2 EXPERIMENTAL

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1) Gas chromatograph, HP 5890 series II plus, manufactured by Hewlett Packard, U.S.A, consisting of
   a) Autoinjector, HP 7673
   b) Electron capture detector (ECD) with ^{63}Ni β-emitter
   c) Hewlett - Packard Autoinjector Controller
   d) Data processing system, Hewlett Packard Vectra 486/33M
   e) Capillary columns :

   HP-608, 30m × 0.53 mm I.D., 0.53 μm film thickness, Hewlett Packard, U.S.A.

   DB-1701, 30m × 0.25 mm I.D., 0.25 μm film thickness, J&W scientific, U.S.A.

2) Gas chromatograph-mass spectrometry, ITS 40 Ion Trap mass spectrometer, manufactured by Finnigan Mat, U.S.A., consisting of
   a) Gas chromatograph, GC 3400 manufactured by Varian, U.S.A.
   b) Data processing system, Compaq Deskpro 386/20e
   c) Capillary column DB-1, 30m × 0.25mm, I.D., 0.25 μm film thickness, J&W Scientific, U.S.A.
3) Air pump, manufactured by Waters, U.S.A.
4) Ultrasonicator, model 2000, manufactured by Branson, U.S.A.
5) Solid phase extraction manifold, manufactured by J. T. Baker Inc., U.S.A.
6) Glass column, 8 ml, manufactured by Merck, Germany
7) Analytical Balance, Satorious Basic BA 210s, manufactured by Sartorious AG, Germany
8) Rotary Evaporator, Buchi Rotavapor F-124, manufactured by Buchi Labortechnik AG, Switzerland , consisting of

a) Water bath, B-480

b) Air pump, KNF Laboport

c) Cooling device, NESLAB , manufactured by NESLAB instruments, Inc., U.S.A.

9) Centrifuge, Cenco, manufactured by Cenco Instrumenten M.U.N.V., The Netherlands

10) Magnetic Stirrer, Heidolph, Germany

11) Autopipette, Merck, Germany

12) Cellulose acetate (filter) 0.45 μm, Satorius, manufactured by Sartorius AG, Germany

2.1.2 Chemicals

1) Bakerbond Octadecyl (C_{18}) 40 μm prep LC packing, manufactured by J.T. Baker Inc., U.S.A.

2) Methanol, HPLC Grade, Carlo Erba, Italy

3) Ethylacetate, AR Grade, BDH, England

4) n-Hexane, AR Grade, BDH, England

5) Isooctane, > 99.5%, Fluka , Switzerland

6) Isopropanol, A.C.S Reagent, J.T. Baker, U.S.A

7) Acetone, A.C.S. Reagent, J.T. Baker, U.S.A

8) Dichloromethane, A.C.S. Reagent, J.T. Baker, U.S.A

9) Water , deionized water

10) Polychlorinated biphenyls standards :

10.1) PCB congeners mixed in isooctane, Supelco, U.S.A, containing

2,6-Dichlorobiphenyl (PCB No. 10) 100% 10 mg l^{-1}

2,4,4,'-Trichlorobiphenyl (PCB No. 28) 99.0% 10 mg l^{-1}

2,2',5,5'-Tetrachlorobiphenyl (PCB No. 52) 99.0% 10 mg l^{-1}

2,2',3,4,4',5'-Hexachlorobiphenyl (PCB No. 138) 99.0% 10 mg l^{-1}

2,2',4,4',5,5'-Hexachlorobiphenyl (PCB No. 153) 98.7% 10 mg l^{-1}

2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB No. 180)100.0% 10 mg l^{-1}

10.2) 2,2'4,5,5'-Pentachlorobiphenyl (PCB No. 101) 99.7%, Accu Standard, U.S.A.

11) Helium gas, 99.99% (HP Grade) , TIG, Thailand

12) Nitrogen gas, 99.999% (UHP Grade), TIG, Thailand

13) Sulphur, solid, Merck, Germany

2.2 Preparation of Standard Solutions

2.2.1 Preparation of 2,2'4,5,5'-pentachlorobiphenyl (PCB No. 101) solution

The PCB No. 101 stock solution of 1000 mg l^{-1} in isooctane was prepared by dissolving 10 mg of PCB No. 101 in isooctane. Then the solution was adjusted to 10 ml in a 10 ml volumetric flask. This stock solution was diluted to 10 mg l^{-1} with isooctane and ready to be used for the preparation of mixed working standard solutions.

