CHAPTER IV

RESULTS AND DISCUSSION

In this study, two groups of model compounds were used according to the difference of their molecular structures and thus their corresponding physicochemical properties. The first group was alkyl-4-hydroxybenzoates or paraben esters and the other was the benzodiazepines. The chemical structures of alkyl-4-hydroxybenzoates and benzodiazepines were illustrated in Table 1 and Table 4, respectively. The alkyl-4-hydroxybenzoates included in the study were methyl-4-hydroxybenzoate (methylparaben), ethyl-4-hydroxybenzoate (ethylparaben), propyl-4-hydroxybenzoate (propylparaben) and butyl-4-hydroxybenzoate (butylparaben); while alprazolam, clonazepam, diazepam and lorazepam were chosen to represent the benzodiazepines. For clarity, the report of observation from each compound group was separately discussed with details outlined as follows.

1. The determination of aqueous and oil solubility and oil-water partition coefficient.
2. The characterization of submicron emulsions with and without incorporated test compounds.
3. The effects of lipophilicity, incorporated compound concentration and incorporation method on the drug distribution into various phases of submicron emulsion.

I The determination of aqueous and oil solubility and oil-water partition coefficient of alkyl-4-hydroxybenzoates

Aqueous solubility of alkyl-4-hydroxybenzoate was determined at 25 °C and performed in triplicate. The results are presented in Table 1. It was found that aqueous solubility was in the increasing order of methylparaben, ethylparaben, propylparaben and butylparaben. Aqueous solubilities of paraben esters were therefore consistent with their chemical structures and polarity. Based on the structures of these four alkyl-4-hydroxybenzoates, the number of carbon atom increased in the order of methyl-, ethyl-, propyl and butylparaben resulting in an increase in molecular weight and lipophilicity. This study found that the aqueous solubility of methylparaben was 2.35, 5.85 and 8.85 times higher than those of ethyl-, propyl- and butylparaben, respectively. However, dipole moment values of all compounds are not different. It was possible that the increase in the number of carbon atom in the straight chain moiety had less effect on the molecular charge distribution resulting in similar vector sum of the individual bond moment in three dimensions. Thus, the dipole moment values could not relate to the polarity of alkyl-4-hydroxybenzoate.

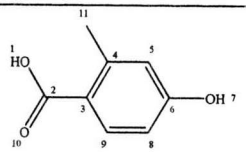
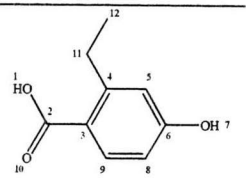
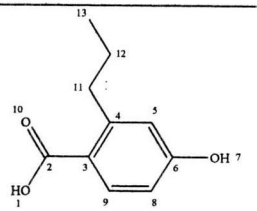
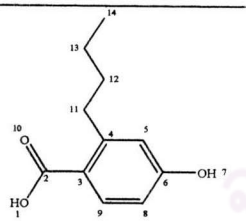
Oil solubility are shown in Table 1. The determination was made in soybean oil at 25 °C and done in triplicate. It was shown that oil solubility of the alkyl-4-hydroxybenzoate series was increased in the opposite order compared to aqueous solubility. The oil solubility of methylparaben was 1.37, 2.00 and 4.09 times lower than that of ethyl-, propyl- and butylparaben, respectively.

The partition coefficient of a substance is influenced by several factors, including the nature of the solvent and surfactant used. In this study, the partition coefficients of these compounds in soybean oil and water were determined at 25 °C. The shake-flask method was used in this experiment. The investigated solute was simply shaken with two immiscible solvents, followed by analyzing the solute concentration in one or both phases. Prior to the partition experiment, soybean oil was saturated with distilled water in order to avoid any volume changes in both phases. The results of oil-water partition coefficient ($P_{o/w}$) are reported in Table 1. It was found that the order of oil-water partition coefficients of investigated compounds was

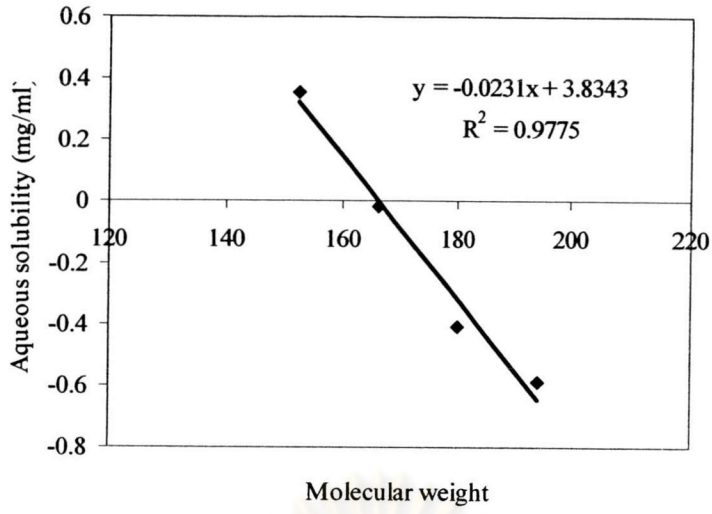
consistent with that of their oil solubilities. The partition coefficient of methylparaben was 1.71, 1.93 and 2.53 times lower than that of ethyl-, propyl- and butylparaben, respectively. Therefore, the lipophilicity of these four compounds was in the increasing order of butyl-, propyl-, ethyl- and methylparaben.

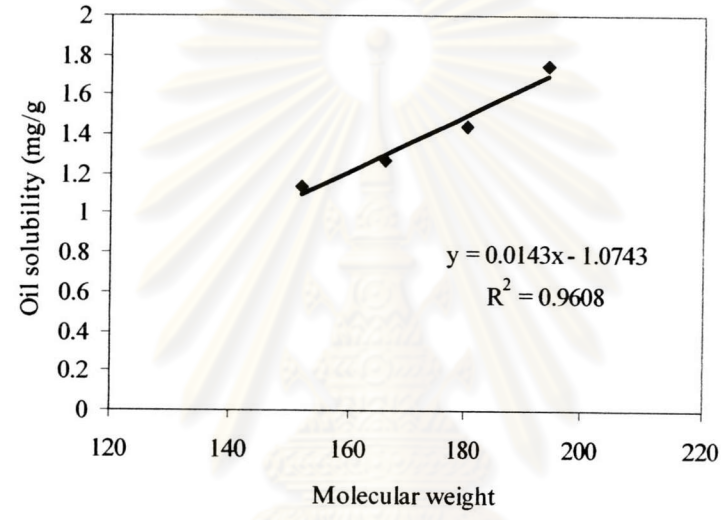
As given in Figure 1, the correlation between molecular weight of alkyl-4-hydroxybenzoates and physicochemical properties was examined. The linear relationship (R^2) between molecular weight of alkyl-4-hydroxybenzoate and physicochemical properties e.g. aqueous solubility, oil solubility and partition coefficient were 0.9775, 0.9608 and 0.9655, respectively. It was noted that the higher molecular weight of paraben esters gave the higher oil solubility and lipophilicity whereas the aqueous solubility was decreased.

Table 1 The physicochemical properties of alkyl-4-hydroxybenzoate at 25°C.

Compound	Chemical structure	Molecular weight	Aqueous solubility (mg/ml) (Mean±SD)	Oil solubility (mg/g) (Mean±SD)	Oil-water partition coefficient (Log $P_{o/w}$) (Mean±SD)	ClogP*	Dipole* Moment (Debye)
methyl-4-hydroxybenzoate		152.14	2.2803 ±0.0283	13.6658 ±0.9937	0.6459 ±0.0278	1.7562	4.278
ethyl-4-hydroxybenzoate		166.2	0.9686 ±0.0203	18.7150 ±0.9462	1.1068 ±0.0595	1.6852	4.325
propyl-4-hydroxybenzoate		180.2	0.3897 ±9.0587×10^{-3}	27.4306 ±0.7851	1.2476 ±0.1068	2.2142	4.514
butyl-4-hydroxybenzoate		194.2	0.2578 ±3.4524×10^{-3}	55.9182 ±0.6304	1.6364 ±0.1872	2.7432	4.492

* Calculated value from MOPAC molecular computation





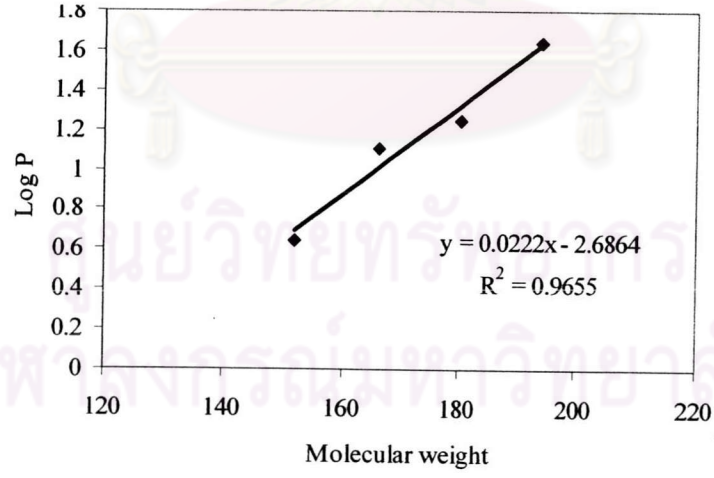


Figure 1 The correlation between molecular weight of alkyl-4-hydroxybenzoate and their physicochemical properties.

A: Aqueous solubility

B: Oil solubility

C: Oil-water partition coefficient (Lipophilicity)

II The characterization of submicron emulsions with and without incorporated alkyl-4-hydroxybenzoate

The physical properties such as particle size distribution, droplet surface charge (zeta potential) and pH of submicron emulsions were examined. These determinations were carried out immediately after prepared and after storing emulsions for 7 days at ambient temperature. Three methods of incorporating interested compound into submicron emulsion were investigated. They were de novo emulsification, extemporaneous addition and shaking. The physical properties of resulted submicron emulsions were compared to differentiate the effects of incorporated compound and its concentration. The results were separately explained according to the methods of incorporations.

2.1 De novo emulsification

De novo emulsification is a method in which compound of interest was dissolved in the oil phase prior to emulsification process. The amounts of model compounds in the submicron emulsions were varied at 30, 40, 50, 60 and 70 % saturated solubility in soybean oil of each compound. During processing, most preparations appeared stable without any coalescence or phase inversion (visual observation) except that of butylparaben at 70 % saturated oil solubility. The physical properties of those formulations are described as following:

2.1.1 Particle size and size distribution

The mean droplet sizes of submicron emulsions containing alkyl-4-hydroxybenzoates were determined by photon correlation spectroscopy which is suitable for the analysis of droplet sizes ranging from 20 nm to 1000 nm. The measurements were reported in terms of effective mean diameter and polydispersity index (PI) to estimate the width of size distribution. A polydispersity index (PI) value of less than 0.25 implies a narrow particle size distribution (Muller and Bohm 1998). As shown in Table 1B, the initial effective mean diameters and the polydispersity indices of all preparations were less than 500 nm and 0.25, respectively. It was shown that de novo emulsification gave small and uniform particle size distribution of

submicron emulsion (Muller and Bohm 1998). The characteristics of particle size distribution seen with de novo emulsified preparations were consistent with the emulsification process which involved a dissolution of incorporated compound in the oil phase and a subsequent droplet size reduction by homogenizing the emulsion under a high pressure of 15,000 psi.

In order to examine the effect of incorporated compound on the particle size of submicron emulsion, the initial effective mean diameter of submicron emulsion base incorporated compound was compared. The initial effective mean diameter of all submicron emulsion bases was 267.65±12.55 nm (255.10 - 280.20 nm) (data from Table 15B, APPENDIX PART II). The effective mean diameter of methylparaben, ethylparaben, propylparaben and butylparaben containing submicron emulsions were around 230-300, 300-480, 315-440 and 350-440 nm, respectively. It appeared that incorporated compounds might have some minor effect on the effective particle size of incorporating submicron emulsion. The effective mean diameter of methylparaben submicron emulsion was smaller than that of emulsion base, whereas those of the ethyl-, propyl- and butylparaben submicron emulsion were larger.

According to the molecular structure of methylparaben, as shown in Table 1, it is likely that this compound will locate at the oil-water interface. This possibility is supported by the study of (Pongcharoenkiat, Wittayanukulluk et al. 2003) which show that methylparaben is able to reduce the interfacial tension between aqueous phase and oil phase. However, the particle sizes of ethylparaben, propylparaben and butylparaben incorporated submicron emulsions were larger than that of submicron emulsion base. This observation would be consistent with those compounds being able to accumulate at the oil-water interface. As such, they may increase the packing parameter of phospholipids and consequently change the curvature of phospholipids monolayer resulting in the increase of mean particle size (Trotta, Pattarino et al. 2002). In addition, the incorporated compound which accumulated at the interface may diminish the reduction of surface tension by the emulsifier (phospholipids) and led to the increased particle size of submicron emulsion.

The effective particle size of submicron emulsion base after keeping for 7 days was 275.01±9.69 nm (265.32 - 284.70 nm). The particle size of submicron emulsion base appeared slightly increased after aging. In Figure 2, it was found the initial effective mean diameter of all paraben ester containing submicron emulsions were around 200-500 nm and higher to 300-550 nm after keeping for 7 days, excepted for that of 40 and 60% methylparaben submicron emulsion. In addition, the particle size of all submicron emulsions was larger as comparing with that of submicron emulsion base when kept for 7 days.

To monitor the larger particle size evidence, the laser diffraction technique was performed. These measured particle sizes were expressed in terms of volume mean diameter D (4, 3). The width of size distribution was reported in term of span. The smaller span becomes, the narrow size distribution occurred. The span is calculated as:

$$\frac{d(0.9) - d(0.1)}{d(0.5)}$$

From Table 16B, the initial volume mean diameter of submicron emulsion base was 280.27±45.15 nm (235.12 - 325.42 nm) and increased to 310.93±55.36 nm (255.57 to 366.29 nm) after keeping for 7 days. The initial volume mean diameters of emulsions containing incorporated paraben esters were larger than that of submicron emulsion base. Moreover, the particle size of most preparations after keeping for 7 days at ambient temperature seemed larger as compared with the initial values (Figure 3).

The particle sizes of ethylparaben submicron emulsions prepared at 40%, 50% and 60% saturated oil solubility were higher than 1000 nm. In addition, higher PI values based on PCS measurement were observed for this group of emulsions. This might be due to the reason that ethylparaben, a moderate lipophilic compound, would be likely to distribute to the interface of submicron emulsion leading to change in the emulsifying property of phospholipids e.g. the reduction of surface tension which might further induce the increase of particle size. In addition, the instability of phospholipids due to hydrolysis might occur during 7-day storage

resulting in the decrease of its emulsifier property. However, it was apparent that the lipophilicity and concentration of incorporated compound did not affect the particle size of submicron emulsion.

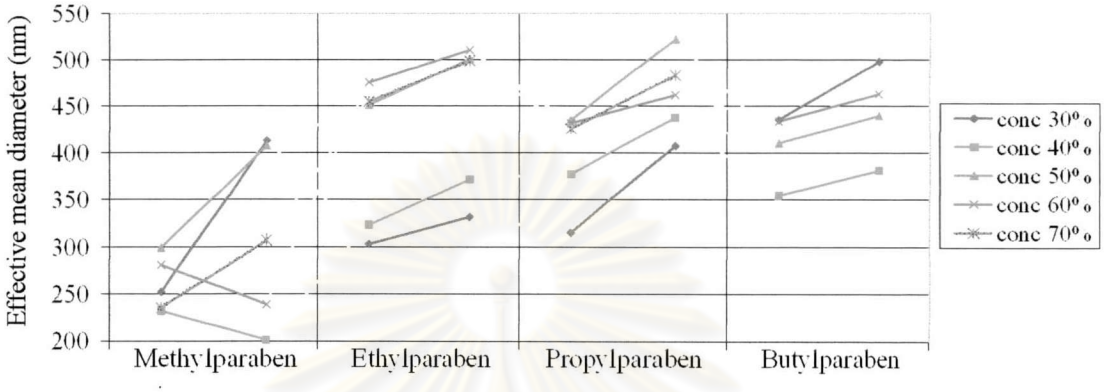


Figure 2 The change in size of paraben submicron emulsion during storage for 7 days at ambient temperature measured by using PCS.

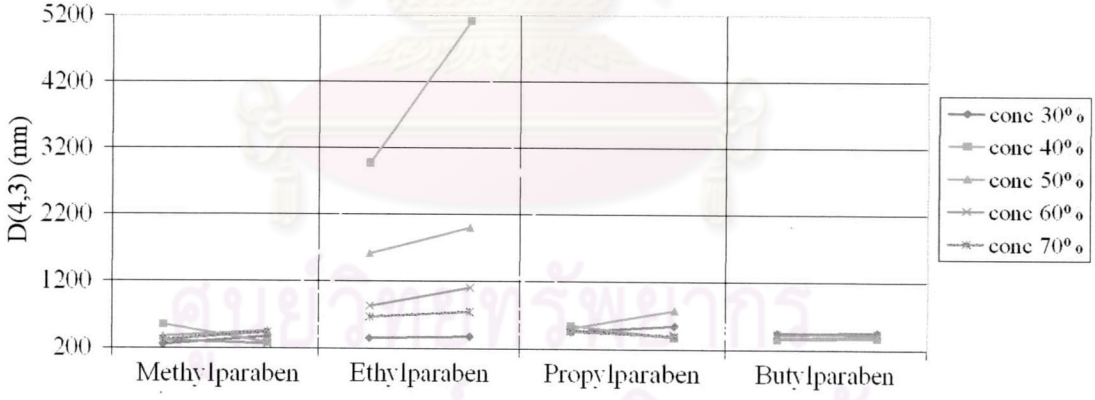


Figure 3 The change in size of paraben submicron emulsions during storage for 7 days at ambient temperature measured by using laser light diffraction.

2.1.2 Zeta potential

The obtained data from Table 17B, it was found that zeta potential of submicron emulsion base was around -36.96±12.75 mV (-24.21 to -49.71 mV) and slightly change to -36.48±9.08 (-27.4 to -45.56 mV) after aging for 7 days. The zeta potential of methyl-, ethyl-, propyl and butylparaben submicron emulsion preparations determined at the initial were varied approximately of -19 to -47, -14 to -27, -36 to -42 and -30 to -39 mV, respectively and seemed to be decreased as comparing with the zeta potential of submicron emulsion base. Figure 4 displays the changes in zeta potential of paraben containing submicron emulsion preparations at the initial preparation and after storage for 7 days at ambient temperature. It was found that the zeta potential of most preparations was slightly increased after keeping for 7 days whereas that of butylparaben containing submicron emulsion was decreased.

The zeta potential values of all preparations were negatively charge of fatty acids in which were yields of hydrolytic products of phospholipids and likely to accumulate at the surface of oil droplets (Rydhag and Wilton 1981). Most of alkyl-4-hydroxybenzoate compounds were mostly in their undissociated forms since the pH of emulsion system was less than pKa. So, charge of model drugs did not affect the change in zeta potential. It was probably that the incorporated compound changed in the charge density of boundary layer of oil droplet resulting in decreasing in zeta potential (Washington 1990). During storage period, it is probable that the hydrolysis of oil phase or phospholipids causing the formation of fatty acid and resulting in the enhancement of zeta potential (more negative value). The zeta potential became more negative due to the accumulation of negatively charged ionized carboxyl groups on the surface (Washington and Davis 1987). In addition, this finding also corresponded with the decrease in pH of the same preparations. However, the zeta potential value did not depended upon the lipophilicity and concentration of incorporated compound.

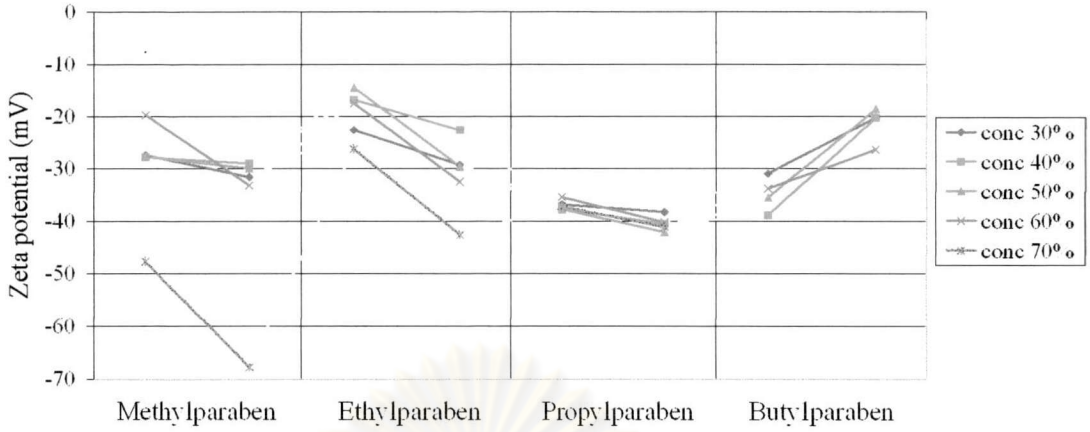


Figure 4 The change in zeta potential of paraben submicron emulsions during storage for 7 days at ambient temperature.

2.1.3 pH

From Table 18B, the initial pH of submicron emulsion base was 5.40±0.34 (5.74 to 5.06) and slightly lower to 5.31±0.35 (5.66 to 4.96) when kept for 7 days. Figure 5 shows pH changes of compound containing submicron emulsion at the initial and after keeping for 7 days at ambient temperature. At initial, the pH values of all preparations were around 5.0 to 6.0 and closed to those of submicron emulsion bases and slightly decreased when keeping for 7 days.

This might indicate that the hydrolysis of oil phase as well as phospholipids occurred resulting in the formation of fatty acid. Such fatty acid could probably interfere the electrical conductivity making the higher value of zeta potential and reduced the pH of submicron emulsion preparations (Washington and Davis 1987; Benita and Levy 1993). However, both of concentrations and lipophilicity did not affect the pH of all preparations.

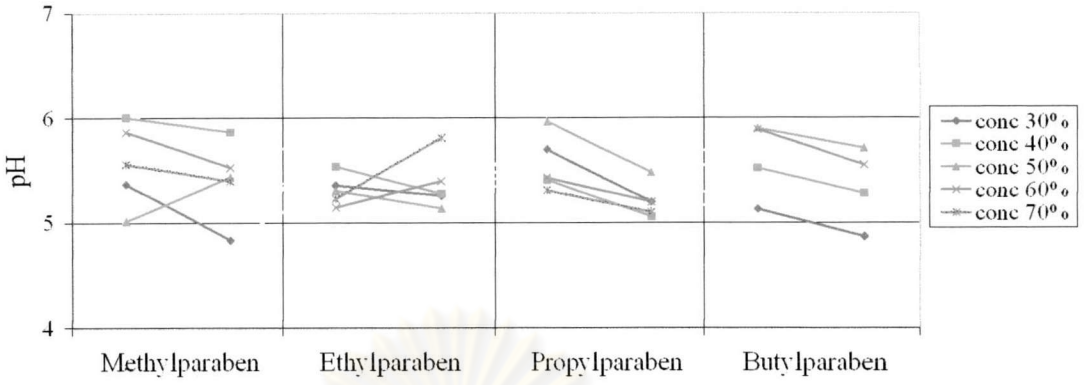


Figure 5 The change in pH of paraben submicron emulsions during storage for 7 days at ambient temperature.

2.2 Extemporaneous addition

This method is to incorporate the investigated compound in the submicron emulsion base in which the preparation was similar to de novo emulsification method. Each model drug was dissolved in 0.45% w/w dimethyl isosorbide and then submicron emulsion base was added in the concentrated drug solution and mixed together.

Dimethyl isosorbide (1, 4: 3, 6 dianhydro-2, 5-di-o-methyl-D-glucitol, Arlasolve® DMI) is an excellent solvent for many poorly water soluble compounds. It is a colourless and practically odourless. The advantage of this solvent is to enhance at least four times of solubility of the muscle relaxant drugs such as methocarbamol, metaxalone, meprobamate and 1-ethylcaramoyl-3-trifluoromethylpyrrholidine comparing with that in PEG-300. In addition, such solutions were contained in intravenous parenteral emulsion giving the synergistic effect when compared with drugs in PEG-300 (US patent 3699230).

However, this method was not suitable for hospitalized routine use due to drug precipitation problem during preparation and storage period. In this study, butylparaben could not be incorporated in submicron emulsion base since the submicron emulsion cracked during mixing. This was due to its higher lipophilicity

and might interact with oil phase and/or oil-water interface disturbing the stability of emulsion system. The comparisons between the physical properties during storage period of submicron emulsion base and compound containing submicron emulsion preparations were determined. The physical properties such as particle size distribution, zeta potential and pH were reported as following:

2.2.1 Particle size and size distribution

The initial effective mean diameter of submicron emulsion base was approximate 267.65±12.55 nm (255.10 to 280.20 nm) while that of methylparaben, ethylparaben and propylparaben submicron emulsions were around 265-302, 254-282 and 285-330 nm, respectively. It was shown that the effective mean diameters of all preparations of any particular compounds in submicron emulsions were slightly increase when compared with their submicron emulsion bases. After keeping for 7 days, the effective mean diameter of methylparaben, ethylparaben and propylparaben submicron emulsion was increase approximate to 270-300, 290-350 and 300-370 nm, respectively. Moreover, the higher lipophilic compound and also its concentrations gave the larger particle size. As shown in Figure 7, it was found that the volume mean diameters of all compounds containing submicron emulsions were higher than that of submicron emulsion bases. After storage for a week, the volume mean diameters of all preparations were increase.

This could be due to the intercalation of compound in the interface making change in packing behavior of phospholipids monolayer resulting in increasing mean particle size (Trotta, Pattarino et al. 2002). In addition, the incorporated compound accumulated at the oil-water interface and also the incident of the hydrolysis of phospholipids during storage period may decrease the reduction of surface tension of emulsifier. Thus, these led to enhance the particle size of compound containing submicron emulsion as comparing with that of submicron emulsion base and after keeping for 7 days.

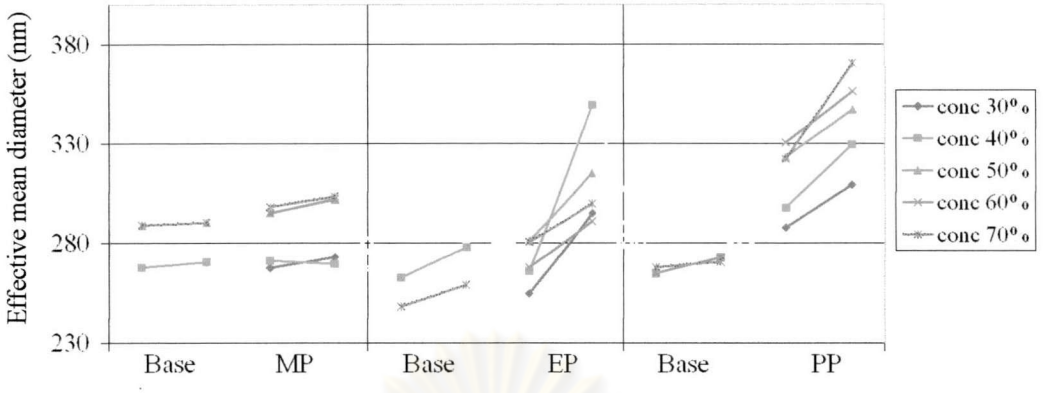


Figure 6 The change in size of paraben submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature measured by using PCS.
(MP=Methylparaben, EP=Ethylparaben, PP=Propylparaben)

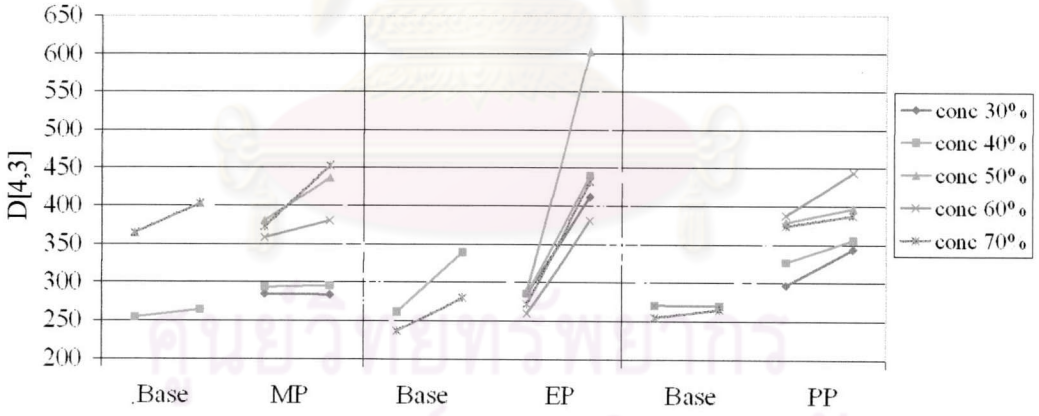


Figure 7 The change in size of paraben submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature measured by using laser light diffraction.
(MP=Methylparaben, EP=Ethylparaben, PP=Propylparaben)

2.2.2 Zeta potential

As given in Figure 8, the initial zeta potential of compound containing submicron emulsions varied around -20 to -40 mV and was lower (less negatively charge) than those of submicron emulsion bases (-24.21 to -49.71 mV). It was possible that the incorporated compounds and also dimethyl isosorbide changed in surface charge density of the oil droplets leading to decreasing of zeta potential. (EL-Gholabzouri, Cabrerizo et al. 1999). In addition, they were slightly increased when keeping for 7 days. It has been attributed to the hydrolysis of oil phase and/or phospholipids leading to produce of free fatty acids and change in zeta potential with age (Washington and Davis 1987).

