MERCURY ANALYSIS MANUAL

March 2004

Ministry of the Environment, Japan

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Foreword

The February 2003 meeting of the Governing Council of the 22nd UN Environmental Program (UNEP) reported on the Global Mercury Assessment and adopted a resolution for future international action.

Developed countries are currently addressing the issue of the adverse health effects of human exposure to low-level methylmercury. In Japan, fish and shellfish are a valuable source of protein as well as a mainstay of the nation's culinary culture; therefore, a careful, prompt, and science-centered response to the risk of mercury exposure through the consumption of fish and shellfish is needed.

To study the adverse health effects of low-level methylmercury exposure on the development of fetuses and infants, Japan initiated a cohort study in 2002 following two preceding cohort studies: one undertaken in the Faroe Islands, the other in the Republic of Seychelles.

Even today, coal-fired thermal power plants in various countries around the world continue to discharge mercury into the surrounding environment, while industrial plants producing chlorine and alkali continue to discharge mercury into water systems. Moreover, developing countries with gold mining operations are suffering from serious pollution caused by the use of mercury in gold refining. Clearly, therefore, it is essential that the state of mercury pollution be monitored.

Against this background, technology capable of highly precise analysis of total mercury and methylmercury must be made available in Japan and elsewhere so that precise risk assessment can be conducted. In this spirit, the Ministry of the Environment has prepared the *Mercury Analysis Manual* in order to put in place proven and internationally accepted analytical methods for more widespread practical application.

As the chairperson of the committee for this manual, I would like to express my appreciation to all who participated in its preparation. I am confident this manual will be widely employed worldwide as a practical aid for mercury analysis.

Tsuguyoshi Suzuki
Chairperson
The Committee for the *Mercury Analysis Manual*

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1. Introduction

Obtaining reliable analytical data for mercury requires the following: appropriate sample collection; pre-treatment for analysis; the selection of a measurement method and preparation method for sample test solutions suited to the samples; experience in their use; and confirmation of the reliability of one's own analytical data. In addition, when performing an analysis, one must regularly pay attention to preventing contamination of the samples by keeping the laboratory clean; providing appropriate ventilation; and adequately washing glassware, tools, and containers.

When evaluating the adverse health effects of mercury and clarifying its dynamics and pathways in humans and in the environment, in addition to performing a quantitative analysis for total mercury, one should also perform separate quantitative analyses for methylmercury and inorganic mercury. This manual first provides a description of sampling followed by descriptions of the analytical methods for total mercury and methylmercury for each target sample.

It should be noted that, for mercury analysis of fish and shellfish, water, air, soil (content and its elutriation) and the like, the following official regulations have been laid down, among others: "Provisional Regulatory Standards for Fish and Other Marine Products" (Director-General Notification No. 99, Environmental Health Bureau, Ministry of Health and Welfare, dated July, 23, 1973); the method listed in the table attached to Environment Agency Notice No. 59 issued in Dec. 1971 (Environmental Quality Standards for Water Pollution); the *Manual of Measuring Methods for Hazardous Air Pollutants* (Air Environment Regulation No. 88, dated March 31, 1999); and Ministry of the Environment Notice No. 19, dated March, 6, 2003. In cases where analysis is a requirement of the official regulation, an analytical method suitable for each case may be used. Further, a number of other methods for mercury analysis have been reported. When using any of these analytical methods, one should of course practice careful quality control/quality

assurance of the obtained data, including simultaneous determination of suitable certified reference materials (CRMs).

Currently, the CRMs prepared for the quality control/quality assurance of analytical values for mercury as well as methylmercury in various biological and environmental matrices are commercially available from several organizations, including the IAEA (International Atomic Energy Agency, Analytical Quality Control Services), NIST (National Institute of Standards and Technology, Office of Standard Reference Materials, USA), NRCC (National Research Council of Canada), and NIES (National Institute for Environmental Studies, Japan). These CRMs may be used as needed.

2. Sampling

2-1 Environmental samples

2-1-1 Biological samples (fish and shellfish)

Water pollution caused by methylmercury can be monitored conveniently by measuring the bioaccumulation of mercury in fish. Further, monitoring the mercury in fish and shellfish eaten most often by people in a particular region is a suitable means of evaluating human exposure levels, because human exposure to methylmercury occurs mostly through the consumption of fish and shellfish. In addition, since most mercury present in fish is in the form of methylmercury, the measurement of total mercury in fish can be used to evaluate methylmercury intake by humans. However, methylmercury should also be measured in cases where extremely high values appear and in cases involving consumption of whale meat and organ tissues whose proportion of methylmercury to total mercury is not always constant.

When collecting fish samples, record the sampling date, location, species, and ages. Also measure the weight and length and the like. For fish, collect 10-20 grams of the edible portion, place it in polyethylene bags, and store it in a freezer. For shellfish, divide the muscle, digestive tract contents, and adductor muscle (for snails, which lack an adductor muscle, divide the edible portion), place the portions in polyethylene bags, and store in a freezer. Since particles of bottom sediment are often contained in the digestive tracts of shellfish, remove these particles before storage.

According to Japan’s Food Hygiene Act, the provisional regulatory standard for mercury in fish and shellfish is 0.4 mg/kg (wet weight) as total mercury. The background level of total mercury in fish and shellfish is considered to be 0.01-0.1 mg/kg (wet weight).

2-1-2 Water

When the source of contamination is directly connected to a river, lake, marsh, or ocean, or when contamination is expected to have spread from a river to a lake, marsh, or ocean, take water samples from the various areas. Use a Bandon water sampler or the like to collect the water samples, preferably at 20-30 cm below the surface. Take great care to prevent bottom sediment from entering water samples collected near the bottom. In principle, collect seawater samples at high tide and avoid windy or rainy days. For lakes, marshes, and ocean regions, clearly state the collection date, location, general water quality, position relative to contamination source, and other information.

Keep water samples in a sealable glass or Teflon container that has been well washed with hydrochloric acid or other agents before being transported. For total mercury, Japan's Effluent Standard and Environmental Quality Standard are 0.005 mg/L (ppm) and 0.0005 mg/L (ppm), respectively, according to Environment Agency Notice No. 64 (September 1974) based on the Water Pollution Control Law in Japan. For alkyl mercury, both the Effluent Standard and Environmental Quality Standard state that it must not be detectable at the detection limit of 0.0005 mg/L (ppm) when analyzed with the official methods provided by the Water Pollution Control Law. When a contamination accident causes a sudden release of wastewater containing high levels of mercury, the levels of total mercury and alkyl mercury must be evaluated according to these official standards. The background levels of total mercury are generally 0.5-3 ng/L (ppt) for ocean water, 2-15 ng/L (ppt) for shore water, and 1-3 ng/L (ppt) for rivers and lakes.

2-1-3 Sediment/soil

When collecting soil samples, vary the frequency of sample collection depending on the plane position of the mercury pollution source and the extent of the suspected contamination. While various methods for collecting soil samples from the site have been proposed, Japan's Soil Pollution Countermeasures Law (Ministry of the Environment, 2003) implemented in 2003 provides detailed descriptions of the methods for collecting soil samples. These methods served as the basis for the sampling methods provided in this manual. Briefly, in most pollution situations, collect one sample per 100 m^2 (based on a 10 m × 10 m grid). At sites where the pollution record suggests the risk of pollution is not extreme, obtain one sample by mixing samples obtained at five spots per 900 m^2 (based on a 30 m × 30 m grid). With this five-spot mixing method, collect individual samples from a total of five spots: the center point of each grid and four subpoints set around it. Combine these five samples to obtain one final composite sample. This enhances the representativeness of the soil samples obtained from each grid. Although the locations of the four subpoints are not precisely set out, it is desirable to collect the four samples at points north, south, east, and west of the center point.

At each sampling point, collect the soil samples between the soil surface and 50 cm below the soil. Specifically, collect the individual samples from two separate regions, one between the soil surface and a point 5 cm below the surface, and the other in the area from 5 cm to 50 cm below the soil surface. After collecting the soil samples, remove most foreign objects (pebbles, roots, etc.) from each sample and homogenize each sample by mixing with the quarter method. After homogenization, mix an equal weight of each sample to obtain a final composite sample. Similarly, for the five-spot mixing method, mix an equal weight of each of the five samples (homogenized with the soil pre-treatment method mentioned above) to obtain one composite sample for the mercury analyses.

For rivers, sampling points allowing easy collection of the bottom sediment are chosen at intervals of 50-200 m downstream from the discharge point of industrial wastewater or city drains; moreover, it is desirable that about two points upstream be set for collection of bottom sediment as the control. The collection spots for the sediment samples are usually specified as both riverbanks and the center of the river. Where the river is wide, increase the number of sampling points.

For lakes, marshes, and ocean areas, radially center the sampling points on the release point or mouth of the river and conduct a grid survey as needed.

As for the sampling methods, the Ekman dredge sampler is used for collection of the surface layer sediment of rivers, lakes, marshes, and seashores, whereas the core sediment sampler is used to collect columnar samples that allow for estimation of the sedimentary state and the history of mercury contamination and accumulation.

Clean the collected bottom sediment of wood pieces, pebbles, shells, and dust and pass it through a 2-mm mesh sieve to prepare a sample. If the sample has a high water content, centrifuge it to remove the supernatant and mix well to homogenize it before submitting it for analysis. Record the date, location, and general conditions (appearance, color, smell, impurities, etc.).

Although glass containers are best for the collected samples, other sealed containers may also be used. Wash the containers well beforehand with hydrochloric acid or another agent. Store the samples in a cool dark place. Samples containing metallic mercury or divalent mercury should be stored in a freezer.

Generally, the mercury level in soil is less than 0.2 mg/kg of dry weight (ppm). When the total mercury level in soil is found to exceed a few mg/kg (ppm), the risk exists that the mercury will migrate from the soil into other environmental sectors. In such cases, mercury contamination in nearby water systems must also be investigated.

2-1-4 Plants

Plants normally exhibit little biological magnification of heavy metals and therefore are not suitable for evaluating contamination. However, lichens have various properties that make them suitable as a biological indicator of air pollutants. As with other rootless air plants, they absorb nutrients directly from the air, accumulate metals efficiently, and exhibit resistance to high concentrations of metals in their tissues.

Lichens are widely distributed geographically, making them suitable for not only domestic but also international evaluations of air pollution. In fact, lichens (including the Parmelia and Usunera species) have often been used in research to evaluate air pollution caused by mercury and various other heavy metal pollutants, and Garty (2001) has reported a related review article. Lichens, which usually grow on trees or branches, are collected, washed well with water, cleaned of wood pieces and dust, and air-dried to make a sample. For mercury analysis, place a few grams of the sample in a vial and cut it into pieces with dissection scissors.

2-1-5 Atmosphere/air

Air samples are collected when mercury pollution is believed to be present in the atmosphere or indoor environment. Since mercury concentrations in the atmosphere vary greatly, sampling points must be selected in order to clarify the mercury distribution with consideration given to prevailing winds and the distance from the contamination source. To obtain air samples from the general indoor environment and indoor environment of workrooms, etc., divide the room into a grid of 3 m square (with the width of the grid adjusted to accommodate the scale of the work environment) and collect samples at the intersections of the grids. In consideration of possible human exposure, set sampling points at 1 to 2 m above ground. To collect mercury in the atmosphere or in the indoor air, place an absorbing solution comprising 20 ml of 0.1% potassium permanganate and 1N sulfuric acid in an impinger or similar bubbler. To sample the air, use a suction pump to draw the air into the absorbing solution from the sampling point at a flow rate of 1 L/min. for a given time.

Because commercially available potassium permanganate often contains mercury, dissolve it in 1N sulfuric acid and boil it to generate a precipitate of MnO_2. Cool and filter it for use as an absorbing solution. This procedure can remove all mercury content and render the absorbance of the blank test solution almost zero. The absorbing solution obtained in this manner can collect mercury vapor efficiently, and one bubbler containing absorbing solution is usually sufficient.

If the absorbing solution has evaporated and decreased in volume after the air samples have been drawn, top up the absorbing solution to a fixed volume to make a test sample. Separately from this sample, take two identical volumes of absorbing solution that have not been aerated. Set aside one volume as a blank test solution; to the other, add a fixed volume of inorganic mercury (II) standard solution to create a standard test solution.

At measurement, add 10% hydroxylamine hydrochloride dropwise to decolorize the potassium permanganate; determine the mercury concentration in the sample test solution by cold vapor atomic absorption spectrometry, as with the other samples. Using the volume of air collected, calculate the mercury concentration in the air sample. This method can be widely applied to tests of the environmental atmosphere, the air of a work environment, and the gas discharged from an emission source or the like.

For mercury in the atmosphere, standard values have not been established in Japan for either the environment or the generating source. However, the Japan Society of Industrial Health recommends a concentration of 0.025 mg/m^3 as the permissible concentration of mercury vapor in the work environment.

2-2 Human samples

Mercury in biological samples -usually hair, blood, urine or umbilical cord- is measured in order to evaluate the level of human exposure and body-burden.

2-2-1 Hair

The mercury concentration in hair is often used as a biomarker for methylmercury exposure because it reflects the concentration in the blood at the time the hair was formed. At the same time, a hair sample provides a simple and noninvasive sampling method as well as a storage method offering good sample preservation. The mercury concentration in hair is generally detected at 250-300 times the blood concentration. Since the hair grows at a rate of roughly 1 cm per month, evaluation of past exposure is possible. However, the mercury concentration in hair can increase as a result of adhesion of external mercury vapor and inorganic mercury, decrease as a result of hair treatments such as permanents, and be influenced by the sample collection site.

In cases of no exposure to external inorganic mercury or mercury vapor, almost all mercury in hair is in the form of methylmercury; therefore, the level of methylmercury exposure from diet can be evaluated by measuring total mercury. However, since people involved in gold mining and gold refining have a high risk of contamination from metallic mercury and mercury vapor, evaluation of actual methylmercury exposure is possible only by measuring methylmercury as well as total mercury in hair. Hair samples should be obtained from the occipital area of the head and should include at least 20 strands of hair measuring 1 cm long (about 10 mg in total) cut with scissors at the hair root. Tie the root ends of the sampled hair strands together with a cotton thread or affix to adhesive tape or the like so that the root ends can be identified. Place the sample in a polyethylene bag and store at

room temperature. Among the general population in Japan, the mercury concentration in hair is in the range of 1-5 ppm and seldom exceeds 10 ppm.

2-2-2 Blood

For people who eat large quantities of fish and shellfish, the mercury concentration ratio of red blood cells to plasma (serum) is approximately 10:1, and most mercury contained in the red blood cells is in the form of methylmercury; therefore, the methylmercury exposure can be evaluated by measuring total mercury in blood. It is believed that 50% of inorganic mercury is present in the plasma and the mercury concentration in the plasma increases in relation to the amount of inorganic mercury accumulated in the kidneys. Thus, the exposure to inorganic mercury/mercury vapor can be evaluated by measuring the total mercury in plasma.

A blood sample in the range of several milliliters is collected as usual from a vein into an injection tube already containing an anticoagulant (heparin) and transferred into a sealed container. The sample is then centrifuged at 3,000 rpm for 10 minutes to separate the red blood cells from plasma. Samples to be stored for a long period of time should be frozen. While the mercury concentration in the blood of the general population in Japan generally does not exceed 40 ng/g, people whose diet is rich in fish sometimes have higher values.

2-2-3 Urine

Most mercury present in the urine is in the form of inorganic mercury. The mercury concentration in the urine increases in relation to the level of inorganic mercury accumulated in the kidneys. Accordingly, the total mercury value in the urine is an important biomarker for evaluating inorganic mercury/mercury vapor exposure. On the other hand, leaking of methylmercury into urine may occur in those with renal disease.

Since the mercury concentration in the urine also varies with the excretion rate, it is necessary to correct it with the creatinine concentration in the urine or to collect the urine sample at a designated time.

Generally, as in usual urinalysis, 50-100 ml of urine is collected as a sample in a paper cup in the early morning. The sample is then stored under refrigeration in a polyethylene container. Samples to be stored for more than one month should be frozen. Since urine contains many inorganic salts, even fresh urine may generate precipitate. Thus, the sample must be homogenized by shaking before analysis. A method also exists where the solubility of the salts is increased by lowering the pH of the urine sample by adding a small amount of hydrochloric acid. Take steps to ensure that microorganisms do not proliferate, as they may cause inorganic mercury to reduce to mercury vapor, which will escape and be lost. It is believed that the average mercury level in the urine of the general population in a region without any particular mercury exposure is less than 10 ng/ml.

2-2-4 Umbilical cord

When food containing methylmercury is ingested by the mother during pregnancy, the methylmercury easily passes through the placenta, transferring from the mother's body to the fetus and exposing the fetus to the methylmercury. One prime example of this was the occurrence of many fetal cases of Minamata disease accompanied by severe cerebral palsy-like symptoms. This was caused by pregnant mothers' ingestion of fish and shellfish highly contaminated with methylmercury when Minamata disease was prevalent in Japan. Mercury in the umbilical cord is used as a suitable biomarker of methylmercury exposure during the fetal period because the mercury concentration in the umbilical cord is highly correlated with that in umbilical cord blood, and most mercury occurring in the umbilical cord is in the form of methylmercury. Several centimeters of the fetus side of the umbilical cord are collected at delivery and washed with physiological saline to remove the blood. The sample is stored frozen until the time of analysis.

