# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Phorbol Esters Extraction Method for Determination by HPLC

### 4.1.1 Determination the optimum condition for extraction methods

As mentioned in the scope of this study Part I that phorbol esters extraction from the Jatropha seeds and meals were evaluated by two techniques; methanol shaking-extraction and methanol ultrasonic extraction. The parameters evaluated for methanol shaking-extraction were shaking rate and contact time; while for methanol ultrasonic extraction, only the contact time was evaluated. The shaking rates were varied at 100, 200 and 300 rpm. For the ultrasonic extraction, the ultrasonic wave at 35 kHz was applied for all experiments. Both extraction procedures were performed for the contact time from 5 to 20 min. The extractants were then analyzed for phorbol esters by using HPLC. The details of the calibration curve and the concentrations of phorbol esters from the extractants are in Appendix A and B.

Figure 4.1 shows the phorbol esters concentration (as TPA) from both extraction procedures at different contact times. For the methanol shaking-extraction, the shaking rate at 300 rpm yielded the highest phorbol esters extraction for all contact time. However, the extraction efficiency from ultrasonic wave at the contact time 20 min was found very close to those from the shaking rate at 300 rpm and contact time 20 min and the statistic test shows not significantly different ($p = 0.05$) for these two conditions (the statistic analysis data is in Appendix F-1). The result was not surprised since the higher rate of shaking provides more contact of solvent (methanol) to phorbol esters in the seeds and meals. Thus, the shaking-extraction at 300 rpm was selected to compare with the ultrasonic extraction in the extended contact times. However, in order to ensure the efficiency of the selected condition of the shaking extraction method with the ultrasonic extraction, the longer contact time was conducted.

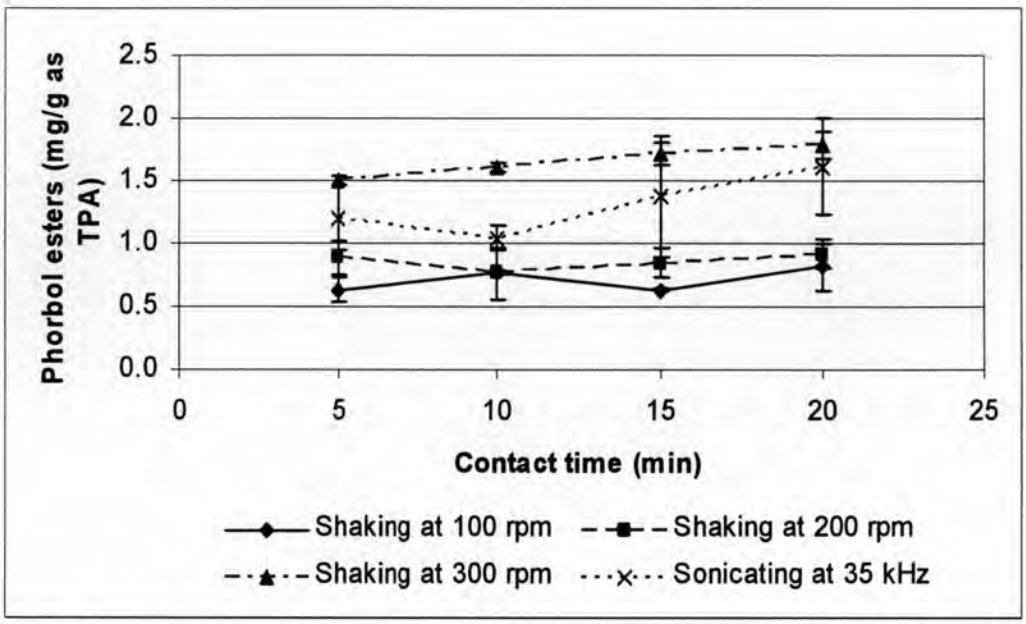



**Figure 4.1** Comparison of phorbol esters extraction efficiency between shaking at different rate and sonicating

The result of time extension for the selected techniques is shown in Figure 4.2. It was clear that the shaking extraction technique was able to extract phorbol esters at higher concentrations than those of the ultrasonic extraction at all contact time from 5 min to 2 hrs. In addition, it was found that the increasing of the contact time from 20 min to 2 hrs, the concentrations of phorbol esters increased from 1.61 and 1.78 mg/g as TPA to 2.18 and 2.35 mg/g as TPA for the ultrasonic extraction and the shaking extraction, respectively. This indicates that only 20 min was not enough time to reach equilibrium for the extraction. Furthermore, the graphs in Figure 4.2 for both methods of extraction seem not to be plateau yet. Thus it was expected that longer time than 2 hrs may increase efficiency of extraction. For Soxhlet extraction generally spent 4 – 6 hrs for the whole process of extraction from the solid sample (Kebbekus and Mitra, 1998) Therefore, to enhance efficiency of the extraction, time for the extraction was extended and some techniques were introduced to modify the selected procedure of extraction. However, due to the lower efficiency of the ultrasonic extraction technique, only ultrasonic wave is not suitable for using as the phorbol esters extraction method.

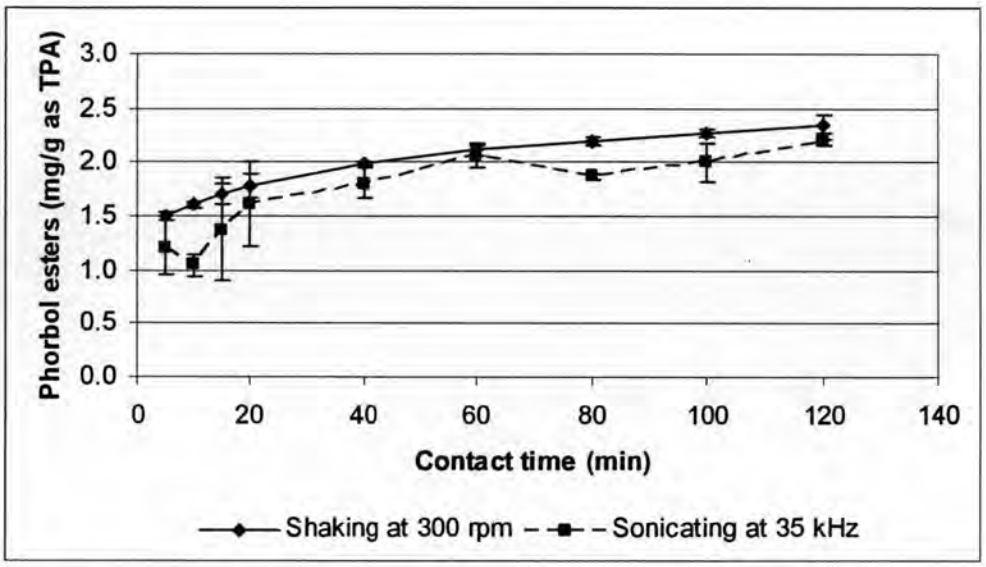



**Figure 4.2** Comparison of phorbol esters extraction efficiency between shaking and sonicating from the duration of extraction time 5 min to 2 hrs.

### 4.1.2 Modification of extraction technique for efficiency enhancement

To modify the method for extraction procedure, combination of sonication and shaking was introduced by pretreatment sample with ultrasonic for 10 min which is considered adequate time for the maximum optimal for ultrasonic extraction, as reported by (Kebbekus and Mitra, 1998). After sonication, the sample was shaken at various times from 1 hr to 6 hrs. In addition, the triple extraction by shaking at rate 300 rpm for 2 hrs each was also performed to evaluate the efficiency of the extractions. It was expected that the repetition of extraction and then combined the extract from each extraction was able to enhance the recovery of analyte due to fresh solvent has ability to dissolve the residue analyte in the sample instead of the saturated solvent (Mitra, 2003). The results from these two modified techniques were compared with the shaking extraction at 300 rpm at various contact time as shown in Figure 4.3. The experimental data is in Appendix B, Table B-2.

The results of the two methods –shaking with and without sonication—were not significantly different ($p = 0.05$) for all contact time (2, 4 and 6 hrs) and at the 6 hrs of extraction, the result of both methods were not significantly different ($p = 0.05$) with the triple of 2 hrs-shaking method either. Even though, the two highest concentrations of phorbol esters found at 6 hrs, 2.96 and 2.83 mg/g as TPA from the

triple of 2 hrs-shaking extraction and 10 min sonication with shaking, respectively, these two values found not significantly different (p = 0.05) with the one obtained from shaking only method at 4 hrs (2.72 mg/g as TPA). Therefore, the shaking-extraction at 4 hrs was selected for used in the next part of the study. The experimental data is in Appendix B, Table B-3 and the statistic analysis data is in Appendix F-2.

