CHAPTER 2

EXPERIMENTAL

2.0 Experimental : Analysis of OC residues in water, sediment and cockles.

2.1 Principles

Analysis is carried out to determine the chemical identity and concentration of the pesticide. The identity and quantity presented as a result in any analytical report would remain meaningless if unaccompanied by efficiency, precision and accuracy data. Efficiency is define as a capability of producing a desired value. Precision is define as a measure of the degree of agreement among replicate analyses of a sample, usually expressed as the standard deviation. Accuracy is the combination of bias and precision of an analytical procedure, which reflects the closeness of a measured value to a true value.

In this project every possibility of interferences, such as impurities in solvent and reagent, contaminated glassware and other sample processing hardware were routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks. The possible interferences from phthalates are avoided by not using plastics in the laboratory.

2.2 Reagents

All solvent were routinely checked for contamination by analyzing solvent blanks. If contamination were detected the reagents were cleaned by distillation from all-glass apparatus. The following solvents and reagents were used :

a) Dichloromethane	BDH, analytical grade (99.5% purity)
b) Hexane	Baker, GC grade and analytical grade (99.8% purity)
c) Methanol	BDH, analytical grade (99.5% purity)
d) Acetone	BDH, analytical grade (99.5% purity)
e) Silica gel	MERCK, Kielselgel 60 GF_{254} 7730
f) Sodium sulphate anhydrous	Fluka, analytical grade (99% purity)
g) Florisil	Sigma, pesticide grade, 100-120 mesh size.
h) Lindane	CHEM SERVICE, Inc., analytical standard (99.0% purity)
i) Formulated lindane (WA lindane 200)	WESCO Agencies (M) Sdn. Bhd, active ingredient gamma-BHC 20.3% w/w, inert material 79.7% w/w.
j) DDT and ^{14}C-DDT	IAEA (International Atomic Energy Agency) (98.0% purity)
k) Purified nitrogen gas	MOX (Malaysian Oxygen), (99.8% purity)

2.3 Glassware

All the glassware used for the analysis were soaked in 10% hydrochloric acid for a least 2 hours, and rinsed thoroughly with tap water, distilled water followed by acetone and finally hexane. The glassware was baked overnight in the oven at 300°C. All glassware was stored in a dust free cabinets and sealed with precleaned aluminum foil when not in use. Glassware was rinsed with analytical grade hexane prior to use.

2.4 Apparatus

The following apparatus were used :

a) Rotary vacuum evaporator	EYELA
b) Drying oven	MEMMERT,maximum temperature 300°C.
c) Freeze dryer	UNITOP 2000
d) Analytical balance	ER-180A
e) Gas chromatography	GC 17A, SHIMADZU
f) Mass spectrometer	QP 5000, SHIMADZU

2.5 Sample extraction.

2.5.1 Water samples

Water samples (500ml) were filtered through a membrane filter and extracted using hexane (50ml x 3). The combined extracts were dried over anhydrous sodium sulphate and the solvent evaporated using rotary evaporator at 40^0C under reduced pressure. A minimum amount of hexane were used to transfer the residue into a 18ml sample vial, followed by evaporation of the solvent under a stream of purified nitrogen. After all the solvent has evaporated 5ml of GC grade hexane was added prior to analysis [1-3].

2.5.2 Sediment sample

Freeze-dried sediment samples (20g) were placed in a precleaned extraction thimble. Hexane (250ml) and precleaned boiling chips were added to the round bottom flask. Extraction was carried out for 8 hours. The hexane extract was evaporated to dryness in a rotary vacuum evaporator, dichloromethane (250ml) was added to the remaining residue and the extraction was then continued for another 8 hours.

The dichloromethane extract was then evaporated to dryness in a rotary evaporator. The residue was then dissolved in hexane (20 - 50 ml) and concentrated in a rotary evaporator to about 15 ml. The hexane extract was then treated with mercury(Hg) to remove sulfur and then concentrated to 1 ml prior to florisil column chromatography clean-up [2].

2.5.3 Tissue sample

Freeze-dried tissue (10-20g) sample was placed in a precleaned extraction thimble. Hexane (250ml) and precleaned boiling chips were added to the round bottom flask. Extraction was carried out for 8 hours. The hexane extract was then concentrated in a rotary evaporator to about 15 ml. The hexane extract was treated with concentrated sulfuric acid for the removal of lipids. The recovered hexane extract was then concentrated in a rotary evaporator to 1ml prior to column chromatography clean-up [4].

