

**APPENDIX A**

**GENERALIZED STANDARD OPERATING PROCEDURE FOR  
METAL SPECIATION BY ELECTRON MICROPROBE ANALYSIS**



**STANDARD OPERATING PROCEDURE**  
**Metal Speciation**

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Date: October 11, 1999 (Rev. #1)

Title: METAL SPECIATION

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**SYNOPSIS:** A standardized method for speciating metals in solid samples using an electron microprobe is described. Equipment operating conditions, sample preparation and handling, and statistical equations for data analysis are included.

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# STANDARD OPERATING PROCEDURE

## Metal Speciation

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### 1.0 OBJECTIVES

The objectives of this Standard Operating Procedure (SOP) are to specify the proper methodologies and protocols to be used during metal speciation of various solid samples, including; soil, house dust, wipes, sediments, tailings, slags, dross, bag house dusts, and paint samples, using an electron microprobe. The metal speciation data generated from this SOP may be used to assess the solid samples, and the provenance associated with the various metal phases. It should be noted that this analysis will establish the mineral forms of a metal or metals in a sample, but will not detect or characterize low concentrations of surface adsorbed metals. Parameters to be characterized during the speciation analyses include particle size, associations, stoichiometry, frequency of occurrence of metal-bearing forms and relative mass of metal-bearing forms. The sample preparation methods and instrument operation parameters to be used during implementation of this SOP are discussed in the following sections.

### 2.0 BACKGROUND

To date, numerous metal-bearing forms have been identified from various environments within western mining districts (Emmons et al., 1927; Drexler, 1991, per. comm.; Drexler, 1992; Davis et al., 1993; Ruby et al., 1992; CDM, 1994; WESTON, 1995), and industrial or agricultural (Drexler, 1999, per. comm.) settings, Table 2-1. This listing does not preclude the identification of other metal-bearing forms, but only serves as an initial point of reference. Many of these forms are minerals with varying metal concentrations (e.g., lead phosphate, iron-lead oxide, and slag). Since limited thermodynamic information is available for many of these phases and equilibrium conditions are rarely found in soil environments, the identity of the mineral class (e.g., lead phosphate) will generally be sufficient for determination of provenance, and exact stoichiometry is not necessary.

It may be important to know the particle-size distribution of metal-bearing forms to assess potential bioavailability. It is believed that particles less than 250 microns ( $\mu\text{m}$ ) are most likely to be ingested by humans (Bornschein, et al., 1987). For this SOP, the largest dimension of any one metal-bearing form is measured and the frequency of occurrence weighted by that dimension. Although not routinely performed, particle area can be determined; it has been shown (CDM, 1994) that data collected on particle area produces similar results. These measurements add a considerable amount of time to the procedure, introduce new sources of potential error and limit the total number of particles or samples that can be evaluated in a study.

Mineral association may affect the solubility of a metal from a particular sample. For example, if a lead-bearing form in one sample is predominantly found within quartz grains, while in another sample it is free in the sample matrix, the two samples are likely to have different lead solubility and different degrees of bioavailability. Therefore, mineral associations are evaluated, and include the following:

- 1) free or liberated
- 2) inclusions within a second phase
- 3) cementing
- 4) alteration rims.

### 3.0 SAMPLE SELECTION

Samples should be selected and handled according to the procedure described in the Project Plan.

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### 4.0 SCHEDULE

A schedule for completion of projects performed under this Metals Speciation SOP will be provided in writing or verbally.

### 5.0 INSTRUMENTATION

Speciation analyses may be conducted at the Laboratory for Environmental and Geological Studies (LEGS) at the University of Colorado, Boulder, or other comparable facilities. Primary equipment used for this work will include:

Electron Microprobe (JEOL 8600) equipped with four wavelength spectrometers, energy dispersive spectrometer (EDS), BEI detector and Geller Microanalytical data processing system. An LEDC spectrometer crystal for carbon and LDE-1 crystal for oxygen analyses are essential.

### 6.0 PRECISION AND ACCURACY

The precision of the EMP speciation and polarized light microscopy (PLM) will be evaluated based on sample duplicates analyzed at a frequency of 10%. The precision of the data generated by the manual PLM particle count and by the "EMP point count" will be evaluated by preparing a graph that compares the original result with the duplicate result. The accuracy of the analyses will be estimated based on a number of methods, depending on the source of the data. Data generated by the "EMP point count" will be evaluated statistically based on the methods of Mosimann (1965) at the 95% confidence level on the frequency data following Equation 1.

$$E_{0.95} = 2P(100-P)/N \quad (\text{Eq. 1})$$

Where:

$E_{0.95}$  = Probable error at the 95% confidence level

$P$  = Percentage of  $N$  of an individual metal-bearing phase based on percent length frequency

$N$  = Total number of metal-bearing grains counted.

In general, site-specific concentrations for these variable, metal-bearing forms will be determined by performing "peak counts" on the appropriate wavelength spectrometer. Average concentrations will then be used for further calculations. Data on specific gravity will be collected from referenced databases or estimated based on similar compounds.

### 7.0 PERSONNEL RESPONSIBILITY

The analysts will carefully read this SOP prior to any sample examination.

It is the responsibility of the laboratory supervisor and designates to ensure that these procedures are followed, to examine quality assurance (QA) samples and replicate standards, and to check EDS and WDS calibrations. The laboratory supervisor will collect results, ensure they are in proper format, and deliver them to the contractor.

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Monthly reports summarizing all progress, with a list of samples speciated to date with data analyses sheets (DAS), will be submitted each month.

It is also the responsibility of the laboratory supervisor to notify the contractor representative of any problems encountered in the sample analysis process.

#### 8.0 SAMPLE PREPARATION

Grain mounts (1.5 inches in diameter) of each sample will be prepared using air-cured epoxy. This grain mounting technique is appropriate for most speciation projects; however, polished thin-sections, paint chips, dust wipes, or filters may be prepared in a similar manner. The grain mounting is performed as follows:

- 1) Log the samples for which polished mounts will be prepared.
- 2) Inspect all disposable plastic cups, making sure each is clean and dry.
- 3) Label each "mold" with its corresponding sample number.
- 4) All samples will be split to produce a homogeneous 1-4 gram sample.
- 5) Mix epoxy resin and hardener according to manufacturer's directions.
- 6) Pour 1 gram of sample into mold. Double check to make sure sample numbers on mold and the original sample container match. Pour epoxy into mold to just cover sample grains.
- 7) Use a new wood stirring stick with each sample; carefully blend epoxy and grains so as to coat all grains with epoxy.
- 8) Set molds to cure at ROOM TEMPERATURE in a clean restricted area. Add labels with sample numbers and cover with more epoxy resin. Leave to cure completely at room temperature.
- 9) One at a time, remove each sample from its mold and grind flat the back side of the mount.
- 10) Use 600-grit wet abrasive paper stretched across a grinding wheel to remove the bottom layer and expose as many mineral grains as possible. Follow with 1,000-grit paper.
- 11) Polish with 15- $\mu\text{m}$  oil-based diamond paste on a polishing paper fixed to a lap. Use of paper instead of cloth minimizes relief.
- 12) Next use 6- $\mu\text{m}$  diamond polish on a similar lap.
- 13) Finally polish the sample with 1- $\mu\text{m}$  oil-based diamond paste on polishing paper, followed by 0.05- $\mu\text{m}$  alumina in water suspension. The quality should be checked after each step. Typical polishing times are 30 minutes for 15  $\mu\text{m}$ , 20 minutes for 6  $\mu\text{m}$ , 15 minutes for 1  $\mu\text{m}$ , and 10 minutes for 0.05  $\mu\text{m}$ .

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NOTE: use low speed on the polishing laps to avoid “plucking” of sample grains.

- 14) Samples should be completely cleaned in an ultrasonic cleaner with isopropyl alcohol or similar solvent to remove oil and fingerprints.
- 15) To ensure that no particles of any metal are being cross-contaminated during sample preparation procedures, a blank (epoxy only) mold will be made every 20<sup>th</sup> sample (5% of samples) following all of the above procedures. This mold will then be speciated along with the other samples.
- 16) Each sample must be carbon coated. Once coated, the samples should be stored in a clean, dry environment with the carbon surface protected from scratches or handling.

### **9.0 GEOCHEMICAL SPECIATION USING ELECTRON MICROPROBE**

All investigative samples will also be characterized using EMP analysis to determine the chemical speciation, particle size distribution and frequency for several target metals.

#### **9.1 Concentration Prescreening**

All samples will be initially examined using the electron microprobe to determine if the number of particles are too great to obtain a representative count. The particle counting will be considered representative if the entire sample (puck) has been traversed about the same time in which the counting criteria are achieved.

If this examination reveals that one metal is abundant (> 1% of total metals concentration), clean quartz sand (SiO<sub>2</sub>) will be mixed with the sample material. The sand should be certified to be free of target analytes. The quartz sand should be added to an aliquot of the investigative sample, then mixed by turning the sample for a minimum of one hour, or until the sample is fully homogenized. The initial mass of the investigative sample aliquot, and the mass of the quartz addition must be recorded on the Data Analysis Sheet (DAS).

#### **9.2 Point Counting**

Counts are made by traversing each sample from left-to-right and top-to-bottom as illustrated in Figure 9-1. The amount of vertical movement for each traverse depends on magnification and CRT (cathode-ray tube) size. This movement should be minimized so that no portion of the sample is missed when the end of a traverse is reached. Two magnification settings generally are used--one ranging from 40-100X and a second from 300-600X. The last setting will allow one to find the smallest identifiable (1-2 micron) phases.

The portion of the sample examined in the second pass, under the higher magnification, will depend on the time available, the number of metal-bearing particles, and the complexity of metal mineralogy. A maximum of 8 hours will be spent on each analysis.

#### **9.3 Data Reduction**

Analysts will record data as they are acquired from each sample using the LEGS software, which places all data in a spreadsheet file format. Columns have been established for numbering the metal-bearing phase particles, their identity, size of longest dimension in microns, along with their association (L =

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liberated, C= cementing, R = rimming, I = included). The analyst may also summarize his/her observations in the formatted data summary files.

