METHOD 4500

MERCURY IN SOIL BY IMMUNOASSAY

1.0 SCOPE AND APPLICATION

1.1 This method provides a screening procedure for the determination of mercury in soils at concentrations as low as 0.5 mg/Kg.

1.2 This procedure describes the analysis of soil samples for the detection of mercury by an Enzyme-Linked Immunosorbent Assay (ELISA). This method is conducted as a screening technique, the sample's concentration is estimated through comparison of the sample to a standard. Other solid matrices (see Table 5) may be analyzed by this technique as long as the QC parameters detailed in this method are achievable.

1.3 In conjunction with this technique, Method 7471 should be used to determine the exact concentration of mercury when required or for confirmatory purposes. This is especially true near regulatory or action levels. A minimum of 10% confirmatory analyses is suggested.

1.4 This method is restricted to use by or under the supervision of trained analysts. Each analyst must demonstrate the ability to generate acceptable results with this method.

1.5 Tubes or reagents from different kits or separate batches are NOT interchangeable.

2.0 SUMMARY OF THE METHOD

2.1 Test kits are commercially available for this method. The manufacturer's directions should be followed.

2.2 In general, the method is performed using an extract of a solid sample. Solid samples are prepared by extraction with a mixture of hydrochloric and nitric acids for ten minutes and then buffered prior to analysis. The sample is added to a tube (treated with BSA-glutathione) and incubated at ambient temperatures for five minutes. The mercuric ions bound to the sulflhydryl groups of the BSA-glutathione are now reacted with a reconstituted antibody specific for mercury and incubated for five more minutes. A peroxidase conjugate is added to the sample, reacting with any mercury specific antibody. The substrate is then added forming a color that is in proportion to the amount of mercury originally present in the sample. The color produced is then spectrophotometrically compared with the control standards.

3.0 DEFINITIONS

3.1 Antibody - A binding protein which is produced in response to an antigen, and which has the ability to bond with the antigen that stimulated its production.

3.2 Cross-Reactivity - The relative concentration of an untargeted substance that would produce a response equivalent to a specified concentration of the targeted compound. In a semi-quantitative immunoassay, it provides an indication of the concentration of cross-reactant that would produce a positive response. Cross-reactivity for individual compounds is often calculated as the ratio of target substance concentration to the cross-reacting substance concentration at 50% inhibition of the immunoassay's maximum signal x 100%.

3.3 Dose-Response Curve - Representation of the signal generated by an immunoassay (y axis) plotted against the concentration of the target compound (x axis) in a series of standards of known concentration. When plotting a competitive immunoassay in a rectilinear format, the dose-response will have a hyperbolic character. When the $\log_{10}$ of concentration is used, the plot assumes a sigmoidal shape, when the log of signal is plotted against the logit transformation (linear representation of calibration data) of concentration, a straight line is produced.

3.4 ELISA - *Enzyme Linked Immunosorbent Assay* is an enzyme immunoassay method that uses an immobilized reagent (e.g., antibody adsorbed to a plastic tube) to facilitate the separation of targeted analytes (antibody-bound components) from non-targeted substances (free reaction components), using a washing step and an enzyme conjugate to generate the signal used for the interpretation of results.

3.5 Enzyme Conjugate - A molecule produced by the coupling of an enzyme molecule to an immunoassay component that is responsible for acting upon a substrate to produce a detectable signal.

3.6 Enzyme Immunoassay - An immunoassay method that uses an enzyme conjugate reagent to generate the signal used for interpretation of results. The enzyme mediated response may take the form of a chromogenic, fluorogenic, chemiluminescent or potentiometric reaction. (see Immunoassay and ELISA)

3.7 False Negatives - A negative interpretation of the sample containing the target analytes at or above the action level. Ideally, an immunoassay test product should produce no false negatives. The maximum permissible false negative rate is 5%, as measured by analyzing split samples using both the test product and a reference method.