2.2.2 Preparation of mixed working standard solutions

The PCBs mixed working standard solution with a concentration of 1 mg l^{-1} consisting of PCBs No. 10, 28, 52, 101, 138, 153, and 180, was prepared by transferring 1 ml of 10 mg l^{-1} solution of PCB congeners and PCB No. 101 standard solution to a 10 ml volumetric flask and the volume was adjusted to the mark with isooctane.

The PCBs mixed working standard solutions in the range of 1 μg l^{-1} – 20 μg l^{-1} were prepared from the 1 mg l^{-1} standard solution above. In the case of concentrations below 1 μg l^{-1} they were prepared by dilution of suitable PCBs standard solutions.

The PCBs mixed working standard solutions in ethylacetate were prepared by using suitable working standard solutions in isooctane. They were gently blown with the aid of nitrogen stream until just dryness then ethylacetate was added and adjusted to a suitable volume.

2.2.3 Preparation of test solutions

2.2.3.1 Test solutions for optimization of solid phase extraction in milk samples analysis

These test solutions used in optimization of solid phase extraction consisting of solutions A, B and C :

- Solution A

A volume of 100 μl of standard solution 500 μg l^{-1} of standard PCBs mixture was transferred to a 125 ml erlenmyer flask and gently blown with nitrogen stream until dryness. The volume of 2 ml of ethylacetate, 4 ml of acetone and 4 ml of in methanol were added to the flask. Finally 10 ml of deionized water was added to the solution and mixed well.

- Solution B

This solution was prepared nearly the same as the solution A except 20 ml of water was added instead of 10 ml.

- Solution C

This solution was prepared nearly the same as the solution A except 30 ml of water was added instead of 10 ml.

2.2.3.2 Test solutions for optimization of solid phase extraction in water samples analysis .

These test solutions used in optimization of solid phase extraction consisting of solutions D-K which were prepared according to the composition listed in Table 2.1. At 0.1 μg l^{-1}spiked level in 50 ml and 20 ml of distilled water, they were prepared by using a volume of 100 μl of 50 μg l^{-1} and 20 μgl $^{-1}$ standard PCBs mixture, prepared in ethylacetate, respectively and spiked in to the distilled water. The solutions were mixed well with the aid of a magnetic stirrer and kept overnight before extraction.

Table 2.1 Composition of solutions D-K

Solution	Composition (ml)				
	Water	Acetone	Methanol	Isopropanol	Ethylacetate
D	50	-	-	-	-
E	50	5	-	-	-
F	50	-	5	-	-
G	50	-	-	5	-
H	50	-	20	-	-
I	50	8	8	-	4
J	20	-	10	-	-
K	20	4	4	-	2

2.2.4 Preparation of standard sulfur solution

The sulfur stock solution of 2000 mg l^{-1} was prepared by dissolving 10 mg of solid sulfur in n-hexane with the aid of ultrasonication. Then the solution was adjusted to 5 ml in a 5 ml volumetric flask. This stock solution was diluted to 10 mg l^{-1} by diluting 25 µl of the stock solution to 5 ml with n-hexane. This solution was injected to GC/MS to use in interference identification of Mae-ping water sample.

2.3 Preparation of Spiked and Nonspiked Milk and Water Samples

2.3.1 Preparation of spiked and nonspiked milk samples

A sample of cow's milk was taken from a convenience store in Chiang Mai City and stored at 4°C before extraction.

The spiked cow's milk samples were prepared at 2 levels, 10 and 1.4 µg l^{-1}, with standard PCBs mixture. At 10 µg l^{-1} level, a volume of 100 µl of 2 mg l^{-1} standard PCBs mixture, prepared in ethylacetate, was spiked to 20 ml of cow's milk and stirred at 37°C for 30 min. Then the spiked cow's milk was kept at 4°C overnight before extraction.