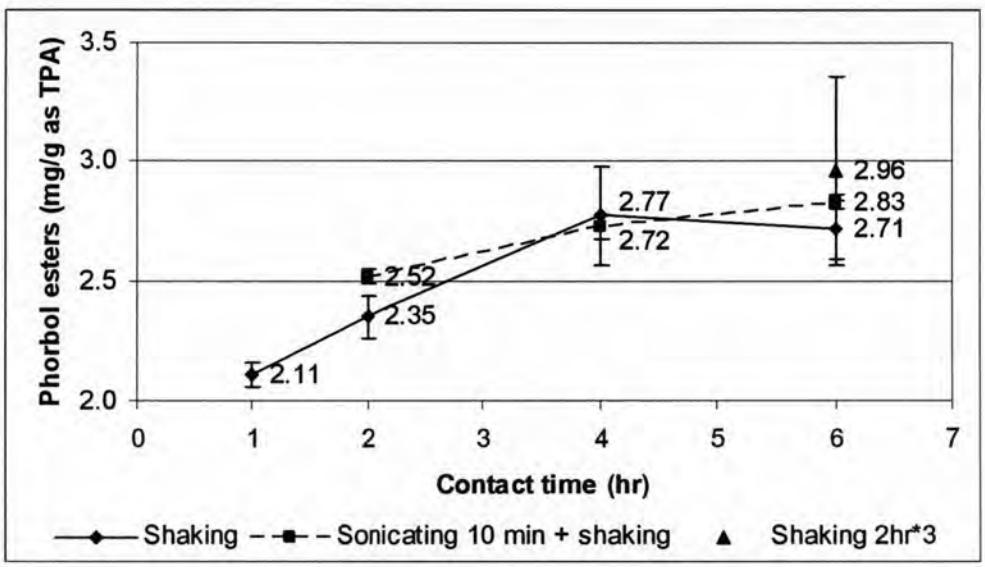



**Figure 4.3** Comparison of phorbol esters extraction efficiency of the shaking extraction and other two modified methods; 1) sonicating 10 min before shaking and 2) triple of 2 hrs-shaking

### 4.1.3 Verification of the selected extraction method

In order to verify the extraction technique selected from the previous part, the experiment on determination of % recovery of phorbol esters by using the selected method was evaluated. The procedure was modified from Mitra (2003) by matrix spiking the samples with known concentration of a similar structure compound with the analyte but not present in the sample. In this study, TPA was used as matrix spike because TPA is a compound in phorbol esters group that not present in the Jatropha seeds. Ten grams of dried kernel are spiked with 4 mg of TPA and then dried them and kept in the desiccators overnight. The concentration of TPA in the spiked kernel was then 0.4 mg/g of kernel. Two grams of spiked kernel were extracted with

methanol at shaking rate 300 rpm for 4 hrs, and then determined the amount of TPA in the extractant by HPLC. Peak of TPA appeared at 15.6 min and was used for calculation the concentration of TPA by the calibration curve and hence concentration of TPA in kernel. The % TPA recovery were calculated and it was assumed that the % recovery of TPA from this experiment represent the phorbol esters that derived from the shake-extraction method. From the result for recovery experiment is shown in Table 4.1, it was found that for the shaking-extraction (300 rpm, 4 hrs) can recover phorbol esters in kernel at around 88.10%. This value was considered high and within the requirement for matrix spike recovery method which is required at least 70% recovery (Kebbekus and Mitra, 1998; Mitra, 2003).

**Table 4.1** Recovery (%) of phorbol esters (TPA) extraction method

| Sample type | Kernel (g) | Peak area (15.6 min) | PEs (mg/g) | %recovery |
|---|---|---|---|---|
| Unspiked | 2.00 | 0 | 0.00 | - |
| | 2.00 | 0 | 0.00 | - |
| | 2.00 | 0 | 0.00 | - |
| | 2.01 | 0 | 0.00 | - |
| | 2.00 | 0 | 0.00 | - |
| | average | | 0.00 | - |
| | SD | | 0.00 | - |
| Spiked 0.4 mg/g | 1.90 | 53121 | 0.34 | 89.84 |
| | 2.00 | 54132 | 0.33 | 86.79 |
| | 2.00 | 53484 | 0.32 | 85.79 |
| | 2.00 | 54595 | 0.33 | 87.57 |
| | 2.00 | 56463 | 0.34 | 90.52 |
| | average | | 0.33 | 88.10 |
| | SD | | 0.0075 | 2.02 |
| | %CV | | 0.29 | 2.29 |

For the conclusion in this step, the selected phorbol esters extraction method for quantitative analysis of phorbol esters was mixing of 2 g *J. curcas* kernels or

meals samples with 20 ml methanol by shaker at 300 rpm, for 4 hours. The extract was analyzed the concentration of phorbol esters by HPLC-UV.

## 4.2 Physical and Chemical Properties of *Jatropha curcas* Seeds, Meals and Oil

### 4.2.1 Physical and chemical properties of seeds

*J. curcas* seeds were studied for their physical properties by random sampling 30 seeds and weighed for an average weight of a seed. Then the seeds were carefully cracked to remove shells, the shells and kernels were weighed and calculated the average weight of shell and kernel. The weight of one seed was ranging from 0.4765 – 0.8737 g and 0.7174 g in average. The weight of kernel from one seed was ranging from 0.2627 – 0.5708 g and 0.4550 g in average. The kernel:shell ratio equal 63.43:36.57. The summary of physical properties is shown in Table 4.2. The experimental data is in Appendix C, Table C-1.

**Table 4.2** Physical properties of *J. curcas* seeds

| Physical properties | Value |
|---|---|
| Average weight per seed (g) | 0.7174 |
| Average weight of kernel per seed (g) | 0.4550 |
| Average weight of shell per seed (g) | 0.2624 |
| Kernel : Shell ratio | 63.43 : 36.57 |

The kernel:shell ratio of our samples was found similar to Makkar et al. (1997) study that the kernel is about 61.3% ± 3.1%wt of a seed. Moreover, Makkar et al. (1998) also revealed that most of the *J. curcas* varieties have kernel:shell ratio with in the range of 60:40 in general.

As mentioned earlier, *J.* curcas is one of interested plant to be developed as an alternative fuel source (Banerji et al., 1985; Kandpal and Madan, 1994; Aderibigbe et al., 1997; Makkar et al., 1997). Even though, their seeds contain high nutrition, some toxins are found in most varieties of Jatropha (Liberalino et al., 1988; Makkar et al., 1997; Makkar et al., 1998; Akintayo, 2004; Chivandi et al., 2005). This leads to this

study that having the main target on removal of phorbol esters which is high toxin from the meals of the defatted Jatropha seeds. In this study, *J. curcas* seeds were obtained from the PTTCH Company. Thus, the three components in the seeds –oil content, crude protein and phorbol esters- focusing in this study were initially analyzed. The result is shown in Table 4.3.

**Table 4.3** Chemical properties of *J. curcas* seeds in this study

| Chemical properties | Seeds | | |
|---|---|---|---|
| | Whole seed | Kernel | Shell |
| Oil content (% wt.) | 36.88 | 58.14 | - |
| Crude protein (% wt.) | 13.01 | 20.51 | - |
| Phorbol esters (mg/g) | 1.59 | 2.49 | 0.036 |

The oil is found mainly in the kernels as expected. Kandpal and Madan (1994) reported from their study of Jatropha seeds in India that the oil proportion is 37.4% and 46% – 48.6% in seeds and kernel, respectively. . Pramanik (2003) also reported 30% – 50% of oil found in seeds and 45% – 60% found in kernels. For the nutrition of the *J. curcas* seeds in this present study, crude protein content were lower than those found in the other studies both in seeds and kernels. Liberalino et al. (1988) reported that crude protein were 26.75% and 19% - 31% in seeds and kernels, respectively. Phorbol esters is generally enclosed within kernels at around 2.49 mg/g which is in the range of 2.17 – 2.70 mg/g of kernels as reported by Makkar et al. (1998) for the toxic variety; whereas, the non-toxic Mexico variety contain phorbol esters around 0.11 mg/g of kernels. The experimental data is in Appendix C, Table C-2 and C-4; Appendix E.

### 4.2.2 Properties of Jatropha oil and residual meals from different oil extraction methods

Oil extraction methods can be classified into two large groups; physical extraction and chemical extraction. For *J. curcas* seeds, the convenient oil extraction method is mechanical pressing which have several different techniques and yield different extraction efficiency (Jongschaap et al., 2007). Solvent extraction is one of

the methods in chemical extraction group. Although, the solvent extraction can recovery most of the oil present in seeds, the process have to evaporate the solvent out to obtain the oil. In this study, instead of using solvent for extraction, surfactant aqueous-based solution was used as a solvent for oil extraction. Because of the amphipathic structure of surfactants, they have capability to separate oil from seeds by reducing the interfacial tension between oil phase and solid phase (Rosen, 2004). There have been researches introduced the surfactant-based solution to extract the oil from the palm kernels and the soybeans. Naksuk (2006) found that this method provided the high efficiency extraction up to 85% compared with the method using hexane extraction by a Soxhlet apparatus. The quality of both palm and soybean oils obtained from different extraction methods--hexane and surfactant-based solution--were similar.