3.8 False Positives - A positive interpretation for a sample is defined as a positive response for a sample that contains analytes below the action level.

3.9 Immunoassay - An analytical technique that uses an antibody molecule as a binding agent in the detection and quantitation of substances in a sample. (see Enzyme Immunoassay and ELISA)

3.10 Immunogen - A substance having a minimum size and complexity, and that is sufficiently foreign to a genetically competent host to stimulate an immune response.

4.0 INTERFERENCES

4.1 Refer to Table 3 for a comparison of the effects of other metals on the procedure.

4.2 Consult the information provided by the manufacturer of the kit used for additional information regarding cross reactivity with other compounds.

4.3 Temperature range in which test can be reliably conducted (refer to test-kit instructions).

5.0 SAFETY

Refer to Chapter Three for guidance.

6.0 EQUIPMENT AND SUPPLIES

6.1 BiMelyze® Soil Extraction Kit and BiMelyze® Mercury Assay Tube Kit for Solid Matrices (BioNebraska, Inc. 3820 NW 46th St., Lincoln, NE 68524) or equivalent. Each commercially available test kit will supply or specify the apparatus and materials necessary for successful completion of the test.

6.2 Analytical balance - capable of measuring 5 g ± 0.1 g.

6.3 Differential photometer or equivalent - capable of reading the absorbance at 405 nm.

6.4 Timer.

6.5 Permanent marking pen.

6.6 Cleaning and waste supplies - lab tissues, disposable gloves, waste container.

6.7 Micropipets - capable of accurate delivery volumes at 105 and 500 µL.

6.8 Squirt bottle - 500 mL or equivalent.

6.9 Graduated cylinder - 500 mL or equivalent.

7.0 REAGENTS AND STANDARDS

7.1 Each commercially available test kit will supply or specify the reagents necessary for successful completion of the test.

7.2 Nitric acid, concentrated - reagent grade or equivalent.

7.3 Hydrochloric acid, concentrated - reagent grade or equivalent.

7.4 Acid mixture - Add 36 mL HCl to 18 mL of reagent water and then add 18 mL of HNO_3 to the HCl/reagent water solution.

7.5 Reagent water - All references to water in this method refer to reagent water unless otherwise specified. Reagent grade water is defined in Chapter One.

8.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 Environmental samples may be contaminated, and should therefore be considered hazardous and handled accordingly.

8.2 All test kits must be stored under the conditions described by the manufacturer.

8.3 Sample Collection - Sufficient sample should be collected to ensure a representative sample. Samples should be collected in pre-cleaned glass or plastic containers.

8.4 All samples that are not immediately analyzed must be stored under the conditions described in Chapter Three.

9.0 QUALITY CONTROL

9.1 Follow the manufacturer's instructions for the test kit being used for quality control procedures specific to the test kit used. Additionally, guidance provided in Method 4000 and Chapter One should be followed.

9.2 Use of replicate analyses, particularly when results indicate concentrations near the action level, is recommended to refine information gathered with the tube kit.

9.3 Do not use test kits past their expiration date.

9.4 Do not use tubes or reagents designated for use with other manufacturer's test kits and **do not use tubes or reagents from separate batches of test kits.**

9.5 Use the test kits within their specified storage temperature and operating temperature limits.

9.6 Although Method 4500 is intended as a field screening method, the appropriate level of quality assurance should accompany the application of this method to document data quality. These include but are not limited to positive and negative controls.

10.0 CALIBRATION AND STANDARDIZATION

10.1 Follow the instrument manufacturer's instruction when conducting the calibration.

10.2 All analyses must be accompanied by at least a reference standard (NIST 2709, NIST 2704, or equivalent).

10.3 All analyses must be accompanied by at least one control. Additional controls may be used to refine the data.

11.0 PROCEDURE

11.1 Follow all of the manufacturer's specific instruction when conducting analyses by the immunoassay technique. A general overview of the technique follows.