2.6 Column chromatography clean-up.

Florisil column chromatography clean-up was used for the OC insecticide by elution with solvents of increasing polarity. Florisil was precleaned by Soxhlet extraction with methanol for 8 hours and with hexane for another 8 hours, then dried in oven at 60^{0}C and then stored in amber glass bottle. The florisil was activated at 650^{0}C for 14 hours and de-activated by adding 5% by weight of distilled water. The florisil was stirred for 3 hours before use.

The column (50cm x 15mm i.d., fitted with a coarse glass frit) was prepared by filling 16g of 5% de-activated florisil and approximately 1.0cm of anhydrous sodium sulfate was placed at the top of the column to absorb any water in the sample or solvent, while the lower end was filled with solvent-washed glass-wool.

The column was pre-eluted with 60ml of hexane to wet and rinse the sodium sulfate and florisil. Just prior to the exposure of the sodium sulfate layer to air, the elution of hexane was stopped and the elute discarded. The sample extract (in the concentrated form; approximately 1ml) was applied to the top of the column and sample container was rinsed twice with 5ml of hexane and was added to the column, allowing the solvent to sink below the sodium sulfate layer. The elution was conducted using three different solvents in increasing polarity viz hexane (70ml) followed by hexane/dichloromethane (70:30) (50ml) and finally with dichloromethane (40ml).

Each elute was collected at a drip of 5ml/min in a clean round bottom flask and was then concentrated using rotary evaporator to the final volume adjusted to 5ml with GC grade hexane. This extract were directly analyze by GCMS.

2.7 Analytical Procedures.

2.7.1 GCMS programming

The separation and quantification of OC compounds were performed with the Shimadzu GC17A gas chromatograph, fitted with a mass spectrometer detector QP 5000. The gas chromatographic conditions employed are summarized in Table 2.1.

2.7.2 Quantification.

The direct comparison technique (using external standards) was chosen for this study because the OC identified in the sample clearly matched with known OC pesticide standards based on their retention times. The gas chromatogram of standard lindane is shown in Figure 2.1.

Table 2.1: Summary of the gas chromatographic operating conditions.

Apparatus:	GC 17A
Detector(type):	QP5000 (mass spectrometer)
Injection technique:	manual
Injection temperature:	250^{0}C
Injection volume:	2µl
Carrier gas:	Helium
Carrier gas flow:	40ml/min
Split ratio:	20
Column(type):	Hicap Fused Silica Capillary column.
Length:	25m
Internal. diameter:	0.2mm
Stationary phase:	OV1701
Film thickness:	0.25µm

Temperature programme:

initial temp:	60^{0}C	for	2min
1st rate:	5^{0}C.min^{-1}	to	100^{0}C
isothermal:	100^{0}C	for	10min
2nd rate	8^{0}C.min^{-1}	to	230^{0}C
isothermal	230^{0}C	for	30min.

After comparison of the retention times of peaks in the sample chromatogram to those of a corresponding standard, peak areas are measured. The concentration of a particular compound is then calculated using the following formula [5].

$$\mathbf{C_{sam} = \frac{a_{sam} \times C_{st} \times V_{st} \times A \times 10^3}{a_{st} \times V_{sam} \times M}}$$

a_{st} = peak area of the compound in the standard (unit area)

C_{st} = concentration of the standard (ng/μL)

V_{st} = volume of the standard injected (μL)

a_{sam}= peak area of the compound in the sample (unit area)

A = total volume of the sample extract (ml)

Water samples;

M = volume of sample extracted (ml)

C_{sam}= concentration of the analyte in the sample (ng/ml)

Sediment and tissue samples;

M = dry weight of the sample extracted (g)

C_{sam}= concentration of the analyte in the sample (ng/g)

2.8 Precision and Accuracy.

2.8.1 Efficiency of the extraction Methods.

To ensure the validity of the analysis the efficiency of the extraction procedures from water, sediment and tissue samples, were determined. Furthermore the precision is also measured by the analysis of replicate samples.

2.8.1.1 Water samples

Distilled water (1L) was spiked with three different concentrations of lindane. 10 replicates of each concentration were prepared. The spiked distilled water was extracted , cleaned and analyzed as described in Sections 2.5.1, 2.6 and 2.7. The results obtained for the recovery and precision of the analysis are tabulated in Table 2.2. The high percentage of recovery ranging from 78.6 - 90.4% with standard deviations ranging from 5.1 - 5.7% indicates that the methodology used for the extraction procedure was acceptable for the present study.

Table 2.2: Efficiency of pesticide extraction from water samples.