In this study, *J. curcas* pressed meals supplied from PTTCH were twice defatted by PTTCH-screw pressing machine of whole *J. curcas* seeds. For other two meals in this study –from hexane Soxhlet and surfactant aqueous-based extractions were prepared in our lab from the seeds obtained from the PTTCH Company. The seeds were carefully cracked to remove shells for getting kernels. The kernels were defatted oil by hexane in Soxhlet apparatus and assumed that the oil content by this extraction was total oil present in the kernels. Thus, hexane Soxhlet method was considered having 100% oil extraction efficiency. For the surfactant-extracted method, 1 g of ground kernels was extracted by 10 ml of mixed surfactant solution that contains 3% Dehydol LS2 TH, 0.02% Alfoterra 145-4PO and 0.6% NaCl.

From Table 4.4, the Soxhlet-hexane extraction yielded the highest amount of oil and was assumed to be 100% efficiency for this study. Moreover, from this extraction method, the phorbol esters were partition into oil phase and hence it was found very low amount in the residual meal. In contrast, crude protein was not found to dissolve or penetrate into oil phase, thus it was found no lose of crude protein in the residual meals. This method may be the most suitable for further use of the residual meal; however, using hexane as a solvent extraction needs some complicated equipments for safety measure and can be apply for only large scale production. In addition, this method has a potential to cause air pollution and health effect if there is leak point in the system. From the conventional pressing method, the oil extraction

efficiency was about 77% which agreed with the result reported by Jongschaap et al. (2007) that the efficiency of mechanized pressing equipment is up to 75%. For the surfactant aqueous-base extraction, even though the oil extraction efficiency cannot competed with hexane extraction, it was considerable equal or better than the pressing technology. Moreover, a surfactant solution not only extracted the oil but also was able to reduce phorbol esters from the residual meal. However, the surfactant-extracted meals showed the highest loss of crude protein when compared with the other methods. The experimental data is in Appendix C, Table C-3 and C-4; Appendix E.

**Table 4.4** Properties of oil and meals from different oil extraction methods

| Properties | Oil Extraction Method | | |
|---|---|---|---|
| | Pressing[a] | Soxhlet[b] | Surfactant[b] |
| Meal | | | |
| - Oil content (% wt.) | 13.42 | 0 | 29.60 |
| - Phorbol esters (mg/g of meal) | 1.45 | 0.014 | 1.12 |
| - Crude protein (% wt.) | 17.94 | 49.18 | 18.94 |
| Oil | | | |
| - Phorbol esters (mg/g of oil) | 1.11 | 6.35 | 2.01 |
| Comparison of oil extraction method | | | |
| - % Oil extraction efficiency | 77.03 | 100.00 | 78.69 |
| - % Phorbol esters reduction in meal | 8.81 | 99.45 | 55.02 |

Note: [a] Pressing whole seeds

[b] Oil extracted only from kernels

### 4.3 Phorbol Esters Removal from Meals using Surfactant Solutions

This part of the study used only the meals from the pressing method because it is the conventional method used for oil extraction from *J. curcas* seeds. PTTCH pressed meals used for this study contained phorbol esters about 1.45 mg/g as TPA and was considered as initial concentration of phorbol esters.

The pressed meals were dried, then ground and sieved for the size of 20 mesh screening. Nonionic surfactant and mixed surfactant solutions were evaluated for phorbol esters removal from pressed meal. Besides the investigation of surfactant system, in order to determine optimum condition for the removal, other physical parameters such as contact time and solid:liquid ratio as well as reused solution were evaluated.

### 4.3.1 Investigation for surfactant solutions

#### 4.3.1.1 Phorbol esters removal by nonionic surfactant systems

Nonionic surfactants were selected for phorbol esters removal because the nonionic surfactants generally have lower CMC than the anionic surfactants and suitable to remove the oily compound from the soil or solid surface (Bourrel and Schechter, 1988; Rosen, 2004). Two groups of nonionic surfactants were evaluated in this study were polyoxyethylene (20) sorbitan or Tween group and fatty alcohol C 12 – 14 ethoxylate group. For Tween group included Tween 20 (T20), Tween 40 (T40), Tween 60 (T60) and Tween 80 (T80). For fatty alcohol C 12 – 14 ethoxylate group included Dehydol LS3, Dehydol LS7 and Dehydol LS9 extended of 3 (LS3), 7 (LS7) and 9 (LS9) ethoxylate group, respectively.

Each surfactant solutions at 40 mM were mixed with 2 g of meals in 20 ml. Then shake for 30 min at 300 rpm. The results are shown in Table 4.5 and Figure 4.4. All experimental data in this section is in Appendix D, Table D-1 to D-7.

From Figure 4.4, for Tween group, the removal efficiency is ranged from 60 to 72 % while the system of fatty alcohol ethoxylate, the removal efficiency is ranging from 30 to 65 %. Tween 40, 60 and 80 have the similar phorbol esters removal and found not significant different ($p = 0.05$). Similarly, results for Tween 20, Dehydol LS7 and Dehydol LS9 have the similar phorbol esters removal and not significantly different with statistical test ($p= 0.05$). From this preliminary result, Tween 60 has the highest efficiency in while LS 3 has the lowest efficiency. Since LS3 showed remarkable low capability on phorbol ester removal, it was ignored for further experiment. The statistic analysis data in this part is shown in Appendix F-3.

**Table 4.5** Properties of nonionic surfactant and phorbol esters removal (%) by 40 mM of various types of nonionic surfactants

| Nonionic surfactant | Properties | | | %PEs removal | | |
|---|---|---|---|---|---|---|
| | C-chain | EON | HLB | %Removal | SD | %CV |
| Dehydol LS3 | C12-14 | 3 | 7.9 | 33.42 | 10.11 | 30.26 |
| Dehydol LS7 | C12-14 | 7 | 12.1 | 58.01 | 2.93 | 5.06 |
| Dehydol LS9 | C12-14 | 9 | 13.4 | 65.63 | 4.85 | 7.39 |
| Tween 20 | C12 | 20 | 16.7 | 61.41 | 4.65 | 7.57 |
| Tween 40 | C16 | 20 | 15.6 | 70.87 | 2.18 | 3.07 |
| Tween 60 | C18 | 20 | 14.9 | 72.14 | 1.87 | 2.59 |
| Tween 80 | C18=1 | 20 | 15.0 | 72.01 | 2.31 | 3.21 |

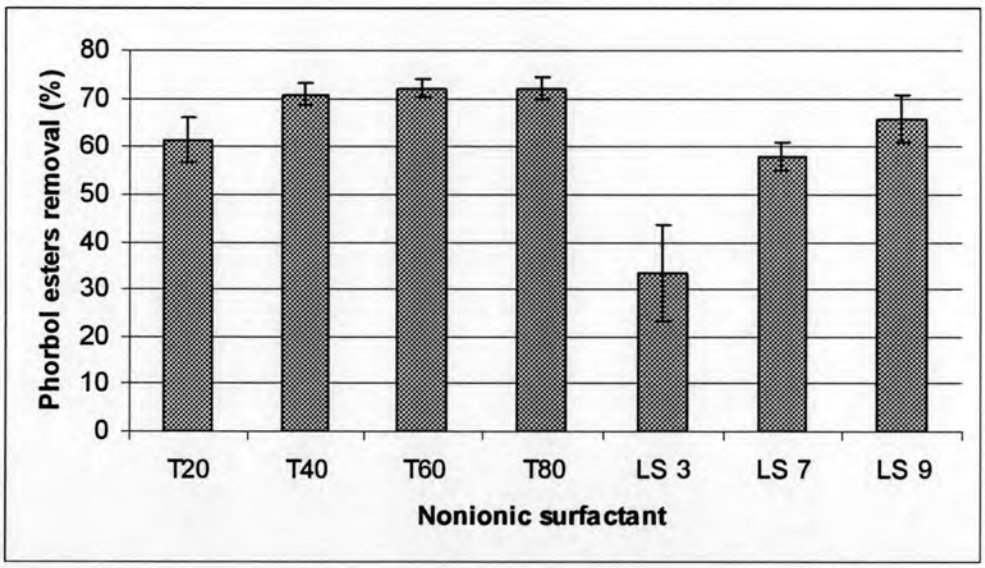


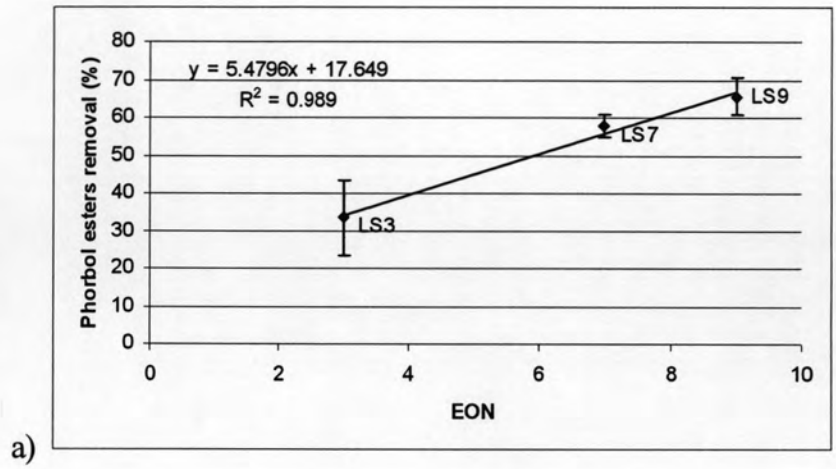

**Figure 4.4** Phorbol esters removal (%) with 40 mM of nonionic surfactant solution: T20, T40, T60 and T80 are Tween 20, Tween 40, Tween 60 and Tween 80, respectively while LS3, Ls 7 and LS9 are Dehydol LS3, Dehydol LS7 and Dehydol LS9, respectively

