CHAPTER III

MATERIALS AND METHODS

Experiment I

3.1 Animals and diets

Ten, 48 weeks old Hisex laying hens were divided into 2 groups (5 hens/group), one hen per cage (18 x 36 x 16 square inch). All hens were reared in an evaporative cooling house. Hens received 16 h light per day (12 h natural light and 4 h fluorescent light). Fluorescent electric light bulb was opened during 05.00-06.00 a.m. and 06.00-09.00 p.m.. Hens were received basal diet containing crude protein 17.5% and energy (Metabolizable energy; ME) 2,750 kcal/kg. Hens in the control group were received 2% lard while treatment group received crude palm oil (CPO) 2% in diet as fat source. The composition and nutrient contents of the basal diet are shown in Table 3.1.

3.2 Experimental procedure

This experiment was designed to examine whether there was an adverse effect of CPO on performance of hens. There were two treatment groups in this experiment.

Group 1 (Control group; Diet I) : hens were received basal diet + lard 2%

Group 2 (CPO group; Diet II) : hens were received basal diet + CPO 2%

3.3 Protocol of the experiment

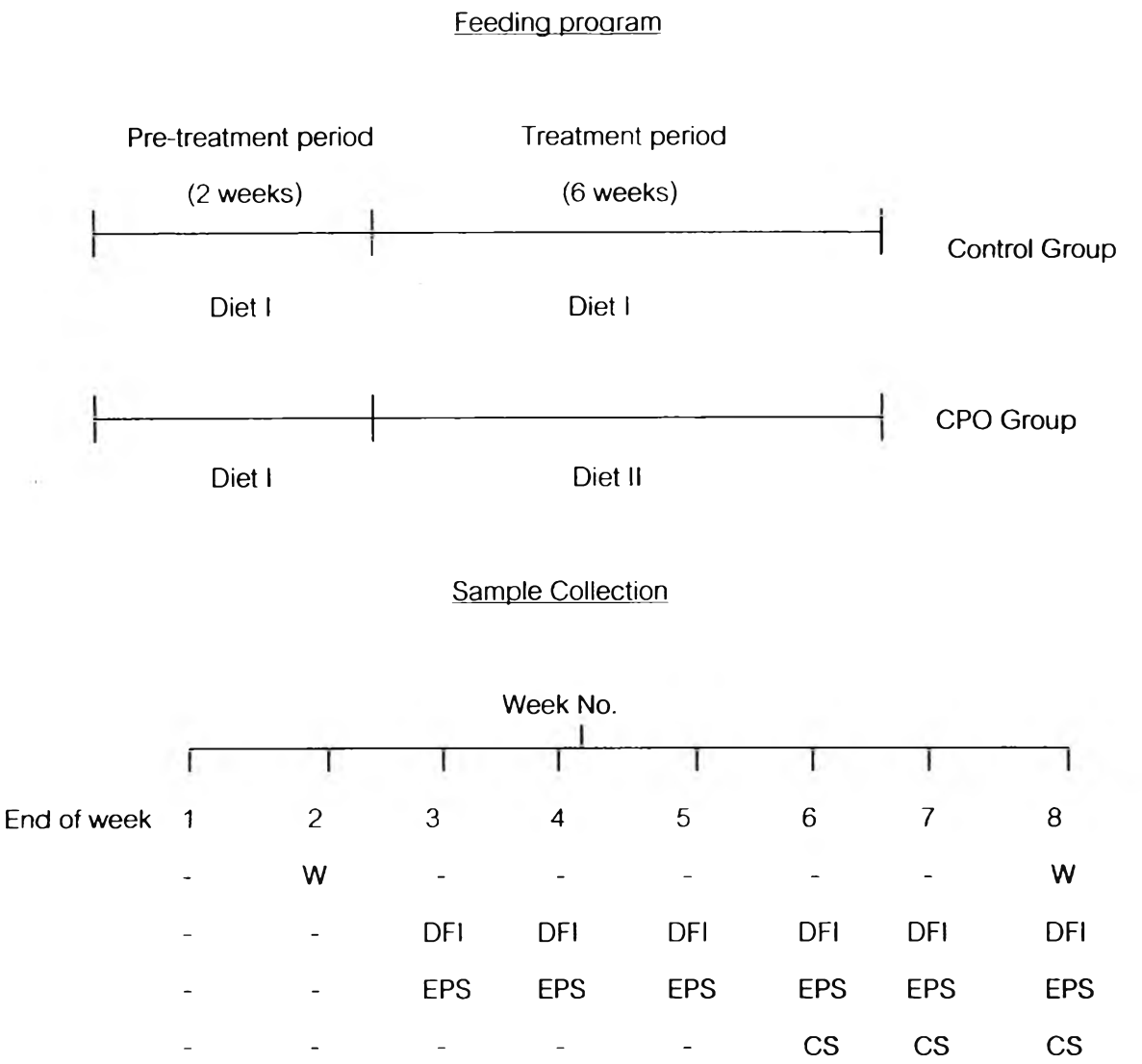

Figure 3.1 Diagram showing the whole period of the experiment I.

Where:

W = hen weighing

DFI = daily feed intake record

EPS = egg performance and egg quality sampling

- hen-day basis (HD; recorded everyday)
- daily egg weight (weighed and recorded everyday)
- egg weight, specific gravity of eggs, yolk color, Haugh unit and yolk weight

CS = egg yolk cholesterol sampling

Table 3.1 Composition and nutrient content of diets in experiment I.

Ingredients	Unit	Control group (Diet I)	CPO group (Diet II)
Corn	%	55.73	55.61
Extracted rice bran	%	6.60	6.76
Lard	%	2.00	0.00
Crude palm oil	%	0.00	2.00
Soybean meal 44%	%	21.00	20.96
Fish meal 60%	%	5.00	5.00
DL-methionine	%	0.06	0.06
Dicalcium phosphate 18%	%	1.14	1.14
Calcium carbonate	%	7.96	7.96
Salt	%	0.26	0.26
Premix*	%	0.25	0.25
Total batch		100.00	100.00
Nutrients	**Unit**	**Control group (Diet I)**	**CPO group (Diet II)**