The frequency of occurrence and relative metal mass of each metal-bearing form as it is distributed in each sample will be depicted graphically as a frequency bar graph. The particle size distribution of metal-bearing forms will be depicted in a histogram. Size-histograms of each metal-bearing form can be constructed from data in the file.

Data from EMP will be summarized using two methods. The first method is the determination of FREQUENCY OF OCCURRENCE. This is calculated by summing the longest dimension of all the metal-bearing phases observed and then dividing each phase by the total.

Equation 2 will serve as an example of the calculation.

$$F_M \text{ in phase-1} = \frac{\Sigma (\text{PLD})_{\text{phase 1}}}{\Sigma (\text{PLD})_{\text{phase-1}} + \Sigma (\text{PLD})_{\text{phase-2}} + \Sigma (\text{PLD})_{\text{phase-n}}} \quad (\text{Eq. 2})$$

Where:

- $F_M$  = Frequency of occurrence of metal in a single phase
- PLD = An individual particle's longest dimension
- $\%F_M \text{ in phase-1}$  =  $F_M \text{ in phase-1} * 100$ .

These data indicate which metal-bearing phase(s) are the most commonly observed in the sample or relative volume percent.

The second calculation used in this report is the determination of RELATIVE METAL MASS. These data are calculated by substituting the PLD term in the equation above with the value of  $M_M$ . This term is calculated as defined below:

$$M_M = FM * SG * \text{ppm}_M \quad (\text{Eq. 3})$$

Where:

- $M_M$  = Mass of metal in a phase
- SG = Specific Gravity of a phase
- $\text{ppm}_M$  = Concentration in ppm of metal in a phase.

The advantage in reviewing the RELATIVE METAL MASS determination is that it gives one information as to which metal-bearing phase(s) in a sample are likely to control the total bulk concentration for a metal of interest. For example, PHASE-1 may comprise 98% relative volume of the sample; however, it has a low specific gravity and contains only 1,000 parts per million (ppm) arsenic. PHASE-2 comprised 2% of the sample, has a high specific gravity, and contains 80,000 ppm of arsenic. In this example it is PHASE-2 that is the dominant source of arsenic to the sample.

Finally, a concentration for each phase is calculated. This quantifies the concentration of each metal-bearing phase. This term is calculated as defined below:

$$\text{ppm}_M = M_M * \text{Bulk metal concentration in ppm} \quad (\text{Eq. 4})$$



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#### 9.4 Analytical Procedure

A brief visual examination of each sample will be made, prior to EMP examination. This examination may help the operator by noting the occurrence of slag and/or organic matter. Standard operating conditions for quantitative and qualitative analyses of most metal-bearing forms are given in Table 9-1. However, it is the responsibility of the operator to select the appropriate analytical line (crystal/KeV range) to eliminate peak overlaps and ensure proper identification/quantification of each analyte. Quality control will be maintained by analyzing duplicates at regular intervals.

The backscattered electron threshold will be adjusted so that all particles in a sample are seen. This procedure will minimize the possibility that low metal-bearing minerals may be overlooked during the scanning of the polished grain mount. The scanning will be done manually. Typically, the magnification used for scanning all samples except for airborne samples will be 40-100X and 300-600X. The last setting will allow the smallest identifiable (1-2  $\mu\text{m}$ ) phases to be found. Once a candidate particle is identified, then the backscatter image will be optimized to discriminate any different phases that may be making up the particle or defining its association. Identification of the metal-bearing phases will be done using both EDS and WDS on an EMP, with spectrometers typically peaked at sulfur, oxygen, carbon and the metal(s) of concern. The size of each metal-bearing phase will be determined by measuring in microns the longest dimension.

As stated previously, a maximum of 8 hours will be spent in scanning and analyzing each mount. For most speciation projects the goal is to count between 100-200 particles. In the event that these goals are achieved in less than 8 hours, particle counting may continue or the analyst may move to another sample in order to increase the sample population.

#### Quantitative Analyses

Quantitative analyses are required to establish the average metal content of the metal-bearing minerals, which have variable metal contents as: iron-(M) sulfate, iron-(M) oxide, manganese-(M) oxide, organic, and slag. These determinations are important, especially in the case of slag, which is expected to have considerable variation in their dissolved metal content.

Results will be analyzed statistically to establish mean values. They may also be depicted as histograms to show the range of metal concentrations measured as well as the presence of one or more populations in terms of metal content. In the later case, non-parametric statistics may have to be used or the median value may have to be established.

#### Associations

The association of the metal-bearing forms will be established from the backscattered electron images. Particular attention will be paid in establishing whether the grains are totally enclosed, encapsulated or liberated. The rinds of metal-bearing grains will be identified. Representative photomicrographs of backscatter electron images establishing the association of the principal metal-bearing forms will be obtained for illustration purposes.

#### 9.5 Instrument Calibration and Standardization

The WDS will have spectrometers calibrated for the metal of concern, carbon, oxygen and sulfur on the appropriate crystals using mineral standards. The EDS will have a multi-channel analyzer (MCA)

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calibrated for known peak energy centroids. Calibration will be performed so as to have both low (1.0-3.0 KeV) and high (6.0-9.0 KeV) energy peaks fall within 0.05 KeV of its known centroid.

The magnification marker on the instrument will be checked once a week. This will be performed by following manufacturer instructions or by measurement of commercially available grids or leucite spheres. Size measurements must be within 4 microns of certified values.

Initial calibration verification standards (ICVs) must be analyzed at the beginning of each analytical batch or once every 48 hours, whichever is more frequent. A set of mineral or glass standards will be run quantitatively for the metal of concern, sulfur, oxygen and carbon. If elemental quantities of the ICVs do not fall within +/- 5% of certified values for each element, the instrument must be recalibrated prior to analysis of investigative samples.

The metal-bearing forms in these samples will be identified using a combination of EDS, WDS and BEI. Once a particle is isolated with the backscatter detector, a 5-second EDS spectra is collected and peaks identified. The count rates for the metal(s) of concern, sulfur, carbon and oxygen can be either visually observed on the wavelength spectrometers or K-ratios calculated.

#### **9.6 Documentation**

Photomicrographs must be taken for each sample, at a rate of 5% (1 photograph per 20 particles counted), for a maximum of 10 per sample and submitted with the results. Particles selected for photography must be recorded on the EMP graph, as well as in the DAS. Any additional photographs should be labeled as "opportunistic".

A positive black-and-white film (Polaroid 52) will be used or a 128x128 (minimum) binary image in ".tif" format may be stored. Recorded on each photomicrograph and negative will be a scale bar, magnification, sample identification, date and phase identification. Abbreviations for the identified phases can be used. Examples are listed in Table 9-2. A final list must be submitted with the laboratory report.

#### **10.0 PERSONAL HEALTH AND SAFETY**

For studies conducted at the University of Colorado, each individual operating the electron microprobe instruments will have read the "Radiation Safety Handbook" prepared by the University of Colorado and follow all State of Colorado guidelines for operation of X-ray equipment.

Latex gloves and particulate masks will be worn during preparation of sample cups. All material that comes in contact with the samples or used to clean work surface areas will be placed in poly-bags for disposal.

#### **11.0 FINAL REPORT**

A final laboratory report will be provided to the Contractor. The report will include all EMP data including summary tables and figures. Individual sample data will be provided on disk.

Speciation results will include: (1) a series of tables summarizing frequency of occurrence for each metal phase identified along with a confidence limit; (2) summary histograms of metal phases identified for each waste type; (3) a summary histogram of particle size distribution in each waste type; and (4) a

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summary of metal phase associations. Representative photomicrographs or .tif images also will be included in the final report.

#### 12.0 REFERENCES

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Davis, A., J.W. Drexler, M.V. Ruby, and A. Nicholson. 1993. The micromineralogy of mine wastes in relation to lead bioavailability, Butte, Montana. *Environ. Sci. Technol.* (In Press).

Mosimann, J.E. 1965. Statistical Methods for the Pollen Analyst. *Handbook of Paleontological Techniques*. In: B. Kummel and D. Raup (Eds.), Freeman and Co., San Francisco, pp. 636-673.

Ruby, M.V., A. Davis, J.H. Kempton, J.W. Drexler, and P.D. Bergstrom. 1992. Lead bioavailability: Dissolution kinetics under simulated gastric conditions. *Environ. Sci. Technol.* 26(6): pp 1242-1248.

WESTON (Roy F. Weston, Inc.). 1995. Metal Speciation Interpretive Report, Leadville, CO. CERCLA Site. March, 1995.

**Table 2-1**

Common Metal-Bearing Forms Found Within Mining, Smelting, Agricultural, Industrial and Residential Media

**OXIDES**

Lead oxide  
Manganese (metal) oxide  
Iron (metal) oxide  
Lead molybdenum oxide  
Arsenic (metal) oxide  
Lead (metal) oxides  
Cadmium oxide  
Copper oxides  
Zinc oxide  
Lead arsenate  
Arsenic trioxide  
Calcium (metal) oxide

**SILICATES**

Slag  
Lead silicate  
Arsenic silicate  
Zinc silicate  
Clays

**SULFATES**

Iron (metal) sulfate  
Lead sulfate  
Lead barite  
Zinc sulfate  
Arsenic sulfate  
Copper sulfate

**CARBONATES**

Lead carbonate  
Zinc carbonate

**PHOSPHATES**

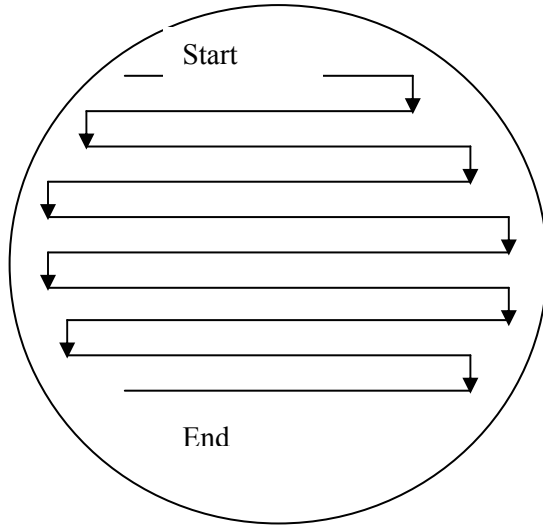
(metal) phosphates

**SULFIDES**

Lead sulfide  
Sulfur-containing salts  
Iron-arsenic sulfide  
Zinc sulfide  
Copper sulfides  
Copper-iron sulfide  
Cadmium sulfide