To gain more understanding of the result, the plots to compare ethoxylate number of fatty acid ethoxylate (EON) and carbon chain length in tails of Tween group with the removal efficiency were drawn in Figure 4.5a and 4.5b. In addition, for comparison the two groups of nonionic surfactant, hydrophile-lipophile balance or HLB is the parameter to normalize two nonionic surfactant groups since the HLB value indicates the property of a surfactant system. The selected surfactant system should have the similar HLB of selected compound that desire to be removed (Rosen, 2004). So, the plot between HLB of all nonionic surfactants and the removal efficiency was drawn as shown in Figure 4.5c.

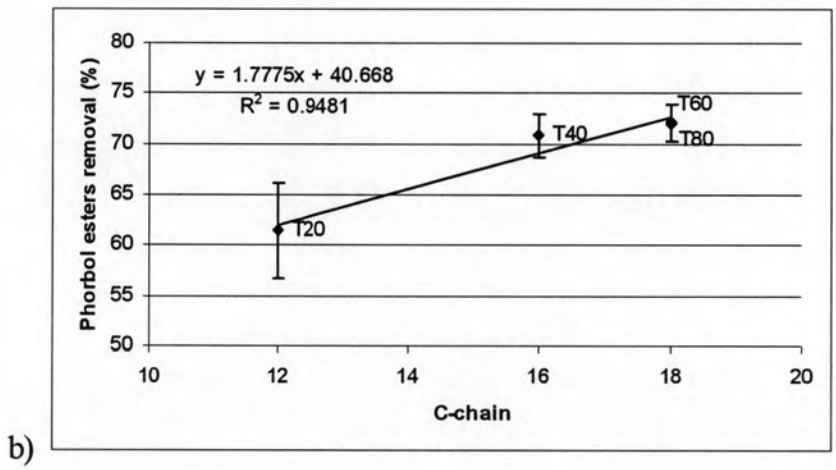
For the fatty alcohol ethoxylate group or Dehydol group, EON of the surfactants used in this study exhibits a linear relationship with the removal efficiency (see Figure 4.5a). The removal efficiency increases when EON increases. This can be explained on the fact that the solubilization of polar compound increases when increases the EON of nonionic with the same hydrophobic portion (Rosen, 2004). Phorbol esters are the hydrophobic compound; however, from their structure of ester, they are considered polar compounds.

On the other hand, C-chain length represents hydrophobicity of the surfactants, the higher C-chain length, and the higher hydrophobicity of the surfactant. However, the result for the Tween groups was the higher C-chain length, the higher removal of phorbol esters. This may due to the hydrophobicity of higher C-chain length is more compatible with phorbol esters structure.

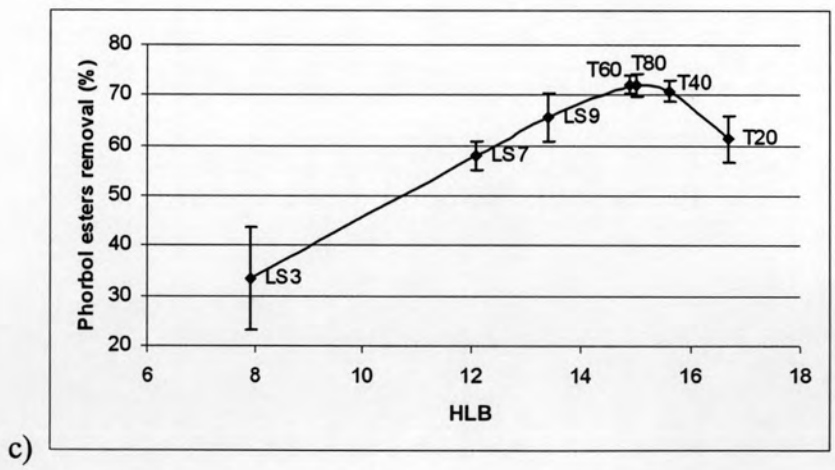
The plot between HLB of nonionic surfactants and phorbol esters removal efficiency is shown in Figure 4.5c. The results found that the increasing of the HLB causes the increasing of the removal until reaches the optimal HLB of solution at around 15 and then the removal efficiency decreased. This indicates that several parameters govern the mechanism of the removal of phorbol esters.




a)




b)




c)

**Figure 4.5** Effect of EON, C-chain length and HLB on the removal of phorbol esters (PEs) of a) fatty alcohol C12-14 EO group, b) Tween group and c) fatty alcohol C12-14 EO and Tween groups

The effect of concentration of nonionic surfactants was evaluated by varying concentration of Tween 20, Tween 40, Tween 60, Tween 80, Dehydol LS7 and LS9 in the range of 10 to 40 mM to remove phorbol esters from the meals at the same removal condition (see Figure 4.6). It was found that Tween 20 and Dehydol LS7 performed the lowest efficiency at all concentrations for the Tween and the fatty alcohol ethoxylate groups, respectively. The result for all surfactants in Tween group reveals clearly that the higher the concentration, the higher efficiency for phorbol esters removal. Even though Tween 80 showed lower performance than others at concentration lower than 40 mM, however at 40 mM Tween 40, 60 and 80, demonstrated similarly result for the phorbol esters removal from the meal. Tween 80 is more commonly used in Thailand compared Tween 40 and 60 due to its lower price. Therefore in the next part of experiments, the selected nonionic surfactants from each group were Tween 80 and Dehydol LS9 TH.