ME of Poultry	Kcal/kg	2,750	2,750
Crude Protein	%	17.50	17.50
Ash	%	3.93	3.95
Crude Fat	%	4.58	4.64
Crude Fiber	%	3.71	3.72
Calcium	%	3.80	3.80
Total phosphorus	%	0.72	0.73
Available phosphorus	%	0.40	0.40
NaCl	%	0.35	0.35
Lysine	%	0.96	0.96
Methionine	%	0.38	0.38
Methionine + Cystine	mg/kg	0.65	0.65
Choline	%	1081.40	1081.98

* commercial premix

3.4. Sample collection and yolk cholesterol preparation

Total pen feed consumption was recorded weekly. The experimental protocol and sample collection are demonstrated in Figure 3.1. Body weight as pen basis was measured at the start and the end of experiment (end of week 2 and week 8). Total numbers of eggs (each treatment group) were collected for weighing and recording everyday. Hen-day basis (HD) were recorded everyday and calculated (North and Bell, 1990). At the end of weeks 3,4,5,6,7 and 8, three eggs from each group were randomly sampled and measured for egg weight, specific gravity, Haugh unit, yolk color and yolk weight. Three eggs from each group were used for measurement cholesterol of yolk. Eggs were weighed, hard-boiled and yolk samples were kept in plastic bags, and stored at –20 °C until analyze.

3.5 Determination of egg performance and quality

3.5.1 Hen-day basis percentage (%HD)

A number of eggs from each group were recorded daily until the end of the experiment and calculated as described by North and Bell (1990).

$$\% \text{ HD per week} = [\text{number of eggs} / \text{number of hens}] \times 100$$

3.5.2 Egg weight

Total eggs were weighed everyday and average egg weights were calculated (North and Bell, 1990).

$$\text{Average egg weight (g/egg)} = \frac{\text{total eggs weight}}{\text{number of total eggs}}$$

3.5.3 Specific gravity

Eggs were dropped onto series of different saline solution; each of them had individual specific gravity. There was adjustment of solution on 11 levels of specific gravity from 1.060 to 1.104. Observed eggs that floated in saline at what level were recorded for calculation of average specific gravity (Thompson and Hamilton, 1982).