**OTHER**

Native: Lead, Copper,  
Cadmium, Mercury, Indium,  
Thallium, Selenium  
Lead/Arsenic/Cadmium/  
Mercury chlorides  
Paint  
Solder  
Organic lead  
Lead vanadate  
Minor telluride, and bismuth-  
lead phases



**Figure 9-1**

**Table 9-1**

**EMP Standard Operating Conditions**

	WDS	EDS
<b>Accelerating Voltage</b>	15 KV	15-20 KV
Beam Size	1-2 microns	1-2 microns
Cup Current	10-30 NanoAmps	10-30 NanoAmps
Ev/Channel	NA	10 or 20
Stage Tilt	NA	Fixed
Working Distance	NA	Fixed
MCA time Constant	NA	7.5-12 microseconds
X-ray lines	S K-alpha PET O K-alpha LDE1 C K-alpha LDEC Zn K-alpha PET As L-alpha TAP Cu K-alpha LIF Cd L-alpha PET Pb M-alpha PET Pb L-alpha LIF In L-alpha PET Tl L-alpha LIF Hg L-alpha LIF Se L-alpha LIF Sb L-alpha PET	S K-alpha 2.31 KeV O K-alpha 0.52 KeV C K-alpha 0.28 KeV Pb M-alpha 2.34 KeV Pb L-alpha 10.5 KeV Zn K-alpha 8.63 KeV Cu K-alpha 8.04 KeV As K-alpha 10.5 KeV As L-alpha 1.28 KeV Cd L-alpha 3.13 KeV In L-alpha 3.28 KeV Tl M-alpha 2.27 KeV Tl L-alpha 10.26 KeV Hg L-alpha 9.98 KeV Hg M-alpha 2.19 KeV Se L-alpha 1.37 KeV Sb L-alpha 3.60 KeV

**Table 9-2****Suggested Abbreviation for Photomicrographs**

Metal-bearing Phase	Abbreviation
In	In
Tl	Tl
Hg	Hg
Se	Se
Sb	Sb
Lead Sulfide	Ga
Lead Sulfate	Ang
Lead Carbonate	Cer
Mn-(M) Oxide	Mn(M)
Fe-(M) Oxide	Fe(M)
(M)Phosphate	(M)Phos
Fe-(M) Sulfate	Fe(M)Sul
Metal Oxide	(M)O
Pb-Mo Oxide	Wulf
Slag	Slag
Metallic Phase	(M)
Metal Silicate	(M)Si
Solder	Sold
Paint	Pnt
Metal-bearing Organic	(M)(Org)
(M) barite	(M)Bar
Pb arsenate	PbAsO
Pb vanadate	PbVan
As-Sb Oxide	AsSbO
Chalcopyrite	Cp
Sphalerite	Sph
Arsenopyrite	Apy





**APPENDIX B**

**STANDARD OPERATING PROCEDURES FOR  
EVALUATING MERCURY SPECIATION IN SOILS**



## **STANDARD OPERATING PROCEDURE:**

### **Evaluating Mercury Speciation in Soils**

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Mercury can occur in soils as elemental mercury in liquid or vapor form, organic mercury compounds, mercuric chloride, or one of several different mineral species, including mercuric oxides, carbonates, and sulfides. In general, organic mercury, mercuric chloride, and elemental mercury in the vapor phase are very soluble and bioavailable; mercuric oxides and carbonates are less soluble; and liquid elemental mercury and mercuric sulfides are relatively insoluble and non-bioavailable.

Recently, several investigators have focused on developing sequential extraction procedures to quantitatively evaluate the speciation of mercury in soils (Revis et al. 1989; Miller 1993; Sakamoto et al. 1992). Application of the procedures of each investigator to mercury-contaminated soils from Oak Ridge, Tennessee, indicated mercury occurring predominantly as elemental mercury and mercuric sulfide minerals (Barnett et al. 1994). However, the relative proportions of the two species did not agree among procedures, indicating that the extractions were either not fully effective in removing specific mercury compounds or not fully specific in extracting individual mercury species. This problem is common to sequential extraction methods (Belzile et al. 1989). All the extraction techniques yielded similar levels of organic mercury in soils. However, the method used by Miller (1993), developed by EPA, generally found much less elemental mercury and mercuric sulfide than did the other two extraction procedures. The method used by Sakamoto et al. (1992) tended to have poor recovery for elemental mercury. The method used by Revis et al. (1993) showed higher recoveries of mercuric sulfide and elemental mercury, but did not include a step for extraction of mercuric oxides and carbonates (acid-soluble mercury). Given these drawbacks to the various methods, a procedure combining the most effective aspects of each was developed. This method has been used to evaluate mercury at a number of sites, and appears to produce reliable results.

#### **Sample Preparation**

Mercury analyses in soils are particularly difficult to reproduce, because elemental mercury commonly occurs as geochemical “nuggets,” where only a small fraction of soil may contain a large proportion of the total mercury in the sample, creating difficulties in obtaining a homogeneous sample. This phenomenon is often evident in field and laboratory duplicates in the form of large relative percent differences (RPDs) between duplicate samples. To minimize this problem as much as possible, all soil samples should be dried thoroughly prior to analysis, and homogenized in a stainless steel bowl. All subsamples should then be prepared using a stainless steel sample splitter.

Soil samples should be air dried beneath a fume hood for 2 days, or until constant weight is achieved. Oven drying should not be used, because it can result in loss of volatile mercury species. The air-dried samples should be desegregated, and sieved through a number 10, 2-mm stainless-steel sieve.

## Sequential Extraction Studies

The intent of this sequential extraction assay is to determine the speciation of mercury in soil for the following mercury species:

- Organic mercury
- Acid-soluble (or bioavailable) mercury, including carbonates, hydroxides, oxides, and chlorides
- Elemental mercury
- Mercuric sulfide.

The sequential extraction of mercury species is outlined in Figure 1. Using air-dried soil samples sieved to less than 2 mm, organic mercury is extracted with chloroform, followed by extraction with a sodium thiosulfate solution. The acid-soluble mercury species are then extracted using sulfuric acid. After extracting these two phases, elemental mercury is determined by the difference between a sample split that has been roasted at 150°C for 5 days and a non-roasted sample split. Mercuric sulfide is assumed to be the mercury that remains after roasting.

## Methods

### Equipment Preparation

Equipment for the organic mercury extraction is prepared by washing four 250-mL glass separatory funnels and four 50-mL Fisher<sup>®</sup> polypropylene centrifuge tubes (with double-start threads) in acid. The separatory funnels are then pre-rinsed with Fisher Scientific<sup>®</sup> HPLC-grade chloroform to purify them of any organic mercury. Once the centrifuge tubes are air dried, they are tared on a balance, and 7.5 g of soil sample (less than 2 mm) is weighed into them.

### Organic Mercury Extraction

The organic mercury extraction begins by adding 30 mL of Fisher Scientific<sup>®</sup> chloroform to the centrifuge tubes and sealing them with the lids. The centrifuge tubes are placed in a wrist-action shaker for 3 minutes and then centrifuged for 3 minutes at 3,000 rpm.

