.90 ± 0.26 | -47.90 ± 0.82 |
| Rx11 | 1.2% EPC + 0.4% Vit E-TPGS | -35.90 ± 0.10 | -38.07 ± 0.06 | -36.90 ± 0.20 | -47.20 ± 1.05 |
| Rx12 | 1.2% EPC + 0.06% sodium oleate | -39.57 ± 0.35 | -39.90 ± 0.10 | creaming | creaming |
| Rx13 | 1.2% EPC + 0.03% sodium oleate | -39.43 ± 0.25 | -39.50 ± 0.26 | creaming | creaming |
| Rx14 | 1.2% EPC + 0.02% sodium oleate | -39.33 ± 0.12 | -39.50 ± 0.26 | creaming | creaming |
| Rx15 | 1.2% EPC + 0.015% sodium oleate | -39.20 ± 0.36 | creaming | creaming | creaming |

### 2.2.3 pH and osmolality

The pH values and osmolality of emulsions are shown in Tables 11 and 12.

**Table 11.** pH of lipid emulsions containing various emulsifiers at room temperature (Mean ± SD, n = 3).

| Rx | Emulsifier | pH | | | |
|---|---|---|---|---|---|
| | | unautoclaved | autoclaved | 1 week | 4 weeks |
| Rx1 | 1.2% EPC | 8.05 ± 0.02 | 6.12 ± 0.17 | 5.64 ± 0.02 | creaming |
| Rx2 | 1.5% EPC | 8.04 ± 0.02 | 5.66 ± 0.07 | 5.29 ± 0.02 | creaming |
| Rx3 | 2.0% EPC | 8.04 ± 0.04 | 5.34 ± 0.07 | 5.28 ± 0.03 | creaming |
| Rx4 | 1.2% EPC + 1.2% Tween® 80 | 8.03 ± 0.01 | 7.07 ± 0.13 | 6.67 ± 0.04 | 6.58 ± 0.04 |
| Rx5 | 1.2% EPC + 0.9% Tween® 80 | 8.03 ± 0.02 | 6.85 ± 0.07 | 6.59 ± 0.02 | 6.71 ± 0.17 |
| Rx6 | 1.2% EPC + 0.6% Tween® 80 | 8.08 ± 0.02 | 7.24 ± 0.01 | 6.92 ± 0.07 | 7.01 ± 0.08 |
| Rx7 | 1.2% EPC + 0.4% Tween® 80 | 8.01 ± 0.02 | creaming | creaming | creaming |
| Rx8 | 1.2% EPC + 1.2% Vit E-TPGS | 8.08 ± 0.06 | 6.89 ± 0.10 | 6.68 ± 0.05 | 6.68 ± 0.04 |
| Rx9 | 1.2% EPC + 0.9% Vit E-TPGS | 8.08 ± 0.05 | 6.74 ± 0.03 | 6.68 ± 0.03 | 6.62 ± 0.04 |
| Rx10 | 1.2% EPC + 0.6% Vit E-TPGS | 8.02 ± 0.01 | 6.84 ± 0.07 | 6.59 ± 0.02 | 6.23 ± 0.14 |
| Rx11 | 1.2% EPC + 0.4% Vit E-TPGS | 8.05 ± 0.01 | 6.67 ± 0.02 | 6.61 ± 0.02 | 6.51 ± 0.02 |
| Rx12 | 1.2% EPC + 0.06% sodium oleate | 8.01 ± 0.02 | 7.91 ± 0.05 | creaming | creaming |
| Rx13 | 1.2% EPC + 0.03% sodium oleate | 8.04 ± 0.01 | 7.37 ± 0.07 | creaming | creaming |
| Rx14 | 1.2% EPC + 0.02% sodium oleate | 8.05 ± 0.02 | 7.40 ± 0.06 | creaming | creaming |
| Rx15 | 1.2% EPC + 0.015% sodium oleate | 8.04 ± 0.02 | creaming | creaming | creaming |

**Table 12.** Osmolality of 10% lipid emulsions containing various emulsifiers at room temperature (Mean ± SD, n = 3).

| Rx | Emulsifier | Osmolality (mOsm/kg) | | | |
|---|---|---|---|---|---|
| | | unautoclaved | autoclaved | 1 week | 4 weeks |
| Rx1 | 1.2% EPC | 324 ± 1 | 315 ± 2 | 287 ± 6 | creaming |
| Rx2 | 1.5% EPC | 321 ± 3 | 312 ± 4 | 320 ± 19 | creaming |
| Rx3 | 2.0% EPC | 333 ± 2 | 309 ± 2 | 351 ± 1 | creaming |
| Rx4 | 1.2% EPC + 1.2% Tween® 80 | 324 ± 1 | 316 ± 5 | 340 ± 2 | 331 ± 2 |
| Rx5 | 1.2% EPC + 0.9% Tween® 80 | 320 ± 1 | 319 ± 1 | 314 ± 1 | 334 ± 1 |
| Rx6 | 1.2% EPC + 0.6% Tween® 80 | 344 ± 4 | 340 ± 1 | 359 ± 1 | 347 ± 3 |
| Rx7 | 1.2% EPC + 0.4% Tween® 80 | 313 ± 4 | creaming | creaming | creaming |
| Rx8 | 1.2% EPC + 1.2% Vit E-TPGS | 322 ± 2 | 319 ± 1 | 334 ± 5 | 324 ± 0 |
| Rx9 | 1.2% EPC + 0.9% Vit E-TPGS | 322 ± 1 | 321 ± 1 | 337 ± 5 | 333 ± 15 |
| Rx10 | 1.2% EPC + 0.6% Vit E-TPGS | 321 ± 2 | 317 ± 5 | 318 ± 0 | 320 ± 1 |
| Rx11 | 1.2% EPC + 0.4% Vit E-TPGS | 326 ± 2 | 320 ± 9 | 323 ± 1 | 324 ± 2 |
| Rx12 | 1.2% EPC + 0.06% sodium oleate | 358 ± 1 | 363 ± 2 | creaming | creaming |
| Rx13 | 1.2% EPC + 0.03% sodium oleate | 336 ± 1 | 345 ± 2 | creaming | creaming |
| Rx14 | 1.2% EPC + 0.02% sodium oleate | 331 ± 0 | 335 ± 1 | creaming | creaming |
| Rx15 | 1.2% EPC + 0.015% sodium oleate | 326 ± 1 | creaming | creaming | creaming |