2.2.3 pH

Figure 9 shows pH values of submicron emulsion base were approximate 5-6, after adding concentrated compound in dimethyl isosorbide, the pH values were decrease by 2 units in all preparations. It was due to the acidic property of solubilizer, dimethyl isosorbide. The pH of methyl- and propylparaben containing submicron emulsions seemed to be unchanged while those of ethylparaben submicron emulsions were lower after keeping for 7 days. It was due to the hydrolysis of phospholipids providing free fatty acid and allowed to decrease in pH. In addition, the desired concentration and lipophilicity did not affect the change in pH of submicron emulsions.

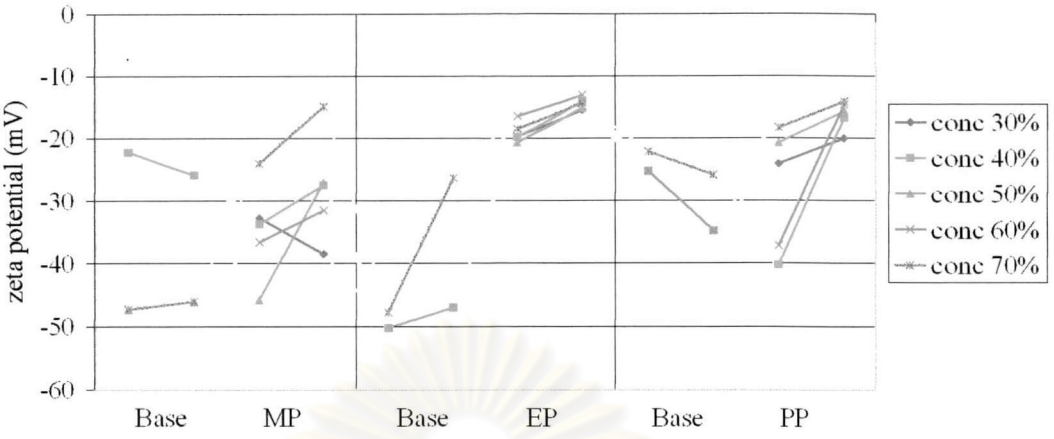


Figure 8 The change in zeta potential of paraben submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature.
(MP=Methylparaben, EP=Ethylparaben, PP=Propylparaben)

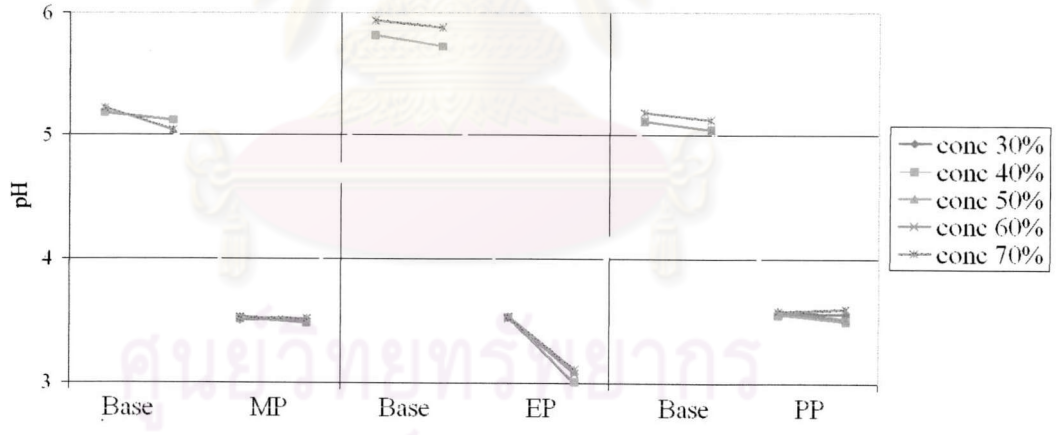


Figure 9 The change in pH of paraben submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature.
(MP=Methylparaben, EP=Ethylparaben, PP=Propylparaben)

2.3 Shaking method

The shaking method is a simple method for incorporating compound or drug in submicron emulsion base. However, ethylparaben could not be prepared by shaking method since the phase separation of submicron emulsion occurred during shaking for a couple hours. According to the moderate lipophilicity of ethylparaben, it was likely to deliver to the inner oil phase and localize at the oil-water interface. This caused the submicron emulsion to break down. The amount of compounds contained in submicron emulsion was examined and presented in Table 2. The content of butylparaben in submicron emulsion was higher than that of the other. It was due to its higher oil solubility and lipophilicity allowed to enhance incorporation in oil droplets and vesicle structures coexisted in aqueous phase.

Table 2 Drug contents of alkyl-4-hydroxybenzoate in submicron emulsion prepared by shaking method.

Drug	Content in submicron emulsion(mg/g)
Methylparaben	1.33±0.02
Propylparaben	1.47±0.19
Butylparaben	2.85±0.37

The physical properties of alkyl-4-hydroxybenzoate submicron emulsion prepared by this method are described below:

2.3.1 Particle size and size distribution

The initial effective mean diameter of submicron emulsion base was 237.40±5.72 nm (231.68 to 243.12 nm) and very slightly increase to 241.4±4.85 nm (236.55 to 246.25 nm) after keeping for 7 days. Figure 10 shows the change in droplet size after storage for 7 days of submicron emulsion base as well as the preparations of paraben submicron emulsion prepared by shaking. The particle size of compounds containing submicron emulsions were higher to 300-400 nm as comparing with submicron emulsion base. After keeping for 7 days, the effective mean diameters

of all preparations were larger than those of the initial. This observation corresponded with the data obtained from laser diffraction technique (Figure 11).

The larger particle size resulted from the incorporated compound which accumulated at oil water interface changing in the emulsifier property e.g. the reduction of surface tension, consequently increased in particle size.

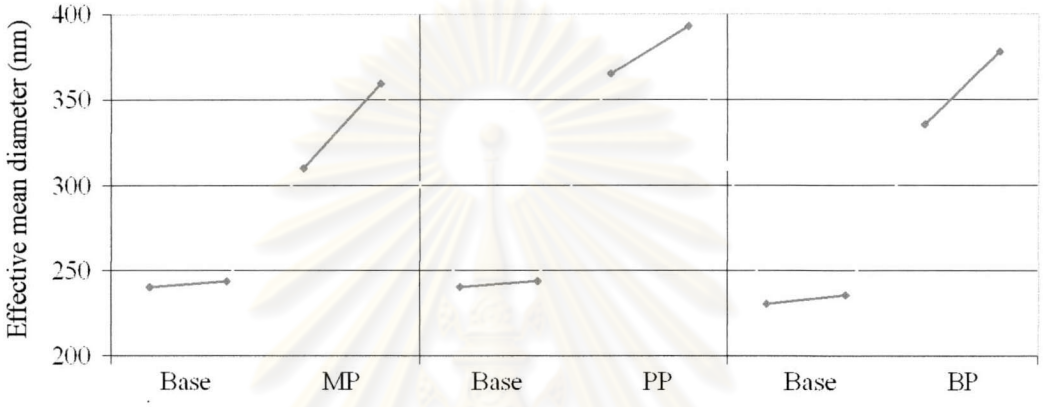


Figure 10 The change in size of alkyl-4-hydroxybenzoate submicron emulsions prepared by shaking during storage for 7 days at ambient temperature measured by using PCS.
(MP=Methylparaben, PP=Propylparaben, BP=Butylparaben)

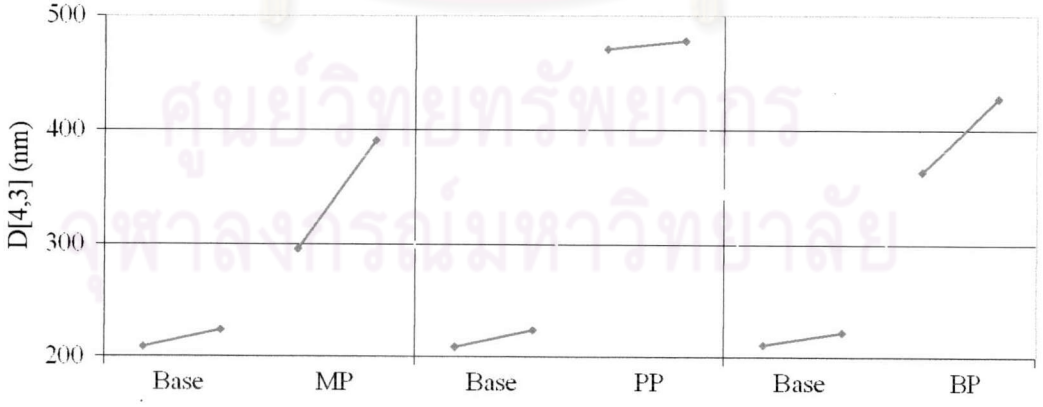


Figure 11 The change in size of alkyl-4-hydroxybenzoate submicron emulsions prepared by shaking during storage for 7 days at ambient temperature measured by using laser light diffraction.
(MP=Methylparaben, PP=Propylparaben, BP=Butylparaben)

2.3.2 Zeta potential

The initial zeta potential of submicron emulsion base was around -43.82±2.96 mV (-40.86 to -46.78 mV). After keeping for 7 days, the zeta potential was slightly increase to -46.47±-0.17 mV (-46.30 to -46.64 mV). The change in zeta potential was displayed in Figure 12. The zeta potential of methylparaben, propylparaben and butylparaben containing submicron emulsion was decreased to 15.10, 17.62 and 19.61, respectively. This caused by the incorporated compounds which might change in charge density surrounding oil droplets resulting in decrease in zeta potential. In addition, the zeta potential was slightly increased after storage for 7 days. It was caused by the hydrolysis of phospholipids given the formation of free fatty acids in emulsion system.

2.3.3 pH

The initial pH of submicron emulsion base was approximate 5.47±0.15 (5.32 to 5.62) and slightly decrease to 5.30±0.16 (5.14 to 5.46) after aging. As shown in Figure 13, pH of submicron emulsion bases and compound containing submicron emulsion preparations were compared. The pH of such preparations was around 5-6 and closed to that prepared by de novo emulsification method and again they were decrease when keeping for 7 days. The hydrolysis of oil phase and/or phospholipids might have occurred and produced the free fatty acids, thereby causing the pH to become lower during aging.

From the results of submicron emulsion characterization, in consideration to the physicochemical properties of drugs such as lipophilicity and also the desired concentration, it is difficult to predict the changes in the physical stability such a mean particle size and particularly zeta potential of this system. Although the zeta potential values were changed when incorporating drug in submicron emulsion and also during storage periods (7 days), all preparations remained stabilized without any remarkable changes in appearance. This was due to a solvation layer surrounded phospholipids head groups given hydration repulsion. The solvation layer has to be dispersed to allow closer contact, which is counteracted by the hydration energy (LeNeveu, Rand et al. 1976).

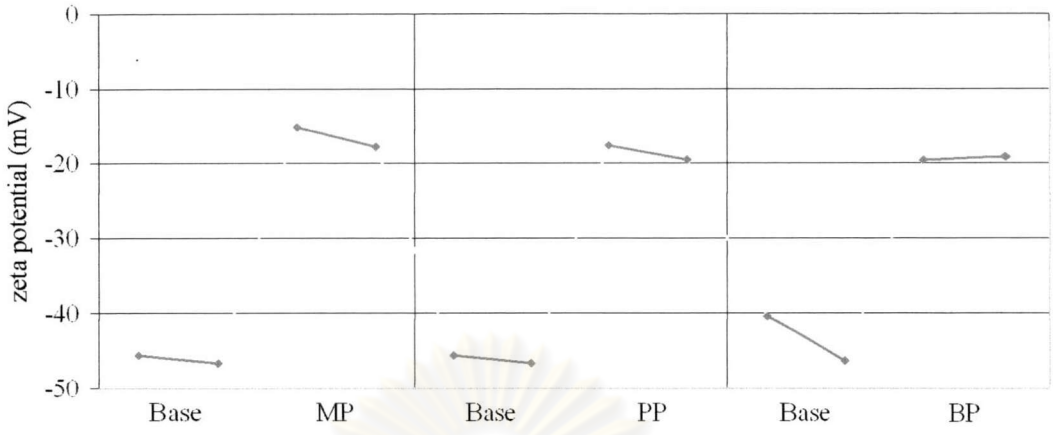


Figure 12 The change in zeta potential of alkyl-4-hydroxybenzoate submicron emulsions prepared by shaking during storage for 7 days at ambient temperature.
(MP=Methylparaben, PP=Propylparaben, BP=Butylparaben)

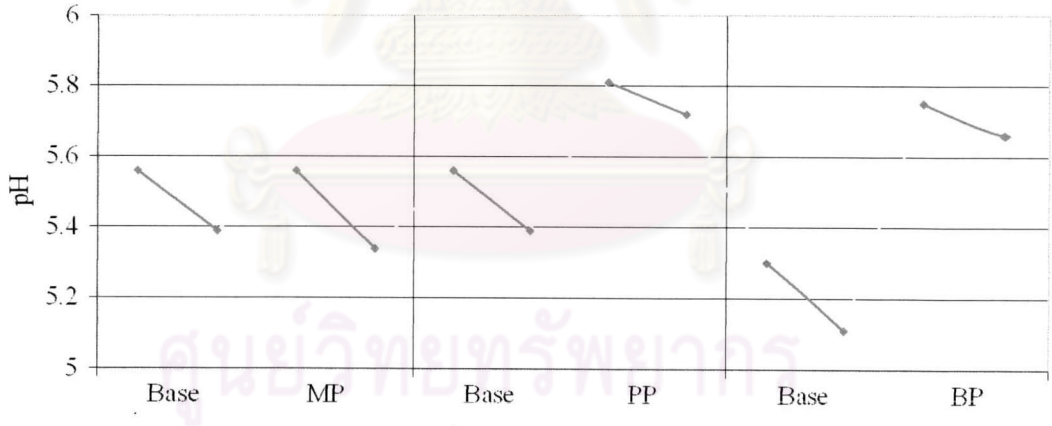


Figure 13 The change in pH of alkyl-4-hydroxybenzoate submicron emulsions prepared by shaking during storage for 7 days at ambient temperature.
(MP=Methylparaben, PP=Propylparaben, BP=Butylparaben)

The comparison of physical properties of alkyl-4-hydroxybenzoate submicron emulsions with different incorporated methods

The physical properties of methyl-, ethyl- and propylparaben submicron emulsions in the concentration of 70% oil saturated solubility prepared by de novo and extemporaneous addition were compared to that of the shaking method and the distinction are presented in Figures 14-16, respectively. De novo emulsification method provided a lower effective mean diameter when a lower lipophilic drug was used such as methylparaben submicron emulsion. The effective mean diameter prepared by extemporaneous addition was closed to that of the shaking method. In addition, the formulations prepared by de novo emulsification gave a high zeta potential. The pH of the preparations prepared by de novo emulsification was similar to that of shaking method while the preparations obtained from extemporaneous addition allowed the lower pH.

After preparing, drug contents of submicron emulsion preparations at various concentrations were examined and data were shown in Figure 17. It was found that the recovery of content was varied around 74-116% and 79-103% as preparing by de novo emulsification and extemporaneous addition, respectively. To compare the drug content in submicron emulsion among different incorporated methods, the percentage drug content of emulsion prepared by de novo emulsification and extemporaneous addition (as 70 % drug saturated oil solubility) were compared with that of prepared by shaking method and data are shown in Table 3. It was found that loading efficacy of methylparaben submicron emulsion prepared by shaking method was slightly higher than that of the others. On the other hand, the contents of propylparaben and butylparaben prepared by shaking method were lower than those prepared by de novo and extemporaneous method.

Table 3 Comparison of % paraben loading between different incorporated methods.

Compound	%drug content in submicron emulsion		
	De novo emulsification (70 %drug saturated in oil phase) (n=1)	Extemporaneous addition (70 %drug saturated in oil phase) (n=1)	By shaking (n=3)
Methyparaben	0.10	0.08	0.13±0.002
Ethylparaben	0.15	0.13	-
Propylparaben	0.22	0.20	0.15±0.02
Butylparaben	0.33*	**	0.28±0.04

*60%saturated in oil phase prepared by de novo emulsification

**could not be prepared due to phase separation

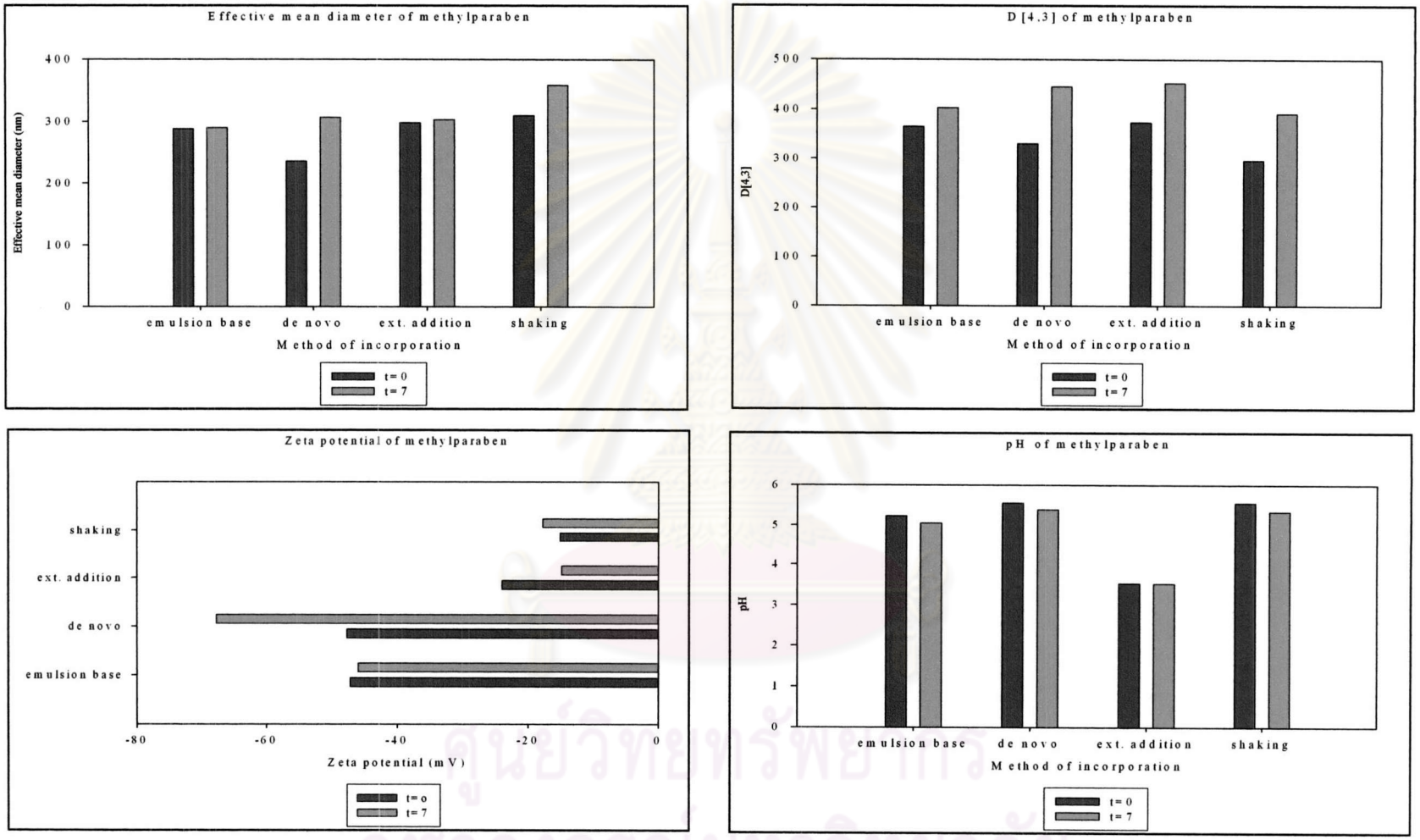


Figure 14 The comparison of physical properties of methylparaben submicron emulsion prepared by different methods.

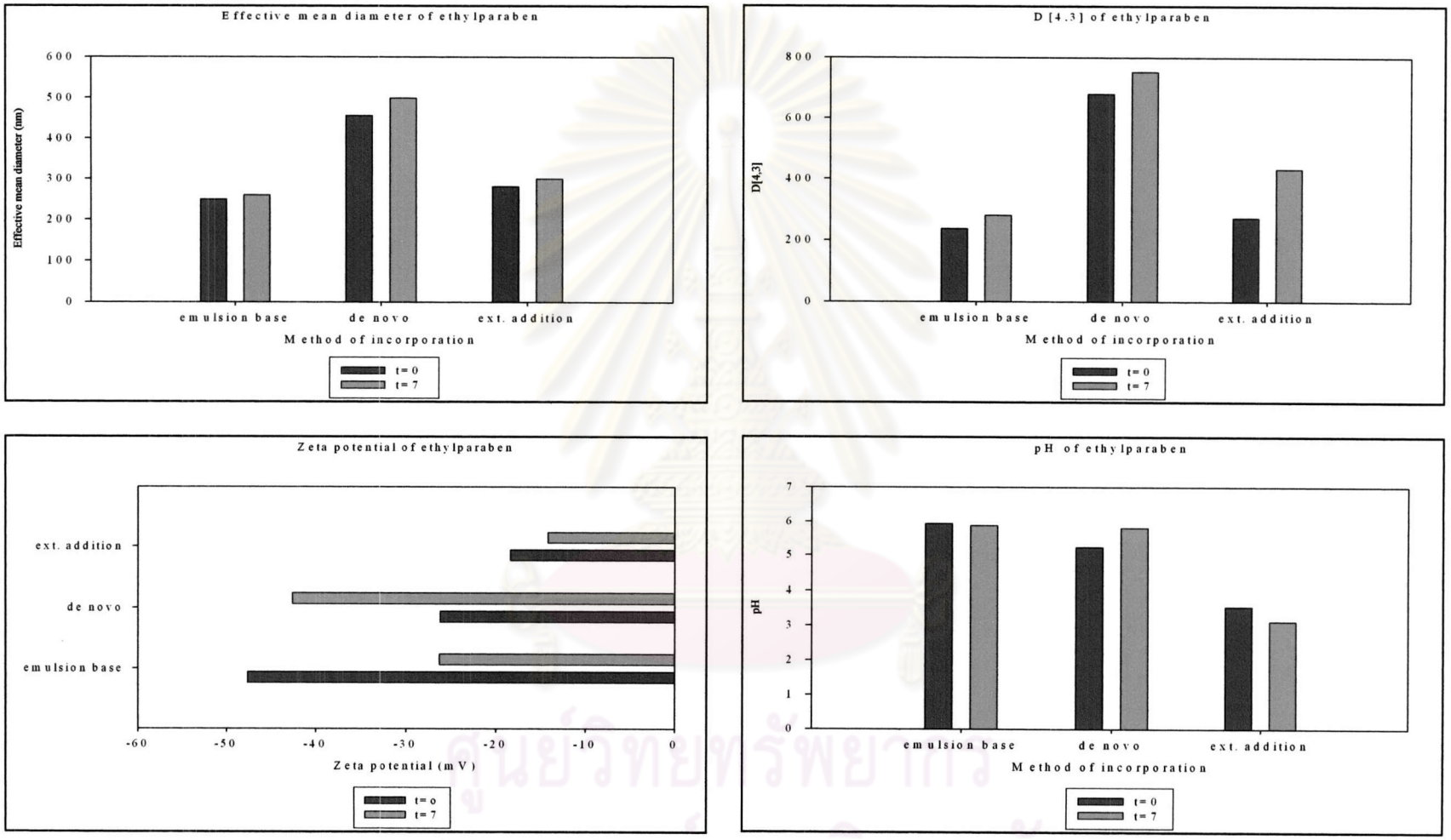


Figure 15 The comparison of physical properties of ethylparaben submicron emulsion prepared by different methods.