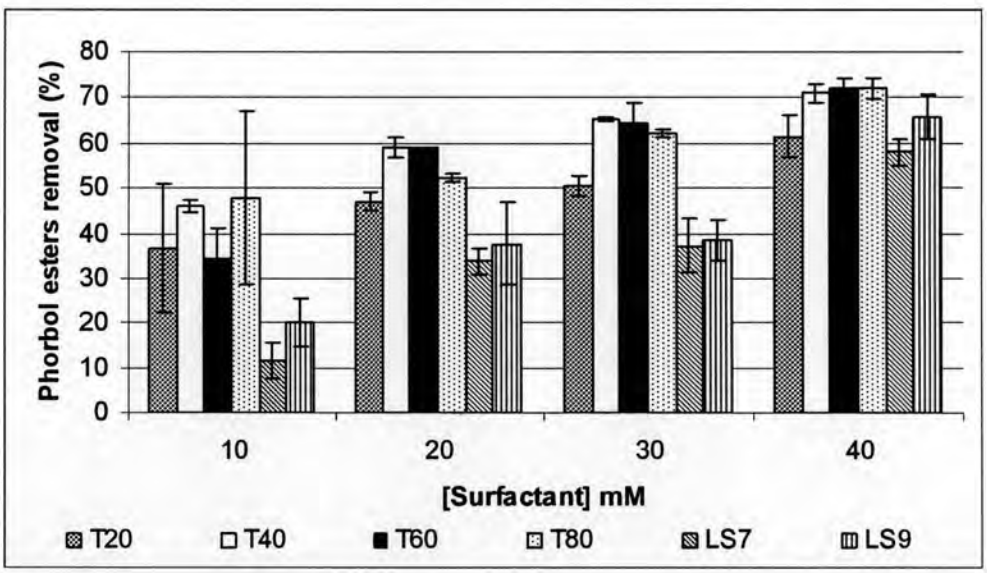



**Figure 4.6** Phorbol esters removal with nonionic surfactant solutions at concentration from 10 to 40 mM

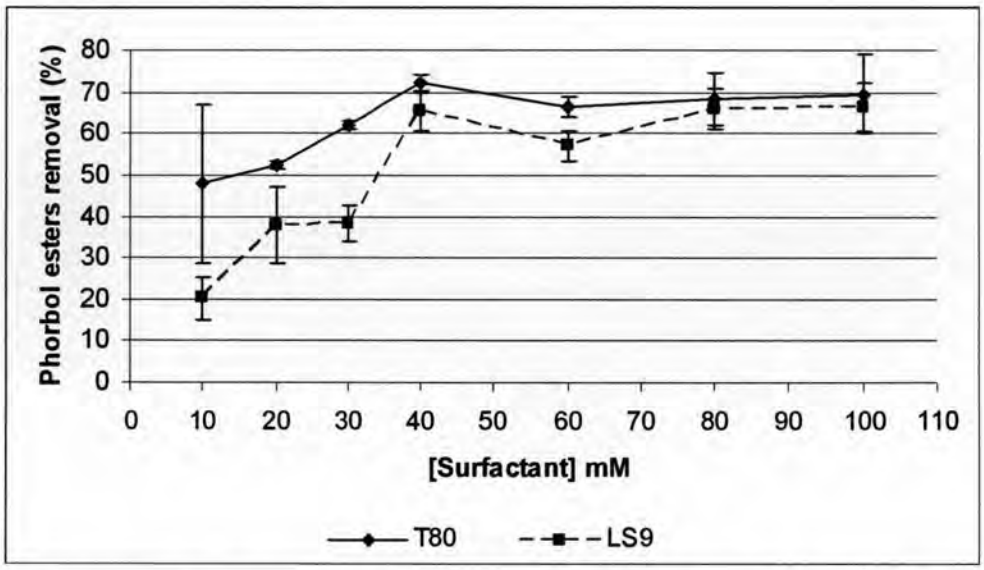



**Figure 4.7** Phorbol esters removal by systems of Tween 80 and Dehydol LS9 at the various concentrations from 10 to 100 mM

To evaluate the efficiency of phorbol esters removal, the concentrations of the selected single nonionic, Tween 80 and Dehydol LS9 were extended to be from 10 mM – 100 mM to find the suitable concentration. The results are shown in Figure 4.7. From the graph in Figure 4.7 for both surfactants, it was confirmed that at 40 mM was the optimum concentration since the graphs tended to be plateau even the concentration increased to 100 mM. Therefore, 40 mM of nonionic surfactants was the optimum concentration and used for evaluation other parameters. The systems of single Tween 80 and single Dehydol LS9 solutions at 40 mM showed their ability to removal phorbol esters around 69.5% – 72.5% and 65.5% – 70.5%, respectively.

#### 4.3.1.2 Phorbol esters removal by mixed surfactant solution

A mixed surfactant between nonionic and anionic surfactant is considered temperature insensitive system as compared to the single nonionic system; moreover, it can enhance the salinity tolerance than the single anionic system (Bourrel and Schechter, 1988, p.324 and 327). Therefore, in this part of the study Aerosol OT or AOT was introduced to mix with nonionic surfactant. AOT is a surfactant that is used as food additive. Tween 80 and Dehydol LS9 were fixed at 40 mM from the previous study to mix with AOT and NaCl for finding the optimal ingredients used for

phorbol esters removal in the pressed meals. The experimental conditions were the same as in the previous study. Two grams of meals were mixed with 20 ml solution, shaked at 300 rpm for 30 min, then left it for 30 min to allow the meals to precipitate and then the meals was filtrated for phorbol esters determination.

#### 4.3.1.2.1 Effect of anionic surfactant (AOT) concentration

In general, performance of anionic surfactant is enhanced by adding some salt due to the salt reduce electrostatic affect of the surfactant. In this study, salt was added and kept constant at 100 mM for all concentrations of AOT. The results in Figure 4.8 show that both single Tween 80 and Dehydol LS9 solutions have higher phorbol esters removal efficiency than all systems mixed with AOT. However, the residual meals that detoxified with mixed surfactants were found easy to filter than those extracted by single nonionic surfactant during the experiment. For mixed systems of Tween 80, at 5 mM of AOT adding resulted the highest removal at 70.59% but found not significant different from 72.32% yielded by the single Tween 80 system. For mixed systems of Dehydol LS9, the highest removal was 62.30% when 7.5 mM of AOT was mixed; however; the result was found not significantly different ($p = 0.05$) with the one mixed at 5 mM of AOT (62.18% removal) and with the one of the single Dehydol LS9 system (64.76% removal) either. Thus, the mixed surfactant system required only 5 mM of AOT to enhance or maintain the efficiency removal phorbol esters from the pressed meals. The experimental data is in Appendix D, Table D-9 and D-10. The statistic analysis data see in Appendix F-4 and F-5.

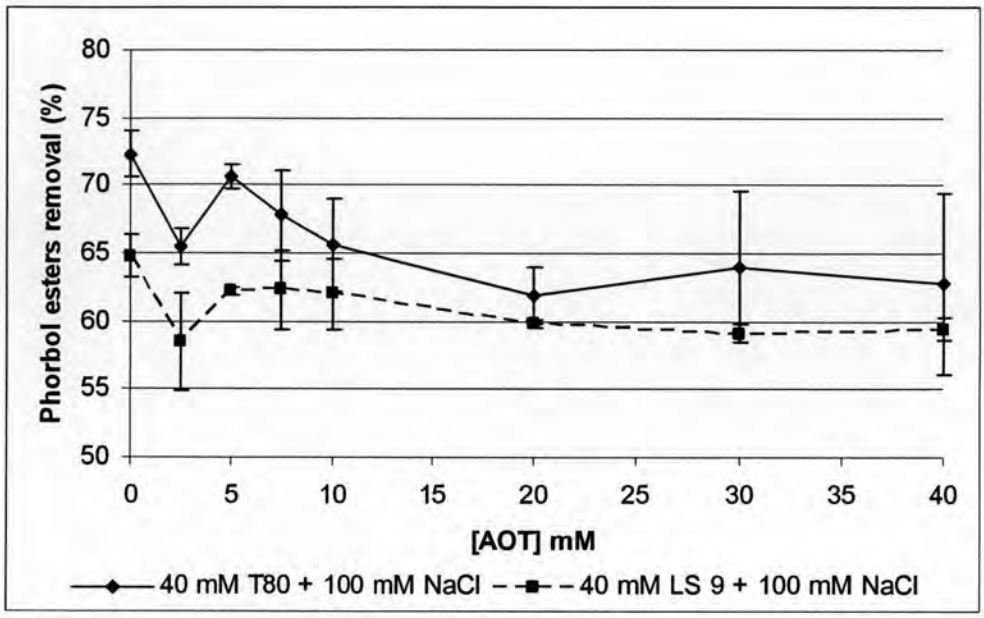



**Figure 4.8** Phorbol esters removal with 40 mM Tween 80 and 40 mM Dehydol LS9 mixed with AOT and 100 mM NaCl

#### 4.3.1.2.2 Effect of electrolyte (NaCl)

NaCl is salt or electrolyte generally used for reducing the ionic strength of negative ion at hydrophilic head of ionic surfactants (Rosen, 2004). The mixed solutions of 40 mM nonionic and 5 mM anionic were evaluated the optimal concentration of NaCl. The results are shown in Figure 4.9.

When the concentration of NaCl increased, the phorbol esters removal slightly increased until reached the optimum point at 100 mM and become plateau for mixed Dehydol LS 9. However, for mixed Tween 80, the removal dropped at 300 mM NaCl. Therefore, 100 mM was selected to be the optimal concentration of NaCl for both mixed surfactant systems. The phorbol esters removal efficiency of the mixed solution of 40 mM Tween 80, 5 mM AOT and 100 mM NaCl ranged from 80.07% to 82.09% with the average at 81.23%. While, the removal efficiency of the mixed solution of 40 mM Dehydol LS 9, 5 mM AOT and 100 mM NaCl ranged from 78.30% to 79.74% with the average at 78.85%. The experimental data is in Appendix D, Table D-11 and D-12. The statistic analysis data see in Appendix F-6 and F-7.

