CHAPTER III

EXPERIMENTAL

3.1 Materials

3.1.1 Chemical reagents All chemicals are of analytical reagent grade.

A standard methylmercury (II) chloride (CH_3HgCl) solution containing 0.433 mgHg cm^{-3} was prepared by dissolving 13.550 mg of methylmercury (II) chloride in 25 cm^3 of benzene. A second stock solution containing 17.32 μgHg cm^{-3} was prepared by diluting 1.0 cm^3 of this solution to 25 cm^3 with benzene. These solutions were prepared and used for four to six months. Standard solutions with lower concentrations were prepared by appropriate dilution of the second stock solution immediately before use.

Spike solution of methylmercury (II) chloride in the concentration of 0.447 mgHg cm^{-3} was prepared by dissolving 5.60 mg methylmercury (II) chloride in 100 cm^3 of absolute ethanol. A stock solution containing 0.45 μgHg cm^{-3} was prepared by diluting 1.0 cm^3 of this solution to 100 cm^3 with absolute ethanol. These solution were prepared and used for four to six months. The stock solution containing 0.45 μgHg cm^{-3} was used for the investigation of optimum digestion time and temperature including the recovery yield of the chemical separation. Benzene

was avoided in order to inchease the solubility of methylmercury (II) chloride in aqueous solution.

A 1% cysteine-sodium acetate solution was prepared by dissolving 1.0 g cysteine hydrochloride and 1.0 g sodium acetate trihydrate in 100 cm^3 of water.

Oxygen-free nitrogen was used as the carrier gas.

3.1.2 Sample preparation Some fish samples were purchased from the market and some were supplied by the Marine Fishery Division, Department of Fishery as shown in Figure 3.1. Four kinds of marine fish stated in Table 3.1 were investigated. A total of 16 samples, 4 samples for each kind, were analyzed. The samples were skinned. and then the edible tissue as shown in ` dark shade in Figure 3.2 was collected and packed in plastic bags. The samples were stored at $-5^{O}C$ in a refrigerator until they were analysed, appropriately five months later. The humidity of the samples was determined by drying a portion of the samples at $110^{O}C$ until a constant weight was obtained. The results are given in Table 3.2
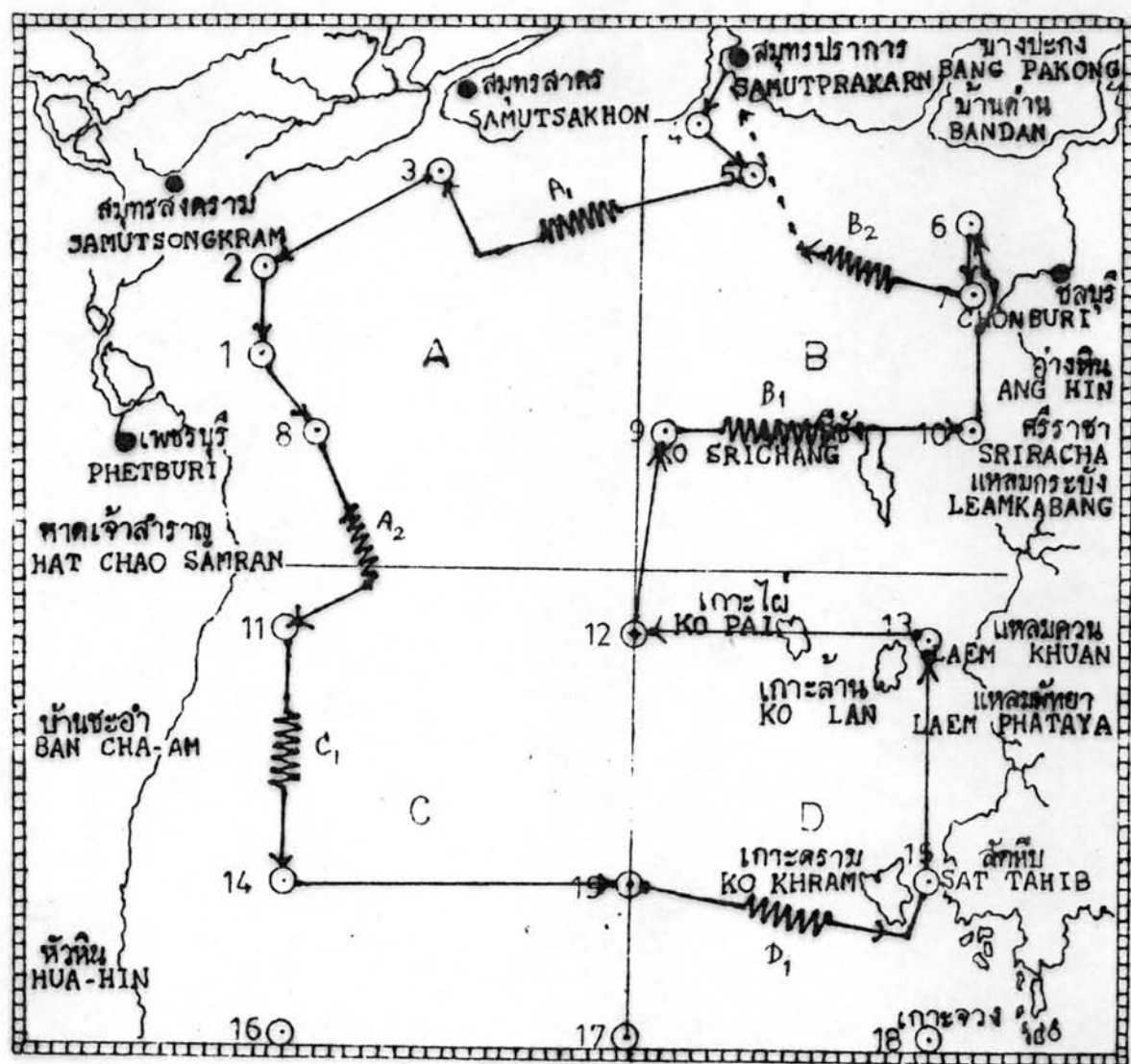
Scale 1 : 480,000

