CHAPTER 2

MATERIALS AND METHODS

2.1 Apparatus

(1) ELISA reader, Tecan, Salzburg, Austria

(2) Freezer –70 °C, Revco, USA

(3) Gas chromatograph (Hewlette Packard 5890 Series II) equipped with electroncapture detector (ECD), autosampler (HP 7673), a fused silica capillary column (Ultra 2.25 m x 0.32 mm i.d. with 0.52 μm film thickness, J&W Scientific, USA), and computerized data handling system (HP 3365 series Chemstation)

(4) Glass tubes with Teflon lined screw caps 16 x 20 mm

(5) High purity helium gas (99.993%), TIG, Bangkok, Thailand

(6) Instrument for lipid analysis, Synchron CX system, Beckman, Germany

(7) Instrument for hormone analysis, Elecsys 1010, Roche, USA

(8) Micro pipette, Pipetman P 25, P 200, and P 1000, Gilson, France

(9) Plate washing machine, SLT 96PW, Germany

(10) Refrigerate centrifuge, IEC International Model K, USA

(11) Ultrasonic cleaner, Cavitator, Mettler, USA

(12) Ultra high purity of nitrogen gas (99.999%), TIG, Bangkok, Thailand

(13) Eluting vacuum, VacElut SPS-24, Varian, USA

(14) Heparinised tubes, Vacutainer 366480, BD, USA

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2.2 Chemicals

(1) Acetone, grade for organic residue analysis, J.T.Baker, USA

(2) Acetonitrile, grade for organic residue analysis, J.T.Baker, USA

(3) Albumin from bovine serum, Fluka Biochemica, Germany

(4) β-estradiol 17-Hemisuccinate BSA, Sigma Chemical Co.,Ltd, USA

(5) Bond-Elut cartridge, octadecyl (C_{18}), 500 mg, Varian, USA

(6) Boeringer Blocking Reagent for ELISA, Roche, USA

(7) Dimethylsulfosid (DMSO), Pro analysis, Merck, Germany

(8) Ethyl acetate, grade for organic residue analysis, J.T.Baker, USA

(9) Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$) for molecular biology, Sigma, USA

(10) Estrogen receptor α/biotinylated clone 1D 5, Dako Cytomation, Denmark

(11) Hexane, grade for organic residue analysis, J.T.Baker, USA

(12) Isooctane, grade for organic residue analysis, J.T.Baker, USA

(13) Methanol, grade for organic residue analysis, J.T.Baker, USA

(14) Peroxidase-biotin (POD-biotin), Fluka, Germany

(15) Polyoxyethylenesorbitan monolaurate (Tween 20), Sigma, USA

(16) Potassium dihydrogen citrate (KH_2-citrate), Pro analysis, Fluka, Germany

(17) Reference organochlorine pesticide standards from Laboratory of Dr.Ehreustorfer, Augburg, Germany: Heptachlor 99.8%, Aldrin 98.3%, Dieldrin 99.3%, *o,p'*-DDE 99.8%, *o,p'*-DDT 99.9%

(18) Reference organochlorine pesticide standards from EPA, Triangle Park, N.C., USA: γ-HCH 99.8%, *p,p'*-DDE 99.47%, *p,p'*-DDT 99%

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(19) Reference organochlorine pesticide standards from Promochen Ltd, Herts, England: *p,p'*-DDD 99.0%, *β*-HCH 99.0%, HCB 99.0%

(20) Sodium carbonate (Na_2CO_3), Pro analysis, Merck, Germany

(21) Sodium chloride (NaCl), Pro analysis, Merck, Germany

(22) Sodium dihydrogenphosphate dihydrate ($Na_2H_2PO_4.2H_2O$), Pro analysis, Merck, Germany

(23) Sodium hydrogen carbonate ($NaHCO_3$), Pro analysis, Merck, Germany

(24) Sodium hydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$), Pro analysis, Merck, Germany

(25) Sorbic acid, Pro analysis, Fluka, Germany

(26) Sulfuric acid (H_2SO_4), Pro analysis, Merck, Germany

(27) 3,3',5,5'-tetramethylbenzidine (TMB), Pro analysis, Fluka, Germany

2.3 Methodology

2.3.1 Methodology of research section 1:

Distribution of POPs in the environment

2.3.1.1 Environmental samples

Environmental samples were pig fats from Mae Sa Mai Village, Mae Rim District, Chiang Mai Province. Twelve pig fat samples were randomized from the pigs fed in this village.