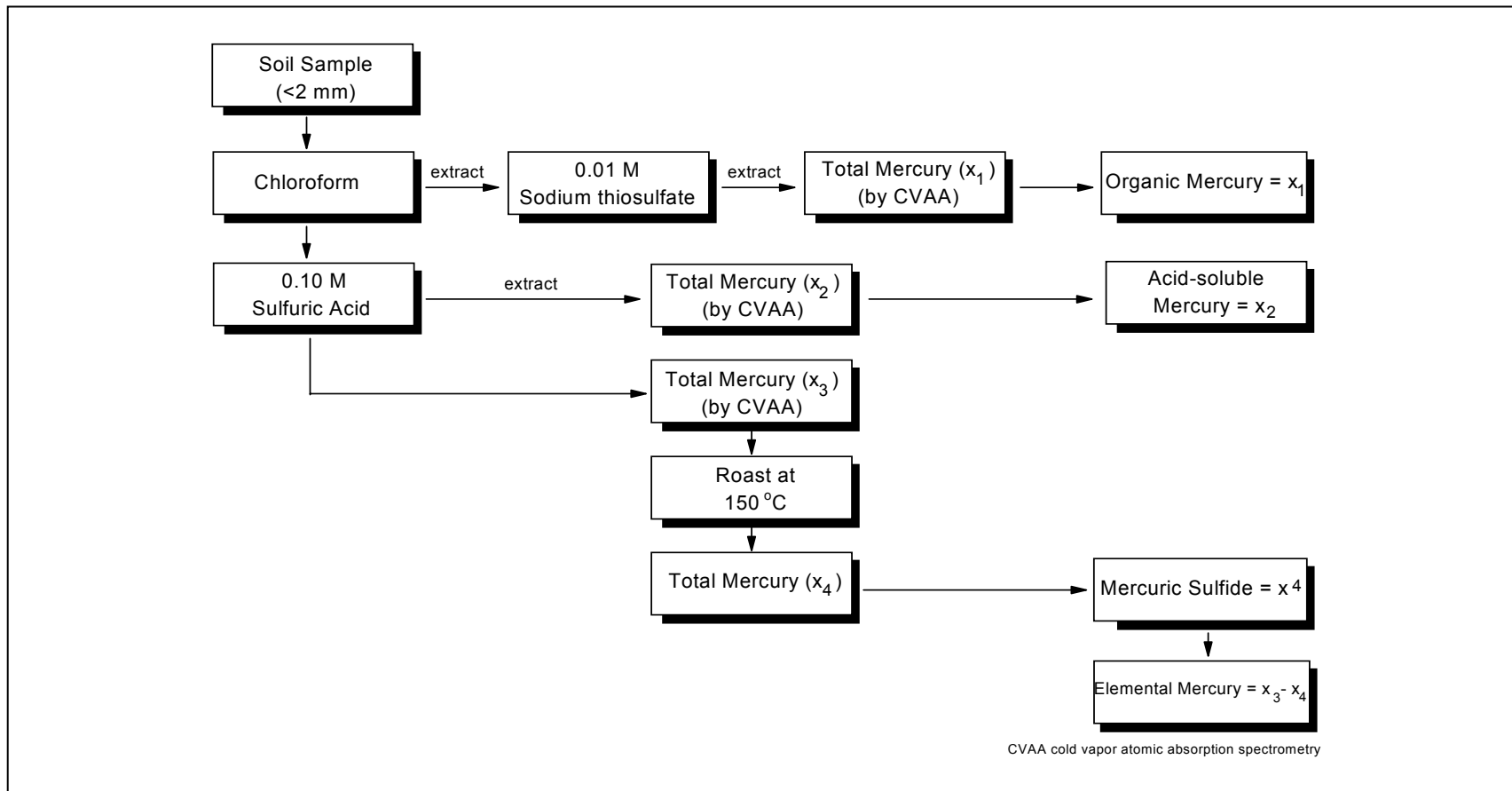


Figure 1. Sequential extraction of mercury phases in soil

After allowing any floating particles to settle, the chloroform phase is decanted into a 250-mL separatory funnel. The extraction is repeated with another 30 mL of chloroform, which is also added to the separatory funnel. Following extraction, 10 mL of 0.01 M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (sodium thiosulfate) is added to the combined chloroform extracts in the separatory funnel to extract the organic mercury from the chloroform. The funnel is hand-shaken for 3 minutes and allowed to settle. Upon separation of the organic and inorganic phases, the chloroform layer is discharged into a large stainless-steel bowl and allowed to evaporate in a hood. The remaining sodium thiosulfate layer and grey layer are filtered through a Whatman<sup>®</sup> GF/C filter. The filtrate is collected in a 15-mL plastic bottle (or 15-mL Fisher Scientific<sup>®</sup> centrifuge tube), and preserved with concentrated trace metal nitric acid (20 μL per 10 mL of sample). This preserved extract should be analyzed for mercury concentration by cold vapor atomic absorption (CVAA) spectroscopy. Using a stainless steel spatula, the solids remaining on the filter paper are returned to the centrifuge tube. The solid remaining in the centrifuge tube is placed under a hood to air dry for the next step of the extraction.

### **Acid-Soluble Mercury Extraction**

The extraction of acid-soluble mercury consists of adding 15 mL of 0.1 M sulfuric acid to the air-dried residue in the centrifuge tube, shaking the tube in a wrist-action shaker for 3 minutes, and centrifuging for 10 minutes, as specified in Sakamoto et al. (1992). The sulfuric acid solution is then aspirated from the centrifuge tube with a 10-mL plastic syringe, the volume of solution is measured, and the solution is filtered through a Corning<sup>®</sup> disposable sterile syringe filter (25 mm, 0.45 μm acrylic, with cellulose acetate membrane). Filtration was added to this step to remove any fine particles that may be suspended in the sulfuric acid solution prior to analysis. The sulfuric acid extract is collected in a 15-mL plastic bottle (or centrifuge tube) and analyzed for total mercury by CVAA. The centrifuge tube is then placed under the hood, and the sample is air dried prior to the next step in the procedure.

### **Elemental Mercury Extraction**

This step of the procedure begins with removing the sample from the centrifuge tube and splitting the sample using a 2-mm sample splitter. This sub-sample is analyzed for total mercury by CVAA. The remaining sample is weighed in tared stainless-steel pans and placed in the oven. Elemental mercury is removed from the samples by oven roasting for 5 days at 150° C. After 5 days of roasting, the sample is removed from the oven, weighed, and analyzed for total mercury by CVAA. All mercury remaining in the roasted sample is assumed to be mercuric sulfide. Elemental mercury is determined by subtracting the mercury concentration of the roasted sample from the concentration of the unroasted sample.

### **Recommended Quality Assurance Samples**

It is recommended that a comprehensive set of quality assurance (QA) samples be analyzed when performing the sequential extraction procedure described above. These samples should include method blanks (e.g., sodium thiosulfate and sulfuric acid extraction solutions, sent blind to the laboratory) and mercury spikes made up the sodium thiosulfate and sulfuric acid extraction solutions (to check mercury recovery from these matrices). Duplicate or triplicate analyses of

solid samples should be used to check for homogeneity of mercury in specific samples, and at least one sample should be evaluated in triplicate in the sequential extraction procedure as a check on reproducibility.

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## **APPENDIX C**

### **IN VITRO METHOD FOR DETERMINATION OF LEAD BIOACCESSIBILITY: STANDARD OPERATING PROCEDURE FOR STOMACH PHASE EXTRACTION**



## **Appendix C**

### ***In Vitro* Method for Determination of Lead Bioaccessibility:**

### **Standard Operating Procedure for Stomach Phase Extraction**

#### **Prepared by:**

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# 1. Introduction

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## 1.1 Synopsis

This SOP describes an *in vitro* laboratory procedure to determine a bioaccessibility value for lead or arsenic (i.e., the fraction that would be soluble in the gastrointestinal tract) for soils and solid waste materials. A recommended quality assurance program to be followed when performing this extraction procedure is also provided.

## 1.2 Purpose

An increasingly important property of materials/soils found at contaminated sites is the bioavailability of individual contaminants. Bioavailability is the fraction of a contaminant in a particular environmental matrix that is absorbed by an organism via a specific exposure route. Many animal studies have been conducted to experimentally determine the oral bioavailability of individual metals, particularly lead and arsenic. During the period 1989–1997, a juvenile swine model developed by EPA Region VIII was used to predict the relative bioavailability of lead and arsenic in approximately 20 soils/solid materials (Weis and LaVelle 1991; Weis et al. 1994; Casteel et al. 1997a,b). The bioavailability determined was relative to that of a soluble salt (i.e., lead acetate trihydrate or sodium arsenate). The tested materials had a wide range of mineralogy, and produced a range of lead and arsenic bioavailability values. In addition to the swine studies, other animal models (e.g., rats and monkeys) have been used to measure the bioavailability of lead and arsenic from soil.

Several researchers have developed *in vitro* tests to measure the fraction of a chemical solubilized from a soil sample under simulated gastrointestinal conditions. This measurement is referred to as “bioaccessibility” (Ruby et al. 1993). Bioaccessibility is thought to be an important determinant of bioavailability, and several groups have sought to compare bioaccessibility determined in the laboratory to bioavailability determined in animal studies (Imber 1993; Ruby et al. 1996; Medlin 1997; Rodriguez et al. 1999). The *in vitro* tests consist of an aqueous fluid, into which soils containing lead and arsenic are introduced. The solution then solubilizes the soil under simulated gastric conditions. Once this procedure is complete, the solution is analyzed for lead and/or arsenic concentration. The mass of lead and/or arsenic found in the aqueous phase, as defined by filtration at the 0.45- $\mu\text{m}$  pore size, is compared to the mass introduced into the test. The fraction liberated into the aqueous phase is defined as the bioaccessible fraction of lead or arsenic in that soil. To date, for lead-bearing soils tested in the EPA swine studies, this *in vitro* method has correlated well with relative bioavailability values.

## 2. Procedure

---

### 2.1 Sample Preparation

All soil/material samples should be prepared for testing by oven drying (<40 °C) and sieving to <250  $\mu\text{m}$ . The <250- $\mu\text{m}$  size fraction is used because this particle size is representative of that which adheres to children's hands. Subsamples for testing in this procedure should be obtained using a sample splitter.

### 2.2 Apparatus and Materials

#### 2.2.1 Equipment

The main piece of equipment required for this procedure consists of a Toxicity Characteristic Leaching Procedure (TCLP) extractor motor that has been modified to drive a flywheel. This flywheel in turn drives a Plexiglass block situated inside a temperature-controlled water bath. The Plexiglass block contains ten 5-cm holes with stainless steel screw clamps, each of which is designed to hold a 125-mL wide-mouth high-density polyethylene (HDPE) bottle (see Figure 1). The water bath must be filled such that the extraction bottles are immersed. Temperature in the water bath is maintained at  $37 \pm 2$  °C using an immersion circulator heater (for example, Fisher Scientific Model 730). Additional equipment for this method includes typical laboratory supplies and reagents, as described in the following sections.

The 125-mL HDPE bottles must have an air-tight screw-cap seal (for example, Fisher Scientific 125-mL wide-mouth HDPE Cat. No. 02-893-5C), and care must be taken to ensure that the bottles do not leak during the extraction procedure.

#### 2.2.2 Standards and Reagents

The leaching procedure for this method uses a buffered extraction fluid at a pH of 1.5. The extraction fluid is prepared as described below.

The extraction fluid should be prepared using ASTM Type II deionized (DI) water. To 1.9 L of DI water, add 60.06 g glycine (free base, Sigma Ultra or equivalent). Place the mixture in a water bath at 37 °C until the extraction fluid reaches 37 °C. Standardize the pH meter using temperature compensation at 37 °C or buffers maintained at 37 °C in the water bath. Add concentrated hydrochloric acid (12.1 N, Trace Metal grade) until the solution pH reaches a value of  $1.50 \pm 0.05$  (approximately 120 mL). Bring the solution to a final volume of 2 L (0.4 M glycine).

Cleanliness of all reagents and equipment used to prepare and/or store the extraction fluid is essential. All glassware and equipment used to prepare standards and reagents must be properly cleaned, acid washed, and finally, rinsed with DI water prior to use. All reagents must be free of lead and arsenic, and the final fluid should be tested to confirm that lead and arsenic concentrations are less than 25 and 5  $\mu\text{g/L}$ , respectively.

