ANALYSIS OF TOTAL MERCURY IN TISSUE BY COLD VAPOR ATOMIC ABSORPTION (CVAA) SPECTROSCOPY

1. SCOPE AND APPLICATION

1.1. This standard operating procedure (SOP) describes the analysis of tissue samples for total mercury content utilizing cold vapor-atomic absorption spectroscopic techniques. It is based on EPA Method 245.6 and is applicable to the determination of mercury in a wide variety of biological tissue including fish, liver, kidney, hair, bird feathers, and brain. Samples are assigned the test identifier (Test ID) T-HG-H.

1.2. Blood samples may also be analyzed using this method. The samples are usually preserved in heparin to prevent clotting, and frozen.

2. SUMMARY OF THE METHOD

Samples are digested using a combination of acids and strong oxidizers which converts all mercury present in the sample to Hg^{+2}. The samples are then treated with hydroxylamine hydrochloride solution to remove excess oxidizing reagents. Each sample digestate is introduced into a gas-liquid separator via an autosampler and peristaltic pump delivery system. The mercuric ions are reduced to atomic mercury vapor by stannous chloride in the gas-liquid separator and purged into the spectrometer's absorption cell by a stream of nitrogen gas or air. Mercury in tissue is measured and reported as mg/kg (wet weight).

3. APPARATUS AND EQUIPMENT

3.1. Nippon RA-3320 Reduced Vaporization Mercury Analyzer; Serial No. 06400552. Software; RA3Win, version 1.2.2 (2000).

3.1.1 Nippon SC-3 autosampler, serial no. 06410266 with a 10 standard sample and 50 test sample rack.

3.1.2 Nippon RD-3 reagent dispenser, serial no. 06420390.

3.1.3 Nippon sample test tubes.

3.2. Finpipette adjustable 2 – 10 mL.

3.3. Eppendorf pipettes, 100 µL fixed volume and 100 – 1000 µL adjustable volume.

3.4. Bottle-top dispensers

3.5. Class A 100 mL graduated cylinder.

3.6. Denver MXX-123, S/N 25955867, top loading balance, max capacity 120 g.

4. REAGENTS AND STANDARDS

Reagents used for analysis 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. The reagents used in analysis are documented on the 'MT-

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HG-RGNT' form located on the Laboratory's computer network, \\FLDEP1\DEAR\Labs\Lab5\Chemistry\InorganicPrep\Mercury\T-HG-H\. Update the form for each analysis and place a copy in the lab report.

The calibration standards used for the analysis must be logged into the LIMS standard prep tracker to generate a serial number and a label, which must be placed on the bottle of calibration standard in the laboratory. The calibration standards used for analysis are documented on the 'MT-HGCAL-STD' form located on the Laboratory's computer network, \\FLDEP1\DEAR\Labs\Lab5\Chemistry\InorganicPrep\Mercury\T-HG-H\. Update the form for each analysis and print a copy to place in the lab report.

Stock standards are documented on the 'MT-HGINT-STD' form, located on the Laboratory's computer network, \\FLDEP1\DEAR\Labs\Lab5\Chemistry\InorganicPrep\Mercury\T-HG-H\. The form should include the LIMS serial number for each standard and the reagent serial numbers used to prepare the standard. Print a copy to include in each lab report.

See DEP SOP MT-069 for reagent and standard documentation requirements and expiration dates.

4.1. Deionized (DI) water

4.2. Trace Metal Grade Nitric Acid (HNO_3) - The purchased acid is stored in the fume hood cabinet at room temperature and the expiration date is provided by the manufacturer.

4.3. Trace Metal Grade Hydrochloric Acid (HCl) - The purchased acid is stored in the fume hood cabinet at room temperature and the expiration date is provided by the manufacturer.

4.4. Trace Metal Grade Sulfuric Acid (H_2SO_4) - The purchased acid is stored in the fume hood cabinet at room temperature and the expiration date is provided by the manufacturer.

4.5. 12 % Hydroxylamine Hydrochloride Solution - Add 60 g of hydroxylamine hydrochloride to a 500 mL Teflon bottle. Dilute to volume with DI water and mix well. This solution expires one year after preparation.