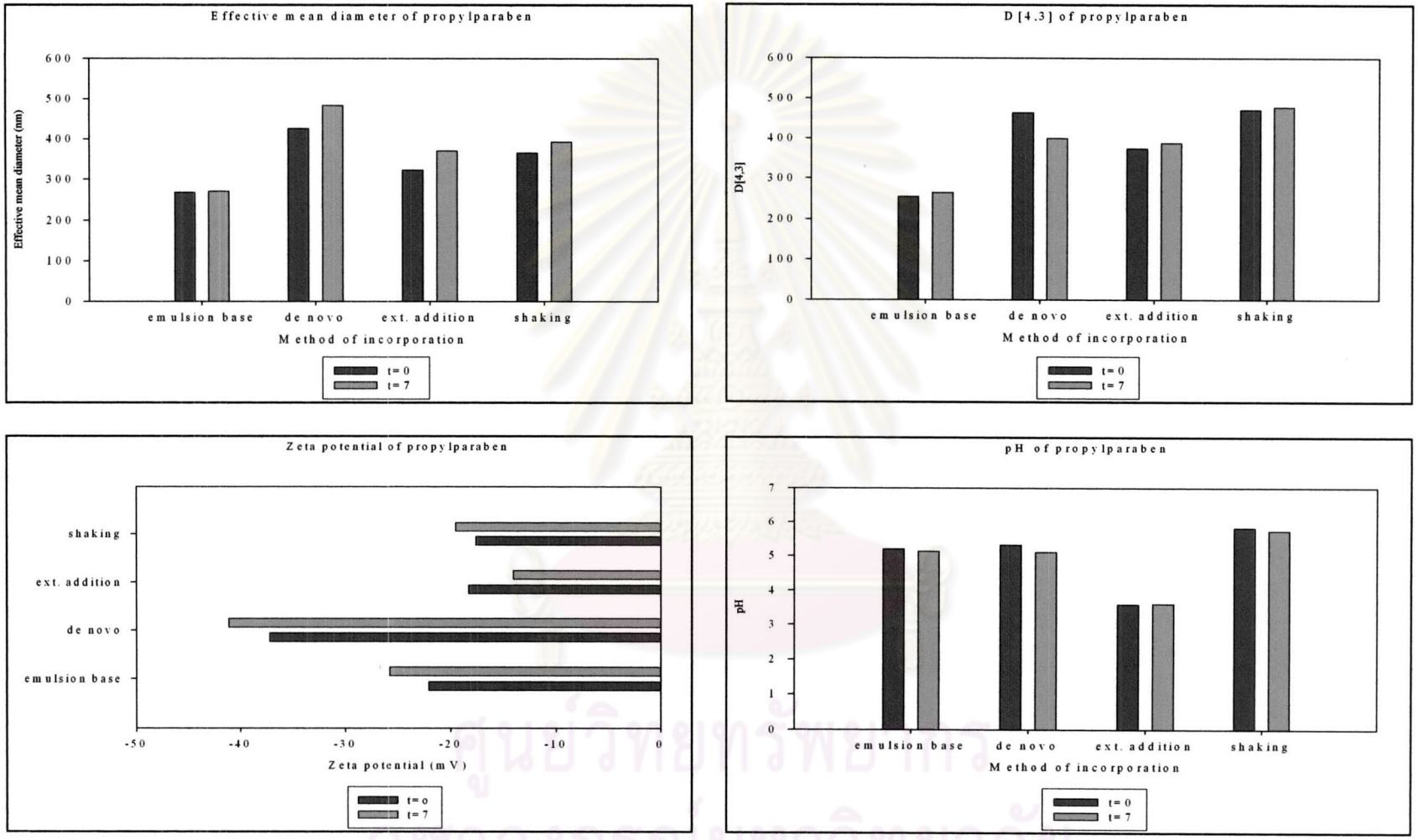
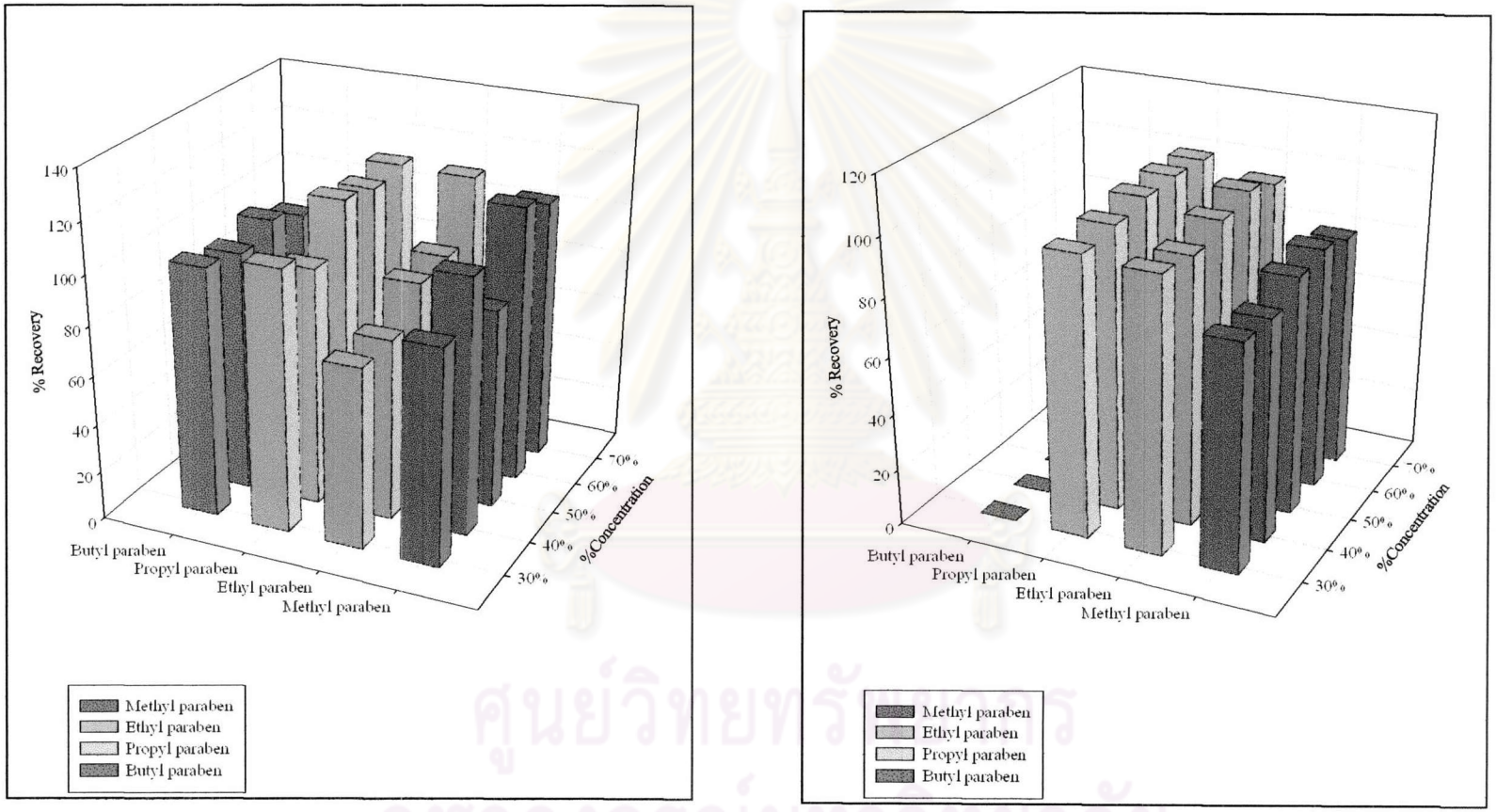


Figure 16 The comparison of physical properties of propylparaben submicron emulsion prepared by different methods.



De novo emulsification **Extemporaneous addition**

Figure 17 Comparison of the percentage recovery content of alkyl-4-hydroxybenzoate between different incorporated methods.

III The effects of lipophilicity, incorporated compound concentration and incorporation method on alkyl-4-hydroxybenzoates distribution into various phases of submicron emulsion

The submicron emulsions for intravenous administration are oil in water emulsions and defined as an oil droplet is dispersed in an aqueous phase and stabilized with phospholipids. The theoretical amount of phospholipids which acted as an emulsifier by forming the monomolecular layer covering the oil droplets could be calculated. Typically a phospholipid stabilized intravenous emulsion has an oil droplet size of around 250 nm. The density of soybean oil is 0.919 g/ml. For 10%w/w of oil phase in submicron emulsion, the number of oil droplets is $65.29/\pi d^3$ droplets and given the corresponding surface area of 2.6×10^6 cm^2. Assuming the cross sectional area per molecule of Epikuron® 200 and its average molecular weight are around 59 $Å^2$ and 770 Da, respectively. A monolayer from 1.2 g phospholipids/100 g emulsion would occupy 5.54×10^6 cm^2. This suggests that there are adequate phospholipids in the submicron emulsion to form a monolayer sufficient to stabilize the oil interface.

The excess of phospholipids formes complicated structures which have been examined by several workers. In 1981, Rydhag and Wilton reported that phospholipids formed liquid crystalline phases and showed lamellar layers at the oil-water interface which enhance emulsion stability. Groves et al. (1985) employed the electron microscopy to examine those structures in 10% Intralipid®. The freeze fractured technique could not indicate the formation of multilayer around the oil droplets. On the other hand, negative staining of undiluted emulsion indicated the presence of multilamellar structure between oil droplets. Moreover, the dynamic exchange between phospholipid molecules at the oil droplets surface and the multilamellar structure might occur especially under the heat stress condition so it was believed that the multilamellar structure acts as a reservoir of stabilizer in this emulsion system. However, the examination of Westesen and Wehler (1992, 1993) disagreed with the previous study. They characterized the infrastructure of phospholipids which stabilize intravenous oil in water emulsion using the combined techniques of PCS, electron microscopy, NMR spectroscopy and small-angle X-ray scattering. They found that the excess phospholipids aggregated as vesicles

(liposomes), which remained in the aqueous phase and might be able to adsorb on the interface. In addition, the particle with bi-and/or oligolayer structures and also double emulsion droplets could be deduced. The results of their studies agreed with those of (Rotenberg, Rubin et al. 1991; Westesen and Wehler 1992; Westesen and Wehler 1993; Ferezou, Lai et al. 1994; Ferezou, Gulik et al. 2001). They found that the excess of phospholipids mostly formed unilamellar liposomes and coexisted in aqueous phase of submicron emulsion. On the other hand, large unilamellar vesicles, micellar structure, and liquid crystal phase were not presented in o/w intravenous emulsion. The formation of small unilamellar vesicles were presented instead of liquid crystalline phases since the energy input during homogenization is high to separate large phospholipids bilayer and multilayer structure from each other and to disrupt them in the continuous phase (Westesen and Wehler 1993).

High speed centrifugation technique was use in this study for phase separation of submicron emulsion. This technique as described by Grove et al. (1985) and Ferezou et al. (1994), was sufficient for separation of oil droplets and most of the aqueous phase. During prolonged centrifugation (6 hr) of model drug containing submicron emulsions, the breakdown of emulsions was achieved and then four phases such as oil phase, phospholipids rich phase, aqueous phase and mesophase were obtained. Figure 18 illustrates the various phases of submicron emulsion after ultracentrifugation. The yellowish oil phase floated on the top of the centrifuged tube while the creamy layer which mainly contained phospholipids was in the middle between oily and aqueous phase. The creamy layer may contain intact vesicles which was observed under the cross-polarized microscope (Figure 19B). It was indicated that the typical birefringence of Maltese crosses from the multilamellar phase can be observed in phospholipids rich phase. The strong coalescence caused the formation of pellet and remained at the bottom of the centrifuged tube. To prove a multilamellar structure for the pellet, the cross-polarized microscopic was employed and shown in Figure 19C. This observation agreed with the study of Grove et al. (1985). The authors observed separation of a yellow layer out from the cream layer with time and also sediment at the bottom of the tube. It is known that liposomes are easily concentrated from the aqueous supernatant by high speed centrifugation to form a pellet. So, it is clearly seen that the vesicle structures in the pellet could only come from the phospholipids. The structure of pellets indicated that the excess of phospholipids was presented in the submicron emulsion system, so it was named

mesophase. Therefore, drug in submicron emulsion may distribute through various phases of submicron emulsion which mentioned above. Figure 20 showed the schematic possibly illustration of drug localization in various phases of submicron emulsion.

The amount of model drug loss after ultracentrifugation was assessed in the term of % recovery by comparing the analytical amount of each phase with the initial theoretical amount. Figure 21 shows the % recovery when using different drug incorporation methods. The recovery was approximate 62-97%, 80-100% and 80-98% for preparing by de novo emulsification, extemporaneous addition and shaking method, respectively. It seemed that the analysis of drug distribution in various phases of submicron emulsion was acceptable. Excepted for the percentage recovery of butylparaben containing submicron emulsions prepared by de novo emulsification were around 62-68%, this might be lost during phase harvesting after ultracentrifugation.

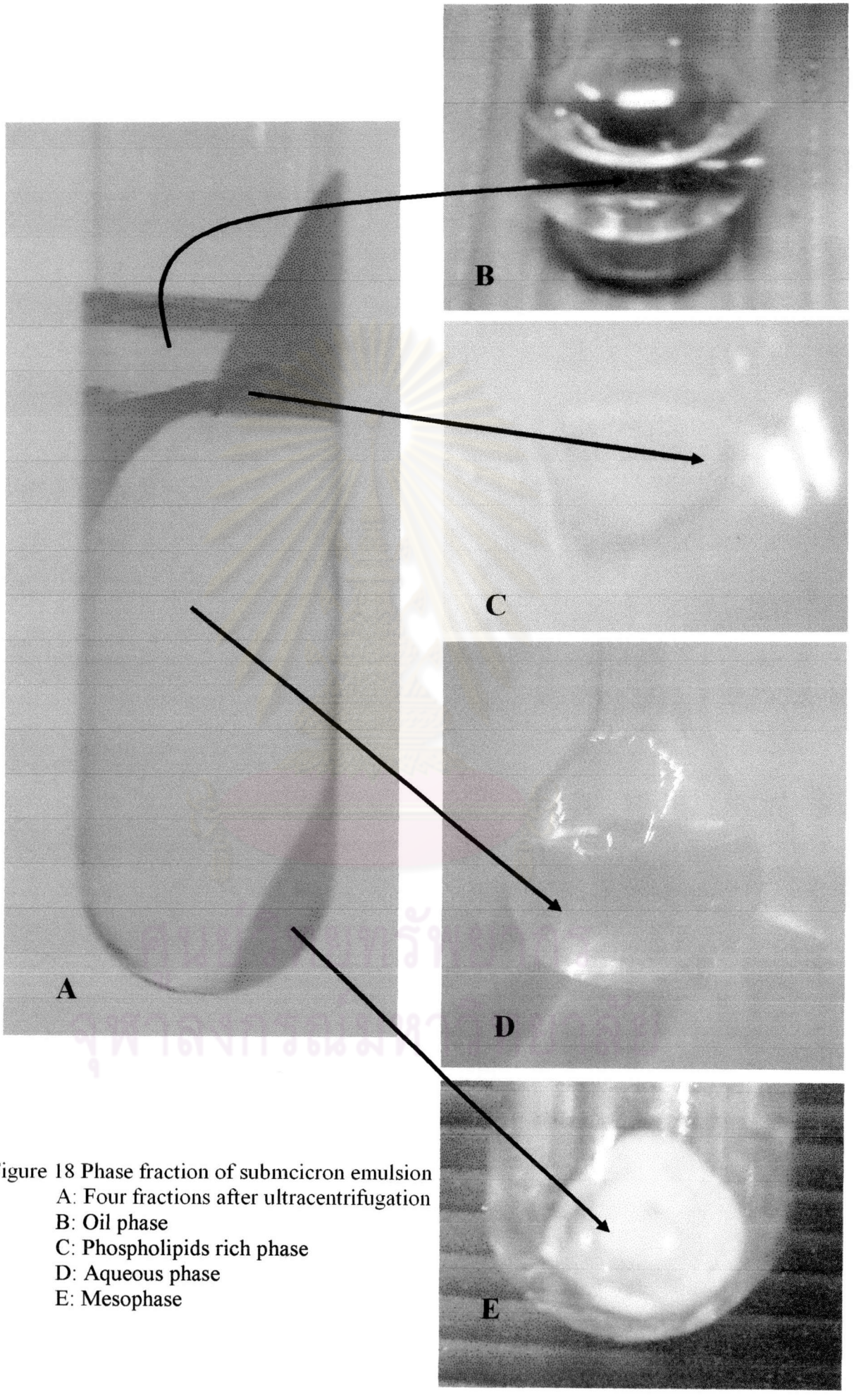


Figure 18 Phase fraction of submcicron emulsion
A: Four fractions after ultracentrifugation
B: Oil phase
C: Phospholipids rich phase
D: Aqueous phase
E: Mesophase

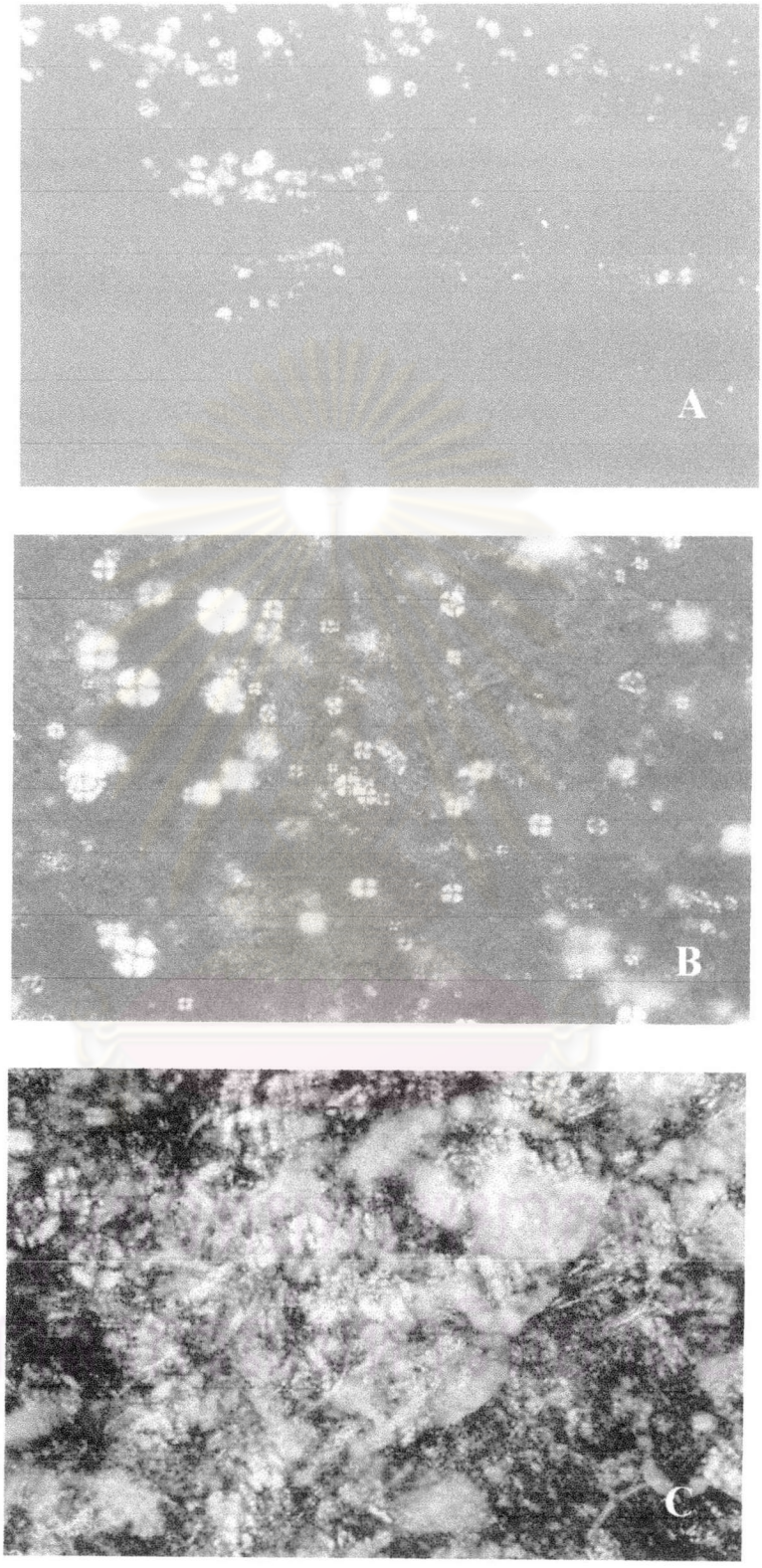


Figure 19 The cross-polarized light microscopic photography of centrifuged fraction (A) oil phase, (B) phospholipids rich phase and (C) mesophase.

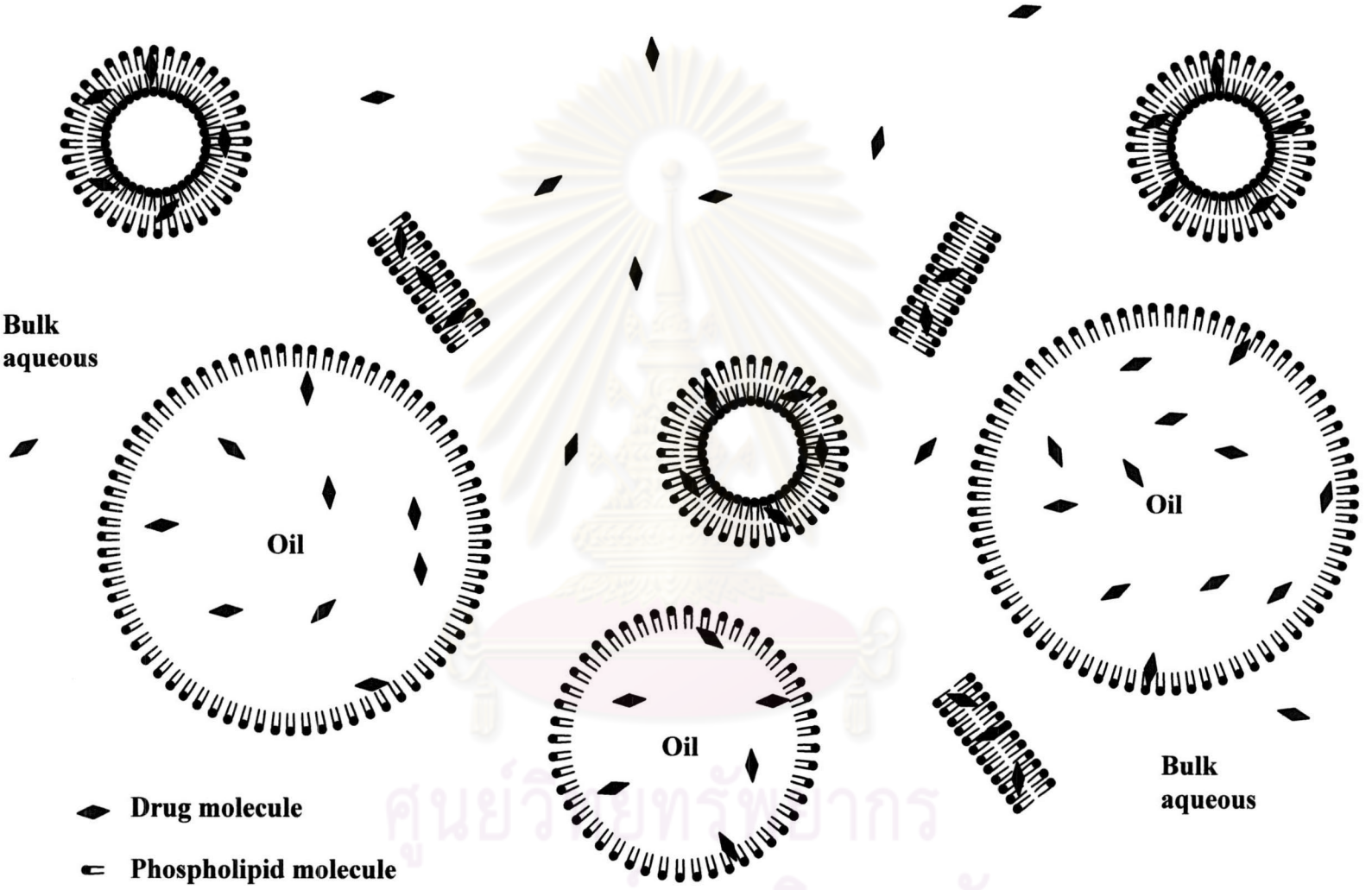
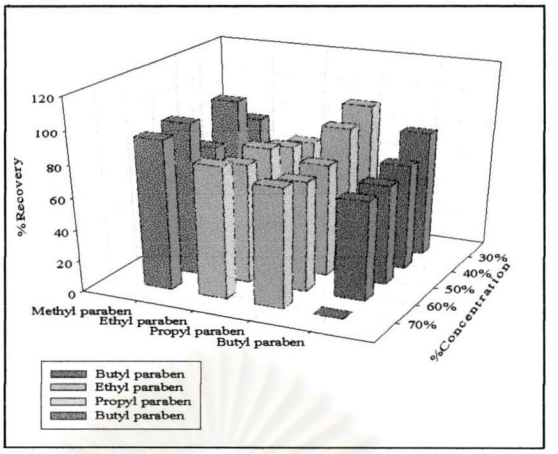
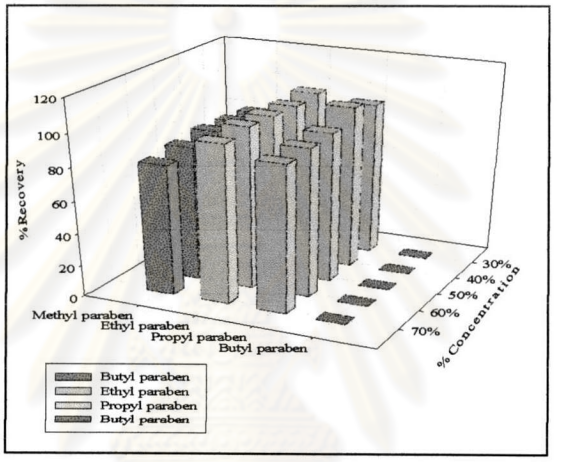


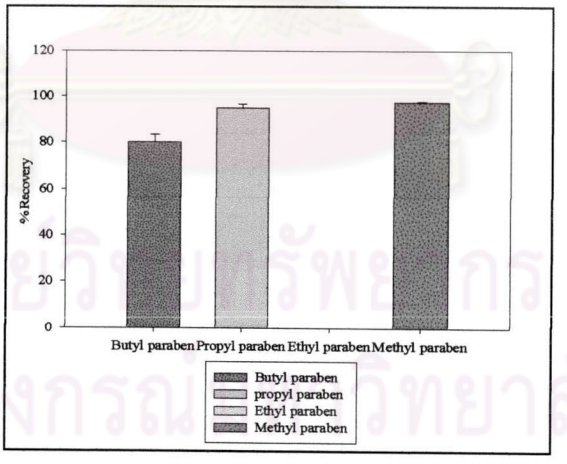
Figure 20 The illustration of oil in water submicron emulsion infrastructure showing the different structure originated from the excess of phospholipids.



A



B



C

Figure 21 Comparison of %mean recovery of alkyl-4-hydroxybenzoate between different incorporated methods.

A: De novo emulsification

B: Extemporaneous addition

C: Shaking

The distribution of alkyl-4-hydroxybenzoate containing submicron emulsions after ultracentrifugation

In this study, the effects of physicochemical properties and concentrations on the drug distribution had been studied. The pKa of methyl-, ethyl-, propyl- and butylparaben were 7.65, 7.68, 7.75 and 8.47, respectively (Sunderland and Watts 1984; Hansch 1990). The pH of these compounds containing submicron emulsion preparations was around 5-6. Most of alkyl-4-hydroxybenzoate compounds, therefore, were mostly in their undissociated forms. This led to obtain the greatest extent of partition between oil and water and to neglect the distribution of anions in emulsion systems. In addition, the method of incorporations had to be considered. Drug distribution of emulsion prepared by these three methods was individually reported and discussed.

4.1 De novo emulsification

Figure 22-25 presents the influence of physicochemical properties (aqueous solubility, oil solubility and oil-water partition coefficient) on the distribution of alkyl-4-hydroxybenzoate in oil phase, phospholipids rich phase, aqueous phase and mesophase, respectively. There were significant differences in distribution of compound in each phase of submicron emulsion and depended on the type of compounds. From the data obtained, the distribution in oil phase was in the increasing order of butyl->propyl->ethyl>methylparaben while the distribution was contrary in aqueous phase. The distribution of these four compounds in either oil phase or aqueous phase, therefore, depended on their chemical structure and polarity. However, the distribution of all compounds in the phospholipids rich phase were widely varied in the range of 11-22%, 15-30%, 15-40% and 5-26% for methyl-, ethyl-, propyl- and butylparaben, respectively. Ethylparaben and propylparaben seemed to be more accumulated at the phospholipids rich phase than the other compounds. It was due to their moderate lipophilicity and likely to intercalate at the oil-water interface allowing the interference at the monolayer film around the oil droplets resulting in larger particle size (Figures 3) and consequently making phase separation. The ionic interactions between theses compounds and charged groups on the phospholipids could be ruled out because at pH of emulsion preparation (pH 5-6), alkyl-4-hydroxybenzoate were unionized and no charge interaction between

phospholipids and alkyl-4-hydroxybenzoate molecule. It is suggested that the observed behavior was the result of hydrophobic interactions between alkyl-4-hydroxybenzoate compounds and the structures created when the phospholipids is adsorbed at the oil-water interface. The distributions of all drugs in mesophase were also varied and the higher lipophilic compounds e.g. ethyl-, propyl- and butylparaben were likely to be located at this phase.

Figure 26 shows the influence of concentration on the distribution of alkyl-4-hydroxybenzoate in various phases of submicron emulsion. The concentrations did not affect the distributions of methylparaben, propylparaben and butylparaben in oil phase, phospholipids rich phase, aqueous phase and mesophase ($p>0.05$). According to the highest and the lowest lipophilic compounds, propylparaben, butylparaben and methylparaben, respectively, they gave the saturated concentration in each phase and their distributions were unchanged despite the higher drug incorporation. However, the concentration of ethylparaben affected its distribution in every phase of submicron emulsion ($p<0.05$). It was found that the higher concentration of ethylparaben, the higher distribution in oil phase and phospholipids rich phase was observed. On the other hand, the higher concentration of ethylparaben gave its lower distribution in aqueous phase and mesophase. This observation indicated that the concentration influenced on the distribution of the moderate lipophilic compound like ethylparaben which could distribute through every portion of submicron emulsion resulting in the incident of unsaturated concentration in various phases. This incident led to influence on the drug distribution when the higher concentration of ethyparaben was incorporated.

