CHAPTER II

MATERIALS AND METHODS

Instruments for analysis

Gas chromatography model GC-15 A, Shimadzu Co.,Ltd., Japan

Auto stream sterilizer model KT-30SD, ALP Co.,Ltd., Japan

pH meter model F-13E, Horiba Ltd., Japan

Microwave oven model NE-7670, Matsushita Electric Industrial Co.,Ltd., Japan

Oven model 240M, Contherm Scientific Ltd., Newzealand

Centrifuge model Z230, Hermle AG Ltd., Germany

Laminar air flow model HS-124,International Scientific Supply Co.,Ltd., Thailand

Analytical Balance model AE240, Metler Instrumente AG Ltd., Switzerland

Rotary evaporator model RE52, Yamato Co.,Ltd., Japan

Aquatherm water-bath shaker model G86, New Brunswick Scientific Co.,Inc, USA

Chemical agents

1. Plant growth regulators used in this study are summarized as follow:

Auxins:

Indole-3-acetic acid (IAA), Sigma Chemical Co., USA

Indole-3-butylic acid (IBA), Fluka Biochemika, Switzerland

α -Napthaleneacetic acid (NAA), Fluka Biochemika, Switzerland

2,4-Dichlorophenoxyacetic acid (2,4-D), Sigma Chemical Co., USA

Cytokinins:

6-Benzylaminopurine (BA), Fluka Biochemika, Switzerland

Kinetin (Kn), Sigma Chemical Co., USA

Gibberellins:

Gibberellic acid (GA_3), Sigma Chemical Co., USA

2. Chemical agents used in this study are included as follow:

Triton X-100, Fluka Biochemika., Switzerland

Absolute Methanol, J.T. Baker Inc., USA

Chloroform, J.T. Baker Inc., USA

Hexane, Mallinckrodt Inc., USA

Toluene, Mallinckrodt Inc., USA

Acetyl chloride, Sigma Chemical Co., USA

Standard fatty acid for GC no. 68-B, NU-CHEK-PREP, Inc., USA

Clorox, Co., Ltd., USA

Plant tissue culture technique

1. Plant materials

Safflower (*Carthamus tinctorius* Linn.) seeds variety Mangira used for the explants through tissue culture works in this study were obtained from open field trial of Office of Agricultural Economics (OAE), Chomthong district, Chiangmai.

2. Nutrient media

Standard basal media used in this study consisted of MS media (Murashige and Skoog, 1962),B5 media (Gamborg, 1970), N6 media (Chu, 1966), HM media (Hilderbrandt, 1962) and LS media (Linsmaier and Skoog, 1965). The components of these media and preparation of stock solution were described in Appendix.

3. Culture conditions

For studying of culture conditions, in some case, *in vitro* cultures of *Carthamus tinctorius* Linn. were separately incubated in the dark or light under cool white fluorescent with 16/8 hrs. of light/dark photoperiod at 25±2° C. The calli were subcultured onto fresh medium for proliferation every 4 weeks.

4. Media preparation

Stock solution of major inorganic nutrients, trace elements, vitamins and plant growth regulators were prepared as indicated in Appendix.

5. Preparation and sterilization of explants

The explants of *Carthamus tinctorius* Linn. were chosen as a starting matenal for callus induction and plant regeneration. The explants comprised of the excised cotyledons, hypocotyls and shoot tips from the clean seedlings stock culture. The clean cultures were grown under aseptic conditions at 25±2°C.

Seeds of *Carthamus tinctorius* Linn. were surface sterilized with 10%(v/v) clorox containing 0.05 %(v/v) triton X-100 for 10 minutes, followed by 4 times rinsing with sterile distilled water, and then germinated on hormone-free full-strength MS medium containing 2.0 %(w/v) sucrose in 0.7 %(w/v) agar. The pH of medium was adjusted to 5.7 prior to autoclaving.