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2.3.1.2 Analytical methods

Types and levels of POPs in pig fats were analysed by using GC-ECD.

2.3.1.3 Statistical Analysis

Descriptive statistics, including arithmetic mean (AR), geometric mean (GM), standard deviation (SD), minimum (Min), and maximum (Max) were analysed for analysing POPs in pig fats.

2.3.2 Methodology of research section 2:

Effect of POPs on reproductive hormone levels in adult men

2.3.2.1 Studied population

Inclusion criteria were adult men, age from 18-45 years old and resided in Mae Sa Mai Village, Chiang Mai Province, for at least 10 years. Ninety-seven adult men who eligible for the inclusion criteria, willing to participate the study, and signed written consents were enrolled as studied population.

2.3.2.2 Observation and analytical methods

Socio-demographic data were collected from the individual participants by trained interviewers after they signed written consents. Data on questionnaire consisted of personal data, years of residence and farm experience, dietary behavior, pesticide use and exposure, and etc. Anthropometric measures, including weight and height, were collected for calculating body mass index (BMI).

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Twelve ml of fasting venous blood sample was collected in heparinised tubes. Plasma was separated and stored in the freezer (–20°C) before analysis of POPs, total lipids, and estrogenic activity of chemicals, and stored in the freezer (–70°C) before analysis of reproductive hormones.

Types and levels of POPs were analysed by using GC-ECD. The method of sample preparation and analysis was presented in elsewhere. Levels of reproductive hormones, including E_2, testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH), were analysed by using chemiluminescense technique. Estrogenic activities of chemicals in plasma extracts were analysed by using ELRA. Triglycerides and total cholesterol levels were analysed by using an enzymatic method. The following conversion formula was used for calculation of total lipids (Phillip et al., 1989).

Total lipids, mg/dl = 2.27(total cholesterol) + triglycerides + 0.623

2.3.2.3 Statistical analysis

SPSS version 11 was performed for data analysis. Descriptive statistics, including GM, AR, SD, Min, Max, and percentile were computed.

Because of non-normal distribution of POPs, years of residence, years of DDT for farming, age, BMI, and estrogenic activities of chemicals, natural logarithm transformation was applied for the variables before testing parametric tests. Pearson correlation coefficient was calculated for the association between plasma levels of POPs and reproductive hormones. Multiple linear regression analysis was computed for the association of POPs with years of residence and DDT use for farming. These regression analysis was also computed for the associations of *p,p'*-DDE and *o,p'*-DDE with E_2, adjusted for confounding variables including age and BMI. Beta (β), standard error (SE), partial correlation coefficient (*partial r*), and level of significance (*P*) were calculated. Student's t-test was used for testing differences in plasma DDT levels

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between the persons who had used DDT for farming and those who had never used it. The level of significance was set at P value < 0.05.

2.3.2.4 Ethical approval

The study was approved by Human Experimentation Committee, Research Institute for Health Sciences, Chiang Mai University (Certificate of Ethical Clearance No. 10/2003, 13 March 2003).

