III Analytical Methods

III.1 Outline of chemical analysis in the environment

III.1.1 Analytical order of chemicals in the environmental samples

In order to be able to determine very small amounts (very low concentrations) of chemicals in the environment, it is necessary to follow a series of operations :

1) isolation (extraction and separation) of the target chemicals from sample matrix (air, water, sediment, living things, etc.).
2) separation and purification of the target chemical from co-extracted, non-target chemicals (sample clean-up).
3) sample concentration.
4) measurement by highly selective and sensitive analytical equipment, such as gas chromatography / mass spectrometry (GC/MS). Occasionally it is also necessary to derivatise (chemically modify) non-volatile and heat degradable target chemicals prior to analysis by the two most commonly used instrumental methods in environmental analysis, GC or GC/MS.

The following sections describe this series of analytical operations in more detail, and introduce some of the main analytical methods for environmental pollutants.

III.1.2 Sample preparation methods

III.1.2.1 Extraction / separation methods

A. Water samples

a) Selection of extraction methods

It is possible to categorise chemical substances and their solubility in water based on simple physico-chemical characteristics, such as their molecular weight, boiling point, and polarity (**Figure III-1-1**). Methods for isolating chemicals from water based on these broad chemical categories are shown in **Figure III-1-2**.

volatility →

	volatile	semi-volatile	non-volatile
polar	alcohols ketones carboxylic acids	alcohols ketones carboxylic acids phenols	high molecular electrolytes carbohydrates fulvic acid
semi-polar	ethers esters aldehydes	ethers esters aldehydes epoxides heterocyclic compounds	protein carbohydrates humic acids
non-polar	aliphatic compounds aromatic compounds	aliphatic compounds aromatic compounds alicyclic compounds	non-ionic polymers lignin
	small	medium	large

polarity ↑

molecular weight →

Figure III-1-1 Classification of chemicals by physicochemical character[1)]

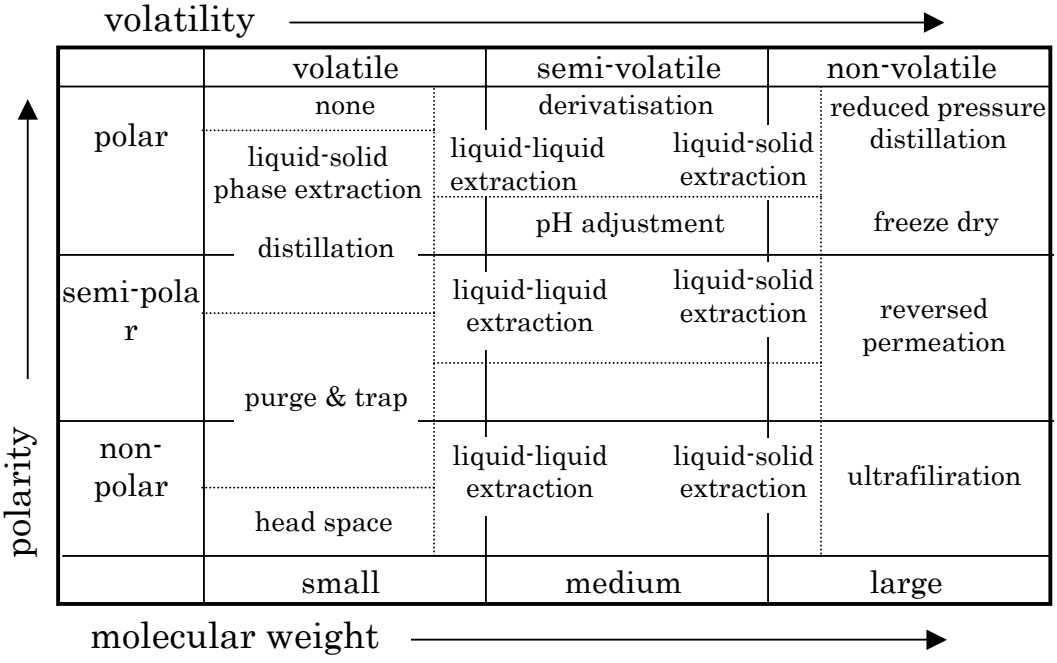

Figure III-1-2 Chemical separation method for water samples[1)]

b) Volatile organic compounds

The two main methods for the isolation of volatile organic compounds from water are the purge and trap method and headspace method. These are described below;

(1) Purge and Trap Method (P&T)

This method, also known as the *dynamic headspace method*, removes (separates) volatile compounds from the sample matrix (in this case, water) by passing an inert gas such as helium or nitrogen through the matrix (purging). The target, volatile compounds are desorbed from the aqueous phase to the gas phase (purged) and are then separated from the stream of gas (trapped) by adsorbent filters. The adsorbent material is then heated in a stream of GC carrier gas (usually pure helium). This releases the trapped substances into the carrier gas, the target analytes are introduced to GC, and analysed. Typical trapping (adsorbent) materials are porous polymer beads, activated charcoal, silica gel, other GC column packing materials, or combinations of such materials.

Practically, this method can only be undertaken using one of a few specified instruments which are fully automated from the purging step, through trapping, releasing the target analytes, and introduction into to the GC.

The P&T method has following features and points:

1) The method is appropriate for micro-level analysis (ppt - ppb concentrations) because it is possible to analyse the whole volatile compounds in water samples.
2) The gas used to purge the sample absorbs significant amounts of water in addition to the volatile chemicals. One must remove this water prior to analysis because water causes interference in the GC/MS system.
3) After the volatile chemicals are released from the adsorbant by heating, one must introduce these compounds into GC column as a narrow band. If wide bore column is used, there is no need for any special treatment because it is possible to pass large carrier gas flows into the GC/MS system. However, when regular bore column is used, the end of column must be cooled (cryo-focused) at -100°C by liquid nitrogen to narrow the width of the band of chemicals.
4) Some volatile compounds are found extensively in the environment. The P&T method is highly sensitive, and analysis tends to be influenced by such extraneous, volatile compounds found in the purge gas and surrounding atmosphere. It is therefore necessary to protect the purified purge gas and the analytical system from contamination. Blank samples should be treated in the same manner as other sample in order to make sure there is no

contamination.

(2) Headspace method (HS)

Also known as the *static headspace method*, this method is less sensitive (ppb level) compared to the purge & trap method, but operation is simple, easily automated. The sample is placed in a sealed container, such as a vial, and left at a constant temperature until the gas and liquid phase are in equilibrium. The target substances in the gas phase (headspace) are collected by gas tight syringe. This is injected into the GC/MS. Calibration curve are made by dissolving the target chemicals in purified water, and then treated in the same manner. However, the air-liquid phase equilibrium is very much affected by matrix in which the sample is dissolved, so in a lot of cases standards dissolved in purified water might not be appropriate surrogates from which to prepare calibration curves. In such cases it is necessary to saturate samples with salt, add an external standard and then quantify the target chemicals (Standard addition method). Also, for samples which exist as ions in water the pH must be adjusted. For example, it is easy to analyse fatty acids in alkaline solution, if first the solution containing the fatty acids is acidified by sulfuric acid. It should be noted that pH adjustment is also often needed for the purge & trap method.

(3) Notes on the analysis of volatile compounds

(i) preparation of purified water

One of the most difficult aspects of trace analysis of volatile compounds is to prepare water blanks not containing extraneous volatile compounds. It is common to use commercial mineral water, such as Evian, because it is so difficult to make one's own blank water. In order to make one's own, the following two methods might be tried:

1) purge purified water with high quality helium
2) boil hard ordinal purified water on a gas fire for more than one hour, and then cool down outdoors in an environment where there is naturally no volatile compound contamination place.

(ii) notes for using cryo focus

There are some trapping materials e.g. silica gel or charcoal, which do not remove enough water. As a result, water will freeze at cryo focus. The GC retention times of the target analytes will change unless sufficient water is removed by another method e.g. dry purge, etc.

c) Semi-volatile chemicals

Methods to extract semi-volatile compounds from water include liquid-liquid extraction, solid phase extraction, and recently developed solid phase microextraction (SPME)[2].

(1) Liquid-liquid extraction

Solvent extraction methods use non-polar solvents which are miscible with water to extract the target compound from water by using the greater solubility of the target compound in the solvent than water. Ideally, one selectively extracts the target compound by using a solvent whose polarity is close to that of the target compound. Volatile solvents such as hexane, benzene, ether, ethyl acetate, and dichloromethane are usually used for the extraction of semi-volatile compounds from water. Hexane is suitable for extraction of non-polar compounds such as aliphatic hydrocarbons, benzene is suitable for aromatic compounds, and ether and ethyl acetate are suitable for relatively polar compounds containing oxygen. Dichloromethane has high extraction efficiency for a wide rage of non-polar to polar compounds. Dichloromethane is suitable for simultaneous analysis because of the following advantages: its boiling point is low and easy to reconcentrate after extraction, it is easy to separate from water because of its higher specific gravity, and it is non-flammable. However, dichloromethane, like benzene, is carcinogenic, and recent trends have been to refrain from using these solvents in liquid-liquid extractions. It is sometimes possible to selectively extract semi-volatile compounds from water by changing the character of samples, not changing solvents. For example, by changing the pH of samples, only acid or basic substances can be extracted. When pH of the water is less than 2, basic compounds become fully ionised and are not extracted by the solvent, allowing selective extraction of acidic and neutral compounds.

When extracting compounds which are relatively soluble in water, salting-out techniques are used in order to increase extraction rates. Adding salt to an aqueous sample decreases the solvation power of the solution and the solubility of target compounds. This is useful not only for liquid-liquid extraction but also for headspace and solid phase extraction methods.

Extraction is commonly achieved by shaking the water sample and solvent in a separating funnel. However, occasionally large amounts of emulsion are formed, and it is difficult to separate the solvent from the aqueous phase. If this occurs, the emulsion is often efficiently dispersed (broken down) by adding either a small amount of ethanol, by sonicating the mixture in ultrasonic bath, or by adding anhydrous sodium sulfate, or continuous liquid-liquid extraction can be performed on samples which form emulsions. Continuous liquid-liquid extraction methods repeatedly circulate solvent in special glassware but, although this method has good extraction efficiency, it is not suitable for thermally unstable compounds because the extraction time is long.

(2) Solid-Phase Extraction (SPE)

Solid phase extraction (SPE) is a more rapid, modern alternative to liquid-liquid extraction. SPE is based on the principle that the components of interest are retained on a special sorbent

contained in a disposable mini-column (cartridge). By using SPE one can remove matrix interferences (these either pass through the cartridge or are subsequently washed off) and then isolate with selective enrichment one's target compounds. Solvent use is small. Common cartridges packing materials (solid phases) are charcoal[3),4)] and XAD[5)] resin, silica gel chemically bonded with ODS[6),7)], and high-polymer resin such as polystyrene[8),9)] and polyacrylate[9)]. Cartridges pre-packed with known quantities of adsorbent are on the market, and they are ready to use after simple conditioning.

There are some disadvantages to solid-phase extraction, which are:

- Although solvent use is small, the solvent flow rate affects the recovery rate.
- For samples which include suspended solid (SS), it is necessary to separate SS composition.
- For samples which are heavily contaminated, it is possible to get analyte break through.
- In order to have high and stable recovery rates, it is important to choose the most appropriate solid phase for the target compounds (refer to solid phase manufacturers' manual). One must undertake recovery tests by adding known quantities of standard into the sample matrix, passing such samples through cartridges, eluting the target compounds, then determining the amount of analyte recovered.

(3) Solid phase microextraction (SPME)[2)]

SPME is a method used to both extract and concentrate organic compounds in which a fibre needle attachment which has been chemically coated with a fused silica equivalent to a GC liquid phase, is dipped directly into liquid samples, or exposed to the headspace vapors from liquid or solid samples. Because SPME has only recently been developed, there are few reports of its use with real environmental samples, and we must wait for the results of future investigation before recommending use. However, the fact that organic compounds can be analysed easily and quickly without using any solvents suggests that this is the direction in which the next generation of analytical methods should proceed.

B. Sediment, soil, and biological samples

a) Purge & trap method (P&T, dynamic headspace method)

The purge & trap method can be used to extract volatile compounds from solid samples such as sediment. The sample (less than 1 g) is placed into the purge bottle, suspended in adding water, then treated and subsequently analysed in the same manner as water samples. Samples which include high concentration of VOC are extracted with methanol, and then a part of extract is analysed by purge & trap after being added to blank water.

b) Organic solvent extraction method

There are three organic solvent extraction methods for semi-volatile compounds from solid samples: 1) Soxhlet extraction, 2) extraction after mechanical mixing such as shaking, homogenisation, or stirring, and 3) ultrasonic extraction.

(1) Soxhlet extraction

In soxhlet extraction, organic components in solid samples are extracted from the matrix by continuously washing the solid with a volatile solvent in a specialised piece of glassware (soxhlet extraction apparatus). This is the most common method for extraction of organic compounds from solid samples, and is used as an extraction rate standard for the newly developed extraction method known as supercritical fluid extraction. Non-polar solvents such as benzene or dichloromethane, polar solvents such as methanol, or mixtures of polar and non-polar solvents whose boiling points are close to those of ethanol / benzene, or acetone / hexane are used. Benzene is known to be an especially efficient extraction solvent for PAHs, and acetone for sulphur-containing compounds. However, soxhlet extraction takes long time to get high extraction efficiency, and is not suitable for organic compounds which are thermally unstable.

(2) Methods extraction after mechanical mixing such as shaking, stirring, or homogenization

Shaking and stirring: These methods are in essence derivations of liquid-liquid extraction. Sample extraction is achieved by simply placing solid samples in centrifuge containers with organic solvents and shaking. After extraction, separate sample matrix and extracts by centrifugation or filtration.

Homogenization: This method is suitable for the extraction of non-polar compounds in biological samples. Anhydrous sodium sulfate is added to the sample, the sample is then homogenised in the presence of a non-polar solvent. After extraction, separate sample matrix and extracts by centrifugation or filtration. Extracts usually include lipids, so defatting processes are required before analysis.

(3) Ultrasonic extraction

Ultrasonic extraction uses ultrasonic vibrations to extract samples with polar solvents in an ultrasonic bath. This is often used for chemical extraction from solid samples because it's simple.

c) Steam distillation (including circulating steam distillation)

In steam distillation, steam is introduced into a macerated solid sample. The target analytes are co-distilled out of the sample container with the steam, and are collected after condensation of the steam to liquid water. In the circulating method, the condensate is extracted by liquid-liquid extraction using a non-polar solvent. Steam distillation is used for compounds with a relatively high vapor pressure which are not soluble in cold water. Steam distillation is an operation which has both extraction and separation / fractionation functions. Because it can separate target compounds from non-volatile compounds and sulfur contained in sediments, purification after extraction is easy.

d) Supercritical Fluid Extraction (SFE)

Gases such as CO_2 and N_2O become fluid when temperature and pressure reach a critical point called the supercritical phase. In supercritical fluid extraction (SFE), this fluid is used to extract target substances in solid samples. This is a relatively new method which has recently begun to be applied extensively. Instruments connecting the SFE apparatus and on line GC have been developed in which the series of steps from extraction to analysis have been automated.

The sample is placed in an extraction chamber through which the supercritical fluid is forced, the target substances are extracted from the samples and trapped in vials by small amounts of methanol or ODS resin. The amount of organic solvent used is much smaller than soxhlet extraction.

The fluids used for SFE include CO_2, N_2O, SF_6, methanol, and water. The fluid used is chosen to meet the analytical purpose. The most commonly used is CO_2. However, supercritical CO_2 is very non-polar, so modifiers are added to improve extraction efficiency for polar compounds. Modifiers for CO_2 include methanol, dichloromethane, acetonitrile, water etc. Modifiers are mixed with the CO_2 using a pump, or spiked directly into the samples.

C. Air Samples

Air samples are categorised as being particulate and gas depending on the phase in which the target substances are found in the air. Since sampling methods for the two types of air sample are different, as mentioned in Chapter 2, sample preparation methods are explained for each sampling method.

a) Solvent Extraction from Filter Papers or Adsorbents

Filter paper is used to trap particulates, while adsorbents are used for samples containing substances intermediate between particulates and gases. Extraction methods for target substances trapped on filter papers and adsorbents are those used for solid samples and sediments etc., such as ultrasonic extraction and soxhlet extraction. However, ultrasonic extraction is not suitable for polymer adsorbents, since they are not strong enough. Use soxhlet extraction for such polymer adsorbents and polyurethane foams. On the other hand, soxhlet extraction is not suitable for volatile and thermally unstable substances. For such compounds, and other adsorbents use ultrasonic extraction. Changing solvents to meet the requirements of different adsorbents or target substances can make for more efficient extraction. When using a polar solvent such as acetone, add salt water after extraction, and then extract by liquid-liquid extraction with a hydrophilic solvent.

b) Thermal Desorption

Adsorption followed by thermal desorption can introduce the whole trapped target substances into GC / MS. This makes such methods suitable for analysing volatile compounds, and samples containing low concentrations of target analytes where it is not easy to be collected large amounts of sample. The basic scheme is shown in **Figure III-1-3**. Adsorbed compounds are thermally desorbed by heating the adsorbent material with a heater. Changing the direction of flow of the GC carrier gas by using a three-way valve allows introduction of these compounds into the GC column. Equipment in which all procedures are automated is on the market. Such equipment generally has a cold trap, where desorbed samples are temporarily trapped in a concentration tube (cold finger) cooled by liquid nitrogen, and then introduced into the column by heating. Such cold trap functions make sample introduction quicker, and gives sharper peaks.

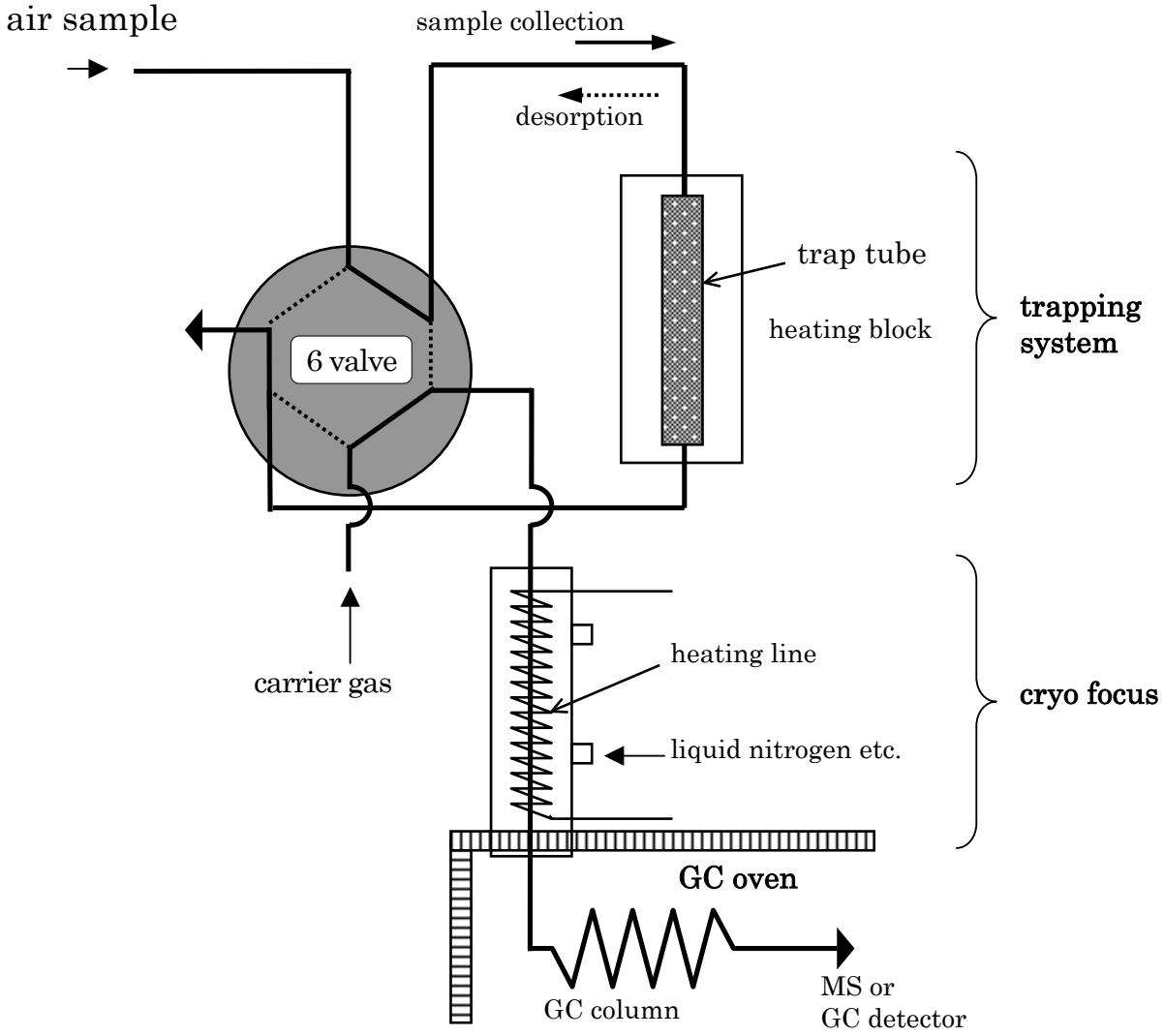

Figure III-1-3 Flow direction in adsorption trap-thermal desorption method

III.1.2.2 Dehydration Methods

Because extracts from liquid-liquid extractions often contain water originating from the sample, sample concentration or clean-up using columns cannot be done directly. Thus, it is necessary to dehydrate the extract. Dehydration is most commonly achieved by using anhydrous sodium sulfate. Samples are dehydrated by adding the anhydrous sodium sulfate directly to the extract and then filtering the dry solvent solution, or passing the samples through a column or funnel packed with anhydrous sodium sulfate.