Figure 3.1 The area for fish sampling in Gulf of Thailand

(upper part)

⊙ – Oceanographic station
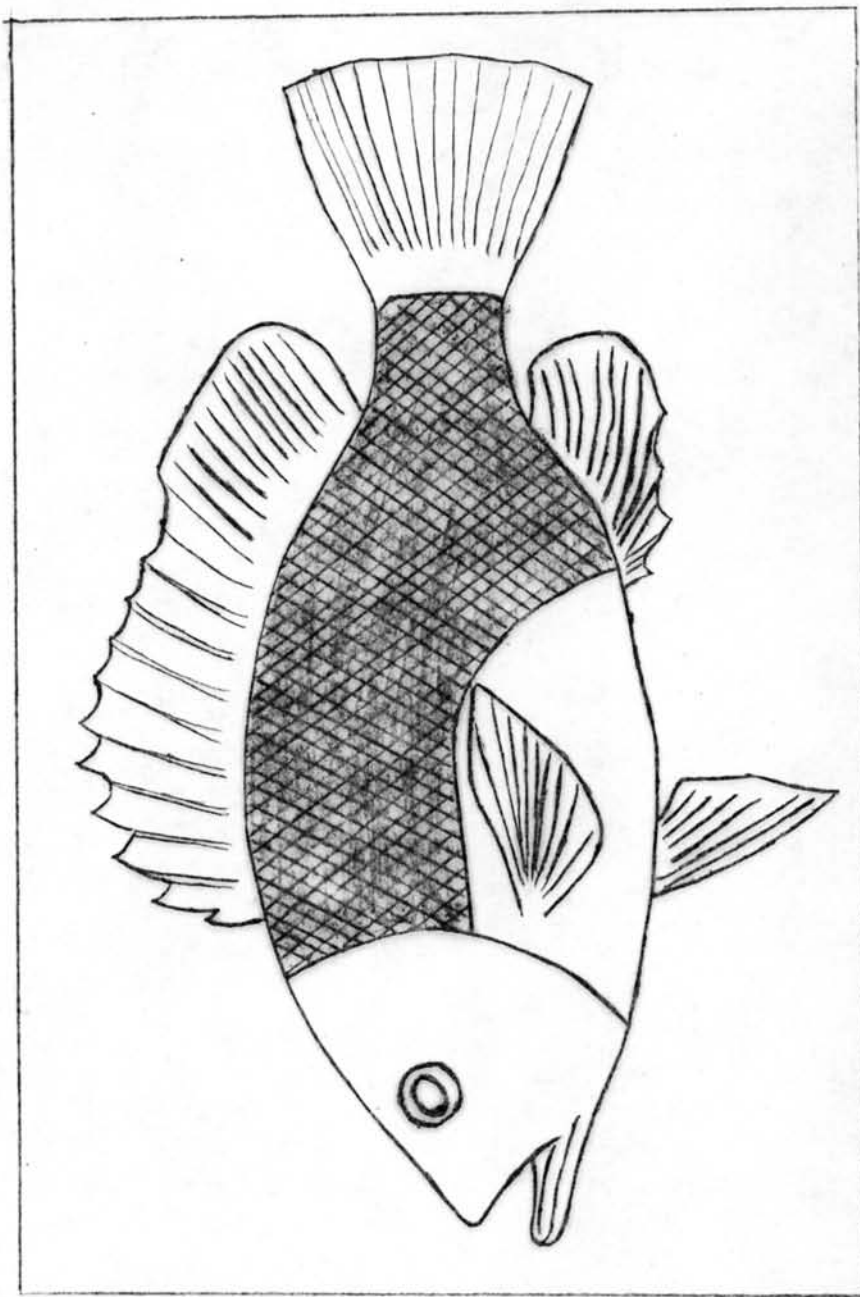
∿∿∿ – Ottertrawl station

Figure 3.2 The tissue in dark shade area was collected for analysis

Table 3.1 List of marine fish subjected to investigation

Local name	English name	Scientific name
ปลาสีกุน	Scad	Caranx spp.
ปลาทรายแดง	Threadfin	Nemipteus spp.
ปลาน้ำดอกไม้	Barracuda	Sphyraena spp.
ปลาดาบเงิน	Hairtail	Trichiurus spp.