The umbilical cord sample can also be stored as a dry sample for a long period if air-dried after collection. In addition, since it is a tradition in Japan for families to store the umbilical cord with care, each child's mercury exposure at birth can be evaluated by measuring the mercury content. Umbilical cords that predate the 1970s, however, often contain much higher amounts of inorganic mercury due to the application of mercurochrome (an antimicrobial containing mercury), which was widely used as an external preparation; therefore, it is necessary to measure methylmercury in such cases. The stored umbilical cord is soaked in water to moisten it, washed with water to remove the blood and other adhering substances, and air-dried to prepare the sample for analysis.

The concentration of methylmercury in the umbilical cord in the general population in Japan is considered to be around 0.1 μg/g (dry weight). It has been

reported that the methylmercury concentration in the umbilical cord can be as high as several μg/g (dry weight) in children born during the Minamata disease outbreak.

Conversion table

1 ppm = 1 mg/kg (L) = 1 μg/g (ml) = 1 ng/mg (μl)

1 ppb = 1 μg/kg (L) = 1 ng/g (ml)

1 ppt = 1 ng/kg (L)

3. Analytical Method for Total Mercury

The conventional methods for measuring total mercury include absorption spectrometry (dithizone colorimetry), neutron activation analysis, and cold vapor atomic absorption spectrometry. In absorption spectrometry, dithizone forms a complex with the metal ions and produces a colored organic solution. The color intensity varies with the mercury concentration. Although this method has been used historically because of the simplicity of the procedures, its use declined greatly with the introduction of highly sensitive atomic absorption spectrometry in the 1960s.

In neutron activation analysis, thermal neutrons in a nuclear reactor are irradiated and gamma radiation from generated ^{197}Hg is measured for comparative quantification with the standard sample. This enables nondestructive analysis in which the sample is analyzed directly without any pre-treatment such as concentration, and it is highly precise and sensitive. However, it is not used frequently due to its high cost, the need for a nuclear reactor, and the need for an expensive counting apparatus, not to mention the safety requirements for handling radioactive materials.

In cold vapor atomic absorption spectrometry, mercury is converted into elemental mercury vapor, which is introduced into an absorption cell and the absorption measured at 253.7 nm for determination of the quantity. It is a much more sensitive method as compared with conventional flame atomic absorption spectrometry. Other advantages include its ability to measure mercury in the samples with a UV spectrophotometer or a simple mercury lamp. It is roughly classified into the reduction/aeration procedure and the sample combustion procedure according to the generation mode for mercury in the elemental form. The former involves wet digestion with a mixture of strong acids followed by the addition of a reducing agent to generate elemental mercury vapor (Hg^0). In the latter, elemental mercury vapor (Hg^0) is generated through direct combustion of the

sample to be analyzed. Currently, the most common method is based on the former technique.

Herein we describe -among these highly sensitive analytical methods- a method involving wet digestion, reduction and cold vapor atomic absorption spectrometry (CVAAS) (the circulation-open air flow system), which offers substantial improvements over the conventional method.

3-1 Determination by the wet digestion/reduction/cold vapor atomic absorption spectrometry (CVAAS) (circulation-open air flow system)

Principle

The present method involving reduction and cold vapor atomic absorption spectrometry (CVAAS) (circulation-open air flow system) is, in principle, similar to the conventional circulation system in that the method includes the following: reduction of Hg^{2+} ions in the sample test solution with stannous chloride to generate elemental mercury vapor (Hg^0); and the introduction of mercury vapor into the photo-absorption cell for the measurement of absorbance at 253.7 nm. However, unlike the conventional closed system in which the elemental mercury vapor generated is continuously circulated with a diaphragm pump through a reaction vessel, a U-shaped tube packed with a drying agent, and the photo-absorption cell, the present method uses a circulation-open air flow system as shown in Figure 1. The apparatus constitutes a closed system and comprises a diaphragm pump, reaction vessel, acid gas trap, moisture trap (ice bath), and a 4-way cock. During its operation, the elemental vapor generated by the addition of stannous chloride is circulated via the 4-way cock at a flow rate of 1-1.5 L/min. for 30 seconds to homogenize the concentration in the gas phase. The 4-way cock is then rotated by 90° to introduce the gas phase into the photo-absorption cell all at once. The measurement is completed within one minute per sample with this apparatus, which can measure even 0.1 ng of mercury with high accuracy.

Additionally, in the method for preparing the sample test solution for the present method, the conventional wet digestion method is improved by the use of a 50-ml flask with a long neck (at least 10 cm), such as a thick-walled volumetric flask[1] with a ground glass stopper, as well as a mixed acid system with an increased rate of sulfuric acid, HNO_3-$HClO_4$-H_2SO_4 (1+1+5), that already contains perchloric acid, for the sample digestion. This is innovative in that sample digestion can be

completed in a relatively short time without loss of mercury. It is a simple method where the sample is subjected to wet digestion on a hot plate at 200-230°C for 30 minutes and cooled followed by topping up to a fixed volume with water. This method can be applied directly to the digestion of biological samples including hair, blood, and fish as well as various solid samples such as sediment and soil. A reflux condenser is not required during heating.

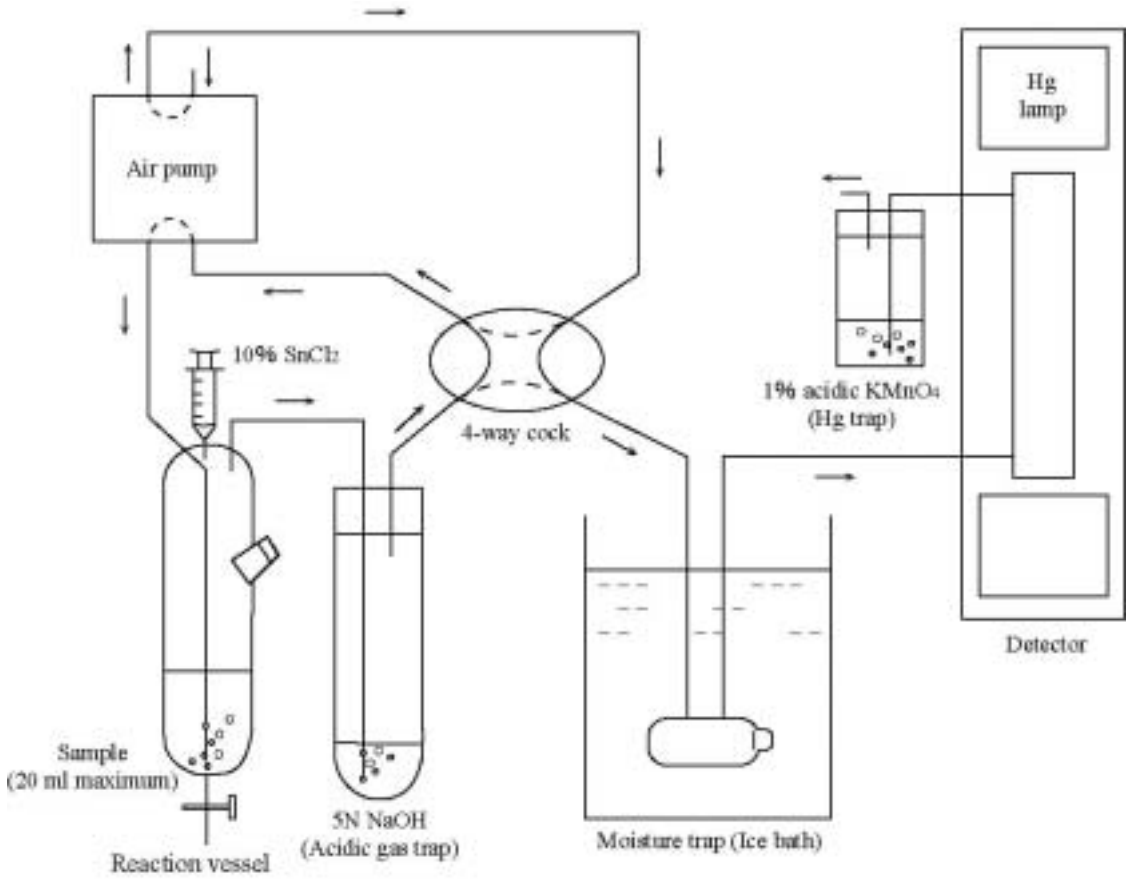


Figure 1. Schematic Diagram of Reduction/Cold Vapor Atomic Absorption Spectrometry (CVAAS) (Circulation-Open Air Flow System)[2]

Procedural Notes

1. A thick-walled volumetric flask made of Pyrex®, available from Koei Co. Ltd., Kumamoto, Japan, is recommended for safety reasons. If it is unavailable, a commercially available volumetric flask made of Pyrex® may be used. In addition, a test tube made of Pyrex® (21 mm in diameter and 200 mm in height) may be used instead of a volumetric flask, and an aluminum block heater may be used instead of a hot plate for wet digestion of the sample using the same procedure as described above.
2. The automated apparatus based on this principle is commercially available as a Model Hg-201 Semi-automated Mercury Analyzer (Sanso Seisakusho Co. Ltd., Tokyo, Japan).

3-1-1 Biological samples (including fish, shellfish, human blood, urine, and tissues such as umbilical cord)

This method is applied to biological samples such as fish, shellfish, human blood, urine and tissues such as umbilical cord. Before being weighed, the sample is placed in a vial, cut into fine pieces with dissection scissors, and homogenized to a rough pasty state to prepare it for analysis. Liquid samples such as blood are mixed well with a Pasteur pipette with a rubber bulb in preparation for analysis.

a. Reagents

(1) HNO_3-$HClO_4$ (1+1): Mix 100 ml of perchloric acid (for measurement of toxic metals) into 100 ml of nitric acid (for measurement of toxic metals). (Store in a cool dark place.)

(2) H_2SO_4: Sulfuric acid (for measurement of toxic metals)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) HCl: Hydrochloric acid (analytical grade)

(5) 10% $SnCl_2$ solution: Dissolve 10 g of tin (II) chloride dihydrate (analytical grade), $SnCl_2 \cdot 2H_2O$, in 9 ml of HCl and dilute to 100 ml with distilled water. Aerate with N_2 gas (100 ml/min., 20-30 minutes) to expel any mercury from the solution.

(6) 5N NaOH: Dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 ml.

(7) 0.1N NaOH: Dilute 5 N NaOH 50-fold with distilled water.

(8) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(9) Methylmercury standard solution[1]: Weigh out 12.5 mg of CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make

a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to obtain a methylmercury standard solution. One ml of this solution contains 1.0 μg of Hg.

(10) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube with a glass stopper. Shake for 3 minutes with a shaker to extract methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(11) 1N H_2SO_4: Gradually add 30 ml of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1,000 ml.

(12) 1% acidic $KMnO_4$ solution for collecting mercury: Dissolve 1 g of potassium permanganate (analytical grade) in 100 ml of 1N H_2SO_4.

(13) 0.5% $KMnO_4$ solution: Dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 ml.

(14) Toluene: $C_6H_5CH_3$ (reagent grade for residual pesticide analyses)

b. Instruments and equipment

(1) Mercury analyzer[2] Model Hg-201 Semi-Automated Mercury Analyzer (Sanso Seisakusho Co., Ltd.)

(2) Hot plate: Capable of attaining a surface temperature of 250°C

(3) Sample digestion flask[3]: 50-ml thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter)

(4) Volumetric flasks: 10, 100, and 1,000 ml

(5) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(6) Vial: 20-ml scintillation vial

(7) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length
(8) Dissection scissors
(9) Multi-flow meter: V4-type flow meter multi-kit (Kojima Instruments Inc.)
(10) Reciprocal shaker
(11) Centrifuge

Note: Prior to use, thoroughly wash all laboratory glassware and sample containers to be used in the analysis with a 0.5% $KMnO_4$ solution. Rinse with water until the color of the $KMnO_4$ solution is no longer visible.

c. Preparation of sample test solution

Precisely weigh out a homogenized sample (0.5 g maximum of wet weight) and place at the bottom of a sample digestion flask. (For dry samples such as umbilical cord, weigh out precisely 0.1 g and add 0.5 ml of distilled water to moisten beforehand.) Add 1 ml of distilled water, 2 ml of HNO_3-$HClO_4$ (1+1), and 5 ml of H_2SO_4 in turn and heat on a hot plate at 200-230°C for 30 minutes Allow to cool, add distilled water to make a fixed volume of 50 ml, mix well, and use the resulting solution as the sample test solution.

For urine samples, add 2 ml of HNO_3-$HClO_4$ (1+1) and 5 ml of H_2SO_4 to a sample digestion flask beforehand. Gradually add a fixed volume (usually 2 ml) of the urine sample while stirring slowly. Heat and treat it in a manner similar to the above procedure to prepare the sample test solution[4]. Separately, transfer 0 and 1.0 ml of methylmercury-cysteine solution (0.10 μg Hg/ml) into two sample digestion flasks (corresponding to 0 and 0.10 μg Hg). Add 1 ml of distilled water only to the former (the blank) followed by 2 ml of HNO_3-$HClO_4$ (1+1) and 5 ml of H_2SO_4 in turn. Follow the same steps as indicated above for preparation of the sample test solutions to make a blank test solution and a standard test solution for the measurement of total mercury.

d. Test procedures and calculations

Test procedures

Gently transfer fixed volumes V ml (usually 5 ml, to a maximum of 10 ml) of each of the blank test solution, the standard test solution, and the sample test solution into the reaction vessel of the mercury analyzer and apply the stopper. Add 1 ml of 10% $SnCl_2$ solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated elemental mercury vapor will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds to homogenize the concentration in the gas phase, while the acidic gas generated from the sample test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of mercury vapor into the photo-absorption cell through an ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the recorder reading begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement.[5]

Calculation

The peak heights (mm) obtained after measurement of fixed volumes V ml[6] of each of the blank, the standard, and the sample test solutions (or their diluted solutions) for the total mercury analysis are labeled Pbl, Pstd, and Ps, respectively. The total mercury concentration in the sample is calculated with the following formula[7]:

Total mercury concentration in the sample (µg/g) = 0.10 µg ×
(Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/sample weight (g)

For blood and urine samples, the mercury concentrations are usually expressed in ng/g and ng/ml, respectively, and thus calculated with the following formula:

Total mercury concentration in blood or urine (ng/g or ml) = 100 ng × (Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/sample amount (g or ml)

Procedural Notes

1. Although a standard solution of inorganic mercury (II) is commonly used as a standard solution for the analysis of total mercury in the sample, the present method uses a methylmercury-cysteine solution as the standard solution, the same used for methylmercury analysis. Similarly to the samples, it is subjected to wet digestion in order to make a standard test solution for total mercury measurement. This is an effort to avoid measurement errors caused by the use of a different standard solution, because most mercury contained in fish and shellfish is in the form of methylmercury, and the total mercury as well as methylmercury in the sample are commonly measured at the same time. Methylmercury in the organic solvent is extremely stable. Even 1 ppm of methylmercury in a toluene solution can be used for several years if stored frozen to prevent volatilization of the solvent. When the preparation of a standard solution for total mercury measurement using the present method unavoidably requires the use of an inorganic mercury (II) standard, the following method is recommended for its stability, good storage characteristics, and other advantages.

   Inorganic mercury standard solution: Weigh out 13.5 mg of mercury (II) chloride (standard) in a 100-ml volumetric flask, dissolve in 4 ml of HNO_3-$HClO_4$ (1+1) and 10 ml of H_2SO_4 added in turn, and top up to the mark with distilled water to make a stock mercury solution (1 ml of the stock mercury solution = 100 µg Hg). The stock mercury solution obtained in such way will

be stable for several years if sealed and stored in a cool dark place. At every use, the stock solution is diluted 1,000 times with the above blank test solution to make a mercury standard solution (1 ml of this solution = 0.10 μg Hg). In addition, when a commercially available standard solution is used, the blank test solution is similarly used to dilute it appropriately.

2. The automated apparatus based on this principle is commercially available as a Model Hg-201 Semi-automated Mercury Analyzer (Sanso Seisakusho Co. Ltd., Tokyo, Japan).
3. A thick-walled volumetric flask made of Pyrex**R,** available from Koei Co. Ltd., Kumamoto, Japan, is recommended for safety reasons. If it is unavailable, a commercially available volumetric flask made of Pyrex**R** may be used. In addition, a test tube made of Pyrex**R** (21 mm in diameter and 200 mm in height) may be used instead of a volumetric flask, and an aluminum block heater may be used instead of a hot plate for wet digestion of the sample using the same procedure as described above.
4. For urine samples, placing the sample in a sample digestion flask and adding HNO_3-$HClO_4$ (1+1) and sulfuric acid in turn -as is the case with the other biological samples- may result in sudden violent reactions accompanied by the risk of overflow of the mixture from the container. To avoid this risk and ensure safe operation, place the acids in the sample digestion flask first and then add the urine sample gradually while swirling the sample digestion flask.
5. Unless sufficient time for purging (at least 15 seconds) has elapsed after measurement of the sample, residues from the former test solution may continue to have an effect. Particularly when measurement of a sample with a low concentration follows measurement of a sample with a high concentration, a measurement should be carried out between them with distilled water in order to confirm that the value has decreased to the background level.