III.1.2.3 Concentration

Use a Kuderna-Danish (KD) concentrator or rotary evaporator to concentrate the extract or column chromatography eluant. Whether the KD method or rotary evaporation is chosen depends on the boiling point of the target compounds, their sublimation character, timeframe for analysis etc.

A. KD Concentration

KD concentration takes longer than rotary evaporation, but there is less loss of target chemical through evaporation, and this method is as applicable to low boiling compounds as to high boiling compounds. This method is able to concentrate samples down to a few mL. For further concentration, one must use a micro-Snyder column or evaporate under a stream of nitrogen. Evaporating under nitrogen gas may cause evaporative loss of low boiling point compounds. However, the micro-Snyder column method can concentrate samples containing low boiling point compounds to volumes of 0.5 mL.

B. Rotary Evaporation

Rotary evaporation can concentrate large volumes of samples in a relatively short period of time. However, it has big evaporative losses and is not suitable for low boiling point compounds.

III.1.2.4 Clean-up Method

Extracts from environmental samples can be complicated mixtures. Components of these mixtures can interfere with GC and HPLC analysis by giving poor separation because of over separation capacity of a column or by containing compounds that elute at the same time as, and interfere with, the target compounds' peaks. Also large amounts of non-volatile compounds or polar compounds can contaminate GC injection ports and columns, which in turn causes problems with analysis. It is, therefore, necessary to clean up, or remove, non target compounds as much as possible. Clean-up methods include acid - alkaline partition, acetonitrile - hexane partition, and column chromatography. Column chromatography separation can involve adsorption, partition, gel permeation, ion exchange etc. The following section describes clean-up methods usually used for environmental analysis.

A. Adsorption Chromatography

Adsorption chromatography is able to separate the constituents of complex samples according to equilibrium between adsorbent (stationary phase), solute (chemicals) and eluent (eluting agent). Generally normal phase chromatography is used. Samples are dehydrated and concentrated, then added to the column. The column is eluted first with a non-polar solvent such as hexane, and then the polarity of the solvent is gradually increased. This has the affect of progressively eluting more polar constituents. Adsorption chromatography is most suitable for the separation of relatively non-polar compounds. Adsorbents typically used are alumina, silica gel, and florisil. The relative strength of adsorption of these materials is: silica gel < florisil < alumina. The suitability or nonsuitability of these materials for the target compounds depends on the strength of adsorption. To find the most appropriate adsorbent, refer to published papers and actual tests. Perform actual sample analysis after confirming adsorption activity level, composition of eluent, and elution pattern because there are some compounds which decompose in the commonly used eluents, such as diethyl ether or acetone.

a) Silica gel

Silica gel is weakly acidic, amorphous silica. Activate this adsorbent by heating at 150 - 160 °C for several hours. It is common to contain 3 - 5 % by weight water in the adsorbent because completely activated silica occasionally does non-reverse adsorption. This material is useful for compounds containing most ionic and non ionic functional groups. However, one must be cautious when using eluents which contain methanol or ethanol because they decrease the adsorbent's adsorption activity.

b) Florisil

Florisil is the commercial name for a magnesium silicate with an acidic character. This material is available on the market from Floridin Co. in a form which has been activated at 667 °C. This material is mostly used for clean-up of samples for GC containing pesticides, organochlorine compounds, esters, ketones, phthalic esters, nitrosoamines, organophosphate pesticides (which include a phosphorus - oxygen bond which can decompose on florisil), separating aliphatic and aromatic hydrocarbons, etc. However, florisil's activity varies between batches, one must watch out for changes in elution patterns. In addition, it is known that some pesticides decompose in ethylether on florisil.

c) Alumina

There are three types of alumina, basic (pH 9-10), neutral, and acidic (pH 4-5). Alumina is used after dehydration at 400 - 450 °C. Water is added to the alumina to adjust activity. Basic

alumina has the strongest adsorption capacity, and is used for basic and neutral compounds, such as alcohols, hydrocarbon, steroid, which are stable in alkaline media. However, ethyl acetate cannot be used as an eluent because esters are unstable in alkaline media and decompose. Acetone cannot be used as an eluent because it causes amidol condensation and produces diacetone alcohol. Neutral alumina has lower activity compared to basic alumina, and is used for aldehydes, ketones, quinones, esters, etc. Acidic alumina is suitable for acidic pigments (dyes) or acidic compounds which are adsorbed by basic and neutral alumina.

B. Partition Chromatography

Partition chromatography is able to separate the constituents of complex samples according to partition equilibrium between a non-polar solid phase and a polar solvent (in this case separation is by *reverse-phase* chromatography). The non-polar solid phase is packed into a column, the sample mixture dissolved in water and loaded onto the column, then the components of the mixture, from most polar to least polar, are eluted with solvent mixtures such as water - methanol, or water - acetonitrile. This method is, therefore, most suitable for separation of high polarity compounds. The most commonly used solid phase is ODS, which has an octadecyl function bonded to the silica particle. This material is commonly used for the fractionation of samples by HPLC.

C. Gel Permeation Chromatography

Gel permeation chromatography separates molecules by size. Compounds are separated when sample mixtures are passed through a column packed with material of a known pore size. Larger molecules elute faster. Gel permeation chromatography is used as a general separation method for semi-volatile compounds. However, the separation ability is worse than other chromatography methods, so GPC is generally used to remove lipids, proteins, and natural resins from samples, not for detailed fractionation.

D. Ion-Exchange Chromatography

Ion exchange chromatography is used to separate compounds which have fully ionisable function groups. Samples are loaded onto the top of a column packed with an ion exchange resin, and eluted by an electrolyte solution. Which ions are separated and the order they are eluted depends on the ion exchange- adsorption character of the molecules with the resin.

E. Acetonitrile - Hexane Partition

Acetonitrile - hexane partition is commonly used to remove lipids from sample extracts.

Target compounds are partitioned into the acetonitrile phase, and lipids partitioned into the hexane phase. However, when the target compound's polarity is extremely small, the target compound too can partition into the hexane phase.

F. Acid - alkaline Partition

Acid-alkaline partition is used to separate neutral, acidic, and basic components by adjusting the pH of aqueous samples. Compounds such as phenol can be extracted into organic solvent when aqueous samples are made acidic (below pH 2). Next, phenols in the extract are reverse extracted by water of pH is 12 - 13. Finally, this aqueous phase is acidified (to below pH 2), and re-extracted by organic solvent. In this manner, only phenols can be extracted from samples. Basic compounds such as amines can also be separated by pH reversal.

G. Sulfur-Cleanup

Elemental sulfur is often found in anaerobic sediment samples. Elemental sulfur can be dissolved in a number of solvents, since its solubility is quite similar to organochlorine and organophosphate pesticides. Sulfur is eluted in the first fraction eluting from florisil clean-up columns. When analysing samples which include sulfur, unless sulfur is removed, an interference peak caused by sulfur is often found between the solvent peak and that of aldrin. To remove sulfur, stir sample solutions well with either copper powder, mercury, or tetrabutyl ammonium solution. Since some compounds which contain sulfur or phosphorus, such as phosphates or pesticides, are unstable, one must check these compounds' stability before treating the samples with sulfur removing materials.

H. Alkaline Decomposition

Alkaline decomposition is used to extract compounds which are stable in alkaline media, such as PCBs, from biological samples which contain lipids. Such samples are refluxed in alkaline ethanolic solution. This saponifies lipids. Salt solution is added to the sample, and the target compounds are extracted by liquid-liquid extraction. It is possible to remove lipids and extract target analytes at the same time using this method.

III.1.2.5 Derivatisation

Derivatisation involves chemically modifying the target compounds. It is common to convert hydroxyl group (phenols), carboxyl groups (fatty acids), amino groups (amines), and organometallic

compounds (organotins) into their trimethylsilyl, ester, acyl, and alkyl derivatives. By derivatising such chemical functional groups, one can make non-volatile compounds volatile. Thus one can analyse by GC compounds which normally cannot be analysed by GC, including compounds which are normally adsorbed by separation columns. It is, however, possible to analyse some polar compounds without derivatisation if one uses a fused-silica capillary column. One should not rush to derivatives target compounds because there are problems with derivatisation which must be carefully considered, such as yield of derivatisation, derivative recovery, stability of a derivative, identification and confirmation.

A. Esterfication

Methyl ester derivatives are generally stable, isolation is usually easy, and it is often possible to confirm the yield and stability of reaction products. After methylation, the increase in molecular weight of the methyl ester derivatives is small, some show molecular ions in mass spectra, and one may therefore use data reference systems. However there are some problems, for example, methylating reagents are often not highly reactive, and many don't react with alcoholic hydroxyl groups and amine groups, although they are used for fatty acids.

B. Silylation

Trimethylsilylation (TMS) shows the highest reactivity of all derivatisation methods for most compounds which have active hydrogens. However, this method should only be chosen when methylation and acetylation cannot be used, since there some disadvantages:

1) products of silylation are easily hydrolysed and cannot be stored for long periods
2) products of silylation often cannot be isolated, and therefore reaction solutions often have to be analysed directly by GC
3) injection of reaction solutions into GC tends to make GC 'dirty' because inorganic silicone compounds are formed
4) polyethylene glycol (PEG) columns react with silylating reagents.

C. Acetylation

This is one of the most often used derivatisation methods, because acylating agents react well with many functional groups, such as alcohols, thiols, and amines. Fluoroacetylation, particularly heptafluoropropionylation, is often used because highly sensitive analysis by ECD or MS is possible.

D. Other Derivatisation Methods

Pentafluorobenzylhydroxylamine (PFBOA) and 2,4-dinitrophenylhydrazine are used for derivatisation of aldehydes. Also "Grignard" reagent is used for alkylation of organotins[10].

III.1.3 Instrumental Analytical Methods

Only GC / MS is discussed hereafter because of the high sensitivity, high selectivity, universality, and the large amount of information available on what is often considered the most appropriate analytical instrument for environmental analysis.

III.1.3.1 GC/MS Analysis

It is not too much to say that whether trace chemical analysis by GC / MS is successful or not depends on whether the set-up of the GC is appropriate for the target compounds or not. Recently because many analyses use fused-silica capillary columns, there has become less need to pay attention to the selection and inactivation of columns (*cf.* when packed columns were used). However, in order to analyse chemicals at sub-ng concentrations efficiently and accurately, it is still necessary to carefully choose columns, sample injection conditions, column temperature conditions, etc. Selection of quantifying ions directly affects MS identification and detection limit. Here follows some basic information which should be known when trace chemicals are to be identified and quantified by capillary GC / MS and things which should be considered when undertaking GC/MS.

III.1.3.2 Factors Affecting Capillary GC Analysis

Good separation in the shortest period of time is the ideal (optimum) for good GC analysis. These criteria are related to the liquid phase of columns, film thickness, length, internal diameter, and temperature.

A. Selection of capillary column

a) Selection of liquid phase of capillary column

Capillary columns are available with many kinds of liquid phase, from non-polar methylsilicones to highly polar polyethylene glycols. For the analysis of most compounds it is sufficient to use four types of liquid phases - methylsilycones, phenylmethylsilicones, cyanopropyls, and polyethylene glycols. Selection of the most appropriate liquid phase, and hence column, to

separate target compounds is dependent on partition coefficients. For instance, to separate non-polar components such as n-alkanes, use non-polar columns - these have the largest partition coefficients for such components. Highly polar liquid phase columns, such as PEGs, are suitable for analysis of highly polar compounds such as alcohols. However, polar columns (especially PEGs) are often thermally unstable and oxygen sensitive, and often have problems with column bleeding. As a result, their operating temperature is limited compared to non-polar columns. These disadvantages make columns with highly polar liquid phases difficult to use. Therefore, if a target chemical shows a sharp peak with no tailing peak, one has to choose a column with a lower polarity liquid phase. Choose columns by referring to analytical method information supplied in column suppliers' catalogues and published analytical methods.

b) Column length

The length of a column length directly affects separation efficiency and analysis time. Although separation efficiency is proportional to the square root of column length, it is not recommended to use very long columns in order to improve separation because analysis time is proportional to column length. For example, when a 30 m column is exchanged for a 60 m column, separation improves 1.4 times but analysis time is doubled. However, simply cutting a few meters off the end of a dirty column doesn't make a big difference to separation efficiency.

c) Column internal diameter

The internal diameter of a column affects column efficiency, retention character, and the amount of sample that may be loaded onto the column while still obtaining analyte separation. The smaller internal diameters, the better separation efficiency, but the less sample may be loaded onto the column. For environmental analysis, column bore of internal diameter 0.2 - 0.35 mm is generally used. Columns whose internal diameter is about 0.25 mm give good separation, although one can load larger samples onto columns whose internal diameter is about 0.32 mm. Wide bore columns, i.e. with an internal diameter of more than 0.5 mm, can be used as substitute for packed columns because the amount of sample that may be loaded is usually several μg for each constituent. However, such columns cannot be used for GC/MS unless the GC/MS has a large vacuum pump (and even so regular bore column resistant tubing is needed), or an enricher (apparatus which separates helium at the interface between the GC and MS).

d) Film thickness

The film thickness affects the column's relative character and amount of sample that may be loaded onto it. The thicker film is, the larger the sample that may be loaded onto the column. Columns with thicker films are suitable for the analysis of low boiling point compounds because

solute constituents are held strongly by the column. Conversely, thinner columns are suitable for the analysis of high boiling point compounds (analysis of high boiling point compounds using thicker film columns requires higher column temperatures, which in turn causes increased column bleeding). For GC/MS, columns should be used which have little column bleeding and unnecessarily thick film columns and non chemical bonding type columns should be avoided. Because small internal diameter and thin film columns can only be loaded with small amounts of sample, it is necessary to take measures to prevent peak shape abnormalities, such as leading, by minimising the amount of sample loaded onto the column or by using a different column.

e) Basic columns

In light of the factors outlined above, the following columns should be considered for chemical analysis of environmental samples:

- For volatile compounds: liquid phase, methylsilicone; length, 60 m; i.d., 0.32 mm, film thickness, 3 μm.
- For semi-volatile compounds : liquid phase, methylsilicone or 5% phenyl 95% methylsilicone; length, 25 - 30 m; i.d., 0.2 - 0.32 mm; film thickness, 0.1 - 0.5 μm.

B. Sample Injection Methods

For capillary columns, there are three principle requirements for any injection method, namely to introduce samples as a narrow width band, to not change sample composition, and to have good reproducibility. Common sample injection methods recently are direct injection, split/splitless injection, and on-column injection methods. In order to select the method that is most suitable for the samples in hand and meet the above criteria, one must understand some of the key features of these methods.

a) Direct injection

In direct injection methods, the whole sample is introduced into the vaporising chamber. This method is used for widebore columns whose internal diameter is more than 0.5 mm, or packed columns. The injection port temperature is typically 20 - 30 °C higher than the GC oven (column) temperature for iso-thermal analysis, and also typically 20 - 30 °C higher than the final oven temperature when temperature gradient programming is used.

b) Split / splitless injection

Split and splitless injection methods can be used at the same injection port.

- **Split method:** In this method, the sample is vaporised instantly in the gas chamber, then a narrow sample band is introduced into the column by introducing only a small part of the

vaporised samples and discharging the rest. This is a useful method for samples with high concentrations of target analytes. It is not suitable for samples with low analyte concentrations such as environmental samples. Also, when analysing compounds which contain a mixture of components with a mixture of boiling points (low, medium, high), such as petrol, fractional distillation (discrimination) may occur in the vaporisation chamber. To prevent this, increase the temperature of the vaporisation chamber, and increase the system's heat capacity putting deactivated glass wool into the insert (injection liner).

- **Splitless method** : In this method, the sample, including any solvent, is introduced onto the column through a modified heated vaporiser. Solvent is removed from the sample by venting the injection to the atmosphere when most of the solvent and essentially all of the sample have entered the column - perhaps 30 sec to 2 min. The correct lag time before venting is critical since too little time causes loss of sample, too long a time period causes a solvent peak larger than necessary and which in turn may cause spectral interference. This method is suitable for environmental samples because it is able to introduce samples with low target analyte concentrations quantitatively into columns. In addition, relatively non-volatile compounds, which often cause damage to columns, are not volatilized and therefore do not penetrate far into the column. Chambered injection port liners are used with the splitless method because this method doesn't need to vaporize samples (*cf.* split method).

c) On-column injection

In on-column injection, the sample solution is injected onto the column as liquid, and then the column temperature is increased. This procedure makes this injection method suitable for the analysis of thermally unstable compounds. This is suitable for trace analysis because the whole sample is injected onto the column. However, if contaminated samples are injected, the column's useful working life span is shortened because in this case relatively non-volatile compounds, which often cause damage to columns, are volatilized and can penetrate far into the column. Therefore, it is usual to attach a guard column to the separation column to prevent column contamination.

Recently the Septum Programmable Injector (SPI) has become popular. This equipment has improved cool on-column methods since with it is possible to control the injection temperature independently of the oven's cooling and heating cycles. It is thus possible to setup injection conditions optimised for producing narrow sample band-widths.

III.1.3.3 Mass Spectrometry

There are two ways to use mass spectrometers in scanning mode or selected ion monitoring mode (SIM). In order to select the appropriate operating mode, one must understand some of the key features of these methods.

A. Scanning method

When operated in the scanning mode, the mass spectrometer records the mass spectrum of all ions detected within a defined mass range by scanning at predetermined mass (m/z) intervals. The record of total ion intensity on each scan with time is called the total ion chromatogram (TIC). The TIC is equivalent to, for instance, a GC-FID chromatogram. The data making up the TIC is recorded electronically, and specific m/z information can be extracted and printed. These latter are called mass chromatograms. The scanning method can change identifying ion freely after analysis, and there is often no need to re-analyse the sample even if there are interfering peaks.

The accuracy and reliability of analyte identification is significantly better than SIM, because one can obtain the mass spectrum of the target compound using the scanning method. However, the method's sensitivity is theoretically much worse than SIM (several to several tens of times) in the case of magnetic field type quadrupole mass spectrometers - low resolution MS cannot perform trace analysis. Finally, the amount data collected is extremely large compared to SIM.

B. SIM method

When operated in the SIM mode, the mass spectrometer monitors specified ions with high sensitivity. This is done by setting up electric or magnetic fields (depending on the instrument design) which focus only ions of a predetermined m/z ratio on the detector. A SIM chromatogram equivalent to the mass chromatogram obtained by the scanning method can also be obtained. As mentioned earlier, the sensitivity of SIM operation is better than the scanning method because the time spent on analysis of the target analyte ions is longer. Recent GC/MS systems have been developed that can routinely detect picogram (pg) levels of organic compounds. However, the number of ions which can be measured at any one time is generally limited to ten. This means the number of compounds which can be analysed at any one time is generally limited to five (at least ten sampling points for each GC peak are needed to get an accurate area for the GC peak. Therefore, if 2 ions per compound are monitored, the number of compounds which can be measured at the same time is limited to a maximum of five). In order to overcome this limitation, a Grouping method which changes the measured ion depending on GC retention time is used.

The accuracy and reliability of analyte identification is lower compared to scanning method because the number of ions measured is limited. Selection of ions to be monitored is important in

order to get high sensitivity and high reliability of identification. Usually multiple ions per compound are measured, and it is necessary to confirm the compounds from relative isotope intensity ratio.

a) Selection of quantifying ion and confirmatory ion for SIM method

In principle one tries to select ions with high sensitivity and high selectivity. First, measure the accurate mass spectrum of the target compound, confirming compound identity by comparison with library spectra. When the intensity of the target compound's molecular ion (parent ion) is high, make that ion the quantifying ion. If the molecular ion is small, choose an ion of high mass number (m/z) and high intensity as the quantifying ion (ions whose mass number (m/z) are less than 100 should be avoided). Choose a confirmatory ion (an ion known to be derived from the target compound and which proves the presence of the target) of high mass number (m/z) and high intensity. Compounds which contain chlorine or bromine produce isotope ions (molecular and fragment ions containing the chlorine or bromine isotopes) in the same ratio as the ratio of naturally occurring chlorine or bromine isotopes. These isotope ions are characteristic and the number produced depends on the number of chlorine or bromine atoms within the molecule. Isotope ions are ideal as confirmatory ions. After deciding which ions to monitor, measure standard solutions, and check if the desired is achieved, if the background noise is small compared to the signal intensity (S/N ratio is fine), or if there are no interferences, etc. If such parameters cannot be achieved, change the measurement ions.

III.1.3.4 Methods for the Calculation of Concentration

A. External Standard Method

The external standard method uses the relationship between peak areas (or height) and analyte concentration. The calibration curve is constructed after multiple, identical injections of a number of standard solutions of known concentration into the GC/MS.

Calibration curves for wide (mega) bore columns can be constructed after manual injection of samples, since several μL of samples is injected at one time and the variation in injection volume is small compared to the total volume injected. However, for regular bore columns, use of an autosampler is desirable, since the volume of sample injected is small and autosamplers are capable of very precise injection of small volumes.

B. Internal Standard Method

The internal standard method can be applied to every type of column. Standard solutions of

the target analyte are prepared in the same way as if using the external standard method, but to each is added a known amount of an internal standard. The solutions are measured. After measurement, calibration curves are constructed using the relationships between target analyte peak intensity and that of the internal standard (target compound peak intensity: internal standard peak intensity ratio) and target analyte concentration and that of the internal standard (ratio of target compound concentration: internal standard concentration). When determining target analyte concentrations in real samples, add the same amount of internal standard to the sample as added to the standard solutions, then calculate the concentration of target analyte from the target compound peak intensity: internal standard peak intensity ratio.

