



## CHAPTER III

### MATERIALS AND METHODS

#### 3.1 Study Area and Location

Samples had been collected at the Map Ta Phut sea coast, Map Ta Phut Sub-district, Muang District, Rayong Province. The coastal extends from Ban Ta Kuan, 5km. eastwards to Ban Pha-la, 5km. westwards of Tambon Map Ta Phut, at latitude of  $12^{\circ} 37' N$  to  $12^{\circ} 40' N$  and longitude  $101^{\circ} 02'$  to  $102^{\circ} 10' E$ . Two of ship-breaking-factories namely Thai International Steel Co. Ltd (TIS). and Thai Hua Lee Co. Ltd. locate in this study area of Ban Nong Faeb. Other build ups in this area are the Map Ta Phut Industrial Estate and the Map Ta Phut Deep Sea Port. The location of the sampling stations and two ship-breaking industries are shown in Figure 3-1.

The schedule for scraping carried out in this study is shown in Table 3-1. The frequency of sampling was designed in accordance with the scraping activity as follows:-

1. October 1989, the period after the last vessel was scraped, seawater, sediment, beach tar and bivalve, Donax spp., samples were collected.

2. June - July 1989, a scraping period, the bulk 32,800 tons of Zarra tanker bought from Ajman was scraped. During the last week of June, an oil leaked from the scraping site was announced by the

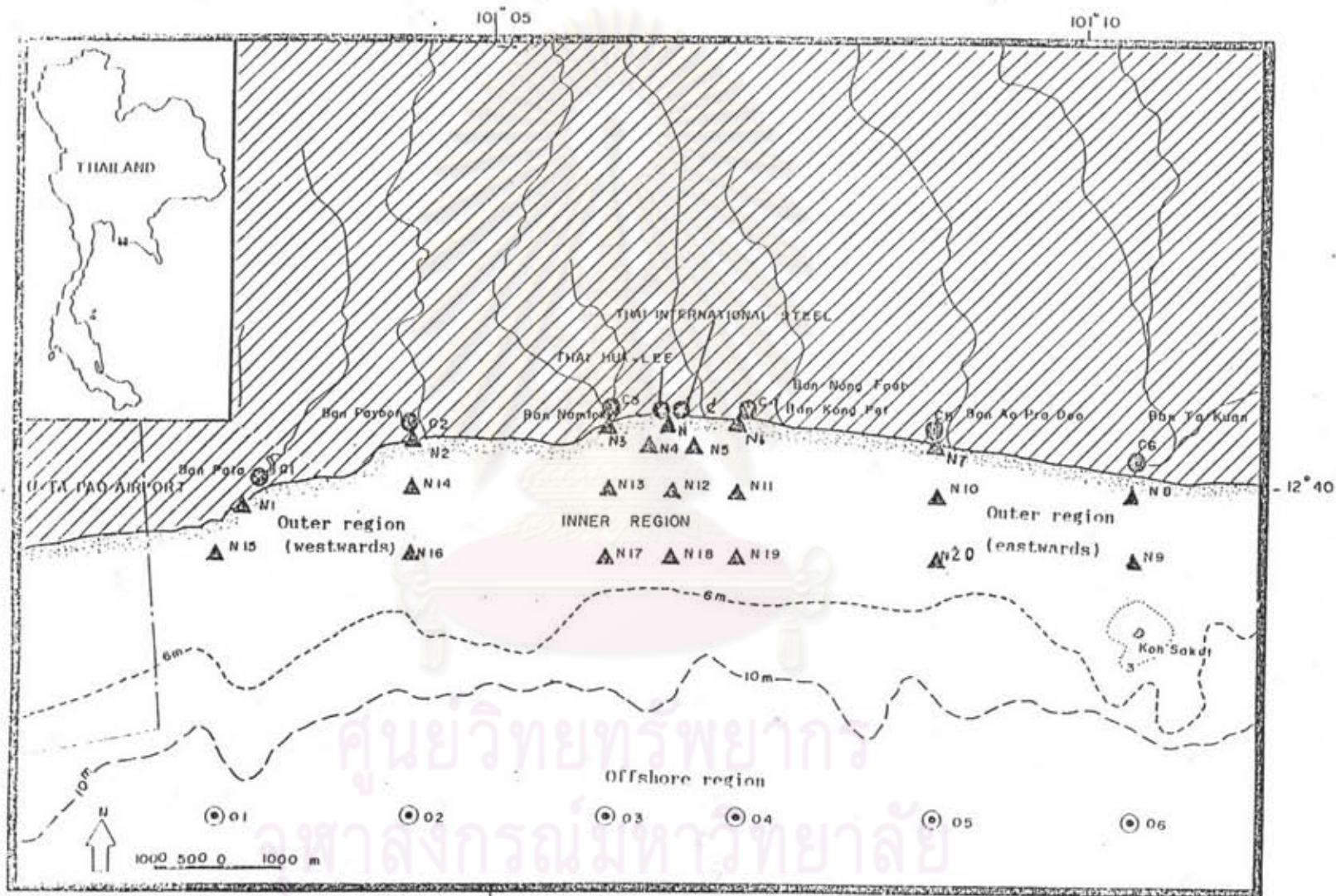


Figure 3-1 Sampling stations set up in this study area around the ship-breaking factories

Inner region	= Stations N, N3, N4, N5, N6, N11, N12, N13, N17, N18 and N19
Outer region	= Stations N1, N2, N14, N15, N16 (eastwards) and N7, N8, N9, N10 and N20 (westwards of the scraping factories).
Offshore region	= Stations O1, O2, O3, O4, O5, and station O6.
Canal	= Stations C1, C2, C3, C4, C5 and C6.

manager of TIS. Sampling at that time was not finished due to intense rain storm and water turbulence. After the second sampling attempt in the second week of July, the samples collected were completed. Seawater, sediment, beach tar and bivalve Donax spp. samples were collected. Twenty-five consecutive sampling hours were conducted on 21-22 June 1988 for current measurement.