At 1.4 µg l^{-1} spiked cow's milk, it was prepared nearly the same as 10 µg l^{-1} spiked level above, except spiking was done with 140 µl of the 200 µg l^{-1} standard PCBs mixture, prepared in ethylacetate.

The powdered milk (25.8% fat content) was reconstituted with water by using 2.7 g of powdered milk and 20 ml of water. The reconstituted milk was kept at 4°C before extraction.

The spiked powdered milk was prepared at 2 levels, 74.1 and 10.4 µg kg^{-1}. At 74.1 µg kg^{-1} spiked level, a volume of 100 µl of the 2 mg l^{-1} standard PCBs mixture, prepared in ethylacetate, was spiked to 20 ml of reconstituted powdered milk and stirred at 37°C for 30 min. Then it was kept at 4°C overnight before extraction.

At 10.4 µg kg^{-1} spiked level, it was prepared nearly the same as 74.1 µg kg^{-1} spiked level above, except spiking was done with 140 µl of the 200 µg l^{-1} standard PCBs mixture, prepared in ethylacetate.

2.3.2 Preparation of nonspiked and spiked water samples

In this work, drinking and natural surface water were analyzed. The drinking water samples were taken from a convenience store in Chiang Mai City and kept at 4°C.

The spiked drinking water at 0.1 μg l^{-1} was prepared by using a volume of 100 μl of the 20 μgl^{-1} standard PCBs mixture, prepared in ethylacetate, spiked into 20 ml of drinking water brand A . Then 10 ml of the solvent mixtures of ethylacetate : acetone : methanol (1:2:2 v/v) was added. The solution was mixed well with the aid of a magnetic stirrer and kept overnight before extraction.

The natural surface water samples were sampled from natural water resources in the northern region of Thailand and kept at 4°C.

The spiked natural surface water at 1.0 μg l^{-1} was prepared by using 20 ml of the water from Mae-ngud Dam filterring through 0.45 μm cellulose acetate. A volume of 100 μl of the 200 μg l^{-1} standard PCBs mixture, prepared in ethylacetate, was spiked into 20 ml of the filtered water. Then 10 ml of the solvent mixture of ethylacetate : methanol : acetone (1:2:2 v/v) was added. The solution was mixed well with the aid of a magnetic stirrer and kept overnight before extraction.

2.4 Preparation of C_{18} Solid Phase Extraction Columns

The C_{18} SPE columns were each prepared by weighing 500 mg of C_{18} and packing it tightly into a 8 ml glass column with a frit at the bottom. Finally another frit was transferred to the top of C_{18} level and packed tightly again.

2.5 Optimization

2.5.1 Optimization of gas-chromatographic condition for milk samples analysis

The HP-608 column was used in this analysis with 1 μl injection volume in the splitless mode. The injector port and detector temperature were held at 250°C and 320°C, respectively. Helium at a flow rate of 2.4 ml min^{-1} was used as the carrier gas whilst nitrogen was used as the auxillary gas. The standard PCBs mixture in isooctane at 10 μg l^{-1} was used in optimization.

According to the EPA 608 method, gas chromatographic temperature program for determination of organochlorine and PCBs was 80°C - 80°C (1 min) - 190°C (30°C min^{-1}) - 280°C (6°C min^{-1}) - 280°C (1 min) - 300°C (20°C min^{-1}) – 300°C (5 min). But in this work, it was found that this temperature program was not suitable. Thus, this temperature program was modified firstly on a trial and error approach.

2.5.2 Optimization of gas - chromatographic condition for water samples analysis

The DB-1701 column was used in this analysis with 2 µl injection volume in the splitless mode. The injector port and detector temperature were held at 250°C and 320°C, respectively. Helium at a flow rate of 1.0 ml min^{-1} was used as the carrier gas whilst nitrogen was used as the auxillary gas. The standard PCBs mixture in isooctane at 1 $\mu g l^{-1}$ was used in optimization. The temperature program was optimized on a trial and error approach.

2.5.3 Limit of detection (LOD)

Limit of detection (LOD) is the least amount of material that the analyst can detect because it yields an instrument response significantly greater than a blank.

Limit of detection was calculated from the Linear Least Squares' Line[104] procedure.