When using the internal standard method, it should be noted that the relative standard deviation (coefficient of variance) of ratios of peak intensities generally becomes larger as the separation (difference in retention times) of the internal standard and the target compounds increases. Therefore, a compound whose retention time is close to that of the target compound should be used as the internal standard, and when many target compounds with wide ranging retention times are being investigated, use of multiple internal standards is desirable. Finally, the chosen as internal standards should obviously not be found in the samples, and should be sufficiently stable to allow analysis by GC/MS. Internal samples synthesized using stable isotopes, such as deuterium or ^{13}C, are ideal compounds.

C. Relative Response Factor method

When a large number of compounds are being investigated simultaneously, it becomes difficult to make calibration curves for each measurement. In such cases, use the relative response factor method. This method is a variant on the internal standard method. First, make calibration curves (a minimum of five points from the detection limit of the instrument to the highest necessary concentration) in the usual manner. Then find linear range of calibration curves for each compound, and calculate the sensitivity against internal standard within the linear range using the following formula.

$$\text{RF of the compound} = \frac{\text{quantifying ion intensity of the compound} \,/\, \text{injection amount of the compound}}{\text{quantifying ion intensity of internal standard} \,/\, \text{injection amount of internal standard}}$$

When using the relative response factor method for sample analysis, analyse a standard solution whose concentration is near the centre of the linear range of calibration curve, or between a standard value and the determination limit, before sample analysis, and check if prescribed result can be obtained (for example, determined value is within ± 30% of the actual concentration). If the prescribed result cannot be obtained, it may be because of functional deterioration of the GC/MS, or decomposition of the target compound in the standard solution. Only begin analysis of the actual samples after solving the problem.

When analysing trace chemicals below the nanogram (ng) level, the smaller injection amount is, the smaller the RF value is. That is, the calibration curve becomes flatter. This symptom is especially notable when the polarity of target compound is increased. In this case determine using not the average RF value but RF value of the concentration which is close to the detection level.

D. Standard addition method

The method of standard additions may be used when components of the sample matrix influence analysis by producing physical or chemical interferences which cause calibration curves to differ from those made using purified water. To use the method of standard addition, first determine the analyte concentration in the samples, then add a small amount of a standard analyte solution of known concentration to the sample, and the analysis is repeated using identical reagents, instrument parameters and procedures. Readings must be corrected for any background signal. The result should be checked by repeating this procedure with at least one other standard addition. By this means, a plot such as **Figure III-1-4** may be obtained, from which the in the samples can be determined.

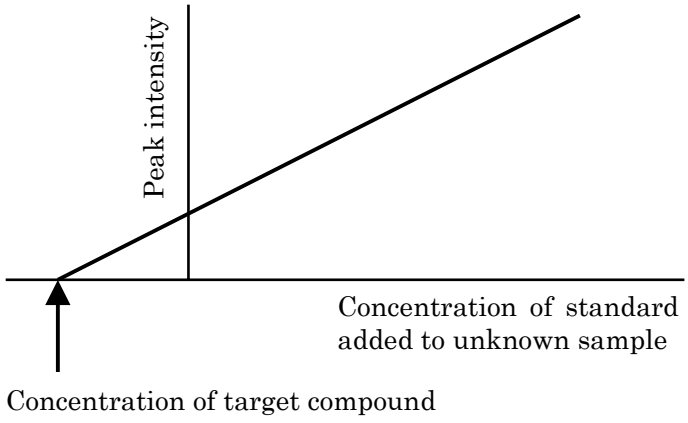

Figure III-1-4 Calibration curve for standard addition method

III.1.4 Procedures for analysis of new compounds

The steps typically required when analysing for the first time compounds whose analytical method is known are as follows.

(1) Collect as mush information as possible from the literature on such things as analytical methods, manuals, physico-chemical characters of the target compounds.

(2) Obtain pure target compounds, internal standards and / or surrogate compounds, and make stock standard solutions of about 1000 mg/L. As mentioned earlier, use as an internal standard a compound whose GC retention time is close to that of the target compound under the anticipated GC conditions, which is stable, and which gives a stable GC peak. Polyaromatic compounds labeled with stable isotopes will be ideal. Also as mentioned earlier, use as a surrogate compound a compound whose physico-chemical character is similar to that of the target compound. Again, target compounds labeled by stable isotopes are ideal. For both internal standards and surrogate compounds, use compounds which are not found in the environment under investigation.

(3) Selection of a GC column. Select a column based on information found in literature analytical methods or manufacturer's catalogues.

(4) Measurement of mass spectrum of target compounds and internal standards. Measure mass spectrum of target compounds, surrogates, and internal standards. The amount of material to be injected is the amount which will definitely give a good mass spectrum (about several ng). At this stage, it is efficient to increase the GC oven temperature from a low temperature because there is no need to repeat measurement. If it is difficult to tell which peak is the base peak because too much material was injected, decrease the amount of material injected and remeasure.

(5) After confirming, by analysis of the collected mass spectrum or by using the instrument's library spectra-matching system, that the mass spectra collected are correct or not, decide which ions will be used for determination and confirmation. Also, finalise GC oven programming to ensure the best measurement conditions in the shortest time.

(6) Prepare a series of low concentration standard solutions for making calibration curves, and make calibration curves of target compounds and surrogates, if used. The amount of internal standard to be used will equate with the minimum amount of compound which produces a stable peak area (0.1 - 1 ng is appropriate). Next determine the instrument's minimum detection limit. This minimum detection limit is the value at which it is possible to measure accurately and precisely the peak area (MS response) of the targets even if the GC/MS system deteriorates. A minimum signal to noise (S/N) ratio of about 10 is required. If the monitor ion is interfered with, or the S/N ratio is bad, change the monitor ion and

remeasure.

(7) Confirm the linear range of the calibration curves from instrumental detection limit to the highest concentration. Determine the volume of a sample and final concentrate for GC/MS analysis which can be analysed in the shortest possible time, while still satisfying the survey's purpose and required detection limit, and using the least solvent.

(8) Determine the recovery and elution pattern by means of the necessary preliminary analytical experiments, such as column chromatography. The concentrations of target compounds used in such preliminary experiments will be that quantity which can be determined accurately and precisely.

(9) Conduct recovery tests, passing spiked reagent water through the full analytical process using identical reagents, instrument parameters and procedures. Conduct at least four recovery tests, as well as a blank. The amount of compounds spiked is generally several times the detection limit.

(10) Evaluate the results of overall recovery tests. Calculate the average recovery and the relative standard deviation (RSD). For water samples, it is acceptable if the recovery is more than 80 % and the RSD is less than 20 - 30 %.

(11) Conduct recovery tests using typical actual samples. The concentrations of spiked analytes should be the same as that found in the real samples (if known to contain target compounds), otherwise the concentration should be several times the detection limit. Blank samples are real samples without addition of compound.

(12) Confirm that the monitor ions are not subject to interference by matrix constituents, and that the recovery of the target analytes when spiked into the sample matrix is almost the same as the results of recovery tests using reagent water.

(13) Conduct actual analysis. Don't forget to use prescribed quality controls.

III.2 Practical analytical methods

This chapter summarises some practical methods for the analysis of chemicals in the environment using GC/MS.

III.2.1 Simultaneous analytical method for toxic chemicals in water and sediment using GC/MS

III.2.1.1 Target compounds

Hexachlorbenzene (HCB), dieldrin, p,p'-DDT and its metabolized products (p,p'-DDD, p,p'-DDE), chlordanes (trans-chlordane, cis-chlordane, trans-nonachlor, cis-nonachlor), HCHs (α-HCH, β-HCH), terphenyls (o-terphenyl, m-terphenyl, p- terphenyl), BHT, benzo(a)pyrene, tributylphosphate

III.2.1.2 Summary of analytical methods

Water samples : add surrogates, extract with hexane, dehydrate, and concentrate. Finally, determine analyte concentrations by GC/MS-SIM.

Sediment samples : add surrogates. Perform acetonitrile-hexane partition by first extracting with acetonitrile, then adding water to the acetonitrile layer, and finally extracting with hexane. Dehydrate, concentrate, and partition by silica gel column chromatography. Finally determine analyte concentrations by GC/MS-SIM.

III.2.1.3 Detection limit

For the purposes of most environmental surveys and most chemicals, the minimum detection limits using these analytical methods will be approximately 0.01 ng/mL for environmental waters, and 1 ng/g (dry weight basis) for sediments.

- For aqueous samples, conduct addition and recovery tests seven times, by adding target compounds into blank samples (samples of the same / similar matrix which don't contain the target compounds) at concentrations 3 to 5 times the lower end (minimum value) of linear portion of the calibration curve, and then calculate detection limit from the following equation.

$$DL=t_{n-1} \times S$$

where t_{n-1} is the value of student t-test (99% confidence level, or 3.143 when measurements are undertaken 7 times), and S is the standard deviation of the results of the 7 measurements

- For sediment samples, calculate the minimum detection limit in the same manner as for aqueous samples (above) , by adding target compounds into blank samples (samples of the same / similar matrix which don't contain the target compounds) so as to give final concentrations in the range 2 - 5 ng/g (dry weight basis).
- When the minimum detection limit is higher than the value required by the environmental survey's purpose, two strategies may be followed which may achieve the desired detection limit : increase the instrument's (GC/MS) capacity, or increase the size (volume or mass) of the sample extracted. This latter step increases the concentration of target analyte in the solution injected into the GC/MS.

III.2.1.4 Reagents and apparatus

A. Reagents

- Organic solvents : pesticide analysis grade
- Target compounds : commercial standard reagents
- Surrogates (HCB-$^{13}C_6$, p-terphenyl-d_4, benzo(a)pyrene-d_{12}) : commercial standard reagents
- Internal standards: commercial standard reagents, such as naphthalene-d_8, fluoranthene-d_{10}, perylene-d_{12}
- Anhydrous sodium sulfate : pesticide analysis grade or reagent special grade, heated at 700 °C for 8 hours then cooled slowly in a desiccator
- Sodium chloride : pesticide analysis grade or reagent special grade, heated at 700 °C for 8 hours then cooled slowly in a desiccator
- Purified water : wash twice with dichloromethane, and finally with hexane
- Purified 5% sodium chloride solution : wash twice with dichloromethane, and finally with hexane
- 5% hydrated silica gel : activate "Wako gel C-200," or equivalent, at 130 °C overnight, then cool slowly in a desiccator. Place 100 g of the silica gel into a Erlenmeyer flask fitted with a glass stopper, add 5 mL of purified water, putting the stopper in, and leave for 4 -5 hours. Shake occasionally until evenly mixed.
- Purified charcoal : wash "Darco G-60 charcoal," or equivalent, with benzene, then wash with

acetone and then benzene again. Filter through glass fibre filter, and wash with a small amount of acetone. Air dry, then dry in an oven at 130 °C, and then grind to a powder in a mixing bowl. Dry again in the oven at 130 °C, then finally store in a desiccator.

- Reduction copper : reduction copper for analysis of organic elements (60 - 80 mesh). Store under nitrogen gas, and wash with the solvent being used in the experiment immediately before use.

B. Apparatus

- Silica gel columns for water samples : use a glass column (length, 30 cm; internal diameter, 1 cm) packed with 1 g of 5% hydrated silica gel. Use hexane to add the silica gel as slurry. Lay 2 cm of anhydrate sodium sulfate onto the top of the silica gel.
- Silica gel columns for sediment samples : use a glass column (length, 30 cm; internal diameter, 1 cm) packed with 5 g of 5% hydrated silica gel. Use hexane to add the silica gel as slurry. Lay 2 cm of anhydrate sodium sulfate onto the top of the silica gel.
- Activated charcoal column : use a glass column (length, 30 cm; internal diameter, 1 cm) packed with 10 g of anhydrous sodium sulfate which contains 2.5 % activated charcoal. Use hexane containing 30 % acetone to load the packing material into the column. Lay 2 cm of anhydrous sodium sulfate onto the top of the packing material.
- Kuderna-Danish (KD) sample concentration apparatus
- Separating funnel
- Ultrasonic irradiation instrument (ultrasonic bath is also OK)
- Centrifuge : use a capable of spinning a 100 mL centrifuge tube at 3,000 rpm
- Gas chromatograph / mass spectrometer (GC/MS) : magnetic field type or quadrupole type with data analysis apparatus, and which have prementioned function.

III.2.1.5 Experimental

A. Sample Preparation (note 1)

a) Water samples

Add 50 g of sodium chloride and the prescribed amount of surrogate (note 2) to 1 L of water sample and mix well. Add 50 mL of hexane and shake for 10 minutes. Separate the hexane layer. Repeat this extraction twice, combine the hexane layers, dehydrate with anhydrous sodium sulfate, filter, then concentrate (reduce) the hexane solution to 5 mL using the KD concentrator. Add the internal standard, then concentrate further to 1 mL by evaporating the solution under a stream of nitrogen. Finally, introduce the concentrated extract into the GC/MS for analysis (note 3).

b) Sediment samples

Put 50 g of wet mud into a 100 mL centrifuge tube, add the prescribed amount of surrogate and mix well. Add 50 mL of acetonitrile and shake for 10 minutes. Conduct an ultrasonic extraction of the mixture by placing the sample in the ultrasonic bath for 10 minutes. Centrifuge the sample at 3000 rpm for 10 minutes, and collect the supernatant liquid. Repeat this extraction procedure three times. Combine the acetonitrile extracts. Place the extracts in a 200 mL separating funnel. Saturate the extracts with hexane by slowly dripping hexane into the mixture. Then add a further 10 mL of hexane and shake for 5 minutes. Collect the acetonitrile layer. Add 20 mL of an aqueous solution of acetonitrile (5 % water, 95% acetonitrile) to the hexane layer. Shake. Separate the acetonitrile layer.

Combine the two acetonitrile extracts, and add this mixture to 500 mL of 5 % sodium chloride solution in a 1 L separating funnel. Add 50 mL of hexane and shake for 5 minutes. Collect the hexane layer. Keep the aqueous phase. Wash the hexane layer with 20 mL of 5 % sodium chloride solution. Collect the hexane layer. Combine the aqueous phase with the aqueous phase collected in the previous step. Extract the combined aqueous phases with another 50 mL of hexane. Collect the hexane layer. Wash this hexane layer with 5 % sodium chloride solution, then combine with the hexane layer collected in the previous step. Dehydrate this hexane solution with anhydrous sodium sulfate, then concentrate (reduce) the hexane solution to 5 mL using the KD concentrator. Add the internal standard, then concentrate further to 1 mL by evaporating the solution under a stream of nitrogen. Finally, introduce the concentrated extract into the GC/MS for analysis.

B. Sample Clean-up (note 4)

a) Water samples (note 3)

Load the concentrated solution obtained after KD concentration in part III.2.1.5 A (a) onto a silica gel column. Elute the column with 20 mL of hexane. Collect the first 20 mL of hexane solution eluting from the column. This first fraction contains all the target compounds mentioned earlier except tributyl phosphate, β-HCH, dieldrin. Elute the column with a solution of hexane : acetone (90 : 10). Collect the next 20 mL of solvent eluting from the column. This second fraction contains tributyl phosphate, β-HCH, dieldrin. Concentrate each fraction up to about 5 mL using a KD concentrator. Add the internal standard, then concentrate further to 1 mL by evaporating the solution under a stream of nitrogen. Finally, introduce the concentrated extract into the GC/MS for analysis (note 5).

b) Sediment samples

Load the concentrated solution obtained after KD concentration in part III.2.1.5 A (b) (note 6) on to a silica gel column. Elute the column with 20 mL of hexane. Collect the first 20 mL of hexane solution eluting from the column (first fraction). Elute the column with 50 mL of hexane : acetone (99 :1) (second fraction). Elute the column with 40 mL of hexane : acetone (90 :10) (third fraction). Concentrate the three fractions using a KD concentrator to a final volume of 1 mL. Load the concentrated third fraction onto an activated charcoal column, and elute with 20 mL of hexane : acetone (70 : 30). Concentrate the eluted solution with a KD concentrator up to a final volume of about 1 mL. Add internal standard to the three fractions, then concentrate further to exactly 1 mL by evaporating the solution under a stream of nitrogen. Finally, introduce the concentrated extract into the GC/MS for analysis (note 5).

C. Preparation of blank samples

Add surrogate into purified water (the same amount as added to samples (25 mL for sediment analysis)) and then treat the blank sample in the same manner as real samples.

D. Preparation of standard solutions

Prepare standard solutions for a calibration curve by mixing target compounds, surrogates, and internal standards in hexane. Surrogates and internal standards should be added in the same amount as that added to samples. Target compounds should be added in amounts that, when the standard solution is injected into the GC/MS will produce an MS response near the high end of the linear region of the calibration curve. (if necessary anticipate this concentration based on instrumental detection limit). Prepare at least five concentrations.

E. Analysis

a) Analytical condition of GC/MS

(1) **GC**

- column : fused silica capillary column (30 m x 0.25 mm i.d., 0.25μm)
- liquid phase : methyl silicone or 5% phenylmethyl silicone
- column temperature : 50 °C (1 min) - 6 °C/min - 100 °C - 15 °C/min - 280 °C
- injection temperature : 230 °C
- injection method : splitless method (1 min for purge-off time), 1μL injection
- carrier gas : He, average linear velocity : 40 cm/sec

- inlet temperature : 280 °C

(2) **MS**

- ionisation method : EI
- ionisation current : 300 μA
- ionisation voltage : 70 eV
- ion source temperature : 220 °C
- detection mode : SIM

(3) **Monitor ions (( ) is m/z of confirmatory ion)**

- Target compounds

  HCB : 284 (286), dieldrin : 263 (277), p,p'-DDE : 246 (318), p,p'-DDD : 165 (235), p,p'-DDT : 165 (235), trans-chlordane : 373 (375), cis-chlordane : 373 (375), trans-nonachlor : 407 (409), cis-nonachlor : 407 (409), α-HCH : 181 (219), β-HCH : 181 (219), o-terphenyl : 230, m-terphenyl : 230, p-terphenyl : 230, BHT : 205, benzo(a)pyrene : 252, tributylphosphate : 99 (151, 211)

- Surrogate compounds

  HCB-$^{13}C_6$: 290 (292), p-terphenyl-d_{14} : 244, benzo(a)pyrene-d_{12} : 264

- Internal standard compounds

  naphthalene-d_8 : 136, fluoranthene-d_{10} : 212, perylene-d_{12} : 264

F. Calibration curves

Make calibration curves for HCB, terphenyls and benzo(a)pyrene by the internal standard method using their stable isotope labelled compounds (HCB-$^{13}C_6$, p-terpneyl-d_{14}, and benzo(a)pyrene-d_{12}). For other compounds, make calibration curves by the internal standard method using internal standards (naphthalene-d_8, fluoranthene-d_{10}, perylene-d_{12}) whose retention times are close to the retention times of target compounds.

G. Determination

Determine by the isotope dilution method when adding HCB-$^{13}C_6$, p-terphenyl-d_4, and benzo(a)pyrene-d_{12} into samples. For other compounds, determine by the internal standard method.

III.2.1.6 Notes

(1) Cover and protect from light during analysis because benzo(a)pyrene is easily degraded by light. In general, protect all standard solutions etc. from light.

(2) Add surrogates to the samples in amounts similar to the anticipated amounts of target compounds in the samples.

(3) Conduct silica gel clean-up before adding internal standards if samples contain compounds that interfere with GC/MS analysis.

(4) Determine the elution pattern and recovery rate of each of the compounds during column chromatography before actual analysis, and change conditions if there are any problems.

(5) May analyse combined fractions if there is no interference with GC/MS analysis. In this case, after collecting all fractions, concentrate, then add internal standards for GC/MS analysis.

(6) When the extracts before clean-up have large amount of precipitate, conduct sample clean-up using silica gel column for water analysis as a preliminary clean-up method.

(7) Elemental sulfur interferes with GC/MS analysis, conduct a reduction copper treatment.

(8) Reduction copper treatment : Add 2 g of reduction copper into the eluate, shake vigorously to mix, then collect the eluate.

III.2.2 Analytical method for volatile compounds in ambient air using canisters

III.2.2.1 Target compounds

Acrylonitrile, vinyl chloride monomer, chloroform, 1,2-dichloroethane, dichloromethane, tetrachloroethylene, trichloroethylene, benzene, 1,3-butadiene

III.2.2.2 Outline of analytical methods

Collect the air samples under conditions of constant flow using sampling containers made of stainless steel. Analyse a specified fraction of the sample by capillary GC/MS.

III.2.2.3 Detection limit and determination limit

The optimum (target) determination limits of the above mentioned target compounds are shown in **Table III-2-1**. Follow all analytical procedures from sample concentration to GC/MS measurement using a sample container which is filled with a humidified mixture of standard gas. This mixture should have a concentration close to the estimated minimum determination limit obtained at the time of making calibration curve. Calculate the concentration of the humidified

mixture of standard gas. Calculate the detection limit and determination limit values for each target compound using the following equations, and values obtained for the standard deviation (s) from more than five standard gas samples. However, in the case of the samples which have operating blanks, measure the operating blank, and then choose whichever of the humidified mixture standard gas or operating blank has the larger standard deviation value. If the minimum determination limit value obtained is higher than required to satisfy the survey's purpose or the optimum determination limit given **Table III-2-1**, adjust to make less than purpose value checking apparatus and instruments. Perform measurements more than once to optimise instrument analysis conditions.

detection limit value = 3s ($\mu g/m^3$)

determination limit value = 10s ($\mu g/m^3$)

Table III-2-1 Optimum determination limits of target compounds

Target compounds	Optimum determination limit ($\mu g/m^3$)
acrylonitrile	0.01
vinyl chloride monomer	0.1
chloroform	0.04
1,2-dichloroethane	0.04
dichloromethane	2.0
tetrachloroethylene	20
trichloroethylene	20
1,3-butadiene	0.004
benzene	0.3

III.2.2.4 Reagents and apparatus

A. Reagents

- Zerogas : Use high purity nitrogen or purified air with concentrations of target compounds lower than optimum (target) determination limit value. Check the concentration of the target compounds before the use. It is important that the zerogas does not contain organic compounds, and it is desirable that for all compounds except target compounds the total hydrocarbon content is less than 0.01 ppm, carbon monoxide less than 0.05 ppm, carbon dioxide less than 0.3 ppm, humidity less than 2 ppm, the dew point less than -70 ◦C, and purity more than 99.999%.
- Humidified zerogas : Prepare humidified zerogas by bubbling zerogas through water (note 1) (relative humidity at 25 ◦C is about 60 - 70 %). Or prepare humidified zerogas by passing zerogas into an evacuated container and inject water by syringe (about 100 μL for 6 L container : relative humidity at 25 ◦C increasing pressure is about 50 %). In either case, be

careful about contamination when humidifying.

