CHAPTER III

METHODOLOGY

3.1 Type of Study

All results in this study were obtained from laboratory experiments. Since the experiment related to natural products; i.e., *Jatropha curcas* seeds and *J. curcas* meals, they were from the same batch in order to reduce error from variation in composition of the specimen. Moreover, all experimental studies were carried out in triplication.

3.2 Workplace

All experiments were conducted at NCE-EHWM Laboratory, Petroleum and Petrochemical College Building, floor 11th, Chulalongkorn University.

3.3 Material

3.3.1 *Jatropha curcas* seeds, pressed meals and oil

Jatropha curcas seeds, pressed meals and oil were supplied by PTT Chemical Public Company Limited. For the preparation of kernels, seeds were cracked and removed shells, then dried at 105°C for 90 min in an oven. Dried kernels were ground and sieved to separate into different sizes using pore screens. The pressed meals were twice defatted by PTTCH-screw pressing machine.

3.3.2 Surfactants

Two types of surfactant, nonionic and anionic surfactant, were used in this study.

3.3.2.1 Nonionic surfactants

For nonionic surfactants, commercial grade fatty alcohol C12-14 ethoxylates (AE) supplied by PTT Chemical Public Company Limited that have 99.8% active (see Table 3.1 and Figure 3.1) and polyoxyethylene (20) sorbitan group, i.e. Tween 20 and Tween 80 purchased from Ajax Finechem, Tween 40 and Tween 60 from Merck. The number 20 following the *polyoxyethylene* part refers to the total number of oxyethylene -(CH_2CH_2O)- groups found in the molecule. The number following the *polysorbate* part is related to the type of fatty acid associated with the polyoxyethylene sorbitan part of the molecule. Monolaurate is indicated by 20, monopalmitate is indicated by 40, monostearate by 60 and monooleate by 80 (see Table 3.2 for their properties and Figure 3.2 for their structure).

Table 3.1 Properties of nonionic surfactants: ethoxylate group

Commercial name	Chemical name	% C-chain		HLB	MW
		C12	C14		
Dehydol LS3 TH	Fatty alcohol C12-14 (3) Ethoxylate	68-78	20-30	7.9	327
Dehydol LS7 TH	Fatty alcohol C12-14 (7) Ethoxylate	68-78	20-30	12.1	503
Dehydol LS9 TH	Fatty alcohol C12-14 (9) Ethoxylate	68-78	20-30	13.4	591

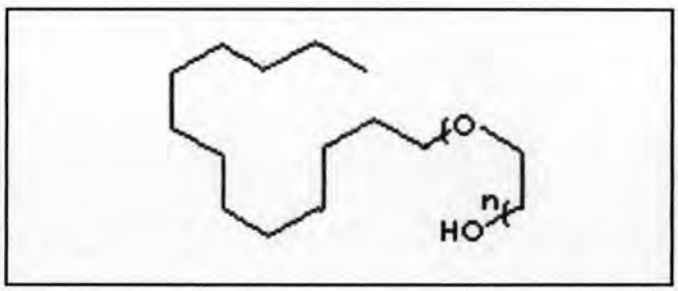


Figure 3.1 Fatty alcohol ethoxylate structure

Table 3.2 Properties of nonionic surfactants: Tween group

Commercial name	Chemical name	C-chain	HLB	MW
Tween 20	Polyoxyethylene (20) sorbitan monolaurate	C12	16.7	1228
Tween 40	Polyoxyethylene (20) sorbitan monopalmitate	C16	15.6	1277
Tween 60	Polyoxyethylene (20) sorbitan monostearate	C18	14.9	1309
Tween 80	Polyoxyethylene (20) sorbitan monooleate	C18=1	15.0	1310