6. The equilibrium concentration between the aqueous phase and the gas phase of reduced and vaporized mercury vapor may differ depending on the acid concentration and volume of the sample test solution at measurement. Therefore, the blank test solution is used for dilution of the sample test solution and both the sample test solution and the standard test solution are measured under the same conditions in every respect (acid concentration and volume).
7. In atomic absorption spectrometry, the multi-point calibration curve method is not always required because the linear range of the calibration curve is wide. Therefore, a one-point calibration curve method is often used as well. Moreover, in addition to the blank solution, choose the most suitable concentration of the standard test solution from, for example, 0.02, 0.05, or 0.10 μg Hg/50 ml for a total mercury measurement with a peak height close to that of the sample test solution. In this case, use the same volume of both the standard test solution and sample test solution during the measurements. This will facilitate quantification.

Biological samples,
(0.5 g max. of wet weight)

Sample digestion flask

Distilled water, 1 ml
HNO_3-$HClO_4$ (1+1), 2 ml
H_2SO_4, 5 ml
Heat at 200-230°C for 30 min.

Digested samples

Cool.
Top up to 50 ml with distilled water.

Test solution, a fixed volume (usually 5 ml)

10% $SnCl_2$ solution, 1 ml

CVAAS

Flow Chart 1. Determination of Total Mercury in Biological Samples (fish, shellfish, human hair, blood, and tissues such as umbilical cord)

Sample digestion flask

HNO_3-$HClO_4$ (1+1), 2 ml
H_2SO_4, 5 ml

Urine samples, 2 ml

Add dropwise while swirling slowly.
Heat at 200-230°C for 30 min.

Digested samples

Cool.
Top up to 50 ml with distilled water.

Test solution, a fixed volume (usually 5 ml)

10% $SnCl_2$ solution, 1 ml

CVAAS

Flow Chart 2. Determination of Total Mercury in Urine

3-1-2 Hair

Weigh 20-30 mg of hair sample in a beaker, wash with neutral detergent (diluted 100-fold) and distilled water by decantation, and wash again with a small amount of acetone to remove the water. Remove the residual acetone under reduced pressure. Transfer the hair sample into a 20 ml vial and cut into an approximately powdery state with dissection scissors to make a sample for analysis.

a. Reagents

(1) Acetone: CH_3COCH_3 (analytical grade)

(2) Ethanol: C_2H_5OH (analytical grade)

(3) HNO_3-$HClO_4$ (1+1): Mix 100 ml of perchloric acid (for measurement of toxic metals) into 100 ml of nitric acid (for measurement of toxic metals). (Store in a cool dark place.)

(4) H_2SO_4: Sulfuric acid (for measurement of toxic metals)

(5) Distilled water: Distill deionized water and store in a clean glass container.

(6) HCl: Hydrochloric acid (analytical grade)

(7) 10% $SnCl_2$ solution: Dissolve 10 g of tin (II) chloride dihydrate (analytical grade), $SnCl_2 \cdot 2H_2O$, in 9 ml of HCl and dilute to 100 ml with distilled water. Aerate with N_2 gas (100 ml/min., 20-30 minutes) to expel any mercury from the solution.

(8) 5N NaOH: Dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 ml.

(9) 0.1N NaOH: Dilute 5 N NaOH 50-fold with distilled water.

(10) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(11) Methylmercury standard solution: Weigh out 12.5 mg of CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to obtain a methylmercury standard solution. One ml of this solution contains 1.0 µg of Hg.

(12) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a stopper. Shake for 3 minutes with a shaker to extract methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly.) One ml of this solution contains 0.1 µg of Hg.

(13) 1N H_2SO_4: Gradually add 30 ml of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1,000 ml.

(14) 1% acidic $KMnO_4$ solution for collecting mercury: Dissolve 1 g of potassium permanganate (analytical grade) in 100 ml of 1N H_2SO_4.

(15) 0.5% $KMnO_4$ solution: Dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 ml.

(16) Toluene: $C_6H_5CH_3$ (reagent grade for residual pesticide analyses)

b. Instruments and equipment

(1) Mercury analyzer: Model Hg-201 Semi-automated Mercury Analyzer (Sanso Seisakusho Co., Ltd., Tokyo, Japan)

(2) Hot plate: Capable of attaining a surface temperature of 250°C

(3) Sample digestion flask: 50-ml thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter)

(4) Volumetric flasks: 10, 100, and 1,000 ml

(5) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(6) Vial: 20-ml scintillation vial

(7) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(8) Dissection scissors

(9) Multi-flow meter: V4-type flow meter multi-kit (Kojima Instruments Inc.)

(10) Reciprocal shaker

(11) Centrifuge

(12) Beaker

Note: Prior to use, thoroughly wash all laboratory glassware and sample containers to be used during the analysis with 0.5% $KMnO_4$ solution. Rinse with water until the color of the $KMnO_4$ solution is no longer visible.

c. Preparation of test solution

Precisely weigh out a finely cut sample (usually around 10 mg) and place in a sample digestion flask. Add 1 ml of distilled water, 2 ml of HNO_3-$HClO_4$ (1+1), and 5 ml of H_2SO_4 in turn. Heat on a hot plate at 200-230°C for 30 minutes. Allow to cool, add distilled water to make a fixed volume, and use the resulting solution as the sample test solution.

Separately, transfer 0 and 1.0 ml of methylmercury-cysteine solution (100 ng Hg/ml) into two sample digestion flasks (corresponding to 0 and 100 ng Hg). Add 1 ml of distilled water to only the former (the blank) followed by 2 ml of HNO_3-$HClO_4$ (1+1) and 5 ml of H_2SO_4 in turn. To obtain a blank test solution and standard test solution for the measurement of total mercury, follow the same procedures as indicated above for preparation of the sample test solution.

d. Test procedures and calculation

Test procedures

Gently transfer a fixed volume, V ml (normally 5 ml, to a maximum of 10 ml) of each of the blank test solution, standard test solution for measurement of total mercury, and the sample test solution into the reaction vessel of the mercury analyzer and apply the stopper. Add 1 ml of 10% $SnCl_2$ solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated mercury vapor will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds to homogenize the concentration in the gas phase while the acidic gas generated from the test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of mercury vapor into the photo-absorption cell through the ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the reading of the recorder begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement.

d. Test procedures and calculation

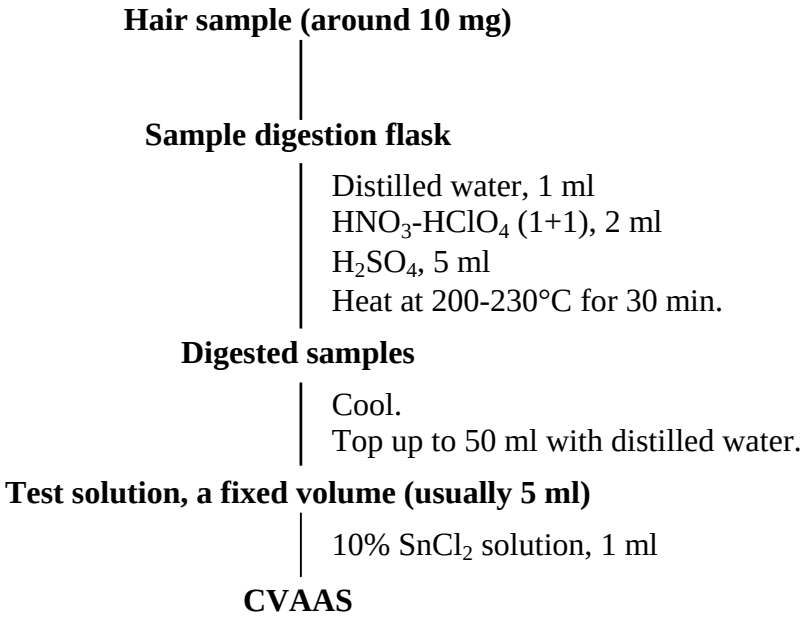
Calculation

The peak heights (mm) obtained after measurement of fixed volumes V ml of each of the blank, the standard, and the sample test solutions (or their diluted solutions) for the total mercury analysis are labeled Pbl, Pstd, and Ps, respectively. The total mercury concentration in the sample is calculated with the following formula:

Total mercury concentration in the sample (ng/mg) =100 ng ×

(Ps–Pbl)/(Pstd–Pbl) × dilution factor ×1/sample weight (mg)

For the basics of total mercury analysis, see pp. 30-32 for the Procedural Notes to “3-1-1 Biological Samples (including fish, shellfish, human blood, urine and tissues such as umbilical cord).”



Flow Chart 3. Determination of Total Mercury in Hair

3-1-3 Sediment/soil

Remove wood pieces, pebbles, shells, and dust from the collected sediment or soil sample. Homogenize the sample with the quarter method and pass it through a 2.0 mm mesh sieve to make a sample for analysis. If the sample has a high water content, centrifuge it to remove the supernatant and mix well to homogenize it before subjecting it to analysis.

a. Reagents

(1) HNO_3-$HClO_4$ (1+1): Mix 100 ml of perchloric acid (for measurement of toxic metals) into 100 ml of nitric acid (for measurement of toxic metals). (Store in a cool dark place.)

(2) H_2SO_4: Sulfuric acid (for measurement of toxic metals)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) HCl: Hydrochloric acid (analytical grade)

(5) 10% $SnCl_2$ solution: Dissolve 10 g of tin (II) chloride dihydrate (analytical grade), $SnCl_2 \cdot 2H_2O$, in 9 ml of HCl and dilute to 100 ml with distilled water. Aerate with N_2 gas (100 ml/min., 20-30 minutes) to expel any mercury from the solution.

(6) 5N NaOH: Dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 ml.

(7) 0.1N NaOH: Dilute 5N NaOH 50-fold with distilled water.

(8) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(9) Methylmercury standard solution: Weigh out 12.5 mg of CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-

fold with toluene to obtain a methylmercury standard solution. One ml of this solution contains 1.0 μg of Hg.

(10) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a stopper. Shake for 3 minutes with a shaker to extract methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(11) 1N H_2SO_4: Gradually add 30 ml of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1,000 ml.

(12) 1% acidic $KMnO_4$ solution for collecting mercury: Dissolve 1 g of potassium permanganate (analytical grade) in 100 ml of 1N H_2SO_4.

(13) 0.5% $KMnO_4$ solution: Dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 ml.

(14) Toluene: $C_6H_5CH_3$ (reagent grade for residual pesticide analyses)

b. Instruments and equipment

(1) Mercury analyzer: Model Hg-201 Semi-Automated Mercury Analyzer (Sanso Seisakusho Co., Ltd.)

(2) Hot plate: Capable of attaining a surface temperature of 250°C

(3) Sample digestion flask: 50-ml thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter)

(4) Volumetric flasks: 10, 100, and 1,000 ml

(5) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(6) Vial: 20-ml scintillation vial

(7) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(8) Dissection scissors

(9) Multi-flow meter: V4-type flow meter multi-kit (Kojima Instruments Inc.)

(10) Reciprocal shaker

(11) Centrifuge

(12) Porcelain crucible

Note: Prior to use, thoroughly wash all laboratory glassware and sample containers to be used during the analysis with 0.5% $KMnO_4$ solution. Rinse with water until the color of the $KMnO_4$ solution is no longer visible.

c. Preparation of sample test solution

Precisely weigh out a homogenized sample (0.5 g maximum of wet weight) in the bottom of a sample digestion flask. Add 1 ml of distilled water, 2 ml of HNO_3-$HClO_4$ (1+1), and 5 ml of H_2SO_4 in turn followed by heat treatment on a hot plate at 200-230°C for 30 minutes. Allow to cool, add distilled water to make a fixed volume of 50 ml, mix well, and use the resulting solution as the sample test solution.

Separately, transfer 0 and 1.0 ml of methylmercury-cysteine solution (0.10 µg Hg/ml) into two sample digestion flasks (corresponding to 0 and 0.10 µg Hg). Add 1 ml of distilled water to only the former (the blank) followed by 2 ml of HNO_3-$HClO_4$ (1+1) and 5 ml of H_2SO_4 in turn. To obtain a blank test solution and standard test solution for the measurement of total mercury, follow the same steps as indicated above for preparation of the sample test solution.

For wet samples, weigh out about 10-20 g of the sample into a porcelain crucible of known weight. Place it in a drying oven at 105°C and dry for 2 to 3 hours. Allow it to cool in a desiccator and weigh it to obtain the ratio of wet weight/dry weight (WW/DW).

d. Test procedures and calculation

Test procedures

Gently transfer a fixed volume, V ml (normally 5 ml, to a maximum of 10 ml) of each of the blank test solution, standard test solution and the sample test solution into the reaction vessel of the mercury analyzer and apply the stopper. Add 1 ml of 10% $SnCl_2$ solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated elemental mercury vapor will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds to homogenize the concentration in the gas phase, while the acidic gas generated from the test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of the elemental mercury vapor into the photo-absorption cell through the ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the reading of the recorder begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement.

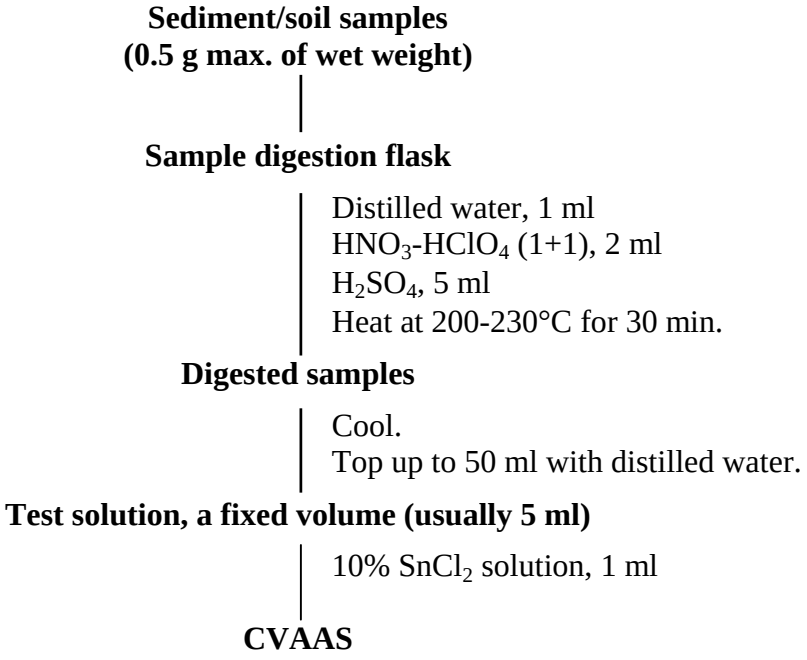
Calculation

The peak heights (mm) obtained after measurement of fixed volumes V ml (usually 5 ml, maximum 10 ml) of each of the blank, the standard, and the sample test solutions (or their diluted solutions) for the total mercury analysis are labeled Pbl, Pstd, and Ps, respectively. The total mercury concentration (µg/g of dry weight) in the sample is calculated with the following formula:

Total mercury concentration in the sample (µg/g) = 0.10 µg × (Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/sample weight (g) × WW/DW

WW/DW: ratio of wet weight/dry weight

Note: For the basics of total mercury analysis, see pp. 30-32 for the Procedural Notes to "3-1-1 Biological Samples (including fish, shellfish, human blood, urine and tissues such as umbilical cord)."



Flow Chart 4. Determination of Total Mercury in Sediment/Soil

3-1-4 Water[1]

After collection of water, the water sample brought to the laboratory is usually filtered with a 0.45-μm membrane filter to make a sample for analysis. It is desirable to analyze the mercury as soon as possible. Total water may be used as a sample for convenience.

a. Reagents

(1) HNO_3-$HClO_4$ (1+1): Mix 100 ml of perchloric acid (for measurement of toxic metals) into 100 ml of nitric acid (for measurement of toxic metals). (Store in a cool dark place.)

(2) H_2SO_4: Sulfuric acid (for measurement of toxic metals)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) HCl: Hydrochloric acid (analytical grade)

(5) 10% $SnCl_2$ solution: Dissolve 10 g of tin (II) chloride dihydrate (analytical grade), $SnCl_2 \cdot 2H_2O$, in 9 ml of HCl and dilute to 100 ml with distilled water. Aerate with N_2 gas (100 ml/min., 20-30 minutes) to expel any mercury from the solution.

(6) 5N NaOH: Dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 ml.

(7) 0.1N NaOH: Dilute 5N NaOH 50-fold with distilled water.