Table 3.2 Humidity content of the fish samples

Sample	wet weight (g)	dry weight (g)	humidity (%)	average humidity (%)
Scad	5.6425	4.1528	26.40	
	6.1830	4.5338	26.67	26.57 $\pm$ 0.14
	6.0730	4.4557	26.63	
Barracuda	6.1721	4.6122	25.27	
	7.9287	5.8858	25.76	
	5.5598	4.2395	23.75	24.62 $\pm$ 1.05
	6.6792	5.0956	23.71	
Hairtail	7.3847	5.5423	24.95	
	7.3486	5.6694	24.89	
	5.1572	3.9469	23.47	24.24 $\pm$ 0.80
	5.4655	4.1756	23.60	

Table 3.2 (continue) Humidity content of the fish sample.

Sample	wet weight (g)	dry weight (g)	humidity (%)	average humidity (%)
Threadfin	4.9006	3.6217	26.09	25.98 $\pm$ 0.20
	5.0537	3.7555	25.69	
	6.2314	4.6030	26.13	
	4.4306	3.2780	26.01	

3.2 Gas chromatographic instrumentation

A Perkin-Elmer gas chromatograph, model F33, with model 56 recorder was used. The schematic diagram of the instrument is shown in Figure 3.3. The injection port, the detector and the column are placed inside the oven. The column is a spiral metal tube with 3.2 mm outside diameter and 2-meter of length. The packing material is 4% w/w polyethyleneglycol succinate (PEGS) on 100 - 200 mesh diatomite "C" AW. The maximum tolerable temperature for this packing material is 190°C. The temperature of the column colud be adjusted between 0°C and 185°C with an accuracy of 2% for dial reading above 100°C. The temperature of the injection port and the detector could be adjusted between 150°C and 300°C with an accuracy of $\pm$ 5°C.
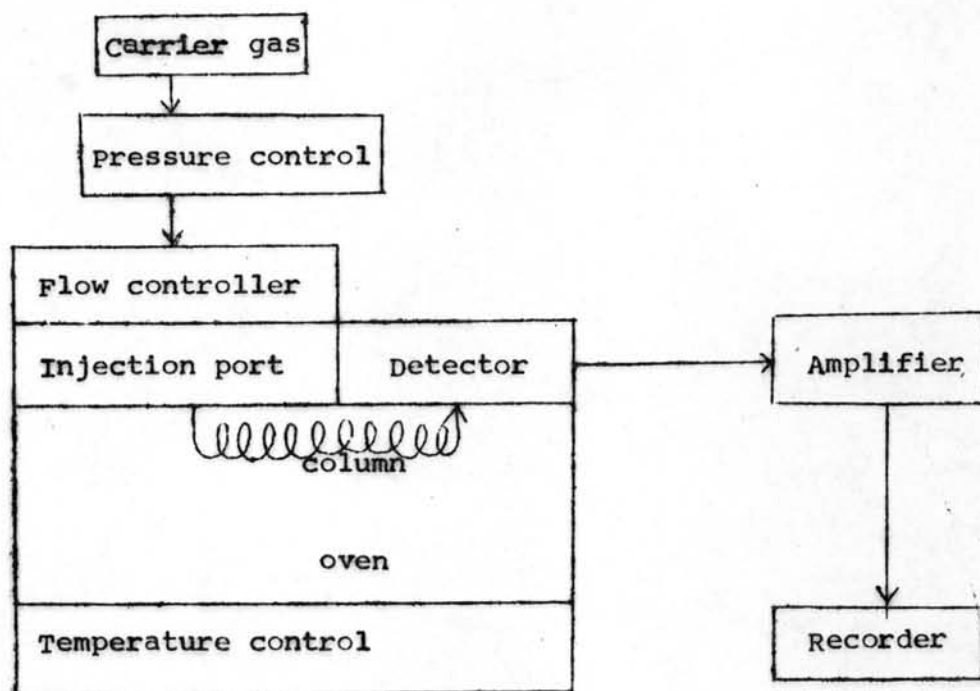
Figure 3.3   Schematic diagram of the model F33 Perkin-Elmer

gas chromatograph with recorder

3.3 Procedure

In order to obtain the optimal conditions for the quan-
titative analysis of methylmercury (II), preliminary experiments
on the effect of temperature in the column, the injection port
and the detector, as well as the flow rate of carrier gas on the
retention time was investigated. As a consequence, the optimum
time and temperature for sample digestion were determined.