No of replicates	Amount spiked(μg)	Amount recovered (μg)[a]	% Recovery	Standard deviation(%)
10	0.14	0.11	78.6	5.4
10	1.40	1.18	84.3	5.1
10	13.96	12.62	90.4	5.7

[a] mean value

2.8.1.2 Sediment samples

The efficiency of the sediment extraction procedure adopted were determined by spiking freeze-dried sediment (1kg) with three different concentrations of lindane. 10 replicates were prepared.

The extraction was carried out in accordance to the analytical procedures as reported in Sections 2.5.2, 2.6 and 2.7. The results obtained for the recovery and precision of the analysis are tabulated in Table 2.3. The high percentage of recovery ranging from 71.7 - 85.5% with standard deviations ranging from 7.3 -8.3% indicates that the methodology used for the extraction of lindane from sediment samples was acceptable for the present study.

Table 2.3: Efficiency of pesticide extraction from sediment samples.

No of replicates	Amount spiked(μg)	Amount recovered (μg)[a]	% Recovery	Standard deviation(%)
10	2.79	2.01	72.0	8.3
10	27.92	23.87	85.5	7.3
10	279.20	200.12	71.7	7.3

[a] mean value

2.8.1.3 Tissue samples

The efficiency of the tissue sample extraction procedure adopted were determined by spiking freeze-dried tissue (10g) with lindane. 10 replicates were prepared. The extraction was carried as described in Sections 2.5.3, 2.6 and 2.7. The results obtained for the recovery and precision of the analysis are tabulated in Table 2.4. The high percentage of recovery ranging from 71.3 - 81.5%

with standard deviations ranging from 6.0 - 8.3% indicates that the methodology used for the extraction of lindane from tissue samples was acceptable for the present study.

Table 2.4: Efficiency of pesticide extraction from tissue samples.

No of replicates	Amount spiked(μg)	Amount recovered (μg)[a]	% Recovery	Standard deviation(%)
10	2.79	1.99	71.3	8.3
10	27.92	22.75	81.5	6.0
10	279.20	200.12	71.7	6.8

[a] mean value

2.8.2 Precision of repeated GC-injections

In determining the error contributed by the GC, 5 different concentrations of standard lindane solutions were repeatedly injected in the GC. The results obtain by repeated analyses of lindane standards are given in Table 2.5.

The standard deviations of repeated GC-injections ranged from 0.9 - 3.9%. There does not appear to be a correlation between standard deviation and the concentration of the injected sample.

Table 2.5: Precision of the GC analysis

Nominal Concentrations (μg/ml)	Recovered percentage(%)					Standard deviation(%)
	1	2	3	4	5	
0.05	75.82	77.86	77.87	75.82	71.13	2.8
0.10	84.84	80.19	84.84	84.80	81.37	2.1
0.15	88.23	81.81	82.37	88.79	80.19	3.9
0.50	93.34	95.27	94.05	95.34	95.27	0.9
1.00	97.31	98.39	98.31	93.30	98.39	2.2

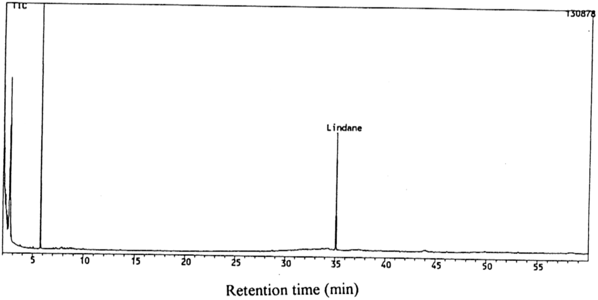

Figure 2.1: Gas chromatogram of a standard solution of lindane.

References

1. Hennion, M.C., Pichon, V. and Barcelo, D. (1994) Surface water analysis (trace-organic contaminations) and EC regulation. *Elsevier Science B.V., Amsterdam, trends in analytical chemistry.*, vol. 13, 9 : 361-372

2. Barcelo, D. (1991) Occurrence, Handling and Chromatographic Determination of Pesticides in the Aquatic Environment. *Analyst.*, 116 : 681-689

3. Barcelo, D. (1993) Environmental Protection Agency and other methods for the determination of priority pesticides and their transformation products in water. *Elsevier Science Publishers B.V., Amsterdam. Journal of Chromatography.*, 643 : 117-143

4. UNEP/FAO/IAEA/IOC: Sampling of selected marine organisms and sample preparation for the analysis of chlorinated hydrocarbons. *Reference Methods For Marine Pollution Studies.*, No. 12 Rev.2., UNEP 1991.

5. UNEP/IOC/IAEA : Determination of DDTs and PCBs by capillary gas chromatography and electron capture detection. *Reference Methods For Marine Pollution Studies.*, No. 40 Rev. 1., UNEP 1986.