(8) Purified 0.01% dithizone-toluene[2]: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml of toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the aqueous phase (bottom phase). After allowing it to settle, transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate). Add 100 ml of toluene and shake to obtain purified 0.01% dithizone-toluene.

Allow the phases to separate, draw off and discard the bottom phase, and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(9) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(10) Methylmercury standard solution: Weigh out 12.5 mg of CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to obtain a methylmercury standard solution. One ml of this solution contains 1.0 μg of Hg.

(11) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a glass stopper. Shake for 3 minutes with a shaker to extract methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(12) 1N H_2SO_4: Gradually add 30 ml of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1,000 ml.

(13) 1% acidic $KMnO_4$ solution for collecting mercury: Dissolve 1 g of potassium permanganate (analytical grade) in 100 ml of 1N H_2SO_4.

(14) 0.5% $KMnO_4$ solution: Dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 ml.

(15) 20N H_2SO_4: Transfer about 350 ml of distilled water into a 1-L volumetric flask. Gradually add 600 ml of sulfuric acid (for measurement of toxic metals) while stirring in ice water. After it returns to room temperature, add distilled water to make a final volume of 1,000 ml.

(16) 10N NaOH: Dissolve 400 g of sodium hydroxide (analytical grade) to make a final volume of 1,000 ml.

(17) 10% $NH_2OH \cdot HCl$ solution: Dissolve 10 g of hydroxylamine hydrochloride (analytical grade) in distilled water to make a final volume of 1,000 ml.

(18) 10% EDTA solution: Dissolve 10 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4 \cdot 4H_2O$, in distilled water to make a final volume of 100 ml.

(19) Toluene: $C_6H_5CH_3$ (reagent grade for residual pesticide analyses)

b. Instruments and equipment

(1) Mercury analyzer: Model Hg-201 Semi-Automated Mercury Analyzer (Sanso Seisakusho Co., Ltd.)

(2) Hot plate: Capable of attaining a surface temperature of 250°C

(3) Sample digestion flask: 50-ml thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter)

(4) Volumetric flasks: 10, 100, and 1,000 ml

(5) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(6) 2-L separatory funnel

(7) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(8) Rotary evaporator

(9) Magnetic stirrer

(10) Multi-flow meter: V4-type flow meter multi-kit (Kojima Instruments Inc.)

(11) Reciprocal shaker

(12) Centrifuge

Note: Prior to use, thoroughly wash all laboratory glassware and sample containers to be used during the analysis with 0.5% $KMnO_4$ solution. Rinse with water until the color of the $KMnO_4$ solution is no longer visible.

c. Preparation of sample test solution

Transfer 2 L of a water sample into a 2 L separatory funnel. Add 10 ml of 20N H_2SO_4 and 5 ml of 0.5% $KMnO_4$ solution, mix by shaking, and let stand for 5 minutes. Neutralize with 20 ml of 10N NaOH, add 5 ml of 10% $NH_2OH \cdot HCl$ solution, and shake. Let stand for 20 minutes.[3] Add 5 ml of 10% EDTA solution to the mixture and mix by shaking. Add precisely 10 ml of purified 0.01% dithizone-toluene followed by vigorous shaking for 1 minute to extract the mercury in the sample. Let stand for at least 1 hour, avoiding direct sunlight. Discard the aqueous phase (lower phase). Transfer the toluene phase preferably into a 10-ml conical centrifuge tube fitted with a glass stopper and centrifuge at 1,200 rpm for 3 minutes with the glass stopper in place. (When an emulsion is formed, add 0.5 g of anhydrous sodium sulfate and shake followed by centrifugation to remove the lower phase.) Transfer a fixed volume (usually 7 ml) of the toluene phase into a sample digestion flask. With a rotary evaporator, evaporate to dryness on a water bath at 60°C. Add 1 ml of distilled water, 2 ml of HNO_3-$HClO_4$ (1+1), and 5 ml of H_2SO_4 and heat on a hot plate at 200-230°C for 30 minutes. Allow to cool and add distilled water to obtain a fixed volume of 50 ml. Mix well and use this as a sample test solution. Separately, choose a sample with a lower mercury content based on the type of water sample. To each of the 2-L water samples chosen, add 0 and 0.2 ml of methylmercury-cysteine solution (100 ng Hg/ml; corresponding to 0 and 20 ng Hg). Follow the above preparation procedures for sample test solutions to obtain a blank test solution and standard test solution for the measurement of total mercury.

d. Test procedures and calculation

Test procedures

Gently transfer a fixed volume, V ml (normally 10 ml) of the blank test solution, standard test solution, and the sample test solution into the reaction vessel

in the mercury analyzer and apply the stopper. Add 1 ml of 10% $SnCl_2$ solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated elemental mercury vapor will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds to homogenize the concentration in the gas phase, while the acidic gas generated from the test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of the elemental mercury vapor into the photo-absorption cell through the ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the reading of the recorder begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement

Calculation

The peak heights (mm) obtained after measurement of fixed volumes V ml (normally 10 ml) of each of the blank, the standard, and the sample test solutions (or their diluted solutions) are labeled Pbl, Pstd, and Ps, respectively. The total mercury concentration in the sample is calculated with the following formula:

Total mercury concentration in the sample (ng/L) = 20 ng × (Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/ sample volume (L)

Procedural Notes

1. Since concentrations of mercury in water samples are extremely low and usually at the ng/L level, pre-concentration of the mercury in the sample is required for measurement of the mercury. In the present method, quantitative and efficient pre-concentration is performed by extracting the mercury with a small volume of 0.01% dithizone-toluene after ionization of all the mercury

species in the sample with potassium permanganate in an acidic medium with sulfuric acid. This uses the chemical properties of dithizone: it combines easily with ionic mercury species to form a complex salt that is insoluble in water but soluble in organic solvents such as toluene. After pre-concentration by extracting the mercury with 0.01% dithizone-toluene, the extract is evaporated to dryness under reduced pressure. Similar to the case of biological samples and the like, the residue is subjected to wet digestion to prepare a sample test solution for the total mercury analysis with CVAAS.

2. Dithizone (diphenylthiocarbazone) is easily oxidized and usually contains its oxidized form, diphenylthiocarbadiazone, as an impurity. As well, it sometimes contains mercury or the like in the form of metal complex, although the amount is minute. These impurities are highly soluble in organic solvents but insoluble in alkaline solutions, whereas pure dithizone has the chemical property of being soluble not only in organic solvents but also soluble in alkaline solutions by forming its salts. This property enables impurities to be removed and dithizone to be purified for use.

3. For samples containing large amounts of Cl^- ions such as seawater, the treatment of the sample with a combination of potassium permanganate and sulfuric acid causes oxidation of Cl^- ions to Cl_2 to occur during the treatment, and the resulting Cl_2, once generated, is difficult to reduce by treatment with hydroxylamine hydrochloride solution. This results in oxidation of dithizone in the subsequent dithizone-toluene extraction step. Therefore, particularly for seawater samples, it is important to maintain the 5 minutes treatment time with potassium permanganate. Further, after the addition and mixing of the hydroxylamine hydrochloride solution, allow at least 20 minutes of reaction time before the EDTA treatment and dithizone-toluene extraction procedures.

For the basics of total mercury analysis, see pp. 30-32 for the Procedural Notes to “3-1-1 Biological Samples (including fish, shellfish, human blood, urine and tissues such as umbilical cord).”

Sample, 2 L (2 L separatory funnel)

Add 10 ml of 20N H_2SO_4 and mix.
Add 5 ml of 0.5% $KMnO_4$ solution and mix.
Let stand for 5 min.
Add 20 ml of 10N NaOH and mix to neutralize.
Add 5 ml of 10% $NH_2OH \cdot HCl$ solution, mix, and allow to stand for 20 min.
Add 5 ml of 10% EDTA solution and mix.
Add 10 ml of purified 0.01% dithizone-toluene and vigorously shake for 1 min.
Allow to stand for at least 1 hr.

Organic phase(10-ml conical centrifuge tube) **Aqueous phase**

(When an emulsion is formed, add 0.5 g of Na_2SO_4 and shake.)
Centrifuge at 1,200 rpm for 3 min.

Organic phase, 7 ml (sample digestion flask)

Evaporate to dryness.

Residue

Distilled water, 1 ml
HNO_3-$HClO_4$ (1+1), 2 ml
H_2SO_4, 5 ml
Heat at 200-230°C for 30 min.

Digested sample

Allow to cool.
Top up to 50 ml with distilled water.

Test solution, a fixed volume (usually 10 ml)

10% $SnCl_2$ solution, 1 ml

CVAAS

Flow Chart 5. Determination of Total Mercury in Water

4. Analytical Method for Methylmercury

For measurement of organic mercury, gas-liquid chromatography with electron capture detection (GLC-ECD) is used for selective analysis of methylmercury and other organomercury compounds. Because this technique provides good separation and superior sensitivity for analyzing organomercury halides, it has been widely used for the determination of methylmercury in various kinds of biological and environmental samples.

Briefly, the analytical procedure involves the extraction of methylmercury in the samples as its halide into an organic solvent after acidification; the back-extraction into a cysteine- or glutathione-aqueous solution; the re-extraction into an organic solvent; and measurement of methylmercury by GLC-ECD. As an alternative, methylmercury can be determined by CVAAS, which measures elemental mercury vapor generated from a heated sample test solution obtained from similar methylmercury extraction procedures. However, in this direct extraction procedure with organic solvent, a solid emulsion is often formed during the extraction process, particularly with fish and other biological samples. This makes the following steps complicated and causes the extraction efficiency of methylmercury to vary with the type of sample. While several pre-treatment methods are proposed to overcome the above drawbacks, we describe herein the following two methods: determination by the dithizone extraction/GLC-ECD method, which is suitable for methylmercury in various types of biological and environmental specimens; and the hydrochloric acid leaching/toluene extraction/GLC-ECD method for the determination of methylmercury in hair.

4-1 Determination by the dithizone extraction/gas-liquid chromatography with electron capture detection (GLC-ECD) method

Principle

The dithizone extraction/GLC-ECD method was established as an analytical method for methylmercury in various biological and environmental matrices. It is based on the following two advantages of dithizone extraction, which was widely used for colorimetry of inorganic and organic mercury species prior to the introduction of atomic absorption in the late 1960s: it has much higher extraction efficiency than that of direct solvent extraction, facilitating the extraction separations of trace amounts of mercury from samples with a small portion of the solution; and alkyl mercuric dithizonate, as soon as it is injected into GLC, reacts with Cl^- in the column to give its chloride form for quantitative detection.

Briefly, this method involves the following steps: pre-treatment of the sample, dithizone extraction, back-extraction into alkaline sodium sulfide, dithizone re-extraction, and GLC measurement. Appropriate pre-treatment to accommodate the characteristics of the composition of each sample enables efficient extraction of methylmercury with a small portion of dithizone-toluene solution. After dithizone-toluene extraction, test solutions are prepared with all common procedures, followed by measurement with GLC-ECD. To accommodate the principle used for this method, pack several centimeters of sodium chloride at the injection port of the column on top of the packing material for GLC.

4-1-1 Biological samples (fish, shellfish, human blood, and tissues such as umbilical cord)[1]

This method is applicable to protein-rich biological samples such as fish, shellfish, human blood and tissues such as umbilical cord. For a solid sample, place it into a vial, cut it into small pieces with dissection scissors, and homogenize to a pasty state to obtain a sample for analysis. For liquid samples such as blood, mix well with a Pasteur pipette with a bulb or the like so that it is homogenized for analysis.

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Hexane: $CH_3(CH_2)_4CH_3$ (pesticide analysis grade)

(3) Ethanol: C_2H_5OH (analytical grade)

(4) Distilled water: Distill deionized water and store in a clean glass container.

(5) 1N KOH-ethanol: Dissolve 56.11 g of potassium hydroxide (analytical grade) in ethanol to make a final volume of 1,000 ml. (Store in a cool dark place.)

(6) 1N HCl: Mix 90 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(7) 20% EDTA solution: Dissolve 20 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4 \cdot 4H_2O$, in distilled water to make a final volume of 100 ml.

(8) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(9) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(10) Purified 0.01% dithizone solution[2]: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml of toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the

aqueous phase (bottom phase). After allowing the phases to separate, transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate). Add 100 ml of toluene and shake to obtain purified 0.01% dithizone-toluene. Allow the phases to separate, draw off and discard the bottom phase, and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(11) Alkaline sodium sulfide solution: Dissolve 0.15 g of $Na_2S \cdot 9H_2O$ (analytical grade) in 10 ml of distilled water in a 10-ml conical centrifuge tube fitted with a glass stopper to make the sodium sulfide stock solution. (Prepare a fresh solution monthly. Store in a cool dark place.) At each use, transfer 0.1 ml of the stock solution into a 100-ml glass container fitted with a glass stopper, add 50 ml of 0.1N NaOH and 50 ml of ethanol, and mix to obtain an alkaline sodium sulfide solution. (One ml of this solution contains 5 µg of Na_2S.)

(12) Walpole's buffer: Mix 200 ml of 1M CH_3COONa and about 200 ml of 1N HCl to adjust to pH 3.0.

(13) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(14) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as a stock solution. Dilute the stock solution 100-fold with toluene to obtain the methylmercury standard solution. One ml of this solution contains 1,000 ng of Hg.

(15) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a stopper. Shake for 3 minutes with a shaker to extract the methylmercury to the aqueous phase. Centrifuge at 1,200

rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(16) Anhydrous sodium sulfate: Anhydrous sodium sulfate (pesticide analysis grade) heated at 500°C for 2-3 hours (stored in a desiccator)

(17) N_2 gas

Note: For the above reagents (6)-(9), (11), and (12), prepare the required amounts in advance, add a 1/2 volume of toluene, wash by shaking in a separatory funnel. Confirm beforehand that no peaks appear that could interfere with the measurement by GLC-ECD.

b. Instruments and equipment

(1) Gas-liquid chromatograph with electron capture detector (GLC-ECD)

(2) Multi-flow meter: Model V4 flow meter multi-kit (Kojima Instruments Inc.)

(3) Centrifuge

(4) Reciprocal shaker

(5) Isothermal bath

(6) Magnetic stirrer

(7) Aspirator

(8) Vortex mixer

(9) pH mater

(10) Volumetric flasks: 10, 100, and 1,000 ml

(11) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(12) Pasteur pipettes

(13) Separatory funnels: 100, 200, and 1,000 ml

(14) Glass containers with glass stoppers: 100, 200, and 500 ml

(15) Glass container with screw cap: 1,000 ml

(16) 40-ml conical centrifuge tube with a glass stopper

(17) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(18) Vial: 20-ml scintillation vial

(19) Dissection scissors

Note: Thoroughly wash all glassware with toluene prior to use. Confirm in advance that no peaks appear that could interfere with measurement by GLC-ECD.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).

ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).

iii) 3.0 mm × 2.0 m glass column packed with 5-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solution

Methylmercury extraction

Precisely weigh out a homogenized sample (usually 0.2-0.5 g as wet weight, about 0.1 g for a dry sample) and place in the bottom of a 40-ml screw-capped conical centrifuge tube. (For a dry sample, add 0.5 ml of distilled water to moisten after weighing.) Add 10 ml of 1N KOH-ethanol. Seal tightly and heat in an isothermal bath at 100°C for 1 hour and occasionally swirl gently.[3] Allow to cool,

add 10 ml of 1N HCl and 5 ml of hexane in turn, and shake for 3 minutes with a reciprocal shaker. Centrifuge at 2,500 rpm for 3 minutes. Draw off and discard the hexane phase (upper phase) to remove fatty materials.[4] Add 2 ml of 20% EDTA and mix well.[5] Add 5 ml of purified 0.01% dithizone-toluene. Shake for 3 minutes to extract methylmercury as its dithizonate (complex) into the toluene phase. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 2,500 rpm for 3 minutes and further draw off and discard as much of the remaining aqueous phase (lower phase) as possible.

Clean-up

Add 3 ml of 1N NaOH to the toluene phase and shake for 3 minutes to wash and remove excess dithizone. (For a blood sample, add 0.5 g of anhydrous sodium sulfate to the toluene phase and shake for 3 minutes prior to this step, followed by washing with 3 ml of 1N NaOH).[6] Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 2,500 rpm for 3 minutes to obtain a clear toluene phase. Transfer a fixed volume (usually 3 ml) of the toluene phase into a 10-ml conical centrifuge tube with glass stopper. Add 2 ml of alkaline sodium sulfide solution and shake for 3 minutes to back-extract the methylmercury into the aqueous phase.[7] Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase) carefully. Add 2 ml of toluene to the aqueous phase and shake for 3 minutes to wash the aqueous phase. Centrifuge again at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase). Add 1N HCl (3-5 drops) to make the solution slightly acidic.[8] With a Pasteur pipette, aerate the solution with N_2 gas through a multi-flow meter for 3 minutes (50 ml/min.) to expel the excess sulfide ions as hydrogen sulfide gas. Subsequently, add 2 ml of Walpole's buffer while washing the tip of the Pasteur pipette. Mix well with a vortex mixer. Add a fixed volume of purified 0.01% dithizone-toluene (0.2-1.0 ml,

usually 0.5 ml) and shake to extract methylmercury. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Add 3 ml of 1N NaOH to the toluene phase and shake for 3 minutes to wash and remove the excess dithizone. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Again draw off and discard as much of the remaining aqueous phase (lower phase) as possible. Acidify the solution by adding 2 drops of 1N HCl to the toluene phase and mix with a vortex mixer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the hydrochloric acid phase (lower phase). Use the resulting solution as the sample test solution.