3.3.1 Effect of column temperature on retention time

A 1.0 μl of 1.0 μgHg cm^{-3} standard methylmercury (II)
chloride solution was injected into the column which temperature
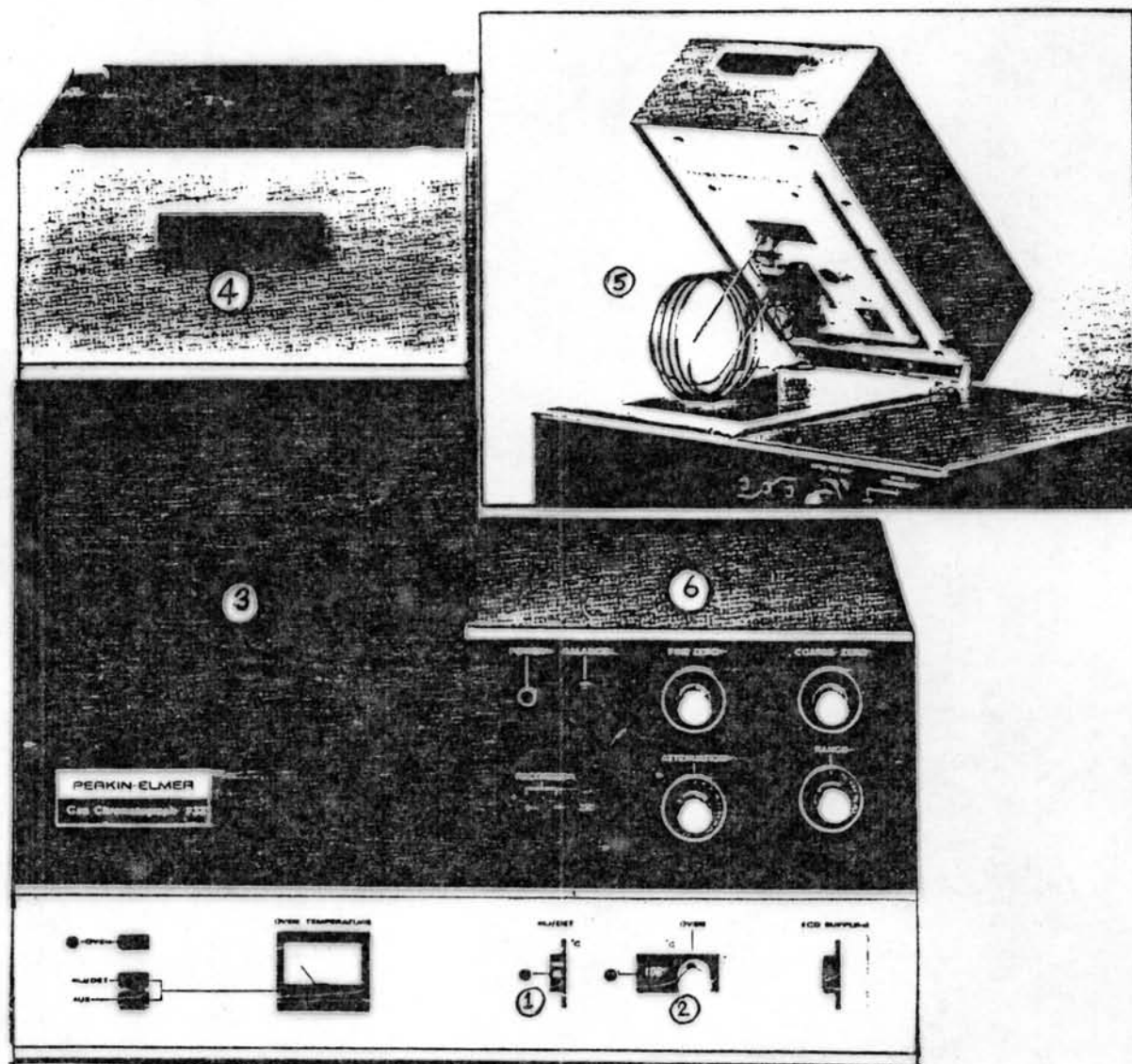was varied between 130°C and 185°C. The temparature of the

Figure 3.4   The Perkin-Elmer model F33 gas chromatograph

(1) — Temperature control of injection port and detector

(2) — Temperature control of oven

(3) — Oven

(4) — Oven lid

(5) — Column

(6) — Amplifier

injection port and the detector was kept constant at 150°C. The flow rate of the carrier gas was 50 $cm^3 min^{-1}$. The retention time of the methylmercury (II) chloride peak was measured.

3.3.2 Effect of temperature of injection port and detector on retention time

A 1.0 μl of 1.0 μgHg cm^{-3} standard methylmercury (II) chloride solution was injected into the column which temperature was kept constant at 135°C. The temperature in the injection port and the detector was varied between 150°C and 300°C. The flow rate of the carrier gas was 50 $cm^3 min^{-1}$. The retention time of the methylmercury (II) chloride peak was measured.

3.3.3 Effect of carrier gas-flow rate on the retention time

A 1.0 μl of 1.0 μgHg cm^{-3} standard methylmercury (II) chloride solution was injected into the column which temperature was kept constant at 135°C. The temperature of the injection port and the detector was kept constant at 150°C. The flow rate of the carrier gas was varied between 10 $cm^3 min^{-1}$ and 120 $cm^3 min^{-1}$. the retention time of the methylmercury (II) chloride peak was measured.