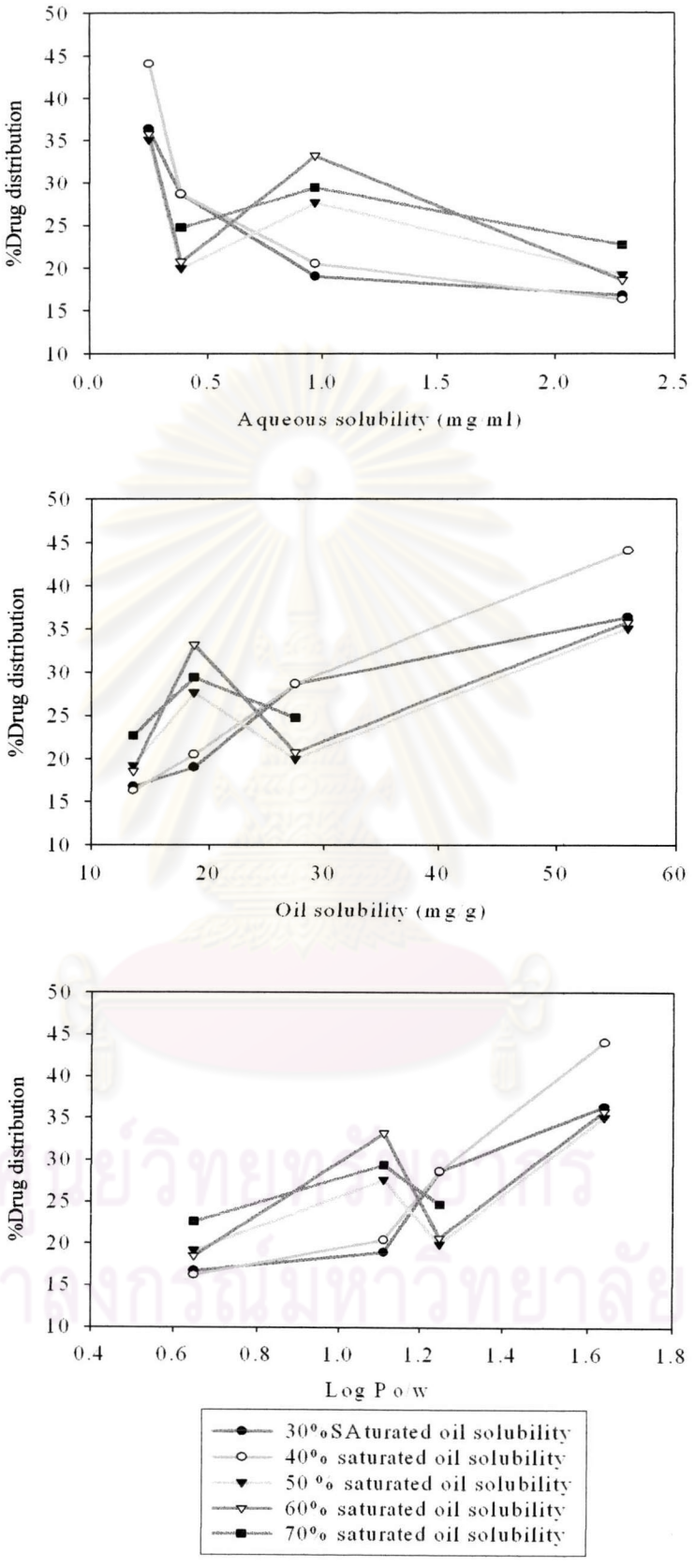


Figure 22 The effect of physicochemical properties on the distribution of alky-4-hydroxybenzoate in oil phase of submicron emulsion prepared by de novo emulsification.

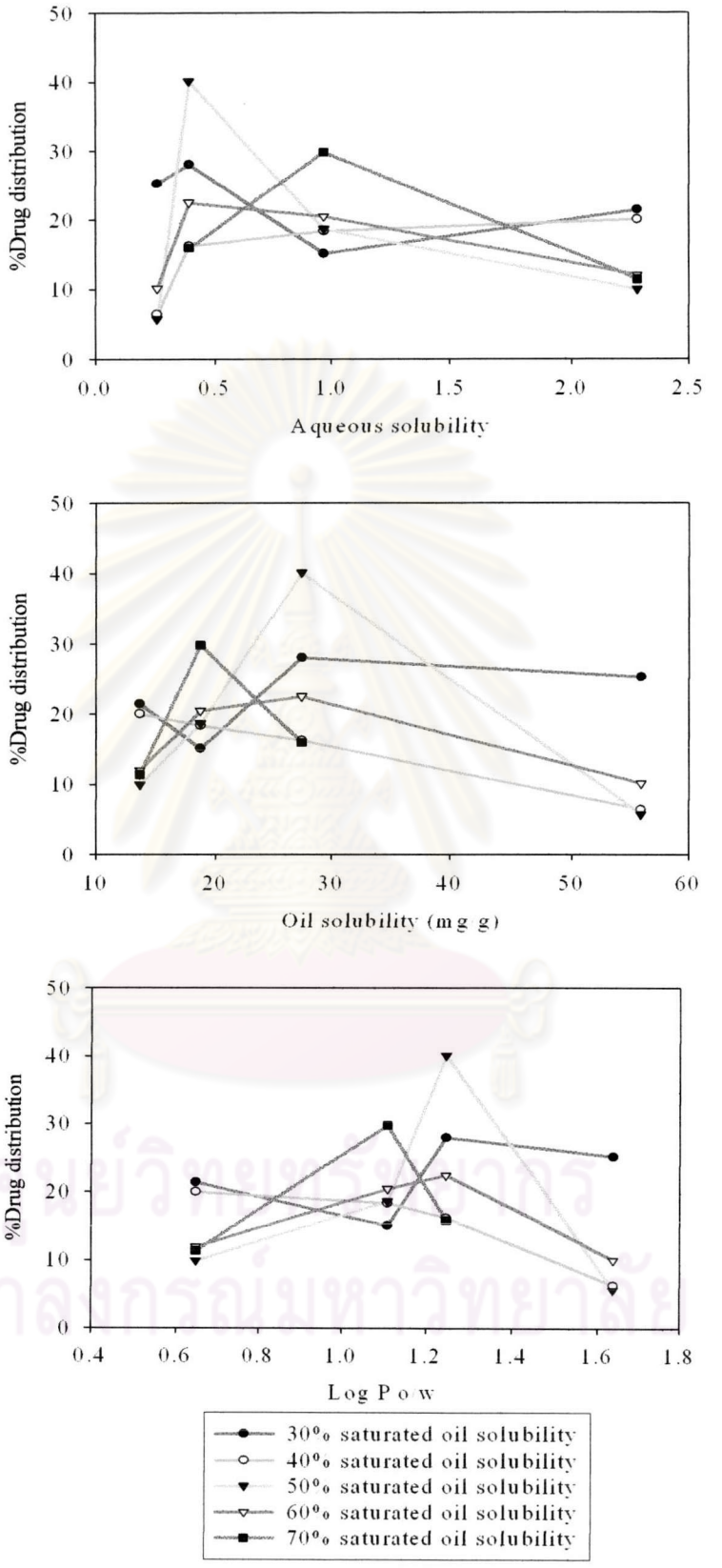


Figure 23 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in PC rich phase of submicron emulsion prepared by de novo emulsification.

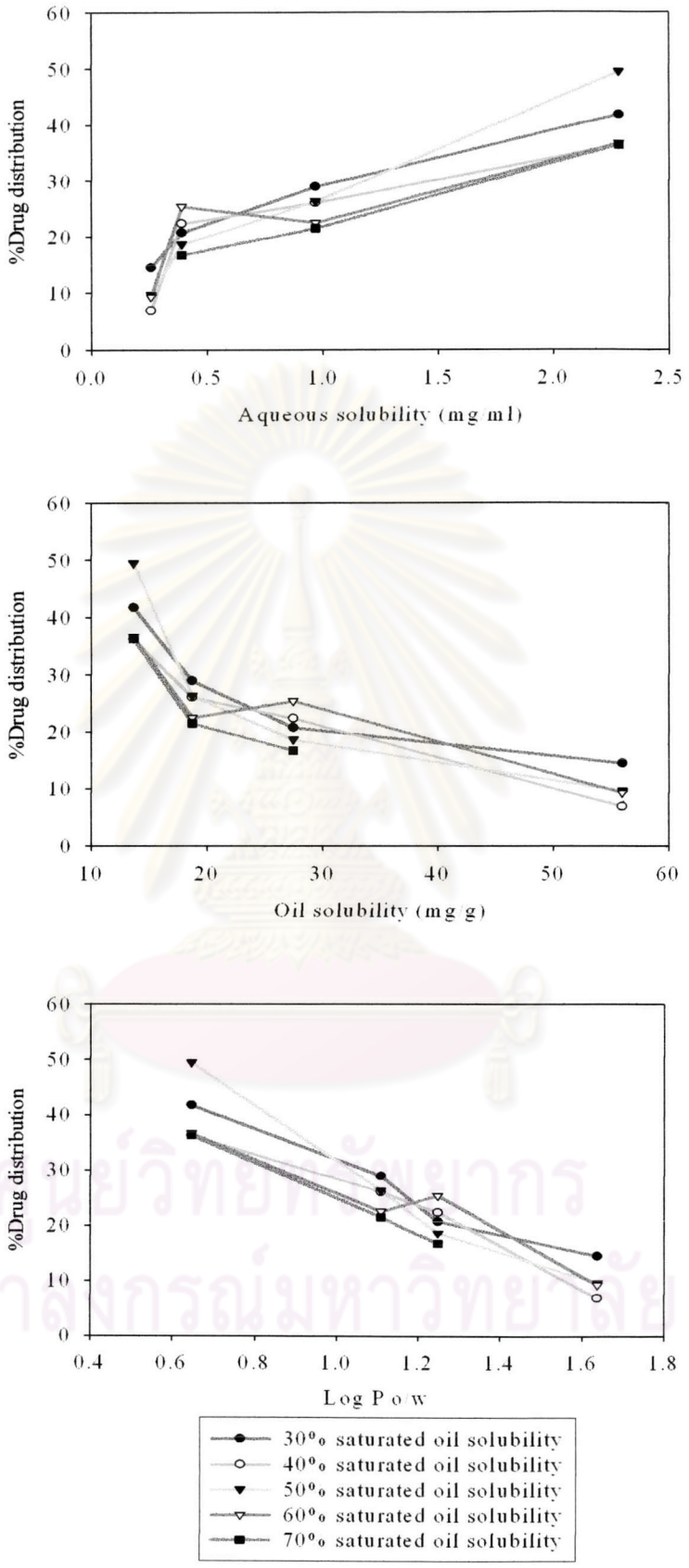


Figure 24 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in aqueous phase of submicron emulsion prepared by de novo emulsification.

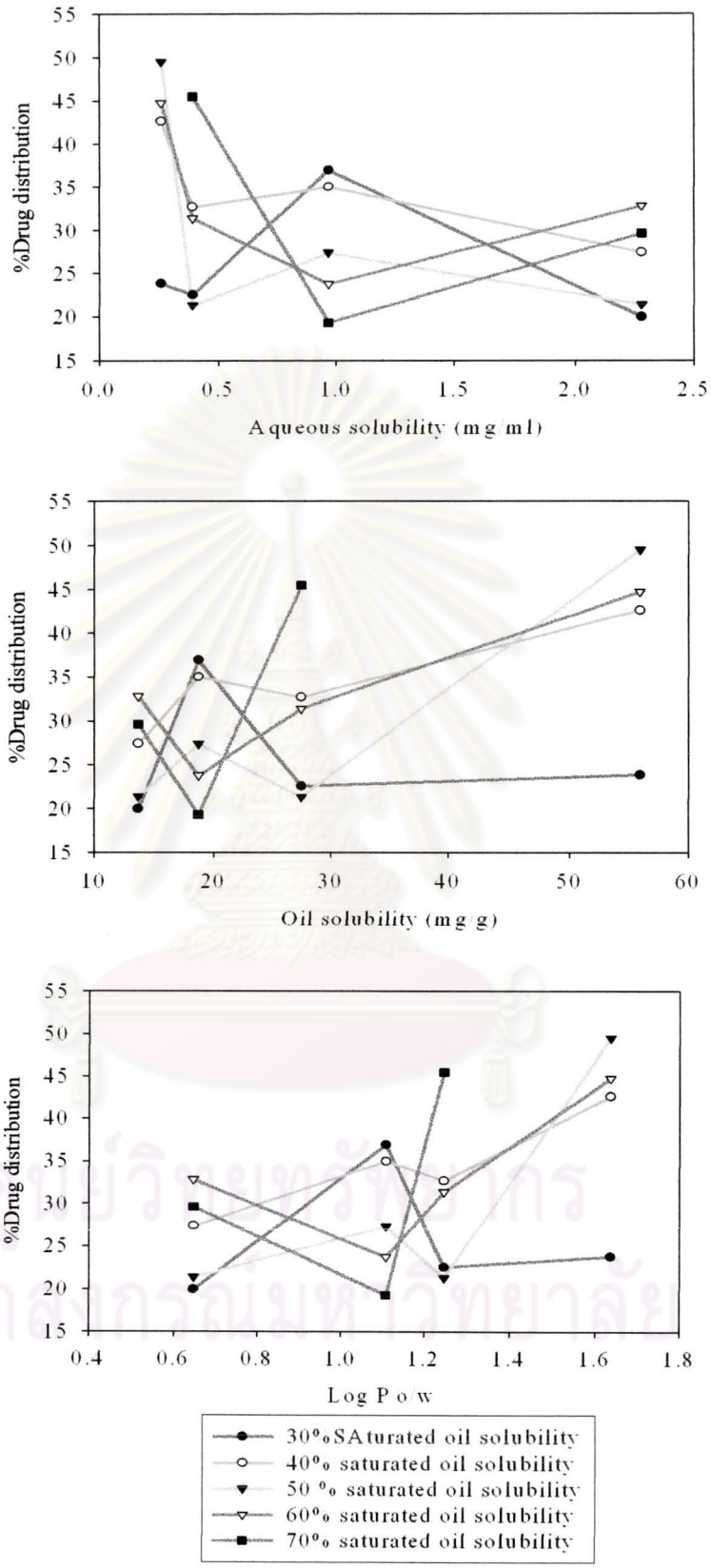


Figure 25 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in mesophase of submicron emulsion prepared by de novo emulsification.

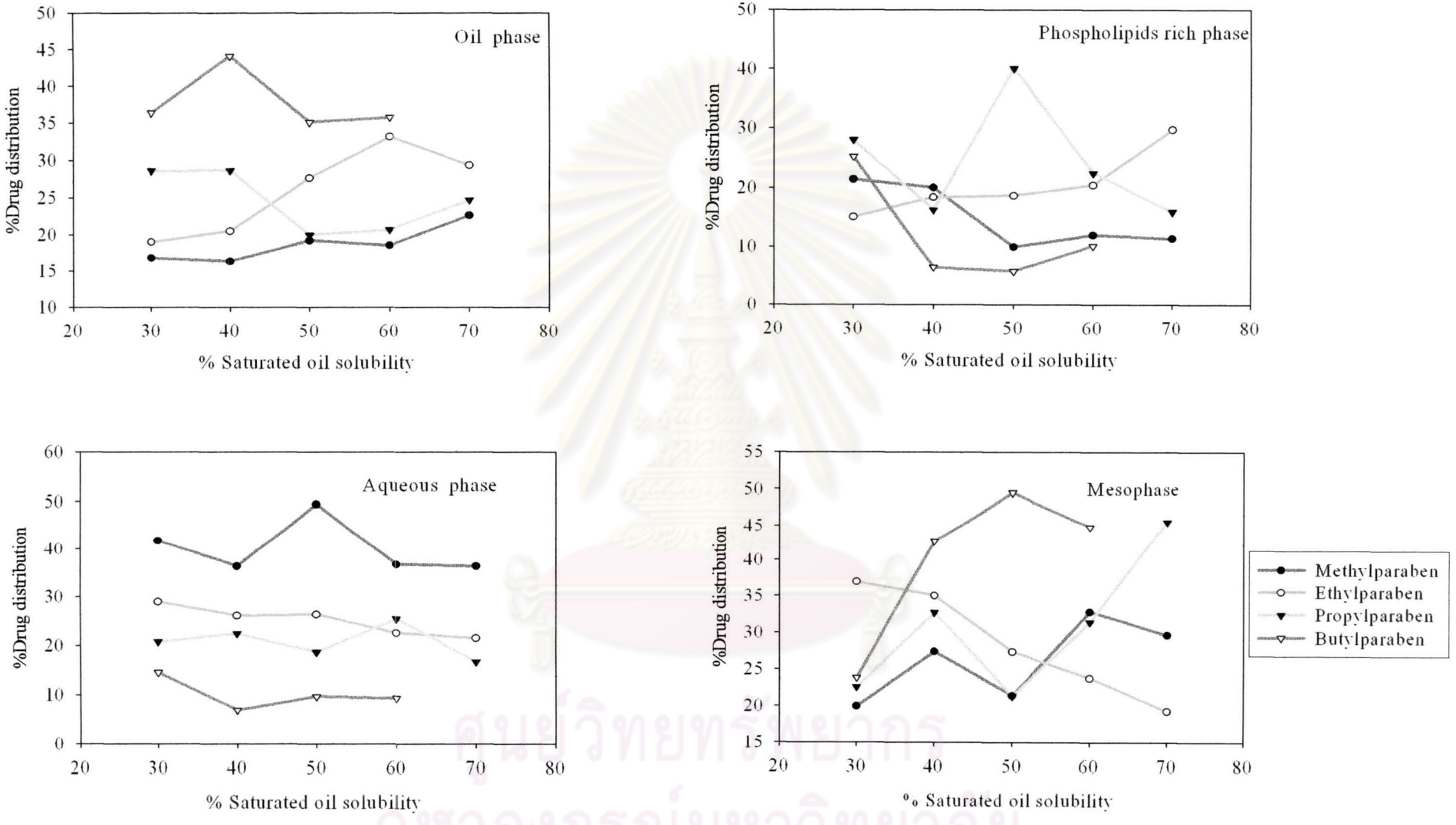


Figure 26 The effect of concentrations on the distribution of alkyl-4-hydroxybenzoate in various phases of submicron emulsion prepared by de novo emulsification.

4.2 Extemporaneous addition

As displayed in Figure 27 and 29, the distribution tendency of four compounds in the oil phase and aqueous phase were contrary. The increasing distribution in oil phase was in the order of propyl-, ethyl- and methylparaben whereas this observation was contrastive in aqueous phase. Propylparaben was mostly localized at the interface. However, all compounds were likely to be deposited in the mesophase.

The effect of concentration on the distribution of alkyl-4-hydroxybenzoate was presented in Figure 31. It was found that the concentration of methylparaben and propylparaben influenced on their distributions in oil phase and mesophase ($p<0.05$). It was due to the presence of liposomes structure in mesophase given drug solubility enhancement. This led to the unsaturated concentration of drug in mesophase, thus, the increasing of drug incorporation, the distributions in oil phase and mesophase were increase. However, the concentration did not affect the distribution of methylparaben, ethylparaben and propylparaben in phospholipids rich phase and aqueous phase ($p>0.05$), this might result from their saturated concentrations in both phases. So, the increasing in drug incorporation did not change in the distribution of all three drugs in phospholipids rich phase and aqueous phase.

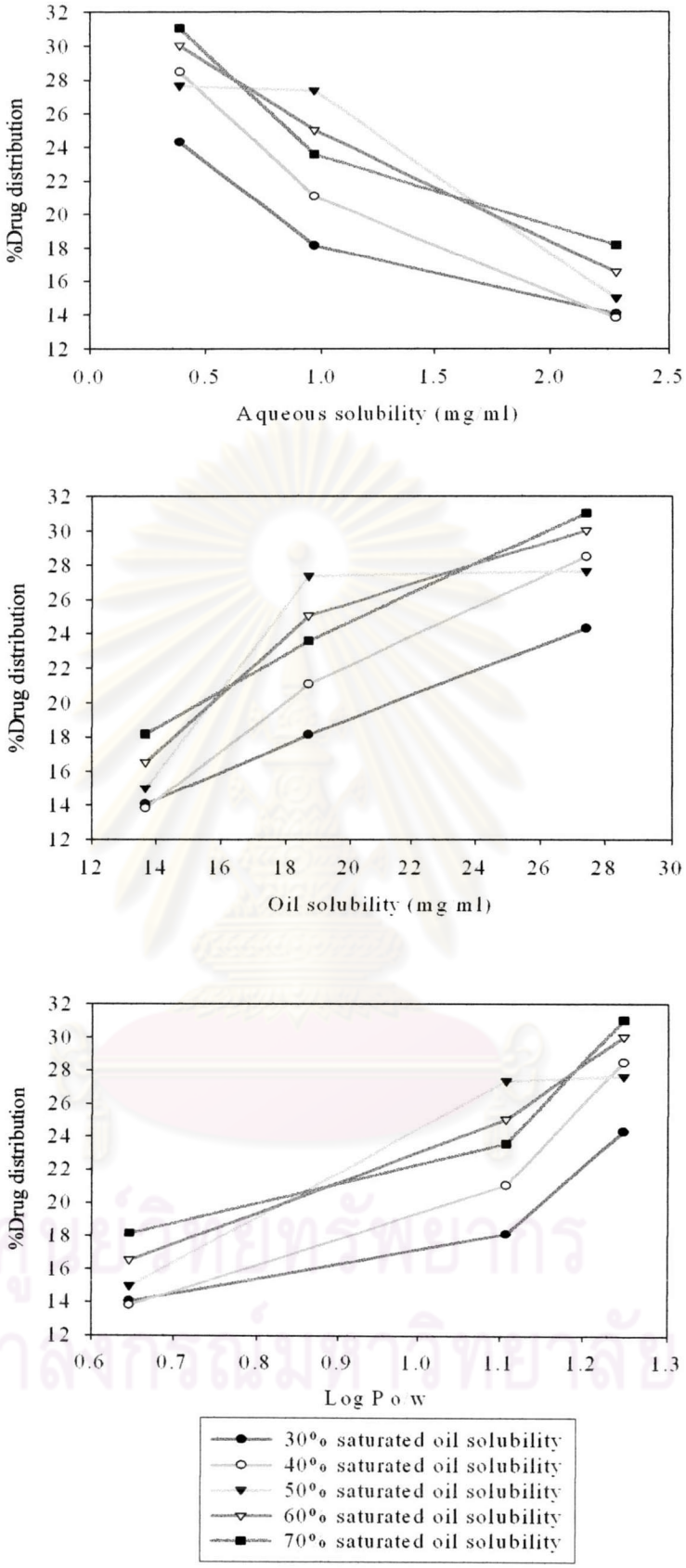


Figure 27 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in oil phase of submicron emulsion prepared by extemporaneous addition.

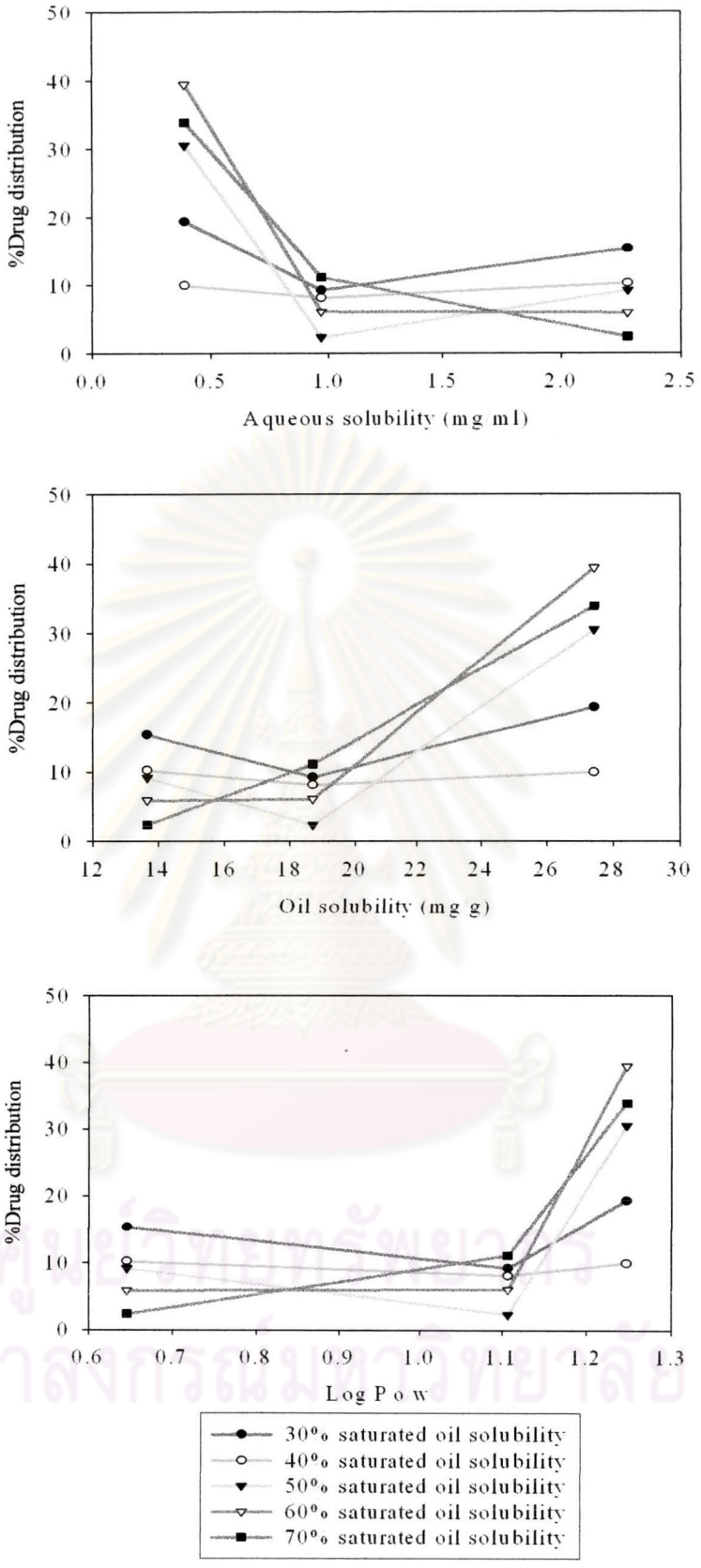


Figure 28 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in PC rich phase of submicron emulsion prepared by extemporaneous addition.

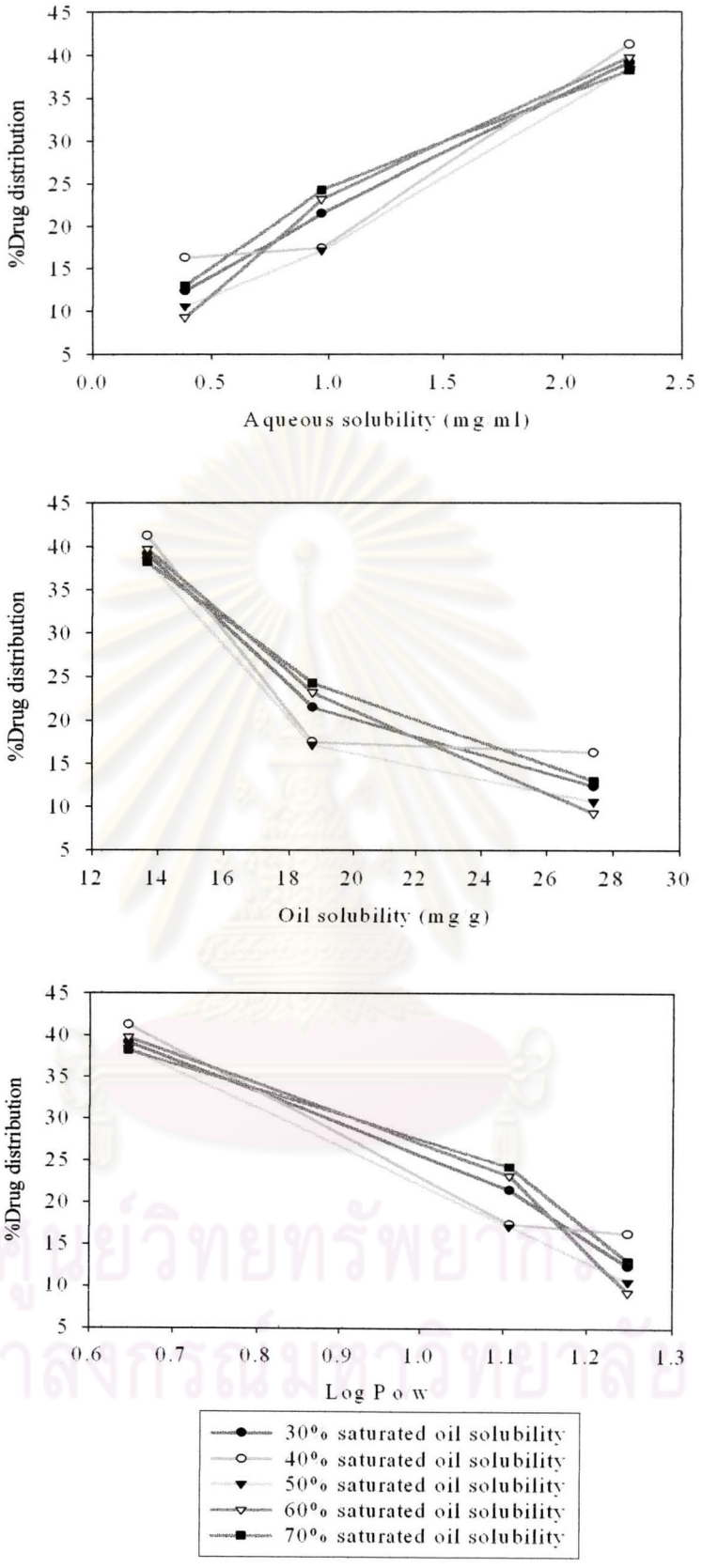


Figure 29 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in aqueous phase of submicron emulsion prepared by extemporaneous addition.