2.3.3 Methodology of research section 3:

Effect of POPs on reproductive and thyroid hormone levels in infants

2.3.3.1 Studied population

Inclusion criteria were the pregnant women who had lived in three subdistricts of Mae Rim District (Pong Yang, Mae Ram, and Sun Kha Yom) and eight subdistricts of Chiang Dao District (Maung Na, Maung Ngai, Mae Na, Tung Khoa Paung, Aru No tai, Pang Feung, Pang Ma Yao, and Bann Na Wai) of Chiang Mai Province for at least 5 years. All had normal delivery and full term gestation. Because iodine deficiency during pregnancy has a strong impact on thyroid status in infants, therefore, the pregnant women who had urinary iodine levels lower than 100 μg/l or who were diagnosed as hypothyroidisms were excluded.

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One hundred and twenty-seven pregnant women, 39 women from Mae Rim District and 88 women from Chiang Dao District, who eligible the inclusion criteria and willing to participate the study, and signed written consents, were enrolled as studied population.

2.3.3.2 Observation and analytical methods

Socio-demographic data were collected from individual participants by trained interviewers after they signed written consents. Data on questionnaire consisted of personal data, years of residence and farm experience, and pesticide use and exposure, delivery history, prenatal care, and etc.

Ten ml of maternal blood was collected by venipuncture 2-5 hours before delivery. Serum was separated and stored at –20°C before analysis of POPs, total lipids, and estrogenic activities of chemicals. Twelve ml of umbilical cord blood was collected immediately when umbilical cod was cut. Serum was separated and stored at –20°C before analysis of POPs, total lipids, and estrogenic activities of chemicals, and stored at –70°C before analysis of reproductive and thyroid hormones.

Types and levels of POPs were analysed by using GC-ECD. Levels of total lipids were analysed by using gravimetric method, according to Blight and Dyer method (1959). Levels of reproductive hormones, including E_2 and testosterone, were analysed by using chemiluminescense technique. Levels of thyroid hormones, including TT_4, FT_4, and TSH, were analysed by using radioimmunoassay (RIA). Estrogenic activities of chemicals in serum extracts were analysed by using ELRA.

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Anthropometric measures at birth, including weight, height, and head circumference, were obtained from hospital delivery records.

2.3.3.3 Statistical analysis

SPSS for window version 11 was used for data analysis. Descriptive statisticals, including AR, GM, SD, Min, Max, and percentile were analysed. Because of non-normal distribution of serum POPs, E_2, testosterone, TSH, and estrogenic activities of chemicals, natural logarithm transformation was applied for the variables before testing parametric tests.

Independent-samples T-test was used to compare mean levels of POPs and hormones between the mothers-infant pairs from Mae Rim and Chiang Dao Districts. Pair-samples T-test was used to compare mean levels of POPs in maternal and cord serum. Pearson correlation coefficient was used to investigate the association of cord serum POPs with maternal serum POPs, hormones, and estrogenic activities of chemicals. Multiple linear regression analysis was conducted for the association of POPs with reproductive and thyroid hormones, adjusted for confounding variables. Birth weight was confounding variable for the association between POPs and reproductive hormones. Gender of infants was confounding variables for the association between POPs and thyroid hormones. β, SE, *partial r*, and *P* value were calculated. The level of significance was set at P value < 0.05.

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2.3.3.4 Ethical approval

The study was approved by Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University (Certificate of Ethical Clearance No. 10/2003, 13 March 2003).

2.4 Analysis of POPs

2.4.1 Preparation of POP stock standard

Steps of Preparation for POP stock standard were as follows:

(1) POP standard was dried in oven at 65°C overnight, unscrewed cap but still place on the bottle.

(2) All volumetric flasks were cleaned with 3 times isooctane, rinsed with 3 times methanol, and 3 times acetone.

(3) Individual POPs were prepared approximately 0.5 mg/ml in isooctane (0.005 g/10 ml isooctane), except *β*-HCH was in toluene.

(4) Individual intermediate and working standards were prepared from stock standards and series of working standard mixture are presented in Table 2.1.