3.5.4 Egg yolk color

After measurement of specific gravity, eggs were broken out and egg yolk color was measured by comparing with standard egg yolk color fan ranging from 1 (yellow) to 15 (red) by used Roche Color Fan (Vuilleumier, 1969).

3.5.5 Haugh unit

After measurement of yolk color, Haugh Unit Gauge (Figure 3.2) was used to measure the height of egg white and care must be taken to get a reading with the contact arm not touching the chalaza; otherwise the reading will be too high and collecting measurement data and application of the formula (Haugh, 1937).

$$\text{Haugh Unit} = \frac{100 \log [\, H - \sqrt{G}\,(30\, W^{0.37} - 100) + 1.9\,]}{100}$$

Where:

H = the height of egg white

G = 32.2 (constant value)

W = egg weight (g)

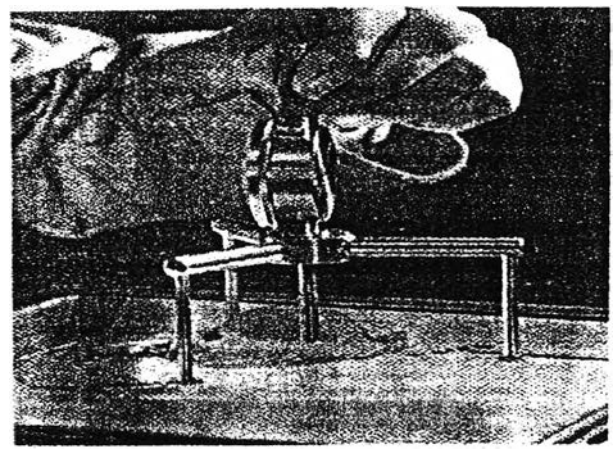

Figure 3.2 Haugh Unit Gauge.

3.5.6 Yolk weight

After measurement of Haugh unit, egg yolk was separated using hand and weighed for calculation of average yolk weight.

$$\text{Yolk weight (g)} = \frac{\text{total yolks weight}}{\text{number of total eggs}}$$

3.6 Determination of cholesterol concentration in egg yolk using colorimetric method

3.6.1 Cholesterol extraction and saponification

Egg yolk cholesterol was extracted by a procedure modified from Bitman and Wood (1980). Sample of 0.2 g yolk (hard cook at 100 ° C for 10 min) was weighed and homogenized with 4.5 ml petroleum ether-ethanol (2:1) in a 15 ml test tube with a Teflon-lined screw cap, sample was vortexed for 3 min and laid down in room temperature for 18 hours. Distilled water (1.5 ml) was added, the tubes were vortexed for 1 min, phases separated by centrifuging at 2,500 rpm for 10 min and a 2 ml aliquot of the petroleum ether phase was transferred to evaporate with nitrogen. Subsequently 2.5 ml of 2.5% KOH in ethanol was added into the dried residue, heated at 60 ° C in water bath (shake for every 5

min) for 60 min to saponify cholesteryl ester and laid down to cool. Petroleum ether (2.5 ml) and distilled water (2.5 ml) were added in sample tubes, vigorously shake for 1 min and laid down for separation of petroleum ether layer. Aliquot of petroleum ether layer (2 ml) were added into test tube, 0.5 ml aliquot petroleum ether was pipetted and added with 9.5 ml petroleum ether, vortexed for 1 min. Aliquots of sample solution (1 ml) was evaporated with nitrogen and cholesterol concentration was analyzed. For standard cholesterol measurement, 0.75 ml of cholesterol standard were used instead of yolk sample.

