DIGESTION OF TISSUE SAMPLES FOR TOTAL MERCURY ANALYSIS

1. SCOPE AND APPLICATION

This SOP describes the digestion of tissue samples for the determination of total mercury content. This method is applicable to all biological tissue assigned the test ID T-HG-H and is based on EPA method 245.6.

2. SUMMARY OF THE METHOD

2.1 Mercury in tissue exists predominately as methyl mercury (organic mercury) with a small amount of inorganic mercury. In the case of fish tissue, virtually all of the mercury (>99 %) is in the form of methyl mercury.

2.2 In order to analyze for total mercury content, the organic mercury must be oxidized to mercuric ions (Hg^{+2}) before analysis. In order to accomplish this, each tissue sample is heated in sulfuric and nitric acids to denature and dissolve the tissue matrix. The dissolved tissue is then allowed to react with potassium permanganate and potassium persulfate to completely oxidize mercury to the ionic mercuric (Hg^{+2}) state.

3. APPARATUS AND EQUIPMENT

3.1 125 mL Nalgene straight-side wide mouth polypropylene jars with screw caps (pre-digestion vessel)

3.2 125 mL Nalgene high-density polyethylene wide-mouth bottles with screw caps (digestion vessel)

3.3 125 mL glass jars (re-usable)

3.4 Fisher Scientific Isotemp water bath

3.5 Pipettes:

3.5.1 10-100 μL adjustable Eppendorf digital

3.5.2 100-1000 μL adjustable Eppendorf digital

3.5.3 2-10 mL adjustable Finnpipette

3.6 Bottle-top dispensers

3.7 Analytical or Top-loading Balance

4. REAGENTS AND CHEMICALS

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See DEP SOP MT-069 for reagent documentation requirements and expiration dates. Reagents used for sample preparation must be logged in to the LIMS standard prep tracker to generate a serial number. The label printed from the LIMS standard prep tracker must be placed on the bottle of reagent in the laboratory. Document the reagents used for sample preparation in the LIMS prep worksheet manager. The certificate of analysis for each lot number of each purchased reagent must be scanned to the network and saved in folder: //FLDEP1/DEAR/Labs/Lab5/Chemistry/InorganicPrep/Certificates of Analysis for Reagents and Standards/Reagents.

4.1 Trace Metal Grade Nitric Acid (HNO_3) - If not in laboratory acid storage area, then order from the chemistry stockroom. The expiration date is provided by the manufacturer.

4.2 Trace Metal Grade Sulfuric Acid (H_2SO_4) - If not in laboratory acid storage area, then order from the chemistry stockroom. The expiration date is provided by the manufacturer.

4.3 6 % Potassium Permanganate Solution ($KMnO_4$) - Add 30 g of permanganate crystals to a 500 mL Nalgene bottle half full of deionized (DI) water, dilute to volume with DI water and mix well. Other volumes of this reagent may be prepared as long as the concentration remains the same. The expiration date for this solution is one day after preparation.

4.4 6 % Potassium Persulfate Solution ($K_2S_2O_8$) - Add 15 g of potassium persulfate crystals to a 250 mL Nalgene bottle half full of DI water and dilute to volume with DI water. Mix well. Other volumes of this reagent may be prepared as long as the concentration remains the same. The expiration date for this solution is one day after preparation.

4.5 Standards and Spiking Solutions:

Preparation of all Hg standards must be documented in the LIMS standard prep tracker. A label printed from the standard prep tracker that shows the identity of the standard, preparation date, expiration date, and serial number must be placed on the container of standard. Document the standards used during sample preparation on the LIMS prep worksheet. The certificate of analysis for each lot number of each purchased standard must be scanned to the network and saved in folder: //FLDEP1/DEAR/Labs/Lab5/Chemistry/InorganicPrep/Certificates of Analysis for Reagents and Standards/Standards and Standard Reference Materials. See DEP SOP MT-069 for standard documentation requirements and expiration dates.

4.5.1 **MHG-3** (1.252 mg Hg/L) - Organic Hg spike solution. This standard is used for the preparation of matrix spikes and laboratory fortified blanks (LFBs) for fish tissue. See DEP SOP HG-012 for preparation instructions. The expiration date for this prepared standard is one year from preparation.

4.5.2 **DORM-4** (0.410 mg/kg) - Commercially available fish tissue standard reference material from the National Research Council of Canada. Store in a

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desiccator in the laboratory. The expiration date is provided by the manufacturer.