3. November 1989, a breaking of 39,500 tons of BONI tanker bought from West Germany was under way. Water samples and beach tars were collected. Current measurement was also conducted.

4. May 1990. No breaking activity was observed after the last BONI operation. Water samples and beach tars were collected.

Table 3-1 Schedule for sample collection in this study.

DURATION	SAMPLING COLLECTION	REMARKS
October 1988	- Water samples - Sediment samples - Beach tars - Bivalve <u>Donax</u> sp.	- before scraping /or 4 months after breaking of the last vessel - in South West monsoon of rainy season (May-October)
June-July 1989	- Water samples - Sediment samples - Beach tars - Bivalve <u>Donax</u> spp. - Current measurement (21-22 June, 1989)	- scraping period (Zarra Tanker from Ajman) - in South West monsoon
November 1989	- Water samples - Beach tars - Current measurement (24-25 November, 1989)	- scraping period (Boni Tanker from West Germany) - in North East monsoon of dry season (Nov-Feb)
May 1990	- Water samples - Beach tars	- 5 months after the Boni Tanker scraping - in South West monsoon

For the study of the dissolved/dispersed petroleum hydrocarbons around the ship-breaking area, the sampling stations were divided into four groups.

1. Inner region : This group of stations covered an area within 2 kms. distance from the two factories on shore. The stations included are N, N3, N4, N5, N6, N11, N12, N13, N17, N18 and N19.

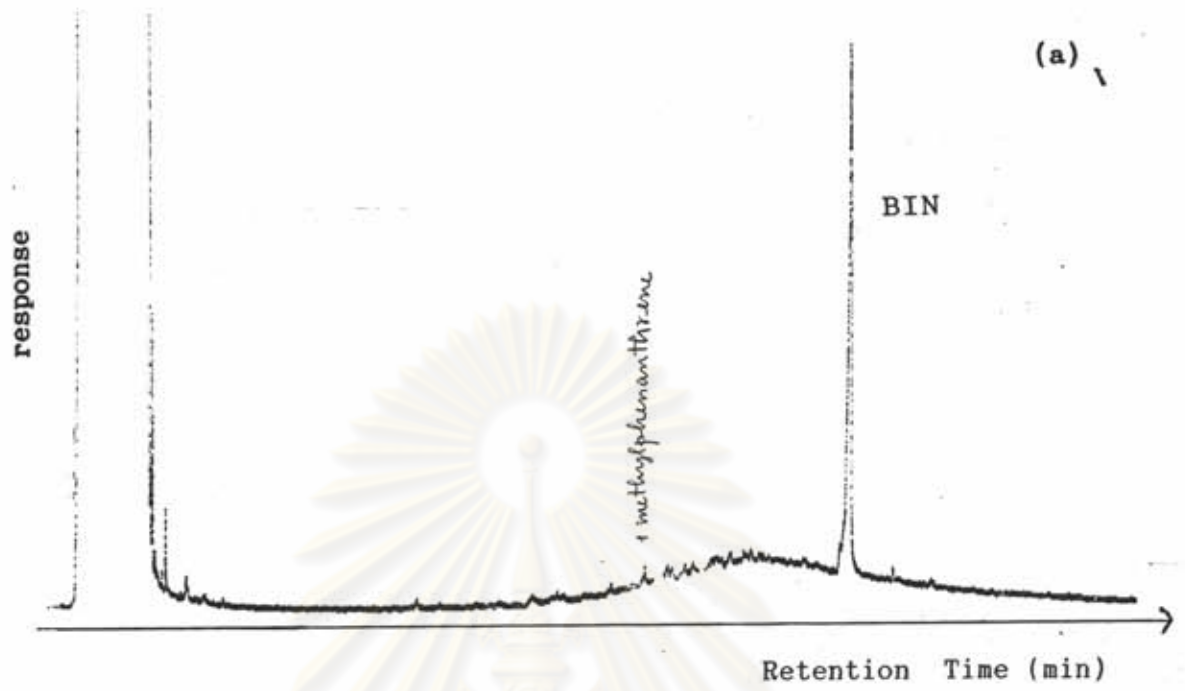
2. Outer region : Ten stations around the factories were assigned in this region, i.e. N1, N2, N14, N15, N16 (eastwards) and N7, N8, N9, N10 and N20 (westwards of the scraping factories).