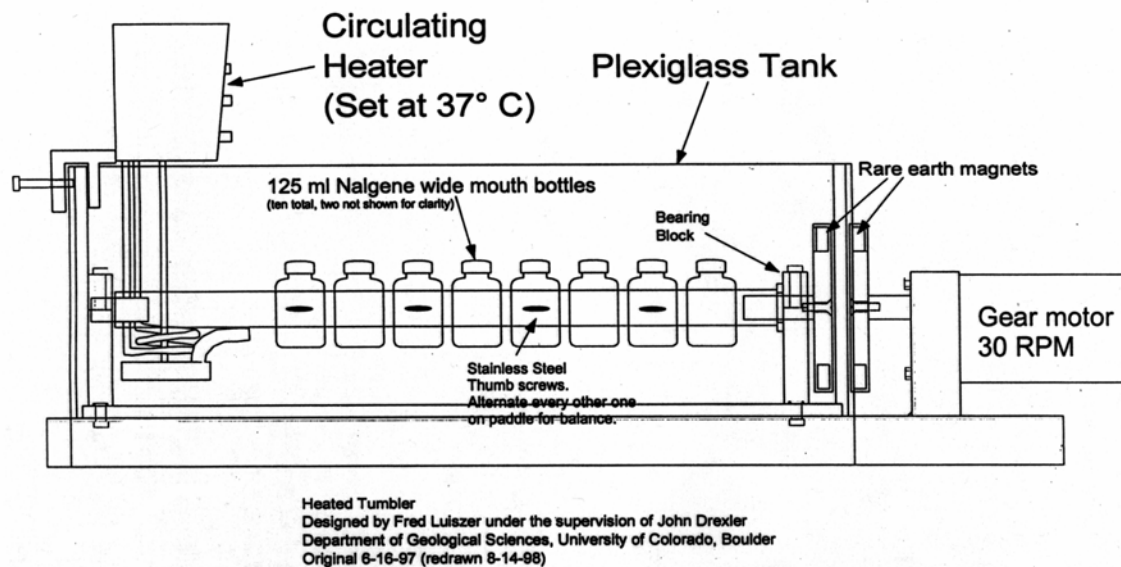


Figure 1. Extraction device for performing the SBRC *in vitro* extraction

### 2.3 Leaching Procedure

Measure  $100 \pm 0.5$  mL of the extraction fluid, using a graduated cylinder, and transfer to a 125-mL wide-mouth HDPE bottle. Add  $1.00 \pm 0.05$  g of test substrate ( $<250 \mu\text{m}$ ) to the bottle, ensuring that static electricity does not cause soil particles to adhere to the lip or outside threads of the bottle. If necessary, use an antistatic brush to eliminate static electricity prior to adding the soil. Record the volume of solution and mass of soil added to the bottle on the extraction test checklist (see Attachment A for example checklists). Hand-tighten each bottle top, and shake/invert to ensure that no leakage occurs, and that no soil is caked on the bottom of the bottle.

Place the bottle into the modified TCLP extractor, making sure each bottle is secure and the lid(s) are tightly fastened. Fill the extractor with 125-mL bottles containing test materials or Quality Control samples.

The temperature of the water bath must be  $37 \pm 2$  °C. Record the temperature of the water bath at the beginning and end of each extraction batch on the appropriate extraction test checklist sheet (see Attachment A).

Rotate the extractor end over end at  $30 \pm 2$  rpm for 1 hour. Record start time of rotation.

When extraction (rotation) is complete, immediately remove bottles, wipe them dry, and place them upright on the bench top.

Draw extract directly from reaction vessel into a disposable 20-cc syringe with a Luer-Lok attachment. Attach a  $0.45\text{-}\mu\text{m}$  cellulose acetate disk filter (25 mm diameter) to the syringe, and filter the extract into a clean 15-mL polypropylene centrifuge tube or other appropriate sample vial for analysis. Store filtered sample(s) in a refrigerator at 4 °C until they are analyzed.

Record the time that the extract is filtered (i.e., extraction is stopped). If the total elapsed time is greater than 1 hour 30 minutes, the test must be repeated.

Measure and record the pH of fluid remaining in the extraction bottle. If the fluid pH is not within  $\pm 0.5$  pH units of the starting pH, the test must be discarded and the sample reanalyzed as follows.

If the pH has dropped by 0.5 or more pH units, the test will be re-run in an identical fashion. If the second test also results in a decrease in pH of greater than 0.5 s.u., the pH will be recorded, and the extract filtered for analysis. If the pH has increased by 0.5 or more units, the test must be repeated, but the extractor must be stopped at specific intervals and the pH manually adjusted down to pH 1.5 with dropwise addition of HCl (adjustments at 5, 10, 15, and 30 minutes into the extraction, and upon final removal from the water bath [60 minutes]). Samples with rising pH values must be run in a separate extraction, and must not be combined with samples being extracted by the standard method (continuous extraction).

Extracts are to be analyzed for lead and arsenic concentration using analytical procedures taken from the U.S. EPA publication, *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. SW-846*. (current revisions). Inductively coupled plasma (ICP) analysis, method 6010B (December 1996 revision) will be the method of choice. This method should be adequate for determination of lead concentrations in sample extracts, at a project-required detection limit (PRDL) of 100  $\mu\text{g/L}$ . The PRDL of 20  $\mu\text{g/L}$  for arsenic may be too low for ICP analysis for some samples. For extracts that have arsenic concentrations less than five times the PRDL (e.g.,  $<100 \mu\text{g/L}$  arsenic), analysis by ICP-hydride generation (method 7061A, July 1992 revision) or ICP-MS (method 6020, September 1994 revision) will be required.

## 2.4 Calculation of the Bioaccessibility Value

A split of each solid material ( $<250 \mu\text{m}$ ) that has been subjected to this extraction procedure should be analyzed for total lead and/or arsenic concentration using analytical procedures taken from the U.S. EPA publication, *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. SW-846*. (current revisions). The solid material should be acid digested according to method 3050A (July 1992 revision) or method 3051 (microwave-assisted digestion, September 1994 revision), and the digestate analyzed for lead and/or arsenic concentration by ICP analysis (method 6010B). For samples that have arsenic concentrations below ICP detection limits, analysis by ICP-hydride generation (method 7061A, July 1992 revision) or ICP-MS (method 6020, September 1994 revision) will be required.

The bioaccessibility of lead or arsenic is calculated in the following manner:

$$\text{Bioaccessibility (\%)} = \frac{(\text{concentration in in vitro extract, mg/L}) (0.1\text{L} - \text{fluid})}{(\text{concentration in solid, mg/kg}) (0.001\text{ kg} - \text{soil})} \times 100$$

## 2.5 Chain-of-Custody/Good Laboratory Practices

All laboratories that use this SOP should receive test materials with chain-of-custody documentation. When materials are received, each laboratory will maintain and record custody of samples at all times. All laboratories that perform this procedure should follow good laboratory practices as defined in 40 CFR Part 792 to the extent practical and possible.



## **2.6 Data Handling and Verification**

All sample and fluid preparation calculations and operations should be recorded in bound and numbered laboratory notebooks, and on extraction test checklist sheets. Each page must be dated and initialed by the person who performs any operations. Extraction and filtration times must be recorded, along with pH measurements, adjustments, and buffer preparation. Copies of the extraction test checklist sheets should accompany the data package.

### 3. Quality Control Procedures

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#### 3.1 Elements of Quality Assurance and Quality Control (QA/QC)

A standard method for the *in vitro* extraction of soils/solid materials, and the calculation of an associated bioaccessibility value, are specified above. Associated QC procedures to ensure production of high-quality data are as follows (see Table 1 for summary of QC procedures, frequency, and control limits):

- Reagent blank—Extraction fluid analyzed once per batch.
- Bottle blank—Extraction fluid only run through the complete extraction procedure at a frequency of no less than 1 per 20 samples or one per extraction batch, whichever is more frequent.
- Blank spikes—Extraction fluid spiked at 10 mg/L lead and/or 1 mg/L arsenic and run through the extraction procedure at a frequency of no less than every 20 samples or one per extraction batch, whichever is more frequent. Blank spikes should be prepared using traceable 1,000-mg/L lead and arsenic standards in 2 percent nitric acid.
- Duplicate—duplicate extractions are required at a frequency of 1 for every 10 samples. At least one duplicate must be performed on each day that extractions are conducted.
- Standard Reference Material (SRM)—National Institute of Standards and Technology (NIST) material 2711 (Montana Soil) should be used as a laboratory control sample (LCS).

Control limits for these QC samples are delineated in Table 1, and in the following discussion.

**Table 1. Summary of QC Samples, Frequency of Analysis, and Control Limits**

<b>QC Sample</b>	<b>Minimum Frequency of Analysis</b>	<b>Control Limits</b>
Reagent Blank	Once per batch (min. 5%)	<25 $\mu\text{g/L}$ lead <5 $\mu\text{g/L}$ arsenic
Bottle Blank	Once per batch (min. 5%)	<50 $\mu\text{g/L}$ lead <10 $\mu\text{g/L}$ arsenic
Blank Spike	Once per batch (min. 5%)	85–115% recovery
Duplicate	10%	$\pm 20\%$ RPD
SRM (NIST 2711)	2%	9.22 $\pm$ 1.50 mg/L Pb 0.59 $\pm$ 0.09 mg/L As

### 3.2 QA/QC Procedures

Specific laboratory procedures and QC steps are described in the analytical methods cited in Section 2.3, and should be followed when using this SOP.

#### 3.2.1 Laboratory Control Sample (LCS)

The NIST SRM 2711 should be used as a laboratory control sample for the *in vitro* extraction procedure. Analysis of 18 blind splits of NIST SRM 2711 (105 mg/kg arsenic and 1,162 mg/kg lead) in four independent laboratories resulted in arithmetic means  $\pm$  standard deviations of 9.22  $\pm$  1.50 mg/L lead and 0.59  $\pm$  0.09 mg/L arsenic. This SRM is available from the National Institute of Standards and Technology, Standard Reference Materials Program, Room 204, Building 202, Gaithersburg, Maryland 20899 (301/975-6776).