3.6.2 Analysis of yolk cholesterol concentration (Jung et al., 1975)

After saponification process, samples and standard residue were added with 5.0 ml of ferric acetate/uranyl acetate solution, mixed and vortexed for 5 min and centrifuged at 2,500 rpm for 5 min. Then 3 ml of the supernatant were added into test tube certainly 2 ml of the sulfuric acid regent by pouring the supernatant slowly to prevent heat production from the interaction of sulfuric acid and supernatant. For the blank tube, 3.0 ml of ferric acetate/uranyl acetate solution was transferred into test tube and 2.0 ml sulfuric acid reagent was added. The contents of each tube were mixed for 30 sec and allowed to cool down in room temperature for 30 min. The absorbance of each solution was measured at wavelength (λ) 560 nm after adjusted the instrument to zero absorbance with the blank solution in the light path and calculated according this below formation.

$$\text{Cholesterol concentration (mg)} = \frac{\text{AU} \times \text{CS}}{\text{AS}}$$

Where:

AU = absorbance of unknown (sample)

AS = absorbance of cholesterol standard

CS = cholesterol standard concentration (200 mg/100 ml)

Experiment II

3.7 Animals and diets

One hundred and forty four, 49 weeks old, Hisex hens were divided into 4 groups (36 hens/group; 4 replicates/group; 4 hens/replicate). The venue of the experiment, cages, basal diet and housing were similar to experiment 1. Hens in the control group were fed on diet supplemented with 2% lard whereas hens in CPO1, CPO2 and CPO3 groups received diets supplemented with crude palm oil at 2%, 3% and 4%, respectively. The composition and nutrient contents of the experimental diets are shown in Table 3.2. Crude palm oil in this study contained α-tocopherol (398 ppm) and γ-tocopherol (35 ppm) and concentrations of α-, γ- and δ-tocotrienol were 429, 738 and 94 ppm, respectively. Vitamin E concentration in CPO was determined by HPLC (mobile phase was hexane (97%):Isopropyl (3%); flow rate 1 ml/min and retention time 20 min).

3.8 Experimental procedure

The whole experiment period (8 weeks) were divided into 2 periods.

Pre-treatment period (2 weeks) : Hens were divided into 36 hens/treatment with four replicates of nine hens per treatment. Hens was randomly selected and killed at the end of pre-treatment period.

Treatment period (6 weeks) : The rest of the hens were divided as followed in the pre-treatment period. There were 8 hens per replicate. A hen in each replicate were killed at the end of the experiment.