The pH of all preparations were adjusted to 8.0 before autoclaving. Table 11 shows that after autoclaving the pH of all formulations slowly decreased with time to weakly acidic. The lowest pH, 5.28 ± 0.03 was found in formulation containing 2.0% EPC. It was possibly due to the hydrolysis of some lipid in the emulsions leading to the formation of free fatty acids which gradually reduced the pH of the system (Hansrani, Davis and Groves, 1983; Herman and Groves, 1992).

The osmolality of all emulsions examined were rather constant with a period of time. The results are shown in Table 12. All preparations had osmolality between 287-363 mOsm/kg. The osmolality of emulsions containing either EPC alone or EPC blended with Vitamin E-TPGS or Tween® 80 was slightly lower than

those containing EPC with sodium oleate. The osmolality seemed to be independent on the storage time. The range of osmolality values of the 10% and 20% commercial parenteral lipid emulsions were in between 290-330 mOsm/kg (see appendix C). It could imply that the osmolality of the formulations in this study were in the same range of the commercial products.

### 2.3 Effect of oil concentration

From the previous studies, the emulsions containing cosurfactants i.e., Tween® 80 and Vitamin E-TPGS, showed better physicochemical properties and physical stability than those containing EPC alone or EPC mixed with sodium oleate. Thus, they were chosen to study the influence of oil concentration. The weight ratio of EPC to Tween® 80 was varied from 1:1 to 2:1 and the weight ratio of EPC to Vitamin E-TPGS was varied from 1:1 to 3:1 while the amount of oil was increased up to 20% w/w. The emulsion preparation was followed the previous experiment. The stability and physicochemical properties of emulsion were determined immediately after preparation and being kept for 1 week and 4 weeks. The significant difference between physicochemical properties of emulsions were calculated using one-way ANOVA ($p<0.05$).

**Table 13.** Physicochemical properties of 20% lipid emulsion containing various surfactants (Mean ± SD, n = 3).

| Rx | Emulsifier | Time | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|---|
| Rx16 | 1.2% EPC + 1.2% Tween® 80 | a0 | 0.204 ± 0.001 | -40.30 ± 3.52 | 391 ± 2 | 8.03 ± 0.02 |
| | | b0 | 0.205 ± 0.000 | -43.33 ± 0.12 | 394 ± 4 | 6.84 ± 0.09 |
| | | b1 | 0.206 ± 0.000 | -37.20 ± 0.10 | 398 ± 1 | 6.83 ± 0.03 |
| | | b4 | 0.205 ± 0.001 | -40.73 ± 3.52 | 417 ± 3 | 6.59 ± 0.09 |
| Rx17 | 1.2% EPC + 0.9% Tween® 80 | a0 | 0.212 ± 0.002 | -41.23 ± 1.39 | 386 ± 0 | 8.05 ± 0.02 |
| | | b0 | 0.211 ± 0.002 | -44.60 ±0.10 | 386 ± 1 | 6.73 ± 0.06 |
| | | b1 | 0.213 ± 0.002 | -37.40 ± 0.30 | 394 ± 1 | 6.64 ± 0.09 |
| | | b4 | 0.215 ± 0.002 | -45.13 ± 0.42 | 414 ± 2 | 6.63 ± 0.11 |
| Rx18 | 1.2% EPC + 0.6% Tween® 80 | a0 | 0.224 ± 0.001 | -43.00 ± 0.10 | 383 ± 2 | 8.05 ± 0.02 |
| | | b0 | 0.305 ± 0.004 | -44.80 ± 0.46 | 380 ± 2 | 6.63 ± 0.11 |
| | | b1 | 0.309 ± 0.007 | -36.97 ± 0.15 | 390 ± 1 | 6.76 ± 0.03 |
| | | b4 | 0.311 ± 0.004 | -47.47 ± 1.24 | 404 ± 3 | 6.61 ± 0.06 |
| Rx19 | 1.2% EPC + 1.2% Vit E-TPGS | a0 | 0.205 ± 0.001 | -36.47 ± 2.50 | 374 ± 0 | 8.05 ± 0.01 |
| | | b0 | 0.206 ± 0.001 | -39.13 ± 0.76 | 379 ± 1 | 6.67 ± 0.04 |
| | | b1 | 0.206 ± 0.001 | -41.07 ± 0.98 | 395 ± 3 | 6.59 ± 0.04 |
| | | b4 | 0.206 ± 0.001 | -59.27 ± 0.15 | 380 ± 1 | 6.50 ± 0.02 |
| Rx20 | 1.2% EPC + 0.9% Vit E-TPGS | a0 | 0.213 ± 0.001 | -38.33 ± 1.60 | 369 ± 1 | 8.06 ± 0.01 |
| | | b0 | 0.214 ± 0.001 | -39.87 ± 0.97 | 374 ± 1 | 6.68 ± 0.01 |
| | | b1 | 0.215 ± 0.001 | -41.30 ± 0.35 | 389 ± 1 | 6.70 ± 0.02 |
| | | b4 | 0.216 ± 0.002 | -59.83 ± 1.38 | 371 ± 1 | 6.62 ± 0.03 |
| Rx21 | 1.2% EPC + 0.6% Vit E-TPGS | a0 | 0.230 ± 0.001 | -38.33 ± 1.42 | 371 ± 1 | 8.06 ± 0.01 |
| | | b0 | 0.230 ± 0.001 | -40.27 ± 1.12 | 373 ± 1 | 6.97 ±0.10 |
| | | b1 | 0.233 ± 0.002 | -39.70 ± 0.26 | 383 ± 2 | 6.70 ± 0.04 |
| | | b4 | 0.229 ± 0.002 | -61.40 ± 2.55 | 364 ± 1 | 6.61 ± 0.08 |
| Rx22 | 1.2% EPC + 0.4% Vit E-TPGS | a0 | 0.251 ± 0.006 | -37.90 ± 1.25 | 371 ± 2 | 8.04 ± 0.03 |
| | | b0 | 0.358 ± 0.003 | -39.30 ± 0.95 | 372 ± 1 | 6.76 ± 0.05 |
| | | b1 | 0.364 ± 0.003 | -40.63 ± 4.15 | 381 ± 2 | 6.59 ± 0.01 |
| | | b4 | creaming | creaming | creaming | creaming |