5.1 Testing for seed germination

Methods for seed germination were tested as described by International Seed Testing Association; ISTA. Some characteristics of seedlings including normal, abnormal, hard, dormant and dead seed were tested and recorded

after growing on the filtered papers for 3 and 7 days in culture room. The percentages of seed germination were tested every 4 months.

5.2 Optimal conditions for seed surface sterilization

Various concentrations of clorox and treatment times used for seed surface sterilization were tested. Seeds were soaked in 10, 15, 20 or 30% (v/v) of clorox in 0.05% (v/v) triton X-100 for 10, 15, 20 or 30 minutes. After completing the treatment, seeds were rinsed throughly 4 times with sterile distilled water. The seeds were germinated aseptic in the dark on hormone-free full-strength MS medium. The cultivated seeds were checked for any contamination on the fourth weeks. The sterile samples will be used as explants for callus induction and explants regeneration.The percentage of contamination were calculated as followed:

$$\% \text{ contamination} = \frac{\text{contaminated tissues} \times 100}{\text{total experimental tissues}}$$

6. Factors affecting callus culture

Studying of various factors involved in callus induction and propagation of *Carthamus tinctorius* Linn. was carried out by using the methods as described below:

6.1 Light and plant growth regulators

The cotyledons from 2 weeks old seedlings were excised horizontally and cultured on full-strength MS medium supplemented with various levels of 0.2, 0.5, 1.0 and 2.0 mg/l Benzyladenine (BA) and fixed concentration at 0.5 mg/l of α-Napthaleneacetic acid (NAA). The medium contained 2.0% (w/v) sucrose and 0.7%

(w/v) agar. Calluses were previously induced by incubation in the dark room for the period of 2 and 4 weeks before transfering to the culture room and maintained at 25±2°C in the white fluorescent light (5000 lux) for 16/8 hrs. photoperiod. Callus induction efficiency were observed and determined from the number of explants forming callus after 30 days.

$$\% \text{ callusing} = \frac{\text{Number of explants forming callus} \times 100}{\text{Total number of explants}}$$

6.2 Light and types of the explants

Cotyledons and hypocotyl segments (cut end below 1.0 cm. from cotyledons) from 2 weeks old seedlings were cultured on full-strength MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA containing 2.0% (w/v) sucrose in 0.7% (w/v) agar. The cultured were previously kept in the dark at 0, 1, 2, 3 and weeks period before transferring to cultured in the conditions as described in the previous section. The percentage of callus induction efficiency and regeneration were examined weekly. Subcultured was carried out at the period of 4 weeks.

6.3 Direction of explant excision

Cotyledons and hypocotyls from 2 weeks old seedlings were cut into small pieces with different directions such as, upper cut transversely, lower cut transversely , longitudinal sections passing through the mid rib of cotyledons, top up-bottom down hypocotyls, bottom up-top down hypocotyls and the outer surface cut horizontally hypocotyls (Fig. 2). The explants were placed on full-strength MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA containing 2.0% (w/v)