4.5.3 **THG-4** (200 µg Hg/L) - Inorganic Standard Solution. This standard is used for the preparation of the practical quantitation limit (PQL) check standard. See DEP SOP HG-012 for preparation instructions. The expiration date for this prepared standard is one year from preparation.

4.5.4 **MHG-2** (10 mg Hg/L) - Organic Hg Spike Solution. This standard is used for the preparation of matrix spikes and LFBs for hair, feathers, and blood tissue. See DEP SOP HG-012 for preparation instructions. The expiration date for this prepared standard is one year from preparation.

4.6 9 % Acid Cleaning Solution - See DEP SOP HG-021 for preparation details.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1 Disposable polyethylene vessels should be rinsed 4-5 times with tap water and shake all excess water from the vessel. Non-disposable glassware should be cleaned in accordance with DEP SOP HG-021.

5.2 Samples are preserved by storage in the bio-freezer at < -20 °C. Samples must be removed from the freezer and allowed to thaw completely prior to digestion.

5.3 The holding time for fish tissue stored in the above condition is 1 year from collection.

6. SAMPLE PREPARATION PROCEDURE

Note: Prior to use on each working day, the water bath temperature must be checked using a NIST traceable thermometer to ensure it is in the range of 90° C ± 2° C. This temperature check must be documented on the LIMS Prep Worksheet Manager worksheet of the batch that is being digested.

6.1 Generate a backlog report using the LIMS and choose a batch of tissue samples for digestion. Similar matrices should be batched together. Fish tissue, hair, crayfish tissue, blood, bird tissue, and feathers should all be batched separately. For more guidance on sample batching, contact your supervisor.

6.2 Sample Custody - Samples are checked out from the Receiving Room (C301) using the LIMS to transfer custody of the samples from the Receiving Room to the preparation analyst.

6.3 Generate a worksheet in the LIMS Prep Worksheet Manager. Document the reagents and standards used for the preparation. Document the temperature of the water bath and ensure the calibration of the thermometer has been checked within the last 12 months. Document the balance used for sample preparation and ensure

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the calibration has been checked and documented each day prior to use.

6.4 Turn on the water bath and set the temperature to 90 °C on the control panel. Fill the water bath with DI water up to the fill mark on the bath. Replace the water in the water bath weekly.

6.5 Prepare the glass sample digestion vessel used for the **DORM-4** SRM by discarding the 10 % aqua regia cleaning solution that has been stored in the vessels. The vessel should then be rinsed 6-8 times with tap water. Rinse the cap as well with tap water and then replace on the digestion vessel.

6.6 Weighing Samples:

6.6.1 If the sample is unclotted blood or blood that has been diluted in the field with acid, homogenize the sample by sonicating for 5 minutes. After the blood has been sonicated, vortex each vial to ensure complete mixing and homogeneity. Pipette between 0.1 mL and 1 mL of blood into a **tared**, pre-labeled digestion vessel. Record the exact weight and also record the volume of the blood pipetted.

6.6.2 If the sample is bird tissue or clotted blood, homogenize sample with a micro tissue grinder, razor blade, or sonicator and weigh 0.1 - 0.25 g of sample into a **tared**, pre-labeled digestion vessel. Record the exact weight on the digestion worksheet.

6.6.3 For fish tissue, use a clean wooden applicator stick to transfer the entire tissue sample (or a ~ 10 g sub-sample if the sample is inordinately large) to a disposable weigh boat. Use a clean scalpel to carefully chop the tissue into very small pieces to homogenize the sample. Carefully transfer 1.0 to 3.0 g of chopped tissue to the **BOTTOM** of a pre-labeled digestion vessel. (Tissue tends to stick to the sides of the vessel; it is important that the tissue be as close to the bottom as possible.) Record the exact weight transferred on the digestion worksheet.

6.6.4 If the sample requires a pre-digestion (crayfish, hair, feathers, etc.) see Appendix A.

Note: Make sure that NO sample has spilled onto the balance outside of the beaker. Repeat for each tissue sample to be digested. When weighing replicate portions of sample for spikes, make sure that the sample weights are within ± 0.005 g of each other. Return the chopped tissue sample back to the original container and discard the weigh boat.

6.7 Matrix spikes are prepared in order to evaluate matrix interference. Five percent of samples analyzed are spiked. For a batch of 20 samples, one sample is chosen for the preparation of duplicate matrix spikes. Three 1.0 – 3.0 g aliquots of the same sample are placed in pre-labeled digestion vessels. One aliquot is prepared unfortified and the other two are fortified with the MHG-2 or MHG-3 standards according to the table below.