a0) = unautoclaved and 24-hour storage ; b0) = autoclaved and 24-hour storage ; b1) = autoclaved and 1-week storage; b4) = autoclaved and 4-weeks storage

From the experiments, it was found that a slight change in the white color of emulsion to soymilk-like emulsion after autoclaving was observed in Rx 18 and Rx 22. Table 13 shows that there were bigger particle size of 20% oil emulsion

containing EPC and Tween® 80 (i.e., Rx16; 0.204 ± 0.001 μm and Rx17; 0.212 ± 0.002 μm) compared to 10% oil emulsion (i.e., Rx4; 0.193 ± 0.000 μm and Rx5; 0.195 ± 0.001 μm) when using the same emulsifier. However, at the weight ratio of 2:1 EPC to Tween® 80, particle size of 20% oil autoclaved emulsion at 24 hours (Rx18) was higher (0.305 μm) when compared to 10% oil emulsion (Rx6; 0.199 μm). Similarly at the weight ratio of EPC to Vitamin E-TPGS being 1:1 to 2:1, particle size of 20% oil emulsion (Rx19 and Rx21, respectively) at 24 hours was slightly bigger than those composed of 10% oil emulsion. Unlikely, at the weight ratio of 3:1 EPC to Vitamin E-TPGS, particle size of 20% autoclaved emulsion (Rx22) at 24 hours was sharply increased up to 0.358 μm while particle size of 10% oil emulsion (Rx11) is only 0.222 μm. The increase in particle size distribution may result from an impoverishment of the surfactant at the interface with increasing surface of the dispersed oil phase. Moreover, the data showed that no significant changes in the droplet size of the emulsions were observed upon storage for 1 and 4 weeks except for Rx22 which was creaming after storage for 4 weeks. This may be due to the insufficient amount of cosurfactant that is not enough to stabilize the emulsion in long term.

The zeta potential of 20% oil emulsion was also higher than 10% oil emulsion. However, there were differences in zeta potential with the storage time. The results revealed that the zeta potential was increased after storage for 4 weeks. Conversely, the pH of emulsions decreased after storage for 4 weeks due to the hydrolysis effect of phospholipids as explained previously in 2.1. The findings were similar to the studies of Yamahuchi *et al.*(1995) which was revealed that zeta potential is pH dependent. They found that at pH 4, 5, 6 and 8 the purified (99%)

lecithin emulsion had zeta potential of 5, -3, -8 and -30 mV respectively, reflecting the zwitterionic nature of PC.

In all previous experiments, it showed that the weight ratio of EPC to Tween® 80 and Vitamin E-TPGS had no significant effect on the physicochemical properties of the emulsions. The weight ratio of 2:1 of EPC to Tween® 80 and Vitamin E-TPGS were selected for further studies as being the ratio representing lowest amount of cosurfactant which could form stable emulsions for both cosurfactants and their physicochemical properties were in the range of parenteral product requirements. In addition, the weight ratio of EPC to Vitamin E-TPGS at 3:1 was also chosen for further studies as it contained lowest amount of cosurfactant.

### 2.4 Optimization of total emulsifier concentration

The next parameter investigated was the total amount of the emulsifier systems used to stabilize the emulsion containing 10% oil. Emulsions were prepared with varying amounts of total emulsifiers from 1.5% to 3.0% w/w at three different ratios of EPC to cosurfactant (i.e., EPC and Tween® 80 at a weight ratio of 2:1, EPC and Viatmin E-TPGS at weight ratio of 2:1 and 3:1). The samples were determined for their physicochemical properties after being kept at $4^{O}C$, $40^{O}C$ and room temperature for 1 week and 4 weeks.

#### 2.4.1 Emulsion containing EPC to Tween® 80 at the weight ratio 2:1

The emulsions containing EPC to Tween® 80 at the weight ratio of 2:1 were prepared using the previous emulsification process and their physical

stability and physicochemical properties were determined. The results showed that there were slight changes from white emulsion to soymilk-like emulsion after autoclaving of the formulations which contained 2.5% and 3.0% total emulsifier. Finally, the creaming was occurred after storage for 4 weeks both at room temperature and 40° C. It can be supported by the results of their physicochemical properties as shown in Tables 14-15 which illustrate that the particle size of emulsion containing 2.5% and 3.0% were subjected to markedly increase after autoclaving. Such a significant change negligible in emulsions with total concentration of emulsifier of 1.5% and 2.0%. This may be related to the amount of surfactant present. The solubility of surfactant in both the disperse and the continuous phases maintain the stability of the surfactant film at the interface from their reservoir created in each phase. Because Tween® 80 tends to soluble in water more than oil, consequence is the lost of balance at the interface film and the HLB value of the system is increased to the value higher than the required HLB of soybean oil, 8 (Krishna, Wood and Shet, 1998; Lund, 1994). As a result of the improper of HLB value, the emulsion showed instability.