Separately, depending on the expected mercury concentrations in the samples, transfer 0-0.20 ml of methylmercury-cysteine solution (corresponding to 0-0.020 µg Hg) into 40-ml screw-capped conical centrifuge tubes and add 10 ml of 1N KOH-ethanol. Follow the same steps as indicated above for preparing the sample test solution to make methylmercury standard test solutions for the calibration curve. Protect the test solutions from light after preparation.

d. Test procedures and calculations

With a micro-syringe, inject into the GLC a fixed volume (usually 2-5 µl) of each of the sample test solutions (or their toluene-diluted solutions), the blank and methylmercury standard test solutions for the calibration curve. Calculate the methylmercury concentration in the sample (µg/g) by comparing the peak height of the sample test solution with the calibration curve obtained from the standard test solutions. [9]

Procedural Notes

1. This method involves the digestion of proteins in the sample by heating with 1N KOH-ethanol, removal of free fatty materials by washing with hexane under a slightly acidic condition, and quantitative extraction separation of methylmercury in the sample into the toluene phase as its dithizonate, followed by clean-up with an alkaline sodium sulfide solution and re-extraction with a small portion of 0.01% dithizone-toluene.
2. Dithizone (diphenylthiocarbazone) is easily oxidized, and its oxidized form (diphenylthiocarbadiazone) is contained as an impurity that causes interfering peaks on the gas chromatogram. Therefore, utilizing pure dithizone's unique chemical property of forming a water-soluble salt that dissolves in alkaline solution, prepare a fresh dithizone-toluene solution for each analysis.
3. If not tightly sealed, the solution may boil during heating. In such a case, remove the container, cool well with tap water, close the screw cap again so that the gas does not leak, and continue the heating. If the volume of the solution has been reduced without any apparent boiling, repeat the procedure from the beginning.
4. To remove the upper phase or lower phase from the test tube, use the Suction-Removal System with a Pasteur pipette connected with a flexible tube through a waste liquid collector to an aspirator, as shown in Figure 2. Briefly, to remove the upper phase (organic phase), perform suctioning by positioning the tip of the Pasteur pipette on the surface of the upper phase down along the inside wall of the test tube in order to draw off most of the upper phase. When only a little upper phase remains, keep the tip of the Pasteur pipette a few mm above the surface of the organic phase and continue to draw off. With this technique, only the organic phase, which has a lower specific gravity than that of the lower phase (aqueous phase) and high volatility, is drawn off together with air, allowing for almost complete removal of the organic phase. To draw off and discard the lower of the two phases separated in the test tube, squeeze

the flexible tube with the fingers to stop the suction of the Pasteur pipette. Position the tip of the Pasteur pipette at the bottom of the test tube and adjust the pressure on the tube to slowly draw up the lower phase. When the lower phase is almost completely removed, squeeze the flexible tube to stop the suction and remove the Pasteur pipette. This makes it possible to remove only the lower phase. Because these procedures require some skill and precision, practice each procedure beforehand.

5. Before dithizone extraction, add the EDTA solution and mix by shaking in order to mask Fe ions and other metal ions contained in the samples, particularly blood samples and the like.
6. This procedure allows the methylmercury-dithizone complex (methylmercury dithizonate) to remain in the toluene phase; however, excess dithizone in the toluene phase forms water-soluble salt in the alkaline solution, which can be transferred into the aqueous phase. This dithizone removal procedure maintains the methylmercury-dithizonate in the toluene phase without any loss. One such dithizone-removal procedure with alkali washing is usually sufficient. However, if a clear toluene phase is not obtained, repeat this procedure.

   For blood samples, black suspended matter generated during dithizone-toluene extraction colors the final sample test solution, often resulting in the appearance of interfering peaks on the gas chromatogram. To prevent this, add 0.5 g of anhydrous sodium sulfate and shake, before performing the alkali washing.
7. Methylmercury dithizonate in the toluene phase reacts with excess sulfide ions, forming a water-soluble complex that can be transferred into the aqueous phase. In order to perform an efficient one-time extraction procedure, use an alkaline ethanol solution of sodium sulfide.

8. To confirm the volume of 1N HCl required to be added for slight acidification of the solution, transfer 2 ml of alkaline sodium sulfide solution into another 10-ml test tube in advance and add a few drops of 0.01% dithizone solution as a pH indicator to obtain a yellow or orange color. Add 1N HCl to this dropwise until the color changes to blue. The number of drops of 1N HCl added is the amount of 1N HCl required to acidify the solution slightly.
9. Generally, in GLC measurement, the linear range of the calibration curve is narrow. Take notice of this, particularly when taking measurements, and undertake appropriate dilution so that the peak height of the sample test solution is in the linear range. When linearity is confirmed through measurement of the methylmercury standard test solution for preparation of the calibration curve, the methylmercury concentration in the sample may be calculated according to the following equation using the peak height (mm) of the standard test solution (Pstd) of, for example, 0.020 µg Hg.

    Methylmercury concentration in the sample (µg/g or ml) = 0.020 µg × (Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/sample weight (g)

    Ps: peak height (mm) of the sample test solution

    Pbl: peak height (mm) of the blank test solution

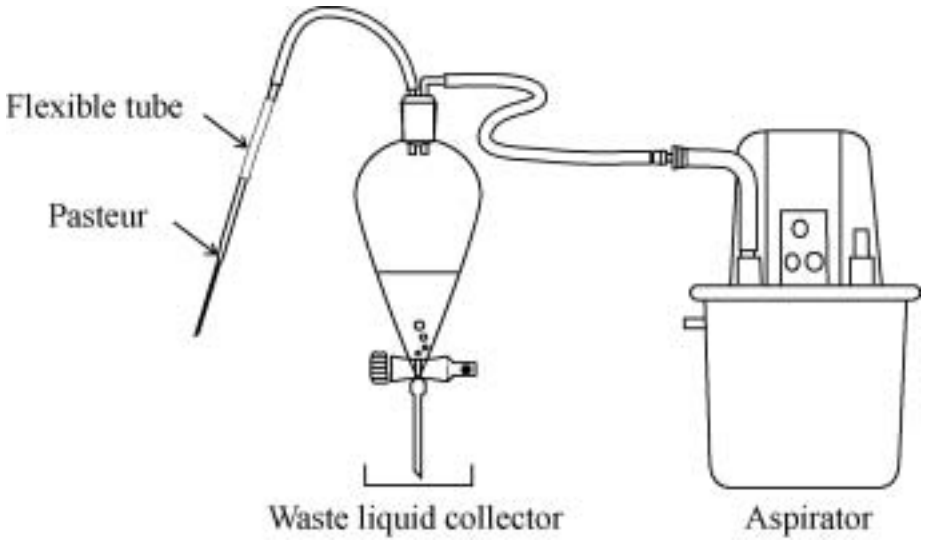
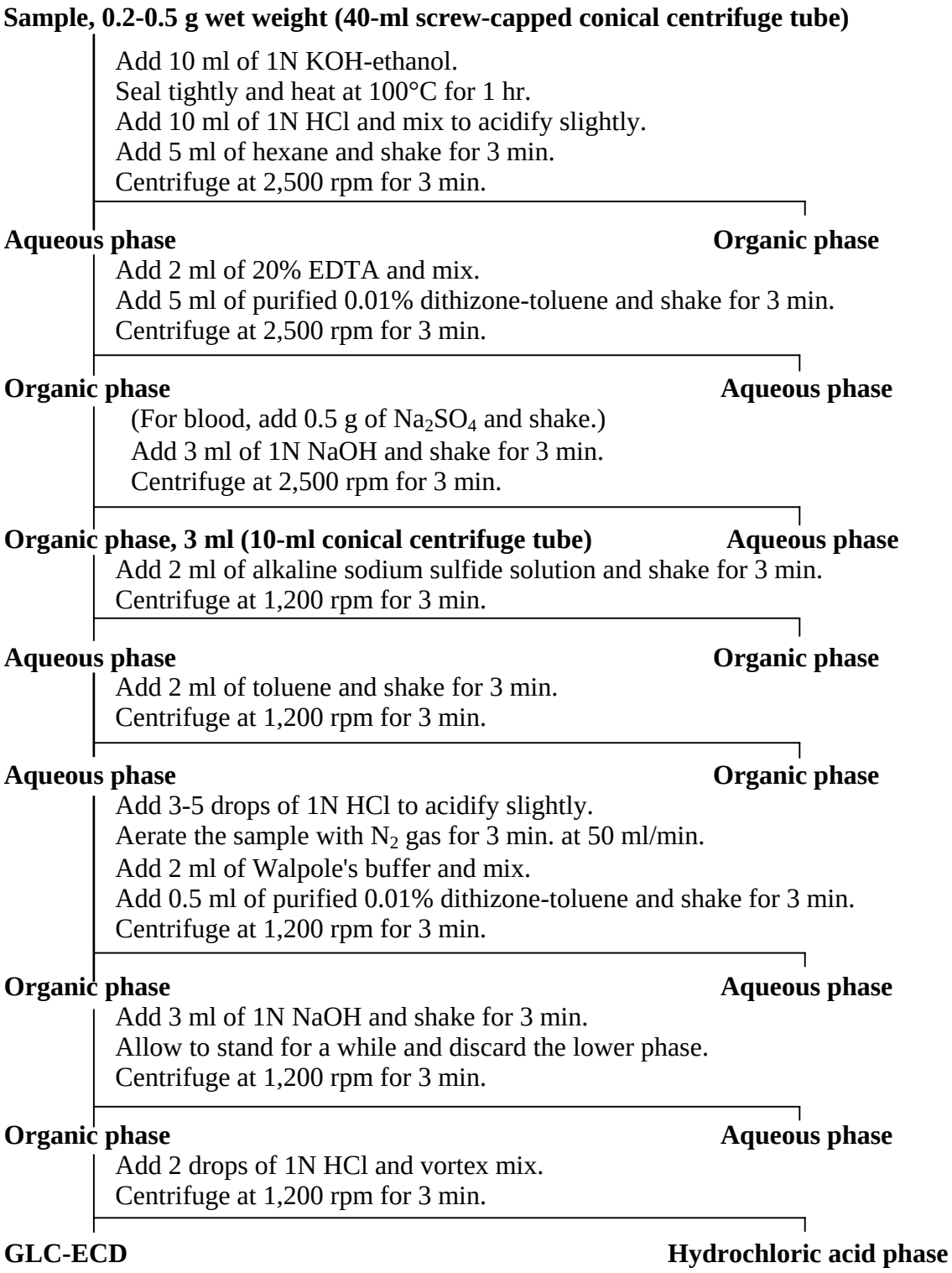


Figure 2. Suction-Removal System for Separation of Liquid Phases



Flow Chart 6. Determination of Methylmercury in Biological Samples (fish, shellfish, human blood, and tissues such as umbilical cord)

4-1-2 Biological samples containing relatively high concentrations of mercury, particularly fish and shellfish (Simplified method)[1]

As described above, the analytical method for methylmercury in biological samples using the dithizone extraction/GLC-ECD method is widely applied for quantitative determination of trace amounts of methylmercury in proteinous samples that include fish, shellfish, blood, and human tissues. However, when the sample test solution is prepared according to this method for fish, shellfish, and the other biological samples containing relatively high concentrations of mercury, substantial dilution of the sample test solution is unavoidable at measurement in most cases. For such samples, a method is available wherein the collected volume of the toluene phase used in back-extraction is reduced in the clean-up step. The following method, in which the above analytical procedures are partially simplified, can also be applied when a high mercury concentration is expected from the measurement of total mercury.

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Hexane: $CH_3(CH_2)_4CH_3$ (pesticide analysis grade)

(3) Ethanol: C_2H_5OH (analytical grade)

(4) Distilled water: Distill deionized water and store in a clean glass container.

(5) 1N KOH-ethanol: Dissolve 56.11 g of potassium hydroxide (analytical grade) in ethanol to obtain a final volume of 1,000 ml. (Store in a cool dark place.)

(6) 1N HCl: Mix 90 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(7) 20% EDTA solution: Dissolve 20 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4 \cdot 4H_2O$, in distilled water to make a final volume of 100 ml.

(8) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(9) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(10) Purified 0.01% dithizone-toluene: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the aqueous phase (bottom phase). After allowing the phases to separate, transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate). Add 100 ml of toluene and shake to obtain purified 0.01% dithizone-toluene. Allow the phases to settle, draw off and discard the bottom phase, and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(11) Alkaline sodium sulfide solution: Dissolve 0.15 g of $Na_2S{\cdot}9H_2O$ (analytical grade) in 10 ml of distilled water in a 10-ml conical centrifuge tube fitted with a glass stopper to make the sodium sulfide stock solution. (Prepare a fresh solution monthly. Store in a cool dark place.) At each use, transfer 0.1 ml of the stock solution into a glass container fitted with a glass stopper, add 50 ml of 0.1N NaOH and 50 ml of ethanol, and mix to obtain an alkaline sodium sulfide solution. (One ml of this solution contains 5 µg of Na_2S.)

(12) Walpole's buffer: Mix 200 ml of 1M CH_3COONa and about 200 ml of 1N HCl to adjust to pH 3.0.

(13) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH{\cdot}HCl{\cdot}H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(14) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as a stock solution.

Dilute the stock solution 100-fold with toluene to obtain the methylmercury standard solution. One ml of this solution contains 1.0 μg of Hg.

(15) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a stopper. Shake for 3 minutes with a reciprocal shaker to extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(16) N_2 gas

Note: For the above reagents (6)-(9), (11), and (12), prepare the required amounts in advance, add a 1/2 volume of toluene, and wash by shaking. Confirm beforehand that no peaks appear that could interfere with the measurement by GLC-ECD.

b. Instruments and equipment

(1) Gas-liquid chromatograph equipped with electron capture detector (GLC-ECD)
(2) Multi-flow meter: Model V4 flow meter multi-kit (Kojima Instruments Inc.)
(3) Centrifuge
(4) Reciprocal shaker
(5) Isothermal bath
(6) Magnetic stirrer
(7) Aspirator
(8) Vortex mixer
(9) pH mater
(10) Volumetric flasks: 10, 100, and 1,000 ml
(11) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml
(12) Pasteur pipettes

(13) Separatory funnels: 100, 200, and 1,000 ml

(14) Glass containers with glass stoppers: 100, 200, and 500 ml

(15) Glass container with screw cap: 1,000 ml

(16) 40-ml conical centrifuge tube with glass stopper

(17) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(18) Vial: 20-ml scintillation vial

(19) Dissection scissors

Note: Thoroughly wash all glassware with toluene before use. Confirm in advance that no peaks appear that could interfere with measurement.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).

ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).

iii) 3.0 mm × 2.0 m glass column packed with 5%-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solution

Methylmercury extraction

Precisely weigh out a homogenized sample (usually 0.2-0.5 g as wet weight, about 0.1 g for a dry sample) and place in the bottom of a 40-ml screw-capped conical centrifuge tube. (For a dry sample, add 0.5 ml of distilled water to moisten after weighing.) Add 10 ml of 1N KOH-ethanol. Seal tightly and heat in an isothermal bath at 100°C for 1 hour, occasionally swirling gently. Allow to cool, add 10 ml of 1N HCl and 5 ml of hexane in turn, and shake for 3 minutes with a reciprocal shaker. Centrifuge at 2,500 rpm for 3 minutes. Draw off and discard the hexane phase (upper phase) to remove fatty materials. Add 2 ml of 20% EDTA and mix well. Add 5 ml of purified 0.01% dithizone-toluene. Shake for 3 minutes to extract methylmercury as its dithizonate (complex) into the toluene phase. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 2,500 rpm for 3 minutes to obtain a clear toluene phase.

Clean-up

Transfer 1.0 ml of the toluene phase into a 10-ml conical centrifuge tube with glass stopper.[2] Add 2 ml of alkaline sodium sulfide solution and shake for 3 minutes to back-extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase) carefully. Add 2 ml of toluene to the aqueous phase and shake for 3 minutes to wash the aqueous phase. Centrifuge again at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase). Add 1N HCl (3-5 drops) to make the solution slightly acidic. With a Pasteur pipette, aerate the solution with N_2 gas through a multi-flow meter for 3 minutes (50 ml/min.) to expel the excess sulfide ions as hydrogen sulfide gas. Subsequently, add 2 ml of Walpole's buffer while washing the tip of the Pasteur pipette. Mix well with a vortex mixer. Add 1.0 ml of toluene and shake for 3 minutes (for re-extraction of methylmercury together with excess dithizone).[3] Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Add 3 ml of 1N NaOH to the toluene

phase and shake to wash and remove the excess dithizone. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard as much of the remaining aqueous phase (lower phase) as possible. Acidify the solution by adding 2 drops of 1N HCl and mix with a vortex mixer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the hydrochloric acid phase (lower phase). Use the resulting solution as the sample test solution.