From the Linear Least Squares' Line procedure,

$$y = a + bx \quad (2.1)$$

y = instrument signals

x = normally are concentrations

a = intercept

b = slope of the straight line

$$Y_L = Y_B + k\,S_B \quad (2.2)$$

Y_L = lowest detectable instrument signals

Y_B = blank signal

≅ intercept, a

k = constant depending on definition such as k = 3, 1.5 or 10 According to IUPAC, in calculation of LOD, k = 3 was used and in this work this value was used.

S_B = blank signal standard deviation

$\cong$ $S_{y/x}$

$S_{y/x}$ can be calculated from the equation

$$S_{y/x} = \{\Sigma(Y_i - \hat{Y}_i)^2/(n-2)\}^{1/2} \quad (2.3)$$

Y_i = response value from the instrument corresponding to the individual x - values

$\hat{Y}_i$ = value of y on the calculated regression line corresponding to the individual x-values

n = number of points on the calibration line

From equation (2.2)

$$Y_L = a + 3\,S_{y/x} \quad (2.4)$$

Thus the concentration at limit of detection (C_L) can be calculated by using the equation (2.6)

$$Y_L = a + bC_L \quad (2.5)$$

Thus

$$a + 3\,S_{y/x} = a + b\,C_L$$

$$C_L = 3S_{y/x}/b \quad (2.6)$$

Thus, the limit of detection can be calculated according to the equation (2.6) (for more detail and an example of calculation, see the Appendix B).

In the milk samples analysis, calculation of the limit of detection was based on the external calibration curve in the concentration range 0.5 – 7 $\mu g\ l^{-1}$.

In the water samples analysis, calculation of the limit of detection was based on the external calibration curve in the concentration range 0.1 - 1.5 $\mu g\ l^{-1}$.

2.5.4 Optimization of solid phase extraction

2.5.4.1 Optimization of solid phase extraction for milk samples analysis

- Study of the volume of water added to the standard solution

500 mg amount of C_{18} sorbent was packed in each of 8-ml glass columns and ended with frits. The columns were first conditioned with 5 ml of dichloromethane, followed with 5 ml of methanol with a flow rate of about 1-2 ml min^{-1}. Then the solutions A, B and C (10, 20 and 30 ml of water added, respectively) were loaded into each column at a flow rate of about 2-3 ml min^{-1} by using the air

pump. Air was pulled through the columns with the aid of an air pump until dryness, for about 30 min. In the final step SPE columns were eluted with 4 ml of isooctane with a gravity flow rate. The eluate was adjusted to 5 ml with isooctane and then analyzed by GC/ECD.

- Study of the effect of conditioning solution to the percent recoveries of PCBs

500 mg amount of C_{18} sorbent was packed in each of 8 -ml glass and ended with frits. The columns were first conditioned with 5 ml of dichloromethane, followed with 5 ml of methanol and finally with 5 ml of solvent mixtures which were used to prepare solutions A, B and C, with a flow rate of about 1-2 ml min^{-1}. Then the solutions A, B, and C were loaded to each column with flow rate about 2-3 ml min^{-1}. Air was pulled through the columns with the aid of air pump until dryness, for about 30 min. In the final step, SPE columns were eluted with 4 ml of isooctane with gravity flow rate. The eluate was adjusted to 5 ml with isooctane and then analyzed by GC/ECD.

- Study of the effect of drying step to the percent recoveries of PCBs

500 mg amount of C_{18} sorbent was packed in each of 8- ml glass column and ended with frits. The column was first conditioned with 5 ml of dichloromethane, followed with 5 ml of methanol and finally with 5 ml of solvent mixtures, water : ethylacetate : methanol : acetone (10:1:2:2 v/v), with a flow rate of about 1-2 ml min^{-1}. When the column had just been dried, the 100 μl of 500 μg l^{-1} of PCBs mixture, PCBs No. 10, 28, 52, 101, 138, 153 and 180, prepared in isooctane, was added to the top of the sorbent. Then the column was dried with the air pulled through for about 30 min. Finally the SPE column was eluted with 4 ml of isooctane with a gravity flow rate. The eluate was adjusted to 5 ml with isooctane and then analyzed by GC/ECD.