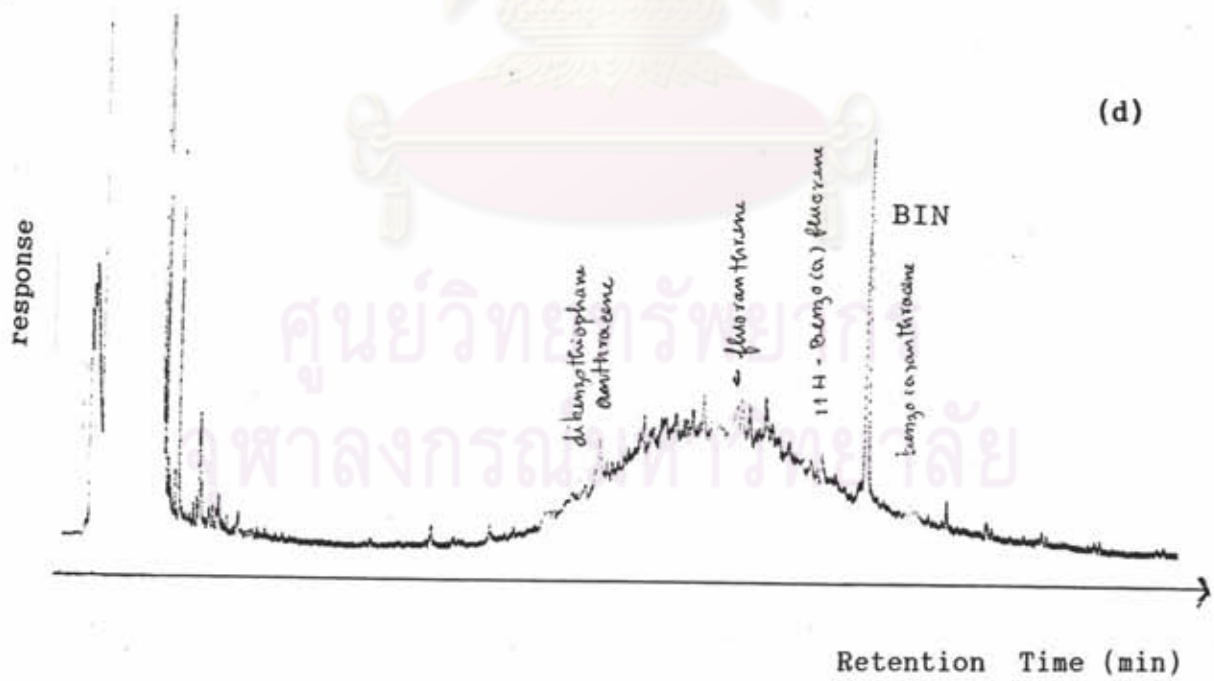
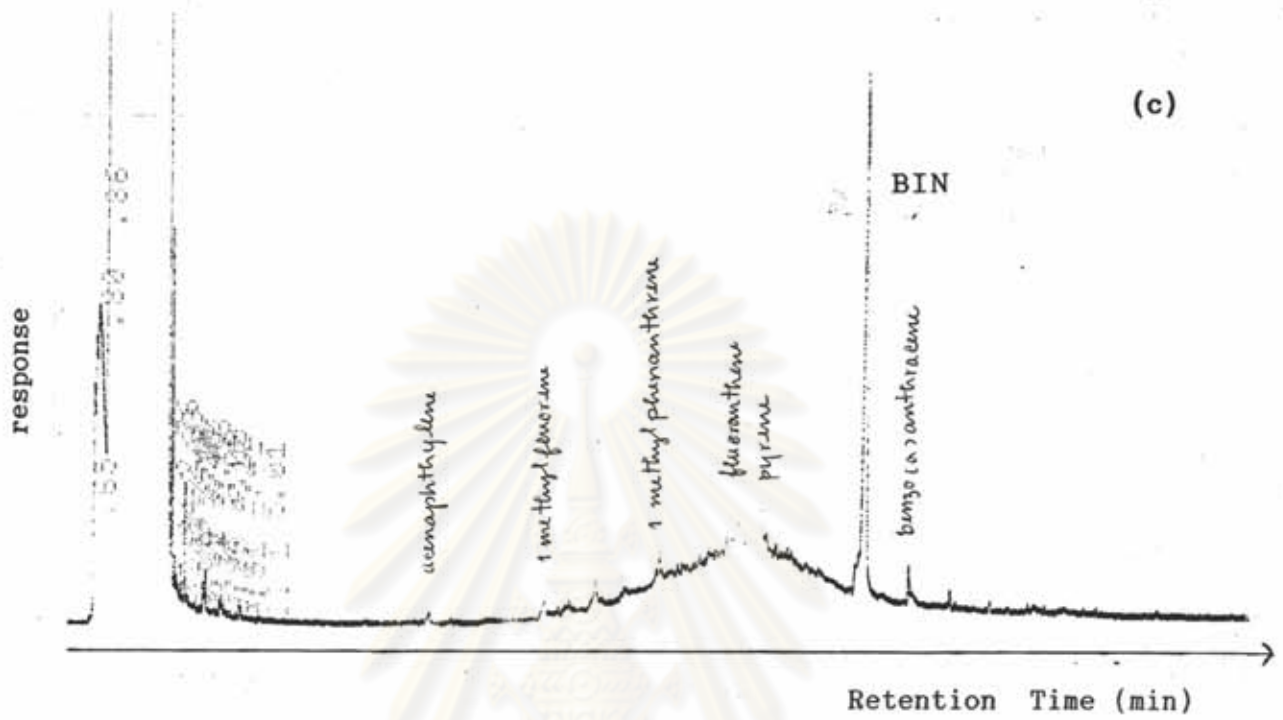
3. Offshore region : This group included 6 stations located at about 6-7 kms. from the coastline of the study area, namely O1, O2, O3, O4, O5, and station O6.

4. Canal : There are 7 small creeks draining to the coastal water. A sampling station was set up in each creek. The stations were C1, C2, C3, C4, C5 and C6. From the field observations, the details characteristic of each canals are as follows:-

C1, C2, C3, C4, and C6 are stations for natural small canals. Some time the canal mouths were closed with transported sand.

C5 is a station for natural small canal. During the first two sampling in October 1988 and June 1989, the canal was not contaminated by human activities. However, during the last two sampling periods (November 1989 and May 1990), the Map Ta Phut Industrial Estate started to operate and artificial canal was





constructed to drain the treated effluent from factories. Therefore, station C5 was later set up in this artificial canal.

### 3.2 Sampling Methods

Different types of sample were collected during this study. Methods of sampling for each sample are described below.