- Standard reagent : special grade reagents of purity more than 98 % or similar grade.
- Standard compounds : special grade reagents of purity more than 98 % or similar grade. When target compounds are gasses, permeation tubes can be used.
- Stock standard gas (1 μg/mL) : Use commercially available standard gases in cylinders. Convert gas concentration from ppm (μL/L) on the commercial gas cylinder into weight / volume concentration (μg/L) using the equation 273xM(22.4x(273+t)) (M : molecular weight, t : temperature). The concentration of stock standard gas (1 μg/mL) is a rough standard - it can be changed for each target compound, bearing in mind the compound's sensitivity or atmospheric concentration. (note 2)
- Humidified standard gas mixture (0 - 0.1 ng/mL) : Use thoroughly washed, non-contaminated sampling containers, and prepare the humidified standard gas mixture at five levels of concentration between 0 - 0.1 ng/mL by diluting the stock standard gas (1 μg/mL) with humidified zerogas (using pressure dilution, capacity ratio mixture, or air flow ratio mixture methods to meet the determination range of each target compound). Prepare humidified standard gas mixture by pressurising (about 200 kPa). (note 3)
- Internal standard compounds : toluene-d_8, fluorobenzene, chlrorobenzene-d_5 etc.
- Internal standard stock gas (1 μg/mL) : Use commercial standard gases.
- Humidified internal standard gas (0.01 ng/mL) : dilute internal standard stock gas with humidified zerogas. Use a different container. Dilute with sufficient gas to meet the optimised concentrations as it is used. (note 4)

B. Apparatus and instruments

a) Sampling equipment

Collect samples using sample containers which are under reduced pressure (less than 13 Pa (about 0.1 mmHg)). Draw the samples into the container under conditions of constant flow using either a mechanical mass flow controller or a thermal mass flow controller. These instruments' have different functions which depend on the sampling methods. There are essentially two methods : subatomospheric pressure sampling method, where sampling is terminated while the container is still at less than atmospheric pressure, and the pressurized sampling method, where sample is pumped into the container to above atmospheric pressure. Pressures of up to 200 kPa (about 1500 mmHg) are attained.

Those parts of the sampling equipment which touch the target compounds, or the tubing, are made of stainless steel inactivated by electrolytic polishing or aluminium coating or an equivalent process. Where non-metallic material is used, it is necessary to check that there is no interaction

with target compounds beforehand. Such materials, except fluorine resin or polyimide, should be avoided where possible.

Prior to sampling, wash the apparatus thoroughly to avoid contamination. When sampling, check for leak after setting up the equipment, wash the sampling apparatus and replace the internal atmosphere by sample air. Endeavour to minimise all risk of contamination and adsorption.

(1) Subatomospheric pressure sampling apparatus

This comprises a filter, mass flow controller, valve, sample container and a pressure meter with which one can check the pressure inside the sample container. The pressure at the end of sampling should be within the range at which the mass flow controller can maintain a constant flow, and this pressure is generally about 80 kPa (80 % of atmospheric pressure).

(2) Pressurized sampling apparatus

This comprises a filter, pump, mass flow controller, valve, pressure meter sample container and a pressure meter with which one can check the pressure inside the sample container. The pressure at the end of sampling is about 200 kPa (about 1500 mmHg).

(i) Sample container

The container capacity should be between 3 - 15 L. Use stainless steel containers whose interior surfaces have been inactivated by electrolytic polishing or aluminium coating. If such stainless steel containers are not available, use any other container made of material of similar or better quality. Use containers from which target analyte recovery rates and storage quality have been confirmed. Ensure the containers do not leak, and can withstand pressurisation to about 300 kPa (about 2200 mmHg), and depressurisation to about 13 Pa (about 0.1 mmHg).

(ii) Mass flow controller

Use a stainless steel mass flow controller whose interior surfaces have been inactivated by electrolytic polishing or aluminium coating. If such stainless mass flow controllers are not available, use any other flow controller made of material of similar or better quality. Use a mass flow controller which can control the flow rate to within 2 - 50 mL/min, control the accuracy of the flow rate at more than 20 kPa (about 150 mmHg), and can control pressure differences to within ± 10 % of the set flow rate. Ensure the flow controller does not leak, and can withstand pressurisation to about 300 kPa (about 2200 mmHg), and depressurisation to about 13 Pa (about 0.1 mmHg). Connection parts are made of stainless or aluminium coated by oxidation or similar or better quality.

(iii) Pump : for analysis of harmful air pollution compounds

(iv) Valve : for analysis of harmful air pollution compounds

(v) Filter : made of stainless, and mesh size is less than 7 μm, generally around 2 μm.

(vi) Pressure meter : for analysis of harmful air pollution compounds

b) Sample introducing apparatus

(1) Concentrator (adsorption concentrator tube or low temperature concentrator tube)

Use adsorption concentrator tubes when one wants to concentrate target compounds by means of adsorption onto a sorbent material. Adsorption concentrator tubes are generally glass tubes (1 - 3 mm i.d.), glass lined stainless tubes, or stainless steel tubes packed with porous polymer beads or carbon adsorbent (either singly or combined). Both ends of the tube are usually packed with deactivated quartz wool. Adsorption concentrator tubes can be heated at 180 °C for desorption. However the maximum heating temperature depends on the adsorbent.

Low temperature concentrator tubes are used when one wants to concentrate target compounds by means of adsorption onto a sorbent material at low temperatures. Low temperature concentrator tubes can be heated more than 90 ◦C for desorption. Low temperature concentrator tube are generally glass tubes (1 - 6 mm i.d.), glass lined stainless tubes or stainless tubes packed with deactivated glass beads (250 - 500 μm diameter), quartz beads (250 - 500 μm diameter), quartz wool or deactivated diatomaceous earth (250 - 500 μm diameter) etc. (note 5)

(2) Cryo focus

The cryo-focus is a trap attached to the front of the capillary column. The cryo-focus is used to narrow the band of target compounds entering the column. Generally, the cryo-focus is a fused silica or deactivated stainless steel chambered tube (0.3 - 0.6 mm i.d.), the temperature of which can be controlled at below -100 °C, and yet heated to more than 80 °C instantly.

(3) Dehumidifier

The dehumidifier, as its name suggests, dehumidifies the samples before concentration. Dehumidification is achieved using high molecular membranes which selectively transport water (e.g. Nafion Dryer - Perma Pure Products), dry purge methods, or other methods which can exclude volatile compounds selectively from water (e.g. by purge & trap theory, etc). Polar compounds such as acrylonitrile shouldn't be effected by the dehumidifier.

III.2.2.5 Experimental

A. Sampling

Sample by either the subatomospheric pressure sampling method or the pressurized sampling method. At the same time, make a travel blank and for 10% of samples, collect two samples for analysis (note 7). For example, when 10 samples are taken, prepare at least one travel blank, and for at least one sample collect two samples for analysis. If samples are taken by the subatomospheric pressure sampling method, record the pressure before and after pressurising samples, and calculate dilution rate after pressurising.

B. Concentration of samples

If the samples were collected by the subatomospheric pressure sampling method, pressurise the sample container with humidified zerogas. Connect the sample containers to the sample introduction apparatus, and concentrate the samples at a constant flow rate. If the samples were collected by the pressurized sampling method, connect the sample containers directly to the sample introduction apparatus, and concentrate the samples at a constant flow rate. Control the flow rate by using the mass flow controller, and end the concentration procedure after a prescribed period of time has elapsed. Determine the amount of samples which has been concentrated by using the concentration of target compounds and the sensitivity of the analytical instruments. At this time concentrate a known amount of humidified internal standard gas. The amount of gas used should be the same as the amount gas used when making calibration curves.

Desorb target compounds by heating the concentrator (e.g. at 180 °C for the adsorption concentrator, at 90 °C for the low temperature concentrator), and re-concentrate on the cryo-focus. However, if there is leakage (a pressure difference of more than ±10 kPa between the end of sampling and analysis inside the container), stop analysing.

C. Preparation of blank samples

a) Operational blank

After washing, conduct the whole procedure from sample concentration to GC/MS measurement using sample containers pressurised up to 200 kPa (about 1500 mmHg) with humidified zerogas, and gain operational blank value. (note 8)

b) Travel blank

Conduct the whole procedure from sample concentration to GC/MS measurement using sample containers for travel blank experiments, and measure target compound quantity in the

concentrated samples. (note 9)

D. Measurement

Measurement is done by SIM method or the scanning method using the following GC/MS conditions. Monitor ions are shown in **Table III-2-2**. When target compounds are detected, obtain the peak area or height of the primary ion of the target compounds and internal standards, and calculate the weight of the target compounds from their respective calibration curves.

An example of GC/MS conditions

- column : methylsilicone capillary column, 0.25 mm i.d., length 60 m, film thickness 0.25 μm
- column temperature : 40 °C (5 min) - 4 °C/min - 140 °C
- interface temperature : 220 °C
- carrier gas : He, 1 - 3 mL/min
- ion source temperature : 200 °C

Table III-2-2 Monitor ions

Compounds	Primary ions	Secondary ions
Acrylonitrile	52	53
Vinyl chloride monomer	62	64
Chloroform	83	85
1,2-Dichloromethane	62	64
Dichloromethane	84	86, 49
Tetrachloroethylene	166	164, 129
Trichloroethylene	130	132, 95
Benzene	54	53
1,3-Butadiene	78	77
Toluene-d_8	98	-
Fluorobenzene	96	-
Chlorobenzene-d_5	117	-

E. Calibration curves

(a) Connect a container of minimum concentration humidified standard gas to the sample introduction apparatus, and concentrate 100 mL of that mixture in the concentrator. Then add 100 mL of humidified internal standard gas into the concentrator and concentrate together. Thereafter, operate all analytical procedures from sample concentration to GC/MS analysis to record chromatograms of each target compounds. Repeat the same procedure using a container of humidified standard gas mixture of a higher concentration. (note 10)

(b) Choose an injection volume which will produce concentrations in the middle of the GC/MS calibration curve. Obtain this information from the standard gas mixture calibration curves obtained from procedure (a) above. Obtain the ratio of the peak intensities of the primary ion and secondary ion of each target compound. (note 11)

(c) Obtain the ratio of intensities of the peak intensities of the primary ion and secondary ion of each target compound at each concentration, and check if they coincide with the intensity ratio of each target compound obtained using procedure (b) above. (note 12)

d) Calculate the ratio of peak intensities of the primary ion of each target compound and the internal standard, and make calibration curves from the ratio of peak intensity and quantity of each target compound.

F. Determination and calculation

Calculate the concentration of each target compound in the air from the results obtained during analysis of samples and travel blanks by using the following formula.

$$C = \frac{n \times (As - At)}{v \times 293/(273 + t)} \times \frac{Pa}{101.3}$$

C : concentration of the target compound in the air at 20 °C (μg/m^3)

n : dilution magnification (for subatomospheric pressure sampling method. n=1 for pressurized sampling)

As: weight of each target compounds in the concentrated samples (ng)

At: travel blank of each target compounds (ng)

v : concentrated amount of samples for analysis. use operational blank value if it is the same.

t : temperature at sample analysis

Pa: atmosphere pressure at sample analysis

III.2.2.6 Notes

(1) Be careful to use commercial mineral water because salts can be deposited at connections and on the inside of the sample containers. When operational blank values are not stable, the use of purified water (water which has been boiled to half of its original volume on a gas burner, and cooled evaporative cooling in a stream of helium gas) is recommended.

(2) To prepare stock standard gas, accurately weigh around 100 mg of a target compound standard (either individually or as part of a mixture) and inject the standard material into a 1 L glass vacuum bottle (whose internal volume is accurately measured by replacing with high quality nitrogen and back to atmospheric pressure). Heat the vacuum bottle to more than 60 °C to evaporate the standard compounds. Dilute the thoroughly volatilised, thoroughly mixed gas mixture 100 times in a second vacuum bottle to make the standard stock gas. If the target compound standard is a gas contained in a cylinder, the 100 mg of target compound can be measured using a gas-tight gas syringe. In this case, the amount of material injected is determined using the following formula : v (mL) = 100x(22.4x(273+t)/273M (M : molecular weight, t : atmospheric temperature), If the target compound standard is a liquid, the 100 mg of target compound can be measured using a micro syringe. In this case, the amount of material injected is determined using the following formula : v (μL) = 100/ρ (ρ: specific gravity or density).

(3) Pressure dilution is a kind of capacity ratio mixing. The dilution ratio is calculated from pressure increments. In the case of dilution using humidified zerogas prepared in the container, be careful that the relative humidity does not become lower because of dilution.

(4) To prepare internal standard stock gas, accurately weigh around 100 mg of the standard and inject the standard material into a 1 L glass vacuum bottle (whose internal volume is accurately measured by replacing with high quality nitrogen and back to atmospheric pressure). Heat the vacuum bottle to more than 60 °C to evaporate the standard compounds. Dilute the thoroughly volatilised, thoroughly mixed gas 100 times in a second vacuum bottle to make the standard stock gas. If the internal standard is a gas contained in a cylinder, the 100 mg of the standard can be measured using a gas-tight gas syringe. In this case, the amount of material injected is determined using the following formula : v (mL) = 100x(22.4x(273+t)/273M (M : molecular weight, t : atmospheric temperature). If the internal standard is a liquid, the 100 mg of the standard can be measured using a micro syringe. In this case, the amount of material injected is determined using the following formula : v (μL) = 100/ρ (ρ: specific gravity or density).

(5) Liquid nitrogen (b.p.=-196 °C) and liquid oxygen (b.p.=-183 °C) can be used as the refrigerant for low temperature concentration at concentrator. However, liquid nitrogen

may clog the tubing because of oxygen condensation from samples. Also, check the tubing regularly, because tubing clogging may be caused by water or carbon dioxide etc. at the time of low temperature concentration.

(6) Be careful about trap tube because water or carbon dioxide may cause clog tubing. Use of the trap tubing may omitted if sample recovery from the concentrator is rapid, and the component peaks which come out at the beginning show enough shape to be determined.

(7) Confirm that the difference in determined concentration of target compounds between the two analyses is less than 30 %, and more than determination limit value. If the difference is more than 30 %, as a matter of principle record the analyses as 'not determined', check the reason, and repeat the sampling. Perform this operation before sample measurement. If the value when converted to atmospheric concentration is over target determination limit value, repeat the measurement after re-washing and machine adjustment, to obtain operational blank values of low enough concentration, and measure samples.

(8) If a value of a target compound in travel blank is the same (or smaller) as an operational blank value, calculate concentration by subtracting the operational blank value from a measurement value of a sample.

(9) If a travel blank value is larger than an operational value; confirm that (a) the determination limit value (10s : converted into atmospheric concentration) calculated from standard deviation (s) of travel blank values from more than three samples is smaller than the optimum determination limit in Table III-2-1, or (b) even if the determination limit value calculated from the travel blank values is larger than the optimum determination limit, a measured value for the sample is larger than the determination limit value from travel blank values. If either of these is satisfied, calculate concentration by subtracting the travel blank value from the sample measurement value. However, if none of these, as a matter of principle treat as 'not determined'. In this case repeat sampling after removing causes of contamination.

(10) In the case which it is confirmed that recovery from container is from 80 to 120 %, standard stock gas can be injected into concentrator directly using a gas syringe.

(11) This operation is in order to confirm target compounds and use every calibration making.

(12) If the intensity ratio of any of the target compounds exceeds 90 - 110 % of the value which is calculated from calibration curves, repeat the analysis of the concentration of the standard gas.

III.2.3 Analysis of dioxins in sediments and biological samples

III.2.3.1 Target compounds

Dioxins (polychlorinated dibenzo-*p*-dioxin (PCDDs) and polychlorinated dibenzofuran (PCDFs))

III.2.3.2 Outline of analytical methods

Extract the dioxins from sediments and biological samples, treat the extracts with sulfuric acid and then clean-up the extracts by column chromatography. Finally, determine PCDD and PCDF concentrations in the extracts by high resolution gas chromatography / mass spectrometry (HRGC/HRMS).

III.2.3.3 Detection limit and determination limit

Target (optimum) determination limits are shown in **Table III-2-3**.

Table III-2-3 Target determination limits

Compounds	Target determination limits
Tetra CDD	0.1 pg/g
Tetra CDF	0.1 pg/g
Penta CDD	0.1 pg/g
Penta CDF	0.1 pg/g
Hexa CDD	0.2 pg/g
Hexa CDF	0.2 pg/g
Hepta CDD	0.2 pg/g
Hepta CDF	0.2 pg/g
Octa CDD	0.5 pg/g
Octa CDF	0.5 pg/g

Sediments : per dry weight
Living things : per wet weight

Measure 1 μL of standard solution of a concentration near the lowest concentration which could be determined at the time of making calibration curves (the standard concentration should be around detection limit). Determine the value of this solution (A pg). Calculate the concentration of samples from the concentration formula. Repeat this measurement five times and calculate standard deviation(s) of the measurements. Obtain detection limits and limits of determination of dioxins from the following formula.

Detection limits = 3s (ng/g) (sediment : per dry weight, living things : per wet weight)

Determination limits = 10s (ng/g) (sediment : per dry weight, living things : per wet weight)

If the value for the operational blank is not zero, measure operational blank in the same way, calculate the standard deviation, and then calculate detection limits and determination limits using whichever standard deviation from the standard solution or the operational blank is the larger. If the determination limits are larger than the target determination limits, check the apparatus and instruments, and adjust conditions to make the determination limits less than the target values.

III.2.3.4 Reagents and apparatus

A. Reagents

- methanol, acetone, hexane, toluene, dichloromethane : pesticide residue analysis grade or PCB residue analysis grade
- nonane, decane, isooctane : pesticide residue analysis grade
- hexane washed water : distilled water thoroughly washed with hexane
- sulfuric acid, hydrochloric acid : reagent special grade or higher grade
- anhydrous sodium sulfate : pesticide residue analysis grade or PCB residue analysis grade
- potassium hydroxide, sodium hydroxide : reagent special grade
- silica gel : wash silica gel for column chromatography with methanol, place in a beaker to a maximum depth (thickness) of 10 mm thickness, dry the layer of silica gel at 130 °C for about 18 hours, then cool in a desiccator over 30 minutes.
- basic alumina : aluminum oxide 90 (activity 1) 70 - 230 mesh (Merck)
- copper powder : Merck, washed with hexane beforehand
- silica gel with activated charcoal : for dioxin analysis
- target compounds : target compounds of dioxins are shown in **Table III-2-4**. Standard mixtures are commercially available.
- internal standard : internal standard for the use is shown in **Table III-2-5**. Standard mixtures are commercially available.

Table III-2-4 Dioxins for analytical target

number of chlorines	dioxins	dibenzofurans
tetra chlorinated	1,3,6,8/1,3,7,9-T_4CDD Isomer Pair 2,3,7,8-T_4CDD	1,3,6,8-T_4CDF 2,3,7,8-T_4CDF
penta chlorinated	1,2,3,7,8-P_5CDD	1,2,3,7,8-P_5CDF 2,3,4,7,8-P_5CDF
hexa chlorinated	1,2,3,4,7,8-H_6CDD 1,2,3,6,7,8- H_6CDD 1,2,3,7,8,9- H_6CDD	1,2,3,4,7,8- H_6CDF 1,2,3,6,7,8- H_6CDF 1,2,3,7,8,9- H_6CDF 2,3,4,7,8,9- H_6CDF
hepta chlorinated	1,2,3,4,6,7,8-H_7CDD	1,2,3,4,6,7,8-H_7CDF 1,2,3,4,7,8,9-H_7CDF
octa chlorinated	1,2,3,4,6,7,8,9-O_8CDD	1,2,3,4,6,7,8,9-O_8CDF

Table III-2-5 Internal standards

Number of chlorines	Internal standards for dioxins	Target dioxins	Internal standards for dibenzofurans	Target dibenzofurans
tetra chlorinated	$^{13}C_{12}$2,3,7,8-T_4CDD	for determination of T_4CDD	$^{13}C_{12}$2,3,7,8-T_4CDF	for determination of T_4CDF
penta chlorinated	$^{13}C_{12}$1,2,3,7,8-P_5CDD	for determination of P_5CDD	$^{13}C_{12}$1,2,3,7,8-P_5CDF	for determination of P_5CDF
hexa chlorinated	$^{13}C_{12}$1,2,3,6,7,8-H_6CDD	for determination of H_6CDD	$^{13}C_{12}$1,2,3,6,7,8-H_6CDF	for determination of H_6CDF
hepta chlorinated	$^{13}C_{12}$1,2,3,4,6,7,8-H_7CDD	for determination of H_7CDD	$^{13}C_{12}$1,2,3,4,6,7,8-H_7CDF	for determination of H_7CDF
octa chlorinated	$^{13}C_{12}$1,2,3,4,6,7,8,9-O_8CDD	for determination of O_8CDD	$^{13}C_{12}$1,2,3,4,6,7,8,9-O_8CDF	for determination of O_8CDF

B. Apparatus

a) Extraction apparatus

- glassware : separating funnels, Erlenmeyer flasks with ground-glass joints and stoppers, round bottom flasks, chromatography columns, test tubes for concentration. All glassware should be washed with hexane beforehand.
- rotary evaporator
- mechanical shaker

b) Apparatus for clean-up

- silica gel chromatography : chromatography column (i.d., 10 mm; length, 300 mm) packed with 3 g of activated silica gel. Mix the silica gel with sufficient hexane to form a free flowing slurry and load into column. Add a 10 mm layer of anhydrous sodium sulfate onto the top of the silica gel. Wash the packed material thoroughly with hexane.
- alumina column chromatography: chromatography column (i.d., 10 mm; length, 300 mm)

packed with 15 g of basic alumina. Mix the alumina with sufficient hexane to form a free flowing slurry and load into column. Add a 10 mm layer of anhydrous sodium sulfate onto the top of the alumina. Wash the packed material thoroughly with hexane.