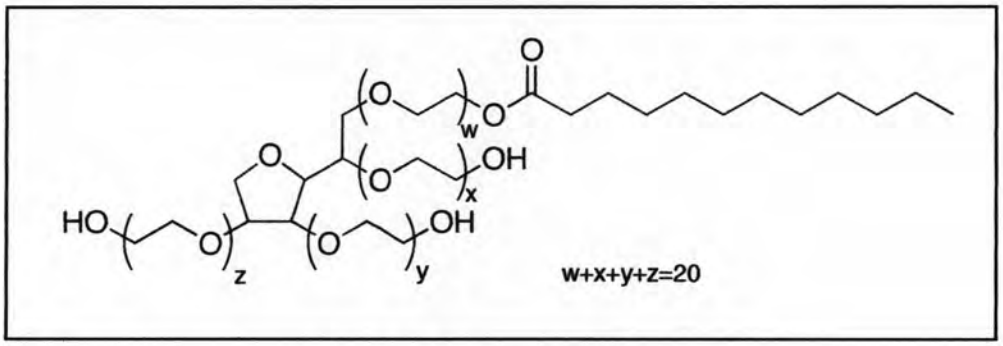
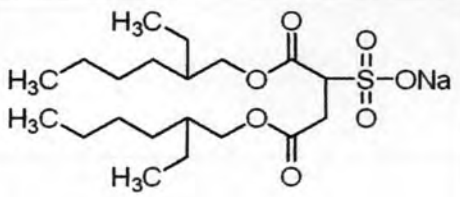


Figure 3.2 Tween 20 structure

3.3.2.2 Anionic surfactants

Sodium bis(ethylhexyl) sulfosuccinate (AOT) purchased from Fluka, 96% active (see Table 3.3)

Table 3.3 Structure of AOT, anionic surfactant used in this study

Commercial name	Chemical name	Structure	MW
AOT	Sodium bis(ethylhexyl) sulfosuccinate ($C_{20}H_{37}NaO_7S$)		444.57

3.3.2 Chemical

- Methyl alcohol: HPLC grade from CARLO ERBA, purity 99.9%
- Acetonitrile: HPLC grade from LAB-SCAN, purity 99.9%
- Sodium chloride (NaCl): Analytical grade from UNIVAR, purity 99.9%
- 12-o-tetradecanoyl-phorbol-13-acetate or TPA from Fluka, purity 98%
- 18 Ω De-ionized water

3.4 Methodology

3.4.1 Jatropha seeds preparation

Prior to the experiment, the average weight of a seed, shell and kernel, and water content were determined by random sampling. An average weight of seed was calculated from dividing total weight of seeds by the number of seeds.

The seeds were carefully cracked and removed shells followed by weighting shells and kernels. Shells and kernels were dried in an oven at 105°C for 90 min; they were kept in a desiccator to cool down to room temperature, and then weighed them again. The kernel:shell ratio was calculated by dividing the average weight of kernel by the average weight of shell.

3.4.2 Phorbol esters extraction for quantitative determination

The ground *J. curcas* kernels were spiked with TPA of known concentration. The spiked kernels were extracted by methanol in the procedures of shaking-extraction and ultrasonic extraction. In each procedure of the extractions, two grams of kernels was added with 20 ml methanol, then shakes at 300 rpm or sonicates in different contact time. The extractants were analyzed for phorbol esters by HPLC-UV. The known TPA concentration was spiked on samples and the TPA concentration of spiked sample after extraction was calculated for the % recovery of each extraction techniques. The method yielded the highest % recovery was then selected as the sample preparation technique for phorbol esters determination by HPLC.

3.4.3 The oil extraction by Soxhlet

The oil extraction by Soxhlet was conducted following the method of the Association of Official Analytical Chemists (2006). The oil yield from this method was used as the reference for the total oil of the kernel and for the calculation the oil extraction efficiency. The defatted meal was used for the phorbol esters reduction experiment.