3.3.4 Determination of the minimum detectable quantity of methylmercury (II) chloride

Since the electrical output of a detector can be increased

to almost any desired value by electronic amplification, unfortunately the electrical noise inherent in the detector and electronics is also amplified. The noise may be high enough to hide the response of the detector. The minimum detectable quantity is defined as the amount of component which gives a detector response equal to twice the average noise level. The minimum detectable quantity ofmethylmercury (II) chloride under optimum operation conditions of the instrument was determined by injection of 2.0 - 5.0 μl portion of the standard methylmercury (II) chloride solution, with a concentration varied between 0.026 μgHg cm^{-3} and 0.26 μgHg cm^{-3}, into the column. The peak height was measured. The temperature of the column was set at 140°C, and the temperature of the injection port and the detector was kept constant at 150°C. The flow rate of the carrier gas was 70 cm^{3}min^{-1}.

3.3.5 Determination ofoptimum time for sample digestion

Methylmercury (II) in fish samples which is normally bound to tissue- proteins was first liberated by alkaline digestion. Methylmercury (II) hydroxide (CH$_3$HgOH) is formed through the digestion process (13, 14). The optimum time for complete digestion was observed.

The wet fish tissue was minced with motar, two grams was weighed into a beaker. A 1.0 cm^{3} of the 0.45 μgHg cm^{-3} standard methylmercury (II) chloride in ethanol solution was added as spike. After making a weight ratio of 1 : 1 between sodium

hydroxide and fish (13) by the addition of 5 cm^3 of 8 mol/1 sodium hydroxide solution, the beaker was covered with a watch glass and placed on a steam bath. The digestion time was varied between 15 and 60 minutes. After digestion, the clear solution was acidified by the addition of 10 cm^3 7 mol/1 HCl to convert methylmercury (II) hydroxide into methylmercury (II) chloride (CH_3HgCl) which could be extracted into benzene if the acidity of the aqueous phase is approximately 1 N(13, 15, 16). The beaker was cooled in an ice – bath during acidification in order to avoid vigorous reaction. The solution in the beaker was poured into a separating funnel which contained 20.0 cm^3 of benzene and 3 cm^3 of 1 mol/1 $CuCl_2$ (13,16). Copper (II) ions were added to mask the sulfhydryl group (RSH) so that no methylmercury (II) sulfhydryl-complex could form. The separating funnel was shaken vigorously for 5 minutes, and the aqueous layer was then re-extracted with 20.0 cm^3 of benzene to ensure quantitative extraction of methylmercury (II). The benzene extracts were combined and washed with a small portion of water. Methylmercury (II) was stripped from benzene twice by shaking with 3.0 and 2.0 cm^3 portions of 1% cysteine acetate solution (9, 16) for 5 minutes respectively, The cysteine layers (5 cm^3 total) were combined and acidified to approximate 1'N with 5 cm^3 of 7 mol/1 HCl and re-extracted twice with 3.0 and 2.0 cm^3 portions of benzene respectively. The benzene layers were combined and dried with anhydrous sodium sulfate. A 5.0 μl of the combined

benzene was injected into the column which temperature was kept at 140°C. The temperature of the injection port and the detector was 150°C. The flow rate of the carrier gas was $70\ cm^3 min^{-1}$. From the peak height, the content of methylmercury (II) chloride in the benzene extracts could be read directly from a standard calibration curve which was previously obtained by plotting the peak height against the known concentration of the standard methylmercury (II) chloride solutions. Least square analysis was applied so that a statistically reliable line could be obtained. (see Appendix) A typical calibration curve is shown in Figure 3.5. The recovery yield was evaluated by the following equation :

$$\text{recovery yield} = \frac{\text{amount of } CH_3HgCl \text{ found in sample}}{\text{amount of } CH_3HgCl \text{ added to sample}} \times 100 \quad ..(6)$$

The amount of CH_3HgCl found in sample was calculated by the following manner :

$$CH_3HgCl \text{ in } \mu g = \frac{A}{B} \times C \quad \ldots\ldots\ldots\ldots(7)$$