- activated charcoal column chromatography: chromatography column (i.d., 10 mm; length, 300 mm). Packed the column first with a 10 mm layer of anhydrous sodium sulfate, then with 1 g of a mixture of silica gel and activated charcoal. Finally, add 10 mm layer of anhydrous sodium sulfate onto the top of the silica gel. Wash the packed material thoroughly with hexane.

c) GC/MS

- GC : GC for capillary column
- MS : double-focusing type, more than 10,000 resolution

III.2.3.5 Experimental

A. Sample preparation

a) Sediment samples

Weigh 50 - 100 g of air-dried mud (note 2) into a round bottomed flask. Add 0.2 mL of a hexane solution containing 0.01 μg/mL each of 10 kinds of internal standard (note 3). Then add 200 mL of acetone-hexane solution (1:1 v/v) and 2 g of copper powder, and shake for one hour. Filter the extracts, wash the flask and the residue with 100 mL of acetone-hexane solution (1:1 v/v), combine the filtrates, and place into a separating funnel. Add 200 mL of purified water, and shake for 10 minutes. After settling, discard the aqueous phase, add 100 mL of 2 % sodium chloride solution, then shake. After settling, discard the aqueous phase, add 100 mL of 2 % sodium chloride solution, then shake for 10 minutes. After settling discard the aqueous phase, dehydrate hexane layer by passing through the funnel packed with 20 g of anhydrous sodium sulfate, and concentrate by rotary evaporation to about 20 mL using a bath temperature of less than 40 °C. Add 20 mL of 1 M potassium hydroxide solution into the concentrated organic phase, shake, settle, and make up the hexane layer a certain volume (L : mL). Separate about the half amount (s : mL) as a sample for clean-up (note 4).

b) Biological samples

Weigh 100 g of homogenised samples into a round bottomed flask. Add 0.2 mL of a hexane solution containing 0.01 μg/mL each of 10 kinds of internal standard (note 3). Then add 200 mL of

2M potassium hydroxide and 150 mL of methanol, shake or stir, and leave the mixture overnight at room temperature. Transfer the mixture to a separating funnel, add 150 mL of hexane, then shake for 10 minutes. After settling, separate the hexane layer. Wash the aqueous phase with a further 100 mL hexane. After settling, separate the hexane layer. Repeat this procedure twice. Combine the hexane extracts, add 200 mL of 2 % sodium chloride solution, then shake gently (swirl or rotate the mixture). After settling, discard the aqueous phase. Add 100 mL of 2 % sodium chloride to the organic phase (hexane layer), and repeat the wash procedure. After settling discard the aqueous phase, dehydrate hexane layer by passing through the funnel packed with 20 g of anhydrous sodium sulfate, and concentrate by rotary evaporation to about 20 mL using a bath temperature of less than 40 °C. Make up a certain volume (L : mL). Separate about the half amount (s : mL) as a sample for clean-up (note 4).

B. Sample clean-up

(1) Sulfuric acid washing

Take a certain amount (s : mL) of the extracts into a separating funnel, add 50 - 150 mL of hexane and 10 mL of concentrated sulfuric acid, and shake gently. Allow the mixture to settle, then discard the sulfuric acid layer. Repeat this procedure until the sulfuric acid layer is colourless. Wash the hexane layer 3 or 4 times with 10 mL of hexane-washed water, then add 10 mL of 5 % sodium bicarbonate solution, and shake gently. After settling discard the aqueous phase, dehydrate hexane layer by passing through the funnel packed with 10 g of anhydrous sodium sulfate, and concentrate the samples for silica gel column chromatography by rotary evaporation to about 5 mL using a bath temperature of less than 40 °C.

(2) Silica gel column chromatography

Load samples from (1) onto the column, and elute slowly (eluate drip speed of 1 drop per second) with 200 mL of hexane. Concentrate the eluate samples for alumina column chromatography with a rotary evaporator to about 5 mL using a bath temperature of less than 40 °C.

(3) Alumina column chromatography (note 5)

Load sample from (2) onto the column, and elute slowly (eluate drip speed of 1 drop per second) with 30 mL of dichloromethane-hexane solution (2 : 98 v/v) (first fraction). Then elute slowly (eluate drip speed of 1 drop per second) with 200 mL of dichloromethane-hexane solution (6 : 4 v/v) (second fraction). Place the second fraction into a round bottomed flask, and concentrate the samples for activated charcoal column chromatography by rotary evaporation to about 0.5 mL

using a bath temperature of less than 40 °C.

(4) Activated charcoal column chromatography

Wash activated charcoal column with toluene, and replace by hexane thoroughly. Load the concentrate from (3) onto the column, and elute slowly (eluate drip speed of 1 drop per second) with 100 mL of dichloromethane-hexane solution (1:3 v/v). This fraction contains PCBs. Then elute dioxins with 200 mL of toluene. Concentrate the toluene layer by rotary evaporation to about 5 mL using a bath temperature of less than 40 °C. Concentrate further to 1 mL by blowing nitrogen over the surface of the solution, add 0.5 mL of decane and concentrate up to 100 μL (E : mL) for GC/MS analysis (note 6).

C. Preparation of blank samples

Prepare blank samples using the same operating procedures as used for samples (note 7). Also analyse some samples twice for quality control (note 8).

D. Preparation of standard solutions

- standard solutions (50ng/mL) : dilute commercial standard mixture solution (50μg/mL) 1,000 times with toluene
- internal standard solutions (50ng/mL) : dilute commercial standard mixture solution (50μg/mL) 1,000 times with toluene

E. Analysis

a) GC/MS conditions

(1) **GC**

- column

(a) fusued silica SP-2331 capillary column, 0.25 mm i.d. x 30 m, 0.2 μm

(b) fused silica DB-17 capillary column, 0.25 mm i.d. x 30 m, 0.15 μm

- column temperature

(a) 150 °C (1 min) - 15 °C/min - 200 °C (5 min) - 3 °C/min - 250 °C (30 min)

(b) 150 °C (1 min) - 15 °C/min - 270 °C (30 min)

- injection port temperature : 260 °C
- injection method : splitless (90s for purge-off time)

(2) **MS**

- resolution : 7000 - 10000

- ionisation method : EI
- ionisation voltage : 30 eV
- ionisation current : 500 μA
- ion source temperature : 260 °C
- detection method : SIM

Introduce the standard for mass adjustment (PFK) into the MS, and adjust the mass pattern, resolution (more than 10000, 10 % Valley), etc. by using the mass adjustment program to meet the analysis purpose. **Table III-2-6** shows monitor ions.

Table III-2-6 List of monitor ions

	Chlorinated substituent	M^+	$(M+2)^+$	$(M+4)^+$
Target compounds	T_4CDDs	319.8965	321.8936	
	P_5CDDs	353.8576	355.8546	357.8516
	H_6CDDs		389.8157	391.8127
	H_7CDDs		423.7766	425.7737
	O_8CDDs		457.7377	459.7348
	T_4CDFs	303.9016	305.8987	
	P_5CDFs		339.8597	341.8567
	H_6CDFs		373.8207	375.8178
	H_7CDFs		407.7818	409.7789
	O_8CDFs		441.7428	443.7399
Internal standards	$^{13}C_{12}T_4CDD$	331.9368	333.9339	
	$^{13}C_{12}P_5CDD$	365.8978	367.8949	369.8919
	$^{13}C_{12}H_6CDD$		401.8559	403.8530
	$^{13}C_{12}H_7CDD$		435.8169	437.8140
	$^{13}C_{12}O_8CDD$		469.7779	471.7750
	$^{13}C_{12}T_4CDF$	315.9419	317.9389	
	$^{13}C_{12}P_5CDF$		351.9000	353.8970
	$^{13}C_{12}H_6CDF$		385.8610	387.8580
	$^{13}C_{12}H_7CDF$		419.8220	421.8191
	$^{13}C_{12}O_8CDF$		453.7830	455.7801

F. Calibration curves

Prepare standard solutions of each chlorinated compound. Make calibration curves using five points, between 0.5 ng/mL and 1 μg/mL (note 9), and include zero. Then add a known amount of internal standards such that the concentration of the internal standard in the standard solutions being used to make the calibration curves is 0.01 μg/mL. Inject 1 μL of standard solution into the GC/MS, and measure. Obtain peak intensity ratio between determinative and confirmative ions of standard solutions which are in the mid-range of the calibration curves for each compound. Check if the peak intensity ratio between determinative and confirmative ions of other concentrations

coincide with those calculated for the mid-range concentration. Obtain peak intensities of each compound and internal standard, and make calibration curves against injection volumes.

G. Determination and calculation

Inject 1 - 2 μL of samples into the GC/MS system. Obtain peak intensities of the determinative ions of each chlorinated compound and the internal standards (note 10). Calculate the weight (As, pg) of each of the chlorinated compounds in the test sample injected from the calibration curves (note 11, 12). Calculate dioxin concentrations in the samples using the following formula.

$$C = \frac{As \times E \times L}{v \times s \times m}$$

where, C is the concentration of each of the chlorinated compounds in sample (ng/g) (sediment - ng/g based on dry weight, living things - ng/g based on wet weight)

As : weight of each chlorinated compound in the sample solution injected into the GC/MS (pg)

E : volume of sample solution for GC/MS analysis

v : volume injected into the GC/MS (μL)

L : original volume of extracts (mL)

s : amount of original extract separated for clean-up (mL)

m : amount of sample (g)

Calculate 2,3,7,8-T_4CDD conversion concentration (TEQ) by multiplying 2.3.7.8-T_4CDD Toxicity Equivalency Factor (TEF) by the measured concentration, and sum up them.

III.2.3.6 Notes

(1) Determine the most appropriate packing materials and solvents for column chromatography after performing fractionation experiments using standard compounds.

(2) Dry (air dry) samples trying not to evaporate dioxins from samples, or contaminate the samples with dioxins.

(3) It is desirable to add all internal standard compounds for determination, but add at least one internal standard for each chlorine numbers. All of these internal standards may at times interfere with analysis depending on the MS resolution set up, so consider and investigate

analytical conditions which don't produce interference. Use $^{13}C_{12}$-1,2,3,4-T_4CDD or $^{37}C1_4$-T_4CDD for confirmation of recovery rate of dioxins during sample preparation, $^{13}C_{12}$-1,2,3,4,6,7,8,9-O_8CDD for internal standard for determination. In addition, as well as $^{13}C_{12}$-1,3,6,8-T_4CDD, it is also necessary to check if dioxin separation on the chromatography column is done properly.

(4) Store original extracts for a while in case reanalysis of some of the samples is necessary.

(5) The activity of alumina varies depending on the age of the alumina (time of preservation after opening the product). 1,3,6,8-T_4CDD and 1,3,6,8-T_4CDF elute in the first fraction when the activity of the alumina goes down. Also O_8CDD may not elute at the defined hexane - dichloromethane ratio (4 : 6 v/v), and it is necessary to confirm this by fractionation tests.

(6) Conduct syringe spikes in order to obtain correct results. Use internal standards except clean-up spike compounds such as $^{13}C_{12}$1,2,3,4-T_4CDD or $^{13}C_{12}$-1,2,3,4,7,8,9-H_7CDF.

(7) If the values obtained from operational blank values exceed target determination limit values, repeat analysis after re-wash and check instruments to make operational blank values as low as possible, then analyse samples.

(8) Check if difference between the values of duplicate analysis whose concentrations are more than determination limit values, is less than 30 %. If the difference is above 30 %, treat as 'not determined' in principle, check the cause, and take samples again.

(9) This concentration range should include concentrations close to detection limit value and concentrations anticipated to be close to the high end of the linear dynamic range of GC/MS.

(10) Determine if the peak area ratio of monitor ions of SIM chromatograms is almost the same as standards, and within ±15 % of the natural isotope ratio (±25 % if around determination limit). In particular, determine 2,3,7,8- chlorinated isomers if SIM chromatogram peaks give good separation and retention times are almost the same as standards, and relative retention times against corresponding internal standards also agree with standards. For isomers which don't have standards, determine by reference to published articles etc.

(11) If recovery of clean-up spike is out of the range 50 % to 120 %, repeat clean-up from sample extracts and re-analyse.

(12) Conduct determination of 2,3,7,8- chlorinated isomers (17 isomers) using their corresponding standards. Determine other isomers using average of 2,3,7,8- chlorinated isomers of each chlorinated compound as standards.

III.2.4 Analytical methods for polycyclic aromatic hydrocarbons in water, sediment, biological organisms, and air

III.2.4.1 Target compounds

Benzo(a)pyrene(B(a)P),benzo(e)pyrene(B(e)P),benzo(b)fluoranthene(B(b)F),benzo(j)fluoranthene (B(j)F),benzo(k)fluoranthene(B(k)F),benzo(ghi)perylene(B(ghi)P),dibenz(a,h)anthracene(B(ah)A), 3-methylcholanthrene(MC)

III.2.4.2 Outline of analytical methods

In general, the analytical methods for polycyclic aromatic hydrocarbons in sediment, biological organisms, and air are similar to those used for water samples, i.e. after liquid-liquid extraction with hexane, concentrate the sample, conduct silica gel chromatography, and finally determine analyte concentrations by HPLC or GC/MS.

- For sediment and biological samples, first digest the sample using an alkaline (1M KOH-ethanol) solution, centrifuge, dilute with water, extract with hexane, and analyse as per water samples.
- For air samples, collect the samples by using an air sampler with a quartz filter paper, perform ultrasonic extraction with organic solvents, clean-up using SEP-PAK silica cartridges, then analyse as per water samples. Note : because GC/MS analysis cannot separate the three isomers of benzofluoranthene, treat these as one chemical when analysing by GC/MS.

III.2.4.3 Detection limits and determination limits

Detection and determination limits for the target chemicals (**III.2.4.1**) in water, sediment and biological samples are shown in **Table III-2-7**, detection limits for air samples in **Table III-2-8**.

Table III-2-7 Detection limits and determination limits of water, sediment and biological samples

units : μg/mL, μg/g

Sample	Sample amount		Analytical instruments	B(a)P	B(e)P	B(b)F	B(j)F	B(k)F	B(ghi)P	B(ah)A	MC
Water	1L	detection limits	HPLC	0.00005	0.00002	0.00006	0.0006	0.00004	0.00005	0.00004	0.00004
			GC/MS	0.00006	0.00001	0.00002	0.00002	0.00002	0.00004	0.00006	0.00004
		determination limits	HPLC	0.0002	0.00008	0.0002	0.002	0.0001	0.0002	0.0001	0.0001
			GC/MS	0.0002	0.00004	0.00007	0.00007	0.00007	0.0002	0.0002	0.0001
Sediment & living things	20 g	detection limits	HPLC	0.001	0.0006	0.0005	0.030	0.002	0.002	0.002	0.002
			GC/MS	0.003	0.0004	0.001	0.001	0.001	0.002	0.003	0.002

Table III-2-8 Detection limit of air samples

Compounds	Detection limits (pg)
B(a)P	26
B(e)P	14
BF	19
B(g,h,i)P	31
B(a,h)A	53
MC	24

III.2.4.4 Reagents and apparatus

A. Reagents

- pesticide residue analysis grade hexane, benzene, acetone, ethanol, anhydrous sodium sulfate
- HPLC grade methanol, distilled water
- KOH : special reagent grade
- target compounds : commercial standards
- p-terphenyl-d_{14}, perylene-d_{12} : Cambridge Isotope Laboratory
- 4% Na_2SO_4 solution : Add anhydrous sodium sulfate into purified water to make a 4 % w/v solution, and wash with hexane.
- silica gel : Activate silica gel S-1 at 130 °C for more than 8 hours.
- quartz filter paper : Watman Ltd. QM-A (8 x 10 inch). Heat filter papers in an electric furnace at 600 °C for four hours. Store the heat-treated filter papers in a desiccator.
- SEP-PAK silica cartridges : or similar products

B. Apparatus

- silica gel column : Pack 3 g of silica gel into a glass column (i.d. 1 cm, length 30 cm) with hexane, and lay about 2 cm height of anhydrous sodium sulfate on the top.
- rotary evaporator (with water bath) : for concentration of extracts
- KD concentrator : for concentration of extracts
- centrifuge : for separation-extraction of sediment and biological samples
- mechanical shaker : for liquid-liquid extraction
- aluminium foil : for covering columns etc during column chromatography
- glassware : separating funnels, round bottom flasks (amber), beaker, centrifuge tubes with stoppers, graduated test tubes (amber), condensers
- low volume air sampler or high volume air sampler
- filter paper folder : glass or stainless (teflon packing)
- electric furnace
- ultrasonic extractor (or sonicator)

III.2.4.5 Experimental

A. Sample preparation (note 1)

a) Water samples

Place 1 L of water sample into a separating funnel. Add 100 mL of hexane, and shake for five minutes, allow to settle, and collect the hexane layer. Add 50 mL of hexane to the water layer again, and repeat the procedure. Combine the hexane layers, dehydrate with anhydrous sodium sulfate, then reduce the volume of the solution to 5 mL using a rotary evaporator (keep the bath temperature below 50 °C). This extract is now ready for clean-up.

b) Sediment and biological samples

Place 20 g of sample (wet mud or crushed fish) into a round bottomed flask (amber). Add 100 mL of 1 M KOH-ethanol solution, and reflux the mixture for one hour. Allow to cool. After cooling, centrifuge at 4000 rpm, and collect the supernatant liquid. Transfer the supernatant liquid into a separating funnel. Add 400 mL of 4 % sodium sulfate solution. Extract the aqueous mixture first with 100 mL of hexane, then with 50 mL of hexane. Dehydrate the combined hexane layers with anhydrous sodium sulfate, then reduce the volume of the solution to 5 mL using a rotary evaporator (keep the bath temperature below 50 °C). This extract is now ready for clean-up.

c) Air samples

(1) Sampling

Attach a filter paper to the filter paper folder (high volume : 20 x 25 cm, low volume : 47 mmϕ) (note 3), connect a suction pump and a gas meter, pass air through the filter paper at the rate of 1300 L/min for high volume, 30 - 40 L/mm for low volume, for 12 - 24 hours to take air samples. Avoid having direct sunlight fall on the surface of the sampling area. Seal (wrap) the filter papers in aluminium foil after sampling.

(2) Extraction

Shred the filter paper. Put the shredded filter paper (note 4) into a centrifuge tube, add 1 mL of ethanol, then add 3 mL of benzene, and conduct ultrasonic extraction for 20 minutes. Immediately after the extraction, centrifuge at 3000 rpm for 15 minutes, and collect the supernatant for clean-up.

B. Sample clean-up

a) Water, sediment and living things

Load the 5 mL extract onto a silica gel column. First, elute the column with 100 mL of hexane (1st. Fr.). Discard the eluate. Next, elute the column with 50 mL of hexane- benzene (85 : 15 v/v), and elute target compounds (2nd Fr.) (note 5). Concentrate this 2nd fraction with a KD concentrator at atmospheric pressure to a volume of 2 mL. Reduce the volume further by blowing nitrogen gas across the surface of the solution until the sample solution reaches a final volume of 0.5 - 1 mL. This sample is used for measurement. If samples are measured by GC/MS, add 1 μg each of p-terphenyl-d_{14} and perylene-d_{12} as internal standards.

b) Air samples

Condition Sep-Pak cartridges by washing with 10 mL of hexane-benzene (9 : 1 v/v). Load 2 mL of extract onto the cartridge. Elute target compounds with 20 mL of hexane-benzene (9 : 1 v/v). Concentrate the eluates with a KD concentrator to about 2 mL. Reduce the volume further by blowing nitrogen gas across the surface of the solution until the sample solution reaches a final volume of 0.5 mL. This sample is used for measurement. If samples are measured by GC/MS, add 1 μg each of p-terphenyl-d_{14} and perylene-d_{12} as internal standards.

C. Preparation of blank samples

Blank samples should be prepared by subjecting purified water (1L for water, 20mL for

sediment and biological samples) to the same procedures used when examining analytical samples. For air samples, simply use the same kind of filter papers.

D. Preparation of standard solutions

Weigh 100 mg of each standard chemical accurately; add sufficient acetone to make an accurate 100 mL solution. This procedure gives a 1000 μg/mL standard stock solution.

- For HPLC analysis of B(j)F, dilute the standard stock solutions with methanol to make four different concentrations (1, 2, 3 and 4 μg/mL) which contain three standards of B(j)F, B(b)F and B(k)F.
- For HPLC analysis of the other target compounds, prepare four different methanolic solutions containing 0.05, 0.1, 0.15 and 0.2 μg/mL of B(e)P, and 0.1, 0.2, 0.3 and 0.4 μg/mL of the other seven compounds.
- For GC/MS analysis, prepare acetone solutions containing the eight compounds at five different concentrations, and add 1 μg of each p-terphenyl-d_{14} and perylene-d_{12} (add 0.1 mL of 10 μg/mL standard solutions).