#### 3.2.1 Seawater Samples

In order to study the distribution characteristics of petroleum hydrocarbons, seawater samples were collected. Research vessels provided by the Eastern Marine Fisheries Development Center, Department of Fisheries were used during sample collection. Two different collection methods were utilized as follows:-

**3.2.1.1 Drop-bottle technique** This technique is recommended by IOC for a monitoring of oil and dissolved/dispersed petroleum hydrocarbons (DDPH) in seawater (IOC, 1984). Sampling was done by using a 4-l amber glass chemical reagent bottle suspended by a nylon rope in the stainless steel wire cage weighted by a lead bottom. The set up apparatus was thrown as far as possible from the vessel and immediately sank to the depth of 1 m from the water surface where it was filled up. A small buoy, attached to the cage on a 1 - m long line, prevented it from sinking and assured that collected water was drawn exactly 1 m below the surface. After filling, 200 ml aliquot was removed from each sample bottle and 50 ml of redistilled hexane

was added. The samples were shaken for five minutes on site, and kept in a dark and cool place for laboratory analysis.

**3.2.1.2 Triangular Screen Technique :** An analysis of petroleum hydrocarbons at surface microlayer was carried out in this study. As we know, the surface microlayer of coastal water serves as an important habitat for many kinds of marine organisms, eggs and larvae of many commercially important fish and shellfish. The hydrophobic nature of petroleum and organic matter usually associate with floatable particles at the surface microlayer. The concentrations of petroleum hydrocarbons in the surface microlayer, however can determine the degree of aquatic surface microlayer pollution.

In this study the surface microlayer samples were collected and handled as recommended by IOC Procedure for sampling the sea-surface microlayer (IOC, Manuals and Guides No. 15). A 16-wires per inch (2.54 cm) triangular stainless steel which was connected by a lines through a bridles of a three-point attachment to the corner of the screen frame was used. The screen was horizontally lowered to contact the sea surface microlayer. The seawater was collected within the screen and later transferred to a 4-l amberglass bottle.

This procedure was repeated 30 times for each sampling. Then 50 ml of redistilled hexane was added. Each sample bottle was shaken for five minutes on site and stored in a dark and cool place for laboratory analysis.



This triangular screen technique was used for all sampling stations except the nearshore stations (N1, N2, N3, N, N6, N7, and N8). This technique is mostly appropriate for sampling from vessels. Therefore, the nearshore water samples were collected only by drop-bottle technique. The screen technique was carried out for all stations except stations N1, N2, N3, N, N6, N7, and N8.

For creek water samples, the 4-l amber glass bottle was filled at the surface of the running water during a low tide period. After filling to about 3.5 l of creek water, 50 ml of redistilled hexane was added. Each sample bottle was shaken for five minutes and stored for further analysis.

In order to compare the concentrations of petroleum hydrocarbons observed at the surface and at 1-m depth, an enrichment factor (CF) was computed. The enrichment factor is a ratio of the surface to 1-m depth concentrations. This factor indicates the trend of pollution intensity within the study area. The CF value shows a trend of oil pollution.

### 3.2.2 Sediments

Sediment samples were collected with a Van Veen grab and immediately placed in a wide mouth glass jars with Teflon lined caps. The samples were kept in an ice box until returned to the laboratory. They were then frozen and stored until analysis.

In June 1989, a core sample was also taken at Station C in order to understand the accumulation characteristics of petroleum hydrocarbons in sediment by depth. The sample was collected by a 50-cm plastic core sampler. Each subsample of 10-cm length was separated and placed in cleaned glass jars for further analysis.

### 3.2.3 Bivalves

Donax spp. were collected along the shoreline. The samples were kept in plastic bags and frozen until analysis in laboratory. Concentrations of petroleum hydrocarbons observed in the bivalve indicate the contamination of oil in living organisms.

### 3.2.4 Beach Tar

Due to the fact that tar ball represent another form of oil contamination /pollution, therefore beach tars were collected during low tide at seven stations along the coast (Ban Pha-la, Ban Payoon, Ban Nam Tok, Ban Nong Faeb (factory location), Ban Kong Pet, Ban Ao Pradoo and Ban Ta Kuan. At each station, tar balls presented in a three randomly chosen narrow strips of 3 meter width were collected, The narrow strips run across the beach from the backshore to the low-tide mark. Visible solid and semi-solid pieces of tar were picked up by small forceps and then transferred into a precleaned bottle. The beach width was also recorded.

### 3.3 Analytical Procedures

In order to minimize the possibility of contamination, only purest solvents available were used for the analysis (redistilled by a 2 meter long distillation tower). All glassware was pre-washed with detergent, and rinsed several times with tap water, distilled water and acetone respectively. Finally, all glassware excepted volumetric flask, were then oven dried at 180-200 °C and wrapped with clean aluminum foil before stored in a dust free container. Prior to use, the glassware was again rinsed with the solvent specially prepared for the analysis.

#### 3.3.1 Seawater Samples

The determination of petroleum hydrocarbons in water samples was conducted using Ultra Violet Fluorescence spectrometry (UVF).

In the laboratory, water samples were extracted three times with hexane. After concentrated to 5 ml by rotary evaporator, extracts were analyzed by fluorescence spectroscopy (Perkin - Elmer, Model 3000 Fluorescence Spectrometer, with 1-cm quartz cells). The conditions of the instrument used were set as follows:-

Slid width 10 nm.

Excitation wavelength 310 nm

Emission wavelength 360 nm (This wavelength was obtained by exciting the chrysene solvent at 310 nm and recording its maximum emission wavelength)

For the UVF analysis samples were dissolved in hexane, therefore, blank of hexane solvent had to be reported periodically during the concentration measurements. Concentrations of a series of chrysene standard were also determined, and the calibration curves were then established for each series of chrysene standard.