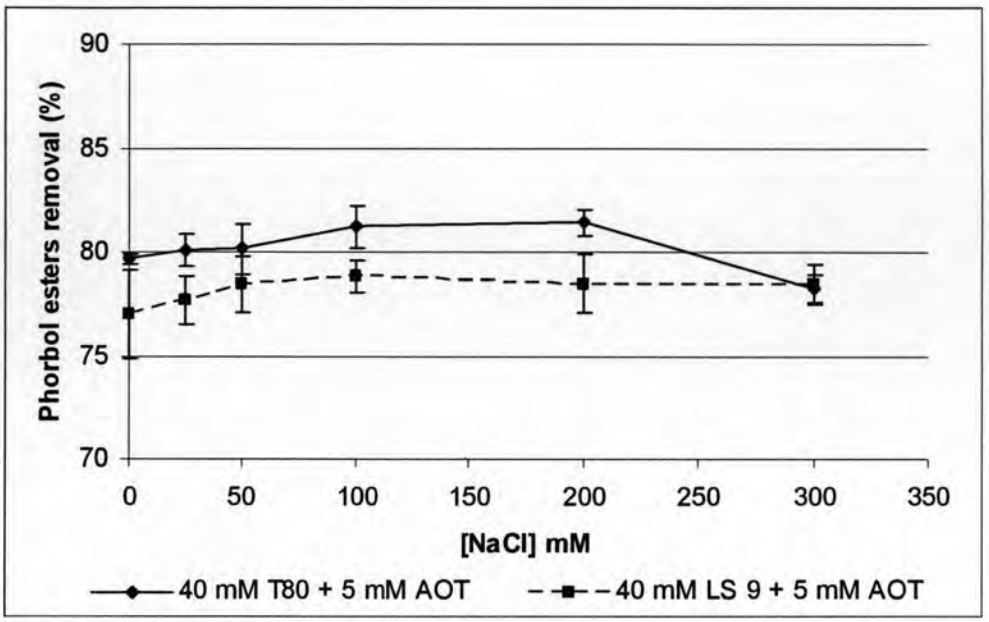



**Figure 4.9** Phorbol esters removal with 40 mM Tween 80 and 40 mM Dehydol LS9 mixed with 5 mM AOT and NaCl

It should be noted here that % phorbol esters removals obtained from this experimental step were found higher than the result from AOT selection step. For mixed Tween 80, the average of phorbol esters removal efficiency increased from 70.59% to 81.23% which is more than 10% enhancing efficiency. Even higher than the mixed Dehydol LS9 that the average efficiency increased from 62.18% to 78.85%. The increasing of removal efficiency was suspected to possibly be from the age of pressed meals. The previous study used the pressed meals that kept for more than 10 months; whereas, this experimental step used the new pressed meals that recently pressed not more than 2 weeks. Regarding to Mitra (2003), the organic compound in the older meal has longer time for organic compound to react with solid phase and hence the stronger bond between chemicals can be created is compared to the newer one. For this reason, the new meals can be extracted the phorbol esters more easily than the old one. However, this should be evaluated to confirm this suspicion in the future study.

#### 4.3.1.3 Comparison of single nonionic surfactant and mixed surfactants systems

The ingredients in percent weight of the selected solutions are shown in Table 4.6. The phorbol esters removal efficiencies were 22.49%, 81.43%, 81.23%, 81.87% and 78.85% from the extraction by using water, single T80, mixed T80, single LS9 and mixed LS9, respectively (Figure 4.10). The results show that some compounds in phorbol esters group were dissolved in the water and hence the concentration after extraction with D.I. water was reduced from the initial concentration. The four selected systems both single and mixed surfactant solutions were not found significantly different for the removal. However, although mixed surfactant systems did not improve the removal efficiency, it was found that the mixed systems facilitated the residual meals to precipitate well and was easy to separate the meals from the solutions. The experimental data by D.I. water is in Appendix D, Table D-8. The statistical analysis data is in Appendix F-8.

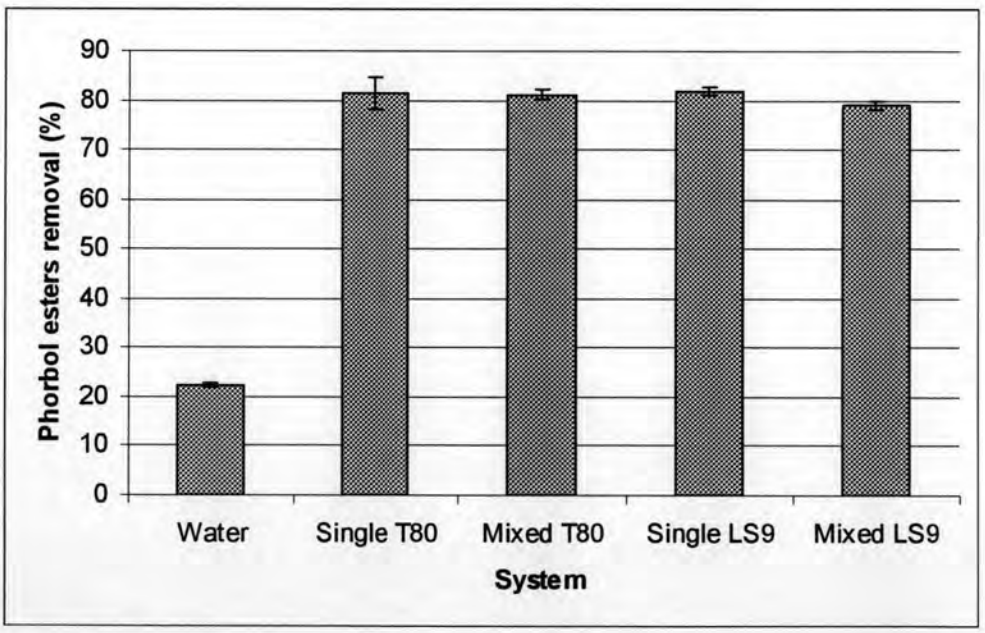



**Figure 4.10** Phorbol esters removal using selected single nonionic surfactant (40 mM) and mixed surfactant (40 mM nonionic surfactant and 5 mM AOT with 100 mM NaCl) systems compared with only D.I.water

**Table 4.6** The ingredient of surfactant solutions in percent weight

| Ingredient (g/100 ml) | Single T80 | Single LS9 | Mixed T80 | Mixed LS9 |
|---|---|---|---|---|
| T80 | 5.24 | - | 5.24 | - |
| LS9 | - | 2.36 | - | 2.36 |
| AOT | - | - | 0.22 | 0.22 |
| NaCl | - | - | 0.58 | 0.58 |

### 4.3.2 Effect of physical parameters on phorbol esters removal

After known the optimal composition of solutions to remove phorbol esters from the pressed meals, the removal efficiencies of the four selected systems of single T80, single LS9, mixed T80 and mixed LS9 was evaluated the removal efficiency by varying the physical parameters. The parameters expected to affect on the efficiency and selected for this study were contact time and solid:liquid ratio. In addition, in order to enhance the removal efficiency, the re-extraction and reuse solution were investigated for reduction the cost of process.

#### 4.3.2.1 Contact time

The previous experiments used 30 min extraction time, in order to evaluate if the efficiency still maintain if the contact time is shorter than 30 min. The extractions were conducted with reduced the time for extraction in the range of 2 min to 30 min. The system of mixed T80 solution was selected for this study. The results in Figure 4.11 demonstrated clearly that the optimum contact time was at 15 min. The statistic analysis found that the removal from 15, 20 and 30 min contact time were not significantly different at 95% confident. Therefore, the system required 15 min to have well mixed between meals and solution. This was expected to provide an opportunity for surfactant to remove phorbol esters from the meals. The experimental data is in Appendix D, Table D-13. The statistical analysis data is in Appendix F-9.

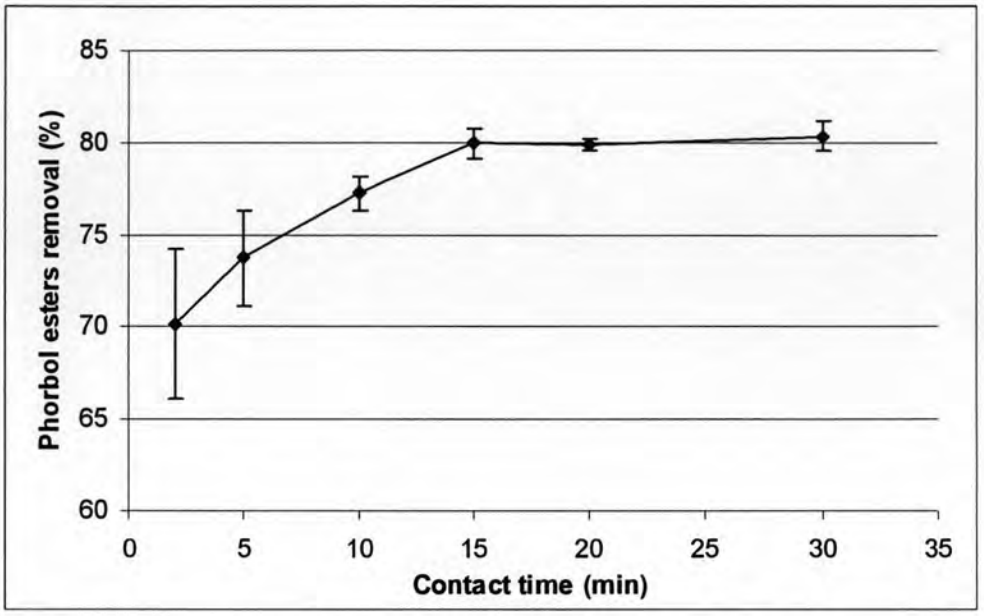



**Figure 4.11** Effect of contact time to phorbol esters removal by Mixed of 40 mM Tween 80 and 5 mM AOT with 100 mM NaCl

#### 4.3.2.2 Solid:liquid ratio

Solid:liquid ratio were evaluated in this experiment by varying the solid (g): liquid (ml) ratio from 0.5:10, 1:10, 1.5:10 and 2:10. It is expected that the less ratio provides the more space for meal to contact surfactant monomers and hence reduce interfacial tension of solid and phorbol esters. This mechanism would lead the phorbol esters to detach and solubilize into surfactant solution. The system of mixed 40 mM LS9 and 5 mM AOT with 100 mM NaCl was used for this experiment. Addition to the varying of solid liquid ratio, contact time was also varied at 15, 20 and 30 min. The results are shown in the Figure 4.12.