3.9 Protocol of experiment

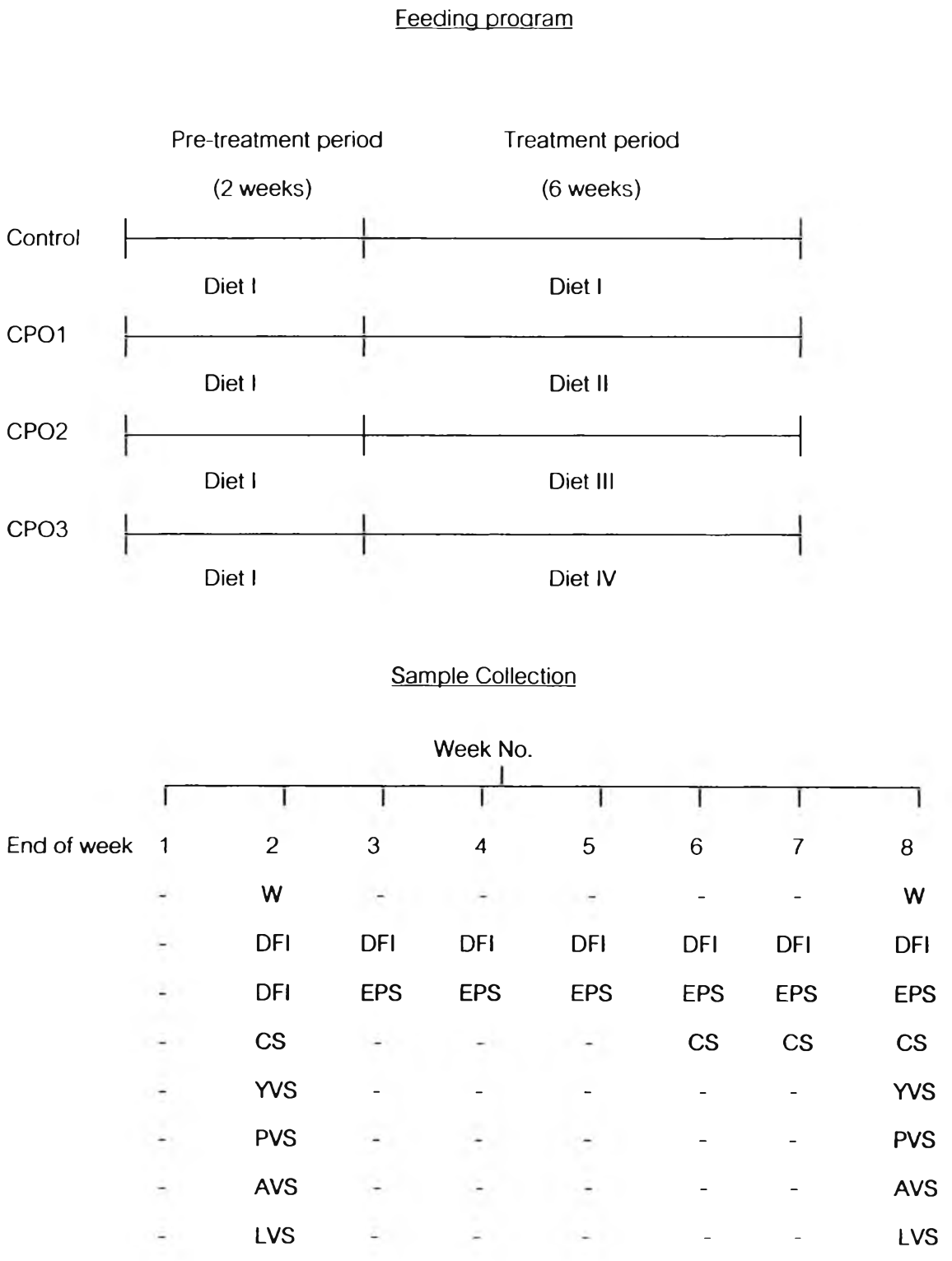

Figure 3.3 Diagram showing the whole period of the experiment II.

Where:

W = hen weighting

DFI = daily feed intake record

EPS = egg performance and egg quality sampling

- Hen-Day Basis (HD; recorded everyday)
- daily egg weight (weighed and recorded everyday)
- egg weight
- specific gravity of eggs
- egg yolk color
- Haugh unit
- egg yolk weight
- egg white weight
- eggshell weight

CS = egg yolk cholesterol sampling

YVS = egg yolk vitamin E sampling

PVS = plasma vitamin E sampling

AVS = adipose tissue vitamin E sampling

LVS = liver vitamin E sampling

3.10 Sample collection and yolk cholesterol preparation

Total pen feed consumption; schedule for sample collection are shown in Figure 3.3. Body weight, egg weight and hen-day basis (HD) were recorded as in experiment I. At the end of weeks 2, 3, 4, 5, 6, 7 and 8 was sampling 2 eggs/replicate (8 eggs/group) were sampled for measurement of egg performance and egg quality. Eight eggs from each group were sampled for measurement of yolk cholesterol with procedure described in experiment I.

Table 3.2 Composition and nutrient content of diets in experiment II.