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6.8 Spikes:

Matrix	Spike Solution	Amount
Blood and Feathers High Low	 MHG-2, 10 mg/L Organic Spike MHG-2, 10 mg/L Organic Spike	 400 µL 100 µL
Fish, Crayfish, & Shrimp	MHG-3, 1.252 mg/L Organic Spike	300 µL

6.9 Prepare a tissue reference standard by weighing 0.500 ± 0.002 g of **DORM-4** into a pre-labeled digestion vessel. Record the exact weight on the digestion worksheet. Prepare one standard reference sample for every batch of 20 samples or less analyzed.

Note: Plastic digestion vessels build up static electricity easily, which can adversely affect the ability to weigh dry powders accurately. Standard reference materials such as **DORM-4** are freeze dried, homogenized powders that are susceptible to the effects of static electricity. The polyethylene digestion vessels also cannot withstand the heat produced from the reaction of the freeze-dried tissue and H_2SO_4. Therefore, glass containers are used for digesting standard reference materials.

6.10 Prepare a digestion blank by measuring 40 mL of DI water into a pre-labeled vessel. Add ~ 0.5 g of pre-cleaned perforated glass boiling beads to the vessel. Prepare one digestion blank for every batch of 20 samples or less. Since 'mercury-free' tissue does not exist, pre-cleaned perforated glass beads are used as the solid mercury-free matrix for the preparation of the digestion blank, PQL, and LFB.

6.11 Prepare a 0.375 µg/L PQL standard by adding 75 µL of 200 µg/L standard **THG-4** into a pre labeled digestion vessel filled with 40 mL of DI water. Add ~ 0.5 g of pre-cleaned perforated glass boiling beads to the vessel. Prepare one PQL for every batch of 20 samples or less.

6.12 Prepare one LFB by measuring 40 mL of DI water into a vessel and spiking with the same spike solution used for the tissue matrix spikes (see spike table in Section 6.8). Add ~ 0.5 g of pre-cleaned perforated glass boiling beads to the vessel.

6.13 Add 5.0 mL of H_2SO_4 to **all** sample vessels. Be aware of possible violent reactions with the tissue samples. If foaming occurs, gently tap the bottom of the vessel to break foam bubbles.

6.14 Add 2.0 mL of HNO_3 to **all** sample vessels. Allow **all** the samples to react for at least 5 minutes.

6.15 For each sample, tighten the Nalgene cap and gently swirl the vessel to expose all tissue to acids. If there is tissue stuck to the sides of the vessel, try to swirl the acids over the tissue to loosen it. Quickly loosen the cap to vent the built-up gases. Repeat for **all** samples.

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6.16 Loosen all caps and place samples in a **90 °C water bath for 45 minutes**. Carefully watch the samples for the first 5 minutes for violent reactions or foaming. Remove those vessels from the water bath and tap to break foam bubbles. Return to the water bath and continue to watch.

6.17 Remove samples and allow to cool. At this point, the tissues should be in liquid form. **Add 40 mL of DI water to TISSUES and DORM-4 REFERENCE MATERIAL ONLY**. DO NOT add water to the digestion blank, LFB, or PQL.

Note: If all of the tissue has not liquefied, return the sample to the water bath until the tissue is totally dissolved. Note the added digestion time on the preparation sheet. Then, allow the samples to cool to room temperature.

6.18 CAREFULLY add 10 mL of 6 % $KMnO_4$ solution to **all** tubes. If permanganate purple color does not persist for at least 15 minutes, add sufficient excess 6 % $KMnO_4$ to the tube in 1 mL increments until color persists for at least 15 minutes. RECORD HOW MUCH EXTRA $KMnO_4$ SOLUTION HAS BEEN ADDED in the $KMnO_4$ reagent column on the LIMS Prep Worksheet Manager worksheet.

6.19 Add 4 mL of 6 % $K_2S_2O_8$ to **all** sample tubes.

6.20 Tighten Nalgene caps and gently invert the digestion vessels several times to mix. Loosen all caps to relieve any pressure that may have built as a result of the mixing. Return all samples, including QC samples to the water bath and heat the samples at **90 °C for 90 minutes** to allow reaction with the potassium permanganate and the potassium persulfate to go to completion. Allow the samples to cool. The samples are now ready for analysis.

6.21 Complete the LIMS prep worksheet to change the status of the samples from W to V which signals the analyst the samples are ready for analysis.

7 SAMPLE ANALYSIS PROCEDURE

The digested samples are analyzed using cold vapor atomic absorption spectrometry. The analysis is described in DEP SOP HG-006.