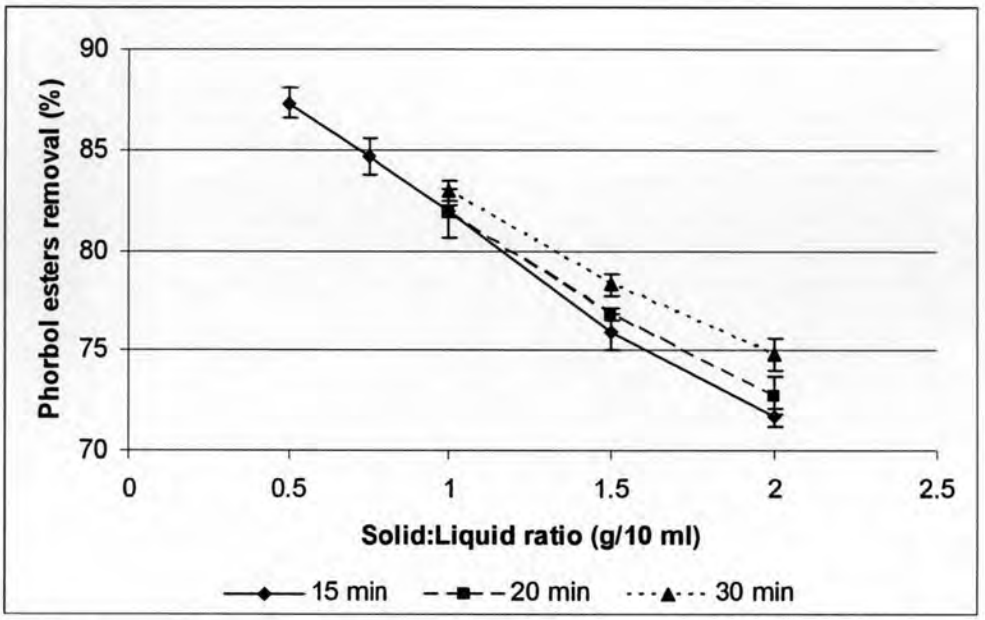



**Figure 4.12** Phorbol esters removal by mixed of 40 mM Dehydol LS9 and 5 mM AOT with 100 mM NaCl in solid:liquid ratio and varies contact time

The result was as expected that at the lowest ratio of the solid liquid ratio yield the highest efficiency of removal. The same trend was found for all sets of different contact time. When focus at the same ratio, the result agreed with the previous experiment that contact time after 15 min has little affect on the efficiency, even though the slightly increase of the removal was observed. On the other hand, the solid:liquid ratio exhibited significantly affect on phorbol ester removal.

#### 4.3.2.3 Re-extraction

This work intended to raise up the removal efficiency by extraction the same meals with fresh solution of the surfactant. Two g of meals were extracted with 20 ml of surfactant solutions for 15 min, and then waited 30 min to allow the residual meals to precipitate, the clear solution was then pouring out and 10 ml of fresh solution was added, then the same extraction procedure was performed. The result of re-extraction is shown in Figure 4-13.

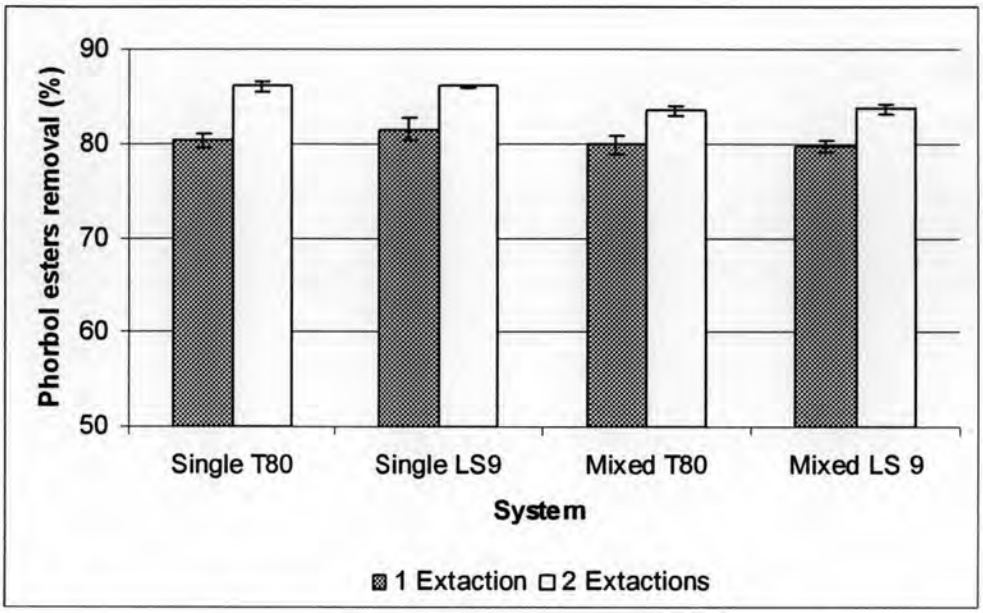



**Figure 4.13** Phorbol esters removal by accumulate from once and twice extractions by new solution, using same meals at 1 g:10 mL ratio; Single T80 as 40 mM Tween 80, Single LS9 as 40 mM Dehydol LS9, Mixed T80 as mixed of 40 mM Tween 80 and 5 mM AOT with 100 mM NaCl, and Mixed LS9 as mixed of 40 mM Dehydol LS9 and 5 mM AOT with 100 mM NaCl

From Figure 4.13, the re-extractions improved the phorbol esters removal by single T80 from 80.28% to 86.06%, single LS9 from 81.52% to 86.06%, mixed T80 from 79.87% to 83.52% and mixed LS9 from 79.80% to 83.74%. The overall shaking is 30 minutes, 2 g of meals per 30 ml of solutions. The experimental data can be seen in Appendix D, Table D-14.

One time extraction with 0.5 g:10 ml in 15 minutes provide the higher the removal efficiency than the re-extraction from the previous study. Therefore, this removal condition was used for next work for all solution system. The solid:liquid ratio equals 0.5 g:10 ml, 1 g per 20 ml in real. The contact time of shaking is 15 minutes. The experimental data see in Appendix D, Table D-15, D-16 and D-17.

#### 4.3.2.4 Reuse solution study

After an extraction, it was assumed that none or small amount of surfactant adsorbed on the meal, thus most of surfactant(s) expected to remain in the solution. Consequently, to indirectly prove this, the solutions were reused with the

new meals for phorbol esters removal. This experiment did not aimed only to indicate what phase the surfactant(s) existed but it also could gain more benefit in term of lower cost of spent surfactant if the used solutions performed well in the second use. The results show that four solutions were able to reuse and have similar efficiency for phorbol esters removal (Figure 4.14).

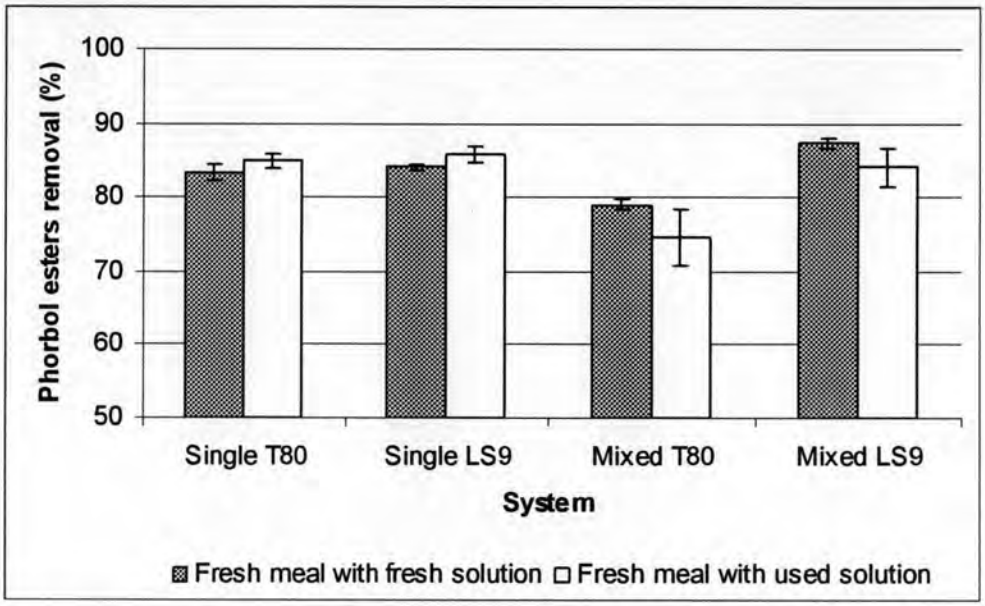



**Figure 4.14** Comparison phorbol esters removal from new surfactant solutions and the reused surfactant solutions; Single T80 as 40 mM Tween 80, Single LS9 as 40 mM Dehydol LS9, Mixed T80 as mixed of 40 mM Tween 80 and 5 mM AOT with 100 mM NaCl, and Mixed LS9 as mixed of 40 mM Dehydol LS9 and 5 mM AOT with 100 mM NaCl