4.6. 10 % Stannous Chloride Solution ($SnCl_2$) – Add 50 mL of DI water to a 100 mL Teflon bottle. Working under a fume hood, slowly add 5 mL of H_2SO_4 and mix gently. Weigh out 10 g of anhydrous stannous chloride crystals OR 12 g of di-hydrated stannous chloride ($SnCl_2 \bullet 2H_2O$) and add to the acidic solution. Dilute to volume and mix well. This solution expires two days after preparation.

4.7. 6 % Potassium Permanganate Solution ($KMnO_4$) - Add 30 g of potassium permanganate crystals to a 500 mL Teflon bottle containing 250 mL of DI water, dilute to volume with DI water, and mix well. The expiration date for this prepared reagent is one day after preparation.

4.8. 6 % Potassium Persulfate Solution ($K_2S_2O_8$) - Add 15 g of potassium persulfate crystals to 250 mL Teflon bottle containing 125 mL of DI water and dilute to volume with DI water. Mix well. The expiration date for this prepared reagent is one day after preparation.

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4.9. Potassium Bromate ($KBrO_3$, 0.05 M)/Potassium Bromide (KBr, 0.1 M) Solution – Add 8.4 g of potassium bromate ($KBrO_3$) and 11.9 g of potassium bromide (KBr) to an acid cleaned 1 L Teflon bottle. Dilute and dissolve both salts with DI water to 1 L. The solution is prepared as needed and is stored at room temperature. The expiration date is 1 year from preparation. The solution is purged with nitrogen for at least two hours before use to remove traces of mercury. Bromine monochloride is prepared in situ by adding HCl and the bromate/bromide solution to the sample.

4.10. Commercial Stock Standard 1000 ug/mL - Commercially available inorganic mercury stock from SPEX, Accustandard, Baker, Baxter, Fisher, VSR, etc. The expiration date is assigned by the manufacturer.

4.11. THG-1 - Instrument Calibration Stock Solution 100 mg/L. Pipette 10.0 mL of commercial 1000 mg/L stock standard into a 100 mL volumetric flask containing 50 mL of DI water. Add 5.0 mL of HNO_3, 3.0 mL of HCl and 3.0 mL of the bromide/bromate solution to it. Dilute to volume with DI water and mix well. The expiration date for this prepared standard is the same as the commercial stock standard from which it was made.

4.12. THG-2 - Instrument Calibration Intermediate Solution 500 ug/L. Pipette 2.5 mL of 100 mg/L stock standard THG-1 into a 500 mL volumetric flask containing 250 mL of DI water. Add 25 mL of HNO_3, 15.0 mL of HCl and 15.0 mL of the bromide/bromate solution to it. Dilute to volume with DI water and mix well. The expiration date for this prepared standard is the same as the commercial stock standard from which it was made.

4.13. CCB - Calibration Blank/Continuing Calibration Blank. The calibration blank and CCB is DI water. Prepare the CCB at the same time and with the same type of container as the calibration standards.

4.14. Calibration Standards: The calibration standards are prepared from intermediate standard, THG-2. The expiration date for prepared calibration standards is 7 days after preparation. See 6.2. for preparation procedure.

4.14.1 T-STD1 (0.50 µg/L) - This standard corresponds to the practical quantitation limit (PQL).

4.14.2 T-STD2 (2.50 µg/L)

4.14.3 T-STD3 (6.25 µg/L) - Also serves as the continuing calibration verification (CCV) standard for the analysis. This standard is analyzed after every 10 samples throughout analysis.

4.14.4 T-STD4 (12.50 µg/L) - Also serves as the initial calibration verification (ICV).

4.14.5 T-STD5 (25.00 µg/L)

5. SAMPLE COLLECTION, PRESERVATION AND HANDLING

Tissue samples (wet tissue) are collected and stored in vials or plastic bottles with air-tight closures. The samples are preserved by storing the samples in a freezer at -20 ^{o}C. Prior to digestion, the samples are removed from the freezer and allowed to thaw to room temperature. Samples consisting of hair or feathers can be stored at room temperature.