Table 2.1 Working standard mixture ($M_1 - M_{10}$) of POPs (ng/ml in isooctane)

POPs	M_1	M_2	M_3	M_4	M_5	M_7	M_{10}
	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
β-HCH	1	2	3	4	5	7	10
HCB	1	2	3	4	5	7	10
γ-HCH	1	2	3	4	5	7	10
Heptachlor	2	4	6	8	10	14	20
Heptachlor epoxide	2	4	6	8	10	14	20
o,p'-DDE	4	8	12	16	20	28	40
p,p'-DDE	3	6	9	12	15	21	30
Dieldrin	3	6	9	12	15	21	30
p,p'-DDD	3	6	9	12	15	21	30
o,p'-DDT	3	6	9	12	15	21	30
p,p'-DDT	5	10	15	20	25	35	50
Aldrin (IS)	10	10	10	10	10	10	10

IS = internal standard

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2.4.2 Sample preparation for POP analysis

Sample preparation was modified from The Prapamontol and Stevenson Method (1991). Extraction and cleaned up steps were as follows:

(1) Two ml of plasma /serum samples (or 1 g of homogenized pig fat samples) was prepared.

(2) 50 μl of aldrin (0.2 μg/ml) as an internal standard was added in each sample and then vortex for 30 seconds.

(3) Each sample was extracted with 2 ml of ethyl acetate (EA), 4 ml of methanol, and 4 ml of acetone, and then vortex for 1 minute.

(4) The extraction reaction was activated in a warm sonicator for 20 minutes, and then centrifuged at 2,000 rounds per minute (rpm) for 15 minutes.

(5) The supernatants were diluted with 13 ml of distilled water.

(6) For pre-condition of C_{18}-cartridges, they were conditioned with 2 x 1 ml of isooctane, 2 x 1 ml of EA, 2 x 1 ml of methanol, and 2 x 1 ml of distilled water.

(7) The supernatants were loaded in the cartridge at 8 –10 mm Hg pressure.

(8) The cartridges were washed with 2 x 1 ml of 25% acetonitrile and dried at 15 mm Hg pressure for 7 minutes.

(9) Finally, the cartridges were eluted with 2 x 0.75 ml on 8 – 10 mm Hg pressure. The step of extraction and clean up is presented in Figure 2.1.

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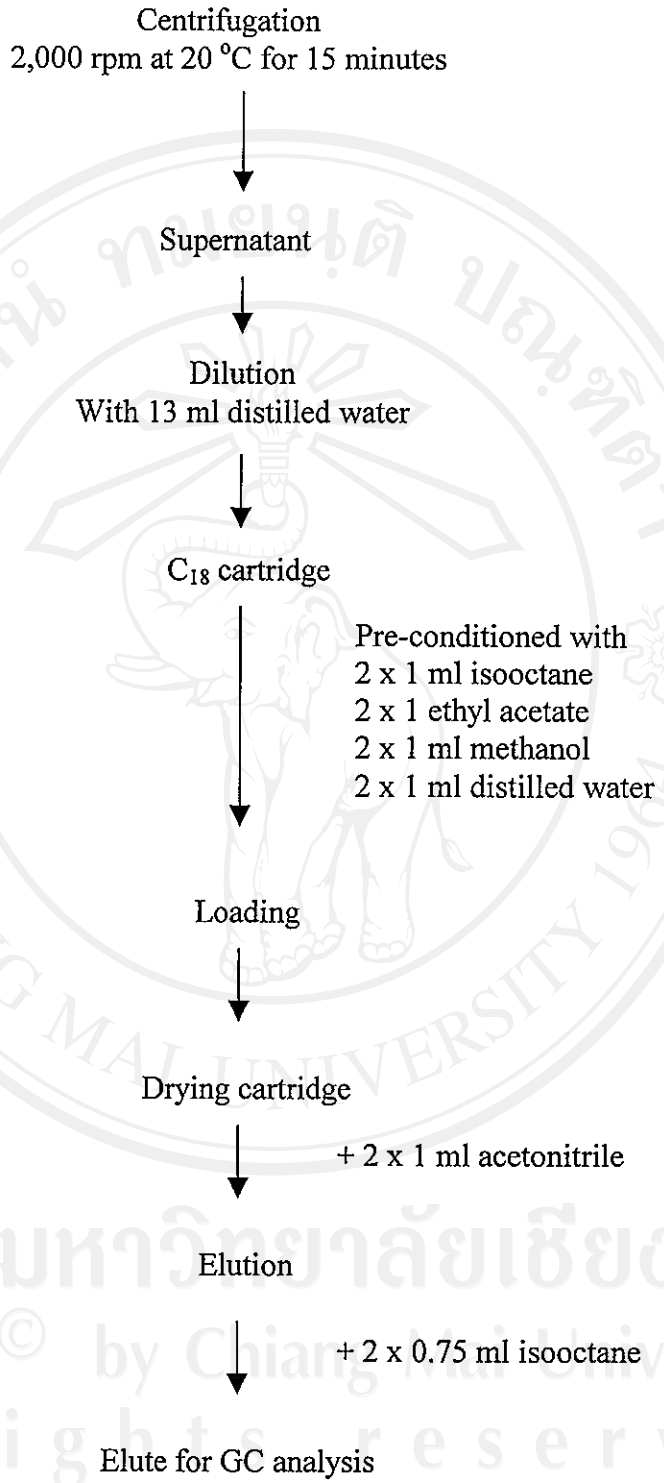