#### 3.2.2 Reagent Blanks/Bottle Blanks/Blank Spikes

Reagent blanks must not contain more than 5  $\mu\text{g/L}$  arsenic or 25  $\mu\text{g/L}$  lead. Bottle blanks must not contain arsenic and/or lead concentrations greater than 10 and 50  $\mu\text{g/L}$ , respectively. If either the reagent blank or a bottle blank exceeds these values, contamination of reagents, water, or equipment should be suspected. In this case, the laboratory must investigate possible sources of contamination and mitigate the problem before continuing with sample analysis. Blank spikes should be within 15% of their true value. If recovery of any blank spike is outside this range, possible errors in preparation, contamination, or instrument problems should be suspected. In the case of a blank spike outside specified limits, the problems must be investigated and corrected before continuing sample analysis.

## 4. References

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**Attachment A:**  
**Extraction Test Checklist Sheets**

## Extraction Fluid Preparation

Date of Extraction Fluid Preparation: \_\_\_\_\_

Prepared by: \_\_\_\_\_

Extraction Fluid Lot #: \_\_\_\_\_

Component	Lot Number	Fluid Preparation		Acceptance Range	Actual Quantity	Comments
		1L	2L			
Deionized Water		0.95 L (approx.)	1.9 L (approx.)	---		
Glycine		30.03±0.05 g	60.06±0.05g	---		
HCl <sup>a</sup>		60 mL (approx.)	120 mL (approx.)	---		
Final Volume	---	1 L (Class A, vol.)	2 L (Class A, vol.)	---		
Extraction Fluid pH value (@ 37°C)	---	1.50±0.05	1.50±0.05	1.45–1.55		

<sup>a</sup> Concentrated hydrochloric acid (12.1 N)

Required Parameters:

Volume of extraction fluid (V) = 100 ±0.5 mL  
 Mass of test substrate (M) = 1.00 ±0.05 g  
 Temperature of water bath = 37 ±2 °C  
 Extraction time = 60 ±5 min

Extractor rotation speed = 30 ±2 rpm  
 Maximum elapsed time from extraction to filtration = 90 minutes  
 Maximum pH difference from start to finish (ΔpH)= 0.5 pH units  
 Spike solution concentrations: As = 1 mg/L; Pb = 10 mg/L

Date of Extraction: \_\_\_\_\_  
 Extraction Fluid Lot #: \_\_\_\_\_  
 Extracted by: \_\_\_\_\_

As Spike Solution Lot #: \_\_\_\_\_  
 Pb Spike Solution Lot #: \_\_\_\_\_

Extraction Log:

Sample ID	Sample Preparation		Extraction							Filtration		
	V (mL)	M (g)	Start Time <sup>a</sup>	End Time <sup>a</sup>	Elapsed Time (min)	Start pH	End pH	ΔpH	Start Temp (°C)	End Temp (°C)	Time <sup>a</sup>	Time Elapsed from extraction (min)
Acceptance Range	(95.5–100.5)	(0.95–1.05)	---	---	(55–65 min)	---	---	(Max = 0.5)	(35–39)	(35–39)		(Max = 90 min)
Bottle Blank												
Duplicate												
Matrix spike												

<sup>a</sup> 24-hour time scale

C-11

**Analytical Procedures**QC Requirements:

QC Sample	Minimum Analysis Frequency	Control Limits	Corrective Action <sup>a</sup>
Reagent blank	once per batch (min. 5%)	< 25 µg/L Pb <5 µg/L As	Investigate possible sources of target analytes. Mitigate contamination problem before continuing analysis.
Bottle blank	once per batch (min. 5%)	< 50 µg/L Pb <10 µg/L As	Investigate possible sources of target analytes. Mitigate contamination problem before continuing analysis.
Blank spike	once per batch (min. 5%)	85–115%	Re-extract and reanalyze sample batch
Duplicate	10% (min. once/day)	±20% RPD	Re-homogenize, re-extract and reanalyze

RPD – Relative percent difference

a – Action required if control limits are not met



## **APPENDIX D**

### **IN VITRO BIOACCESSIBILITY TEST: STANDARD OPERATING PROCEDURES FOR SEQUENTIAL STOMACH AND SMALL INTESTINAL PHASE EXTRACTION**



## **Appendix D**

### **In Vitro Bioaccessibility Test: Standard Operating Procedures for Sequential Stomach and Small Intestinal Phase Extraction**

*The in vitro extraction test presented in this appendix, which involves sequential simulated stomach and small intestinal phases, is based on the method of Ruby et al. (1996), but incorporates the test cell and mixing method developed by Dr. John Drexler at the Department of Geological Sciences, University of Colorado at Boulder.*

The *in vitro* test is designed to determine the fraction of an inorganic element that is solubilized and available for absorption in the gastrointestinal tract. Development of the test, and the rationale for selection of representative parameters, are described in detail in the literature cited in Appendix C (In Vitro Method for Determination of Lead Bioaccessibility: Standard Operating Procedure for Stomach Phase Extraction). The *in vitro* method was designed to replicate gastrointestinal-tract parameters for a human child, including stomach and small-intestinal pH and chemistry, soil-to-solution ratio, stomach mixing, and stomach emptying rate. The method is implemented in two phases, simulating the passage of ingested soil from the acidic environment of the stomach to the near-neutral conditions of the small intestine.

The reaction is carried out in a sealed container (Figure 1), to minimize interactions between the reaction fluid and atmospheric oxygen, and the potential for cross contamination. Argon gas is introduced into the reaction vessel at the beginning of the *in vitro* assay to purge it of atmospheric oxygen to simulate the anoxic conditions present in the gastrointestinal tract.

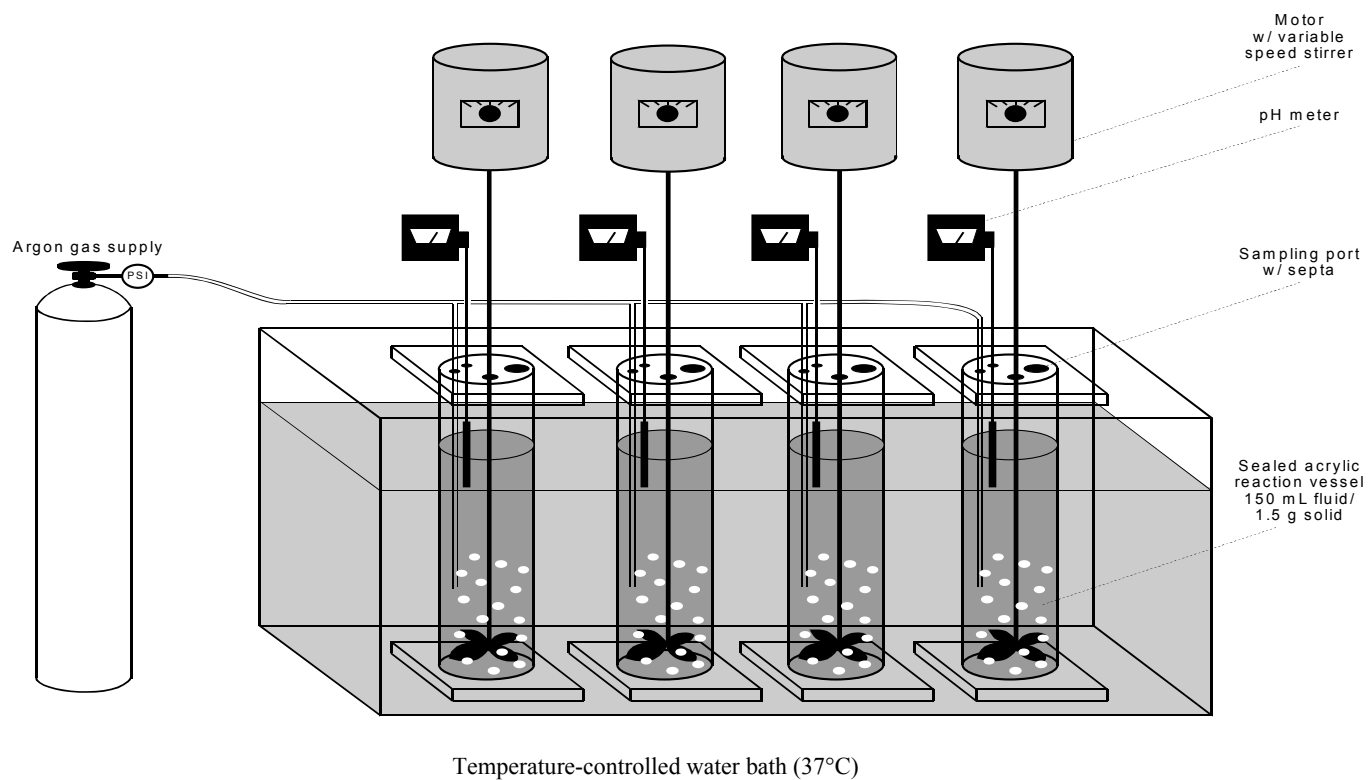


Figure 1. Schematic of *in vitro* experimental system.