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6. SAMPLE AND CALIBRATION STANDARD PREPARATION PROCEDURE

6.1. Refer to DEP SOP HG-016 for sample preparation details.

6.2. Calibration standards are prepared according to the following procedure:

6.2.1 Prepare a set of calibration standards using clean, dry, and pre-labeled Teflon bottles. Add DI water with a calibrated bottle-top dispenser and use a calibrated pipette for addition of the THG-2 standard.

6.2.2 Using Table 1 as a guide, pipette the appropriate aliquots of THG-2 (500 ug/L) stock standard into each culture tube. The calibration levels are 0.50, 2.50, 6.25, 12.50 and 25.00 ug/L.

TABLE 1.

Standards preparation

Level	Concentration (μg/L)	Volume of THG-2 (μL)	Volume of DI water (mL)
T-STD1	0.50	120	120.0
T-STD2	2.50	600	119.5
T-STD3 (CCV)	6.25	1500	118.5
T-STD4 (ICV)	12.50	3000	117.0
T-STD5	25.00	6000	114.0

6.2.3 Add 15.0 mL of H_2SO_4 to the standard vessels. Add 6.0 mL HNO_3 to the standard vessels. Gently swirl vessels to mix. Solution may become hot. Allow the standard to cool if necessary.

6.2.4 Add 30.0 mL 6 % $KMnO_4$ to the standard vessels. Add 12.0 mL of 6 % $K_2S_2O_8$ to the standard vessels. Tighten cap and invert standard vessels to mix. Loosen caps and allow to cool.

6.2.5 Preparation of all calibration standards must be documented in the LIMS standard prep tracker module. A label should be printed from standard prep tracker that shows the identity of the standard, preparation date, expiration date, and serial number. Expiration date for the working calibration standards is 7 days after preparation.

7. SAMPLE ANALYSIS PROCEDURE

7.1. Prepare the digested samples for analysis by adding 4 mL of the 12 % hydroxylamine hydrochloride to each sample. This reagent reduces the excess permanganate oxidant in the digested sample. Tighten the bottle caps and gently swirl and invert the bottles to mix the reagents. Loosen the cap to release pressure. Make sure to mix all visible particulates into the liquid. The purple color of the digestates should disappear leaving behind a clear colorless to pale yellow liquid.

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7.2. If the purple color persists, add additional hydroxylamine to the sample in 1 mL increments, and mix until the solution turns colorless. Record the amount of additional hydroxylamine added to the sample in the comment section of the LIMS Prep Worksheet Manager. Notify the supervisor to edit the prep worksheet. This information will be used during calculations to compensate for changes in volume.

8. INSTRUMENT SETUP AND OPERATION

8.1. **Nippon RA-3320:** Start the instrument by turning on the switches at the rear of the RA-3 analyzer and at the front of the SC-3 autosampler. A standby screen will appear on the instrument display for 20 minutes while the system is warming up and stabilizing.

Note: The instrument can be left on overnight. However, if it will not be used on consecutive days it should be shut down following the procedure in section 8.1.12.

8.1.1 While the instrument is warming up, prepare a 50 % H_2SO_4 solution and a 10 % $SnCl_2$ solution. Reagent preparation instructions can be found in section 4.

8.1.2 Fill the bottles on the RD-3. The large bottle should be filled with 1 L DI water. The middle bottle should be filled with 50 mL of 50 % H_2SO_4 solution and the front bottle should be filled with 50 mL of 10 % $SnCl_2$ solution. Connect the reagent tube from each pump to its respective bottle.

8.1.3 At the end of the stabilization period the instrument display will show two options, SC/RD and Maintenance. Select the SC/RD by touching it on the screen. On the next screen select “Measurement Preparation” and hit “ENT”. Place a tube in the waste position of the autosampler and a rinse test tube in the cleaning holder. Push the start button. When reagent filling is complete press esc to return to the main screen.

8.1.4 Double click on the “RA3Win” icon on the desktop to launch the software.

8.1.5 The online profile window displays a peak profile of absorbance during measurement. At the same time, it also indicates an instantaneous absorbance value, a peak value, and an integrated value. The calibration window shows the created calibration curve.