11.2 Prepare all assay solutions, standards, and controls prior to beginning the analysis. Appropriately label all vials.

11.3 Soil Extraction

11.3.1 Weigh out 5 ± 0.1 g of soil sample and place into the extraction vessel.

11.3.2 Add a 4 mL volume of the acid mixture to the extraction vessel for all samples, standards, and controls.

11.3.3 Cap the vessels and swirl the samples for 15 seconds of each minute during the 10-minute extraction period and then add 7 mL of the buffer included with the test-kit.

11.3.4 Place bottle filter tops firmly onto extraction bottles. Squeeze bottle and discard the first few drops. Add three drops (105 µL) into the corresponding dilution bottles.

11.4 Sample Analysis

11.4.1 Add diluted samples to the black line on each mercury assay tube (500 µL) Incubate tubes for five minutes at ambient temperature. Occasionally swirl and after five minutes empty and then rinse tubes three times with approximately 2 mL of reagent water.

11.4.2 Add antibody to the black line on each mercury assay tube (500 µL), incubate for 5 minutes at ambient temperature, wash and rinse tubes with buffer three times.

11.4.3 Add conjugate to the black line on each mercury tube (500 µL), incubate for 5 minutes at ambient temperature, wash and rinse tubes with buffer three times.

11.4.4 Add substrate to the black line on each mercury tube (500 µL), and incubate for 5 minutes at ambient temperature. Add three drops (105 µL) of stop solution to each tube in the same order as the substrate was added.

11.4.5 Read absorbance of each sample and standard at 405 nm.

11.4.5.1 Samples with absorbances less than that of a control should be reported as "<[control concentration]." For example, if the control is 4 ppm and the sample's absorbance is less than that of the 4 ppm control then the result should be reported as "<4 ppm."

11.4.5.2 Samples with absorbances greater than that of a control should be reported as ">[control concentration]." For example, if the control is 4 ppm and the sample's absorbance is greater than that of the 4 ppm control then the result should be reported as ">4 ppm."

11.4.5.3 Results may be bracketed between two controls to further refine the data. For example, if a 4 ppm control and a 20 ppm control are used, potentially three results are possible: <4 ppm, 4-20 ppm, and >20 ppm.

12.0 DATA ANALYSIS AND CALCULATIONS

Ensure that all sample containers, dilution bottles, extraction vials, and tubes have been labeled properly prior to analysis. Proper laboratory protocols, including documentation and notetaking, should be conducted according to good laboratory practices.

13.0 METHOD PERFORMANCE

13.1 Table 1 displays data obtained from analyzing samples using both the tube kit and CVAA at a Superfund Site. Sixty-nine samples were analyzed, nine samples analyzed by the tube kit had results greater than that of the CVAA analyses. This gives a false positive rate of 13%. Two of the samples analyzed by the tube kit gave results lower than the CVAA analyses. This gives a false negative result of 2.8%.

13.2 Table 2 displays data concerning false positives and false negatives at the detection limit of 0.5 ppm. Twenty samples were prepared using NIST Standard Reference Material 8407 (soil standard) diluted gravimetrically with mercury free soils to levels of 1.0, 0.50, 0.25, and 0.00 ppm. Four sets of the soil samples were extracted in five separate experiments. Within each of the five experiments, a 0.5 ppm sample that gave an absorbance greater than that of the 0.5 ppm

standard would be considered a false positive. An absorbance greater than that of a 0.5 ppm standard would be determined to be a false negative. None of the experiments yielded false positives or false negative results.

13.3 Table 3 displays the effects of other metals on the quantitation of mercury by ELISA and the concentration at which cross-reactivity may be observed. Elements with values designated "as greater than" displayed no interference at the levels shown in the table.

13.4 Table 4 provides comparison of the tube kit with eight different certified reference materials.

13.5 Table 5 provides data for a set of analyses conducted at an abandoned battery reclamation site.

13.6 Figure 1 displays the tube-kit's 10 month stability claims at 4°C with a control (fresh kit every analysis period), a kit stored at room temperature (22-25°C), and a kit stored at 4°C.