The concentrations of dissolved/dispersed petroleum hydrocarbons in seawater samples were calculated by the formula:

$$\mu\text{g petroleum hydrocarbons/l} = \frac{\begin{array}{l} \mu\text{g/ml read from calibration curve} \\ \times 5 \text{ ml final volume of extract} \end{array}}{\begin{array}{l} \text{(chrysene equivalent)} \\ \text{1 of water sample} \end{array}}$$

**Emission and Synchronous Scanning.** Further informations on the sample type were obtained using emission scanning as well as synchronous scanning spectro fluorometry. The emission scans of samples when compared to hexane indicate presence of other compounds extracted from samples. The synchronous scans of extracted samples show the numbers of aromatic hydrocarbons contained in the samples. For emission scanning, excitation wavelength was held constant at 310 nm with the emission wavelengths scanned from 250 to 500 nm. For synchronous scanning, the excitation and emission wave lengths were scanned simultaneously (excitation wavelength 230-550 nm, emission wavelength 253-573 nm). A summary of the analytical procedure is shown in Figure 3-2.

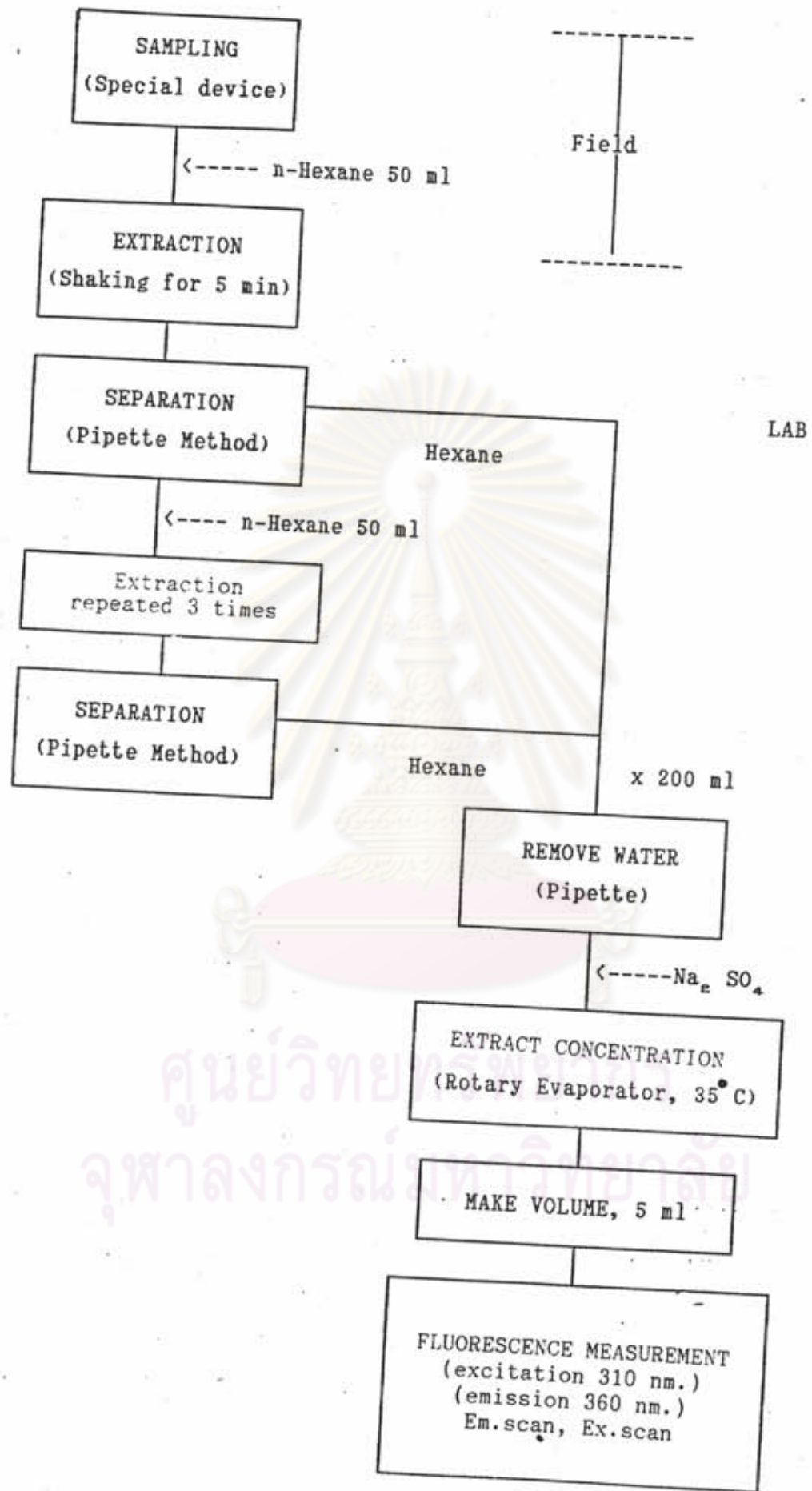


FIGURE 3-2 PROCEDURE FOR PETROLEUM HYDROCARBONS ANALYSIS IN WATER SAMPLES.

### 3.3.2 Sediment Samples

The determination of petroleum hydrocarbons in sediment samples were carried out by this stepwise procedure (adapted from IOC/UNESCO, 1982):-

#### 3.3.2.1 Sample Preparation and Extraction

Prior to analyses, the sediment samples were thawed, placed in precleaned stainless steel trays and then freeze-dried in a Virtis 10 MR TR freeze-drier for about 48 hours. The freeze-fried sediments were gently homogenized with a mortar and pestle and transferred to a clean teflon-capped jars before storing and kept in a freezer until extraction. Sediment samples of 130 to 150 g dry weight were spiked with 2 - methyl octadecane and 1,1-binaphthyl as internal standards and then soxhlet-extracted with 300 ml dicholoromethane for 24 hours. The extract were reduced to approximately 3 ml by rotary evaporation and transferred to a 15 ml centrifuge tube and evaporated gently under a nitrogen stream to 1 ml.

An internal standard is a compound added to the sample in order to correct the results for recovery through the steps of analytical procedure. It behaves in an identical manner to the compounds to be identified. In this study, 2-methyl octadecane and 1,1-binaphthyl are used as internal standards. 2-methyl octadecane will elude with the saturated hydrocarbons and 1,1-binaphthyl will elude in aromatic fraction process.