8.1.6 In the Table window click on the “STD” tab. Type in the standard value, and check the “Mes” cell for each line that is being used in the table. The first two standards run should be DI water blanks. Press start.

8.1.7 After pressing start, the calibration sequence initiates. Five calibration standards of varying concentration are run plus a blank. The calibration curve is calculated by the instrument software using the equation $y = ax + 0$.

8.1.8 In the Table window click on the “SMP” tab. Type in the sample names. Check the “Mes” cell for each line that is being used in the table. Every 10 samples, a CCV and every 5 samples a CCB must be analyzed. In the "SMP" table window, the CCV and CCB can be typed in the sequence every 10 and 5 samples respectively.

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8.1.9 Additional samples can be added to the run while samples are running by typing the sample information into the "SMP" table and being sure to check the "Mes" cell.

8.1.10 Low room temperatures (< 20 °C or 68 °F) might adversely affect the recoveries of the DORM-4 SRM. This is because the DORM-4 tissue standard is freeze dried and has a higher fat content than normal "wet-tissue" samples. In this case briefly warm the sample by immersing the tube in a 90°C water bath for ~5 minutes.

8.1.11 Measurement conditions can be found by selecting the Measurement Condition option in the Run Menu. Table 3 contains these parameters.

TABLE 3.

Instrument Parameters for Nippon RA-3320

Parameter	Value
Common Tab	
Measurement Time (seconds)	120
Time axis (seconds)	180
Analog out range	0.05
Upper limit (Absorbance)	1.50000
Purge time (seconds)	180
Measurement	Continue
Control Tab	
Std (ug/L)	5
Max Dev (%)	10
Abnormal Conditions	Stop
Table Conditions	
Calibration curve	y = ax + 0
Peak/Integ	Integ
Unit	ug/L
Decimal places	3
Drift correction	no
Description of Minus	Minus as it is

8.1.12 When the run is complete select the "SC/RD" screen on the instrument display. Select the "Measurement Complete" option and follow the onscreen directions for rinsing the reagent tubes. When the "SC/RD" screen returns, the instrument can be shut down. Flip the switch to off on the back, right side of the instrument and also flip the switch to off on the back, left side of the autosampler.

8.1.13 Nippon test tubes must be cleaned after each run. This is accomplished by rinsing the tubes with water followed by soaking them for a minimum of two hours in 5 % aqua regia. If stannous chloride residue is observed in the test tubes after the acid rinse, prepare a base bath using NaOH. Dissolve 50 g solid NaOH in a bucket with 1 L of DI water and soak the test tubes for at least 2 hours.

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9. DATA ARCHIVAL

The Nippon instrument software does not automatically save the results of an analytical run. After measurements are complete, click File>Save and save the instrument software file (RA3 Data File) on the hard drive of the instrument computer with the format N2016aa0216t1. Then, the worksheet needs to be saved as a comma delimited "CSV" text file. This is done by selecting "save as text file" in the edit menu. The text file is saved to \\FLDEP1\DEAR\Labs\Lab3\Chem_Data\Inorganic\Mercury\Nippon_AA\. A file with the name N2016aa0216t1 would be the first tissue run on February 16, 2016 using the Nippon RA-3320.

10. QUALITY CONTROL

Table 4 shown below lists the quality control parameters and their acceptable limits for any tissue analysis using this method.

TABLE 4.

QC for Tissue AND Biofluid

CALIBRATION	**R** ≥ 0.995		
MDL	0.125 ug/L		
CCBs	-MDL < CONCENTRATION < MDL		
	LEVEL	**RECOVERY**	**RPD**
PQL	0.50 ug/L	70 % - 130 %	
DORM-4	0.410 mg/kg	85 % - 115 %	
DUP. SPIKE	9.375 ug/L	80 % - 120 %	≤ 20 %
DUP. SPIKE	9.375 ug/L	80 % - 120 %	≤ 20 %
AT SPIKE (low)	25.0 ug/L	80 % - 120 %	
AT SPIKE (high)	100.0 ug/L	80 % - 120 %	
	LEVEL	**RECOVERY**	**RPD**
ICV	12.5 ug/L	85 % - 115 %	
CCV	6.25 ug/L	85 % - 115 %	

For calculation equations see section 4 of DEP SOP MT-058.