Figure 2.1 Diagram of sample preparation for GC analysis (Prapamontol and Stevenson, 1991)

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2.4.3 Gas chromatographic condition

One μl of eluate was injected and analysed by using GC-ECD. The GC analysis consisted of a Hewlett-Packard model 5890 Series II equipped with a ^{63}Ni ECD, an autosampler (HP 7673), a fused silica capillary column (Ultra 2: 25 m x 0.32 mm i.d. with 0.52 μm film thickness), and computerized data handling data system (HP 3365 Series Chemstation). Programming temperature was 300 °C for detection port and 200 °C for injection port (splitless mode). Temperature programming of oven was as follows: initial temperature 80 °C for 10 minutes, first ramp 30 °C/minute ramp rate to 190 °C, second ramp 4 °C /minute ramp rate to 250 °C and final temperature hold at 250 °C for 10 minutes. High purity helium (99.993%) was used as a carrier gas and ultra high purity nitrogen (99.999%) was used as a make up gas.

2.4.4 Qualitative and Quantitative analysis of POPs

Individual POPs were qualified by the retention time and quantified by peak area. Aldrin solution was used as an internal standard.

2.4.5 Quality control of POP analysis

2.4.5.1 Levels of detection

Limit of detection (LOD) and limit of quantification (LOQ) were analysed. Series of working standard mixture, including M_1, M_2, M_3, and M_4, were analysed 7 times for calculation of the standard deviation (S.D.). POP levels (X axis) and S.D. (Y axis) in individual series were plotted as a linear curve for determining Y-intercept. Limit of detection was calculated as described below:

LOD = 3 x Y-intercept

LOQ = 10 x Y-intercept

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2.4.5.2 Recovery

Pooled serum samples were unspiked and spiked with known levels of individual POPs in some samples for analysis of recovery. It was calculated as described below:

$$\text{Recovery (\%)} = \left((Cs - Cu) \ / \ Ck \right) \times 100$$

where Cs = the individual POPs levels in spiked serum (ng/ml)

Cu = the individual POPs levels in unspiked serum (ng/ml)

Ck = the known spiked levels of individual POPs (ng/ml)

2.4.5.3 Intra- and inter-batch variations

For intra-batch analysis, five replicates of pooled serum samples were analyzed at the same time for calculating AR, SD, and coefficient of variation (% CV) of POPs.

For inter-batch analysis, at least one aliquot pooled serum sample was applied in each batch (n = 22) and analysed POP levels for monitoring POP variations.