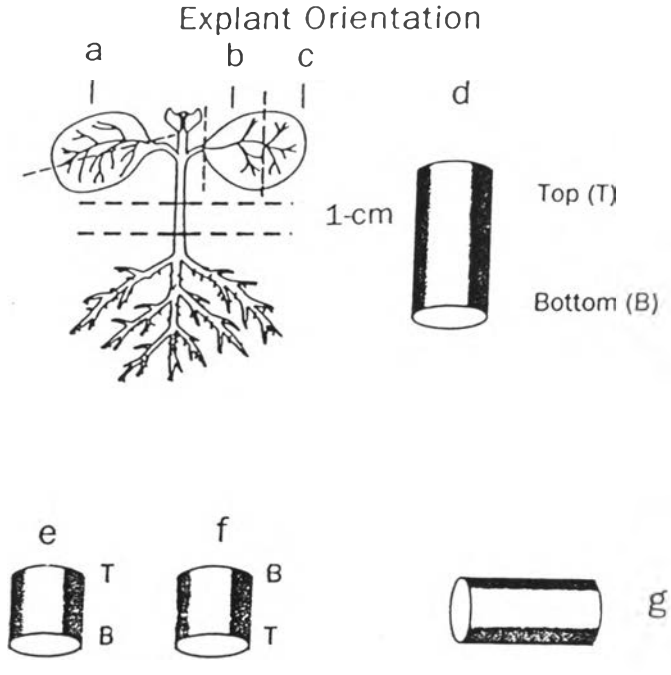

Figure. 2 The illustration of excised cotyledons* and hypocotyl segments** for studying the effect of explant excision direction on callus induction and plant regeneration.

* excised cotyledons

a - longitudinal sections

b - transversely cut - lower part

c - transversely cut - upper part

** hypocotyl segments

e - top up-bottom down T - top

f - bottom up-top down B - bottom

g - outer surface horizontally

sucrose in 0.7% (w/v) agar. The percentage of callus induction efficiency and shoot regeneration were recorded at 4 weeks before subcultured.

6.4 Maturity of seedlings

Cotyledons from the different age of seedlings (4, 7, 10, 14, 17 and 21 days) were cut transversely and cultured on full-strength MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA containing 2.0% (w/v) sucrose in 0.7% (w/v) agar. The cultures were maintained at 25±2°C in the presence of 16/8 hrs. photoperiod. The percentage of callus induction and plant regeneration were examined weekly.

6.5 Types of media

Cotyledon segments from 2 weeks old seedlings were cut transversely and then transferred to culture on various type of media such as; MS (Murashige and Skoog, 1962), B5 (Gamborg, 1970), N6 (Chu, 1966) HM (Hilderbrandt, 1962) and LS (Linsmaier and Skoog, 1965). Each media were supplemented with 1.0 mg/l 2,4-D containing 2.0% (w/v) sucrose in 0.7% (w/v) agar. The pH of medium was adjusted to 5.7, sterilization and cultured as the previously described. The percentage of callus induction was examined weekly for the period of 4 weeks prior to subcultured.

6.6 Types of plant growth regulators

Cotyledons from 2 week old-seedlings were cut transversely and transferred onto full-strength MS medium. The medium was supplemented with fixed concentration (0.5 mg/l) of different types of auxins (2,4-D, NAA, IAA and IBA) in combination with cytokinins (BA and kinetin) which was also fixed at the same

concentration. The medium was consisted of 2.0% (w/v) sucrose in 0.7% (w/v) agar. Results were observed weekly within 4 weeks. In addition to the percentage of callus induction efficiency, the induction of plant regeneration, the relative size of callus and the color of the tissues were recorded.

6.7 Concentrations of plant growth regulators

The cotyledons were removed from 2 weeks old seedlings and cultured on full-strength MS medium. The medium was supplemented with 2.0% (w/v) sucrose in 0.7% (w/v) agar containing different concentrations of the auxin, NAA (0.2, 0.3, 0.5, 0.8 and 1.0 mg/l) in combination with different concentration of cytokinin, BA (0.2, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l). The cultures were maintained in the condition as described in the previous section. The percentage of callus induction and plant regeneration were scored weekly and subcultured within every 4 weeks.

6.8 Ammonium nitrate and potassium nitrate

Cotyledons from 2 weeks old seedlings were cut transversely and cultured on full-strength MS medium. The medium was supplemented with 0.5 mg/l NAA, 0.5 mg/l BA, 2.0% (w/v) sucrose in 0.7% (w/v) agar. Various combinations of NH_4NO_3 1.65, 3.0, 5.0 and 7.0 g/l and KNO_3 1.9, 3.0, 5.0 and 7.0 g/l were added to the medium before the final pH was adjusted to 5.7 prior to autoclaving at 121^{o}C for 15 minutes. Cultivation was carried out as described in the previously section. The percentage of callus induction, shoot regeneration, relative size of callus and the morphology of the tissues were recorded in every 4 weeks period.