E. Measurement

a) HPLC conditions

- column : Perkin-Elmer make PAH 2.6 mm x 250 mm
- guard column : Nucleosyl C18 4.6 mm x 33 mm
- column temperature : 30 °C
- mobile phase : methanol : water = 85 : 15 (note 6)
- flow rate : 0.8 mL/min
- detector : Ex 365 nm, Em 430 nm → B(a)P, B(b)F, B(k)F, B(ghi)P, [MC]
  Ex 300 nm, Em 393 nm → B(e)P, [B(k)F], B(ah)A, MC
  Ex 330 nm, Em 509 nm → B(j)F, [B(b)F], [B(k)F] (note 7)

b) GC/MS conditions (note 8)

(1) **GC : capillary column GC**

- column : ULTRA-2 (5 % phenylmethylsolicone chemical bonded type, 25 m x 0.32 mm i.d., film thickness 0.17 μm) or similar type of column
- column temperature : 50 °C (1 min) → 25 °C/min → 160 °C → 15 °C/min → 250 °C → 2 °C/min → 290 °C (5 min)

- injector temperature : 250 °C
- carrier gas : He (flow rate 40 cm/s)
- injection method : splitless (purge after 1 min)

(2) **MS : quadrupole type or double focusing type**

- transfer line temperature : 290 °C
- ionisation method : EI
- ionisation voltage : 70 eV
- ionisation current : 300 μA
- ion source temperature : 230 °C

(3) **monitor ion**

- target compounds : B(a)P, B(e)P, B(b)F, B(j)F, B(k)F = 252, B(ghi)P = 276, B(ah)A = 278, MC = 268
- internal standards : p-terphenyl-d_{14} = 244, perylene-d_{12} = 264

F. Calibration curves

For HPLC, inject 20 μL of the standard mixtures onto the column and record the detector response. Make calibration curves using the external standard method by plotting compound concentration against and peak areas (or peak heights). For GC/MS, inject 1 μL of standard mixtures onto the column. Make calibration curves using the internal standard method.

G. Determination and calculation

HPLC : inject 20 μL of sample and determine analyte concentrations by using the calibration curves (extrapolate from sample peak areas (or peak heights)) to sample concentration). GC/MS : inject 1 μL of sample and determine analyte concentrations by using the calibration curves of the internal standards and target compounds.

Calculate concentrations of each target compound from the following formulas.

a) Water , sediment, and living things

$$\text{concentration (mL or μg)} = \text{detected amount (ng)} \times \frac{\text{sample solution amount (mL)}}{\text{injection amount (μL)}} \times \frac{1}{\text{sample amount (mL or g)}}$$

b) Air

$$\text{concentrat ion (ng/m}^3\text{)} = \text{detected amount (ng)} \times \frac{1000 \times (273 + t)}{V \times (273 + 20)} \times \frac{760}{P} \times \frac{Vc}{I}$$

t : average temperature at the time of sampling (°C)

V : sampling air amount (L)

P : atmospheric pressure at the time of sampling (mmHg)

Vc : sample solution amount (mL)

I : injection amount (μL)

III.2.4.6 Recovery tests results

A. Results of recovery tests undertaken using the target compounds and water, sediment and biological samples are shown in **Table III-2-9**.

Table III-2-9 Recovery and relative standard deviation (RSD) of water, sediment and biological samples

Samples	recovery (%) (RSD (%))							
	B(a)P	B(e)P	B(b)F	B(j)F	B(k)F	B(ghi)P	B(ah)A	MC
river water	92 (4.8)	95 (5.7)	96 (3.8)	92 (4.5)	92 (4.0)	93 (3.4)	92 (4.3)	90 (3.6)
Seawater	94 (5.3)	95 (8.5)	93 (4.3)	94 (4.0)	85 (3.5)	95 (2.5)	94 (3.7)	94 (4.2)
Sediment	89 (3.7)	88 (4.0)	87 (5.0)	85 (8.3)	88 (7.5)	89 (8.0)	89 (6.8)	90 (6.7)
living things	90 (4.2)	87 (6.2)	89 (4.5)	90 (7.0)	92 (6.7)	90 (6.6)	88 (7.1)	87 (7.4)

water : n=4, sediment and living things : n=7

B. Results of recovery tests using the target compounds in air samples

Recovery measurements were undertaken by placing 0.5 μg of each target compounds onto a quartz filter papers(*1), and sucking indoor air through the filter paper (*2). Results are shown in **Table III-2-10**. The table shows that the recovery of these compounds decreases as amount of aeration increases. This is especially true for B(a)P and MC.

The recovery of B(a)P and MC was measured by aerating 3.0 m^3 of highly purified nitrogen gas at an aerating rate of 20 L/min. Also 3.0 m^3 of indoor air was aerated in the same way as a control. The results are shown in **Table III-2-11**. Though the recovery was 80 % when nitrogen gas was aerated, it was only around 50 % when indoor air was aerated.

(*1) 0.5 mL of standards (1 mg/L per one PAH) were added by using pipettes.

(*2) Particles in the air are removed by filter papers attached in front of filter papers for recovery.

Table III-2-10 Recovery and relative standard deviation of target compounds from filter papers depends on difference of aeration amount

aeration amount	B(a)P	B(e)P	BF	B(ghi)P	B(ah)A	MC
0.0 m^3	95.0 % (3.1 %)	93.5 % (12.0 %)	93.5 % (12.0 %)	91.0 % (12 %)	92.0 % (11.9 %)	78.0 % (11.7 %)
9.7 m^3	24.8	92.6	83.5	86.6	88.9	12.6
17.3 m^3	26.3	91.2	81.1	95.7	97.2	13.5
39.5 m^3	20.4	87.5	73.8	95.5	97.5	8.5

(Relative standard deviation (n=3))

Table III-2-11 Recovery of the target compounds from filter papers depends on aerating gases

aerating gases		B(a)P	MC
air (indoor)	recovery (%)	51.2	46.3
	RSD (%)	9.5	4.0
nitrogen	recovery (%)	85.5	80.2
	RSD (%)	2.5	3.1

aerating amount : 3.3 m^3, n=3

III.2.4.7 Notes

(1) B(a)P, MC etc. in aqueous solution or organic solvents are degraded by light. Therefore, samples and standard solutions should be shielded from light during storage and chromatography. For instance, MC in acetone at 100 μg/mL will be totally degraded in six months even if stored in amber test tubes.

(2) If fatty acids etc. from biological samples are deposited when concentrating the extract to 5 mL, in order to avoid clogging of columns centrifuge and collect the supernatants for clean-up.

(3) Carry filter papers wrapped in aluminium foils, and attach the filter paper folder at the sampling sites.

(4) Cut out the necessary size of filter papers for high volume air sampler for analysis and store the rest in the desiccator in a dark and cold place. Use the whole 47 mmϕ quartz filter paper for low volume air sample.

(5) It is necessary to check elution patterns beforehand. The eight target compounds can be completely eluted with 50 mL of 15 : 85 benzene-hexane mixture.

(6) If the separation of three isomers of benzofluoranthene is not sufficient, increase the amount of water in the mobile phase to improve resolution (for example, 20 : 80

H_2O-methanol). Methanol gives better separation than acetonitrile in the mobile phase.

(7) Divide the samples into three groups to minimise analysis time. Where it is possible to set the most appropriate wavelengths for each of the target compounds, use the following for measurement.

The most appropriate wavelength : B(a)P → Ex = 370 nm, Em = 407 nm, B(e)P → Ex = 335 nm, Em = 379 nm, B(b)F → Ex = 350 nm, Em = 395 nm, B(k)F → Ex = 370 nm, Em = 406 nm, B(ghi)P → Ex = 385 nm, Em = 419 nm, B(ah)A → Ex = 298 nm, Em = 395 nm, MC → Ex = 365 nm, Em = 420 nm

(8) The retention time order of the three isomers of benzofluoranthene is B(b)F, B(j)F, and B(k)F, but it is impossible to separate the baseline. If the presence of these three compounds in the sample is confirmed by GC/MS, then conduct quantitative analysis by HPLC.

III.2.5 Analytical method for aldehydes and ketones in air

III.2.5.1 Target compounds

acetaldehyde, formaldehyde, crotonaldehyde, acetone, 4-methyl-2-pentanone, 2-butanone

III.2.5.2 Outline of analytical methods

Collect air samples by sucking air into sample trap cartridges (trap agent : silica gel dipped with 2,4-dinitrophenylhydrazine (2,4-DNPH)). Target compounds react with the trapping agent and are held as hydrazones in the cartridges. Elute hydrazone derivatives from the cartridges by ethyl acetate, treat with cation exchange resin, and add internal standards after dehydration and concentration, then determine by GC-FTD (NPD).

III.2.5.3 Detection limits

Detection limits of this analytical method are shown in **Table III-2-12**.

Table III-2-12 Detection limits of this analytical method

compounds	detection limits (ng)
acetaldehyde	42[1)]
formaldehyde	4.4[1)]
crotonaldehyde	2[2)]
acetone	2[1)]
4-methyl-2-pentanone	2[2)]
2-butanone	2[2)]

1) values determined from the blank value change ($3\sqrt{2}\sigma$)

2) values determined from S/N ratio against interferences (S/N=3)

III.2.5.4 Reagents and apparatus

A. Reagents

- acetaldehyde : MERCK
- formaldehyde : special reagent grade
- crotonaldehyde : special reagent grade
- acetone : for spectroscopic analysis
- 4-methyl-2-pentane : special reagent grade
- 2-butanone : for ultraviolet absorption spectrum
- 2,4-DNPH form of target compounds : (note 1)
- diphenylamine (internal standard) : special reagent grade
- ethyl acetate : for HPLC (note 2)
- anhydrous sodium sulfate : special reagent grade. Heated in an electric furnace at 400 °C before use.
- hexane : for HPLC
- purified water : for trihalomethane analysis

B. Apparatus

- ozone scrubber : Waters chromatography Division / Millipore Corporation products. Form copper pipe (46 mm i.d., 1 m length) into coil (coil diameter is not specified), fill the coil with potassium iodide solution (dilute 1 g of potassium iodide into 0.7 mL purified water), and leave for 10 minutes. After 10 minutes remove the potassium iodide solution from the coil, and dry by nitrogen gas.
- sample trap cartridges : Waters Chromatography Division / Millipore Corporation products Sep Pak ® DNPH-Silica Cartridges (Short Body) (note 3). Silica gel which is filled up in the

cartridges is coated with 1.0 mg 2,4-DNPH. Discard the cartridges after use because the blank value of the cartridges increase depends on the storage duration (recommended storage length by the manufacturer is about one month at 20 - 25 °C, 6 months at 4 °C).

- cation exchange resin : TOSHO Corp. products TOYOPAK IC SP-M

III.2.5.5 Experimental

A. Sampling method

Connect the ozone scrubber, sample trap cartridge, suction pump, and gas meter, then suck the air sample through the apparatus and trap the target compounds. Sucking speed is at 0.1 - 1.0 L/min, and the sampled volume is 20 - 200 L. During sampling, shield cartridges by aluminium foil to avoid light. After sampling seal the both ends of the cartridges, place into glass containers with stoppers, and wrap with aluminium foil. Return the trap cartridges to the laboratory and conduct extraction procedures as soon as possible.

B. Sample preparation

After sampling, connect a sample trap cartridge to cation exchange resin, inject ethyl acetate by using a glass syringe, and elute the hydrazone derivatives from the cartridge (note 4). Place the eluate into an Erlenmeyer flask, add anhydrous sodium sulfate to dehydrate, filter into test tubes, and concentrate the hexane solution up to 1 mL by blowing nitrogen gas across the surface of the solution. Add 40 μL of internal standard for analysis.

C. Preparation of standard solutions

To prepare the stock solutions, weigh the masses of the hydrazone derivatives of each of the target compounds shown in **Table III-2-13**, and dissolve in ethyl acetate (50 mL). One mL of stock solutions contain 50 μg of each target compounds. Dilute and mix these stock solutions to make each target compound concentration 0.5 - 10 μg/mL. An internal standard stock solution is prepared by dissolving 20 mg of diphenylamine in 100 mL of ethyl acetate. Store both stock solutions and standards in light tight glass containers in a cold and dark place.

Table III-2-13 Amount of standards for stock solutions

compounds	amount (mg)
acetaldehyde-2,4-DNPH	12.7
formaldehyde-2,4-DNPH	17.5
crotonaldehyde-2,4-DNPH	8.9
acetone-2,4-DNPH	10.3
4-methyl-2-pentanone-2,4-DNPH	7.0
2-butanone-2,4-DNPH	8.8

D. Measurement

a) Analytical conditions of GC-FTD

- detection method : GC-FTD (NPD)
- column : fused silica capillary column ULTRA#1 (length 25 m x 0.32 mm i.d. x film thickness 0.17 μm) or similar column
- carrier gas : He, column head pressure 73 kPa
- column temperature : 50 °C (1 min) → 30 °C/min → 200 °C (15 min)
- injector temperature : 260 °C
- detector temperature : 280 °C
- injection method : splitless (purge after 1 min)

b) Analytical conditions of GC/MS (note 5)

- column : fused silica capillary column DB-1 (length 30 m x 0.25 mm i.d. x film thickness 0.25 μm) or similar column
- carrier gas : He, column head pressure 40 kPa
- column temperature : 50 °C (1 min) → 30 °C/min → (15 min)
- injector temperature : 260 °C
- separator temperature : 280 °C
- ionisation current : 300 μA

E. Calibration curves

Prepare standard solutions for calibration curves by adding 40 μL of internal standard to 1 mL of standard solution, inject 2 μL of the mixture into the GC, and make calibration curves by the internal standard.

F. Determination

Inject 2 μL of sample solutions into GC, determine peak area ratio of target compounds and internal standard, and decide detected amount (ng) from the calibration curves.

Calculate concentration of each target compound C (ng/m^3) from the following formula.

$$C\ (ng/m^3) = C' \times 1000 \times \frac{273 + t}{V + (273 + 20)} \times \frac{101.3}{P}$$

C' : detected amount (ng)

t : average temperature at the sampling time

V : collected air volume (m^3)

P : atmospheric pressure at he sampling time

III.2.5.6 Results of recovery tests

After connection of a trap cartridge and glass T-tube, 10 μL of hexane solution of each standard (concentration : 0.2 - 0.4 μg/μL, water solution for formaldehyde only) was injected. Nitrogen gas was passed through for more 15 minutes at the rate of 400 mL/min, then recovery was determined. **Table III-2-14** shows the results.

Table III-2-14 Recovery and relative standard deviation of recovery tests (n=5)

compounds	recovery (%)	RSD (%)
acetaldehyde	97	10
formaldehyde	95	22
crotonaldehyde	95	7.0
acetone	82	10
4-methyl-2-pentanone	81	4.0
2-butanone	84	4.1

III.2.5.7 Notes

(1) Commercial hydrazone derivative standards are sold by RADIAN (all 6 compounds) and Tokyo Kasei Corp. (5 compounds except 4-methyl-2-pentanone-2,4-DNPH).

(2) Check blank values of ethyl acetate and use ethyl acetate with minimum impurities (the blank values vary depending on manufacturers, grade or lot). Commercial reagent for pesticide residue analysis contains 0.08 - 1.2 μg/mL of acetaldehyde.

(3) Sep Pak ® DNPH-Silica Cartridge has a long body type which contains double the amount of adsorbent, and can be expected to provide more trap amount. However, it is recommended to use a short body type because of increase of impurities from solvent accompanied by increase of amount of solvent used.

(4) Reverse elution direction of cartridges from sample loading. Allow gravity and the weight of the syringe cylinder to inject ethyl acetate into cartridges. If solvent cannot be injected automatically, it is recommended to put a light weight on the cylinder. When silica gel becomes colourless after elution, extraction has completed (because 2,4-DNPH, which coats silica gel in the cartridge, moves as a yellow band).

(5) Conditions of GC/MS are for peak confirmation.

III.2.6 Analytical method for volatile compounds in water

III.2.6.1 Target compounds

Dichloromethane, dibromochloromethane, tetrachloromethane, trichloromethane (chloroform), tribromomethane (bromoform), bromodichloromethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,1-dichloroethylene, cis-1,2-dichloroethylene, trans-1,2-dichloroethylene, tetrachloroethylene, trichloroethylene, 1,2-dichloropropane, 1,3-dichloro-1-propene, 1,4-dichlorobenzene, xylene, benzene, toluene

III.2.6.2 Outline of analytical method

Transport the target compounds from the aqueous phase into the gaseous phase by bubbling an inactive gas through the sample solutions. Trap the target compounds specialised trap cartridges, then heat the cartridges to desorb the target compounds, conduct cryo-focussing at the cooling and condensing apparatus, and introduce the target compounds into the GC/MS for measurement. Alternatively, after trapping the target compounds in the trap cartridges, heat the trap cartridges, then introduce the target compounds into the GC/MS directly (without cryo-focus).

III.2.6.3 Detection limits and determination limits

Table III-2-15 shows determination limits and repeat analysis accuracy of this analytical method.

Table III-2-15 determination limits and the accuracy of repeated analysis when using this analytical method

target compounds	range of determination (ng)	repeatability (%)
all compounds	0.5 – 250	10 - 20

III.2.6.4 Reagents

- target compounds : special grade or more, high purity reagents
- methanol : of a grade known not to contain target compounds
- blank water : water which doesn't contain target compounds (note 1)

III.2.6.5 Experimental

A. Sample preparation

Collect water samples directly into P&T vial bottles (note 2) at the sampling site, add 5 μL of p-bromofluorobenzene internal standard (20 mg/L) to the sample solutions.

B. Preparation of control

Use blank water, and conduct the analysis using the same procedures as used on the samples.

C. Preparation of standard solutions

a) Target compounds

Weigh 100 mg of each target compounds accurately, add methanol to make a precise 100 mL solution. This gives a 1000 mg/L standard stock solution. Dilute this stock solution with methanol, and prepare mix standard solution of concentrations 2, 20, and 50 mg/L.

b) Internal standards

Weigh 100 mg of p-bromofluorobenzene accurately; add methanol to make a precise 100 mL solution. This gives a 1000 mg/L standard stock solution. Dilute this stock solution with methanol, and prepare a standard solution of concentration 20 mg/L.

c) Preparation of standard solutions for measurement

Place about 30 mL of blank water into a P&T vial, add 10 μL of 2, 20, 50, and 100 mg/L

standards, then add 5 μL of p-bromofluorobenzene internal standard, and dilute with a known volume of blank water to produce standard solutions for measurement.

D. Measurement

a) Measurement conditions of P&T

- machine : ex. TEKMAR LSC-2000
- purge : 10 min at room temp
- dry purge : 4 min
- cryo cooldown : -150 °C
- desorb : 2 min at 220 °C
- inject : 3 min at 220 °C
- bake : 20 min at 260 °C

b) Measurement conditions of GC/MS

- machine: quadrupole MS, ex. JEOL-Automass 50 type
- column : ex. VOCOL (SUPELCO) 60 m x 0.32 mm i.d. x 3 μm

(1) **Conditions of GC**

- init. temp : 40 °C
- init. time : 5 min
- rate : 7.0 °C/min
- final temp : 230 °C
- final time : 5 min
- inject temp : 180 °C
- carrier gas flow rate: 1.0 mL/min (He)

(2) **Conditions of MS**

- emission current : 300 μA
- ionisation energy : -70 eV
- detection gain : -0.6 kV
- source temp : 210 °C
- interface temp : 230 °C

(3) **Monitor ions are shown in Table III-2-16.**

Table III-2-16 Monitor ions

target compounds	monitor ions
dichloromethane	84, 86, 49
dibromochloromethane	129, 127, 131
tetrachloromethane	117, 119, 121
trichloromethane (chloroform)	83, 85, 47
tribromomethane (bromoform)	173, 171, 175
bromodichloromethane	83, 85, 47
1,2-dichloroethane	62, 64
1,1,1-trichloroethane	97, 99, 61
1,1,2-trichloroethane	97, 83, 99
1,1-dichloroethylene	96, 61
cis-1,2-dichloroethylene	96, 61, 98
trans-1,2-dichloroethylene	96, 61, 98
tetrachloroethylene	166, 164, 129
trichloroethylene	130, 132, 95
1,2-dichloropropane	63, 76, 62
cis-1,3-dichloropropene	75, 110, 49
trans-1,3-dichloropropene	75, 110, 49
1,4-dichlorobenzene	146, 148, 111
o-xylene	106, 91, 105
m-xylene	106, 91, 105
p-xylene	106, 91, 105
benzene	78, 77, 52
toluene	92, 91
p-bromofluorobenzene	174, 176, 95

E. Calibration curves

Analyse standards by GC/MS by injecting 5 mL of standard solution into the P&T apparatus, and make calibration curves according to the relations between purge amount (μg) and peak area ratio of standards and internal standards.

F. Determination and calculation

Analyse samples by GC/MS by injecting 5 mL of standard solution into the P&T apparatus, and determine the detected amount from the calibration curves after determining the peak area ratio of target compounds and internal standards.

$$\text{calculated amount (μg/L)} = \frac{\text{detected amount (ng)}}{\text{purge amount (mL)}}$$

III.2.6.6 Notes

(1) To make blank water, boil purified water to about half its initial volume using a gas stove, and cool down by blowing helium gas across the surface of the water. When commercial water is used, check that target compounds are not detected.
(2) Check the capacity of the P&T vials before use because there are small differences around 43 mL in the case of Tekmar Co. products. Remove vials which have big error.