Separately, depending on the expected mercury concentrations in the samples, transfer 0-1.0 ml of methylmercury-cysteine solution (corresponding to 0-0.10 μg Hg) into 40-ml screw-capped conical centrifuge tubes and add 10 ml of 1N KOH-ethanol. Follow the same steps as indicated above for preparing the sample test solution to obtain methylmercury standard test solutions for the calibration curve. Protect the test solutions from light after preparation.

d. Test procedures and calculations

With a micro-syringe, inject into the GLC a fixed volume (usually 2-5 μl) of each of the sample test solutions (or their toluene-diluted solutions), the blank and methylmercury standard test solutions for the calibration curve. Calculate the methylmercury concentration in the sample (μg/g) by comparing the peak height of the sample test solution with the calibration curve obtained from the blank and methylmercury standard test solutions.

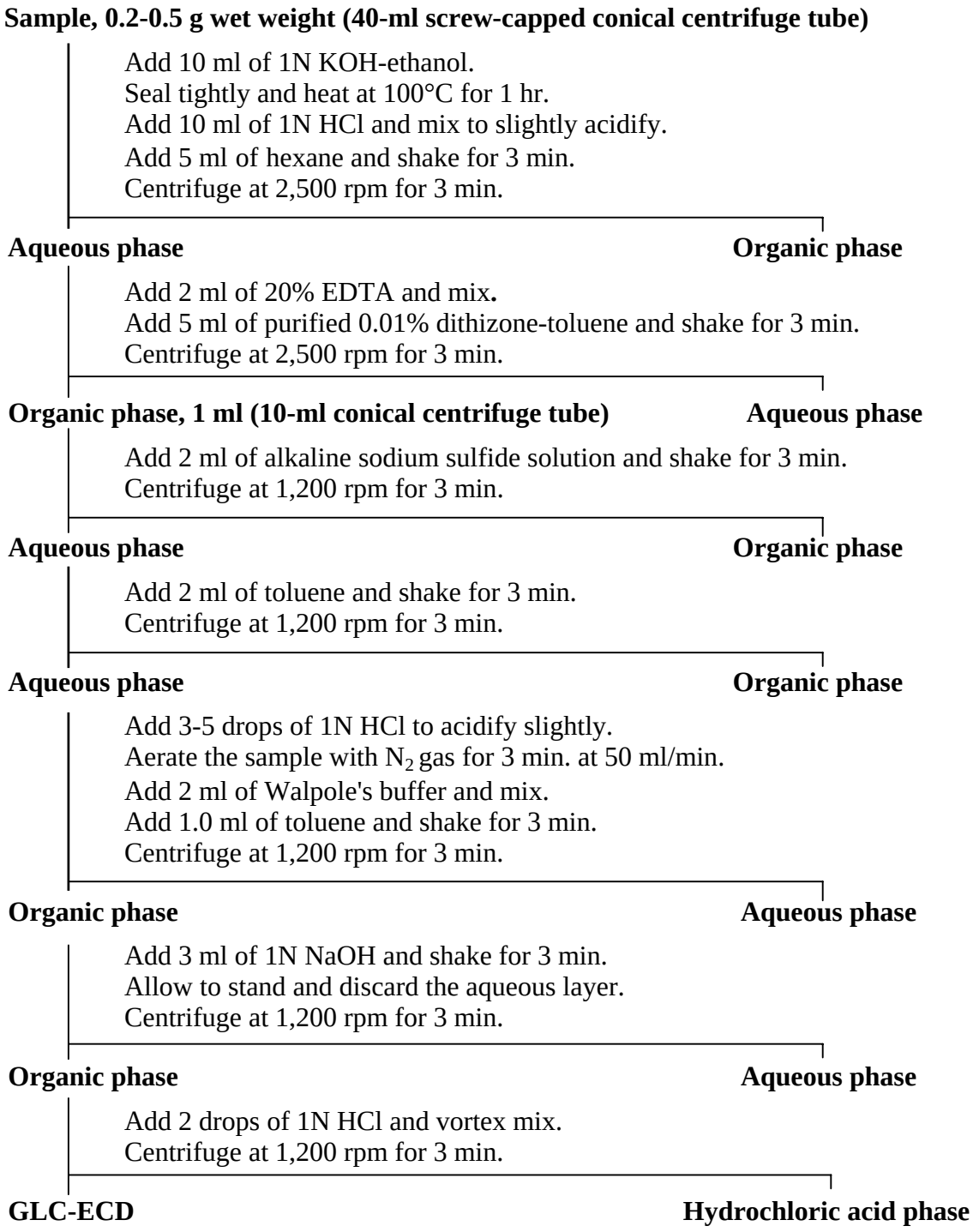
Procedural Notes

1. This method is applied to determination of methylmercury in biological samples (particularly those of fish and shellfish) containing relatively high concentrations of mercury (exceeding 0.1 ppm). This method differs from that described in 4-1-1 in the following points: after 0.01% dithizone-toluene

extraction, omit 1N NaOH washing and the subsequent centrifugal separation; in the methylmercury re-extraction step, the extraction can be performed with toluene alone instead of 0.01% dithizone-toluene. In addition, this method has the advantage that an obvious change of color in the step of slight acidification readily indicates the need to expel excess sulfide ions because dithizone, which serves as a pH indicator, has already been extracted into the aqueous phase and colored the solution during the preceding back-extraction with the alkaline sodium sulfide solution.

2. In this process, methylmercury dithizonate as well as free dithizone in 0.01% dithizone-toluene are transferred at the same time into the alkaline sodium sulfide solution; therefore, the volume of the 0.01% dithizone-toluene extract to be used for back-extraction into alkaline sodium sulfide is limited to 1 ml.
3. In this procedure, methylmercury together with dithizone, which has been in the free form in the aqueous phase, is re-extracted into toluene. Therefore, conduct re-extraction with this method using only toluene in a volume of 1 ml, the same as that of the 0.01% dithizone-toluene extract used.

For the basics of methyl mercury analysis, see pp. 60-62 for the Procedural Notes to “4-1-1 Biological samples (fish, shellfish, human blood, and tissues such as umbilical cord)”



Flow Chart 7. Determination of Methylmercury in Biological Samples Containing Relatively High Concentrations of Mercury, Particularly Fish and Shellfish (Simplified Method)

4-1-3 Urine

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Ethanol: C_2H_5OH (analytical grade)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) 1N KOH-ethanol: Dissolve 56.11 g of potassium hydroxide (analytical grade) in ethanol to obtain a final volume of 1,000 ml. (Store in a cool dark place.)

(5) 1N HCl: Mix 90 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(6) 20% EDTA solution: Dissolve 20 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4 \cdot 4H_2O$, in distilled water to make a final volume of 100 ml.

(7) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(8) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(9) Purified 0.01% dithizone-toluene: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml of toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the aqueous phase (bottom phase). After allowing the phases to separate, transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate), add 100 ml of toluene, and shake to obtain purified 0.01% dithizone-toluene. Allow the phases to settle, draw off and discard the bottom phase, and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(10) Alkaline sodium sulfide solution: Dissolve 0.15 g of $Na_2S \cdot 9H_2O$ (analytical grade) in 10 ml of distilled water in a 10-ml conical centrifuge tube fitted with

a glass stopper to make the sodium sulfide stock solution. (Prepare a fresh solution monthly. Store in a cool dark place.) At each use, transfer 0.1 ml of the stock solution into a glass container fitted with a glass stopper, add 50 ml of 0.1N NaOH and 50 ml of ethanol, and mix to obtain an alkaline sodium sulfide solution. (One ml of this solution contains 5 μg of Na_2S.)

(11) Walpole's buffer: Mix 200 ml of 1M CH_3COONa and about 200 ml of 1N HCl in 600 ml of distilled water to adjust to pH 3.0.

(12) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(13) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to obtain the methylmercury standard solution. One ml of this solution contains 1,000 ng of Hg.

(14) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a stopper. Shake for 3 minutes with a reciprocal shaker to extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.1 μg of Hg.

(15) Anhydrous sodium sulfate: Anhydrous sodium sulfate (pesticide analysis grade) heated at 500°C for 2-3 hours (stored in a desiccator)

(16) N_2 gas

Note: For the above reagents (5)-(8), (10) and (11), prepare the required amount before use, add a 1/2 volume of toluene, and wash by shaking. Confirm beforehand that no peaks appear that could interfere with the measurement by GLC-ECD.

b. Instruments and equipment

(1) Gas-liquid chromatograph equipped with electron capture detector (GLC-ECD)
(2) Multi-flow meter: Model V4 flow meter multi-kit (Kojima Instruments Inc.)
(3) Centrifuge
(4) Reciprocal shaker
(5) Magnetic stirrer
(6) Aspirator
(7) Vortex mixer
(8) pH mater
(9) Volumetric flasks: 10, 100, and 1,000 ml
(10) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml
(11) Pasteur pipettes
(12) Separatory funnels: 100, 200, and 1,000 ml
(13) Glass containers with glass stopper: 100, 200, and 500 ml
(14) Glass container with screw cap: 1,000 ml
(15) 50-ml round-bottom centrifuge tube with glass stopper
(16) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

Note: Thoroughly wash all glassware with toluene before use. Confirm in advance that no peaks appear that could interfere with the measurement by GLC-ECD.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).

ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).

iii) 3.0 mm × 2.0 m glass column packed with 5%-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solution

Methylmercury extraction

Transfer a urine sample (usually 20 ml) into a 50-ml round-bottom centrifuge tube fitted with a glass stopper. Add 10 ml of 1N KOH-ethanol and shake for 5 minutes. Add 10 ml of HCl and mix to acidify the sample slightly. Add 2 ml of 20% EDTA and mix well. Add 5 ml of purified 0.01% dithizone-toluene and shake for 3 minutes to extract methylmercury in the sample. Centrifuge at 1,200 rpm for 3 minutes with the attached grass stopper. Draw off and discard the aqueous phase (lower phase). Add 0.5 g of anhydrous sodium sulfate to the toluene phase and shake for 5 minutes. Centrifuge again at 1,200 rpm for 3 minutes with the attached grass stopper. Draw off and discard as much of the remaining aqueous phase (lower phase) as possible.

Clean-up

Add 3 ml of 1N NaOH to the toluene phase and shake for 3 minutes to remove excess dithizone in the toluene phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Repeat the washing with 3 ml of 1N NaOH for the clean-up. Allow the phases to separate. Draw off and discard the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes to obtain a clear toluene phase. (If the emulsion still remains in

the toluene phase, draw off and discard the lower phase. Add about 0.5 g of anhydrous sodium sulfate and shake. Perform centrifugal separation and discard the lower phase.)

Transfer a fixed volume (usually 3 ml) of the toluene phase into a 10-ml conical centrifuge tube with a glass stopper. Add 2 ml of alkaline sodium sulfide solution and shake for 3 minutes to back-extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Carefully draw off and discard the toluene phase (upper phase). Add 2 ml of toluene to the aqueous phase and shake for 3 minutes to wash the aqueous phase. Centrifuge again at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase). Add 1N HCl (3-5 drops) to make the solution slightly acidic. With a Pasteur pipette, aerate the solution with N_2 gas through a multi-flow meter for 3 minutes (50 ml/min.) to expel the excess sulfide ions as hydrogen sulfide gas. Subsequently, add 2 ml of Walpole's buffer while washing the tip of the Pasteur pipette and mix well with a vortex mixer. Add a fixed volume of purified 0.01% dithizone solution (0.2-1.0 ml, usually 0.5 ml) and shake for 3 minutes to extract methylmercury. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Add 3 ml of 1N NaOH to the toluene phase and shake for 3 minutes to remove the excess dithizone. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Again draw off and discard as much of the aqueous phase (lower phase) as possible. Acidify the solution by adding 2 drops of 1N HCl to the toluene phase and mix with a vortex mixer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the hydrochloric acid phase (lower phase). Use the resulting solution as the sample test solution.

Separately, place 20 ml of distilled water in each of three 50-ml screw-capped round-bottom centrifuge tubes. Add 0, 0.050, and 0.10 ml (corresponding to

0, 5, and 10 ng Hg) of methylmercury-cysteine solution, respectively. Subject these samples to the same procedures indicated above for preparing the sample test solutions to obtain a blank test solution and methylmercury standard test solutions for calibration. Store these test solutions in a cool dark place after preparation.

d. Test procedures and calculations

With a micro syringe, inject into the GLC a fixed volume (usually2-5 µl) of each of the sample test solutions (or their toluene-diluted solutions), blank and methylmercury standard test solutions. Calculate the methylmercury concentration in the urine sample (ng/ml) by comparing the peak height of the sample test solution with the calibration curve obtained from the blank and standard test solutions.

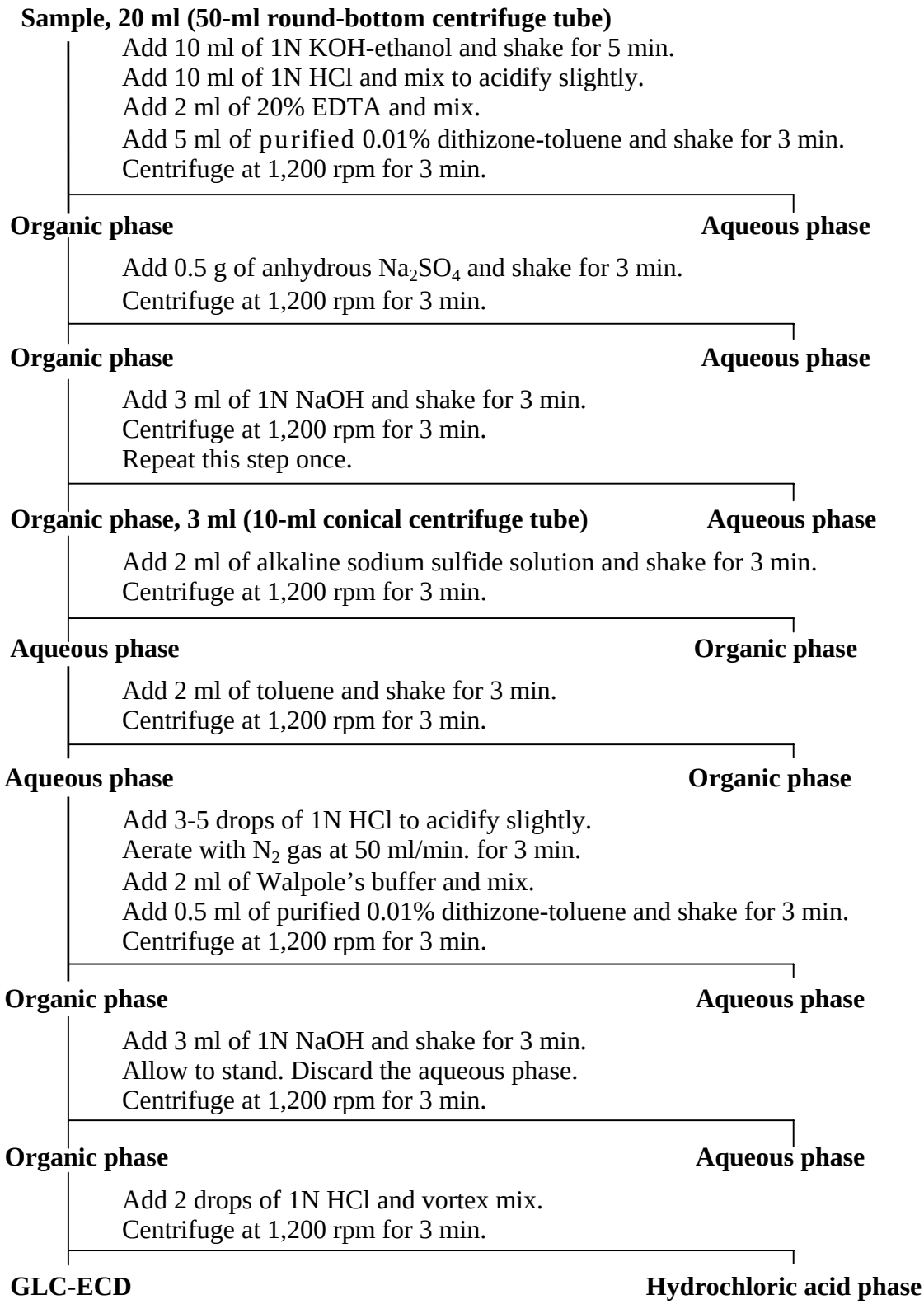
Alternatively, when the linearity is confirmed by measurement of the methylmercury standard test solutions for calibration, calculate the concentration of methylmercury in the sample with the following equation using the peak height(mm) of the standard test solution (Pstd) of, for example, 10 ng Hg.