8 QUALITY CONTROL

8.1 Digestion Blank - One per batch of 20 samples.

8.2 Sample Matrix Spikes - One set of duplicate spikes per batch; the sample that is chosen for the duplicate matrix spikes must also be analyzed unfortified.

8.3 **DORM-4** reference standard - One per batch.

8.4 PQL Check Standard - One per batch.

8.5 LFB - One per batch

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9. SAFETY/HAZARDOUS WASTE MANAGEMENT

9.1 Refer to DEP SOP HG-013 for correct waste disposal procedures.

9.2 Treat all samples as a possible hazard by wearing appropriate laboratory clothing; gloves, safety glasses, lab coat, etc.

9.3 The water bath should be placed in a well-ventilated area.

9.4 All samples should be vented in a hood.

9.5 Watch for possible violent reactions when adding reagents to the digestion vessels. The addition of reagents should be done in a well-ventilated area.

9.6 The organic spike solution (methyl mercuric chloride) is toxic and must be handled with care.

10. REFERENCES

10.1 DEP SOP HG-021, Procedure for High Level Mercury Glassware Cleaning

10.2 DEP SOP HG-006, Analysis of Total Mercury in Tissue by Cold Vapor Atomic Absorption (CVAA)

10.3 Chandrasekhar T. M., Design and Analysis of Experiments – Final Project, 'Robustness in Method Development: The determination of mercury in biological tissue'

10.4 EPA Method 245.6, Mercury in Tissues by Cold Vapor (CV/AAS)

10.5 DEP SOP HG-012, Mercury Group Standards Preparation Procedures

10.6 DEP SOP HG-013, Mercury Waste Disposal

10.7 DEP SOP MT-069, Metals Standard and Reagent Preparation Procedure

10.8 Health and Safety Plan, located on the Division of Environmental Assessment and Restoration intranet.

10.9 Laboratory Quality Manual, located on the Division of Environmental Assessment and Restoration intranet.

10.10 DEP-SOP-001/01 FS 1000 General Sampling Procedure (preservation).

11. APPENDIX A: **Pre-digestion of Hair and Feathers**.

11.1 Feathers and hair must be weighed on an analytical balance. Prior to weighing sample aliquots, consult with your supervisor to determine if the entire sample or an aliquot from the sample will be digested. Weigh the sample into a **tared** pre-digestion vessel, Nalgene straight-sided, wide-mouth, 125 mL polypropylene jar.

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Record the exact weight on the Excel pre-digestion worksheet.

11.2 For samples such as crayfish, cut the sample into little pieces using a razor blade and put the pieces into a **tared** 50 mL graduated centrifuge tube. Record the exact weight on the Excel pre-digestion worksheet.

11.3 **The following steps must be done under a fume hood.** Slowly add 7 mL of a 5:2 solution of H_2SO_4:HNO_3 to each sample using a calibrated Finnpipette or bottle-top dispenser. Also, prepare one blank to monitor the reagents used during pre-digestion. If a pipette is used, note that H_2SO_4 is very viscous and will cling to the sides of the pipette. After delivering each aliquot of acid, allow time for the remaining acid to reach the tip, and then empty it again. Samples will fume at this point, sometimes very heavily. Leave the vessel caps off for at least one hour, swirling the samples in the vessel at 15 minute intervals, to expose all surfaces to the acid. Add additional 7 mL aliquots of the 5:2 acid solution to the sample until the entire sample is in solution. Document the amount of acid added on the Excel pre-digestion worksheet. The samples must sit for at least twenty-four hours from the time acids are added before the next stage of the digestion may be commenced. Larger samples such as some feathers may take up to 4 days and additional acid for complete dissolution. Document the LIMS serial numbers of the H_2SO_4 and HNO_3 used for pre-digestion on the Excel pre-digestion sheet.

11.4 After the sample has been completely liquefied in the acids, gently swirl the liquid in the closed Nalgene jars, trying to wet the walls of the container with the contents. Do not shake the sample. Use the Finnpipette to dispense 7 mL of the pre-digestate into a 125 mL Nalgene wide mouth polyethylene bottle with screw cap and perform step 6 of this SOP, except that no more additional acid is added to the pre-digestates (omit 6.13 and 6.14). The quality control samples for the batch (digestion blank, LFB, DORM-4, and PQL) do get the acid added as per steps 6.13 and 6.14.

Appendix of Significant Changes

06/17/2008 A new section (Section 7) for the sample analysis procedure was added. Other minor revisions to the text were made for format and clarity.

06/15/2009 Minor edits to the format of the SOP.

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