III.2.7 Analytical method of organochlorine pesticides in water, sediment and biological samples

III.2.7.1 Target compounds

Aldrin, α-HCH, β-HCH, γ-HCH (lindane), δ-HCH, p,p'-DDT, p,p'-DDE, p,p'-DDD, dieldrin, endosulfan I, endosulfan II, endosulfan sulphate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide (isomer B), methoxychlor, trans-chlordane, cis-chlordane, trans-nonachlor, cis-nonachlor, toxaphene, hexachlorobenzene (HCB)

III.2.7.2 Outline of analytical method

- Extract water samples with hexane, dehydrate, concentrate, and then analyse by GC/MS-SIM or GC-ECD.
- Extract sediment samples with acetone, add sodium chloride solution, and then extract with hexane. After dehydrating and concentrating the hexane layer, clean-up by florisil column chromatography, and analyse by GC/MS-SIM or GC-ECD.
- Extract biological samples with a mixture of acetone and hexane, then wash with water. Remove lipids by acetonitrile-hexane partitioning, fractionate by florisil column chromatography, and analyse by GC/MS-SIM or GC-ECD.

III.2.7.3 Reagents and apparatus

A. Reagents

- organic solvents : pesticide analysis grade

- target compounds : commercial standard reagents
- internal standards (naphthalene-d_8, fluoranthene-d_{10}, perylene-d_{12}) : Cambridge Isotope Laboratory products
- anhydrous sodium sulfate, sodium chloride : pesticide analysis grade or reagent special grade, heated at 700 °C for 8 hours then cooled
- purified water and 5% sodium chloride water solution : wash twice with dichloromethane, and finally with hexane
- florisil : pesticide analysis grade (60 / 100 mesh), heated at 130 °C for 8 hours then cooled slowly in a desiccator
- 5 % hydrated silica gel : activate "Wako gel C-200", or equivalent, at 130 °C overnight, then cool slowly in a desiccator. Place 100 g of the silica gel into a Erlenmeyer flask fitted with a glass stopper, add 5 mL of purified water, replace the stopper, and leave for 4 - 5 hours. Shake occasionally until evenly mixed.
- charcoal : for column chromatography
- minute crystal powder cellulose : for column chromatography
- reduction copper : reduction copper for analysis of organic elements (60 - 80 mesh). Store under nitrogen gas, and wash with the solvent being used in the experiment immediately before use.
- other reagents : reagent special grade

B. Apparatus and instruments

- silica gel column : use a glass column (length, 30 cm; internal diameter, 1 cm) packed with 1 g of 5 % hydrated silica gel. Use hexane to add the silica gel as slurry. Lay 2 cm of anhydrous sodium sulfate onto the top of the silica gel. Wash with 20 mL of hexane which contains 2 % acetone before use.
- florisil column : use glass column (length, 30 cm; internal diameter, 1 cm) packed with 10 g of florisil. Use hexane to add the florisil as slurry. Lay 2 cm of anhydrous sodium sulfate onto the top of the florisil. Wash with 100 mL of hexane before use.
- activated charcoal column : use a glass column (length, 30 cm; internal diameter, 1 cm) packed with 5 g of cellulose which contains 10 % activated charcoal onto the 2 cm layer of anhydrous sodium chloride. Use hexane to add the charcoal as slurry. Lay 2 cm of anhydrous sodium sulfate onto the charcoal. Wash with 100 mL of hexane before use.
- Kuderna-Danish (KD) concentration apparatus
- rotary evaporator

- separating funnel
- homogeniser
- ultrasonic irradiation instrument (ultrasonic bath is also OK)
- centrifuge
- gas chromatograph / mass spectrometer (GC/MS) or GC-ECD : capillary column type GC, double focusing or quadrupole MS

III.2.7.4 Experimental

A. Sample preparation

a) Water samples

Dissolve 50 g of sodium chloride in 1 L of water sample, then extract with 50 mL of hexane by shaking for 10 minutes. Separate the hexane layer. Repeat the extraction. Combine the hexane layers, dehydrate with anhydrous sodium sulfate, concentrate by rotary evaporation up to about 5 mL, then concentrate the hexane solution to about 1 mL by blowing nitrogen gas across the surface of the solution. Use this solution for sample clean-up.

b) Sediment samples

Place 50 g of wet mud into a centrifuge test tube fitted with a glass stopper. Add 50 mL of acetone, and shake for 10 minutes. Conduct an ultrasonic extraction of the mixture by placing the sample in the ultrasonic bath for 10 minutes. Centrifuge the sample at 3000 rpm for 10 minutes, and collect the supernatant liquid. Repeat this procedure three times. Combine the supernatant liquids (the acetone extracts), and add them to 500 mL of 5 % sodium chloride solution in a 1 L separating funnel. Add 50 mL of hexane and shake for 5 minutes. Allow the organic and aqueous layers to separate, then collect the hexane layer. Repeat this procedure twice. Combine the hexane layers. Dehydrate the hexane solution by anhydrous sodium sulfate, concentrate by rotary evaporation up to about 5 mL, then concentrate the hexane solution to about 1 mL by blowing nitrogen gas across the surface of the solution. Use this solution for sample clean-up.

c) Biological samples

Add 50 mL of acetone and 100 mL of hexane to 50 g of homogeneous sample, and homogenise by polytron type homogeniser for 2 minutes. Centrifuge this at 3000 rpm for 10 minutes, then collect the supernatant liquid. Repeat this procedure twice. Combine the extracts in a separating funnel, add 150 mL of purified water, then shake gently (swirl or rotate the mixture). After settling, discard the aqueous phase. Repeat this water wash, then dehydrate the organic

phase by passing it through a column packed with 30 g of anhydrous sodium sulfate. Finally, concentrate by rotary evaporation to 10 mL. Place this concentrate into a separating funnel with 50 mL of acetonitrile saturated by hexane, shake for one minute, then collect the acetonitrile layer. Add 50 mL of acetonitrile saturated by hexane into the hexane layer and shake, then collect the acetonitrile layer. Combine the acetonitrile layers, add 5 mL of water, then shake gently. After settling, collect the hexane layer. Add the acetonitrile layer to 500 mL of 5 % sodium chloride solution in a separating funnel, add 50 mL of hexane, then shake for 5 minutes. Collect the hexane layer. Repeat this extraction twice, and combine the hexane layers. Dehydrate the hexane solution by anhydrous sodium sulfate, concentrate by rotary evaporation up to about 5 mL, then concentrate the hexane solution to about 1 mL by blowing nitrogen gas across the surface of the solution. Use this solution for sample clean-up.

B. Sample clean-up

a) Water samples

Load the concentrated sample onto a silica gel column, and elute target compounds with 30 mL of hexane which contains 3 % acetone (note 1 & 2). Concentrate the eluate samples by KD concentration to about 2 mL, then concentrate the hexane solution for GC-ECD analysis to exactly 1 mL by blowing nitrogen gas across the surface of the solution. For GC/MS analysis, add internal standard to each fraction then concentrate.

b) Sediment samples

Load the concentrated sample onto a Florisil column, and elute target compounds with 100 mL of hexane (Fraction 1) (note 3 & 4), 100 mL of hexane which contains 4 % diethylether (Fr. 2), and 150 mL of hexane which contains 15 % diethylether (Fr. 3) in that order. For GC-ECD analysis, concentrate each fraction to precisely 1 mL by KD concentration under reduced pressure and by blowing nitrogen gas across the surface of the solution (note 5). For GC/MS analysis, add internal standard to each fraction then concentrate (note 5).

c) Biological samples

Load the concentrated sample onto a Florisil column, and elute target compounds with 100 mL of hexane (Fraction 1) (note 4), 100 mL of hexane which contains 4 % diethylether (Fr. 2), and 150 mL of hexane which contains 15 % diethylether (Fr. 3) in that order. For GC-ECD analysis, concentrate each fraction to precisely 1 mL by KD concentration under reduced pressure and blowing nitrogen gas (note 5). For GC/MS analysis, add internal standard then concentrate (note 5).

C. Preparation of blank samples

Prepare blank samples as an analytical control by using the same procedures as described above for sample preparation and clean-up.

D. Preparation of standard solutions

Prepare 1000 mg/L standard stock solutions by dissolving standards of each target compound in hexane. Dilute and mix these with hexane to make standard mixtures of fixed concentrations of each analyte standard. Conduct the same procedure of preparation and storage for internal standard mixture solutions (naphthalene-d_8, fluoranthene-d_{10}, perylene-d_{12}) as used for target compounds.

E. Measurement

a) Measurement conditions of GC/MS

(1) GC

- column : fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm)
- liquid phase is methylsilicone or 5 % phenylmethylsilicone
- column temperature : 50 °C (1 min) - 10 °C/min - 280 °C (5 min)
- injector temperature : 250 °C
- injection method : splitless (purge after 1 min), injection amount 1 μL
- carrier gas : He, average linear velocity : 40 cm/sec
- transfer line temperature : 280 °C

(2) MS

- ionisation method : EI
- ionisation current : 300 μA
- ionisation voltage : 70 eV
- ion source temperature : 220 °C
- detection method : SIM

(3) measurement mass numbers (confirmatory ion)

- target compounds

Aldrin : 263 (265), α-HCH : 181 (219), β-HCH : 181 (219), γ-HCH : 181 (219), δ-HCH : 181 (219), p,p'-DDT : 165 (235), p,p'-DDE : 246 (318), p,p'-DDD : 165 (235), dieldrin : 263 (277), endosulfan I : 195 (241), endosulfan II : 195 (241), endosulfan sulphate : 385 (387), endrin : 81 (263), endrin

aldehyde : 67 (345), heptachlor 100 (272), heptachlor epoxide : 353 (355), methoxychlor : 227 (228), trans-chlordane : 373 (375), cis-chlordane : 373 (375), trans-nonachlor 407 (409), cis-nonachlor : 407 (409), toxaphene : 159 (231), hexachlorobenzene : 284 (286)

- internal standards

naphthalene-d_8 : 136, fluoranthene -d_{10}: 212, perylene-d_{12} : 264

b) Measurement conditions of GC-ECD

- column : fused silica wide bore capillary column (ex. : 30 m x 0.53 mm i.d., 0.5 μm) liquid phase is methylsilicone or 5 % phenylmethylsilicone
- column temperature : 50 °C (1min) - 8 °C/min - 280 °C (5 min)
- injector temperature : 250 °C
- injection method : direct injection, injection amount 3 μL
- carrier gas : He, aerate linear velocity : 40 cm/sec
- detector temperature : 280 °C

F. Calibration curves

When using the internal standard method, add prescribed amounts of internal standards into the standard mixture, take 1 μL from the mixture, and inject into the GC/MS, and make calibration curves based on the peak area (height) ratio of each target compounds and internal standards. When using the external standard method with wide bore columns, inject 3 μL of standard mixture into GC, and make calibration curves for each target compounds from the corresponding peak area (height). The concentration range covered by the calibration curves should be more than 5 orders of magnitude, including the minimum determinable concentration (which is anticipated to be close to detection limit of the analytical method).

G. Determination and calculation

When using the internal standard method for determination, inject 1 μL of sample solution into the GC/MS, and determine the detection amount from the calibration curve of peak area (height) ratio of each target compound and internal standards. When using external standard method for determination, inject 3 μL of samples to GC, and determine the detection amount from peak area (height) and the calibration curves of each target compounds. Then calculate concentrations of target compounds in the samples from detection amount, amount injected into the GC, analysed sample amount, and concentration rate etc.

III.2.7.5 Recovery tests

Conduct overall recovery tests using samples or similar samples per 10 samples or once a day, and calculate recovery rate. After adding acetone solution standards of target compounds at about ten times amount of detection limit and thoroughly mixing, leave for more than 60 minutes, then start the recovery tests.

III.2.7.6 Notes

(1) If there is no interference in analysis, column chromatography can be omitted.
(2) Check elution pattern of column chromatography and recovery rate of each compound. If the recovery rate is poor or interference appears, consider other column clean-up method (such as Florisil, silica gel, or alumina).
(3) Elemental sulfur elutes in the first fraction (Fr. 1) of Florisil column chromatography. If there is interference in GC analysis, add 2 g of reducing copper, shake vigorously for 1 minute, and then collect samples.
(4) PCBs elute in this fraction. If there is interference in GC analysis, conduct the following charcoal column chromatography : Load the concentrate of the fraction onto a charcoal column, elute with 100 mL of hexane and collect the eluate.
(5) Even if combining all fractions doesn't avoid GC interferences, it is OK to combine them. In this case, 150 mL of hexane which contains 15 % ethylether can be used for the elution from the beginning. Also add internal standards to the combined fractions.
(6) If target chemicals are detected by GC-ECD, confirm by using different polarity columns. It is desirable to confirm by mass spectrum in GC/MS analysis, but if it is not possible, measure more than two ions by SIM, then confirm from the peak intensity ratio.

III.2.8 Analytical method for tributyltin (TBT) and triphenyltin (TPT) in water, sediment and biological organisms

III.2.8.1 Outline of analytical method

(1) Water samples : Add tripentyl chloride as internal standard, and extract with hexane under acidic conditions. Separate and dehydrate the organic phase, concentrate, then propylise with bromopropyl magnesium. Clean-up the sample using a Florisil column, concentrate, and determine by GC-FPD.

(2) Solid samples : Add tripentyl chloride as internal standard, and extract with methanol-ethyl acetate under acidic conditions. Then extract the aqueous phase with ethyl acetate - hexane, and clean-up by anion and cation exchange resin. Then propylise in the same manner described above for water samples, and determine by GC-FPD.

III.2.8.2 Detection limits

Table III-2-17 shows detection limits of this analytical method.

Table III-2-17 Detection limits of this analytical method

target compounds	water sample		sediment and biological sample	
	sample amount	detection limits	sample amount	detection limits
TBT	1 L	2 ng/L	10 g	5 ng/g
TPT	1 L	6 ng/L	10 g	10 ng/g

III.2.8.3 Reagents and apparatus

A. Reagents

- organic solvents : pesticide analysis grade. If pesticide analysis grade is not available, use solvents which do not contain a GC interference peak with the retention times of both organotins, i.e. special reagent grade or higher.
- sulfuric acid, hydrochloric acid : special reagent grade or higher
- bromopropyl magnesium : 2 mol/L, tetrahydrofuran solution
- sodium chloride : special reagent grade
- anhydrate sodium sulfate : special reagent grade or PCB analysis grade
- tributyltin chloride (TBT)
- triphenyltin chloride (TPT)
- tripentyl tin chloride (TPeT) : as internal standard
- Florisil cartridge column : cartridge type (ex. Sep Pak Florisil)
- anion exchange resin : commercial cartridge type (note 1). Condition them with 10 mL of 0.2 M NaOH, 20 mL of purified water, and 20 mL of ethanol before use.
- cation exchange resin : commercial cartridge type (note 1). Condition them with 10 mL of 0.2 M HCl, 20 mL of purified water, and 20 mL of ethanol before use.

B. Apparatus

- rotary evaporator
- mechanical shaker
- mixer
- aspirator

III.2.8.4 Experimental

A. Sample preparation

a) Water samples

Measure 1 L of sample accurately. Place in a separating funnel. Add the prescribed amount of internal standard (e.g. 20 ng), 10 mL of hydrochloric acid, and 20 g of sodium chloride, and shake. Extract with 100 mL of hexane by shaking for 10 min. Separate the organic phase. Extract the aqueous phase with another 50 mL of hexane, then combine the hexane layers. Add anhydrous sodium sulfate to the hexane layer to dehydrate, swirl and filter. Concentrate the hexane solution under reduced pressure by rotary evaporation using a water bath at less than 40 °C until the final volume of the solution is about 5 mL. Place the concentrate in a test tube with a glass stopper and while heating gently, blow nitrogen gas over the surface of the solution to further concentrate the solution to about 1 mL. Use this sample solution for propylising.

b) Sediment and biological samples

Place 10 g of homogenised sample (biological sample are evenly ground with a mixer) into a centrifuge test tube, add the prescribed amount of internal standard (e.g. 20 ng) and mix thoroughly. Then add 70 mL of a mixture of methanol-ethyl acetate (1:1 v/v) containing 1 M hydrochloric acid, shake and extract for 30 minutes, then filter using an aspirator (note 2). Wash the test tube with a further 30 mL of the mixture of methanol-ethyl acetate (1:1 v/v) containing 1 M hydrochloric acid, and wash the residue by filtering using aspirator. Combine the filtrates and place them into a separating funnel. Add 100 mL of 10 % sodium chloride solution and 50 mL of ethyl acetate-hexane (3:2 v/v) and shake and extract for 5 minutes. Separate the organic phase. Repeat this procedure using another 30 mL of ethyl acetate-hexane (3:2 v/v). Combine the organic solvent layers in a separating funnel, add 150 mL of hexane, allow to settle for more than 20 minutes, and remove the aqueous phase (note 3). Add 100 mL of 10 % sodium chloride solution, and shake and wash the organic solvent phase. Repeat this procedure until pH of the aqueous phase becomes neutral (about four times) (note 4). After washing, dehydrate the organic solvent

phase by passing the mixture through a funnel packed with 20 g of anhydrous sodium sulfate. Collect the organic solvent in a round bottom flask. Concentrate the hexane solution under reduced pressure by rotary evaporation using a water bath at less than 40 °C until the final volume of the solution is about 1 mL. Then blow nitrogen gas over the surface of the solution until the solvent has evaporated completely. Dissolve the residue in about 10 mL of ethanol, pass through anion and cation ion exchange columns connected in series (anion exchange column first) at a flow rate of 1 mL/min to absorb organotin onto the cation column. Wash both columns with 20 mL of ethanol and remove the anion exchange column. Pass 15 mL of methanol solution containing 1 M hydrochloric acid through the cation column to elute the organotin. Collect the eluate in a separating funnel; add 30 mL of water and 5 mL of a mixture of hexane-cyclohexane (1:1 v/v), then shake and extract for 5 minutes. Separate and transfer the organic solvent layer into a round bottomed flask, and re-extract the aqueous phase with another 5 mL of hexane-cyclohexane (1:1 v/v). Combine the organic layers, concentrate the solution under reduced pressure by rotary evaporation using a water bath at less than 40 °C until the final volume of the solution is about 5 mL. Place the concentrate in a test tube with a glass stopper and while heating gently; blow nitrogen gas over the surface of the solution to further concentrate the solution to about 1 mL. Use this sample solution for propylising.

B. Sample clean-up (propylisation)

Add 1 mL of bromopropyl magnesium to the concentrated sample solution, mix by shaking gently, and allow to settle for 30 minutes at room temperature. Add 10 mL of 0.5 M sulfuric acid gradually and with external cooling. Transfer the mixture into a separating funnel, and add 10 mL of methanol and 10 mL of water. Extract this solution twice with 2.5 mL of hexane containing 5 % diethylether. Separate and combine the organic phase. Wash the organic extracts twice with 10 mL of water, and dehydrate the solution by passing the mixture through a funnel plugged by cotton or quartz wool and packed with anhydrous sodium sulfate. After washing a Florisil cartridge column with 10 mL of hexane, load the sample solution. Elute with 10 mL of hexane containing 5 % diethylether. Collect the eluate in a test tube fitted with a glass stopper (note 5). Concentrate the eluate by blowing nitrogen gas across the surface of the solution while heating gently to a final volume of 0.2 mL.

C. Preparation of blank samples

Prepare blank samples using purified water subjected to the same procedures as described

above for sample preparation and clean-up.

D. Preparation of standard solutions

Accurately and precisely weigh 20 mg of tributyltin chloride and 60 mg of triphenyltin chloride, and dissolve in 100 mL of hexane (standard stock mixture). Accurately dilute this stock solution 100 times with hexane to prepare standard mixtures (1.0 μg/mL tributyltin chloride and 6.0 μg/mL triphenyltin chloride). Prepare internal standard solutions of tripentyltin chloride (1.0 μg/mL) using the same two step procedure.

To a range of small volumes of the standard mixture (e.g. from 0.1 to 0.5 mL), add 0.5 mL of internal standard, and make up the solution to 1 mL with hexane. Then add 1 mL of bromopropyl magnesium solution to propylise, treat with 10 mL of 0.5 M sulfuric acid and 10 mL of purified water, then extract twice with 2 mL of hexane, and combine the extracts. Add hexane to the combined extract to make 5 mL. Use these standard solutions for preparation of calibration curves.

E. Measurement

Measurement conditions of GC

- column : fused silica capillary column (30 m x 0.53 mm i.d., 1.5 μm) liquid phase is methylsilicone
- column temperature : 80 °C (1 min) - 8 °C/min - 290 °C (10 min)
- injector temperature : 290 °C
- injection method : splitless or direct injection
- detector : flame photometric detector (FPD), with filter for tin, detector temperature : 300 °C

F. Calibration curves

Inject the standard solutions prepared for making the calibration curves into the GC. Measure the peak heights of the propylised forms of tributyl tin (TBT), tripentyl tin (TPeT), and triphenyl tin (TPT), then make calibration curves using the internal standard method and TPeT as internal standard.

G. Determination and calculation

Measure a constant amount (e.g. 3 μL) of samples by GC. Measure the peak heights of the propylised forms of TBT, TPeT, and TPT, and calculate the concentrations (as chlorinated compounds) of TBT and TPT in the samples using the following formula.

$$\text{TBT (TPT) (ng/L or ng/g)} = A \times \frac{\text{TPeT spiked amount (ng)}}{\text{sample amount (L or g)}}$$

A : absolute ratio of TBT (TPT) which is calculated from B values by calibration curves against TPeT

B : peak height (mm) of TBT (TPT) in samples / peak height (mm) of TPeT in samples

III.2.8.5 Recovery tests

Table III-2-18 and **Table III-2-19** show the results of recovery tests of water and biological samples.