2.5 Analysis of estrogenic activities of chemicals

2.5.1 Enzyme-linked receptor assay (ELRA)

ELRA was performed in polystyrene 96-microwell plates according to the method of Seifert et al. (1999). Steps of analysis were as follows (Figures 2.2 and 2.3):

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(1) Plates were coated and incubated overnight with 150 μl of E_2-BSA conjugate per well at 4 °C.

(2) After washing plate with PBS washing buffer and blocking for 2 hours, 100 μl of E_2 solution of known concentrations (0-1,000 μg/l) or extract samples were added to each well and incubated together with the 100 μl per well of estrogen receptor diluted 1:30 in PBS-EDTA buffer for 1 hour and 30 minutes.

(3) After further washing step, 200 μl of 1:500 dilution of the biotinylated mouse anti-estrogen receptor antibody was added to each well and incubated for 30 minutes incubation.

(4) After further washing the plates, 200 μl of a streptavidin-POD-biotin complex was added into each well and incubated for 1 hour.

(5) After further washing the plates, 200 μl of a TMB substrate was added. The substrate reaction was terminated after 30 minutes with 50 μl of 2 Molar sulfuric acid.

(6) The absorbance was measured at 450 nm with an ELISA reader. Dose-response curve of E_2 standard solution was presented in Figure 2.4.

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2.5.2 Quality control of ELRA

Pooled serum extracts were unspiked and spiked with known levels of E_2 (0.2 ng/ml E_2 equivalent) in some samples for analysis of recovery. It was calculated as described below:

$$\text{Recovery (\%)} = \left((Cs - Cu) \ / \ Ck \right) \times 100$$

where Cs = estrogenic activities of chemicals in spiked plasma/serum (ng/ml)

Cu = estrogenic activities of chemicals in unspiked plasma/serum (ng/ml)

Ck = the known spiked levels of E_2 (ng/ml)

2.6 Quality control of lipids and hormones

For intra-batch analysis, five replicates of pooled serum samples were analysed for lipids and hormones at the same time for measuring lipid and hormone variations.

For inter-batch analysis, at least one aliquot pooled serum sample was applied in each batch (n = 5) and analysed for lipid and hormone levels for monitoring their variations.

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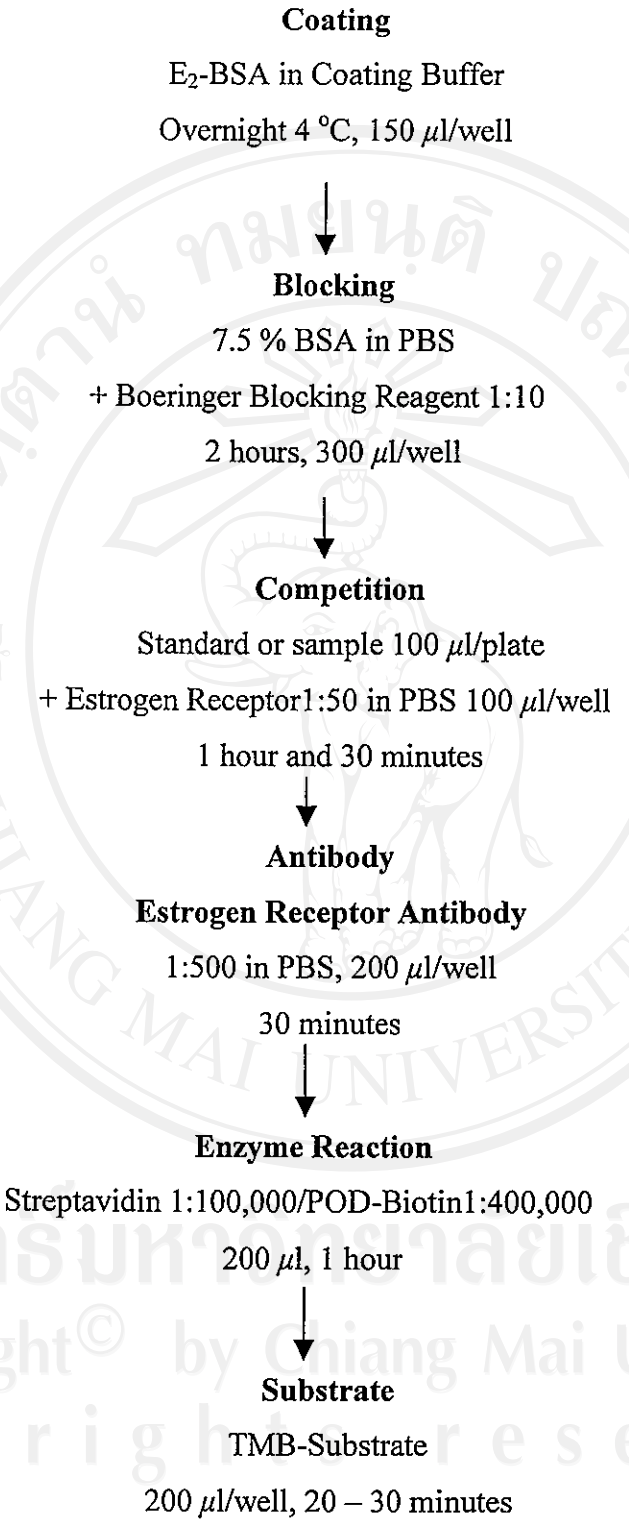