where   A is μg of CH_3HgCl from the calibration curve

B is the injected volume of benzene extract to the column

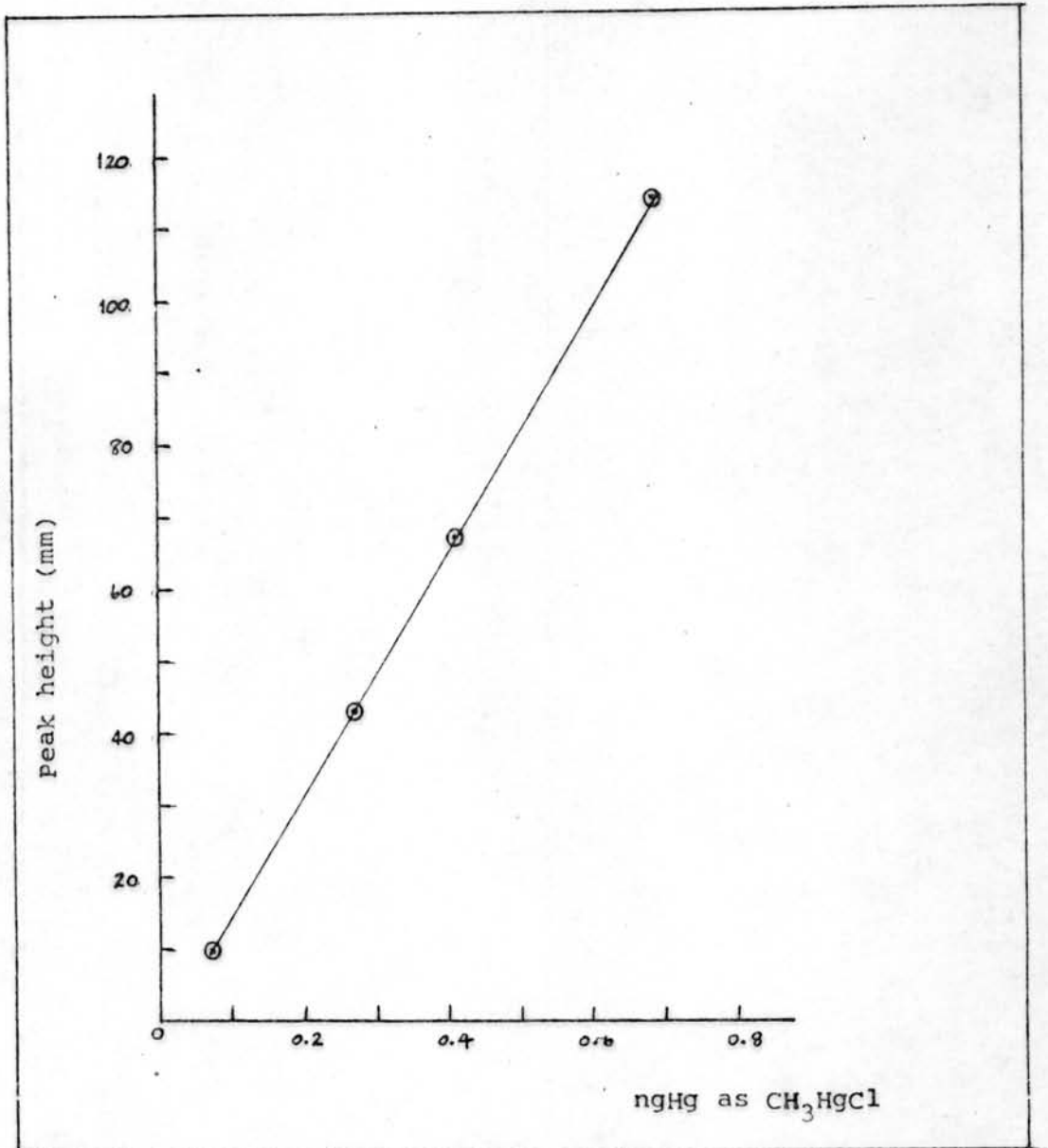C is the total volume of benzene extract.

Figure 3.5 Typical calibration curve of standard methylmercury

(II) chloride solution

3.3.6 Determination of optimum temperature for sample digestion

The optimum temperature for complete digestion was determined. The procedure described under clause 3.3.5 was followed with a constant digestion time of 30 minutes. The temperature of the water bath was varied through a thermoregurator between $30 \pm 2^{\circ}C$ and $100 \pm 2^{\circ}C$. Since the thermoregurator could not control temperature higher than $100^{\circ}C$, the maximum temperature for digestion was limited to $100^{\circ}C$. From the content of methylmercury (II) chloride recovered from the sample after the whole separation procedure was operated, the recovery yield could be evaluated in the same manner as described in clause 3.3.5.

3.3.7 Determination of the recovery of methylmercury (II) chloride added to fish

The effectiveness of the developed method can be tested by evaluating the extent of recovery of the added quanlity. This technique may reveal errors arising from the method of treating the sample or from the presence of other elements or compounds.

All recovery yields obtained under clause 3.3.5 and 3.3.6 were found to be under 70%. The loss was considered to be caused by incomplete of phase separation after extraction. In the following experiments, only a portion of the previous extraction step was used for the following extraction step.

Two grams of minced fish sample was weighed into a beaker followed by the addition of 0.90 μgHg. Five mililitres of 8 mol/l NaOH was added. The beaker was covered with a watch glass and placed on a steam bath for 30 minutes. After the digestion had been completed, 10 cm^3 7 mol/l HCl was added to acidify the clear digestive solution. The beaker was cooled in an ice-bath during acidification in order to avoid vigorous reaction. The methylmercury (II) chloride was extracted twice with 2 x 20.0 cm^3 of benzene in the presence of 3 cm^3 of 1 mol/l CuCl$_2$ for 5 minutes. The benzene extracts, a total of approximately 40 cm^3, were combined and washed with a small portion of water. An exact portion of 30.0 cm^3 of the benzene extract was pipetted into a separation funnel and methylmercury (II) in this portion was stripped twice by shaking with 2 x 5.0 cm^3 of 1% cysteine acetate solution for 5 minutes. The cysteine layers, a total of about 10 cm^3, were combined. Eight mililitres of the combined cysteine was pipetted and aciditied with 5.0 cm^3 of 7 mol/l HCl. The aqueous solution was extracted twice with 2 x 5.0 cm^3 of benzene for 5 minutes. The benzene layer were combined and dried with anhydrous sodium sulfate. A 5.0 μl of the benzene extract was injected into the column which temperature was kept at 140°C. The temperature of the injection port and the detector was kept constant at 150°C. The flow rate of carrier gas was 70 $cm^3 min^{-1}$. From the peak height, the content of methylmercury (II) chloride in the benzene extract was read