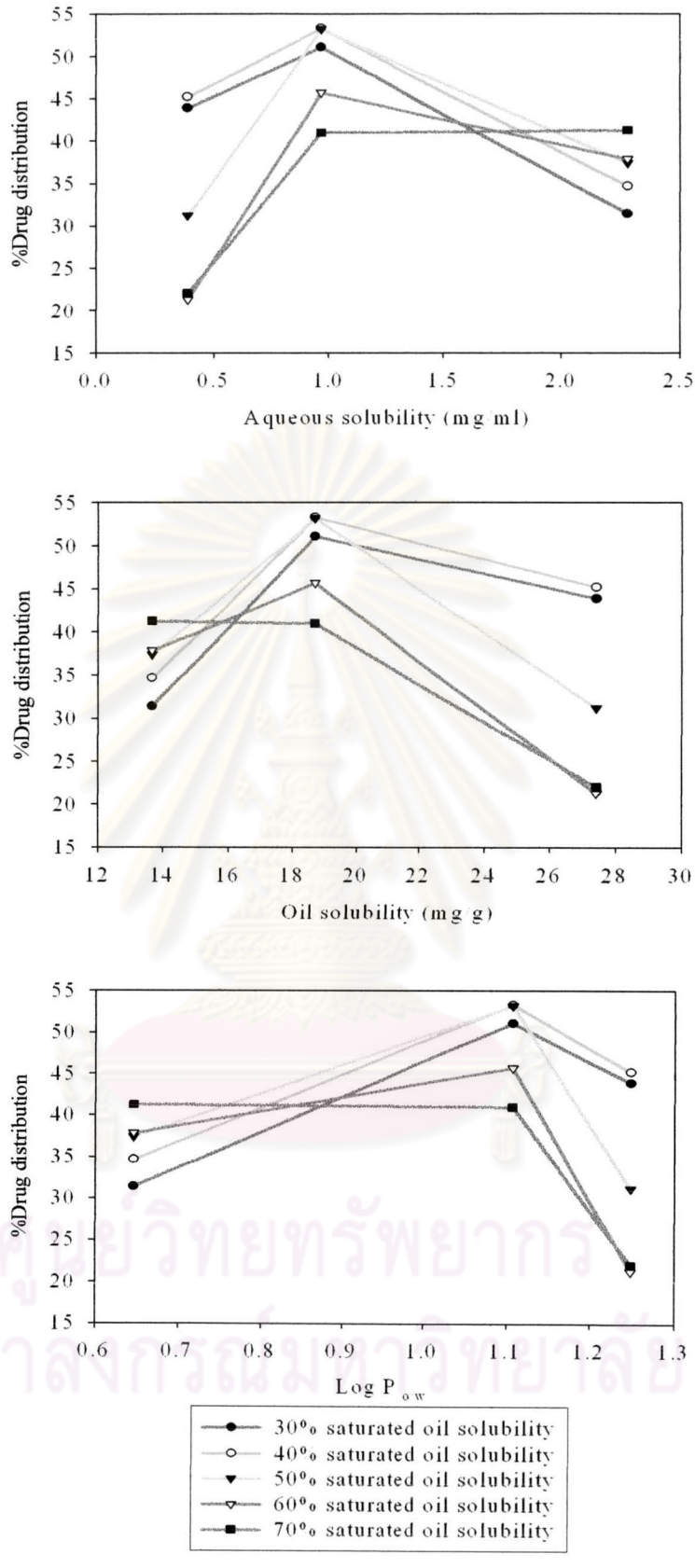


Figure 30 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in mesophase of submicron emulsion prepared by extemporaneous addition.

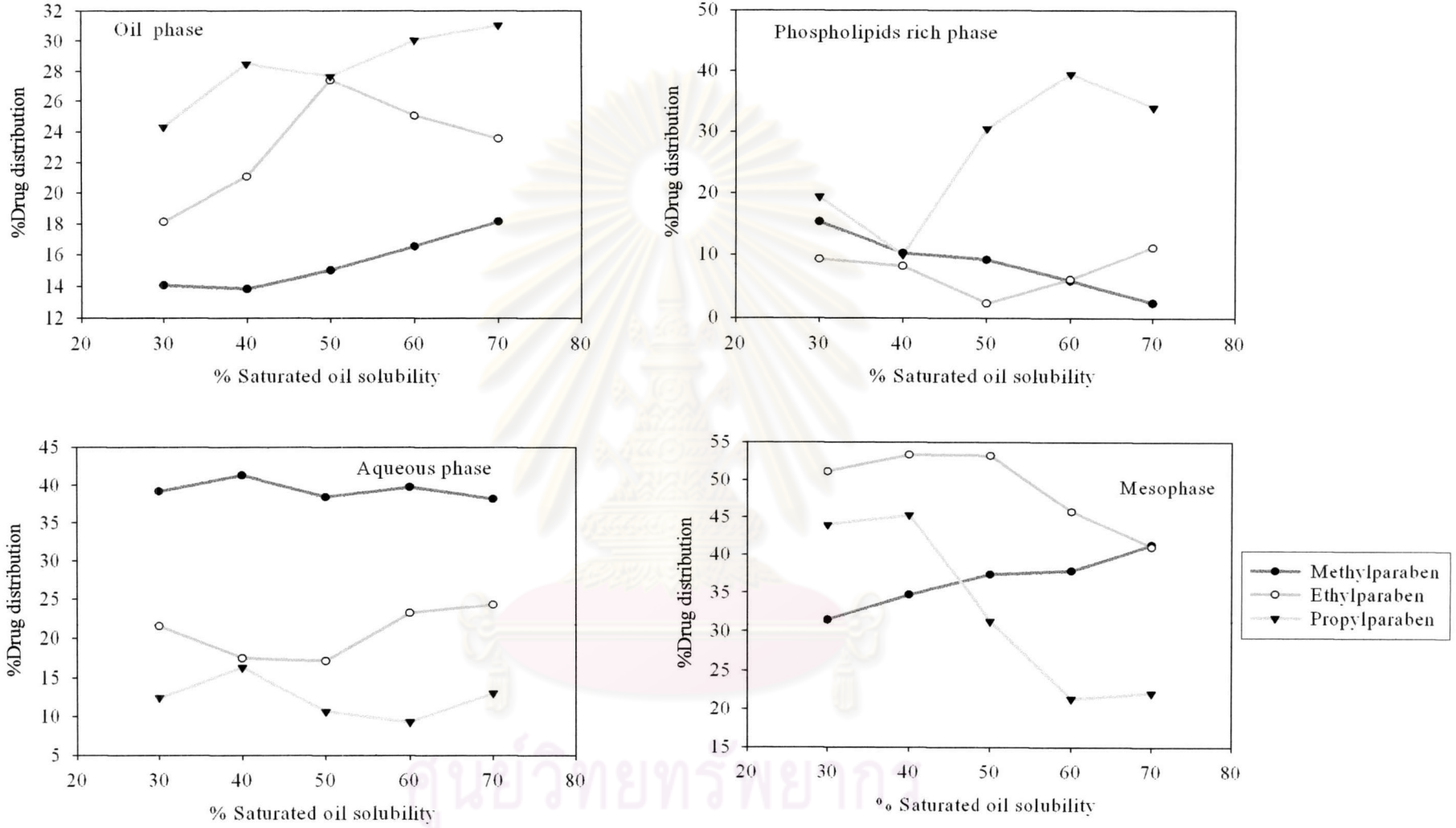


Figure 31 The effect of concentrations on the distribution of alkyl-4-hydroxybenzoate in various phases of submicron emulsion prepared by extemporaneous addition.

4.3 Shaking

From the data obtained as shown in Figure 32, butylparaben was mostly deposited in oil phase but less in aqueous phase. Propylparaben, the moderate lipophilicity, likely distributed through the phospholipids rich phase. However, all compounds were mostly deposited in mesophase.

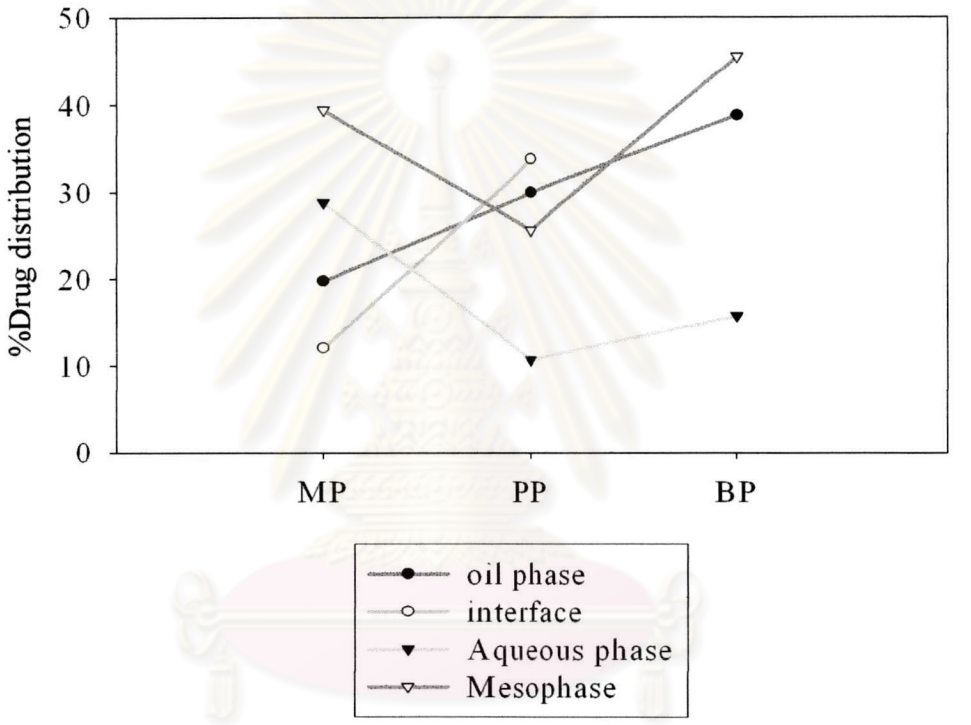


Figure 32 The effect of physicochemical properties on the distribution of alkyl-4-hydroxybenzoate in submicron emulsion prepared by shaking method.

Effect of incorporating methods on the distribution through various phases of submicron emulsion

In this study, three methods of incorporation such as de novo emulsification, extemporaneous addition and shaking were investigated. All three methods have a difference in the phase which firstly contacted to the model drug. For de novo emulsification, oil phase is the phase which firstly contacted to the model drug whereas the solubilizer, dimethyl isosorbide, and aqueous phase (continuous phase) were primary phases for extemporaneous addition and shaking, respectively. Figure 33 presents the influence of incorporated methods on the drug distribution through various phases of submicron emulsion. It was found that the difference in incorporated methods was affected the distribution through the oil phase of alkyl-4-hydroxybenzoate.

In general, the drug retaining in oil phase preparing by de novo emulsification had more potential than the other methods. It was due to the oil phase was a primary phase which contacted to model drug before emulsification process so this could maintain the model drug in the oil phase and then model drug distribute to every portion of submicron emulsion according with the equilibration. From this study, it was found that the distribution of model drugs through oil phase which prepared by extemporaneous addition and shaking method showed the same magnitude as comparing to those prepared by de novo emulsification. The reason was dimethyl isosorbide acted as a drug carrier, this might be expected to result in an increase in drug distribution through oil phase and phospholipid rich phase. Moreover, the shaking force and prolong shaking condition could deliver those drugs to the inner oil phase. In addition, the moderate lipophilicity compound such ethylparaben which prepared by de novo emulsification distributed from the inner oil phase to the phospholipids rich phase while that of propylparaben prepared by extemporaneous addition and shaking methods seemed to distribute to the phospholipids rich phase as well as the inner oil phase. In aqueous phase, the series of the distribution of model drug prepared by de novo emulsification and extemporaneous addition depended on their aqueous solubility. In addition, the distributions to mesophase of higher lipophilicity compounds liked propylparaben and butylparaben were greater than that of the lower ones. Moreover, drug containing submicron emulsion prepared by extemporaneous addition and shaking seemed to ocalize in aqueous phase and mesophase.

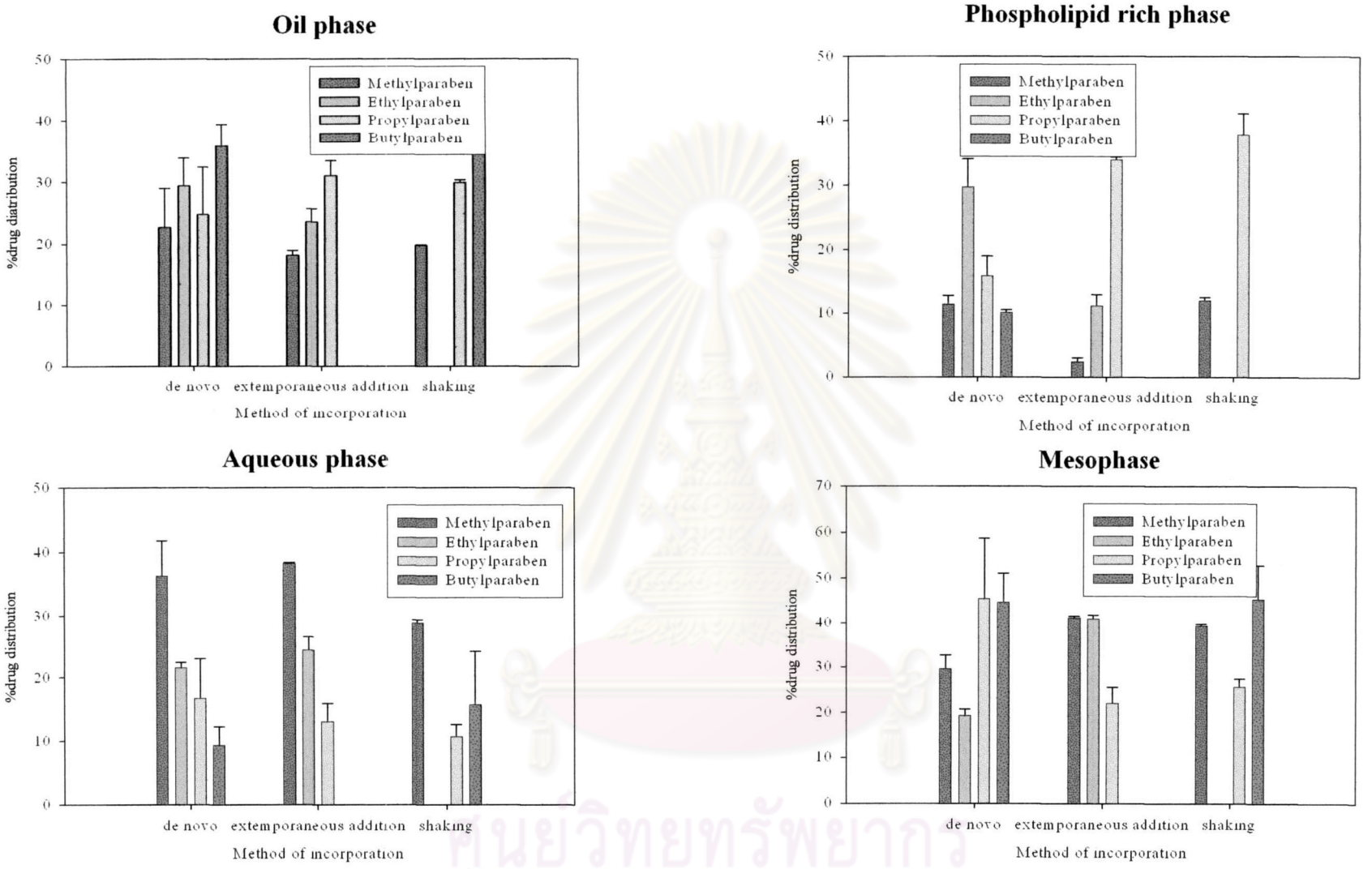


Figure 33 The effect of method of incorporation on the distribution of alkyl-4-hydroxybenzoate in various phases of submicron emulsion.

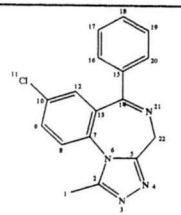
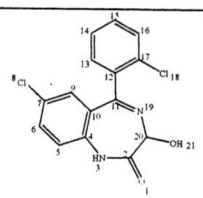
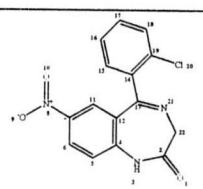
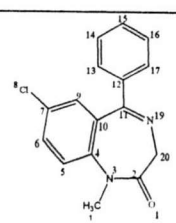
I The determination of aqueous and oil solubility and oil-water partition coefficient of benzodiazepine drug

The physicochemical properties of benzodiazepine drugs such as oil solubility, aqueous solubility and partition coefficient including calculated partition coefficient (ClogP) and dipole moment value are presented in Table 4. ClogP value is a calculated n-octanol/water partition coefficient. ClogP and dipole moment values were calculated by using MOPAC molecular computation in Chem3D Ultra 8.0 program. From the data obtained, it was found that the aqueous solubility values were in the increasing order alprazolam≈lorazepam>diazepam>clonazepam. In general, the polarity of drugs could express in the term of the dipole moment of molecule. The dipole moment measures the asymmetry in the molecular charge distribution and is reported as a vector sum of the individual bond moment in three dimensions. The higher value of dipole moment indicated the greater polarity. From Table 4, the dipole moment value of alprazolam was higher than lorazepam, clonazepam and diazepam. So, alprazolam was more polar than lorazepam, clonazepam and diazepam and these corresponded with the observed aqueous solubility. Considered the chemical structure of all compounds in Table 4, the substitution of a pyrazole moiety in 1, 4 benzodiazepine of alprazolam chemical structure gave more polar than that of hydroxyl, nitro and methyl moiety in the structure of lorazepam, clonazepam and diazepam, respectively. These conferred increased aqueous solubility on the alprazolam molecule. However, the aqueous solubility of clonazepam was unrelated to its dipole moment. It was due to the resonance structure of clonazepam. The intermediate nitroxyl radicals of nitro aromatic moiety were generated and led to increase stability (Montanari, Ciluzo et al. 2001). This may change in the distribution of electron in clonazepam structure and resulting in the stronger the bonds between its molecules. The interaction between clonazepam and solvent molecule was unfavorable, therefore, the aqueous solubility of clonazepam was lower than expected value.

Oil solubility of diazepam was 13-35 times higher than that of lorazepam, alprazolam and clonazepam according to its lowest polarity. Moreover, the influence of resonance structure of clonazepam on its oil solubility was observed and resulting in the lower oil solubility.

The lipophilicity of benzodiazepine drugs has been obtained from determining the partition coefficient ($LogP_{o/w}$) for soybean oil and distilled water mixture (50%w/w). The simple shake-flask method was employed by dissolving the model drug in aqueous phase and the two phases were shaken together. After separation, each phase was analysed for model drugs. It is need to presaturate both phases before making the experiment. Without pre-saturation, the equilibration of drug takes a long time and the phase volume ratio may be affected. In this study, the partition coefficient was in the increasing order of diazepam, clonazepam, lorazepam and alprazolam. This observation agreed with the partition coefficient calculated from MOPAC program. However, the calculated values were higher than the observed ones since the measurement based on the topological distance between potentially interacting fragments but the solvation effects which may change in conformation were neglected. Therefore, it was concluded that diazepam possesses a higher lipophilicity than clonazepam, lorazepam and alprazolam.

Table 4 The physicochemical properties of benzodiazepine drugs at 25°C.

Compound	Chemical structure	Molecular weight	Aqueous solubility (mg/ml) Mean±SD	Oil solubility (mg/g) Mean±SD	Oil-water Partition Coefficient (Log $P_{o/w}$) Mean±SD	CLogP*	Dipole* Moment (Debye)
alprazolam		308.77	0.1162 $\pm 2.0546\times10^{-3}$	0.6687 $\pm 4.9632\times10^{-3}$	0.5374	2.2049	5.705
lorazepam		321.17	0.1166 $\pm 7.9926\times10^{-4}$	1.0354 ±0.0299	0.9872	2.3683	3.692
clonazepam		315.72	0.0084 ±0.1734	0.3772 ±0.0104	1.4606	2.3842	2.104
diazepam		284.75	0.0510 $\pm 2.9012\times10^{-4}$	13.7177 ±0.0461	2.2321	3.1704	1.852

*Calculated value from MOPAC molecular computation

II The characterization of submicron emulsions with and without incorporated benzodiazepine drug

The physical property determinations of drug containing submicron emulsions which prepared by different incorporating methods were determined at initial and after keeping for seven days at ambient temperature. The changes in mean droplet size and droplet size distribution, zeta potential and pH of benzodiazepine drug containing submicron emulsion were described as following:

2.1 De novo emulsification

As presented in Figure 34, the initial effective mean diameter of benzodiazepine containing submicron emulsions preparations were approximately 200-250 nm and slightly increased when keeping for 7 days. This was due to the incorporation of drug providing the increase in packing parameter thus resulting in the formation of the larger droplets (Duzgunes, Wilschut et al. 1981). However, the polydispersity of most preparations were within the acceptable range (<0.25), indicated that submicron emulsion prepared by this method showed a consistency in size distribution (Muller and Bohm 1998).

Figure 36 shows the zeta potental of benzodiazepine drugs containing submicron emulsion preparations comparing between initial and seven days storage. Zeta potential values of preparations were all negatively charge. An increase in negative zeta potential after storage period was observed in clonazepam, diazepam and lorazepam submicron emulsion preparations. It is probable that the hydrolysis of oil phase or phospholipids causing the formation of fatty acid and resulting in an increase of zeta potential. In addition, this finding was also corresponding with the decrease in pH of the same preparations.

The submicron emulsion was stabilized by phospholipids which undergo hydrolysis and yields free fatty acids as a hydrolytic product. Thus, the fatty acids produced by hydrolysis of phospholipids will release protons and yield anions in the emulsion system. Following the increase in free fatty acid concentration, the pH of all

preparations slightly decreased from around 6 at initial to around 5 after keeping for seven days (Figure 37).

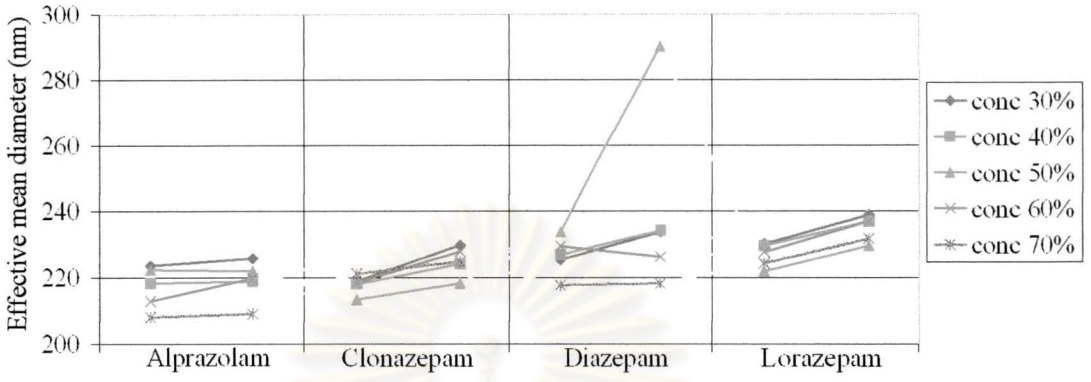


Figure 34 The change in size of benzodiazepine submicron emulsions during storage for 7 days at ambient temperature measured by using PCS.

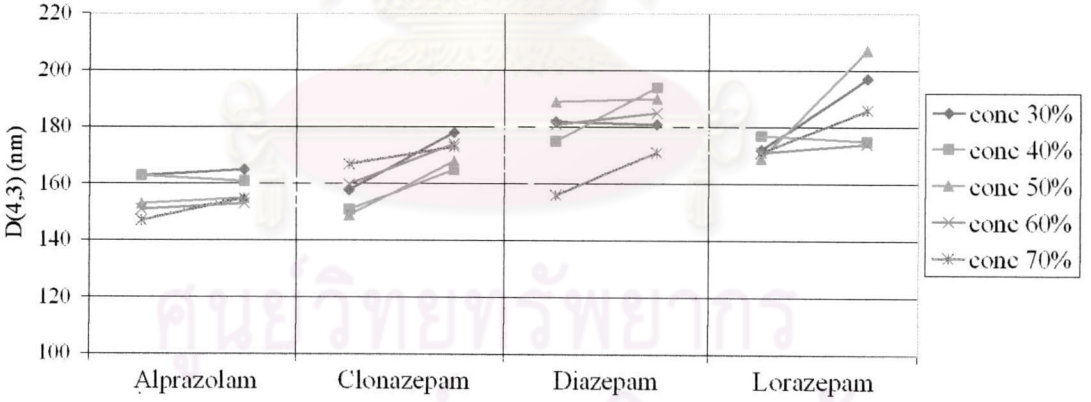


Figure 35 The change in size of benzodiazepine submicron emulsions during storage for 7 days at ambient temperature measured by using laser light diffraction.

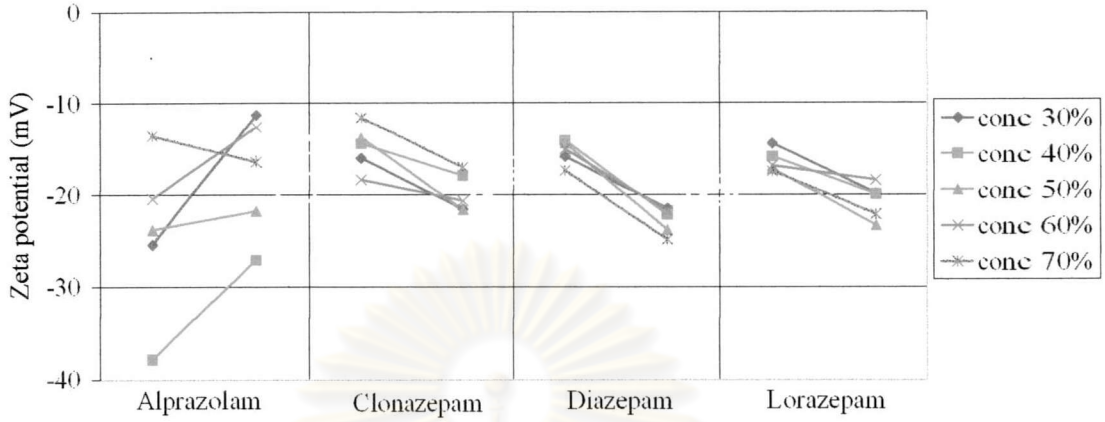


Figure 36 The change in zeta potential of benzodiazepine submicron emulsions during storage for 7 days at ambient temperature.

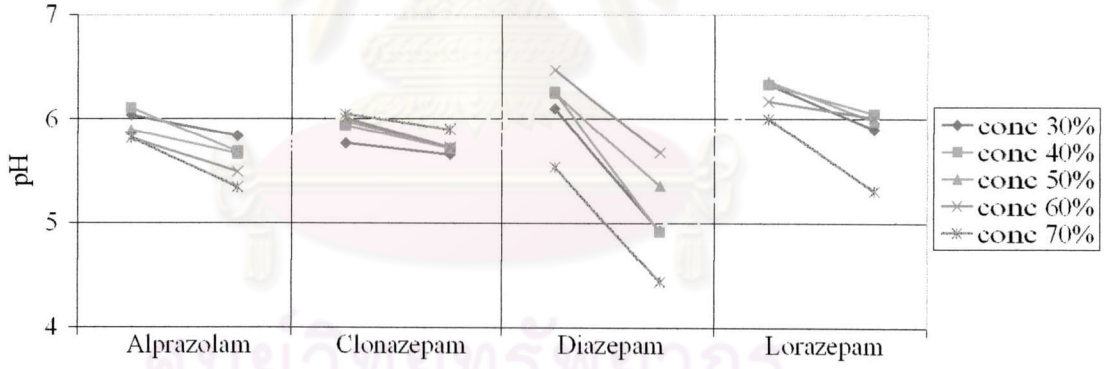


Figure 37 The change in pH of benzodiazepine submicron emulsions during storage for 7 days at ambient temperature.

2.2 Extemporaneous addition

In Figure 38, the effective mean diameter of submicron emulsion bases and benzodiazepine drug containing submicron emulsion preparations were compared. The effective mean diameters of benzodiazepine submicron emulsion preparations were slightly increased as compared with submicron emulsion bases. This was probably that the incorporated drug which accumulated at the oil-water interface affected the emulsifying property of phospholipids e.g. the decreasing in surface tension reduction. In addition, the increasing in drug loading did not change in effective mean diameter of all preparations. However, the larger particle size was observed by laser diffraction technique (Figure 39).