The *in vitro* test is conducted according to the following method (all chemicals from Sigma Chemical Company, unless otherwise noted):

- Prepare the stomach solution by adding the following compounds to 1 L of deionized water (stirred continually on a stir plate)
  - 1.25 g pepsin (50 mg, activity of 800–2,500 units/mg)
  - 0.50 g citrate (Fisher Chemical Co.)
  - 0.50 g malate (Aldrich Chemical Co.)
  - 420  $\mu$ L lactic acid (synthetic syrup 85 percent w/w)
  - 500  $\mu$ L acetic acid (97 percent w/w; Fisher Chemical Co.).
- Adjust the pH of the stomach solution to 2.0 by adding a measured volume of concentrated HCl.
- Add 150 mL of stomach solution to the 200-mL acrylic reaction vessel.
- Sparge the stomach solution with argon for 10 minutes to remove oxygen.
- Add 1.5 g of soil and seal the reaction vessel.
- Submerge the reaction vessel approximately halfway into a temperature-controlled water bath heated to maintain a constant 37 °C in the reaction vessel.
- Allow the soil/stomach solution to stand (no agitation) for 10 minutes.
- Stir the mixture with a plastic propeller stir rod mounted in a rheostat-controlled motor (Arrow Engineering Model 1750 motor on a rheostat setting of 2, resulting in approximately 150 rpm for the stir rod).
- Check the pH at 5-minute intervals, and readjust to pH 2.0 with HCl if necessary.
- Collect 5-mL samples at 30 and 60 minutes, using a stainless-steel hypodermic syringe to pierce the sampling septum. Filter the samples through a 0.45- $\mu$ m acetate syringe filter.
- At 1 hour, titrate the solution to pH 7.0 by adding a 5-in. length of dialysis tubing (8000 MWCO, cellulose ester tubing) containing approximately 2 g of NaHCO<sub>3</sub> to each reaction vessel.
- Allow the pH of the reaction vessel solution to increase slowly to 7.0  $\pm$ 0.2 before removing the dialysis bag.
- Dissolve 260 mg of bile salts and 75 mg of pancreatin in 10 mL of deionized water and inject the fluid into the reaction vessel.
- Using a stainless-steel hypodermic syringe, obtain a 5-mL intestinal-phase sample through the septum at 1.0 hour after the reaction fluid reaches equilibrium at pH 7. Filter the sample through a 0.45- $\mu$ m filter.
- At 3.0 hours after the reaction fluid reaches pH 7, end the test and collect a final 50-mL sample. Filter the sample through a 0.45- $\mu$ m filter.

- After the final sample is collected, measure and record the pH and final volume of the flask contents.
- Preserve the 5-mL stomach-phase samples with 50  $\mu$ L concentrated nitric acid.
- Refrigerate the samples, and ship on ice to the laboratory.
- Analyze each of the two stomach-phase and the two small-intestinal-phase samples for chromium and mercury concentrations, by the analytical method described in the work plan.

### **Reference**

Ruby, M.V., A. Davis, R. Schoof, S. Eberle, and C.M. Sellstone. 1996. "Estimation of Lead and Arsenic Bioavailability Using a Physiologically Based Extraction Test." *Environ. Sci. Technol.*, 30(2): 422-430.

**APPENDIX E**

**TEMPLATE PROTOCOL FOR DETERMINATION OF THE BIOAVAILABILITY  
OF ARSENIC IN SOIL FOLLOWING ORAL ADMINISTRATION IN  
CYNOMOLGUS MONKEYS**





**TEMPLATE PROTOCOL**  
**for**  
**DETERMINATION OF THE BIOAVAILABILITY OF ARSENIC IN SOIL**  
**FOLLOWING ORAL ADMINISTRATION IN CYNOMOLGUS MONKEYS**

**1.0 PROJECT IDENTIFICATION INFORMATION**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVE**

To determine the bioavailability of arsenic in cynomolgus monkeys following oral administration (via capsules) of a test soil containing arsenic. Bioavailability is estimated from both serial blood samples and urinary excretion data collected from animals dosed orally with soil containing arsenic or with a soluble arsenic form. For use in human health risk assessment, it is necessary to estimate the relative oral bioavailability of soil arsenic compared to a soluble arsenic form. A determination of absolute bioavailability is not needed; however, an intravenous dose group is included so that absolute bioavailability may also be determined. [Note: This protocol includes collection of both blood and urine samples, both of which can be used to estimate bioavailability. For a simpler, less expensive study, blood sampling may be eliminated. The intravenous dose group may also be omitted.]

**3.0 TEST/CONTROL ARTICLE INFORMATION**

3.1 Test Substance Identification

The test substance for this study will be soil samples from the test site. Characterization of the concentration of the arsenic in the soil will be done prior to study by EPA SW-846 Method 7060A using graphite furnace atomic absorption spectroscopy (GFAA) or by EPA SW-846 Method 6010B using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (U.S. EPA, 2001). Moisture content of the sample will be determined by weighing and drying a 5 gram sample for 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the weight of the sample before and after drying. The percentage of organic matter in the test substance will be determined using the method of loss-on-ignition at 430°C until the sample reaches constant weight or has been heated for 24 hours (Davies, 1974). Total element content will

be determined by EPA SW-846 Method 6010 (U.S. EPA, 2001) using ICP-AES for determination of 25 elements. Soil pH will be determined by EPA SW846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either American Society for Testing and Materials (ASTM) Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

Sodium arsenate heptahydrate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ , molecular weight 41.6) will be used as the soluble arsenic reference compound.

### 3.2 Dose Analysis

The dosing solution for the intravenous and gavage administration groups will be analyzed for arsenic by graphite furnace atomic absorption spectroscopy or, if the concentration of arsenic is sufficiently high, the analysis will be conducted by inductively coupled plasma atomic emission spectroscopy (ICP-AES). A sample of the dosing solution will be taken at the time of preparation. Triplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than  $\pm 10$  percent.

### 3.3 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the  $<250\text{-}\mu\text{m}$  size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction  $<250\ \mu\text{m}$ . Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.4 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within  $\pm 5$  percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The sodium arsenate to be used for dosing the intravenous and gavage study groups will be formulated into an aqueous solution. A sufficient quantity of sodium arsenate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for intravenous and oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg. A single batch of sodium arsenate dosing solution will be prepared on one day which will be used to dose all of the intravenous and gavage study group animals.

### 3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights). On the day of dosing, the interval of time between each capsule administration for a given animal will be long enough to allow the animal to completely swallow each capsule and to minimize the possibility of expulsion of the soil. If this occurs with one of the monkeys, then a washout period will be required before the animal is dosed again.

For the intravenous study group animals, the dosing solution will be administered into the saphenous vein using a butterfly infusion set over approximately a one- to three-minute time period.

For the gavage study group animals, the dosing solution will be administered through a rubber feeding tube that has been inserted into the stomach of the animal.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification of Animal Species

The test system selected for this study is the cynomolgus monkey. Monkeys have closer anatomical and physiological similarities to humans than most other species, and this species of monkey has been successfully used to estimate the bioavailability of arsenic in humans.

### 4.2 Justification of the Route of Administration

The oral route of administration was selected because this is the most likely route of human exposure to the soil.

### 4.3 Test Systems

Male adult cynomolgus monkeys weighing approximately 3 to 9 kg will be used (*specify vendor here*).

### 4.4 Animal Health and Quarantine

All animals will be quarantined under environmental conditions according to the standard operating procedure of the testing facility. Each monkey will be examined and its health status determined by a laboratory animal veterinarian prior to being released for dosing. During quarantine, blood samples will be collected from all animals, on 3 specified dates for analysis of prestudy arsenic levels.

### 4.5 Animal Housing

Monkeys will be individually housed in stainless steel cages during quarantine period and transferred to metabolic cages during the study. All housing and care will conform to AAALAC and/or ILAR standards and those published in the "Guide for the Care and Use of Laboratory Animals," NIH Publication No. 85-23. The environmental conditions of the animal study room will conform to the standard operating procedures of the testing facility.

#### 4.6 Diet and Water

During quarantine, monkeys will be fed *ad libitum* Primate<sup>®</sup> chow or equivalent, except when fasted prior to dosing. Animals will be fasted for approximately 16 hrs prior to dosing and food will be presented approximately four hrs after dosing. Feed will be analyzed at the testing laboratory. Triplicate samples of feed will be removed from the batch of feed used. The samples will be digested and single aliquots of the digestate removed for arsenic and phosphorous determinations.

Deionized water will be provided to animals *ad libitum* via water bottles fitted with stainless steel sipper tubes. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed prior to the in-life phase and analyzed for arsenic. Triplicate aliquots of water will be analyzed.

#### 4.7 Animal Identification

All of the animals will be uniquely identified by an indelible ink ear marking or tattoo. Also, each cage will be labeled with the number that corresponds with the ear marking or tattoo of the animal in the cage.

### 5.0 EXPERIMENTAL DESIGN

This study involves using two routes of administration (intravenous and oral) and two dosing formulations (aqueous solution and capsule) for characterizing the bioavailability of arsenic in soil following oral administration. Three monkeys will be used in each treatment group. The intravenous study group will be used to determine the extent of elimination of arsenic in bile.

On Day -1, animals should be weighed in order to determine doses to be used. On Study Day 1, animals will be given the soluble arsenic dosing solution by intravenous injection (intravenous group) or orally using a feeding tube (gavage group). The soil-filled capsules will be administered using a capsule applicator (test soil group). For all groups, samples of whole blood, urine, cage rinse, and feces will be collected from each animal at specified time intervals for 48 hrs (2 days) after administration. In addition, clinical observations will be determined daily for each animal. At the end of the 48 hr in-life period, animals will be removed from the study without additional collection of any biological samples for analysis.

The following table summarizes the study treatment groups:

Aqueous Solution (mg of As/kg BW)		Capsule (mg of As/kg BW)			Number of Animals
IV	Oral	Low	Medium	High	
1.95	1.95	0.78	1.95	3.9	3

## **6.0 SAMPLE COLLECTION**

### **6.1 Blood**

Serial samples of whole blood (approximately 1 mL) will be collected from an appropriate blood vessel prior to dosing and at 2, 5, 10, 15, 30, and 60 min and 2, 4, 8, 12, 16, 24, and 48 hr after administration (intravenous dosing) and prior to dosing and at 15, 30, 45, 60, and 90 min and at 2, 4, 6, 8, 12, 16, 24, and 48 hr after administration (oral dosing). Whole blood will be collected into a heparinized container. Serial blood samples will be used to determine arsenic concentrations.

*[Note: Blood analyses are optional and may not be necessary for some studies.]*

### **6.2 Excreta**

Urine and feces will be collected from each animal prior to dosing and thereafter at 24-hr intervals for 120 hrs. The 24-hr samples will be pooled to provide samples for analyses at 0-24, 24-72, and 72-120 hrs after dosing. The total volume (urine) and amount (feces) of each sample will be recorded. The collected samples will be frozen (approximately -20EC) after collection and kept frozen except during preparation for analysis. The pre-dose samples will be saved for possible analysis at a later date.