Figure 2.2 Diagram of enzyme-linked receptor assay (ELRA) (Seifert et al., 1999)

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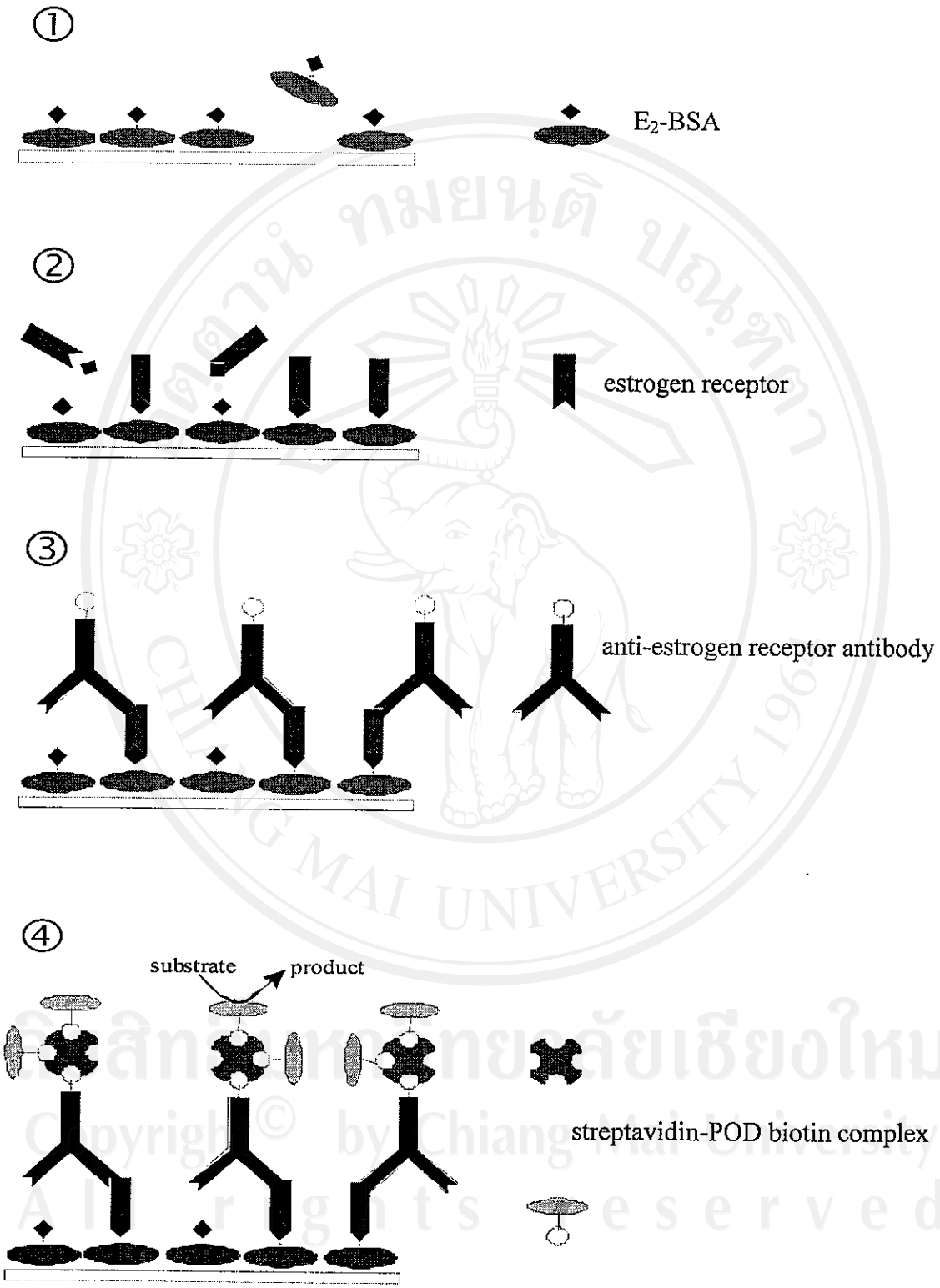