The average zeta potential of submicron emulsion base was about –30 mV while that of drug containing submicron emulsions were varied decreased in the range of -7 to –26 mV (Figure 40). The charge covering the oil droplet arose from the fatty acid which was a hydrolytic product of soybean phospholipids. Since the pKa of alprazolam, clonazepam, diazepam and lorazepam were 2.4, 1.5 and 10.5, 3.3, 1.3 and 11.5, respectively (Moffat 1986; Neil, Smith et al. 2001) and pH of emulsion system was around 3.5-4, thereby model drugs were unionized forms. So, charge of model drugs did not affect the change in surface charge of submicron emulsion. However, the presence of an additional drug might decrease in the charge density surrounding the oil droplets and led to decrease in zeta potential. In addition, after keeping for seven days, zeta potential was increase according to the formation of free fatty acid. Free fatty acid was yielded from the hydrolysis of oil phase and/or phospholipids providing more negative zeta potential and corresponding with the decrease in pH of submicron emulsion.

As shown in Figure 41, the approximate pH of submicron emulsion base was 4-6 whereas that of drug containing submicron emulsion preparations was around 3.5-4. It was due to the acidic property of dimethyl isosorbide, a solubilizer, given a lower pH of submicron emulsion preparation. In addition, the pH of both submicron emulsion base and preparations were lower during ageing. The reason was the hydrolysis of oil phase and/or phospholipids forming free fatty acids in emulsion system.

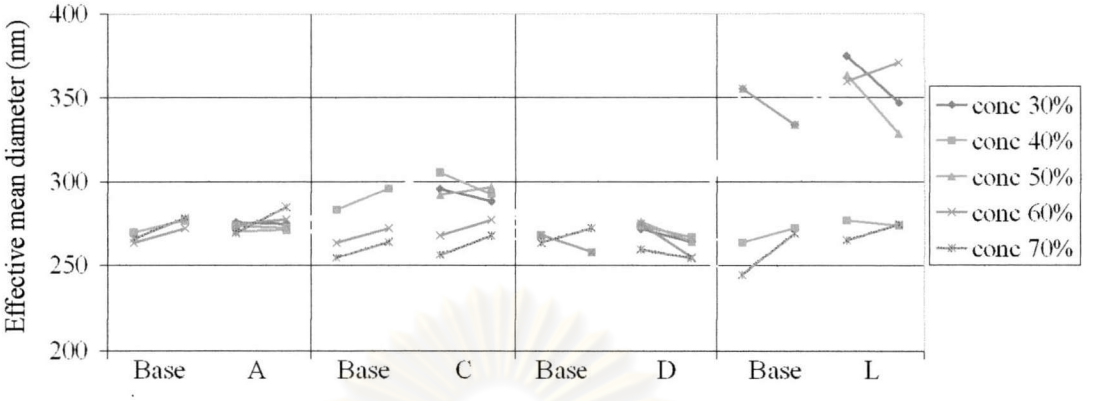


Figure 38 The change in size of benzodiazepine submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature measured by using PCS.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

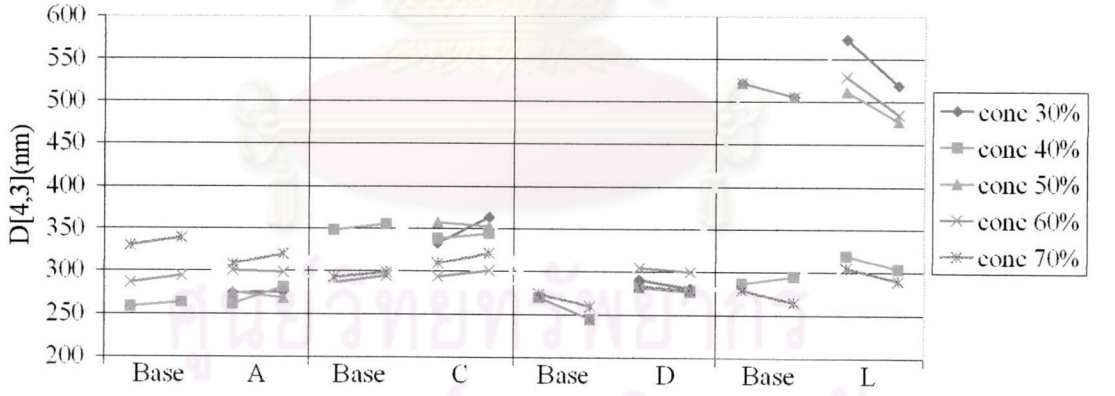


Figure 39 The change in size of benzodiazepine submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature measured by using laser light diffraction.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

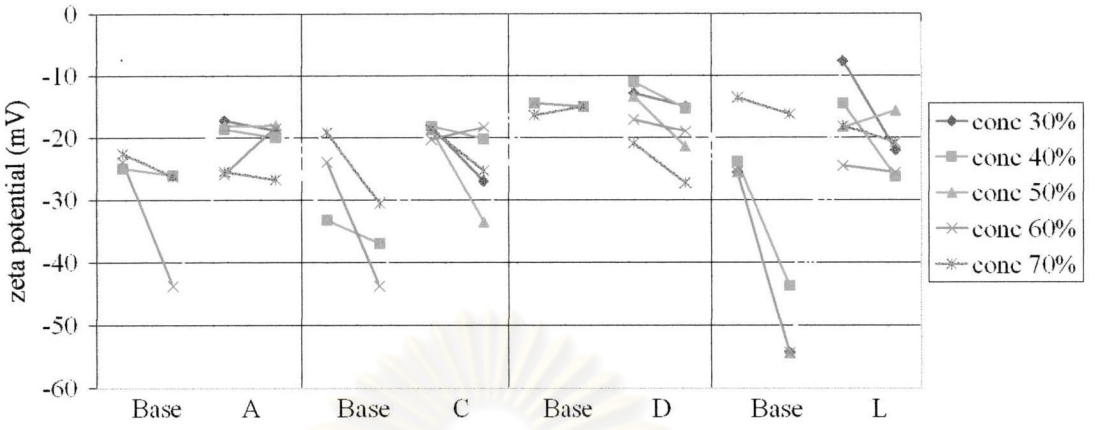


Figure 40 The change in zeta potential of benzodiazepine submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

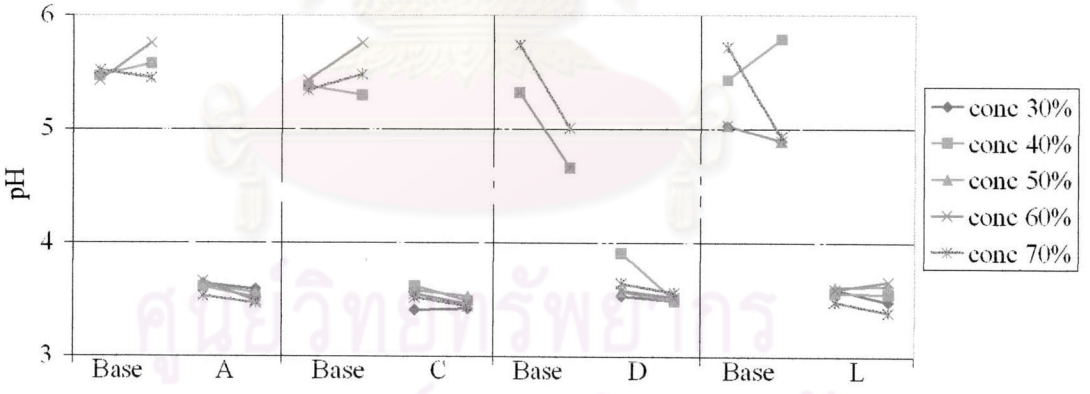


Figure 41 The change in pH of benzodiazepine submicron emulsions containing Arlasolve DMI during storage for 7 days at ambient temperature.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

2.3 Shaking

Figure 42-45 shows the change in physical properties of submicron emulsion bases and benzodiazepine drug containing submicron emulsions prepared by shaking method. Size average of submicron emulsion base was ranging from 270-280 nm and increasing to 280-300 nm when benzodiazepine drugs were incorporated. However, the larger mean particle size was observed by laser diffraction technique. The incorporation of drugs could increase the packing parameter of phospholipids that meant the formation of larger droplets (Trotta, Pattarino et al. 2002). The zeta potential values of all preparations were lower than that of submicron emulsion bases. This might be due to the change in charge density around oil droplets and reduced the magnitude of zeta potential. pH of the all formulations were around 5-6 and closed to that of submicron emulsion bases. However, it was slightly decreased after seven days storage, this might indicate the formation of the fatty acid. Such fatty acid could probably reduced the pH of submicron emulsion preparations (Benita and Levy 1993). However, all preparations remained stabilized without remarkable appearance change during seven days storage period. The content of drug in submicron emulsion was examined and presented in Table 5. It was found that, the loading efficacy of diazepam in submicron emulsion was higher than the other. It was due to its high oil solubility and lipophilicity.

Table 5 Drug contents of benzodiazepine in submicron emulsions prepared by shaking method.

Drug	Content in submicron emulsion (mg/g)
Alprazolam	0.565±0.0168
Clonazepam	$0.213 \pm 8.7706 \times 10^{-3}$
Diazepam	1.658±0.0353
Lorazepam	$1.108 \pm 9.7457 \times 10^{-3}$

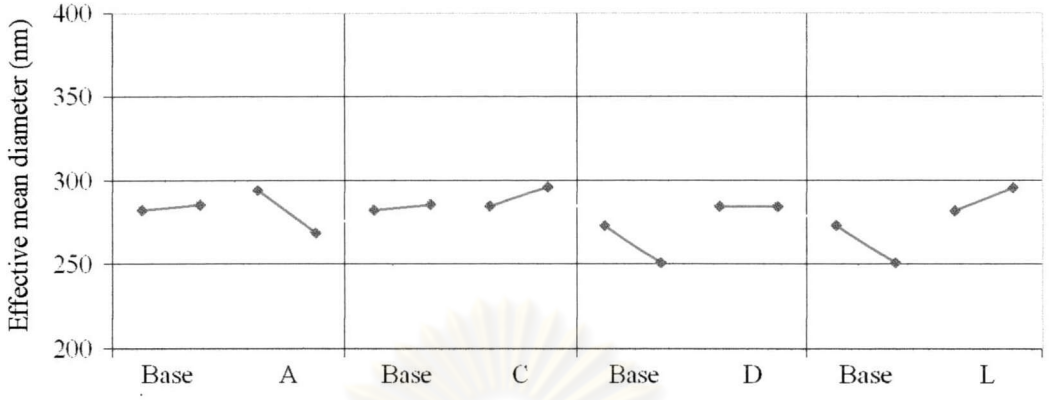


Figure 42 The change in size of benzodiazepine submicron emulsions prepared by shaking during storage for 7 days at ambient temperature measured by using PCS.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

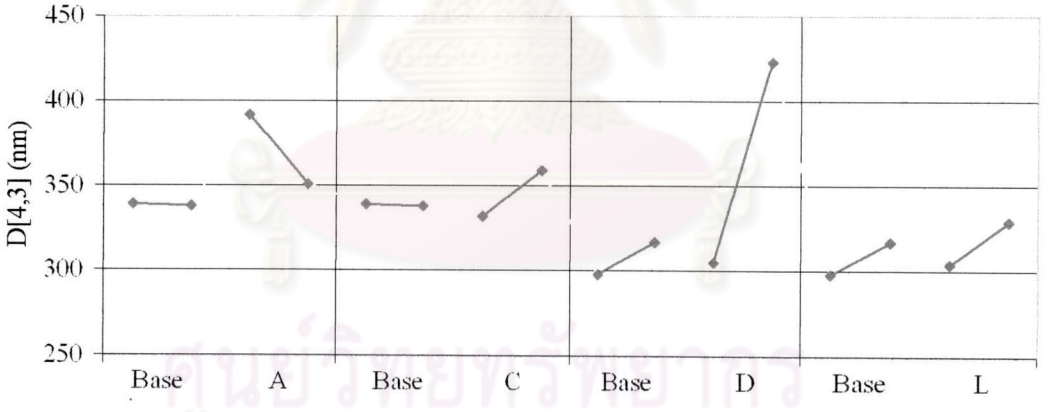


Figure 43 The change in size of benzodiazepine submicron emulsions prepared by shaking during storage for 7 days at ambient temperature measured by using laser light diffraction.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

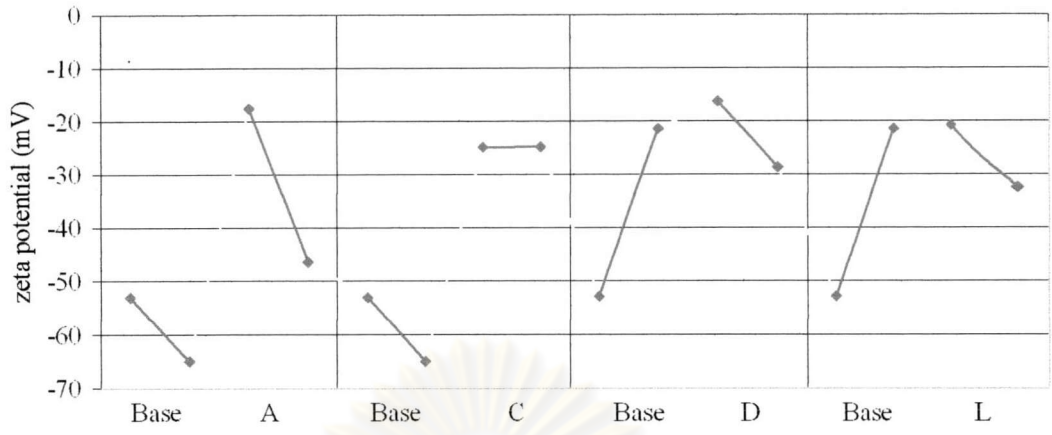


Figure 44 The change in zeta potential of benzodiazepine submicron emulsions prepared by shaking during storage for 7 days at ambient temperature.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

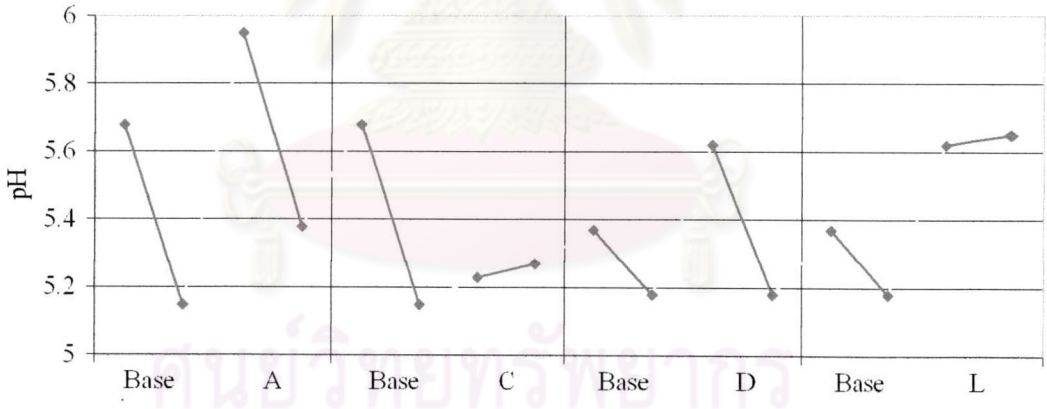


Figure 45 The change in pH of benzodiazepine submicron emulsions prepared by shaking during storage for 7 days at ambient temperature.
(A=Alprazolam, C=Clonazepam, D=Diazepam, L=Lorazepam)

The comparison of physical properties of benzodiazepine drug containing submicron emulsions with different incorporated methods

The physical properties of alprazolam, clonazepam, diazepam and lorazepam containing submicron emulsions in the concentration of 70% oil saturated solubility prepared by de novo and extemporaneous addition were compared to that of the shaking method as presented in Figure 46-49, respectively. De novo emulsification method provided a smaller effective mean diameter. The effective mean diameter prepared by extemporaneous addition was closed to that of the shaking method. In addition, the formulations of alprazolam and clonazepam prepared by de novo emulsification gave a low zeta potential while that of diazepam and lorazepam were not different among incorporating method. The pH of the preparations prepared by de novo emulsification was similar to that of shaking method while the preparations obtained from extemporaneous addition allowed the lower pH.

After preparing by de novo emulsification and extemporaneous addition, the content of drug in submicron emulsion preparation was determined. As shown in Figure 50, it was found that the recovery of content was varied around 90-109% and 84-101% as preparing by de novo emulsification and extemporaneous addition, respectively. To compare the loading efficacy between different incorporated methods, the maximum content (70% drug saturated in oil phase) of drug containing submicron emulsion preparation prepared by de novo emulsification and extemporaneous addition and solubility of these compound in submicron emulsion base (the data obtained from shaking experiment) were assessed. This finding is given in Table 5. It was found that shaking method conferred the higher drug loading than the other two methods. It was probable that this method contained additionally ultrafine nanocrystals resulting in increasing drug loading. The ultrafine nanocrystal may localize in the interfacial area of emulsion due to their poor solubility in water and oil (Akkar and Muller 2003; Akkar and Muller 2003). The shaken efficacy loading of alprazolam, clonazepam, diazepam and lorazepam was approximate 12.5, 12, 1.5 and 14.3 times higher than that of the other two methods, respectively. This indicated that shaking method allowed the lower lipophilicity, alprazolam, clonazepam and lorazepam, to incorporate in emulsifier layer and also remaining phospholipids structure which coexisted in aqueous phase. This corresponded with the

observed distribution of those three drugs preparing by shaking method were higher localized through phospholipids rich phase and mesophase than the other method of incorporations.

Table 6 Comparison of % benzodiazepine loading between different incorporated methods.

Drug	%drug content in submicron emulsion		
	De novo emulsification (70% drug saturated in oil phase) (n=1)	Extemporaneous addition (70% drug saturated in oil phase) (n=1)	By shaking (n=3)
Alprazolam	4.41×10^{-3}	3.39×10^{-3}	0.05±0.02
Clonazepam	2.69×10^{-3}	2.43×10^{-3}	$0.03 \pm 2.33 \times 10^{-3}$
Diazepam	0.10	0.09	0.15±0.04
Lorazepam	7.25×10^{-3}	6.74×10^{-3}	$0.10 \pm 8.32 \times 10^{-3}$

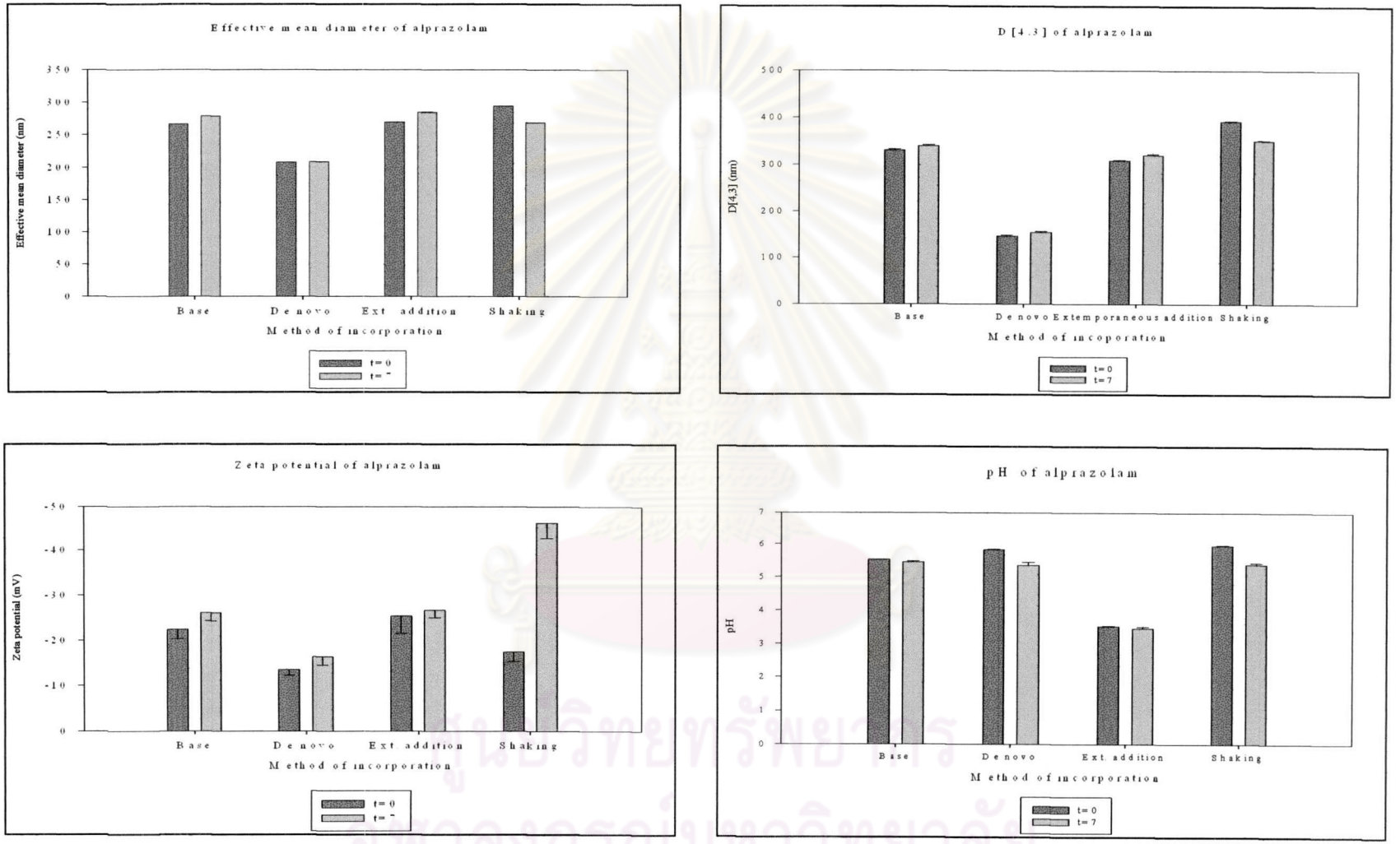


Figure 46 The comparison of physical properties of alprazolam submicron emulsion prepared by different methods.

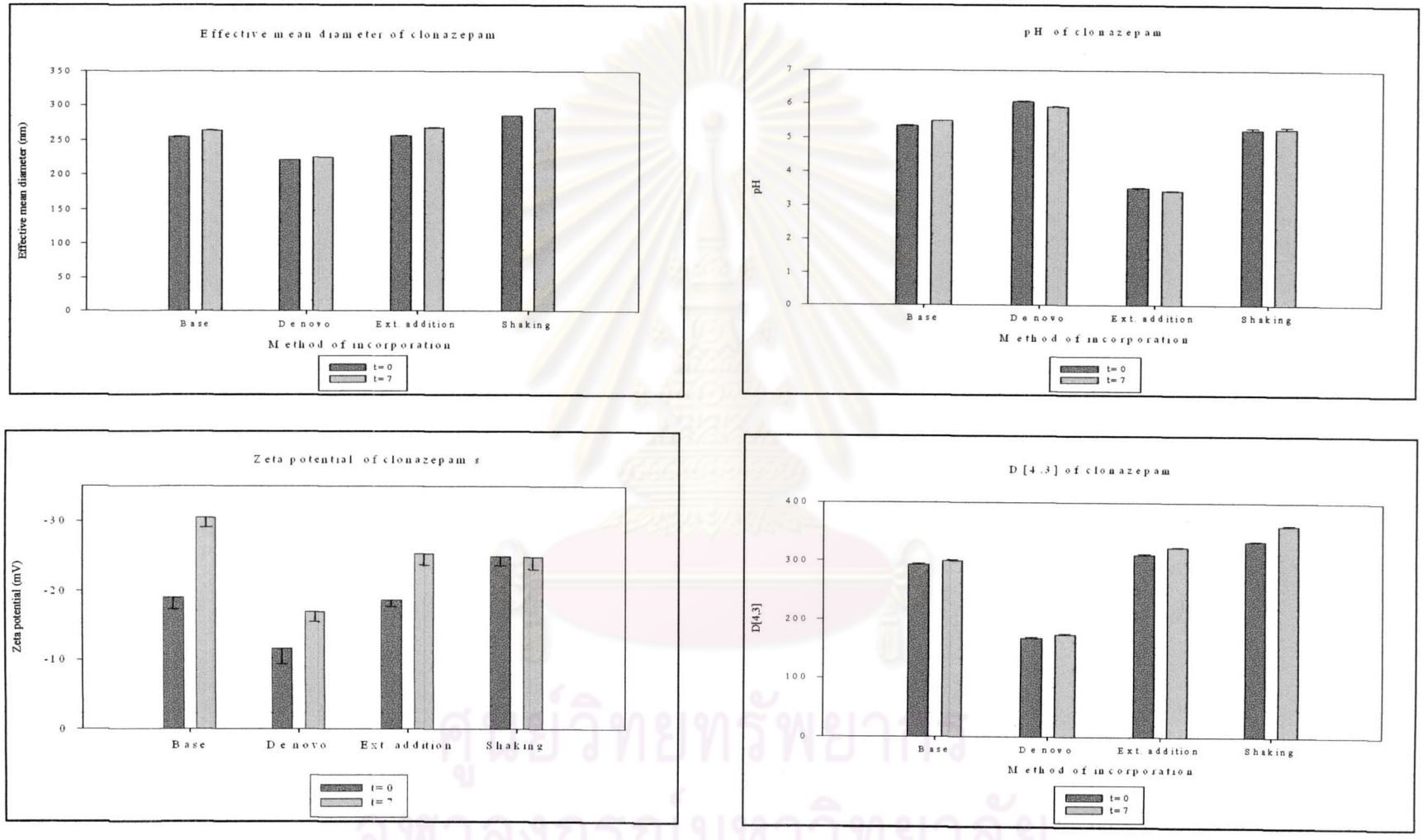


Figure 47 The comparison of physical properties of clonazepam submicron emulsion prepared by different methods.

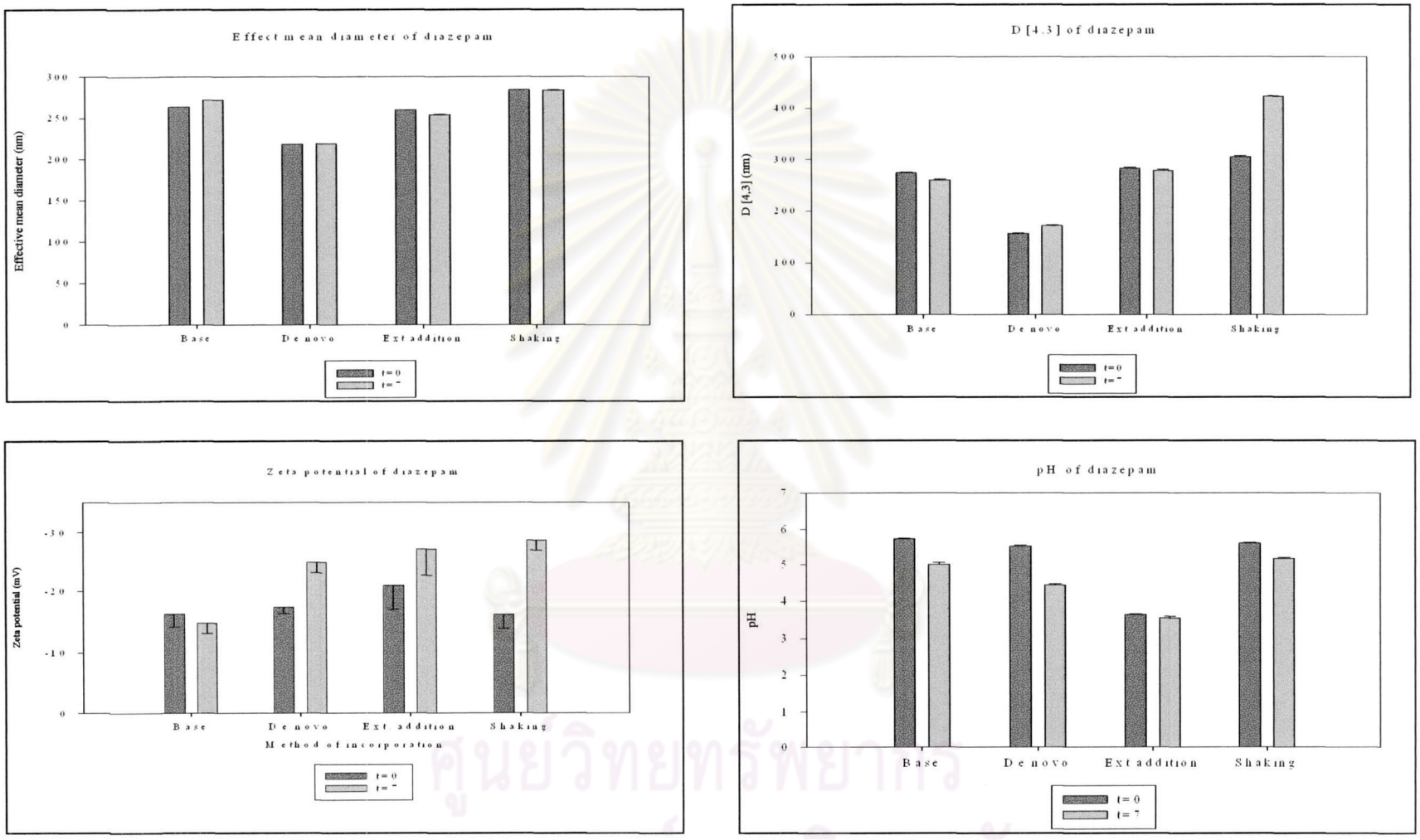


Figure 48 The comparison of physical properties of diazepam submicron emulsion prepared by different methods.