Ingredient (%)	Control (Diet I)	CPO 1 (Diet II)	CPO 2 (Diet III)	CPO 3 (Diet IV)
Corn	55.33	55.39	51.53	49.72
Extracted rice bran	7.99	7.92	11.25	12.00
Lard	2.00	-	-	-
Crude Palm oil	-	2.00	3.00	4.00
Soybean meal 44%	19.24	19.25	18.76	18.84
Fish meal 60%	6.00	6.00	6.00	6.00
DL-methionine	0.05	0.05	0.05	0.06
Dicalcium phosphate (P18%)	0.93	0.93	0.91	0.90
Calcium carbonate	7.97	7.97	7.99	7.99
Salt	0.23	0.23	0.23	0.23
Premix*	0.25	0.25	0.25	0.25
Total batch	100.00	100.00	100.00	100.00
Nutrients	**Control**	**CPO 1**	**CPO2**	**CPO3**
ME for Poultry (Kcal/kg.)	2750.00	2750.00	2750.00	2781.83
Crude Protein (%)	17.50	17.50	17.50	17.50
Crude Fat (%)	4.64	4.71	5.58	6.50
Crude Fiber (%)	3.75	3.74	4.01	4.06
Ca (%)	3.80	3.80	3.80	3.80
Total phosphorus (%)	0.72	0.72	0.76	0.76
Available phosphorus (%)	0.40	0.40	0.40	0.40
NaCl (%)	0.35	0.35	0.35	0.35
Lysine (%)	0.97	0.97	0.97	0.97
Methionine (%)	0.39	0.39	0.39	0.39
Methionine + Cystine (%)	0.65	0.65	0.65	0.65

* Commercial premix

3.11 Determination of egg performance, egg quality and egg yolk cholesterol concentration.

Egg performance, egg quality and egg yolk cholesterol concentration were determined with the same procedure described in experiment I. In this experiment, egg white and eggshell weight were also determined, after measurement of yolk weight, shell residue was kept air-dried for 24 hours and weighed. Egg white weight was calculated as in the formula below.

Egg white weight (g) = egg weight – (yolk weight + shell weight)

3.12 Analysis of tocopherols and tocotrienols in egg yolk (Ikeda et al., 2001).

Egg yolk (fresh yolk) samples (0.5 g) (1 sample/replicate) were weighed into centrifuge tube, and 0.1 ml of 20 g/L sodium chloride, 1 ml of ethanol containing 60 g/L pyrogallol. Then, 0.2 ml of 600 g/L potassium hydroxide was added, vortexed for 1 min and saponified at 70°C for 30 min. Afterwards, 4.5 ml of 20 g/L sodium chloride was added and tocopherols and tocotrienols were extracted with 3 ml of hexane containing 10% (v/v) ethyl acetate and vortexed for 1 min. The sample tubes were centrifuged at 2,500 rpm for 10 min. The hexane layer (1 ml) was pipetted into test tube and evaporated under a stream of nitrogen (Ikeda et al., 2001). The residue samples were redissolved with hexane (1 ml) and 20 µl of the solution was used to inject into a HPLC. Concentrations of tocopherols and tocotrienols were determined using HPLC (High performance Liquid Chromatography) (Ueda and Igarashi, 1987). Recovery of tocopherol and tocotrienol contrations in this study was 97%. Sets of instrumentation were consisted of a Shimadzu LC-10AS CLASS VP (Shimadzu, Tokyo, Japan) with a Shimadzu RF-10AXL fluorescence detector (excitation 298 nm, emission 325 nm). The analytical column was a Develosil 60-5 (4.6 x 250 mm), the mobile phase was hexane containing 1% (v/v) dioxane and 0.2% (v/v) isopropyl alcohol, flow rate was 1 ml/min and run time 50 min.