13.7 The MDL at the 99% confidence level was derived from the data in Table 2. The standard deviation of the lowest standard analyzed (0.067 = SD for the 0.25 ppm standard) was multiplied by the t-statistic for 20 samples (2.54). The calculated MDL is 0.17 ppm. For the purposes of this methodology the detection limit will be listed as 0.5 ppm.

13.8 The following documents may provide additional guidance and insight on this method and technique:

13.8.1 Wylie, D.E., Lu, D., Carlson, L.D., Carlson, R., Babacan, K.F., Schuster, S.M., and Wagner, F., "Monoclonal Antibodies Specific for Mercuric Ions", *Proc. Natl. Acad. Sci.*, Vol. 89, pp 4104-4108, May 1992.

13.8.2 Wylie, D.E., Lu, D., Carlson, L.D., Carlson, Schuster, S.M., and Wagner, F., "Detection of Mercuric Ions in Water by ELISA with a Mercury-Specific Antibody", Analytical Biochemistry 194, 381-387 (1991).

14.0 POLLUTION PREVENTION

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity and/or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions consult *Less is Better: Laboratory Chemical Management for Waste Reduction* available from the American Chemical Society as listed in.

15.0 WASTE MANAGEMENT

The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories

to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* available from the American Chemical Society, Department of Government Relations and Science Policy, 1155 16th Street, NW, Washington, D.C., 20036, (202) 872-4477.

16.0 REFERENCES

1. BiMelyze® Mercury Assay Kit and BiMelyze® Mercury Assay Soil Extraction Kit, BioNebraska, Inc.

2. Schweitzer, Craig, et. al.; "Enzyme-Linked Immunoassay (ELISA) for the Detection of Mercury in Environmental Matrices."

3. Letter to Frank Calovini, SAIC; data submission from Craig Schweitzer, BioNebraska, Inc.; August 16, 1995.

4. Letter and data submission attachments, O.M. Fordham, USEPA; from Craig Schweitzer, BioNebraska, Inc.; March 20, 1995.

5. California Environmental Protection Agency, Department of Toxic Substances Control, Environmental Technology Certification Program, "BiMelyze® Field Screening Assay for Mercury ("Tube Assay") and Soil Extraction Kit, with a Partial Evaluation of BiMelyze® Laboratory Screening Test for Mercury ("Plate Assay")", BioNebraska, Inc. Lincoln, NB; Contract No. 93-T0470, June 1995.

17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

The pages to follow contain Tables 1 through 5, Figure 1, and a flow diagram for this method's procedure.

TABLE 1

CORRELATION OF IMMUNOASSAY AND CVAA ANALYSES
FOR MERCURY IN SOIL AT A SUPERFUND SITE

Hg conc. by CVAA (ppm)	Hg conc. by Immunoassay (ppm)	Agreement [a]
1.0	<5	Y
0.2	<5	Y
0.02	<5	Y
0.03	<5	Y
<0.02	<5	Y
<0.02	<5	Y
0.02	<5	Y
36.2	>15	Y
7.4	5-15	Y
0.03	<5	Y
0.3	<5	Y
0.03	<5	Y
0.03	<5	Y
0.1	<5	Y
<0.03	<5	Y
0.9	<5	Y
0.03	<5	Y
0.04	<5	Y
<0.02	<5	Y
39.4	>15	Y
46.5	>15	Y
18.2	>15	Y
139	>15	Y
106	>15	Y
4.7	>15	N - FP
0.4	<5	Y

(Continued)

TABLE 1 (Continued)