- Investigation of optimal elution volume

500 mg of C_{18} sorbent was packed in a 8-ml glass column and ended with frits. The column was first conditioned with 5 ml of dichloromethane, followed with 5ml of methanol and finally with 5 ml of solvent mixtures, water :

ethylacetate : acetone : methanol (10:1:2:2 v/v) , with flow rate of about 1-2 ml min^{-1}. Then the solution B was loaded onto the column with a flow rate of about 2-3 ml min^{-1}. Air was pulled through the column with the aid of air pump until dryness for about 30 min. Finally the SPE column was eluted with 4×1 ml of isooctane with gravity flow and each milliliter of the eluate was collected in a vial. Then each fraction of the eluate was analyzed by GC/ECD.

2.5.4.2 Optimization of solid phase extraction for water samples analysis

- Study of the effect of type and amount of wetting solvent and sample volume to the percent recoveries of PCBs

500 mg of amount C_{18} sorbent was packed in each of 8-ml glass column and ended with frits. The columns were first conditioned with 5 ml of dichloromethane, followed with 5 ml of methanol and finally with 5 ml of solvents or solvent mixtures which were used in the preparation of solutions D-K with a flow rate of about 1-2 ml min^{-1}. Then the solutions D-K were loaded onto each column with a flow rate of about 2-3 ml min^{-1} with the aid of air pump. Air was pulled through the columns with the aid of air pump until dryness, for about 30 min. In the final step, SPE columns were eluted with 3 ml of isooctane with a gravity flow rate. In the solutions D-I, the final volume was adjusted to 5 ml before injection to GC. In the solutions J and K, the final volume was adjusted to 2 ml with the aid of gentle nitrogen stream before injection to GC.

2.5.5 Investigation of the number of extraction in milk samples analysis

The spiked cow's milk at 10 μg l^{-1} was used in optimization of the number of extraction. The experiments were done according to the scheme in Figures 2.1 and 2.2.

thaw the spiked cow's milk at 37 °C and stir for 30 min

2 ml of spiked cow's milk + 10 ml of ethylacetate : methanol : acetone (1:2:2 v/v)

mix 1 min

↓

sonicate 15 min

↓

centrifuge 10 min

↓

collect the supernatant

↓

add 18 ml of water to the supernatant

↓

mix well

and load onto C_{18} SPE column already condition with

5 ml of dichloromethane, 5 ml of methanol and 5 ml of the solvent mixtures, water : ethylacetate : acetone: methanol (10:1:2:2 v/v)

with a flow rate of about 2-3 ml min^{-1}

↓

wash the column with 5 ml of water

↓

dry with the aid of air pump for about 30 min

↓

elute with 2 ml of isooctane

↓

adjust the final volume to 2 ml and analyze by GC/ECD

↓

recovery assay

Figure 2.1 Scheme for a single extraction of 10 μg l^{-1} spiked cow's milk.

thaw the spiked cow's milk at 37 °C and stir for 30 min

↓

2 ml of spiked cow's milk + 10 ml of ethylacetate : acetone : methanol (1:2:2 v/v)

↓

mix 1 min

↓

sonicate 15 min

↓

centrifuge 10 min

↓

collect the supernatant

↓

add with 1 ml of water, 1 ml of ethylacetate, 2 ml of acetone and 2 ml of methanol into the residue of extraction

↓

mix 1 min

↓

sonicate 10 min

↓

centrifuge 10 min

↓

collect the supernatant

↓

pool the supernatant with the first portion

↓

add 27 ml of water into the supernatant

↓

mix well

↓

load onto C_{18} SPE already condition in the same manner as in Figure 2.1

↓

wash the column with 5 ml of water

↓

dry with the aid of air pump for about 30 min

↓

elute with 2 ml of isooctane

↓

adjust the final volume to 2 ml and analyze by GC/ECD

↓

recovery assay

Figure 2.2 Scheme for a double extraction of 10 μg l^{-1} spiked cow's milk.