The pH values of all stable preparations were slightly decreased during the period of time as previously described in 2.1. The pH values of autoclaved emulsions after 4-week storage at room temperature were in between 6.98 and 7.10 and between 6.58 and 7.17 for systems stored at 40°C which were still in the range of parenteral emulsion. The osmolalities were not affected by autoclaving process and the storage time at any temperature. The osmolality of the system was rather constant for all storage period.

In contrast, the zeta potential tended to increase in negativity during storage at condition except at 4°C that the values were slightly increased.

From the results, the physical stability and physicochemical properties of emulsion containing total emulsifier at 1.5% and 2.0% showed the better stability than those containing 2.5% and 3.0%. These could be advantage of preparing emulsion with low concentration of emulsifier.

**Table 14.** Physicochemical properties of 10% lipid emulsions containing EPC to Tween® 80 at 2:1 at room temperature (Mean ± S.D., n = 3).

| Total emulsifier (%w/w) | Time | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|
| 1.5 (LE4) | 24 hrs (U) | 0.200 ± 0.001 | -37.83 ± 1.17 | 328 ± 2 | 8.03 ± 0.02 |
| | 24 hrs (A) | 0.207± 0.002 | -41.97 ± 0.12 | 325 ± 1 | 7.09 ± 0.01 |
| | 1 wk | 0.207 ± 0.001 | -46.87 ± 0.50 | 320 ± 1 | 6.94 ± 0.07 |
| | 4 wks | 0.207 ±0.001 | -47.00 ± 4.55 | 322 ± 2 | 6.98 ± 0.13 |
| 2.0 (LE5) | 24 hrs (U) | 0.194 ± 0.000 | -36.03 ± 0.60 | 328 ± 1 | 8.02 ± 0.02 |
| | 24 hrs (A) | 0.194 ± 0.001 | -42.10 ± 0.17 | 329 ± 3 | 7.26 ± 0.05 |
| | 1 wk | 0.194 ± 0.001 | -47.70 ± 0.85 | 322 ± 1 | 7.20 ± 0.02 |
| | 4 wks | 0.194 ± 0.001 | -64.70 ± 4.16 | 328 ± 2 | 7.10 ± 0.02 |
| 2.5 | 24 hrs (U) | 0.191 ± 0.000 | -34.95 ± 0.21 | 334 ± 2 | 8.08 ± 0.02 |
| | 24 hrs (A) | 0.264 ± 0.003 | -41.50 ± 0.17 | 336 ± 0 | 6.99 ± 0.03 |
| | 1 wk | 0.276 ± 0.010 | -55.03 ± 0.15 | 331 ± 3 | 6.94 ± 0.03 |
| | 4 wks | Creaming | | | |
| 3.0 | 24 hrs (U) | 0.190 ± 0.000 | -35.95 ± 0.21 | 337 ± 1 | 8.13 ± 0.03 |
| | 24 hrs (A) | 0.362 ± 0.002 | -52.13 ± 0.31 | 335 ± 1 | 6.92 ± 0.03 |
| | 1 wk | Creaming | | | |
| | 4 wks | Creaming | | | |

U = Unautoclaved, A = Autoclaved

**Table 15.** Physicochemical properties of 10% lipid emulsions containing EPC to Tween® 80 at 2:1 at 4 and 40°C (Mean ± S.D., n = 3).

| Total emulsifier (%w/w) | Time | Temperature (°C) | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|---|
| 1.5 (LE4) | 1 wk | 4 | 0.209 ± 0.001 | -40.53 ± 3.90 | 322 ± 2 | 7.11± 0.06 |
| | | 40 | 0.207 ± 0.003 | -47.85 ± 1.13 | 322 ± 1 | 6.86 ± 0.01 |
| | 4 wks | 4 | 0.209 ± 0.001 | -41.07 ± 0.90 | 327 ± 2 | 7.21 ± 0.10 |
| | | 40 | 0.206 ± 0.001 | -52.47 ± 5.58 | 326 ± 1 | 7.17 ± 0.03 |
| 2.0 (LE5) | 1 wk | 4 | 0.194 ± 0.000 | -46.70 ± 0.50 | 324 ± 1 | 7.28 ± 0.06 |
| | | 40 | 0.194 ± 0.001 | -54.07 ± 1.36 | 324 ± 2 | 7.24 ± 0.09 |
| | 4 wks | 4 | 0.194 ± 0.000 | -47.37 ± 1.53 | 329 ± 1 | 7.30 ± 0.03 |
| | | 40 | 0.194 ± 0.001 | -59.50 ± 2.75 | 324 ± 1 | 6.58 ± 0.07 |
| 2.5 | 1 wk | 4 | 0.289 ± 0.014 | -53.67 ± 0.40 | 328 ± 5 | 6.89 ± 0.06 |
| | | 40 | 0.271 ± 0.013 | -56.43 ± 0.47 | 329 ± 4 | 6.91 ± 0.09 |
| | 4 wks | 4 | 0.289 ± 0.010 | -59.30 ± 4.96 | 329 ± 1 | 6.78 ± 0.04 |
| | | 40 | Creaming | | | |
| 3.0 | 1 wk | 4 | 0.345 ± 0.027 | -54.10± 0.53 | 355 ± 1 | 6.99 ± 0.09 |
| | | 40 | Creaming | | | |
| | 4 wks | 4 | 0.335 ± 0.005 | -57.00 ± 3.44 | 347 ± 3 | 7.12 ± 0.09 |
| | | 40 | Creaming | | | |

### 2.4.2 Emulsion containing EPC to Vitamin E-TPGS at the weight ratio of 3:1 and 2:1

The emulsion containing EPC to Vitamin E-TPGS at the weight ratios of 3:1 and 2:1 were prepared using the previously studied emulsion preparation and their physical stability and physicochemical properties were investigated. The results are shown in Tables 16-19.