6.9 Silver nitrate and cobalt chloride

The cotyledons were cut transversely into 2 pieces. The upper parts were transferred to a full-strength MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BA, 2.0% (w/v) sucrose in 0.7% (w/v) agar. Various concentrations of $AgNO_3$ (1.0, 3.0, 5.0 and 10.0 mg/l) and $CoCl_2$ (1.0, 3.0, 5.0 and 7.0 mg/l) were used in each cultured medium. $AgNO_3$ and $CoCl_2$ were added to the medium before adjusting the final pH to 5.7. Sterilization and culture conditions were performed as previously described in the above sections. Subculturing were performed every 4 weeks. The percentage of shoot regeneration was determined from the number of explants forming shoots with two leaves. The morphogenetic response of calluses were also observed.

6.10 Sucrose concentration

Full-strength MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BA in 0.7% (w/v) agar were used in these experiments. The excised cotyledons from 2 weeks old seedlings were placed on MS medium containing different concentration of sucrose (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v)). The cultures were maintained at $25\pm2^{o}C$ with 16/8 hrs. photoperiod. The percentage of callus formation and morphogenetic response were examined after 4 weeks, prior to subculturing onto fresh media.

7. Study on the growth rate of callus

Original stock cotyledon calli were removed from cotyledons explants after subculturing at 4 weeks to the standard medium as mentioned in the section 6.1. Two sets of callus tissues were separately incubated in the dark (0 lux) and light (5000 lux) at $25\pm2^{o}C$. A dry weight of callus tissues were obtained each week.

Three replicate of samples were obtained at one week interval, and weighed for growth curve and growth index before lipid analysis.

8. Regeneration of shoot from callus cultures

Shoot regeneration of *Carthamus tinctorius* Linn. from its callus were observed on the standard conditions described in sections 6.1- 6.10. The percentage of plant regeneration was calculated as follow:

$$\%\text{Plant regeneration} = \frac{\text{the number of callus to form organs} \times 100}{\text{the number of all callus}}$$

9. Root induction

The regenerated shoots obtained as described in sections 6.1 - 6.10 were grafted and transferred onto the media for rooting at $25\pm2^{o}C$ under 16/8 hr. photoperiod of cool white fluorescent (5000 lux). The medium tested for root induction was included as follow:

1) full-strength MS medium without plant growth regulators.
2) half-strength MS medium without plant growth regulators.
3) half-strength MS medium supplemented with 0.5 mg/l NAA.
4) half-strength MS medium supplemented with 0.5 mg/l NAA and 0.1 mg/l BA
5) half-strength MS medium supplemented with 7.0% (w/v) sucrose
6) half-strength MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l GA_3 plus 1.0 g/l charcoal added.

7) grafting the regenerated shoots and immersed in 1.0 mg/l IBA solution for 2 hrs. then transferred to culture on MS medium without plant growth regulators.

Quantitative analysis of total lipids and fatty acids in *Carthamus tinctorius* Linn.

1. Preparation of plant materials

1.1 Lipid synthesis during growth stage of explants

Total lipid content and some fatty acid compositions was determined at the following stage:

(a) soaked seeds without seed coat

(b) cotyledons after germination

- 7 days old seedlings
- 14 days old seedlings

(c) cotyledons with visible callus

- 14 days after cultured
- 28 days after cultured

(d) excised callus from cotyledon segments

- subculturing I
- subculturing II
- subculturing III
- subculturing IV
- subculturing V

Callus initiation and growth was occurred on MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BA, 2.0% (w/v) sucrose in 0.7% (w/v) agar. Each

treatment was performed in triplicates. The methods for extraction and esterification of total lipid contents were described in sections 3.1 and 3.2.