It was surprising that for both single nonionic surfactant solutions, the efficiency of reuse solutions was slightly higher than the first using. The first using efficiency of single T80 was 83.39% and when reused this solution with the new meal, the removal raised up to 84.85%. For the single LS9 system, the efficiency was also increased from 84.04% to 85.86%. While the mixed solutions, the reuse solutions lose ability to remove phorbol esters about 3 – 4 %. Mixed T80 solution was able to remove 79.07% in the first using and reduced to 74.66% when reused the solution. Similar result found with the mixed system of LS9 that the removal

efficiency reduced from 87.32% in the first to 84.13% when it reused. However, the results of removal efficiencies from the first using and the reused solution with fresh meals of each solution are not significantly different ($p = 0.05$) in statistic test. The experimental data is in Appendix D, Table D-18 and the statistic test is in Appendix F10 – F11.

### 4.3.3 Up-scale the removal process

This work expanded the scale of experiment for all four selected systems from 1 g meals/20 mL solution in 40 I-CHEM test tubes to 8 g meals/ 160 mL solution in 250 mL Erlenmeyer flask. The removal conditions were kept in the same condition, i.e. 300 rpm and 15 min contact time also same solid:liquid ratio equal 0.5 g:10 ml. Interestingly that the results found in the larger scale were better than test tube scale for all cases. The removal efficiencies were raised up from 83.39% to 86.64% for the single T80 system; from 84.04% to 89.94% for single LS9; from 79.07% to 82.47% for the mixed T80 system; and from 87.32% to 87.61% for the mixed LS9 system (see Figure 4.15). This probably related to mass transfer (Mitra, 2003) since the Erlenmeyer flask provides well mixed compared to those in the test tube and allowed more coalescence among all components in the systems. Therefore, the surfactant technology has the opportunity for phorbol esters removal in a large-scale; for example, industrial scale. The experimental data is in Appendix D, Table D-19.

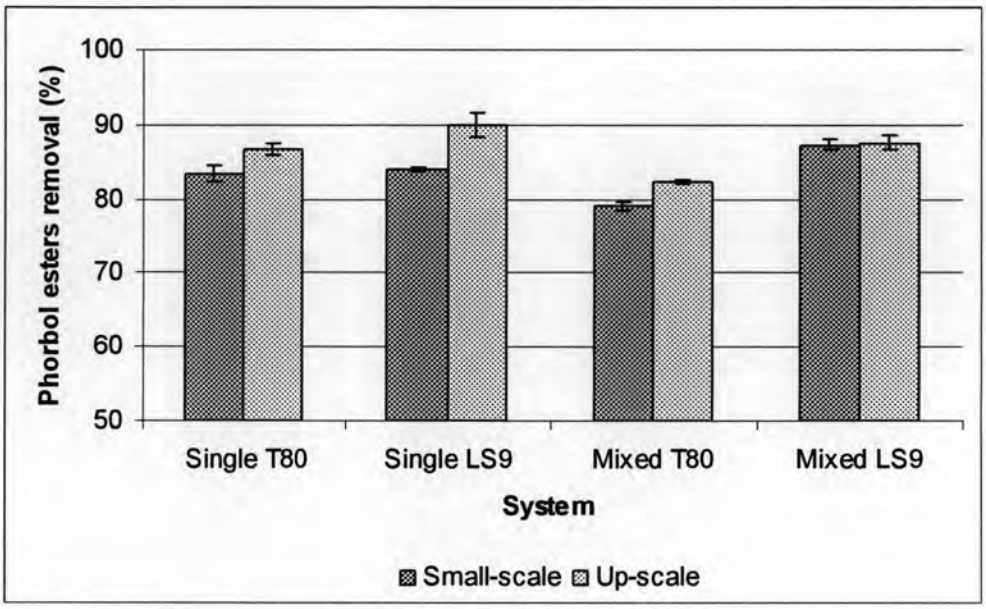



**Figure 4.15** Comparison of phorbol esters removal between a experimental test-tube - scale of 1 g meals/20 ml solution in 40 with 8 times larger scale in 250 mL Erlenmeyer flask; Single T80 as 40 mM Tween 80, Single LS9 as 40 mM Dehydol LS9, Mixed T80 as mixed of 40 mM Tween 80 and 5 mM AOT with 100 mM NaCl, and Mixed LS9 as mixed of 40 mM Dehydol LS9 and 5 mM AOT with 100 mM NaCl

### 4.3.4 Effect of phorbol esters removal process on crude protein in residual meals

The pressed meals and detoxified meals from the four surfactant solutions were determined for crude protein by Kjeldahl's method. The result is in Table 4.7. This experiment was done by the Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University (Appendix E).

From Table 4.7, the T80 system caused the protein loss higher than the LS9 systems both single and mixed solutions. The mixed surfactant solutions affected on the loss of protein lower than those of the single solutions both T80 and LS9. The protein loss from meals found more in the single nonionic surfactant system may be because protein is considered polar organic compound that generally prefer to be solubilized at outer or palisade region of a micelle. For the single nonionic system, it is generally having more space in this regain due to steric effect of head group.

Consequently, it provides more space for protein to solubilize. While the mixed surfactants system, the palizade layer is more packed from the interaction between anionic and nonionic surfactants, thus the space is smaller than those of the nonionic system (Rosen, 2004). Therefore, more protein loss was found in the single nonionic system.

**Table 4.7** Crude protein content in initial pressed meals and residual meals after phorbol esters removal by surfactant solution

| Sample | Crude protein (%wt.) | % Crude protein loss* |
|---|---|---|
| Pressed meals | 17.94 | - |
| Single T80 meals | 14.46 | 19.40 |
| Single LS9 meals | 15.10 | 15.83 |
| Mixed T80 meals | 15.21 | 15.22 |
| Mixed LS9 meals | 16.24 | 9.48 |

Note: * the loss (%) was calculated by assuming from initial content in pressed meal

In fact, *J. curcas* seeds contain the high nutrient in protein and have the essential amino acid and meet FAO standard (Liberalino et al., 1998). However, they also contain some toxin and antinutrient, i.e. trypsin inhibitor activity, saponins, phytate, lectin activity, tannins, also phorbol esters. The other toxin except phorbol esters are destroyed by moist heat treatment (Makkar et al., 1997; Makkar et al., 1998; Chivandi et al., 2005; Martínez-Herrera et al,. 2006). Thus, the meals are not suitable for feedstock if no detoxification. Because of phorbol esters are thermal persistent, these compounds are the important toxin from *J. curcas* meals. Only heat treatment cannot use to reduce this toxin due to the other techniques were investigated.

According to Aregheore et al. (2003), they found that heat treatment can only inactivate lectin but not for phorbol esters. However, heat treatment at 121°C with 66% moisture for 30 min then washing 4 times with 92% methanol, 95% of phorbol ester can be removed from residue meal (from 1.78 mg/g to 0.09 mg/g). Martínez-Herrera et al. (2006) found that the meals passed ethanol washing and follows with

0.07% $NaHCO_3$ can decrease phorbol esters down to 0.08 mg/g. Nokkaew et al. (2008b) found that washing with 2–3 %wt. of potassium hydroxide then followed with 95 % ethanol for one night can eliminate phorbol esters from the pressed meals to be as low as 0.11 mg/g which is the same content found in a non-toxic *J. curcas* variety (Makkar et al., 1998).

In this study, the phorbol esters are removed from 1.45 mg/g of pressed meals down to 0.20 – 0.30 mg/g depending on the surfactant solutions. The overall removal efficiency is range from 75% to nearly 90%. Even though, using surfactant solutions may not be able to eliminate the phorbol esters from the toxic meals to be as low as those found in the non-toxic variety, the phorbol esters removal by surfactant aqueous-based solution is considered clean technology. Furthermore by this technique, surfactants do not destroy the structure of phorbol esters as it may happen in the active chemical, thus to recovery phorbol esters for further use may be another possibility for further research trends.