directly from a standard calibration curve. The methylmercury (II) chloride content was evaluated in the following manner :

$$CH_3HgCl \text{ in } \mu g = \frac{a}{b} \times \frac{c}{d} \times \frac{e}{f} \times g \qquad \cdots\cdots\cdots\cdots (8)$$

where a = the total volume of the benzene extract

b = the portion of the benzene extract for stripping

c = the total volume of the cysteine extract

d = the portion of the cysteine extract for analysis

e = the total volume of the final benzene extract

f = the injected volume of the benzene extract to the GLC column

g = the μg of methylmercury (II) chloride from the calibration curve

3.3.8 Quantitative analysis of methylmercury (II) in fish samples

A weight of 8 to 10 g of fish sample was used for each digestion. The separation procedure described under clause 3.3.7 was followed. The procedure is presented in the form of flow diagram in Figure 3.6. The content of methylmercury (II) was determined by the standard addition method. Standard solutions of methylmercury (II) chloride with a concentration of 0.04, 0.09 and 0.13 $\mu gHg \text{ cm}^{-3}$ were used as spike. Two mililitres each of the final combined benzene extract (a total of 10 cm^3) were pipetted into four separate volumetric flasks after which 1.00 cm^3 of the spike solute were added
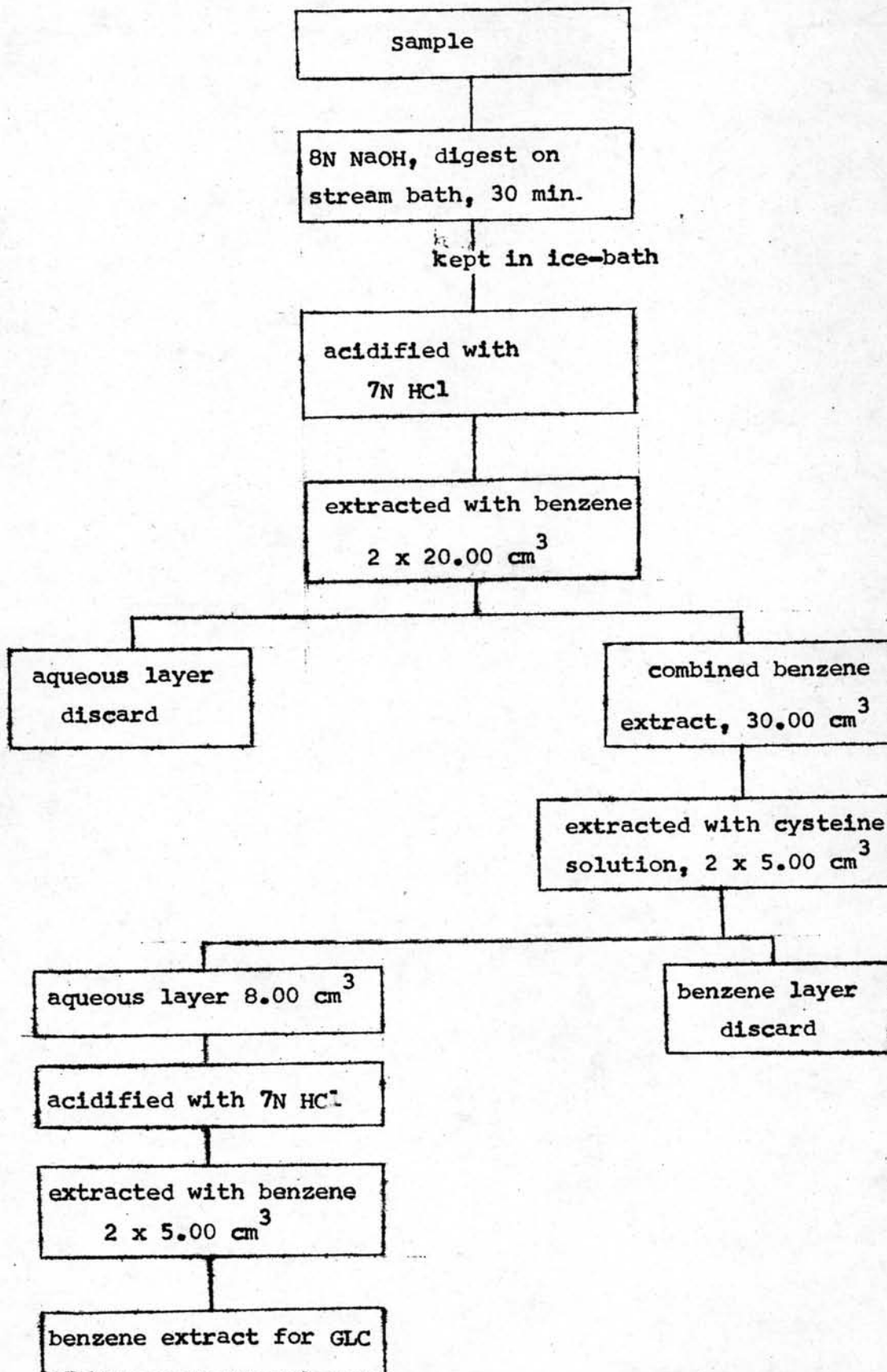
Figure 3.6 The flow diagram for the determination of methylmercury