Methylmercury concentration in the urine sample (ng/ml) = 10 ng × (Ps–Pbl)/(Pstd–Pbl) × 1/sample volume (ml)

Ps: peak height (mm) of sample test solution

Pbl: peak height (mm) of blank test solution

Note: For the basics of methyl mercury analysis, see pp. 60-62 for the Procedural Notes to “4-1-1 Biological samples (fish, shellfish, human blood, and tissues such as umbilical cord)”



Flow Chart 8. Determination of Methylmercury in Urine

4-1-4 Sediment/soil

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Ethanol: C_2H_5OH (analytical grade)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) 1N KOH-ethanol: Dissolve 56.11 g of potassium hydroxide (analytical grade) in ethanol to obtain a final volume of 1,000 ml. (Store in a cool dark place.)

(5) 1N HCl: Mix 90 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(6) 20% $NH_2OH{\cdot}HCl$ solution: Dissolve 20 g of hydroxylamine hydrochloride in distilled water to make a final volume of 100 ml.

(7) 20% EDTA solution: Dissolve 20 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4{\cdot}4H_2O$, in distilled water to make a final volume of 100 ml.

(8) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(9) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(10) Purified 0.01% dithizone-toluene: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml of toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the aqueous phase (bottom phase). After allowing the phases to separate, transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate). Add 100 ml of toluene and shake to obtain purified 0.01% dithizone-toluene. Allow the phases to settle, draw off and discard the bottom phase, and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(11) Alkaline sodium sulfide solution: Weigh out 0.15 g of $Na_2S \cdot 9H_2O$ (analytical grade) in a 10-ml conical centrifuge tube with a glass stopper and dissolve in 10 ml of distilled water to make the sodium sulfide stock solution. (Prepare a fresh solution monthly. Store in a cool dark place.) At each use, transfer 0.1 ml of the stock solution into a glass container with a glass stopper, add 50 ml of 0.1N NaOH and 50 ml of ethanol, and mix to make an alkaline sodium sulfide solution. (One ml of this solution contains 5 µg of Na_2S.)

(12) Walpole's buffer: Mix 200 ml of 1M CH_3COONa and about 200 ml of 1N HCl in 600 ml of distilled water to adjust to pH 3.0.

(13) Anhydrous sodium sulfate: Anhydrous sodium sulfate (pesticide analysis grade) heated at 500°C for 2-3 hours (stored in a desiccator)

(14) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(15) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as a stock solution. Dilute the stock solution 100-fold with toluene to make the methylmercury standard solution. One ml of this solution contains 1,000 ng of Hg.

(16) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube with a stopper. Shake for 3 minutes with a reciprocal shaker to extract the methylmercury to the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 0.10 µg of Hg.

(17) Florisil: Florisil for column chromatography (60-100 mesh) heated at 130°C for 2-3 hours (stored in a desiccator)

(18) Florisil column: A glass column packed with 0.5 g of Florisil (60-100 mesh) and 0.5 g anhydrous sodium sulfate in turn

(19) N_2 gas

Note: For the above reagents (5)-(9), (11), and (12), prepare the required amounts in advance, add a 1/2 volume of toluene, and wash by shaking. Confirm beforehand that no peaks appear that could interfere with the GLC measurement of methylmercury.

b. Instruments and equipment

(1) Gas-liquid chromatograph equipped with electron capture detector (GLC-ECD)

(2) Multi-flow meter: Model V4 flow meter multi-kit (Kojima Instruments Inc.)

(3) Centrifuge

(4) Reciprocal shaker

(5) Magnetic stirrer

(6) Aspirator

(7) Vortex mixer

(8) pH mater

(9) Volumetric flasks: 10, 100, and 1,000 ml

(10) Measuring pipettes: 0.2, 0.5, 1.5, and 10 ml

(11) Pasteur pipettes

(12) Separatory funnels: 100, 200, and 1,000 ml

(13) Glass containers with glass stoppers: 100, 200, and 500 ml

(14) Glass container with a screw cap: 1,000 ml

(15) 50-ml screw-capped round-bottom centrifuge tube

(16) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

(17) Cotton wool

(18) Porcelain crucible

Note: Thoroughly wash all glassware with toluene before use. Confirm in advance that no peaks appear that could interfere with the GLC measurement of methylmercury. If a peak appears, perform heat treatment at 300°C for 30 minutes.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).

ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).

iii) 3.0 mm × 2.0 m glass column packed with 5%-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solution

Methylmercury extraction

Precisely weigh out a homogenized sample (usually 0.2-0.5 g as wet weight, around 0.1 g for a dry sample) in the bottom of a 50-ml screw-capped round-bottom centrifuge tube. (For a dry sample, add 0.5 ml of distilled water to moisten after weighing.) Add 10 ml of 1N KOH-ethanol. Stir and crush well with a glass rod and

shake for 20 minutes with a reciprocal shaker and add 10 ml of 1N HCl to acidify the sample solution slightly. Aerate the sample solution with N_2 gas at 100 ml/min. for 5 minutes while stirring with a magnetic stirrer. Add 2 ml of a 20% $NH_2OH \cdot HCl$ solution and 2 ml of a 20% EDTA solution in turn, and mix by shaking.[1] Add 5 ml of purified 0.01% dithizone solution and shake for 3 minutes to extract methylmercury in the sample. Centrifuge at 2,500 rpm for 3 minutes. Using a 5-ml measuring pipette provided with a small amount of cotton wool wound around the tip, collect at least 4 ml from the toluene phase, being careful not to mix the lower phase, and pass it through the Florisil column. Receive the eluate into a 10-ml conical centrifuge tube with a glass stopper.

Clean-up

Add 3 ml of 1N NaOH to the toluene phase and wash by shaking for 3 minutes to remove excess dithizone into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard as much of the lower phase as possible. Add another 3 ml of 1N NaOH. Shake and wash similarly. Allow the phases to separate. Draw off and discard the lower phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Transfer a fixed volume (usually 3 ml) of the toluene phase into another 10-ml conical centrifuge tube with a glass stopper. Add 2 ml of alkaline sodium sulfide solution and shake for 3 minutes to back-extract the methylmercury into the aqueous layer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached and draw off and discard the toluene phase (upper phase) carefully. Then, add 2 ml of toluene to the aqueous phase and shake for 3 minutes to wash the aqueous phase. Centrifuge again at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase). Add 1N HCl (3-5 drops) to make the solution slightly acidic. With a Pasteur pipette, aerate the solution with N_2 gas through a multi-flow meter at 50 ml/min. for 3 minutes to expel the excess sulfide

ions as hydrogen sulfide gas. Subsequently, add 2 ml of Walpole's buffer while washing the tip of the Pasteur pipette in turn and mix well with a vortex mixer. Add a fixed volume of purified 0.01% dithizone solution (0.2-1.0 ml, usually 0.5 ml) and shake to extract methylmercury. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Add 3 ml of 1N NaOH to the toluene phase, shake, and wash to remove the excess dithizone. Allow the phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached, and again draw off and discard as much of the aqueous phase (lower phase) as possible. Acidify the solution by adding 2 drops of 1N HCl to the toluene phase and stir with a vortex mixer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the hydrochloric acid phase (lower phase). Use the resulting solution as the sample test solution.

Separately, depending on the expected mercury concentrations in the samples, transfer 0-0.20 ml of methylmercury-cysteine solution (corresponding to 0-0.020 µg Hg) into at least three 50-ml screw-capped round-bottom centrifuge tubes. Add 10 ml of 1N KOH-ethanol and follow the same procedures as indicated above for preparing the sample test solutions to obtain the blank test solution and methylmercury standard test solutions for the calibration curve. Store all these test solutions in a cool dark place.

For wet samples, at collection of the samples for analysis, precisely weigh about 10-20 g of the sample into a porcelain crucible of known weight. Place it in a drying oven at 105°C and dry for 2-3 hours. Allow to cool in a desiccator, then weigh to obtain the wet weight/dry weight ratio (WW/DW).

d. Test procedures and calculations

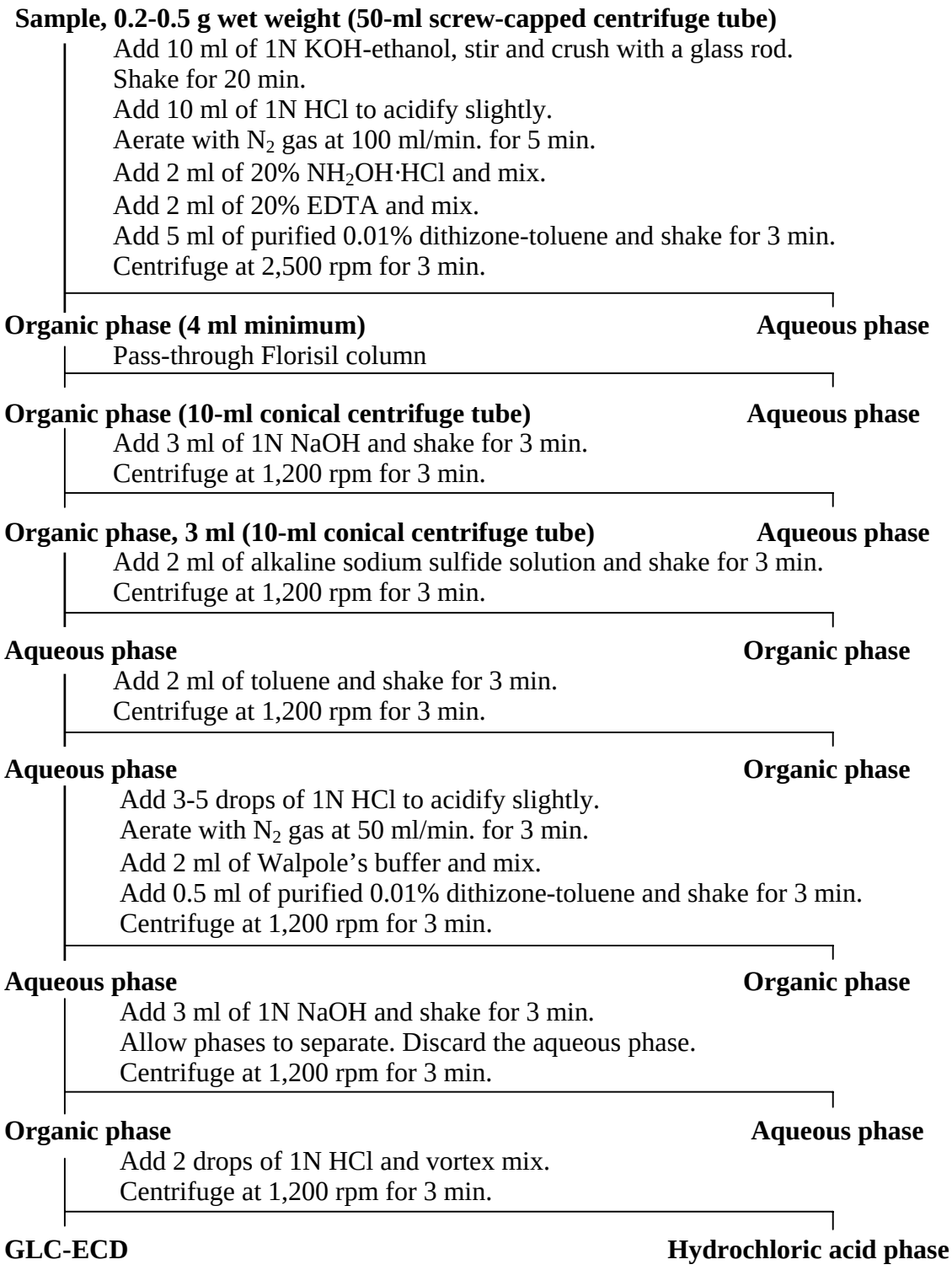
With a micro-syringe, inject into the GLC a fixed volume (usually 2-5 µl) of each of the sample test solutions (or their toluene-diluted solutions), the blank and

the methylmercury standard test solutions for the calibration curve. Calculate the methylmercury concentration in the sample (µg/g wet weight) by comparing the peak height of the sample test solution with the calibration curve obtained from the blank and standard test solutions. Convert the result to a dry weight (µg/g dry weight) with the wet weight/dry weight ratio determined for the sample with the procedure indicated above.

Procedural Notes

1. Before dithizone extraction, add hydroxylamine hydrochloride and EDTA solution and mix by shaking to reduce oxidative substances and mask other metal ions contained in the sample. This prevents dithizone from unnecessary consumption/decomposition during the dithizone-toluene extraction process.

For the basics of methyl mercury analysis, see pp. 60-62 for the Procedural Notes to “4-1-1 Biological samples (fish, shellfish, human blood, and tissues such as umbilical cord)”



Flow Chart 9. Determination of Methylmercury in Sediment/Soil

4-1-5 Water

Similar to the method for total mercury analysis of water samples, this method involves the ionization and liberation of mercury compounds through treatment with potassium permanganate under sulfuric acid-acidification; pre-concentration of the mercury by dithizone-toluene extraction; back-extraction of the methylmercury in the extract into alkaline sodium sulfide solution; re-extraction with dithizone-toluene to clean up; and measurement by GLC-ECD.

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Ethanol: C_2H_5OH (analytical grade)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) 20N H_2SO_4: Transfer about 350 ml of distilled water into a 1-L volumetric flask. Gradually add 600 ml of sulfuric acid (for measurement of toxic metals) while stirring in ice water. After it returns to room temperature, add distilled water to a final volume of 1,000 ml.

(5) 10N NaOH: Dissolve 400 g of sodium hydroxide (analytical grade) to make a final volume of 1,000 ml.

(6) 0.5% $KMnO_4$ solution: Dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 ml.

(7) 10% $NH_2OH{\cdot}HCl$ solution: Dissolve 10 g of hydroxylamine hydrochloride in distilled water to make a final volume of 100 ml.

(8) 10% EDTA solution: Dissolve 10 g of tetrasodium ethylenediaminetetraacetate (analytical grade), $C_{10}H_{12}N_2O_8Na_4{\cdot}4H_2O$, in distilled water to make a final volume of 100 ml.

(9) 1N HCl: Mix 90 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(10) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(11) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(12) Purified 0.01% dithizone-toluene[1]: Dissolve 0.011 g of diphenylthiocarbazone, $C_6H_5N{:}NCSNHNHC_6H_5$, in 100 ml of toluene in a 200-ml separatory funnel. Add 50 ml of 0.1N NaOH and shake briefly to extract the dithizone into the aqueous phase (bottom phase). Allow the phases to separate. Transfer the bottom phase into a glass container fitted with a glass stopper. Add 1N HCl dropwise to make the solution slightly acidic (blackish-green crystals will precipitate), add 100 ml of toluene, and shake to obtain purified 0.01% dithizone-toluene. Allow the phases to separate. Draw off and discard the bottom phase and seal. Store in a cool dark place. (Prepare a fresh solution for each analysis.)

(13) Alkaline sodium sulfide solution: Weigh out 0.15 g of $Na_2S{\cdot}9H_2O$ (analytical grade) in a 10-ml conical centrifuge tube fitted with a glass stopper and dissolve in 10 ml of distilled water to make a sodium sulfide stock solution. (Prepare a fresh solution monthly. Store in a cool dark place.) At each use, transfer 0.1 ml of the stock solution into a glass container fitted with a glass stopper, add 50 ml of 0.1N NaOH and 50 ml of ethanol, and mix to make an alkaline sodium sulfide solution. (One ml of this solution contains 5 µg of Na_2S.)

(14) Walpole's buffer: Mix 200 ml of 1M CH_3COONa and about 200 ml of 1N HCl in 600 ml of distilled water to adjust to pH 3.0.

(15) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH{\cdot}HCl{\cdot}H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(16) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve

in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to make a methylmercury standard solution. One ml of this solution contains 1,000 ng of Hg.

(17) Methylmercury-cysteine solution: Transfer 0.5 ml of the methylmercury standard solution and 5 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube fitted with a glass stopper. Shake for 3 minutes with a reciprocal shaker to extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached and draw off and discard the organic phase (upper phase). Seal the tube and store as a stock solution in a cool dark place. (Prepare a fresh solution monthly). At each use, dilute the stock solution 10-fold with distilled water to make a methylmercury-cysteine solution for preparing calibration samples for water methylmercury analysis. One ml of this solution contains 10 ng of Hg.

(18) Anhydrous sodium sulfate: Anhydrous sodium sulfate (pesticide analysis grade) heated at 500°C for 2-3 hours (stored in a desiccator)

(19) N_2 gas

Note: For the above reagents(5), (7)-(11), (13), and (14), prepare the required amounts in advance, add a 1/2 volume of toluene, and wash by shaking in a separatory funnel. Confirm beforehand that no peaks appear that could interfere with the GLC measurement of methylmercury.

b. Instruments and equipment

(1) Gas-liquid chromatograph equipped with electron capture detector (GLC-ECD)

(2) Multi-flow meter: Model V4 flow meter multi-kit (Kojima Instruments Inc.)