**Table16.** Physicochemical properties of 10% lipid emulsions containing 2:1 EPC to Vitamin E-TPGS after storage at room temperature (Mean ± S.D., n = 3).

| Total emulsifier (%w/w) | Time | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|
| 1.5 (LE2) | 24 hrs (U) | 0.201 ± 0.001 | -38.63 ± 1.53 | 329 ± 1 | 8.01± 0.01 |
| | 24 hrs (A) | 0.199 ± 0.000 | -41.77 ± 0.40 | 324 ± 2 | 6.97 ± 0.07 |
| | 1 wk | 0.197 ± 0.006 | -47.53 ± 0.85 | 331 ± 3 | 6.68 ± 0.03 |
| | 4 wks | 0.196 ± 0.006 | -47.47 ± 0.67 | 321 ± 3 | 6.73 ± 0.04 |
| 2.0 (LE3) | 24 hrs (U) | 0.196 ± 0.000 | -39.53 ± 0.59 | 330 ± 1 | 8.06 ± 0.02 |
| | 24 hrs (A) | 0.194 ± 0.000 | -42.93 ± 0.85 | 327 ± 2 | 6.77 ± 0.05 |
| | 1 wk | 0.192 ± 0.005 | -49.83 ± 0.68 | 328 ± 0 | 6.57 ± 0.03 |
| | 4 wks | 0.191 ± 0.005 | -52.97 ± 0.60 | 325 ± 8 | 6.62 ± 0.03 |
| 2.5 | 24 hrs (U) | 0.195 ± 0.001 | -41.80 ± 0.30 | 340 ± 2 | 8.04 ± 0.02 |
| | 24 hrs (A) | 0.193 ± 0.000 | -47.03 ± 0.64 | 332 ± 2 | 6.67 ± 0.02 |
| | 1 wk | 0.190 ± 0.005 | -52.83 ± 2.40 | 333 ± 1 | 6.45 ± 0.04 |
| | 4 wks | 0.190 ± 0.005 | -52.67 ± 1.53 | 332 ± 1 | 6.56 ± 0.02 |
| 3.0 | 24 hrs (U) | 0.195 ± 0.001 | -43.07 ± 0.76 | 331 ± 3 | 8.08 ± 0.03 |
| | 24 hrs (A) | 0.193 ± 0.000 | -47.27 ± 1.69 | 325 ± 1 | 6.67 ± 0.06 |
| | 1 wk | 0.190 ± 0.005 | -56.67 ± 1.52 | 321 ± 2 | 6.56 ± 0.04 |
| | 4 wks | 0.190 ± 0.005 | -56.63 ± 1.29 | 319 ± 1 | 6.54 ± 0.05 |

U = Unautoclaved, A = Autoclaved

**Table17.** Physicochemical properties of 10% lipid emulsions containing 2:1 EPC to Vitamin E-TPGS after storage at 4 and 40$^{O}$C (Mean ± S.D., n = 3).

| Total emulsifier (%w/w) | Time | Temperature ($^{o}$C) | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|---|
| 1.5 (LE2) | 1 wk | 4 | 0.200 ± 0.001 | -46.97 ± 1.12 | 331 ± 2 | 6.66 ± 0.02 |
| | | 40 | 0.200 ± 0.000 | -48.33 ± 0.75 | 330 ± 3 | 6.65 ± 0.05 |
| | 4 wks | 4 | 0.200 ± 0.000 | -47.03 ± 1.50 | 327 ± 1 | 6.87 ± 0.03 |
| | | 40 | 0.199 ± 0.001 | -49.37 ± 3.20 | 318 ± 2 | 6.65 ± 0.04 |
| 2.0 (LE3) | 1 wk | 4 | 0.195 ± 0.001 | -49.57 ± 0.93 | 330 ± 1 | 6.65 ± 0.05 |
| | | 40 | 0.194 ± 0.001 | -50.17 ± 0.95 | 327 ± 2 | 6.54 ± 0.05 |
| | 4 wks | 4 | 0.194 ± 0.000 | -52.57 ± 0.21 | 324 ± 1 | 6.68 ± 0.01 |
| | | 40 | 0.194 ± 0.001 | -54.70 ± 0.35 | 325 ± 3 | 6.54 ± 0.04 |
| 2.5 | 1 wk | 4 | 0.193 ± 0.000 | -53.67 ± 1.86 | 335 ± 2 | 6.57 ± 0.03 |
| | | 40 | 0.193 ± 0.000 | -54.30 ± 2.69 | 329 ± 2 | 6.42 ± 0.04 |
| | 4 wks | 4 | 0.193 ± 0.000 | -57.53 ± 0.45 | 329 ± 1 | 6.62 ± 0.04 |
| | | 40 | 0.193 ± 0.000 | -55.67 ± 0.91 | 329 ± 3 | 6.54 ± 0.04 |
| 3.0 | 1 wk | 4 | 0.193 ± 0.000 | -52.93 ± 0.25 | 319 ± 1 | 6.58 ± 0.03 |
| | | 40 | 0.193 ± 0.000 | -63.53 ± 1.17 | 323 ± 2 | 6.50 ± 077 |
| | 4 wks | 4 | 0.193 ± 0.000 | -53.63 ± 1.53 | 320 ± 1 | 6.59 ± 0.05 |
| | | 40 | 0.192 ± 0.001 | -64.17 ± 5.35 | 325 ± 1 | 6.43 ± 0.03 |

**Table 18.** Physicochemical properties of emulsions containing 3:1 EPC to Vitamin E-TPGS after storage at room temperature (Mean ± S.D., n = 3).