separately into 3 flasks. One mililitre of benzene was pipetted into the fourth flask to make a final volume of 3.00 cm^3. A portion of 5.0 μl from each flask was injected into the column which temperature was kept at 140°C. The temperature of the injection port and the detector was kept at 150°C. From the standard addition curve (using least square analysis) in which the height of the methylmercury (II) chloride peak was plotted against the spiked concentration, the amount of methylmercury (II) in the sample could consequently be evaluated. A typical standard addition cureve was shown in Figure 3.7. The amount of methylmercury (II) in the sample was calculated by using the following equation :

$$CH_3HgCl \text{ in } \mu g = \frac{a}{b} \times \frac{c}{d} \times \frac{e}{f} \times \frac{g}{h} \times j \qquad \cdots\cdots\cdots(9)$$

where   a   =   the total volume of the benzene extract

         b   =   the portion of the benzene extract for stripping

         c   =   the total volume of the cysteine extract

         d   =   the portion of the cysteine extract for analysis

         e   =   the total volume of the final benzene extract

         f   =   the volume of the final benzene extract for standard addition

         g   =   the total volume of benzene solution after spiking the standard methylmercury (II) chloride

         h   =   the injected volume of the spiked benzene solution

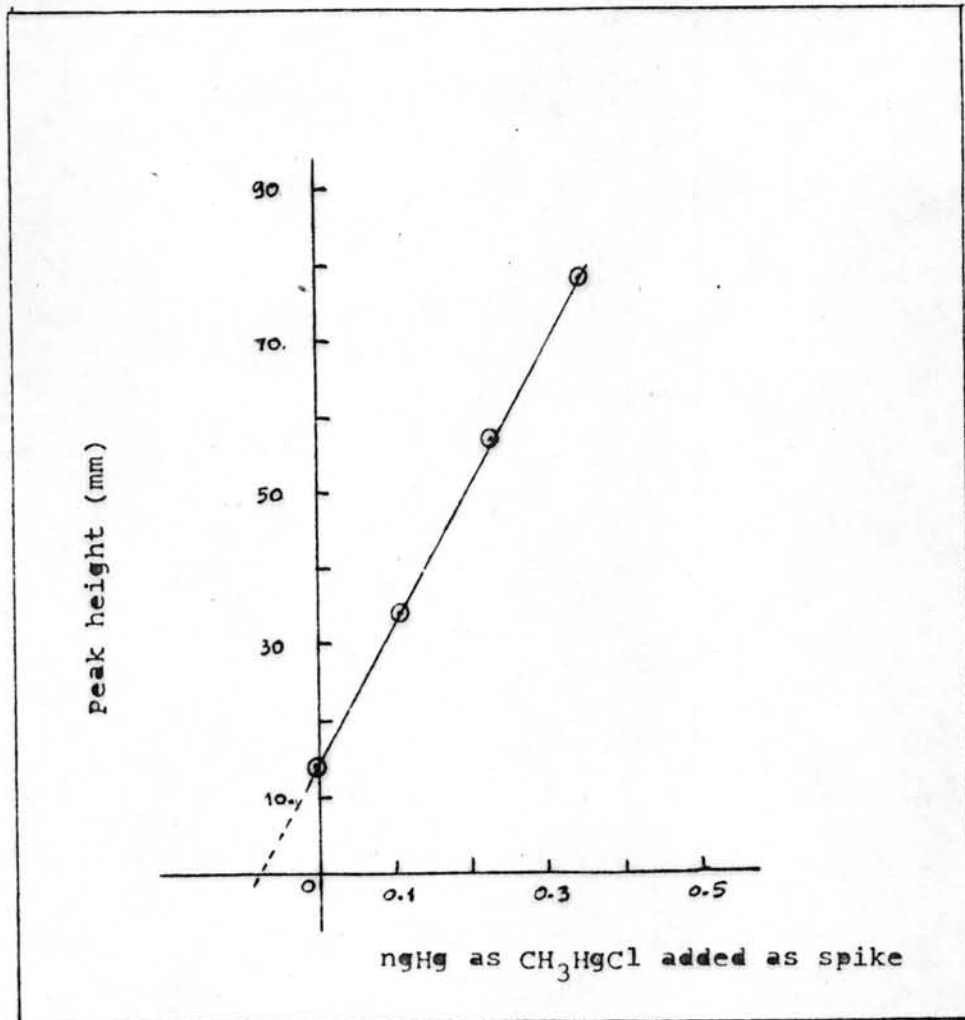   j   = μg of CH$_3$HgCl from the standard addition curve.

Figure 3.7   The Standard addition curve

The μg of methylmercury (II) chloride from the standard addition curve is obtained by dividing the response at zero concentration by the slope of the line.