3.4.4 The oil extraction by surfactant aqueous-base

One gram of ground kernels was placed in 15 ml-test tube with screw cap then 10 g of the selected surfactant solution was added and gently shaken to mix them thoroughly. Simultaneously centrifuge after shaking to let free oil separate from surfactant aqueous phase and allow residue meal to settle. The free oil was sucked from the tube and weighed for determination of extraction yielded. Selected surfactant aqueous system with different salinities were tested to investigate the optimum condition. Other parameters such as solid-liquid ratio, grain size of kernel, contact time, and centrifuge rate were carried out for determination of the highest extraction efficiency. The settled meals was filtered and used to phorbol esters extraction study

3.4.5 Phorbol ester extraction in meal

The kernel and the oil extracted kernel or meal were determined the amount of phorbol ester for calculation the removal of phorbol esters in the oil extraction process. Then, the meals were dried and ground to prepare for the phorbol esters elimination study. The surfactants and some co-surfactants were evaluated to determine their optimum extraction of phorbol esters.

Extraction procedure

Two grams of ground meals was put in 40 ml-test tube with screw cap then 20 ml of surfactant solution (in different concentrations and types) was added and gently shaken to mix them thoroughly. After that it was kept for 30 min to settle meals from surfactant aqueous phase then filtered the residual meals. The filtered meals were dried and analyzed for the phorbol esters content and calculated the phorbol esters removal efficiency. Three replications were carried out for each

condition. Selected surfactant aqueous systems with different salinities were tested to evaluate the optimum condition. The experiments with varying parameters such as solid-liquid ratio and contact time were carried out for determination of the highest removal efficiency.

3.4.6 Nutrient study

The amount of crude protein in the kernels, the meals and the detoxified meals were determined and compared the amount of crude protein loss from different oil extraction process and also the loss in the phorbol ester elimination process.

3.5 Analytical method

3.5.1 Quantitative of oil contain by Soxhlet (AOAC, 2006)

Two grams of kernels were extracted by hexane in Soxhlet apparatus for 24 hours then evaporated hexane by evaporator. The oil content was calculated by deducting the weight of round-bottom flask after evaporated hexane with the weight of round-bottom flask before extraction than divided with the total raw kernel.

3.5.2 Quantitative of oil yield

The free oil that derived from the oil extraction was weight by 4 digit balance by surfactant aqueous solution and recorded. Oil weight was divided by the total weight of kernel that used in experiment to calculate the yeild, express as g/g of kernel.

Calculation efficiency of oil extraction

The oil extraction efficiency was calculated by the following the equation [3.1]. The oil extraction efficiency was reported in percent.

$$\%oil\ extraction\ efficiency = \frac{W_{surf}}{W_{soxhlet}} \times 100 \qquad [3.1]$$

W_{surf} and $W_{soxhlet}$ represent the weight of oil (g/g kernel) by surfactant aqueous solution extraction unit and the weight of oil (g/g kernel) by Soxhlet, respectively.

3.5.3 Quantitative of middle phase height

The height of middle layer was measured in millimeter-scale of 2 digits.

3.5.4 Quantitative of crude protein (AOAC, 2006)

The protein in sample was converted to ammonia in the Kjeldahl digestion apparatus and was analyzed as ammonia content. Then amount of ammonia will be converted in the crude protein content.

3.5.5 Quantitative of phorbol esters

Phorbol esters in meals were extracted with methanol and analyzed by HPLC-UV detector following the method proposed by Hass and Mittelbach (2000). The standard for determination of the phorbol esters that generally used is 12-o-tetradecanoyl-phorbol-13-acetate (TPA) even though this compound is not found in Jatropha, it is only available standard for determination of phorbol esters groups (Liberalino et al., 1988; Makkar et al., 1997; Becker and Makkar, 1998; Haas and Mittelbach, 2000; Aregheore et al., 2003; Chivandi et al., 2005; Makkar et al., 2007Nokkaew et al., 2008b).

HPLC analysis condition

- Reversed column: octadecyl (C_{18}) as the functional group, control at 25°C
- Mobile phase: isocratic acetonitrile:water ratio 80:20 (v/v) at the flow rate of 1 ml/min
- Detector: UV adsorption detector at wavelength 280 nm
- Sample injected volume 20 µl
- Calibration curve: used TPA as an external standard dissolved in methanol

Calculation concentration

The concentration of phorbol esters was calculated from the calibration curve.