| Total emulsifier (%w/w) | Time | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|
| 1.6 | 24 hrs (U) | 0.203 ± 0.001 | -35.90 + 0.10 | 326 ± 2 | 8.05 ± 0.01 |
| | 24 hrs (A) | 0.222 ± 0.001 | -38.07 + 0.06 | 320 ± 9 | 6.67 ± 0.02 |
| | 1 wk | 0.221 ± 0.003 | -36.90 ± 0.20 | 323 ± 1 | 6.61 ± 0.02 |
| | 4 wks | 0.221 ± 0.001 | -47.20 ± 1.05 | 324 ± 2 | 6.51 ± 0.02 |
| 2.0 (LE1) | 24 hrs (U) | 0.197 ± 0.000 | -41.33 ± 0.74 | 325 ± 2 | 8.09 ± 0.02 |
| | 24 hrs (A) | 0.215 ±0.002 | -46.27 ± 0.50 | 318 ± 1 | 7.02 ± 0.05 |
| | 1 wk | 0.213 ± 0.002 | -48.40 ± 0.46 | 321 ± 2 | 6.63 ± 0.06 |
| | 4 wks | 0.216 ± 0.002 | -57.50 ± 0.72 | 330 ± 1 | 6.56 ± 0.02 |
| 2.5 | 24 hrs (U) | 0.194 ± 0.000 | -39.70 ± 0.26 | 327 ± 4 | 8.03 ± 0.03 |
| | 24 hrs (A) | 0.337 ± 0.007 | -45.53 ± 0.21 | 326 ± 2 | 6.42 ± 0.08 |
| | 1 wk | 0.333 ± 0.002 | -51.60 ± 0.30 | 327 ± 2 | 6.20 ± 0.05 |
| | 4 wks | Creaming | | | |
| 3.0 | 24 hrs (U) | 0.193 ± 0.000 | -40.07 ± 0.84 | 362 ± 1 | 8.05 ± 0.05 |
| | 24 hrs (A) | 0.254 ± 0.006 | -51.60 ± 0.70 | 360 ± 1 | 6.84 ± 0.11 |
| | 1 wk | 0.249 ± 0.013 | -44.70 ± 0.96 | 377 ± 1 | 6.70 ± 0.01 |
| | 4 wks | Creaming | | | |

U = Unautoclaved, A = Autoclaved

**Table19.** Physicochemical properties of emulsions containing 3:1 EPC to Vitamin E-TPGS after storage at 4 and 40$^{O}$C (Mean ± SD, n = 3).

| Total emulsifier (%w/w) | Time | Temperature ($^{O}$C) | D[4,3] (μm) | Zeta potential (mV) | Osmolality (mOsm/kg) | pH |
|---|---|---|---|---|---|---|
| 2.0 (LE1) | 1 wk | 4 | 0.211 ± 0.001 | -48.17 ± 0.65 | 322 ± 1 | 6.70 ± 0.04 |
| | | 40 | 0.209 ± 0.001 | -48.50 ± 0.82 | 318 ± 1 | 6.62 ± 0.04 |
| | 4 wks | 4 | 0.213 ± 0.005 | -64.60 ± 5.15 | 332 ± 1 | 6.82 ± 0.02 |
| | | 40 | 0.212 ± 0.001 | -63.63 ± 3.59 | 327 ± 1 | 6.41 ±0.02 |
| 2.5 | 1 wk | 4 | 0.333 ± 0.006 | -52.43 ± 0.15 | 325 ± 1 | 6.32 ± 0.05 |
| | | 40 | Creaming | | | |
| | 4 wks | 4 | 0.345 ± 0.008 | -64.27 ± 2.47 | 330 ± 1 | 6.30 ± 0.03 |
| | | 40 | Creaming | | | |
| 3.0 | 1 wk | 4 | 0.261 ± 0.004 | -43.03 ± 0.38 | 380 ± 1 | 6.71 ± 0.02 |
| | | 40 | 0.272 ± 0.007 | -44.90 ± 0.26 | 377 ± 1 | 6.46 ± 0.04 |
| | 4 wks | 4 | 0.270 ± 0.009 | -57.93 ± 3.00 | 376 ± 8 | 6.71 ± 0.04 |
| | | 40 | Creaming | | | |

Since there was no report on using Vitamin E-TPGS as an emulsifier in intravenous lipid emulsion, however, Mu and Feng (2002) proposed that Vitamin E-TPGS could be a more effective and safer emulsifier with easier usage in fabricaiton and characterization of polymeric nanospheres for drug delivery. In addition, Sokol (1993) revealed that Vitamin E-TPGS is a safe and effective form of vitamin E for reversing or preventing vitamin E deficiency. It can also improve the oral bioavailability of vitamin E.

This experiment was tried to find out the optimum ratio of EPC to Vitamin E-TPGS and the total concentration of emulsifier used. From the results, there was a creaming occurred in the two formulations which contained EPC to Vitamin E-TPGS at a weight ratio of 3:1 at total emulsifier of 2.5% and 3.0% after storage for 4 weeks at $40^{O}C$ and at room temperature (Tables 18 and 19). Compared the particle size of the system using EPC to Vitamin E-TPGS at weight ratios of 3:1 and 2:1 and at total emulsifier of less than 2.5%, the first ratio showed slightly bigger particle size. At a total concentration of 2.5% and 3.0%, the system at the ratio of 3:1 showed a dramatic change in particle size after autoclaving which finally, led to a creaming after storage for 4 weeks. Surprisingly, there were no changes in particle size of all of the emulsions at the ratio of 2:1 were observed at any storage conditions.

Comparing the emulsion contained Vitamin E-TPGS and Tween® 80 at the same weight ratio (2:1), the particle size of emulsion using Vitamin E-TPGS was smaller than those containing Tween® 80 in any concentrations of emulsifier. In addition, the emulsions contained Vitamin E-TPGS were stable up to 4 weeks after storage both at room temperature and in accelerate condition. These may possibly be

due to the effect of ionic stabilization of Vitamin E-TPGS which can be observed in the zeta potential value that tended to be increased in negativity with time.