2. Factors involved lipid biosynthesis in callus cultures

2.1 Effect of light incubation period

Calli cultures were established from excised cotyledons on MS medium which was supplemented with 0.5 mg/l NAA, 0.5 mg/l BA, 2.0% (w/v) sucrose in 0.7% (w/v) agar. The light grown callus were cultivated under cool white fluorescent (5000 lux) for 16/8 hrs. photoperiod. The culltures were transferred onto fresh medium every 30 days. Subculture was repeated for 3 times before being used for lipid analysis in comparison to the cultures which were incubated in the similar conditions without light expressed.

2.2 Growth stages

Calli were removed from excised cotyledons after subculturing in the standard conditions for the period of 4 weeks. They were transferred to a fresh MS medium which was supplemented with 0.5 mg/l NAA, 0.5 mg/l BA, 2.0% (w/v) sucrose in 0.7% (w/v) agar and cultivated according to the previous method described in section 6.1. Callus tissues were pooled and analyzed at the interval of 1, 2, 3, 4, 5, 6 and 7 weeks. Triplicate samples were taken to determine total lipid contents and fatty acid composition as mentioned in section 3.

2.3 Plant growth regulators

Cotyledons from 2 weeks old seedlings were dissected out to cultivate on the MS medium. Each medium was supplemented with constant concentration (0.5 mg/l) of cytokinins BA, kinetin and auxins (NAA, IBA, IAA and 2,4-D). The calli

were incubated in the cultured room for 30 days at 25±2°C under 16/8 hrs. photoperiod of cool white fluorescent (5000 lux). Three replicates of callus tissues were maintained for each treatment conditions. The method for extraction and esterification of total lipid contents were described in the section 3.

2.4 Sucrose concentration

In this study, the effect of sucrose-mediated regulation on fatty acid composition in callus tissues was determined essentially as described in the methods section 6.10. Calli were harvested in one week interval for the period of 8 weeks. Each treatment was analyzed in triplicate. The method for extraction, esterification and determination of total lipid were described in the section 3.

3. Sample preparation for GC analysis

3.1 Extraction of total lipids

Various parts of *Carthamus tinctorius* Linn. seedlings and callus tissues were investigated for total lipids using the method as described by Ways and Hanahan (1964). Callus tissues were homogenized with a binary solvent mixture ($2CHCl_3$:$1CH_3OH$ v/v) and proceeded as schematic shown in figure 3.

3.2 Preparation of fatty acid methyl esters

The dry weight of total lipids sample (section 3.1) were dissolved in methanol/toluene (3:2 v/v). The esterification process was carried out essentially as described in figure 4.