Hg conc. by CVAA (ppm)	Hg conc. by Immunoassay (ppm)	Agreement[a]
1.0	≤5	Y
0.2	<5	Y
0.3	<5	Y
4.1	5-15	N - FP
<0.02	<5	Y
0.05	<5	Y
56.6	>15	Y
0.5	<5	Y
0.2	<5	Y
0.1	<5	Y
0.3	<5	Y
0.02	<5	Y
0.04	<5	Y
0.08	<5	Y
0.03	<5	Y
0.02	<5	Y
<0.01	<5	Y
0.2	<5	Y
0.06	<5	Y
<0.01	<5	Y
<0.01	<5	Y
28.1	5-15	N - FN
51.8	>15	Y
21.8	5-15	N - FN
7.7	5-15	Y
0.4	<5	Y
18.0	>15	Y

(Continued)

TABLE 1 (Continued)

Hg conc. by CVAA (ppm)	Hg conc. by Immunoassay (ppm)	Agreement [a]
0.8	<5	Y
2.2	<5	Y
4.4	5-15	N - FP
1.1	5-15	N - FP
0.05	<5	Y
1.3	5-15	N - FP
0.06	<5	Y
<0.02	<5	Y
<0.01	<5	Y
3.1	5-15	N - FP
3.4	5-15	N - FP
0.3	<5	Y
3.4	5-15	N - FP
2.0	5-15	N - FP
0.13	<5	Y
0.06	<5	Y

[a] Y = Yes, N = No, FN = False Negative, FP = False Positive

Source: Reference 4

TABLE 2

MERCURY ANALYSIS OF SOIL NEAR THE IMMUNOASSAY DETECTION LIMIT OF 0.50 PPM

		ABSORBANCE OBTAINED BY IMMUNOASSAY				CONCENTRATION BY CVAA			
		SAMPLE CONC. (ppm)				SAMPLE CONC. (ppm)			
		1.0	0.50	0.25	0.00	1.0	0.50	0.25	0.00
#1)	A)	0.97	0.75	0.53	0.08	0.99	0.48	0.24	0.00
	B)	0.93	0.74	0.50	0.06	1.00	0.50	0.25	0.00
	C)	0.84	0.73	0.51	0.08	0.98	0.53	0.25	0.00
	D)	0.88	0.70	0.51	0.08	0.98	0.49	0.24	0.00
#2)	A)	1.05	0.81	0.58	0.10	1.02	0.52	0.24	0.00
	B)	0.99	0.83	0.43	0.10	0.90	0.50	0.24	0.00
	C)	1.00	0.65	0.57	0.10	0.97	0.49	0.25	0.00
	D)	1.08	0.88	0.48	0.12	0.94	0.53	0.25	0.00
#3)	A)	1.17	0.80	0.58	0.08	1.06	0.55	0.26	0.00
	B)	1.11	0.82	0.52	0.11	1.03	0.54	0.28	0.00
	C)	0.95	0.62	0.43	0.10	1.00	0.52	0.28	0.00
	D)	0.99	0.80	0.51	0.11	1.03	0.54	0.27	0.00
#4)	A)	0.91	0.76	0.36	0.09	1.07	0.57	0.28	0.00
	B)	0.87	0.66	0.49	0.07	1.15	0.58	0.31	0.00
	C)	0.78	0.67	0.42	0.06	1.26	0.57	0.30	0.00
	D)	0.90	0.69	0.39	0.06	1.16	0.57	0.28	0.00
#5)	A)	1.15	0.61	0.46	0.07	0.88	0.47	0.24	0.00
	B)	1.11	0.67	0.35	0.07	0.94	0.48	0.23	0.00
	C)	1.07	0.66	0.48	0.07	0.90	0.47	0.26	0.00
	D)	1.09	0.54	0.50	0.09	0.88	0.47	0.24	0.00