From the results, the zeta potential of all formulations showed an increase in negativity required for long term stability while the osmolality was rather constant in all preparations. The pH values of all autoclaved preparations were slowly decreased with storage time.

Finally, the most suitable formulation was 10% soybean oil emulsified with EPC and Vitamin E-TPGS at the ratio of 2:1 at total concentration of 1.5%. The formulation was considered to contain less amount of emulsifiers and was stable upon storage both at room temperature and accelerate condition. Furthermore, the formulation containing EPC to Tween® 80 at the weight ratio of 2:1 at total concentration of 1.5% is also recommended. Even though its particle sizes were slightly bigger than the former formulation, the results illustrated no change in physicochemical properties during storage in all conditions.

**3. Sterility test**

The sterility test was assured to the sterility of emulsion after sterilization by autoclaving at 121° C for 15 minutes. The five sterile emulsions from previous study were selected.

| Rx | Emulsifier | total emulsifier concentration (%w/w) |
|---|---|---|
| LE1 | EPC:Vitamin E-TPGS at 3:1 | 2.0 |
| LE2 | EPC:Vitamin E-TPGS at 2:1 | 1.5 |
| LE3 | EPC:Vitamin E-TPGS at 2:1 | 2.0 |
| LE4 | EPC:Tween® 80 at 2:1 | 1.5 |
| LE5 | EPC:Tween® 80 at 2:1 | 2.0 |

The test included an evaluation of the number of viable aerobic microorganisms present and for freedom from designated microbial (USP27, 2004). The results showed in Table 20 indicated that all formulations were free from microorganisms and molds. Hence, any effect on the stability and properties of emulsions was not the result of microbial activities. Robins, Watson and Wilde (2002) revealed that emulsion science may provide a tool to unravel the interaction of bacteria with surfaces: mixing *E.coli* cells with an oil-in-water emulsion stabilized with certain emulsifiers can cause droplet coalescence and flocculation, due to surface charges on both bacteria and droplets. This suggested yet another way, over and above microbial spoilage, that bacteria can wreck an emulsion

**Table 20.** Microbial limit test of 10% emulsion containing various emulsifiers.

| Test items | Units | LE1 | LE2 | LE3 | LE4 | LE5 |
|---|---|---|---|---|---|---|
| Total plate count | CFU/ml | Less than 1 | Less than 1 | Less than 1 | Less than 1 | Less than 1 |
| Total anaerobic plate count | CFU/ml | Less than 1 | Less than 1 | Less than 1 | Less than 1 | Less than 1 |
| Yeast & Mold | CFU/ml | Less than 1 | Less than 1 | Less than 1 | Less than 1 | Less than 1 |
| *Staphylococcus aureus* | Per 10 ml | Not found | Not found | Not found | Not found | Not found |
| *Pseudomonas aeruginosa* | Per 10 ml | Not found | Not found | Not found | Not found | Not found |
| *Samonella spp.* | Per 10 ml | Not found | Not found | Not found | Not found | Not found |
| *Escherichia Coli* | Per 10 ml | Not found | Not found | Not found | Not found | Not found |

LE1: emulsion contained EPC to Vitamin E-TPGS at 3:1 (2.0% total emulsifier)
LE2: emulsion contained EPC to Vitamin E-TPGS at 2:1 (1.5% total emulsifier)
LE3: emulsion contained EPC to Vitamin E-TPGS at 2:1 (2.0% total emulsifier)
LE4: emulsion contained EPC to Tween® 80 at 2:1 (1.5% total emulsifier)
LE5: emulsion contained EPC to Tween® 80 at 2:1 (2.0% total emulsifier)

**4. Hemolysis study**

The hemolytic activity has been suggested as a toxicity screen *in vitro*, serving as a simple and reliable measure for estimating the membrane damage caused by formulations *in vivo*. The prepared lipid emulsion, referred as LE1-LE5 from 3 were tested for the hemolytic effect on human red blood cells in order to assure the suitability of synthetic cosurfactant used in the formulation. Lipid emulsion were studied after freshly prepared and compared to commercial product such as 10% Intralipid®, 20% Intralipid® and Vitalipid® N Infant.

**Table 21.** The hemolysis induced by the different types of emulsion (mean ± S.D., n = 3).

| Rx | Emulsifier | % Hemolysis |
|---|---|---|
| LE1 | EPC:Vitamin E-TPGS at 3:1, 2.0% | 18.76 ± 0.67 |
| LE2 | EPC:Vitamin E-TPGS at 2:1, 1.5% | 22.42 ± 3.62 |
| LE3 | EPC:Vitamin E-TPGS at 2:1, 2.0% | 24.29 ± 1.52 |
| LE4 | EPC:Tween® 80 at 2:1, 1.5% | 24.18 ± 2.51 |
| LE5 | EPC:Tween® 80 at 2:1, 2.0% | 23.90 ± 1.49 |
| 10% Intralipid® | *EPC 1.2% | 24.12 ± 0.82 |
| 20% Intralipid® | *EPC 1.2% | 25.05 ± 2.76 |
| Vitalipid® N Infant | *EPC 1.2% | 24.51 ± 1.36 |

* as reported on the label

The results indicated that the hemolysis was found in prepared emulsions investigated. The hemolytic effect was also found in commercial products with the value similar to the prepared emulsion. The emulsion contained Tween® 80 seemed

to be higher in hemolytic effect than those contained Vitamin E-TPGS. This may be explained by the studied of Jumaa, Kleinebudde and Müller (1999) which showed higher hemolytic effect of Tween® 80 than soy lecithin (Lipoid S75) and nonionic copolymer, Synperonic F68. The addition of S75 or F68 led to a remarkable decrease in the hemolytic activity induced by Tween® 80.