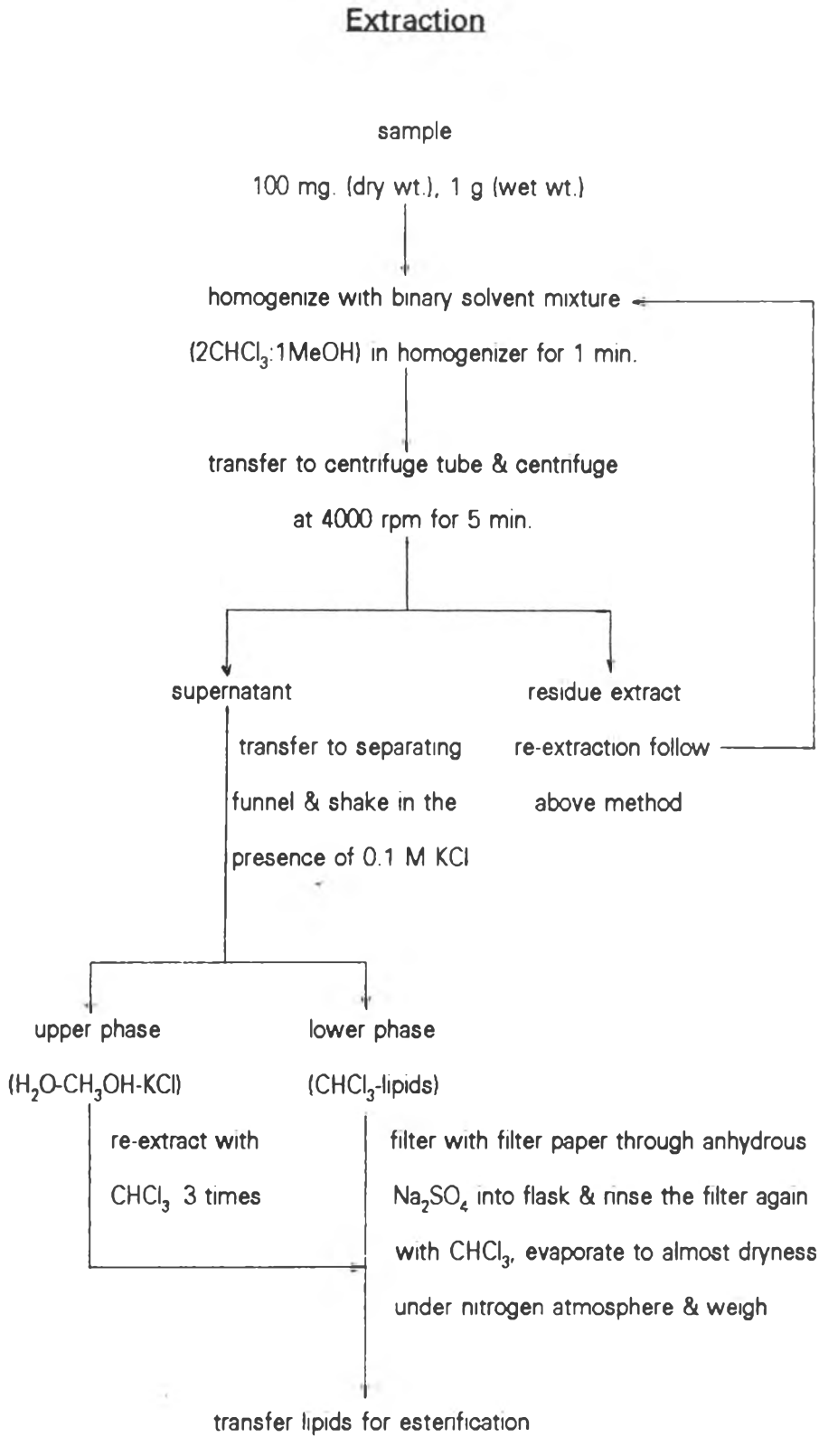

Figure. 3 The method for lipid extraction from plant tissues.