11. INTERFERENCES

11.1. Volatile compounds that absorb at the same wavelength as mercury (253.7 nm) constitute positive interferents for this method. Removal of interfering volatile materials may be accomplished by thoroughly sonicating the sample before transferring it to the autosampler rack.

11.2. Positive interference has been also observed in samples containing high levels of sulfides, copper and chlorides, probably due to the formation of free chlorine.

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11.3. Addition of sulfuric acid may induce the formation of a heavy precipitate. If this is present, the affected sample should not be analyzed by this method or results must be qualified.

12. CORRECTIVE ACTIONS FOR COMMON PROBLEMS

Corrective actions for common QC failures are listed in Table 5.

TABLE 5.

Corrective Actions

Problem	Corrective Action
Calibration curve correlation coefficient out of range.	Remake the standards and recalibrate the instrument.
Failure of CCB.	Change the filter on the autosampler bubble. Change the pump tubing. Check the volume of waste disposal bottle.
Failure of CCV.	Check the detector for signal drift. Recalibrate if necessary.
Failure of external SRM (DOLT 4), PQL, LFB, matrix spikes, or replicate RPD.	Redigest the sample and repeat the analysis.

13. MAINTENANCE

13.1. Pump Tubing should be replaced once per year.

13.2. Filter on the autosampler bubbler should be changed every 3 months.

13.3. Light source lamp needs to be replaced if instrument display shows an alarm message of “Lamp Life Exceeded” or “Lamp Broken”. The lamp life error message will appear when the total operating time exceeds 20,000 hours.

13.4. The warning message “Carbon Filter Full” appears when the activated carbon filter in the RA-3 unit has absorbed more than 1.5 g of mercury. Section 4.1.3 in the RA-3000 Maintenance Manual explains how to replace this filter.

13.5. If contamination or reduced sensitivity become a problem or the error message “Cell Dark” appears the I joint, L joint, Teflon tubes, dehumidifier tube and joints, cell body, and quartz lenses need to be cleaned. Specific instructions are giving in section 5 of the RA-3000 Maintenance Manual.

14. SAFETY/HAZARDOUS WASTE MANAGEMENT

14.1. See DEP SOP HG-013 for correct waste disposal procedures.

14.2. Always wear a lab coat and safety glasses or protective eyewear while working in the lab. Powder free latex gloves and a lab coat should be worn while handling chemicals. When disposing the digested blank and PQL samples, carefully collect the glass beads for proper disposal. Concentrated acids should be handled in a well-ventilated hood.

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Samples should be sonicated in a hood, since the sonication process may result in the sample/s releasing acidic vapors.

15. REFERENCES

15.1. EPA Method 245.6, "Methods for the Determination of Metals in Environmental Samples", U.S. EPA/600/4-91/010

15.2. Nippon Mercury/RA-3000 system measurement manual.

15.3. Nippon Mercury/RA-3 Data Analysis software manual.

15.4. Nippon Mercury/RA-3000 system maintenance manual

15.5. DEP SOP HG-013, Mercury Waste Disposal

15.6. DEP SOP HG-016, Digestion of Tissue Samples for Total Mercury Analysis

15.7. DEP SOP LB-027, Standard Operating Procedure for Reporting Qualified Data and Correcting Quality Control Problems

15.8. DEP SOP MT-058, Procedure for Metals Data Review and Authorization DEP SOP

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

15.10. DEP Laboratory Health and Safety Manual located on the Laboratory's intranet site.

15.11. Quality Manual, DEP Laboratory

Appendix of Significant Changes

07/07/2008 A couple of older sections were consolidated into Section 6. Other minor revisions to the text were made for format and clarity.

02/23/2009 The Cetac instrument was surplused. The Nippon RA-3320 sample analysis procedure was added (Section 8.2).

12/22/2015 The Varian SpectraAA-220 was surplused. Equipment and procedure related to the Varian AA instrument was removed from the SOP.

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