Source: Reference 5

TABLE 3

CROSS-REACTIVITY OF ELEMENTS WITH THE MERCURY IMMUNOASSAY

ELEMENT	SOIL EQUIVALENT CONCENTRATION REQUIRED TO YIELD A POSITIVE RESULT (ppm)
Mercury	0.36
Arsenic	>55,000
Barium	>100,000
Cadmium	>82,000
Chromium	38,000
Copper	47,000
Gold	144,000
Iron	>41,000
Lead	>150,000
Nickel	>43,000
Silver	79,000
Sodium	>17,000
Strontium	>64,000
Thallium	>150,000
Zinc	>48,000

Source: Reference 3

TABLE 4

ANALYSIS OF CERTIFIED REFERENCE SOILS USING IMMUNOASSAY

REFERENCE SAMPLE	[Hg] (ppm)	ABSORBANCE @ 405-nm exp.1	exp.2	exp.3	INTERPRETATION
ERA Inorganic Blank Soil	<0.10	0.12	0.05	0.08	----[3]
NIST 2709	1.40	1.01	0.64	0.47	<4
NIST 2704	1.47	0.78	0.41	0.47	<4
ERA CLP Lot #216	2.36	1.54	0.84	0.93[2]	<4
ERA Custom Mercury Std.[1]					
dil. 1	4	1.76	1.01	0.83	----[3]
dil. 2	15	1.99	1.45	1.59	----[3]
dil. 3	50	2.04	1.73	2.02	>15
NIST 8408	122	2.55	2.55	2.55	>15

Source: Reference 2

[1] dilutions from 107 ppm.
[2] only value that gives incorrect conclusion.
[3] standard reference point, no interpretation.