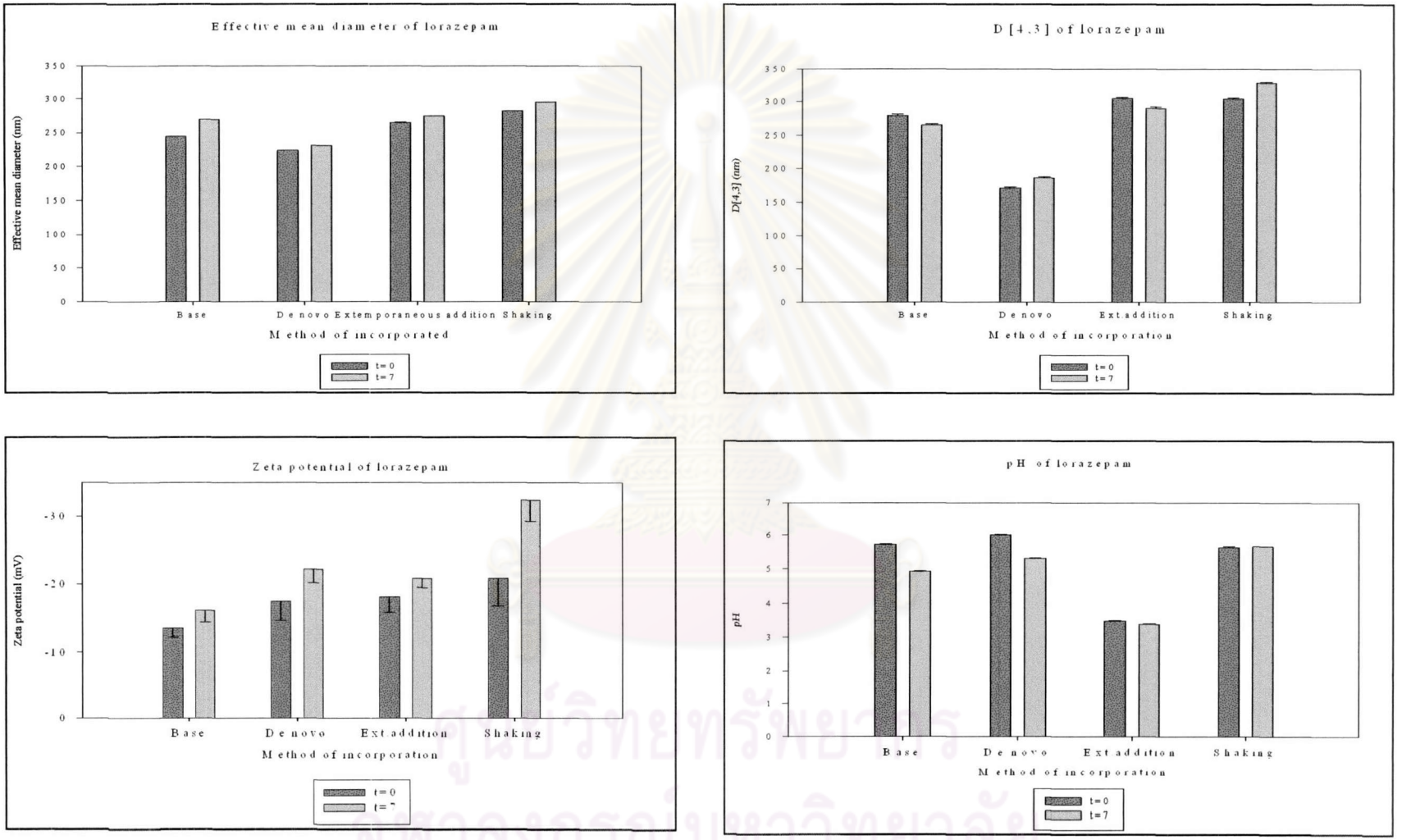
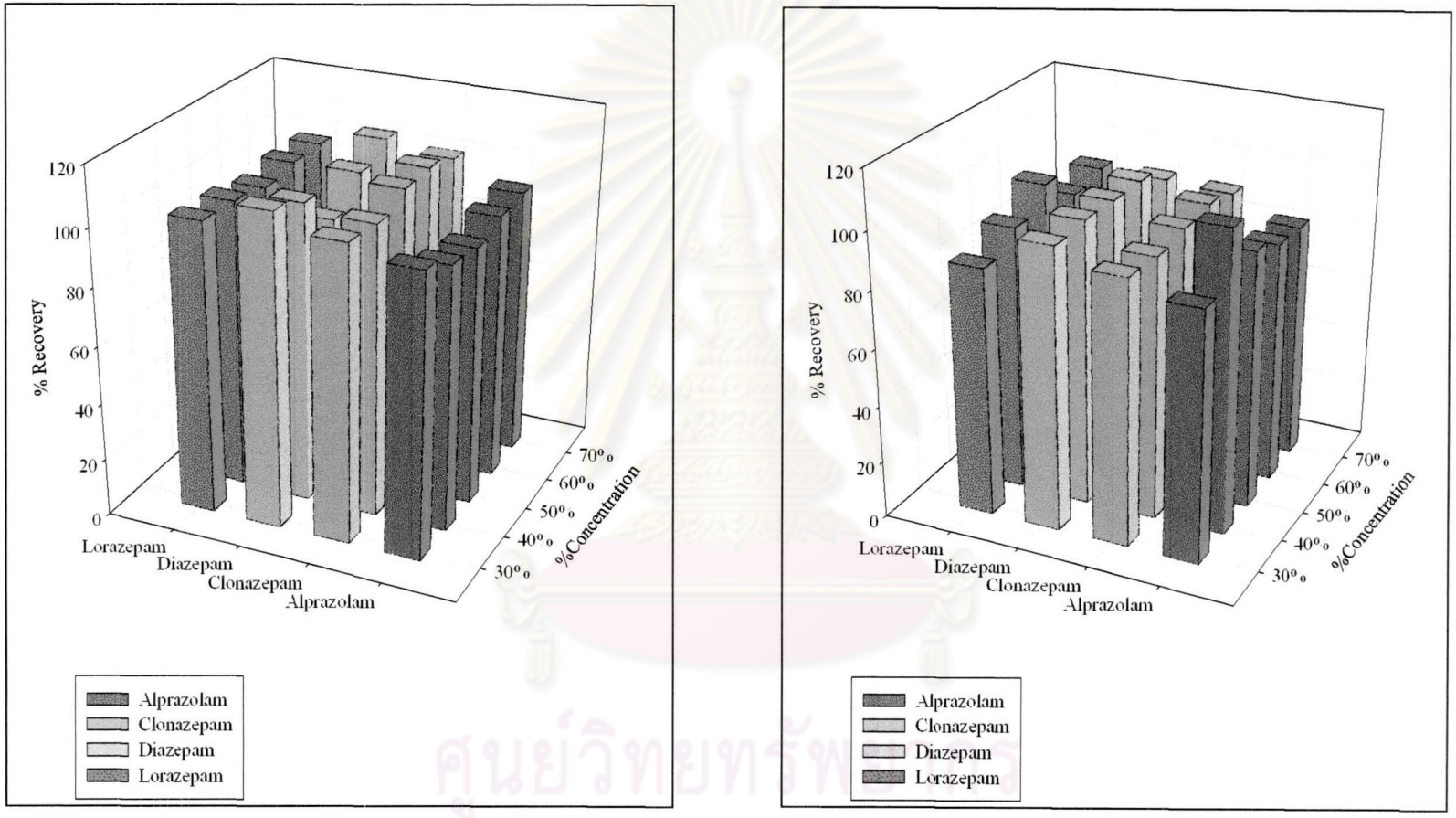


Figure 49 The comparison of physical properties of lorazepam submicron emulsion prepared by different methods.

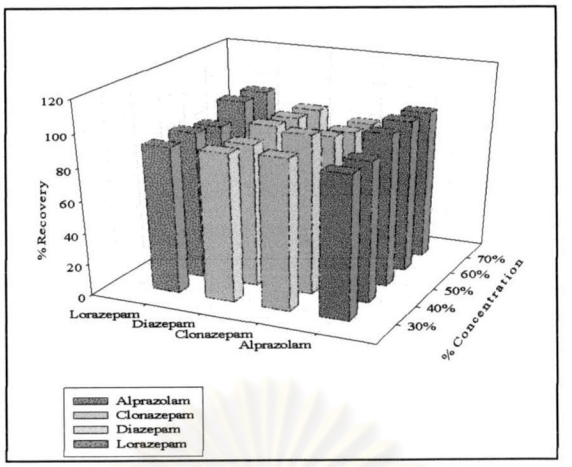


De novo emulsification **Extemporaneous addition**

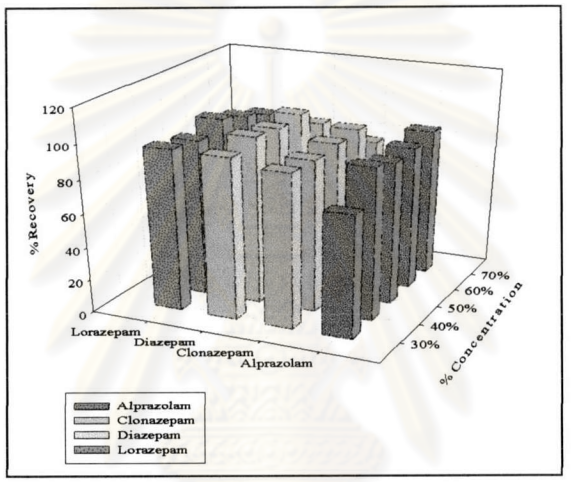
Figure 50 Comparison of recovery content of benzodiazepine drugs between different incorporated methods.

III The effects of lipophilicity, incorporated compound concentration and incorporation method on benzodiazepines distribution into various phases of submicron emulsion

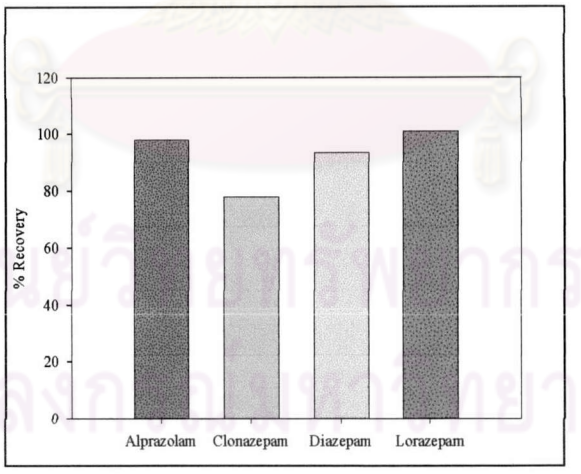
The infrastructure of submicron emulsion, method of phase separation and also phase collection are similar to the previous study of alkyl-4-hydroxybenzoate. However, the amount of model drug loss after ultracentrifugation was assessed in the term of %recovery which comparing the analytical amount of each phase to the initial theoretical amount. Figure 51 showed the percentage of mean recovery of different incorporating methods. The recovery was approximate 82-99%, 73-100% and 75-100% for preparing by de novo emulsification, extemporaneous addition and shaking method, respectively. It seemed that the analysis of drug distribution in various phases of submicron emulsion was acceptable.



A



B



C

Figure 51 Comparison of %mean recovery of benzodiazepine drugs between different incorporated methods.
A: De novo emulsification
B: Extemporaneous addition
C: Shaking

The distribution of benzodiazepine drug in submicron emulsions

4.1 De novo emulsification

As shown in Figure 52-55, the influence of physicochemical properties of drug on the distribution of alkyl-4-hydroxybenzoate in oil phase, phospholipids rich phase, aqueous phase and mesophase was presented. There were significant differences in distribution among each phase of submicron emulsion as well as different type of model drugs. From the data obtained, diazepam, the highest oil solubility and partition coefficient drug, mostly accumulated in oil phase and contrarily distributed in aqueous phase. Clonazepam, the moderate partition coefficient drug, likely distributed from oil phase through phospholipids rich phase. The distributions in aqueous phase were in the increasing order of alprazolam, lorazeoam, clonazepam and diazepam and correspond with their aqueous solubility. The distribution of these four drugs in either oil phase or aqueous phase, therefore, depended on their chemical structure and polarity. The distributions of all drugs in mesophase were also varied and the moderate lipophilic drugs e.g. clonazepam and lorazepam were likely located through this phase.

The effect of drug concentration on the distribution through various phases of submicron emulsion was examined (Figure 56). The increasing of diazepam concentration gave a higher distribution in oil phase whereas those of the other drugs were varied. Moreover, it was found that the increasing concentration of alprazolam, the higher distribution through phospholipids rich phase and mesophase was observed but this behavior was contrastive in aqueous phase. It was notably that the distribution of clonazepam and diazepam in aqueous phase was higher as increasing in concentration.

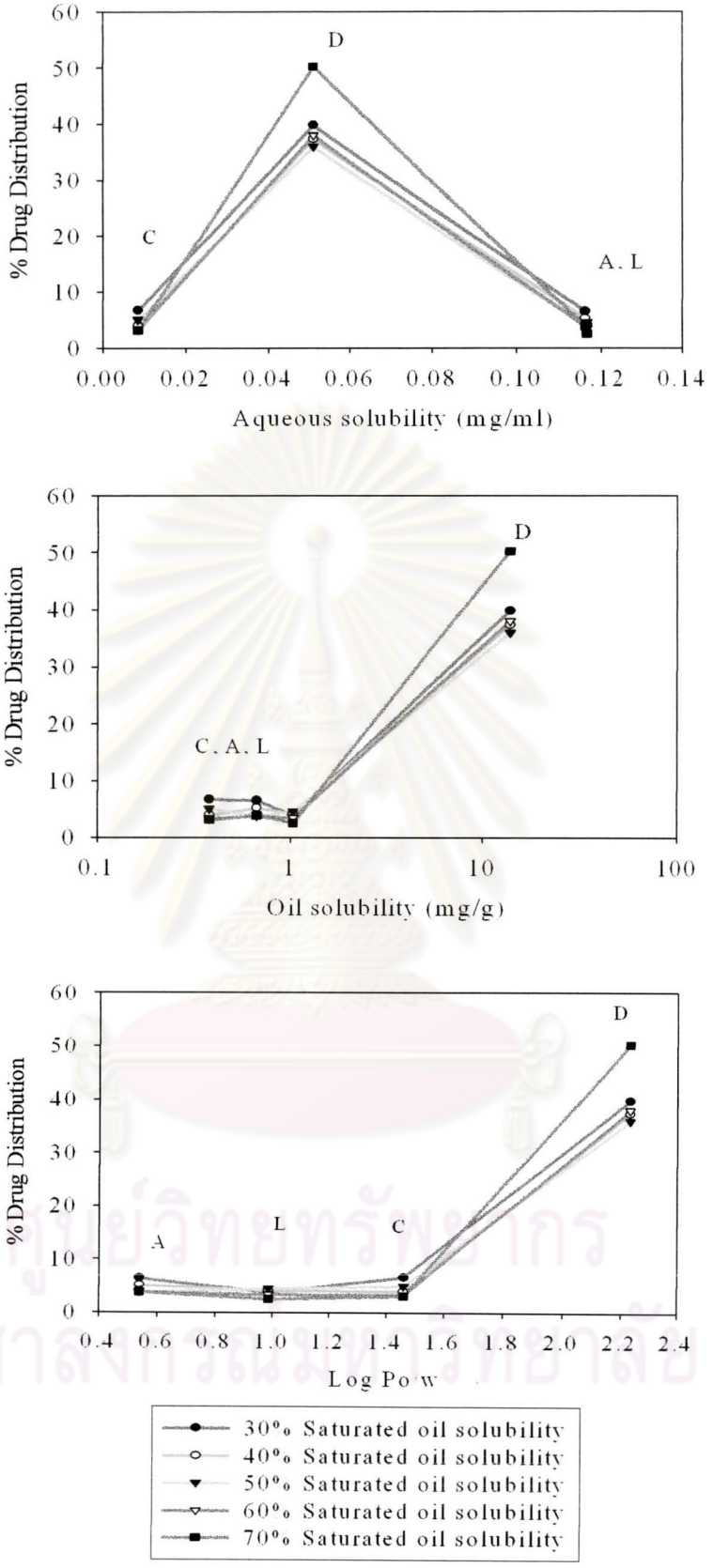


Figure 52 The effect of physicochemical properties on the distribution of benzodiazepine drugs in oil phase of submicron emulsion prepared by de novo emulsification.

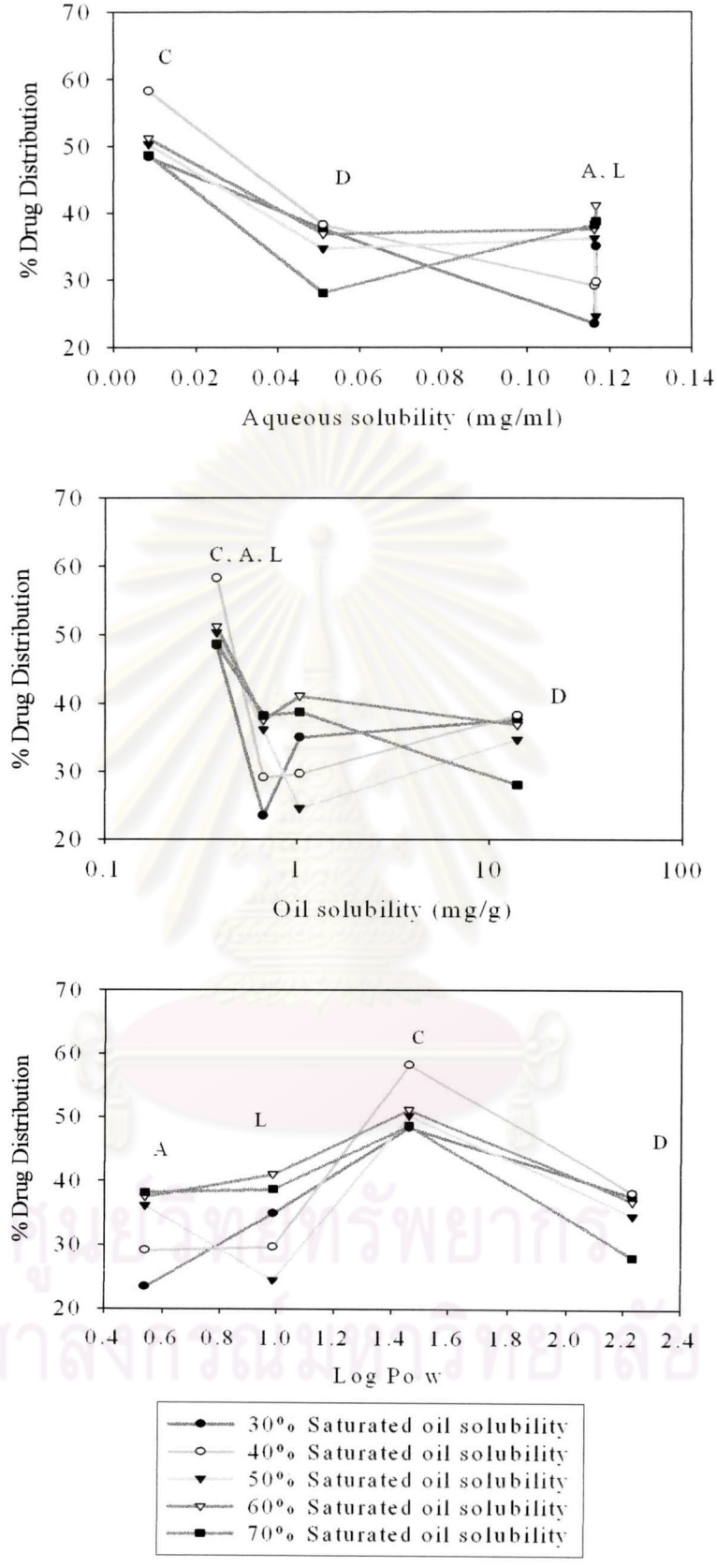


Figure 53 The effect of physicochemical properties on the distribution of benzodiazepine drugs in PC rich phase of submicron emulsion prepared by de novo emulsification.

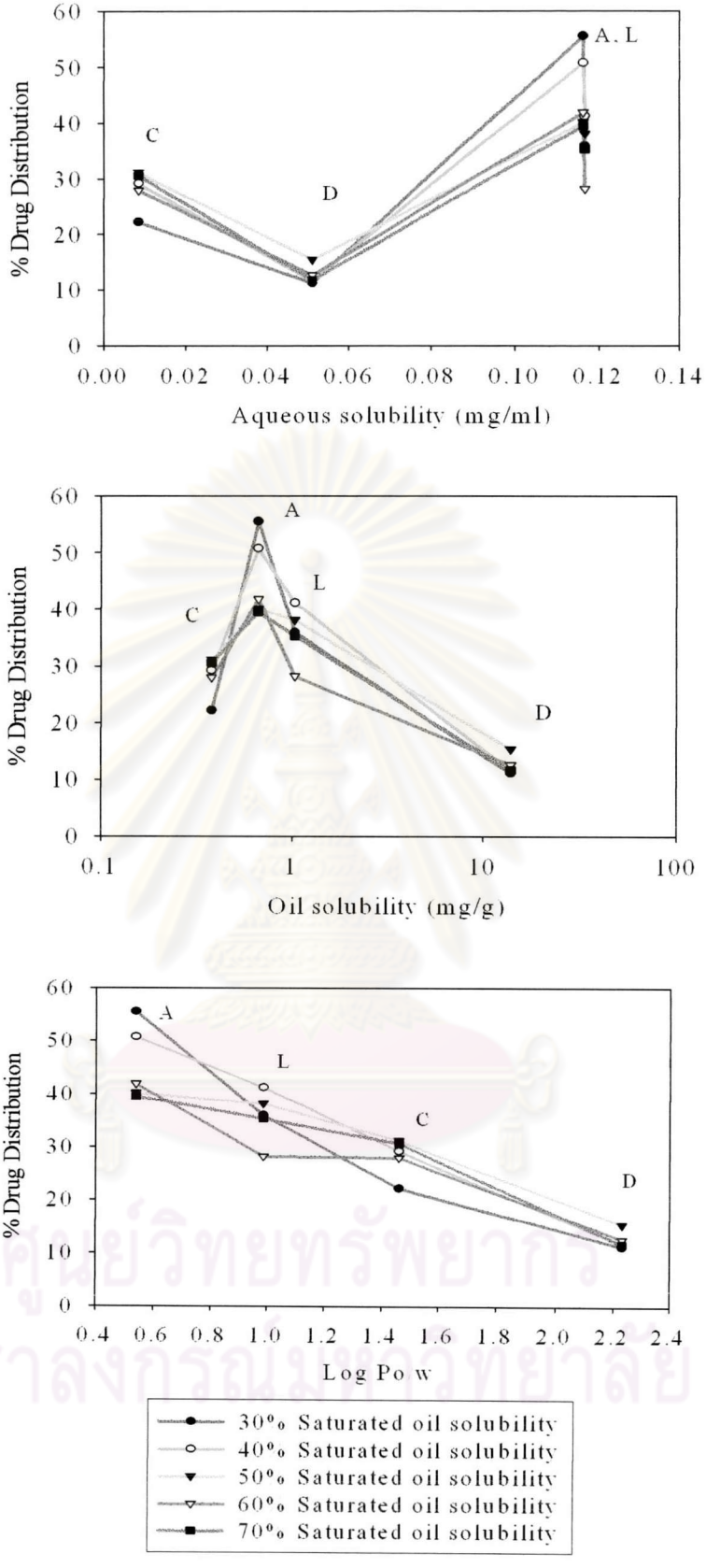


Figure 54 The effect of physicochemical properties on the distribution of benzodiazepine drugs in aqueous phase of submicron emulsion prepared by de novo emulsification.

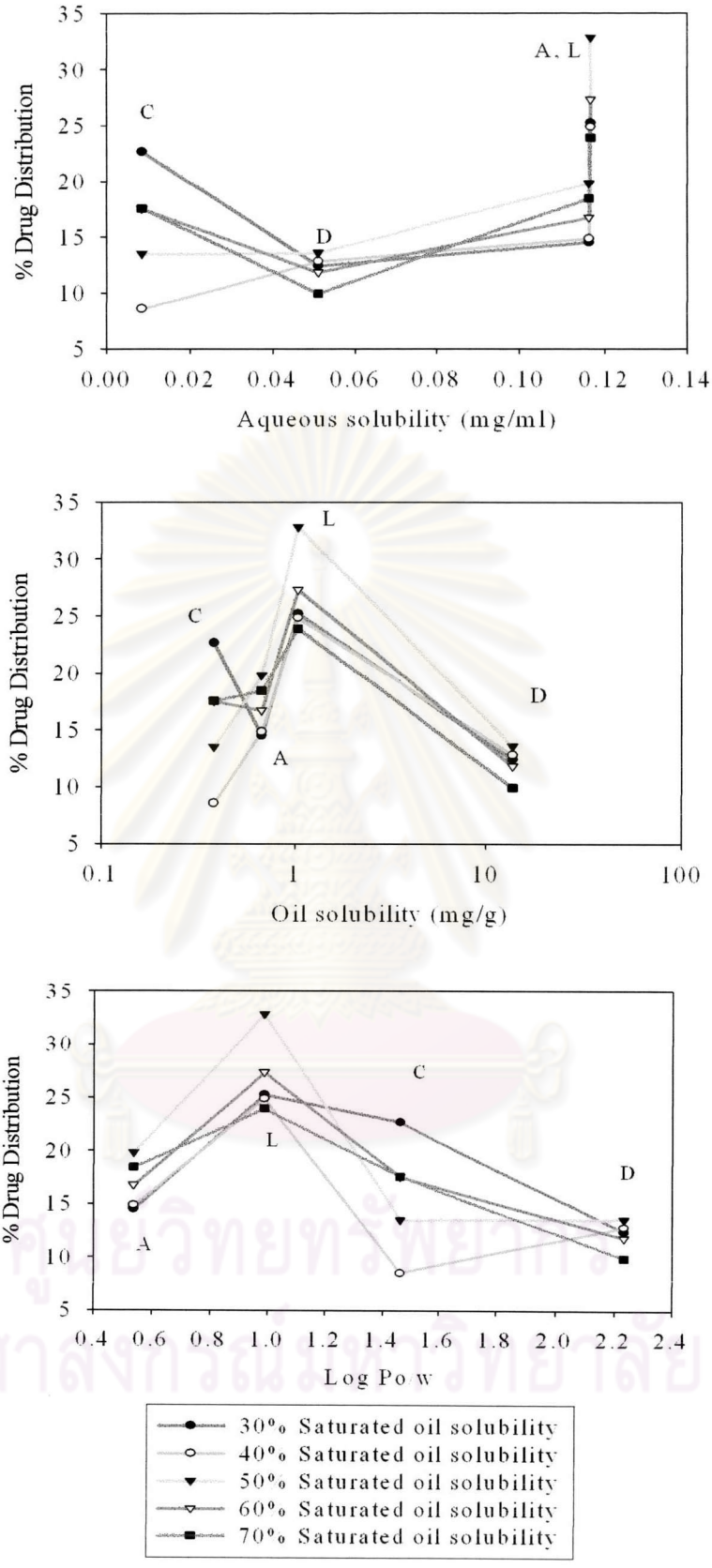


Figure 55 The effect of physicochemical properties on the distribution of benzodiazepine drugs in mesophase of submicron emulsion prepared by de novo emulsification.