### 3.3.2.2 Adsorption Chromatography

An adsorption chromatography analysis was conducted to remove interfering lipids and to fractionate hydrocarbon extracts into aliphatic and aromatic fractions. Silica-gel (100 - 200 mesh) was soxhlet-extracted with hexane for 24 hours, then allowed to air dry. The pre-extracted silica-gel was activated by heating at 220 C for 16 hours before use. Activated copper was prepared by mixing with conc. hydrochloric acid in order to eliminate copper oxide. After that, it was rinsed with distilled water following by acetone and hexane and then kept in hexane solution.

A glass column (1 cm internal diameter x 30 cm length) with pre-extracted glass wool plugged at the narrow bottom was rinsed with hexane. Fifteen grams of activated silica-gel was slurred with 50 ml hexane, transferred to the column and the solvent was allowed to drain. The gel was settled to a height of 17.5 cm. Activated copper layer of 1.5 cm thick was then applied on top of the column to remove any sulfur presented in the samples. The column was washed with 50 ml of hexane with a solvent flow rate of about 2 ml per minute.

The extract was quantitatively transferred into the column with a pasture pipette and eluded with 15 ml hexane and followed by 35 ml 40/60 (V/V) dichloromethane/hexane. Two fractions were collected, one for each was an eluding solvent. The first 5 ml of the hexane fraction was discarded (Sunwanich, 1991). Before analyzed

by gas-liquid chromatography, the volume of both fractions were reduced further to 0.2 ml by blowing a gentle nitrogen stream over the surface of the solution. The first and the second fractions are aliphatic and aromatic hydrocarbon fractions, respectively.

#### 3.3.2.3 Determination of Petroleum Hydrocarbons in Sediment by Using UVF Technique

Extracted sediment samples from fraction 2 were determined the hydrocarbon concentration by using fluorescence spectroscopy at excitation and emission wavelengths of 310 and 360, respectively. The technique employed was the same as 3.3.1 for water samples.

#### 3.3.2.4 Determination of Petroleum Hydrocarbons in Sediment by Using Gas Chromatography with Flame Ionization Detector

The hexane fraction containing mostly aliphatic hydrocarbons and the dichloromethane/hexane fraction containing mostly aromatics, were analyzed by a gas liquid chromatography, Varian 3700 GC, capillary column with a flame ionization detector (FID). Residual solvent was removed under a nitrogen stream and both fractions were dissolved in 0.1 or 0.2 ml toluene prior to injection. Peak areas for each chromatogram were computed by CI4100 computing integrator according to their respective retention times and reported by an IBM PC/AT turbo computer. The Milton Roy integrator was used.



The conditions of the GC were as follows:-

Column : 25 m, 0.25 mm i.d. SE-54 bonded fused silica capillary column

Injector temperature : 240 °C

Detector temperature : 280 °C

Temperature program : from 70 ° to 280 ° C at 8 ° C/min

Carrier gas : hydrogen gas approximately 20 psi

Air : 300 ml/min

Make up gas : 30 ml/min

Splitter : 30 ml/min

Injection volume : 1-2 ul

Injection technique : splitless injection

For a compound identification, individual aliphatic hydrocarbons and isoprenoid pristane were identified by comparing with Kovats Index of the standard aliphatic hydrocarbon compounds. The Kovates Index (KI) of the each compound was calculated using the equation below : (Lee and Vassilaros, 1979)

$$KI = 100 \frac{T_{R(\text{substance})} - T_{R(Cz)}}{T_{R(Cz+1)} - T_{R(Cz)}} + 100$$

where KI is Kovats index,  $T_{R(\text{substance})}$  is the retention time of the substance for which the retention index is to be determined,  $T_{R(Cz)}$

and  $T_{R(Cz+1)}$  are retention times for the n-alkane standards which

bracket the substance of interest, and z is the number of carbon atoms in the n-alkan standard that eluded prior to the substances of interest. Since authentic standard of phytane are unavailable, the identification is base on phytane in the samples. The compound identification have been done by comparing the Kovate indices of samples to those of the standards.

The Aromatic Retention Indices (ARI) (Lee and Vassilaros, 1979) of a number of standards PAHs have been established using the same approach. Seven nonbiogenic aromatic hydrocarbons of naphthalenes, biphenyls, phenanthrenes, pyrenes, chrysenes, perylenes and benzo(ghi)perylene which are dominating aromatics in samples polluted by petroleum hydrocarbons were selected and the ARI of those PAHs are assigned to be 0, 100, 200, 300, 400, 500 and 600, respectively. The ARI of standard aromatics that eluded between those seven substances of interest have been calculated the same way as the Kovate Index for the aliphatic hydrocarbons. The compound identification have been done by comparing the ARI of samples to those of the standards.

Concentrations of identified compounds were calculated by the internal standard method as follows:-

$$\text{ng compound/g sample} = \frac{\text{peak area of identified compound}}{\frac{\text{peak area/ng of internal standard}}{\text{g of sediment sample}}}$$

Recovery studies were performed to determine the efficiency of the analytical method using 2-methyloctadecane for aliphatic fraction and 1,1-binaphthyl for aromatic fraction. By spiking known amount of the standards into the sediment samples and let them go through the analytical procedures, the concentrations of the standard were calculated. Percentage recovery can be calculated

using the formula:

$$\% \text{ recovery} = \frac{\text{Area / ng of internal std} \times \text{ul of final volume}}{\text{ul of injection volume}} \times 100$$

$$\text{Area / ng of external standard}$$

### 3.3.2.5 Determination of Petroleum Hydrocarbons by using Gas Chromatography/Mass Spectroscopy (GC/MS)

Some selected sediment extracts were analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) technique in order to confirm the indentification of aliphatic as well as aromatic compounds. The GC/MS analysis was performed at the Faculty of Agriculture, Kagawa University, Japan, with the help of Dr. Takeshi Katayama and Dr. Gullaya Wattayakorn. The conditions are as follows:

GC-MS : JEOL JMS-DX300 mass spectrometer equipped with a JEOL MS-GCG06 gas chromatograph.  
 Data system : a JEOL JMA-DA5000 data system  
 Ionizing voltage : 70 eV  
 Ionizing current : 300 uA  
 Mass range : 35-500  
 Scanning rate : 1 scan/sec

### 3.3.2.6 Oxidizable Organic Carbon Content in Sediment Samples

Organic carbon content in the sediment was analyzed by using Walkley-Black wet oxidation method (Jackson,1960). Potassium dichromate and sulfuric acid were added in the freeze-dried sediment to oxidize the organic carbon. The amount of oxidizable organic matter in the sediment was calculated after back titration with ferrous ammonium sulfate.

### 3.3.2.7. Grain Size Analysis of Sediment Samples

The hydrometer technique was used for grain size analysis according to the Manual of Physical and Chemical Analysis of Sediment (Agriculture Department, 1988). The organic matter in the sediment sample is eliminated by using hydrogen peroxide ( $H_2O_2$ ). 5% calgon was added into the sediment in the container, and sediment was transferred to a sedimentation cylinder. The hydrometer value of the density read at the designed time scale, in this step, have been used for % sand, silt and clay calculation.

A summary of the analytical procedure for the analysis of petroleum hydrocarbon in sediments is presented in Figure 3-3.

### 3.3.3 Bivalve Tissue

The analytical procedure for the determination of petroleum hydrocarbons in bivalve tissues was presented in Figure 3-5.

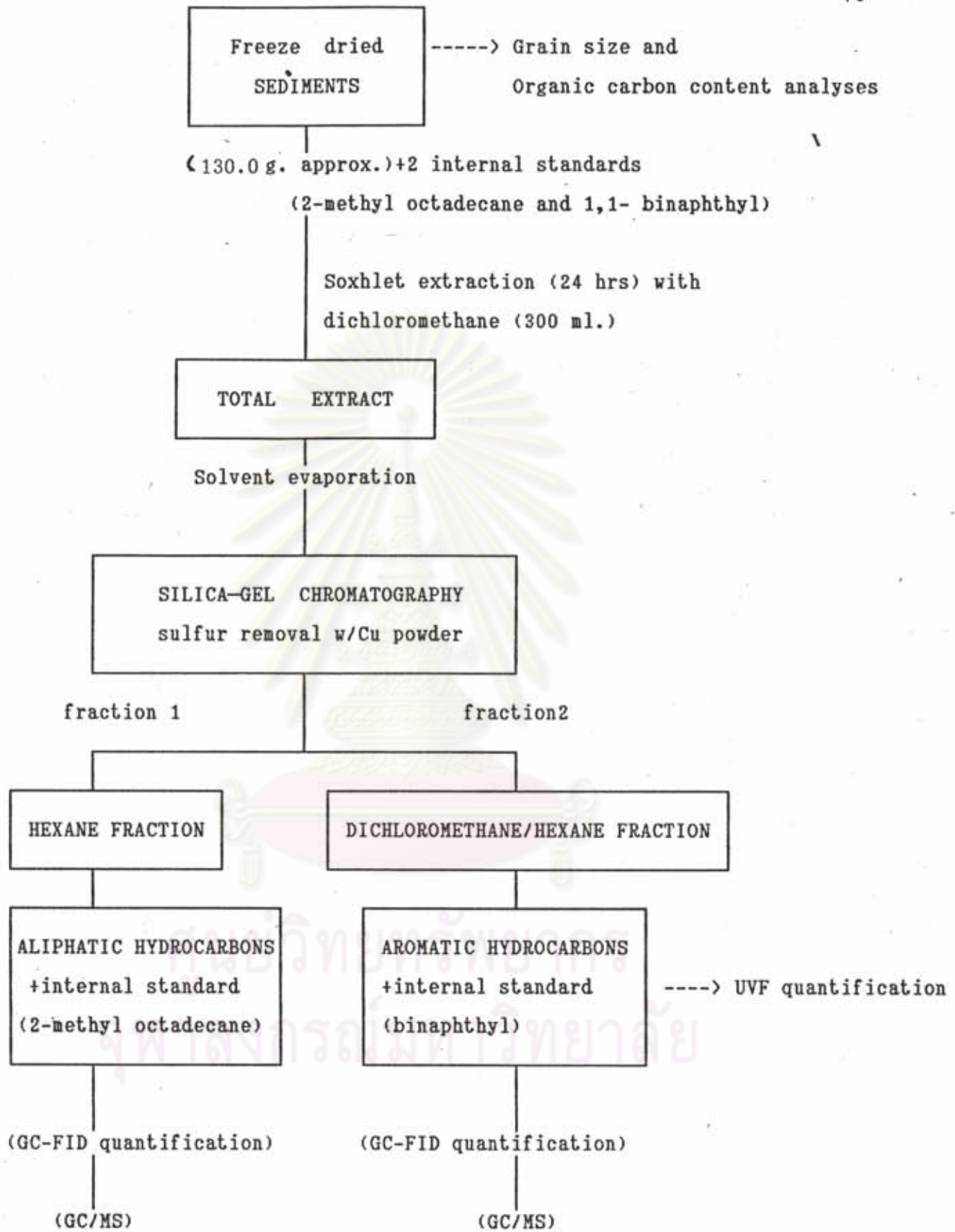


Figure 3-3 A summary of analytical procedure for sediment samples.