Table III-2-18 Result of recovery tests (n=6)

Target compounds	river water			seawater		
	spike amount (ng)	recovery (%)	RSD (%)	spike amount (ng)	recovery (%)	RSD (%)
TBT	10	99.7	3.5	10	100.4	9.8
	100	98.4	2.2	100	96.3	4.2
	1000	99.0	4.3	1000	97.2	5.9
TPT	30	107.6	1.4	30	99.0	2.5
	300	98.7	1.9	300	97.5	7.4
	3000	107.3	4.1	3000	108.5	3.4

water sample amount : 1 L

Table III-2-19 Result of recovery tests (n=3)

Target compounds	biological samples		
	spike amount (μg)	recovery (%)	RSD (%)
TBT	0.5	90	1.9
TPT	1.0	77	3.9

sample amount : 10 g

Recovery of biological samples is measured by spiking propylised tripentyl tin immediately before GC analysis.

III.2.8.6 Notes

(1) Check recovery before hand because there are some differences depending on the kinds of ion exchange resin.
(2) Centrifuge if it is difficult to filter using an aspirator.
(3) Dehydrate with hexane increasing non-polarity because ethyl acetate content is large and it is difficult to dehydrate using anhydrous sodium sulfate.
(4) Wash with water thoroughly because the recovery of cation exchange resin becomes poor when acetic acid resulting from the hydrolysis of ethyl acetate remains.
(5) Florisil column clean-up can be omitted if there is no interference in GC analysis.

III.2.9 Analytical methods for the determination of organophosphorus pesticides in water, sediment, biological samples, and air

III.2.9.1 Target compounds

(1) water, sediment, biological organisms, and air : fenitrothion (MEP), diazinon, fenthion (MPP), edifenphos (EDDP), malathion, isoxathion, EPN
(2) water, sediment and biological organisms: kitazinon P, methidathion, salithion, phosalone, phosmet (PMP), α-chlorfenvinphos (α-CVP), β-chlorfenvinphos (β-CVP)
(3) air : dichlorvos (DDVP), propaphos, piperophos

III.2.9.2 Outline of analytical methods

(1) Extract water samples with dichloromethane, dehydrate, concentrate, add internal standard, then determine by capillary GC/MS-SIM or GC-FPD.
(2) Extract sediment samples with acetone, change solvent to dichloromethane, dehydrate and concentrate to dryness. Clean-up by hydrous silica gel column chromatography, concentrate, then determine by GC/MS-SIM or GC-FPD.
(3) Extract biological samples (e.g. fish) with acetone, change solvent to dichloromethane, dehydrate and concentrate to dryness. Then separate the non polar to relatively polar fractions and the polar fraction which contains lipids by silica gel column chromatography. Remove lipids from polar fraction by polyamide reverse phase chromatography. Extract the eluate from reverse phase chromatography with dichloromethane, and dehydrate. Combine

both fractions and concentrate, and determine by GC/MS-SIM or GC-FPD.

(4) Pass 1000 L of air samples through ODS packed trap cartridge using air sampler, then elute with a mixture of dichloromethane and n-hexane, concentrate and determine by GC/MS-SIM or GC/FPD.

III.2.9.3 Detection limits and determination limits

Table III-2-20 shows the GC/MS-SIM detection and determination limits for a range of organophosphorus pesticides in water, sediment and biological organisms. **Table III-2-21** shows the detection limits in air.

Table III-2-20 Detection limits and determination limits in water, sediment and biological organisms

compounds	water (μg/L)		sediment & biological organisms detection limits (μg/Kg)
	detection limit	determination limit	
salithion	0.013	0.042	2.4
diazinon	0.013	0.043	2.9
kitazinon P (IBP)	0.027	0.090	7.7
fenitrothion (MEP)	0.032	0.110	23.7
malathion	0.044	0.150	23.1
fenthion (MPP)	0.120	0.400	10.6
α-chlorfenvinphos (α-CVP)	0.023	0.078	11.7
β-chlorfenvinphos (β-CVP)	0.024	0.079	10.7
methidathion	0.072	0.240	37.5
isoxathion	0.110	0.380	N/D
edifenphos (EDDP)	0.034	0.110	35.2
phosmet (PMP)	0.048	0.160	27.3
EPN	0.120	0.390	18.6
phosalone	0.073	0.240	20.9

Table III-2-21 Detection limits in air

target compounds	detection limits (ng/m^3)
fenitrothion (MEP)	1.5
diazinon	1.0
fenthion (MPP)	1.0
dichlorvos (DDVP)	5.0
malathion	3.5
isoxathion	50
EPN	10
propaphos	1.5
piperophos	2.5

III.2.9.4 Reagents and apparatus

A. Reagents

- dichloromethane, acetone, hexane and methanol : pesticide analysis grade
- water : commercial mineral water (note 1)
- sodium chloride and anhydrous sodium sulfate : pesticide analysis grade
- azobenzene : reagent first grade
- pesticide standards : commercial pesticide standards
- internal: Cambridge Isotope Laboratory products
- eluent for polyamide column chromatography : methanol : water = 1 : 1
- silica gel : Wako gel C-200
- polyamide : polyamide C-200 (for column chromatography)
- preparation of 5 % and 40 % hydrated silica gel : activate "Wako gel C-200", or equivalent, at 130 °C overnight, then cool slowly in a desiccator. Place 100 g of the silica gel into an Erlenmeyer flask fitted with a glass stopper, add 5 and 40 mL of purified water, putting the stoppers in, and leave for 4 - 5 hours. Shake occasionally until evenly mixed.

B. Apparatus

- homogeniser : for biological sample extraction
- centrifuge : for separation of sediment and biological sample extracts
- Kuderna-Danish (KD) : sample concentration apparatus
- chromatography column : 1 cm i.d., 20 - 30 cm length
- ultrasonic extractor : for sediment sample extraction (ultrasonic bath is also OK)
- preparation of 5 % hydrated silica gel column for sediment : packed with 2 g of 5 % hydrated silica gel. Use hexane to add the silica gel as slurry. Lay 2 cm of anhydrous sodium sulfate onto the top of the silica gel.
- preparation of 40 % hydrated silica gel column for biological analysis : packed with 5 g of 40 % hydrated silica gel. Use hexane to add the silica gel as slurry. Lay 2 cm of anhydrous sodium sulfate onto the top of the silica gel.
- preparation of polyamide column for biological samples : packed with 1.0 g of polyamide in a chromatography column 1 cm i.d. using eluent (methanol : water = 1 : 1) to add the polyamide as a slurry (note 2). Liquid surface comes out about 2 cm on the column head (note 3).
- air sample trap cartridge : Sep Pak tC18 cartridge washed with 5 mL of dichloromethane :

n-hexane (1 : 1) and dried by passing ultrapure nitrogen gas through.

III.2.9.5 Experimental

A. Sample preparation

a) Water samples

Dissolve 50 g of sodium chloride in 1 L of aqueous sample in a separating funnel. Add 100 mL of dichloromethane, shake and extract. Separate the dichloromethane layer. Extract the aqueous phase again with another 50 mL of dichloromethane. Separate the organic phase and combine the dichloromethane layers. Dehydrate the dichloromethane layer with anhydrous sodium sulfate, and concentrate by KD concentrator (at atmospheric pressure) to a final volume of 3 to 5 mL.

b) Sediment samples

Place 10 g of sediment sample in a centrifuge test tube, add 30 mL of acetone, stir with a spatula and thoroughly disperse, and extract by ultrasonic for 10 minutes. After extraction, centrifuge at 3000 rpm for 10 minutes, and separate acetone layer. Add acetone to the residue, repeat extraction and separation, and combine the acetone layers. Add 200 mL of 5 % sodium chloride solution to the acetone layer, extract with 50 mL of dichloromethane twice, and combine the dichloromethane layers. Dehydrate the dichloromethane layer with anhydrous sodium sulfate, concentrate by KD concentrator to a final volume of 3 to 5 mL. Evaporate the solution to near dryness by passing a stream of nitrogen gas over the surface of the solution, then add 2 mL of hexane for clean-up. (note 4)

c) Biological samples

Place 10 g of biological sample in a homogeniser, add 30 mL of acetone, and homogenise for 5 minutes. Transfer the contents of the homogeniser to a centrifuge tube, centrifuge at 3000 rpm for 10 minutes, then separate the acetone layer. Repeat this extraction and separation with another 30 mL of hexane, then combine the organic phases. Add 200 mL of 5 % sodium chloride solution to the organic phase, extract with 50 mL of dichloromethane twice, and combine the dichloromethane layers. Dehydrate the dichloromethane layer with anhydrous sodium sulfate, concentrate by KD concentrator to a final volume of 3 to 5 mL. Evaporate the solution to near dryness by passing a stream of nitrogen gas over the surface of the solution, then add 2 mL of hexane which contains 500 ppm azobenzene for clean-up. (note 5)

d) Air samples

Connect a trap cartridge to a flow meter and an air sampler, pass air through the system at a

flow rate of 0.7 L/min for 24 hours, and sample 1000 L of air. After trapping the sample, connect syringe, pipette, etc. to the trap cartridge, put in 10 mL of a mixture of dichloromethane : hexane (1:1), and elute by passing through at the flow rate of about 1 mL/min without pressuring.

B. Sample clean-up

a) Water samples

Concentrate the prepared sample solution to 0.5 mL by passing a stream of nitrogen gas over the surface of the solution, and add prescribed amount of internal standards for analysis.

b) Sediment samples

Load the concentrate onto a 5 % hydrated silica gel column. Wash the column with 20 mL of hexane. Discard the eluate. Then elute the target compounds with 30 mL of hexane which contains 10 % acetone. Concentrate the eluate by KD concentration to a final volume of 3 to 5 mL. Evaporate the solution to 0.5 mL by passing a stream of nitrogen gas over the surface of the solution, and finally add a prescribed amount of internal standard for analysis.

c) Biological samples

Load the concentrate onto a 40 % hydrated silica gel column. Initially, elute the column with hexane, and note as the eluted volume the amount of hexane required to elute the centre of the yellow azobenzene band (azobenzene was added at the previous step to act as a marker for the column chromatography). Then continue eluting the column with hexane, and collect double the amount of hexane eluate recorded in the previous step (Bio. Fr-1) (note 6). Elute with 50 mL of hexane which contains 10 % acetone (Bio. Fr-2a). Concentrate fraction (Fr-2a) to a volume of 3 to 5 mL by KD concentrator, evaporate the solution to near 0.5 mL by passing a stream of nitrogen gas over the surface of the solution. Add 0.3 g of polyamide to the concentrate and stir with a spatula (note 7). Load this onto a polyamide chromatography column, develop with 120 mL of eluting solution, and collect the eluate in a container containing 100 mL of 5 % sodium chloride solution (note 8) (Bio. Fr-2b). Shake and extract the eluate / sodium chloride mixture with 30 mL of dichloromethane. Separate the dichloromethane phase. Repeat the extraction with another 30 mL of dichloromethane. Combine the dichloromethane phases, dehydrate with anhydrous sodium sulfate and filter. Combine the filtrate with the hexane fraction (Fr-1). Concentrate with a KD concentrator to a final volume of 3 to 5 mL. Evaporate the solution to 0.5 mL by passing a stream of nitrogen gas over the surface of the solution, and finally add a prescribed amount of internal standard for analysis.

d) Air samples

Add a prescribed amount of internal standard to the eluate, evaporate the solution to 0.5 mL by passing a stream of nitrogen gas over the surface of the solution. Use this solution for analysis.

C. Preparation of blank samples

Prepare blank samples using the same procedures as used for sample preparation and clean-up using the same amount of water as samples for the control. For blank air samples, operate the same procedures from the elution of a cartridge.

D. Preparation of standard solutions

Prepare 1000 mg/L standard stock solutions by dissolving each target compound in acetone. Mix prescribed amounts of standard stock solutions and dilute with acetone to make a 10 mg/L standards mixture. Dilute this mixture to prepare 1.0 mg/L solutions in acetone for standard mixture solution.

Internal standard solutions are each 1.0 mg/mL of phenathrene-d_{10}, fluoranthene-d_{10}, chrysene-d_{12} in acetone solution. Store these standards in a cool and dark place.

E. Measurement

a) Measurement conditions of GC/MS-SIM

- machine: ex. (MS) JEOL-DX303 DA-5000, (GC) HP 5790
- column : ex. fused silica capillary column Ultra-2-Crosslinked 5 % phenylmethyl silicone (25 m x 0.32 mm i.d., 0.52 μm) or similar column
- column temperature : 50 °C (1 min) - 5 °C/min - 280 °C (5 min)
- injector temperature : 250 °C
- interface temperature : 280 °C
- injection method : splitless (purge off ; 1.5 min)
- carrier gas : He (10 psi)
- ion source temperature : 250 °C
- ionisation energy : 70 eV
- ionisation current : 300 μA
- ionisation method : EI, Positive

- injection amount 1 μL
- motor ion : **Table III-2-22**

Table III-2-22 Monitor ions

Compounds	Monitored Ions
phenanthrene-d_{10} (internal standard)	188
fluoranthene-d_{10} (i. s.)	212
chrysene-d_{12} (i. s.)	240
fenitrothion (MEP)	277 (260)
diazinon	304 (179)
fenthion (MPP)	278 (169)
Edifenphos (EDDP)	310 (173)
malathion	173 (127)
isoxathion	313 (177)
EPN	185(169)
kitazinon P (IBP)	204 (288)
methidathion	145 (125)
salithion	216 (183)
phosalone	182 (367)
phosmet (PMP)	160 (317)
α-chlorfenvinphos (α -CVP)	323 (325)
β-chlorfenvinphos (β -CVP)	323 (325)
dichlorvos (DDVP)	220 (185)
propaphos	304 (220)
piperophos	320 (140)

(confirmatory ion)

b) Measurement conditions of GC-FPD (note 9)

- column : fused silica wide bore capillary column DB-5 Crosslinked 5 % phenylmethylsilicone (30 m x 0.53 mm i.d., 0.5 μm) or similar column
- column temperature : 50 °C (1min) - 5 °C/min - 280 °C (5 min)
- injector temperature : 250 °C
- injection method : direct injection
- carrier gas : He, flow rate : 10 mL/min
- injection amount : 3 μL
- detector : FPD (P mode)
- detector temperature : 280 °C

F. Calibration curves

For GC/MS analysis, take several volumes of the standard mixture, add prescribed amounts of internal standards, and add acetone or evaporate with nitrogen gas until the solution volume is 1 mL. Inject 1 μL into the GC/MS, and make calibration curves from the ratio of the peak area of the standards and the internal standard with the closest retention time.

For GC-FPD analysis, take several volumes of the standard mixture, add acetone to make exactly 1 mL, and inject 3 μL into the GC-FPD system. Construct calibration curves from peak areas.

G. Determination and calculation

Inject a known amount of sample (1 μL for GC/MS, 3 μL for GC-FPD).

- Internal standard method : determine the amount of target compound in the samples from the ratio of sample peak area and internal standard peak areas.
- External standard method : calculate the amount of target compound in the samples from calibration curves and gained peak areas.

Then calculate the concentrations in water, sediment and biological samples from formula 1. For air samples, use formula 2.

$$C\ (ng/mL\ \text{ or }\ ng/g) = \frac{C_S\ (ng)}{S\ (mL\ \text{ or }\ g)} \qquad \text{formula 1}$$

$$C_A\ (ng/m^3) = C_S\ (ng) \times \frac{1000}{V} \times \frac{273 + t}{273 + 20} \times \frac{1013.25}{P} \qquad \text{formula 2}$$

C : target compound concentration
C_S : target compound amount in the treated sample
C_A : target compound concentration in the air sample
S : Sample amount
V : trapped air amount (L)
t : average atmospheric temperature (°C)
P : average atmospheric pressure (hPa)

III.2.9.6 Recovery tests

A. Table III-2-23 shows the results of recovery tests performed using actual water, sediment, and fish samples spiked with target pesticides at low concentrations.

Table III-2-23 Recovery of water, sediment, and fish samples and relative standard deviation (RSD)

Compounds	river water			seawater		
	spike amount (μg)	recovery (%)	RSD (%)	spike amount (μg)	recovery (%)	RSD (%)
Salithion	0.12	101.5	3.3	0.12	121.3	5.7
Diazinon	0.18	122.0	2.3	0.18	115.8	5.4
Kitazinon P (IBP)	0.21	123.5	5.4	0.21	127.5	6.9
Fenitrothion (MEP)	0.3	116.0	1.2	0.3	115.3	5.4
Malathion	0.6	126.3	3.5	0.6	125.0	5.7
Fenthion (MPP)	0.3	112.5	1.1	0.3	108.3	5.3
α-chlorfenvinphos (α-CVP)	0.3	128.5	8.0	0.3	135.5	8.2
β-chlorfenvinphos (β-CVP)	0.3	125.0	7.2	0.3	128.3	5.8
Methidathion	0.9	121.5	7.5	0.9	125.8	6.0
Isoxathion	1.2	128.5	3.4	1.2	145.0	3.2
Edifenphos (EDDP)	0.9	125.3	2.1	0.9	122.0	4.7
Phosmet (PMP)	0.9	132.2	3.7	0.9	119.5	3.2
EPN	1.5	101.5	2.6	1.5	104.0	1.8
Phosalone	1.2	101.3	4.7	1.2	103.3	0.9
Compounds	sediment			fish		
	spike amount (μg)	recovery (%)	RSD (%)	spike amount (μg)	recovery (%)	RSD (%)
Salithion	1.0	102.3	17.7	1.0	39.7	5.1
Diazinon	1.0	97.5	6.2	1.0	91.8	4.7
Kitazinon P (IBP)	1.0	119.2	13.1	1.0	96.7	4.6
Fenitrothion (MEP)	1.0	113.0	25.8	1.0	99.5	6.6
Malathion	1.0	93.6	14.8	1.0	101.0	7.1
Fenthion (MPP)	1.0	109.2	21.9	1.0	97.5	4.7
α-chlorfenvinphos (α-CVP)	1.0	114.3	16.9	1.0	93.0	4.4
β-chlorfenvinphos (β-CVP)	1.0	116.7	15.7	1.0	90.9	4.2
Methidathion	1.0	133.2	20.4	1.0	94.4	5.0
Isoxathion (note 9)	-	-	-	1.0	66.6	35.3
Edifenphos (EDDP)	1.0	112.4	28.5	1.0	67.3	9.8
Phosmet (PMP)	1.0	113.2	6.4	1.0	11.3	23.9
EPN	1.0	125.6	11.7	1.0	91.1	10.8
Phosalone	1.0	166.2	15.5	1.0	85.9	8.7

water samples : 1000 mL, sediment and fish samples : 10 g

water sample : n=4, sediment and fish sample : n=7

B. Air samples.

Table III-2-24 shows the results of recovery tests in which 500 ng of each pesticide was dissolved in toluene, and then put onto a trap cartridge, then passed 1000 mL of air through for recovery tests.

Table III-2-24 Result of recovery tests of air samples

Compounds	recovery (%)	RSD (%)
fenitrothion (MEP)	104.8	3.8
Diazinon	111.5	4.2
fenthion (MPP)	68.0	4.9
dichlorvos (DDVP)	96.6	1.6
Malathion	109.9	4.2
isoxathion (note 9)	82.8	9.8
EPN	89.2	13.9
Propaphos	95.1	9.6
Piperophos	92.9	10.1

III.2.9.7 Notes

(1) Depending on the tap water source, distilled water made from tap water contains pesticides and other chemicals. Commercial mineral water is recommended. However, 2,6-di-tert-butyl-p-cresol (BHT) and its decomposition product, 2,6-di-tert-butylbenzoquinone, might be detected. These compounds seem to be derived from the container.

(2) Fine particles of polyamide come out and cause turbidity when preparing the column, but it will become clear after settling down.

(3) Leave a space of about 2 cm between the liquid surface and the column head so as to be able to fit in the polyamide sprinkled with sample.

(4) Depending on the sediment, it may be difficult to know when evaporation is complete -some of the contents don't evaporate when blowing nitrogen gas across the solution. In this case recovery may become poor. Try gentle heating during the blowing of nitrogen gas across the solution surface.

(5) When hexane elution has to be stopped right before an interference originating in the organism comes out, it is difficult to see the position of the interference. Therefore azobenzene is used as marker. Azobenzene is observed moving as a yellow band in the column. Graduated test tube (25 mL) is suitable for the collection vessel. Elute the azobenzene with about 7 mL of hexane.

(6) A small amount of target pesticide may be eluted in this fraction depending on solvent condition before column loading or the amount of fat. Besides start to measure amount of hexane at the point of sample loading.

(7) Elute with 120 mL of eluting solution. However, some interferences originating in the organism might be eluted with less than 120 mL depending on the sample conditions. If the sodium chloride solution in the collection vessel becomes cloudy, then stop development immediately.
(8) When GC-FPD seems to detect compounds, it is necessary to confirm this by using a column of different polarity.
(9) Consider insert cleanness or column conditions to analyse isoxathion because sensitivity of isoxathion is remarkably effected by dirtiness of injection port or column ageing.

III.2.10 Measurement method of coarse lipid

For many biological samples, it is desirable to express the concentration of target compound per unit volume or mass of lipid, as well as a simple sample concentration, because some types of target compounds, especially those compounds which have bioconcentration character (such as organic chlorine compounds), tend to be found primarily in lipid. This is done by measuring the total amount of coarse lipid contained in the sample.

Place 5 g of sample into a homogenizer cup. Add 20 mL of chloroform and 40 mL of methanol, and homogenise the sample for 2 minutes. Filter the resulting mixture. Add 80 mL of chloroform : methanol (1:1 v/v) to the residue, rehomogenise, and filter. Combine the filtrates (the organic layers) and transfer into a separating funnel. Add 60 mL of purified water, then mix by shaking gently. Collect the lower chloroform layer, dehydrate with anhyrous sodium sulphate, evaporate the solvent by rotary evaporation, then dry the sample in a desiccator using phosphorus pentoxide as desciccant. Finally, weigh the lipid sample. The results are to be expressed as lipid weight (%).