3.13 Analysis of tocopherols and tocotrienols in plasma and tissues

3.13.1 Plasma tocpherols and tocotrienols

After 16 hour overnight fasting, blood (2 ml) was withdrawn from jugular vein into eppendoefs containing 1% (w/v) EDTA sodium salt solution. Plasma was separated by centrifugation at 3,000 rpm for 10 min. Plasma (200 µl) was pipetted into two 10 ml centrifuge tubes with Teflon coated screw-caps and 6% ehtanolic pyrogallol solution (1 ml) was added to each tube while stirring. The solution was preheated at 70°C for 3 min. 60% KOH solution (0.2 ml) was added and the contents were saponified at 70°C for 30 min. After cooling in ice water, 1% NaCl solution (4-5 ml) was added and whole samples were extracted with 10% ethyl acetate in *n*-hexane (3 ml), shaked vigorously for 1 min. The saponified extracted samples were centrifuged at 2,000 rpm for 10 min. The hexane layer (1 ml) was pipetted into test tube and evaporated under a stream of nitrogen. In each case, 20 µl of the *n*-hexane solution was injected into the HPLC system (Shimadzu LC-10AS CLASS VP, Tokyo, Japan) Detection wavelength was used excitation 297 nm and emission 327 nm. The analytical column Develosil (normal phase) was used (250 mm x 4.5 mmi.d.), for the mobile phase *n*-hexane/isopropyl alcohol (97:3 v/v) was used, with a flow rate of 1 ml/min and retention time was 15 min (Ueda and Igarashi, 1987).

3.13.2 Tissues (adipose tissue and liver) tocpherols and tocotrienols

Laying hens were killed (1 hen/replicate; 4 hens/group) after 16 h overnight fasting overnight, tissues were cut into small pieces (<*ca.* 1 mm) and weighed accurately in 10 ml Teflon coated screw-capped centrifuge tubes (liver approximately 100 mg and adipose tissue (abdominal fat) 30 mg). Then, 1% NaCl solution (*ca.* 100 µl) was immediately added in the tubes used for saponification. 6% Ethanolic pyrogallol (1 ml) and 60% KOH (0.2 ml) were added, without preheating and the contents were saponified at 70°C for 60 min. 1% NaCl Solution (4-5 ml) was added and the whole mixture was extracted with *n*-hexane/ethyl acetate solution (3 ml) as described in the plasma assay using HPLC (Ueda and Igarashi, 1987).

3.14 Preparation of solutions of tocopherols and tocotrienols standards (AOCS, 1993)

3.14.1 α-Tocopherol acetate standard stock solution

A stock solution of α-tocopherol acetate was prepared by accurately weighing 10 mg of the standard into a 100 ml volumetric flask, and making up to volume with hexane. The solution (10 ml) was pipetted into an amber glass round-bottom flask, and hexane was removed using nitrogen. Atmospheric pressure was restored with nitrogen, and the flask was removed from the evaporator as soon as all the solvent dried. Approximately 10 ml of methanol was pipetted into the flask and swirled to dissolve the tocopherol acetate. The absorbance of this solution was measured at wavelength 284 nm, and the concentration (as μg/ml α-tocopherol acetate) was calculated by dividing the absorbance value by 0.0045 (extinction co-efficient).

3.14.2 γ- and δ-Tocopherol standard stock solutions

Similar stock solution and aliquots for UV spectrometry of γ- and δ-tocopherol standards were prepared as described in the α-tocopherol standard stock solution. The absorbance of each solution was measured at the following wavelengths, and the corresponding divisor factors were used for calculation of concentrations:

298 nm γ-tocopherol = 0.0091

298 nm δ-tocopherol = 0.0087

3.15 Calculations of tocopherol and tocotrienol concentration

The α-tocopherol content of the sample in μg/g was calculated by:

$$\frac{C \times a \times D \times 25}{A \times m}$$

Where:

C = concentration of the α-tocopherol standard

A = mean of the peak areas obtained for the α-tocopherol standard

a = mean of the peak areas obtained for the α-tocopherol in test sample

m = mass of test sample taken

D = dilution factor, e.g., for a test solution prepared

The γ- and δ-tocopherol contents of the test sample was calculated in the same way, using the data from chromatography of the corresponding tocopherol standard. The tocotrienol content of a sample can be estimated using the C and A values for the corresponding tocotrienol (C and A value of α-, γ- and δ-tocopherol standard was used in determination for α-, γ- and δ-tocotrienol in test sample, respectively) and reported as the final result the mean of the value obtained as μg/g yolk. Chromatogram standard mixture of tocopherols and tocopherols and tocotrienols of crude palm oil are shown in Figures 3.4 and 3.5.