2.6 Determination of Fat Contents in Milk Samples

The fat contents in milk samples were determined by using hexane : acetone (1:1 v/v) extraction,[105] as shown in Figure 2.3.

add 8 ml of hexane : acetone (1:1 v/v) to 2 ml of cow's milk
or 0.3 g of powdered milk (reconstituted in 2 ml of water)

↓

mix 1 min

↓

sonicate 20 min

↓

centrifuge 10 min

↓

collect the supernatant

↓

extract residue two times with 4 ml of hexane

↓

pool all of the supernatant

↓

evaporate until dryness

↓

weight differentiation

↓

calculate the fat contents

Figure 2.3 Scheme for fat contents determination in milk samples.

2.7 Recovery Assay

The percent recovery was calculated based on the external calibration curve and peak area.

2.7.1 Milk samples analysis

The spiked cow's milk was spiked at 2 levels, 10 and 1.4 μg l^{-1}. The extraction procedure was performed in the same manner as in Figure 2.2 except at 1.4 μg l^{-1} spiked level with the final volume adjusted to 1 ml via the aid of nitrogen stream before analyzing by GC/ECD.

The powdered milk was reconstituted with water (see section 2.3.1) and 2 ml of the spiked sample was extracted. The spiked powdered milk was spiked at 2 levels, 74.1 and 10.4 μg kg^{-1}. The extraction procedure was performed in the same manner as in Figure 2.2 except at 10.4 μg kg^{-1} spiked level, the final volume was adjusted to 1 ml with the aid of nitrogen stream before analyzing by GC/ECD.

The blank samples of cow's milk and powdered milk were performed in the same manner as in the spiked samples.

2.7.2 Water samples analysis

The recoveries study were performed on both drinking and natural surface water samples. The drinking water was spiked at 0.1 μg l^{-1} with the standard PCBs mixture. The extraction procedure is shown in Figure 2.4. The natural surface water was spiked at 1.0 μg l^{-1} with the standard PCBs mixture. The extraction procedure is shown in Figure 2.4.

20 ml of spiked water samples
(see section 2.3.2 for preparation)

↓

load onto C_{18} SPE columns already condition with
5 ml of dichloromethane, 5 ml of methanol and 5 ml of solvent mixtures,
water : ethylacetate : acetone : methanol (10 : 1 : 2 : 2 v/v)
with a flow rate of about 2-3 ml min^{-1}

↓

dry with the aid of air pump for 30 min

↓

elute with 3 ml of isooctane

↓

adjust the final volume to 2 ml with the aid of nitrogen stream

↓

analyze by GC/ECD

↓

recovery assay

Figure 2.4 Scheme for spiked water samples extraction.

2.8 Analysis of Samples

In this work, milk and water were selected to study. In milk samples, one brand of cow's and powdered milk were selected. The extraction procedure was performed in the same manner as in Figure 2.2 except the final volume was adjusted to 1 ml with the aid of nitrogen stream before analyzing by GC/ECD. The samples were analyzed in triplicate.

In the water samples, three brands of drinking water and four sources of natural surface water (Mae-ngud, Mae-ping, Hauy-hong-krai, and Mae-kuang) were selected. A volume of 20 ml of each water sample was added with 10 ml of solvent mixtures, ethylacetate : acetone : methanol (1:2:2 v/v) and left overnight before extraction.

The extraction procedure was performed in the same manner as in Figure 2.4. The samples were analyzed in triplicate.

2.9 GC/MS of Mae-ping Sample and Standard Sulfur Solution

The eluate from C_{18} SPE of Mae-ping sample and standard sulfur solution were injected into the GC/MS in order to identify type of interference compounds. Likewise, a portion of the standard sulfur solution was injected into the GC/MS. The conditions of GC/MS employed are shown in Table 2.2.

Table 2.2 Condition of GC/MS

Operation	Conditions
1. Column	DB-1, 30m × 0.25 mm. I.D., 0.25 μm film thickness
2. Temperature program	100°C – 100°C (1 min) – 190°C (10°C min^{-1}) – 250°C (5°C min^{-1}) – 250°C (10 min)
3. Injector temperature	250°C
4. Injection	1 μl in splitless mode
5. Transfer-line temperature	250°C
6. Ion source temperature	200°C
7. Mass range	40 – 500 amu
8. Ionization mode	EI, 70 eV
9. Head Pressure	13 psi