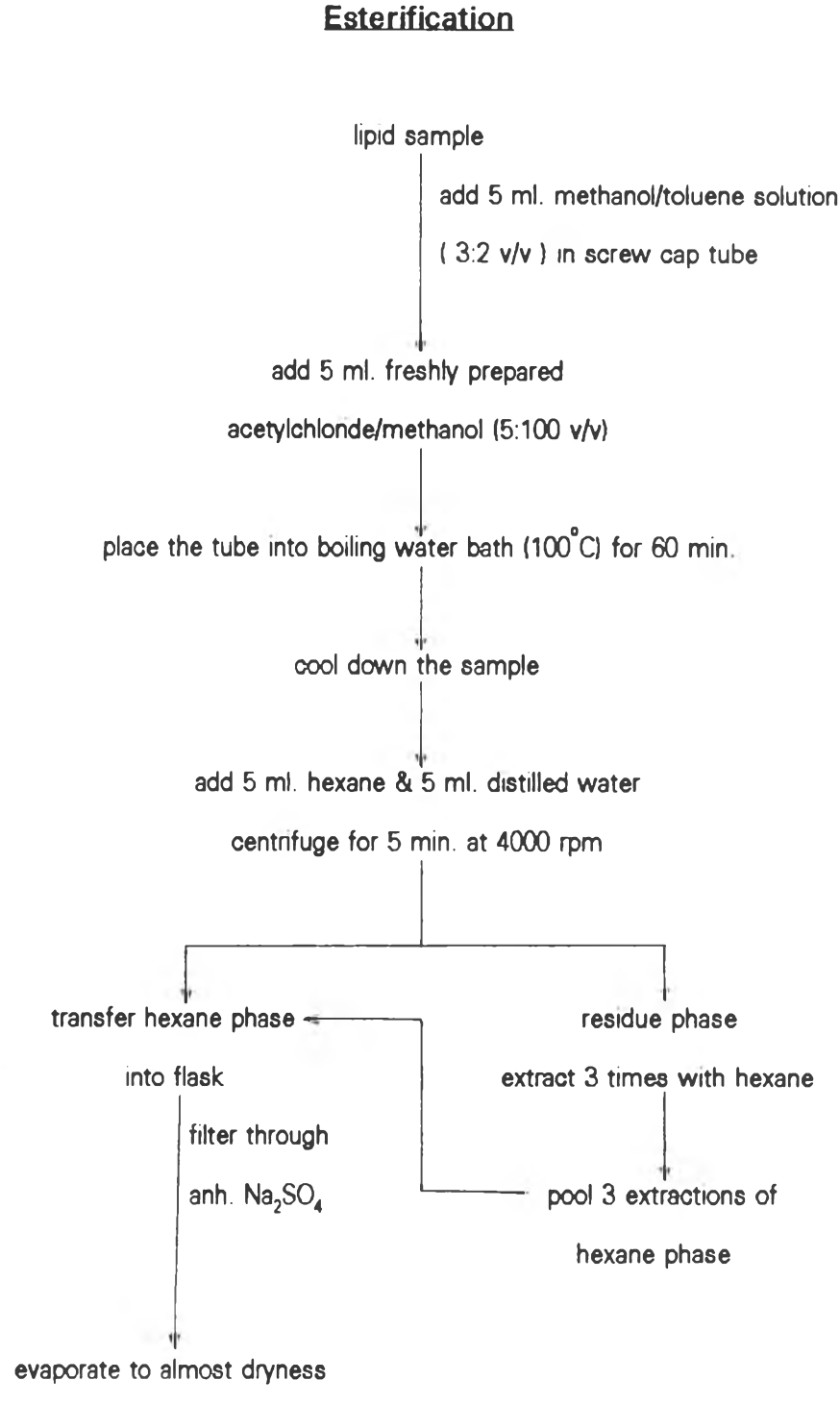

Figure. 4 Lipid esterification method for GC analysis.

3.3 Gas chromatographic conditions

Fatty acid compositions in all treatments of *Carthamus tinctorius* Linn. were analysed by Gas chromatography. The standard conditions of GC analysis were described below:

GC conditions:

Instrumental model	: Shimadzu GC 15-A Gas chromatography
Detector	: FID
Column	: Widebore Fused Silica Capillary Column (30 m x 0.324 mm) packed with dimethylpolysiloxane (J&W scientific)
Column temperature	: Program temperature Initial temp. = 135°C hold 2 min. Prog.rate = 135-235°C rate 2°C/min. Final temp. = 235°C hold 5 min.
Injector temperature	: 250°C
Detector temperature	: 250°C
Nitrogen carrier gas	: pressure 1.0 kg/cm^2
Nitrogen make up gas	: flow rate 40 ml/min.
Hydrogen supply	: 0.6 kg/cm^2
Air supply	: 0.5 kg/cm^2
Sample injection	: 0.5 µl

Fatty acid content and composition were analyzed with Shimadzu GC 15-A gas chromatography. Peak areas of fatty acid methyl esters were determined with a Shimadzu C-R4A chromatopac integrator. Peak areas were identified by comparing retention times of samples with those of the mixture of pure standards mixture. The identity of each fatty acid was confirmed by gas chromatography and mass spectroscopy (GC/MS).