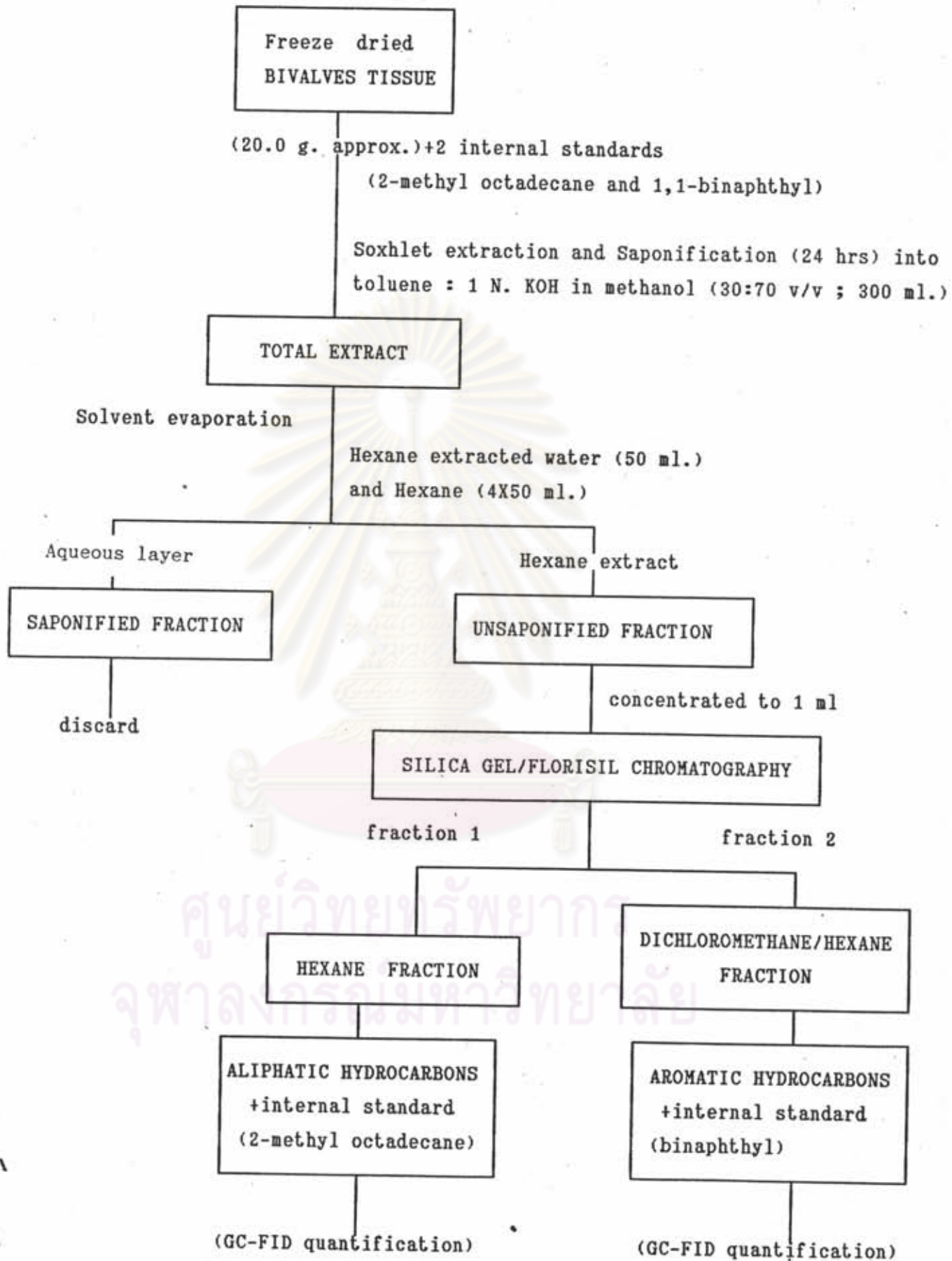


Figure 3-4 A summary of analytical procedure for bivalve tissue.

Bivalve tissues were carefully picked from their shells, homogenized by high speed homogenizer and finally freeze-dried for about 28 hours. Before extraction started, about 20 g. dry weight tissue were spiked with 2-methyl octadecane and 1,1-binaphthyl as internal standards and soxhlet-extracted with 300 ml toluene : 1 N KOH in methanol (30:70 V/V) for 24 hours.

Unaponified fraction was obtained by extraction into hexane (4 times of 50 ml each) and 50 ml of hexane extracted water. After evaporated by rotary evaporator to 3 ml, the hexane xtract was transferred to a 15 ml centrifuge tube and evaporated gently under a nitrogen stream to 1 ml. Lipids were separated by silica-gel/florisil column chromatography. Silica gel and florisil were soxhlet-extracted with hexane for 24 hours, then allowed to air dry in hood before used. These pre-extracted silica-gel and florisil were activated by heating at 220 ° C for 16 hours and deactivated by spraying 5 and 0.5 % hexane extracted water respectively. Fifteen grams of activated silica gel was slurred and settled to a height of 17.5 cm and with solvent flow rate of 2 ml per minute and the florisil slurry was transferred to 5 cm height (about 3 gram). Elution steps of the unaponified fractions, gas chromatography and identification were the same as those of sediment samples mentioned above.

#### 3.3.4 Beach Tar

After sand separation, beach tars were quantified by weighing to two decimal points and reported in weight per narrow strip of 1 meter width (g/m).

#### 3.3.5 Current Measurement

Current speeds and directions were measured from the anchored research vessel "Pramong 5" at Station 03 using Duncan current meter. The measurements were taken at three depths, i.e., near surface (2m below the surface), mid-depth, and near bottom (1-2 m above the bottom). The measurements were conducted at 25 consecutive sampling hours in order to complete a tidal cycle. The hourly current records were plotted as the net tidal drift and the progressive vector Meteorological Department. The current and wind data support an understanding of the distribution patterns of petroleum hydrocarbons from the period of June and November 1989.

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