Moreover, Nagasaka and Ishii (2001) concluded that the hemolysis caused by the interaction between erythrocytes and emulsions was shown to be related to the phospholipids dispersed in the water phase, and was dependent on the PC contents in both the emulsifying agent used for preparation of the emulsion and in the erythrocyte membrane. The sphingomyelin in the erythrocyte membrane was shown to be an important component for the stabilization of erythrocytes against hemolysis induced by lipid emulsions.

**5. Transmission Electron Microscopy (TEM)**

The prepared lipid emulsions containing EPC either with Vitamin E-TPGS or Tween® 80 at the weight ratio of 2:1 at the total concentration of 1.5%w/w (LE2 and LE4, respectively) were selected to run the transmission electron microscope (TEM) for observing their morphology compared to commercial product, 10% Intralipid®. The TEM image of the lipid emulsions (LE 2 and LE4) was illustrated in Figure 21a and Figure 21b, respectively comparing to the TEM image of the commercial emulsion, 10% Intralipid®, Figure 21c. It could be seen that LE2 and LE4 showed a spherical shape with about 200 nm in diameter while 10% Intralipid® showed a spherical shape with about 300 nm in diameter. The TEM micrograph confirmed the results of particle size obtained from Mastersizer® 2000 which were 0.199, 0.200 and

0.289 μm for LE2, LE4 and 10% Intralipid®, respectively. In Figure 21a, the oil droplet surface of emulsion containing EPC and Vitamin E-TPGS was less smooth than either those containing EPC and Tween® 80 (Figure 21b) or 10% Intralipid® (Figure 21c).

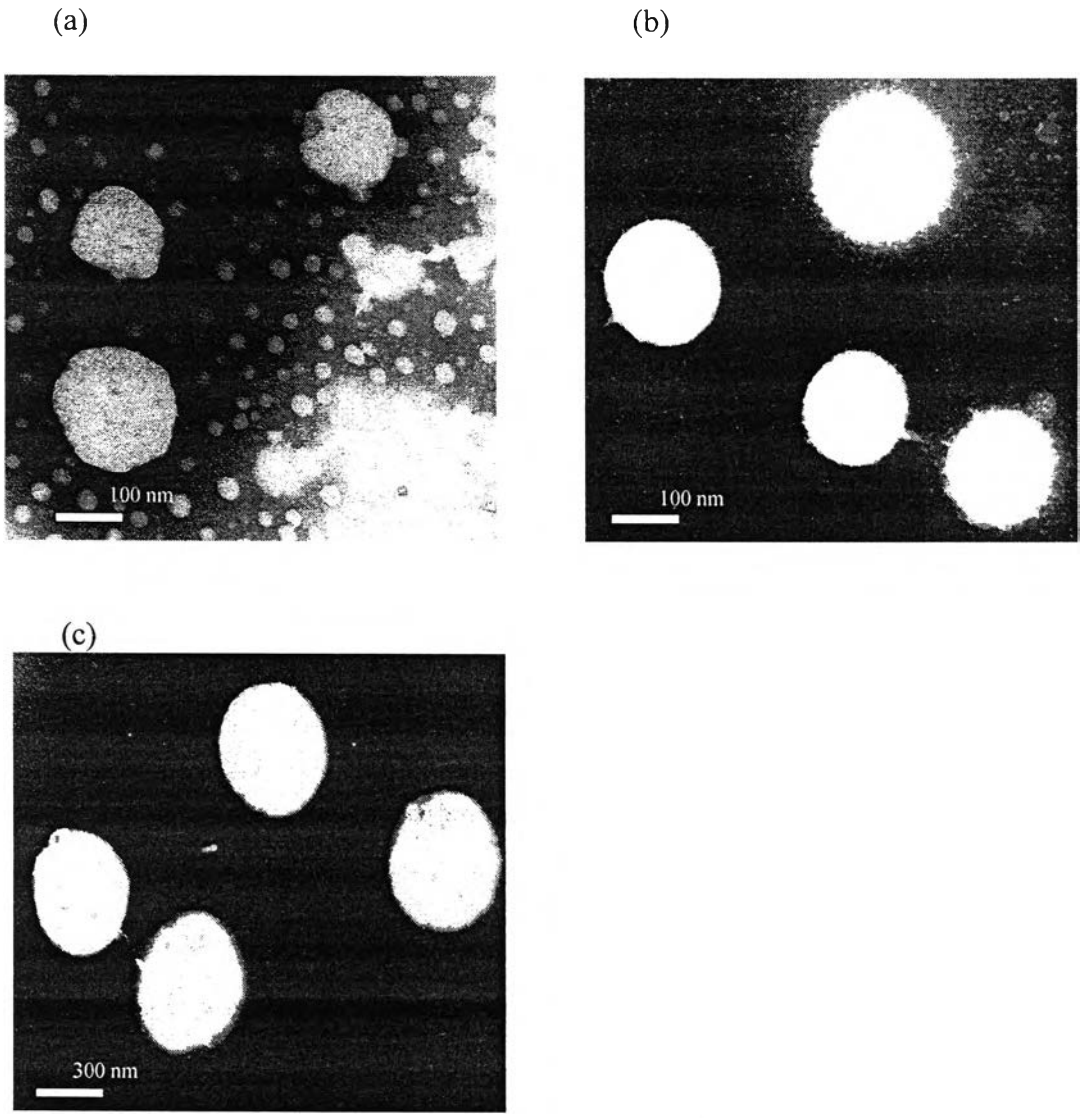


**Figure 21**. Transmission electron micrographs of 10% lipid emulsions using negative stain technique (a) emulsion contained EPC and Vitamin E-TPGS at the weight ratio of 2:1 at the emulsifier concentration of 1.5% w/w (LE2), (b) emulsion contained EPC and Tween® 80 at the weight ratio of 2:1 at the emulsifier concentration of 1.5% w/w (LE4), (c) 10% Intralipid®.