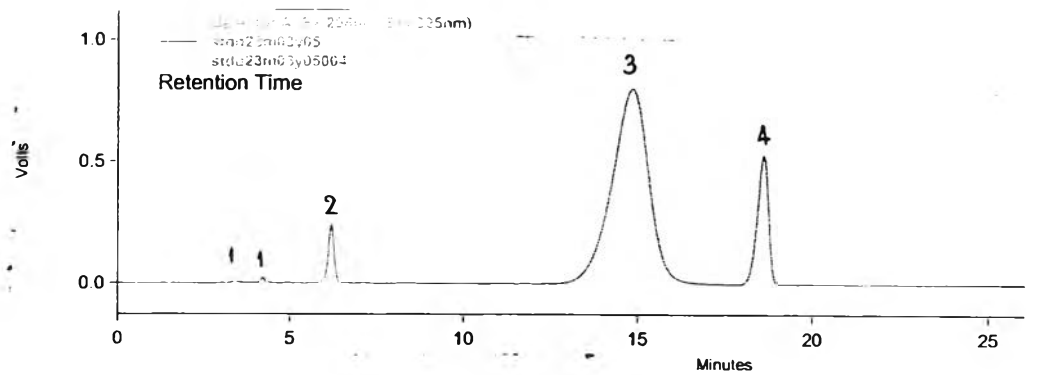

Figure 3.4 HPLC chromatogram of standard mixture: (1), solvent peak; (2), α-tocopherol; (3), γ-tocopherol; and (4), δ-tocopherol. (Ex. 298 and Em. 325; mobile phase, hexane (98.9%):dioxane (1%):isopropyl alcohol (0.2%); flow rate 1ml/min).

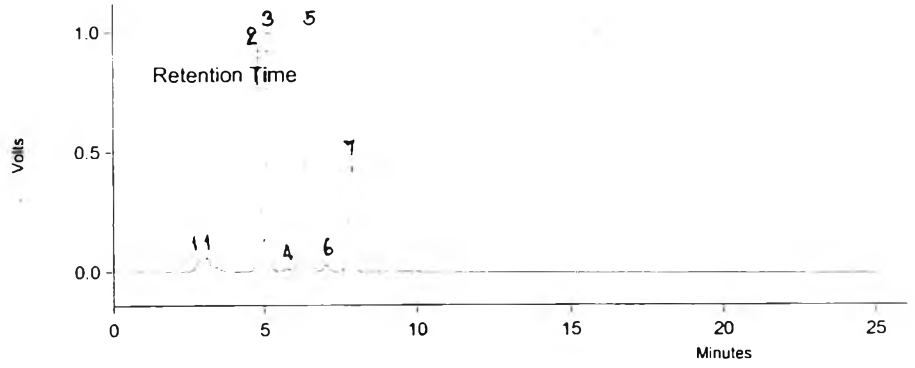

Figure 3.4 HPLC chromatogram of tocopherol and tocotrienol in crude plam oil: (1), solvent peak; (2), α-tocopherol; (3), α-tocotrienol; (4), γ-tocopherol; (5), γ-tocotrienol; (6), δ-tocopherol; and (7), δ-tocotrienol. (Ex. 297 and Em. 327; mobile phase, hexane (97%):isopropyl alcohol (3%); flow rate 1ml/min).

3.16 Statistical analysis

All data were presented as individual mean in each treatment and standard deviation of means. In experiment I, the effect of treatment was analyzed using unpaired t-test and the effect of time on various treatments was analyzed using paired t-test. In experiment II, the effect of treatment was analyzed using One-Way Analysis of Variance (ANOVA) and the effect of time was analyzed using Repeated Measurement in CRD. Individual means were compared with Tukey test. Significant level was set at $P<0.05$ (Steel and Torrie, 1960). Data, which were not complied with the equal variance and homogeneity test, were analyzed using non-parametric methods (Kruskral Wallis and ANOVA with repeated measure on ranks test).