(3) Centrifuge

(4) Reciprocal shaker

(5) Magnetic stirrer

(6) Aspirator
(7) Vortex mixer
(8) pH mater
(9) Volumetric flasks: 10, 100, and 1,000 ml
(10) Measuring pipettes: 0.2, 0.5, 5, and 10 ml
(11) Pasteur pipettes
(12) Separatory funnels: 100, 200, 1,000, and 2,000 ml
(13) Glass containers with glass stoppers: 100, 200, and 500 ml
(14) Glass container with screw cap: 1,000 ml
(15) 35-ml conical centrifuge tube with glass stopper
(16) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter × 100 mm in length

Note: Thoroughly wash all glassware with toluene before use. Confirm in advance that no peaks appear that could interfere with the GLC measurement of methylmercury.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).
ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).
iii) 3.0 mm × 2.0 m glass column packed with 5%-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solution

Methylmercury extraction

Transfer 2 L of a water sample into a 2-L separatory funnel. Add 10 ml of 20N H_2SO_4 and 5 ml of a 0.5% $KMnO_4$ solution. Mix and let stand for 5 minutes. Add 20 ml of 10N NaOH and mix by shaking to neutralize. Add 5 ml of a 10% $NH_2OH{\cdot}HCl$ solution and shake for several seconds to mix. Let stand for 20 minutes. Add 5 ml of 10% EDTA solution and mix by shaking.[2] Add 10 ml of purified 0.01% dithizone-toluene and shake vigorously for 1 minute to extract the methylmercury in the sample. Let stand for at least 1 hour out of direct sunlight. Discard the aqueous phase (lower phase).

Clean-up

Transfer as much of the toluene phase as possible into a 35-ml conical centrifuge tube fitted with a glass stopper. Attach the glass stopper and centrifuge at 1,200 rpm for 3 minutes. Draw off and discard the aqueous phase (lower phase). (For analysis with unfiltered water, if emulsification occurs, remove the lower aqueous phase, add about 0.5 g of anhydrous sodium sulfate, and shake followed by centrifugal separation to remove the lower phase.) Add 5 ml of 1N NaOH and wash by shaking for 3 minutes to remove excess dithizone. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Again add 5 ml of 1N NaOH and repeat this washing procedure. After centrifugal separation, draw off and discard the lower phase. Transfer a fixed volume (usually 7 ml) of the toluene phase to a 10-ml conical centrifuge tube with a glass stopper. Add 2 ml of alkaline sodium sulfide solution and shake for 3 minutes to back-extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Carefully draw off and discard the toluene phase (upper phase). Add 2 ml of toluene to the aqueous phase and shake

for 3 minutes to wash the aqueous phase. Centrifuge again at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the toluene phase (upper phase). Add 1N HCl (3-5 drops) to make the solution slightly acidic. With a Pasteur pipette, aerate the sample solution with N_2 gas through a multi-flow meter at 50 ml/min. for 3 minutes to expel the excess sulfide ions as hydrogen sulfide gas. Subsequently, add 2 ml of Walpole's buffer while washing the tip of the Pasteur pipette. Mix well with a vortex mixer. Add a fixed volume of purified 0.01% dithizone-toluene (usually 0.2 ml) and shake for 3 minutes to extract methylmercury. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the aqueous phase (lower phase). Add 3 ml of 1N NaOH to the toluene phase and wash by shaking for 3 minutes to remove the excess dithizone. Let stand to allow the two phases to separate. Draw off and discard the aqueous phase (lower phase). Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached, and again draw off and discard as much of the remaining aqueous phase (lower phase) as possible. Acidify the solution by adding 2 drops of 1N HCl and mix with a vortex mixer. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached. Draw off and discard the hydrochloric acid phase (lower phase) to make a sample test solution.

Separately, choose the water sample with the lowest mercury concentration and transfer 2 L each to three 2-L separatory funnels. Add 0, 0.10, and 0.20 ml of methylmercury-cysteine solution (corresponding to 0, 1.0 and 2.0 ng Hg), respectively. Subject the samples to the same procedures as indicated above for preparing sample test solutions to make a blank test solution and methylmercury standard test solutions, respectively. Store these test solutions in a cool dark place after preparation.

d. Test procedures and calculations

With a micro-syringe, inject into the GLC a fixed volume (usually 2-5 µl) of each of the sample test solutions (or their toluene-diluted solutions), and the blank and methylmercury standard test solutions. Calculate the methylmercury concentration in the water sample (ng Hg/L) by comparing the peak height of the sample test solution with the calibration curve obtained from the blank and standard test solutions. Alternatively, when the linearity is confirmed by measurement of the methylmercury standard test solutions for preparation of the calibration curve, the methylmercury concentration in the sample can be calculated according to the following equation using the peak height of the standard test solution (Pstd) of, for example, 2 ng Hg.

Methylmercury concentration in the water sample (ng/L) = 2 ng ×
(Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/ water sample (L)

Ps: peak height (mm) of the sample test solution

Pbl: peak height (mm) of the blank test solution

Procedural Notes

1. Dithizone (diphenylthiocarbazone) is easily oxidized and usually contains its oxidized form (diphenylthiocarbadiazone) as an impurity that causes interfering peaks on the gas chromatogram. Therefore, utilizing pure dithizone's unique chemical property of forming a water-soluble salt that dissolves in alkaline solution, prepare a fresh dithizone-toluene solution for each analysis.
2. In preparing sample test solutions for water samples, add hydroxylamine hydrochloride to reduce the remaining potassium permanganate and add EDTA solution to mask other metal ions contained in the sample. Thus, add both to prevent unnecessary consumption of dithizone by metal ions and oxidation during dithizone-toluene extraction.

For the basics of methyl mercury analysis, see pp. 60-62 for the Procedural Notes to “4-1-1 Biological samples (fish, shellfish, human blood, and tissues such as umbilical cord)”

Sample, 2 L (2-L separatory funnel)

Add 10 ml of 20N H_2SO_4 and mix to acidify.
Add 5 ml of 0.5% $KMnO_4$ solution, mix, and let stand for 5 min.
Add 20 ml of 10N NaOH and mix to neutralize.
Add 5 ml of 10% $NH_2OH \cdot HCl$ solution, mix, and let stand for 20 min.
Add 5 ml of 10% EDTA solution and mix.
Add 10 ml of 0.01% dithizone-toluene and shake for 3 min.
Let stand for at least 1 hr.

Organic phase (35-ml conical centrifuge tube) — **Aqueous phase**

(When an emulsion is formed, add 0.5 g of anhydrous Na_2SO_4 and shake.)
Centrifuge at 1,200 for 3 min.

Organic phase — **Aqueous phase**

Add 5 ml of 1 N NaOH and shake for 3 min.
Centrifuge at 1,200 rpm for 3 min.

Organic phase, 7 ml (10-ml conical test tube) — **Aqueous phase**

Add 2 ml of alkaline sodium sulfide solution and shake for 3 min.
Centrifuge at 1,200 rpm for 3 min.

Aqueous phase — **Organic phase**

Add 2 ml of toluene and shake for 3 min.
Centrifuge at 1,200 rpm for 3 min.

Aqueous phase — **Organic phase**

Add 3-5 drops of 1N HCl to acidify slightly.
Aerate with N_2 gas at 50 ml/min. for 3 min.
Add 2 ml of Walpole's buffer and mix.
Add 0.2 ml of purified 0.01% dithizone-toluene and shake for 3 min.
Centrifuge at 1,200 rpm for 3 min.

Organic phase — **Aqueous phase**

Add 3 ml of 1N NaOH and shake for 3 min.
Allow to stand. Discard the aqueous phase.
Centrifuge at 1,200 rpm for 3 min.

Organic phase — **Aqueous phase**

Add 2 drops of 1N HCl and vortex mix.
Centrifuge at 1,200 rpm for 3 min.

GLC-ECD — **Hydrochloric acid phase**

Flow Chart 10. Determination of Methylmercury in Water

4-2 Determination by the hydrochloric acid leaching/toluene extraction/gas-liquid chromatography with electron capture detection (GLC-ECD) method

Methylmercury analysis for hair sampling can be performed more simply with a method different from that presented above. Briefly, this method involves immersion of the sample in 2N HCl, heating at 100°C for 5 minutes to leach out methylmercury from the sample, methylmercury extraction into toluene, and determination by GLC-ECD.

4-2-1 Hair

Transfer several tens of milligrams of the sample into a beaker. Wash with neutral detergent(diluted 100-fold) and distilled water by decantation. Add a small volume of acetone to the sample to remove the remaining water. Remove the acetone under reduced pressure. Transfer the sample into a 20-ml vial and cut it into fine pieces with dissection scissors.

a. Reagents

(1) Toluene: $C_6H_5CH_3$ (pesticide analysis grade)

(2) Ethanol: C_2H_5OH (analytical grade)

(3) Distilled water: Distill deionized water and store in a clean glass container.

(4) 2N HCl: Mix 180 ml of hydrochloric acid (analytical grade) with distilled water to obtain a final volume of 1,000 ml.

(5) 1N NaOH: Dissolve 40 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 1,000 ml.

(6) 0.1N NaOH: Dilute 1N NaOH 10-fold with distilled water.

(7) 0.1% L-cysteine solution: Dissolve 10 mg of L-cysteine hydrochloride, $HSCH_2CH(NH_2)COOH \cdot HCl \cdot H_2O$, in 10 ml of 0.1N NaOH. (Prepare a fresh solution for each analysis.)

(8) Methylmercury standard solution: Weigh out 12.5 mg of methylmercury chloride, CH_3HgCl (authentic standard) in a 100-ml volumetric flask, dissolve in toluene to make a final volume of 100 ml, and store as stock solution. Dilute the stock solution 100-fold with toluene to make the methylmercury standard solution. One ml of this solution contains 1,000 ng of Hg.

(9) Methylmercury-cysteine solution: Transfer 2 ml of the methylmercury standard solution and 2 ml of the 0.1% L-cysteine solution into a 10-ml conical centrifuge tube with a glass stopper. Shake for 3 minutes with a reciprocal shaker to extract the methylmercury into the aqueous phase. Centrifuge at 1,200 rpm for 3 minutes with the glass stopper attached and draw off and discard the organic phase (upper phase). Seal the tube and store in a cool dark place. (Prepare a fresh solution monthly). One ml of this solution contains 1,000 ng of Hg.

Note: For the above reagents (5) and (6), prepare the required amounts in advance, add a 1/2 volume of toluene, and wash by shaking in a separatory funnel. Confirm beforehand that no peaks appear that could interfere with the measurement of methylmercury by GLC-ECD.

b. Instruments and equipment

(1) Gas-liquid chromatograph equipped with electron capture detector (GLC-ECD)

(2) Centrifuge

(3) Reciprocal shaker

(4) Isothermal bath: Use polyethylene glycol 400.

(5) Aspirator

(6) Volumetric flasks: 10, 100, and 1,000 ml
(7) Measuring pipettes: 0.2, 1, 5, and 10 ml
(8) Pasteur pipettes
(9) Separatory funnel: 1,000 ml
(10) Glass container with glass stopper: 500 ml
(11) Beaker: 100 ml
(12) 10-ml screw-capped round-bottom centrifuge tube: 16.5 mm in diameter × 105 mm in length
(13) 10-ml conical centrifuge tube with glass stopper: 16.5 mm in diameter, 100 mm in length
(14) Vial: 20-ml scintillation vial
(15) Glass wool or cotton wool
(16) Dissection scissors

Note: Thoroughly wash all glassware with toluene before use. Confirm in advance that no peaks appear that could interfere with measurement of methylmercury by GLC-ECD. If a peak appears, perform heat treatment at 300°C for 30 minutes.

Gas-liquid chromatographic conditions:

Either of the following three different columns can be used for the analysis:

i) 3.0 mm × 0.75-1.0 m glass column packed with Hg-20A on Uniport HP (AW-DMCS, 60-80 mesh, GL Science Co., Ltd., Tokyo, Japan).
ii) 3.0 mm × 0.75-1.0 m glass column packed with 10% KOCL-Hg on Chromosorb W (AW-DMCS, 60-80 mesh, J-Science Co., Ltd., Kyoto, Japan).
iii) 3.0 mm × 2.0 m glass column packed with 5%-10% poly-diethylene glycol succinate (DEGS) on Chromosorb W (AW-DMCS).

After packing the column, pack about 2-3 cm of NaCl, previously heated at 500°C for 2-3 hours, on top of the packing material (at the injection port).

Temperature: Column oven: 140-160°C, Injection port: 180°C, Detector oven: 200°C

Carrier gas: N_2, 30-40 ml/min.

c. Preparation of sample test solutions

Precisely weigh out a finely cut hair sample (usually around 10 mg) and transfer into a 10-ml screw-capped round-bottom centrifuge tube. Add 2 drops of ethanol to moisten the sample. With a glass rod, insert a small amount of glass wool or cotton wool into the tube and press lightly on the sample to cover it. Gently place 3 ml of 2N HCl onto the glass wool or cotton, taking care to keep the sample below the surface. Seal tightly and heat in an isothermal bath at 100°C for 5 minutes to elute methylmercury from the sample.[1] Allow to cool and invert it to mix. Centrifuge at 1,200 rpm for 3 minutes. Transfer 1 ml of the supernatant into a 10-ml conical centrifuge tube provided with a glass stopper. Add 2 ml of toluene and shake for 3 minutes to extract the methylmercury present in the HCl phase into the toluene phase.[2] Centrifuge at 1,200 rpm for 3 minutes. Draw off and remove the lower phase[3] to make a sample test solution.

Separately, transfer 0, 0.050, and 0.10 ml of a methylmercury-cysteine solution (corresponding to 0, 50, and 100 ng Hg) into three 10-ml screw-capped round-bottom centrifuge tubes, respectively, and add 2N HCl to make a final volume of 3 ml. Subject these solutions to the procedures indicated in the method for preparing test solutions to make methylmercury standard test solutions for the calibration curve. Protect all the test solutions from light after preparation.

d. Test procedures and calculations

With a micro-syringe, inject into the GLC a fixed volume (usually 2-5 µl) of each of sample test solutions (or their toluene-diluted solutions), and the blank and methylmercury standard test solutions. Calculate the methylmercury concentration

in the hair sample (ng/mg) by comparing the peak height of the sample test solution with the calibration curve obtained from the blank and methylmercury standard test solutions.

Alternatively, when the linearity is confirmed by measurement of the methylmercury standard test solutions for preparation of the calibration curve, the methylmercury concentration in the sample can be calculated according to the following equation using the peak height of the standard test solution (Pstd) of, for example, 100 ng Hg.

Methylmercury concentration in the sample (ng/mg) = 100 ng ×
(Ps–Pbl)/(Pstd–Pbl) × dilution factor × 1/sample weight (mg)

Ps: Peak height (mm) of sample test solution

Pbl: Peak height (mm) of blank test solution

Procedural Notes

1. Although the leaching of methylmercury in hair samples with dilute hydrochloric acid proceeds gradually at normal temperatures, it is accelerated by heating. When using 2N HCl, heating at 100°C causes it to elute almost completely within several minutes; exceeding 10 minutes causes other organic substances to elute, resulting in the appearance of interfering peaks on the gas chromatogram. A heating time of 5 minutes is sufficient under the conditions of this method. Do not exceed 10 minutes of heating time.
2. In order to transfer the methylmercury in the HCl eluate quantitatively into the toluene phase in this extraction step, use a toluene volume at least twice that of the HCl eluate for extraction.
3. To remove the upper phase or lower phase in the test tube, use the Suction-Removal System with a Pasteur pipette connected with a flexible tube through a waste liquid collector to an aspirator, as shown in Figure 2. Briefly, to remove the upper phase (organic phase), perform suctioning by positioning the

tip of the Pasteur pipette on the surface of the upper phase down along the inside wall of the test tube in order to draw off most of the upper phase. When only a little upper phase remains, keep the tip of the Pasteur pipette a few mm above the surface of the organic phase and continue to draw off. With this technique, only the organic phase, which has a lower specific gravity than that of the lower phase (aqueous phase) and high volatility, is drawn off together with air, allowing for almost complete removal of the organic phase. To draw off and discard the lower of the two phases separated in the test tube, squeeze the flexible tube with the fingers to stop the suction of the Pasteur pipette. Position the tip of the Pasteur pipette at the bottom of the test tube and adjust the pressure on the flexible tube to slowly draw up the lower phase. When the lower phase is almost completely removed, squeeze the flexible tube to stop the suction and remove the Pasteur pipette. This makes it possible to remove only the lower phase. Because these procedures require some skill and precision, practice each procedure beforehand.

Sample, around 10 mg (10-ml screw-capped centrifuge tube)

Add 2 drops of ethanol.
Cover with a small amount of glass wool or cotton wool.
Slowly add 3 ml of 2N HCl.
Seal tightly and heat at 100°C for 5 min.
Allow to cool and mix.
Centrifuge at 1,200 rpm for 3 min.

Hydrochloric acid phase, 1 ml (10-ml conical centrifuge tube)

Add 2 ml of toluene and shake for 3 min.
Centrifuge at 1,200 rpm for 3 min.

Organic phase | **Hydrochloric acid phase**

GLC-ECD

Flow Chart 11. Determination of Methylmercury in Hair

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