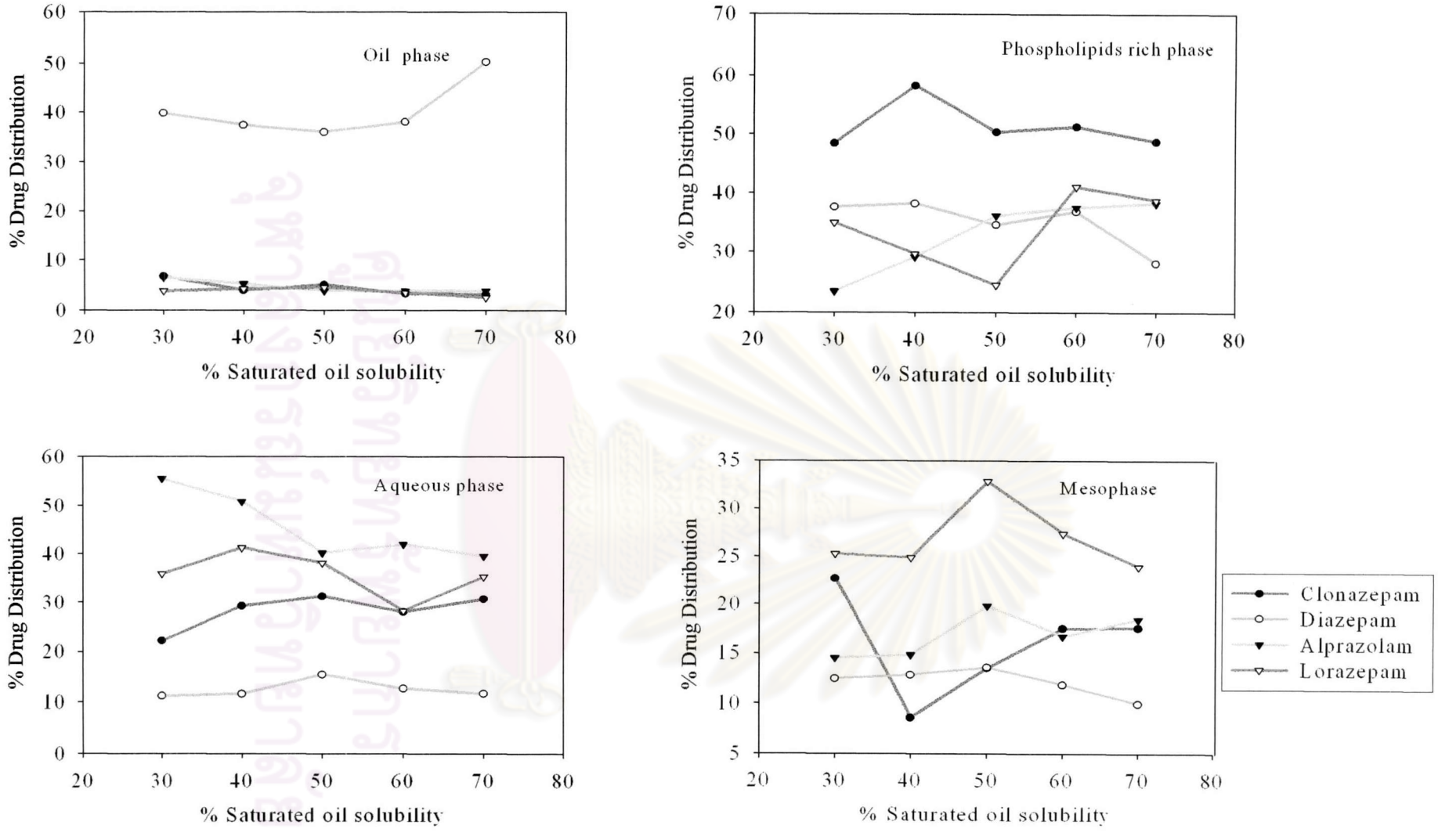


Figure 56 The effect of concentrations on the distribution of benzodiazepine drugs in various phases of submicron emulsion prepared by de novo emulsification.

4.2 Extemporaneous addition

From the data obtained from Figure 57-60, the amount of diazepam and alprazolam were contrary distributed through the oil phase due to the difference in physicochemical properties. However, the amount of clonazepam and lorazepam were not detected in oil phase. In addition, the concentrations of diazepam and alprazolam affected their distributions in oil phase. Clonazepam was mostly located in the phospholipids rich phase. Again, the distribution in aqueous phase was in the increasing order of alprazolam, lorazepam, clonazepam and diazepam. This indicated that the distribution in aqueous phase depended upon their aqueous solubility. However, lorazepam and clonazepam, the moderated lipophilicity compounds, were likely deposited in the mesophase and concentration was less effect on their distribution.

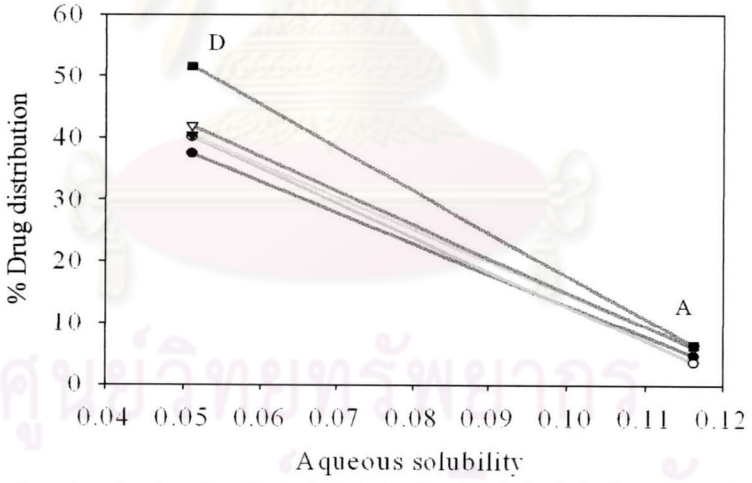


Figure 57 The effect of physicochemical properties on the distribution of benzodiazepine drugs in oil phase of submicron emulsion prepared by extemporaneous addition.

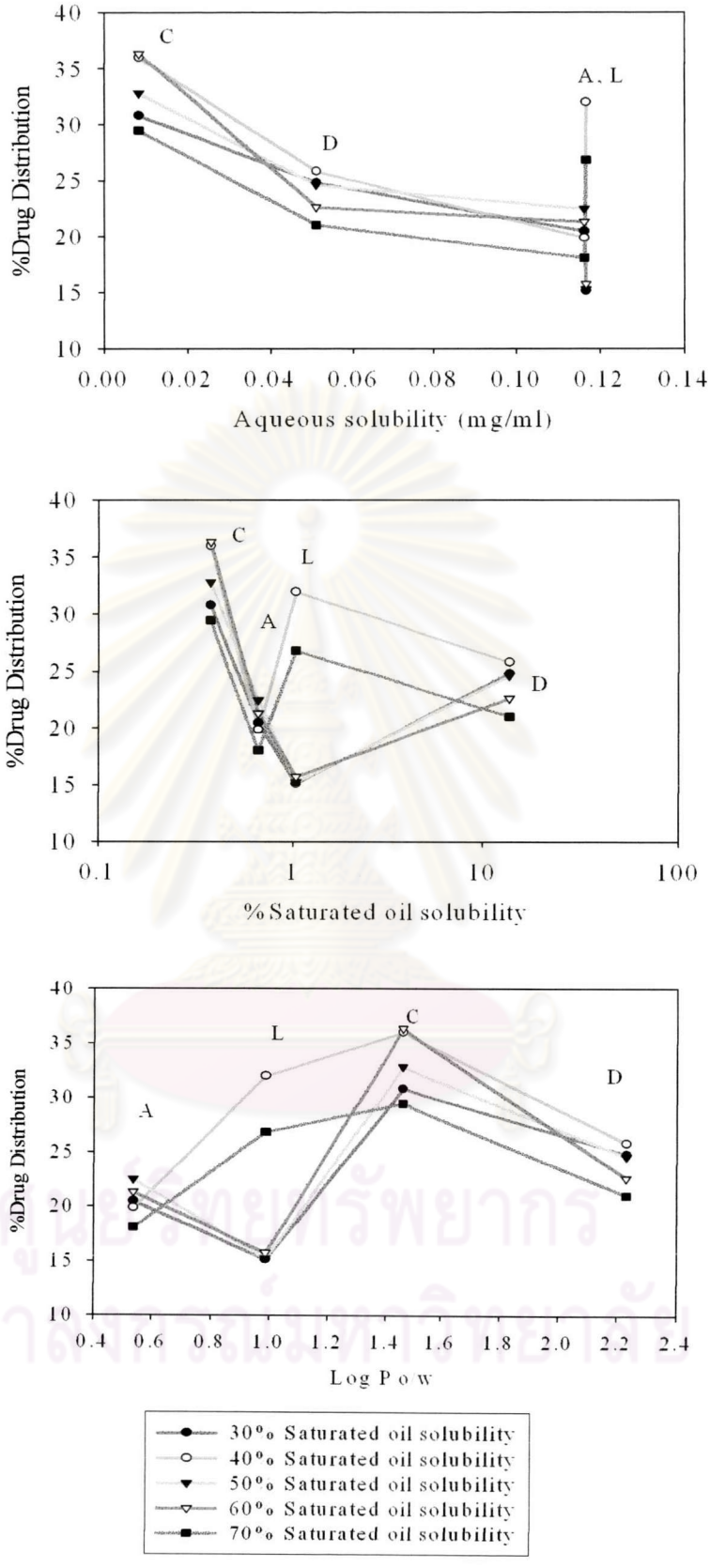


Figure 58 The effect of physicochemical properties on the distribution of benzodiazepine drugs in PC rich phase of submicron emulsion prepared by extemporaneous addition.

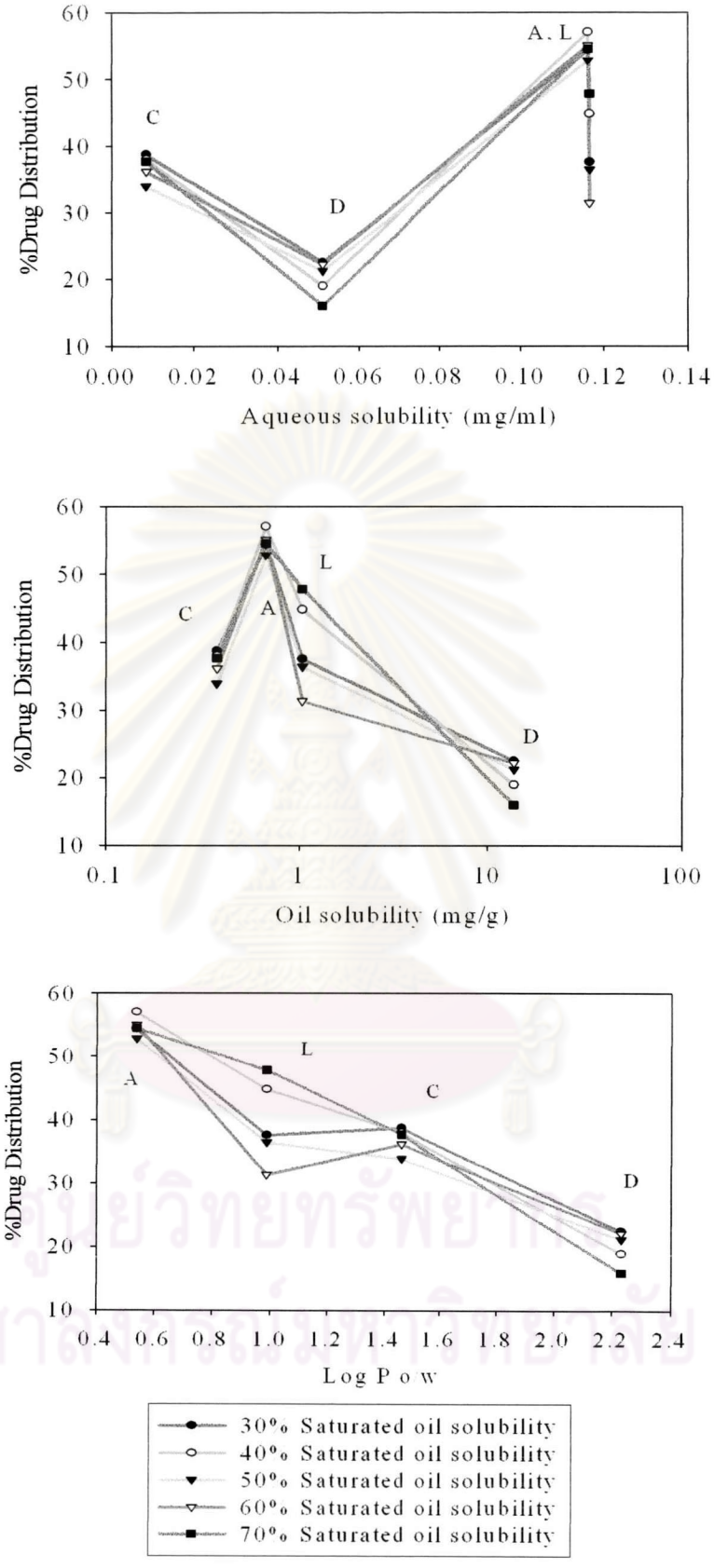


Figure 59 The effect of physicochemical properties on the distribution of benzodiazepine drugs in aqueous phase of submicron emulsion prepared by extemporaneous addition.

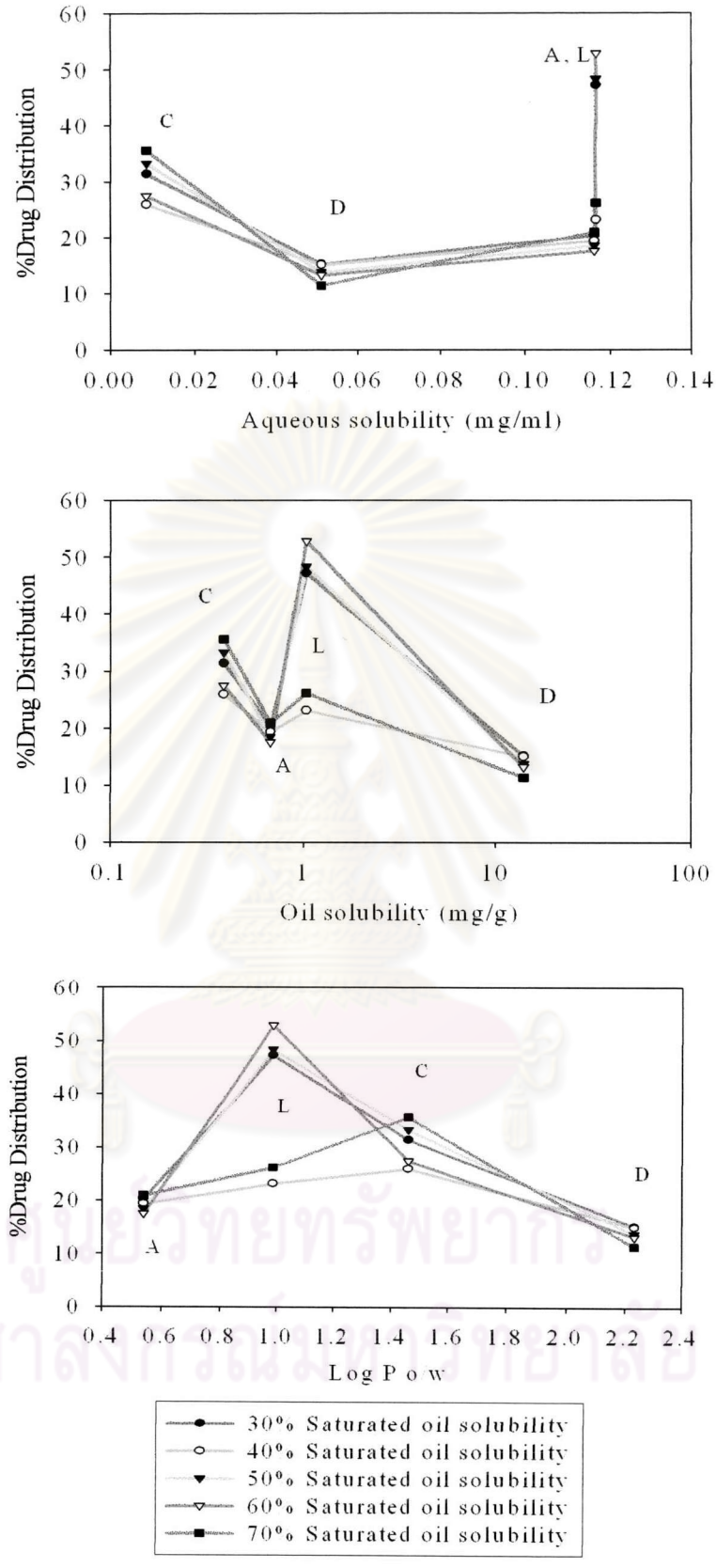


Figure 60 The effect of physicochemical properties on the distribution of benzodiazepine drugs in mesophase of submicron emulsion prepared by extemporaneous addition.

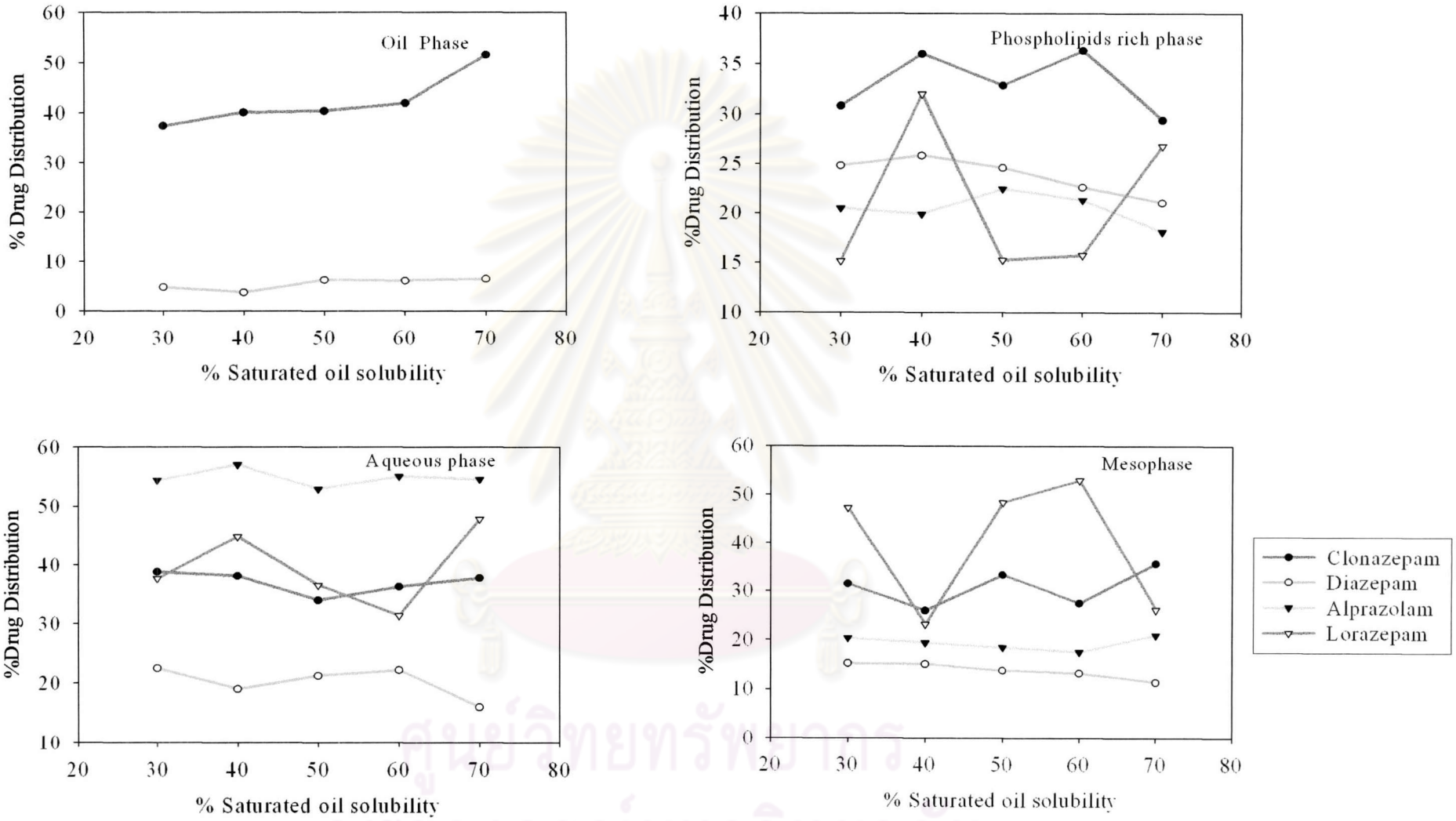


Figure 61 The effect of concentrations on the distribution of benzodiazepine drugs in various phases of submicron emulsion prepared by extemporaneous addition.

4.3 Shaking

Figure 62 displayed the distribution of benzodiazepine drug containing submicron emulsion. It was found that diazepam was more deposited in oil phase than clonazepam, lorazepam and alprazolam while in aqueous phase showed different behavior. Alprazolam, the lowest oil solubility and partition coefficient was less distributed in oil phase but mostly predominated in aqueous phase and mesophase. The moderate lipophilicty, clonazepam and lorazepam were mostly deposited in mesophase.

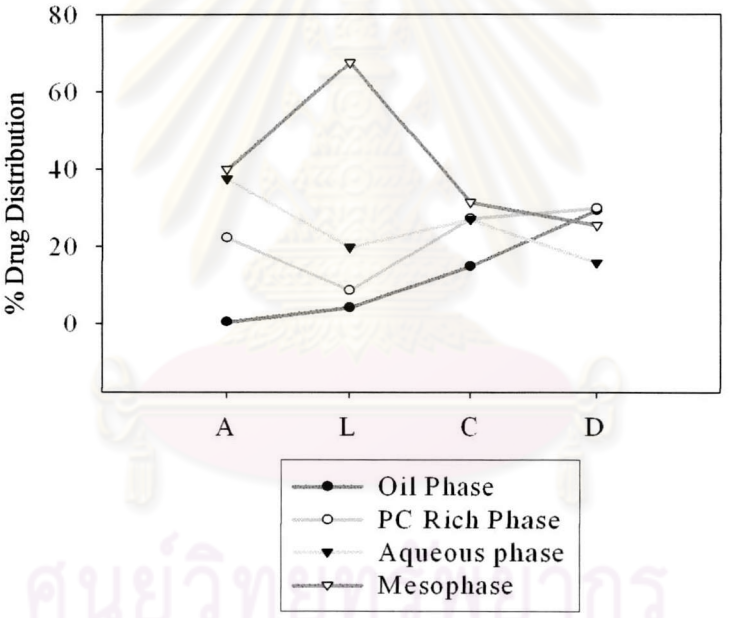


Figure 62 The effect of physicochemical properties on the distribution of benzodiazepine drugs in various phases of submicron emulsion prepared by shaking method.

Effect of incorporated methods on the distribution through various phases

Figure 63 illustrated the distribution of benzodiazepine drugs into various phases of submicron emulsion that prepared by different methods. From the data obtained it was found that amount of benzodiazepine drugs varied notably in the different phases when preparing by different methods. For all incorporation methods, according to the highest oil solubility and partition coefficient, diazepam was distinctly concentrated in oil phase and at least 15 times higher than the other three drugs whereas its amount in aqueous phase was much lower. The amount of alprazolam, clonazepam and lorazepam were not detectable in oil phase when preparing by extemporaneous addition while those by shaking preparation were present. This might cause by the longer shaking period (24 hour) comparing with the mixing period (2 hour) of extemporaneous addition. The distribution to oil phase of alprazolam and diazepam prepared by shaking was lower than that by de novo method. It was indicated that the physicochemical properties such as oil solubility and partition coefficient had more influence on the distribution to oil phase than shaking force for the highest and lowest lipophilic drugs, diazepam and alprazolam, respectively. However, the distribution to oil phase of clonazepam and lorazepam prepared by shaking were higher than that by de novo method, this might be caused by their moderate lipophilicity and also longer shaking period. According to their moderate lipophilicity compounds, clonazepam and lorazepam preparing by de novo emulsification including extemporaneous addition were more localized at interface than the other compound. In aqueous phase, the distribution of all drugs depended on their aqueous solubility, so, alprazolam, the highest aqueous solubility and lowest partition coefficient was prevailingly accumulated in this phase. Moreover, the amount of four drugs in aqueous phase prepared by extemporaneous addition was remarkably higher than that of prepared by de novo and shaking methods. It was revealed that the addition of dimethyl isosorbide for dissolving the model drugs prior to mixing with submicron emulsion base affected the solubilization potential of drug in aqueous phase. For shaking method all drugs seem to be gathered in mesophase according to the difficult partitioning into the inner phase except for diazepam which is the highest oil solubility and partition coefficient.

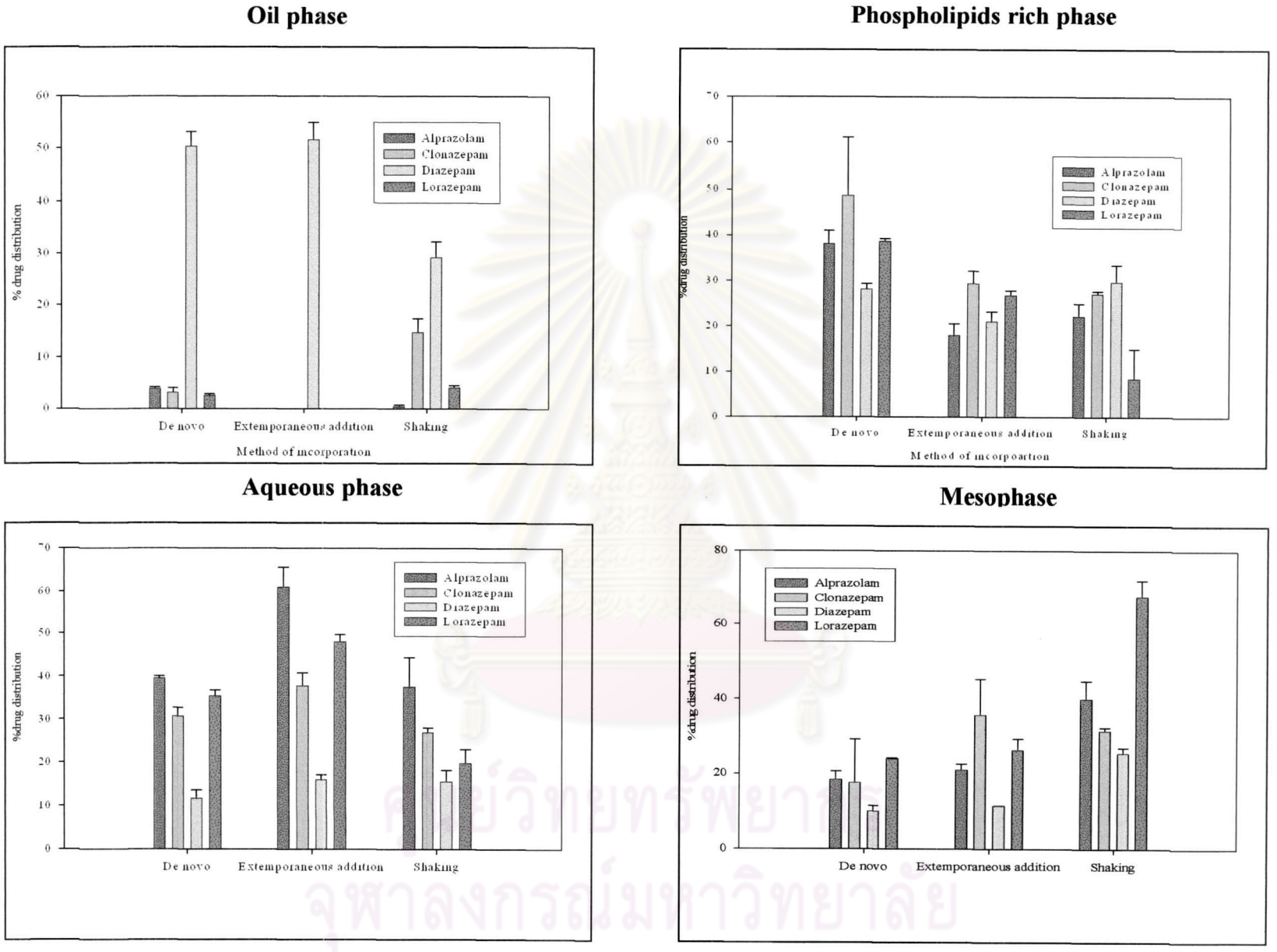


Figure 63 The effect of method of incorporation on the distribution of benzodiazepine drugs in various phases of submicron emulsion.