TABLE 5

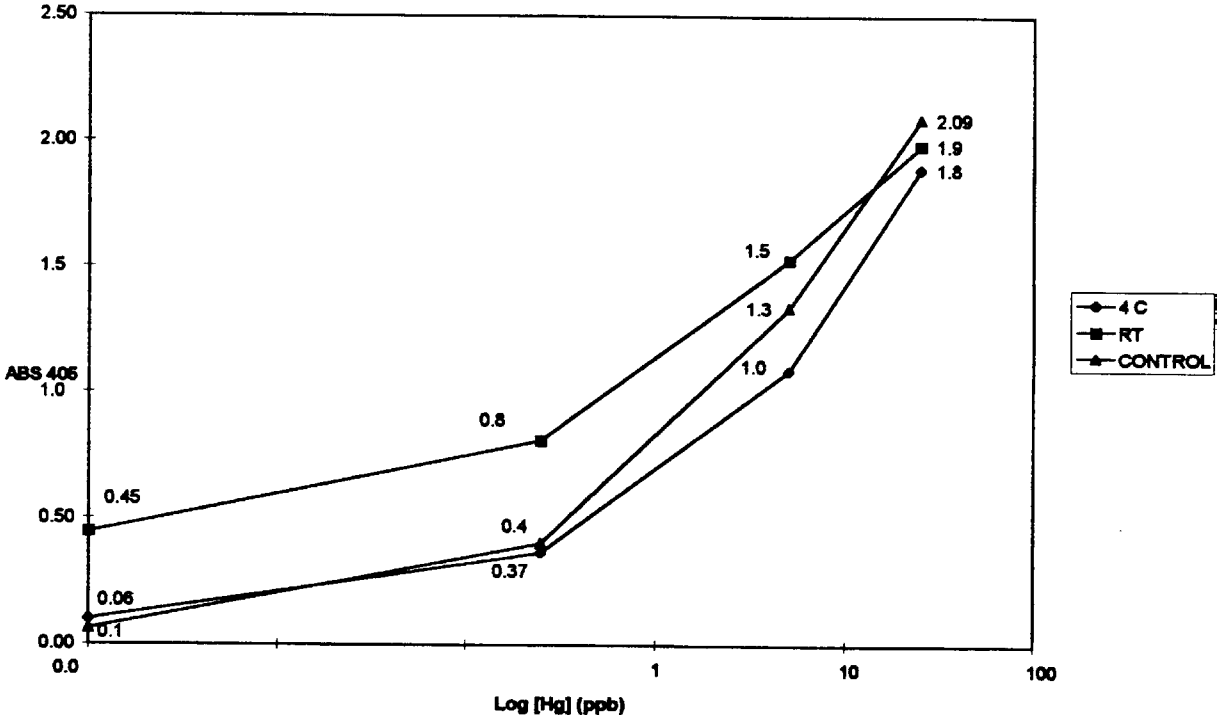
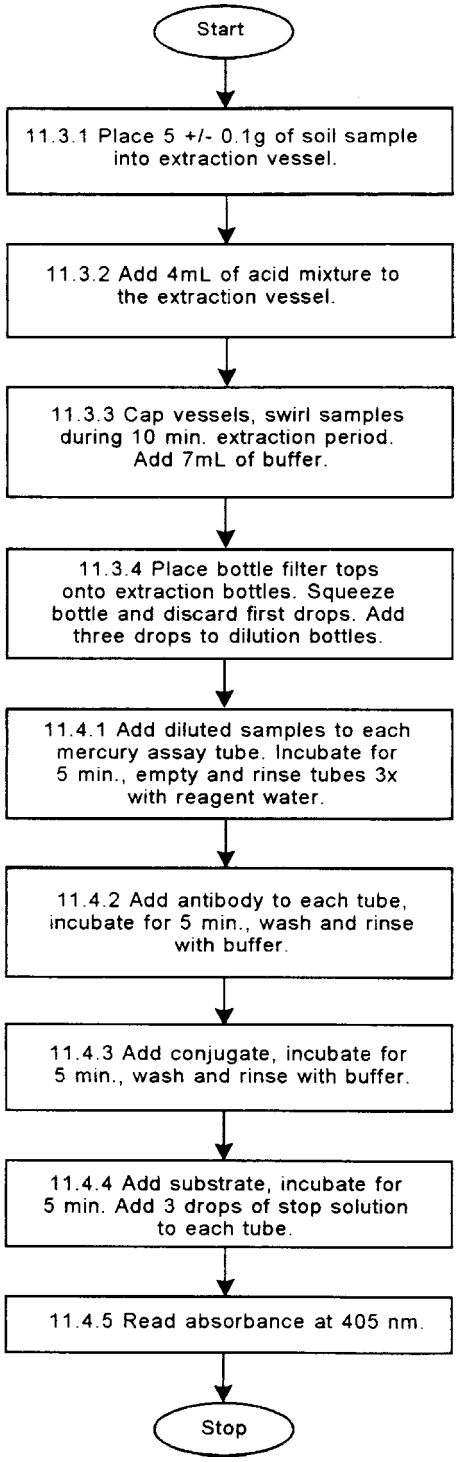
ANALYSIS OF MULTIPLE MATRICES AT AN ABANDONED BATTERY RECLAMATION SITE USING IMMUNOASSAY

SAMPLE DESCRIPTION	IMMMUNOASSAY RESULTS	CVAA RESULTS
Process Room	< 5 ppm	0.83 ppm
Dust from process room	< 5 ppm	> 4.5 ppm
Groundwater - unfiltered	< 0.5 ppb	< 0.4 ppb
Soil, alkaline	< 5 ppm	0.93 ppm
Sludge from tank	> 15 ppm	4,400 ppm
Sump sludge	5 > 15 ppm	14 ppm
Cinder block	< 5 ppm	3 ppm
Cinderblock duplicate	< 5 ppm	
Soil	5 > 15 ppm	14 ppm
Paint	> 15 ppm	34 ppm
Background cinderblock	< 5 ppm	1.4 ppm
Background paint	> 15 ppm	14 ppm
Debris from CO_2 blast	> 15 ppm	19 ppm

Source: Reference 2

FIGURE 1

TEN-MONTH IMMUNOASSAY KIT STABILITY



MERCURY IN SOIL BY IMMUNOASSAY