Source: Seifert et al., 1999

Figure 2.3 Test principle of ELRA

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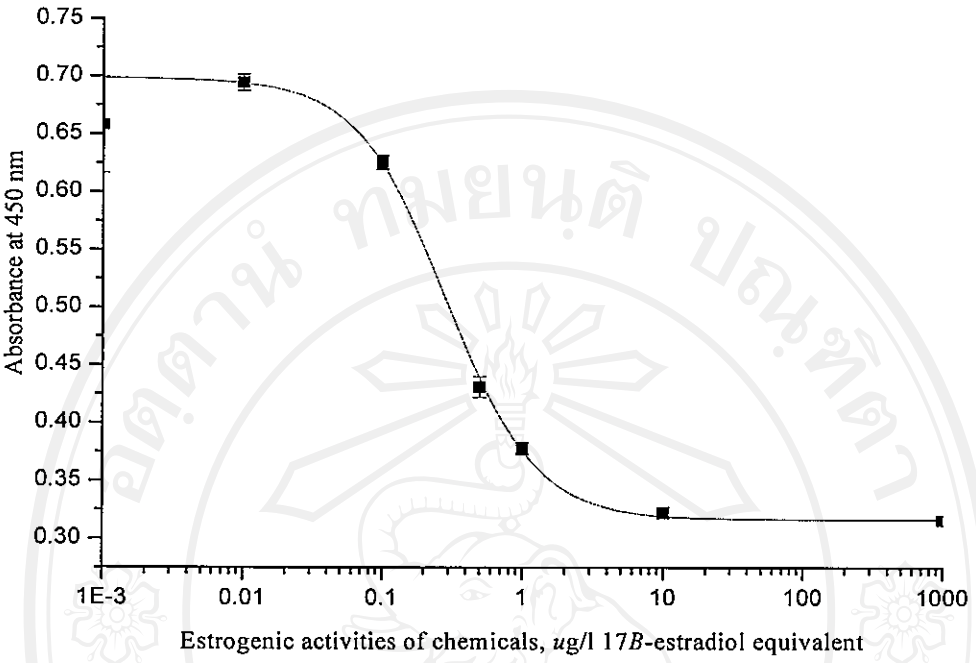

Figure 2.4 Dose-response curve of E_2 standard solution

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