### **6.3 Cage Rinse**

Each metabolism cage will be rinsed after each 24-hr urine and feces samples have been collected. Approximately 500 ml of deionized water will be used to remove any residual excreta that adhere to the surface of the metabolism cage. These cage rinse samples will be pooled to provide samples for analyses at 0-24, 24-72, and 72-120 hrs after dosing. The total volume of cage rinse used to wash the cage at each interval will be recorded. Cage rinse samples will be stored frozen (approximately -20EC) until removed for preparation.

## **7.0 SAMPLE PREPARATION/STORAGE**

The intravenous and gavage dosing solutions will be sampled directly without any preparation prior to analysis. Whole blood, urine, and cage rinse specimens will be acidified using nitric acid to avoid precipitation of any arsenic present in the specimen. Feces samples will be weighed and homogenized in a volume of water equivalent to twice the wet weight of the collected sample to produce a uniform feces mixture.

Whole blood will be stored refrigerated at approximately 5EC until removed for analysis. All other biological samples will be stored in their original collection container in a freezer at approximately -20°C until removed for preparation for analysis.

## **8.0 ANALYSIS OF SAMPLES**

All samples will be analyzed for arsenic using graphite furnace atomic absorption spectrophotometry or ICP-AES if the concentration is sufficiently high to warrant this method. Single analyses of whole blood and duplicate analyses of all other biological samples (urine, cage rinse, and feces) will be conducted. Biological samples will be digested in acid, as necessary,

prior to removal of an aliquot for arsenic analysis. When low concentrations of arsenic are found, a sample may have to be concentrated to measure the arsenic levels. Duplicate analyses will be averaged. An additional single analysis will be repeated if values from the original duplicate analyses are disparate (as a general rule, if the duplicate analyses differ by more than 20 percent of the mean) and the concentration is greater than 1 ppm. If the concentration is less than approximately 1 ppm, then the Study Director in conjunction with the Study Chemist will determine whether or not the disparate duplicates warrant additional analyses.

The following list summarizes the approximate numbers of samples per sample type that will be analyzed for arsenic. *[Specify number of samples for each.]*

- I. Non-Biological Samples
  - A. Test Substance
    - Intravenous Sodium Arsenate
    - Oral Sodium Arsenate
    - Oral Soil
  - B. Dosing Solution
  - C. Diet
  - D. Water
  
- II. Biological Samples (for all groups)
  - A. Urine
  - B. Whole Blood (prestudy and study samples)
  - C. Feces
  - D. Cage Rinse
  
- III. Quality Control Samples

## 9.0 STATISTICS

All individual raw data will be summarized and reported. Absolute bioavailability of arsenic in the soil will be determined in two ways: (a) as the percent of arsenic excreted in the urine of the capsule group animals compared to the intravenous group and (b) by comparing the areas under the plasma concentration time AUC curves for the oral and intravenous routes of administration. Relative oral bioavailability of soil arsenic compared to soluble arsenic may be determined by dividing the absolute bioavailability of soil arsenic (derived by either method) by the absolute bioavailability of soluble arsenic. Alternatively, relative bioavailability may be calculated directly by comparing the AUCs or urinary excretion for the two oral dosage forms, without first calculating absolute bioavailability.

In calculating the AUCs from blood concentrations, values will be corrected for background (predose). The equation that will be used to calculate bioavailability values based on blood will be:

$$\frac{\text{AUC for oral treatment}}{\text{AUC for intravenous treatment}} \times \frac{\text{Total administered dose for intravenous treatment (mg/kg)}}{\text{Total administered dose for oral treatment (mg/kg)}} \times 100$$

Relative bioavailability ( $RAF_x$ ) of soil arsenic (X1) compared to soluble arsenic (X2) may be calculated directly using the following equation with blood data:

$$RAF_x = \frac{AUC_{X1}}{Dose_{X1}} \times \frac{Dose_{X2}}{AUC_{X2}}$$

Bioavailability values based on urine will be determined according to the following equation:

$$\frac{\text{Total amount of As in urine } (\mu\text{g}) \text{ for oral group}}{\text{Total amount of As in urine } (\mu\text{g}) \text{ for intravenous group}} \times \frac{\text{Total administered dose for intravenous group (mg/kg)}}{\text{Total administered dose for oral group (mg/kg)}}$$

Relative bioavailability may be calculated directly from urine data by substituting data from the group receiving a soluble arsenic form orally for the intravenous data in the equation above.

## 10.0 RECORD AND SAMPLE RETENTION

### 10.1 Sample Retention

All samples will be frozen (approximately -20EC) and retained frozen until final report.

### 10.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on arsenic concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis

- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 10.3 Report

A written draft final report of this study will be submitted to the Sponsor within (*specify*) days of the sacrifice date of the last animals.

## 11.0 REFERENCES

Davies, B.W. 1974. Loss-on ignition as an estimate of soil organic matter. Soil Sci. Soc. Am. Proc. 38:150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency. [www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).



**APPENDIX F**

**TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF ARSENIC AND LEAD IN SOIL  
FOLLOWING ORAL ADMINISTRATION USING JUVENILE SWINE**



**TEMPLATE PROTOCOL**  
**for**  
**BIOAVAILABILITY STUDY OF ARSENIC AND LEAD IN SOIL**  
**FOLLOWING ORAL ADMINISTRATION USING JUVENILE SWINE<sup>1</sup>**

**1.0 PROJECT IDENTIFICATION INFORMATION**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVES**

The objectives of this study will be to use juvenile swine as a test system to determine the oral bioavailability of arsenic and lead in soil contaminated with arsenic and lead relative to the bioavailability of soluble forms of arsenic and lead. These relative bioavailability estimates are anticipated to be used in human health risk assessments. The relative bioavailability of arsenic will be determined based on urinary arsenic excretion after 15 days of daily dosing. The relative bioavailability of lead will be determined based on blood and tissue lead concentrations after 15 days of dosing. Relative bioavailability of arsenic and lead in soil will be estimated by comparison to data from swine dosed with sodium arsenate and lead acetate, respectively, for approximately 15 days.

*Note: This template protocol applies to sites where both arsenic and lead concentrations are elevated in soil. Elements of this protocol may be adapted to test only arsenic or only lead, if only one of these metals is of concern at a site.*

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<sup>1</sup> Based on methods developed by Dr. Stan Casteel and others (Casteel et al., 1997).

### 3.0 TEST SUBSTANCES

#### 3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Before the study, soil arsenic and lead will be characterized and concentrations will be determined. If desired, mineral forms of arsenic and lead also will be determined. (*Note: Methods of determining the speciation of metals are discussed in the text of the main document.*)

For the soluble arsenic-dosed study group animals, sodium arsenate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ , MW 41.6) will be used to administer appropriate doses of water-soluble forms of arsenic. For the soluble lead-dosed study group animals, lead (II) acetate trihydrate ( $[\text{CH}_3\text{CO}_2]_2\text{Pb} \cdot 3\text{H}_2\text{O}$ , MW 379.33) will be used to administer appropriate doses of water-soluble forms of lead.

#### 3.2 Test Substance Analysis

##### 3.2.1 Concentration

The concentrations of arsenic and lead in the test substances will be determined. Analysis of the test substances will involve extracting arsenic and lead from a sample of each test substance, digesting the extracted material, and measuring the concentrations of arsenic and lead by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of arsenic and lead in the soil will be determined by digesting triplicate aliquots of the sample and analyzing single aliquots of the digestate.

##### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

##### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

##### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu\text{m}$  size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250  $\mu\text{m}$ . Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

## 3.3 Dosing Formulation Preparation

### 3.3.1 Soluble Arsenic and Lead Formulations

The appropriate amount of sodium arsenate or lead acetate stock solution is mixed with a 5g ( $\pm$  1g) mass of moistened feed (“doughball”). The feed is a special low-lead variety (guaranteed less than 0.2 ppm lead by the manufacturer, Zeigler Brothers, Inc., Gardners, PA). Mixture with the doughball is achieved by placing the test material in a small depression in the doughball. After the stock solution has permeated into the doughball and no free liquid remains, the depression is filled by squeezing the dough ball in on itself, and the doughball is administered to the animal by hand feeding.

All animals in each dose group will receive the same volume of sodium arsenate and lead acetate stock solution, based on the mean body weight of all animals in the group. The precise dose to each animal will subsequently be calculated from the individual measured body weights. The volume of the stock solution placed in the dough balls of each dose group (twice each day) will be calculated using the following equation:

$$Vol = 0.5 \left( \frac{MBW \times Dose}{Conc} \right)$$

where:

Vol = Volume of stock solution ( $\mu\text{L}$ )

MBW = Mean body weight (kg)

Dose = Target dose for the group ( $\mu\text{g}/\text{kg}\cdot\text{d}$ )

Conc = Concentration of arsenic or lead in stock solution ( $\mu\text{g}/\mu\text{L}$ )

Three stock solutions of sodium arsenate will be prepared at concentrations that will result in target dose concentrations of 25, 50, and 125 µg/kg when a volume of stock solution between 20 µL and 100 µL is added to the doughball. The concentration of lead acetate stock solutions will be determined based on target doses of 25, 75, and 225 µg/kg.

### 3.3.2 Soil Formulation

The required mass of soil is placed in a small depression in a 5-g (± 1g) doughball. The depression is filled by squeezing the doughball in on itself, trapping the test material in the center. If the mass of soil required is too large to encapsulate into a single doughball, the soil will be divided into approximately equal portions and placed in the minimum number of doughballs required to contain the soil.

All animals in each dose group will receive the same mass of test material, based on the mean body weight of all animals within the dose group. The precise dose to each animal will subsequently be calculated from the individual measured body weights. The mass of soil placed in the dough balls of each dose group (twice each day) will be calculated using the following equation:

$$\text{Mass} = \frac{1}{2} \left( \frac{\text{MBW} \times \text{Dose}}{\text{Conc}} \right) (1,000 \mu\text{g/kg})$$

where:

Mass = Mass of soil (mg)

MBW = Mean body weight (kg)

Dose = Target dose for the group (µg/kg-d)

Conc = Concentration of arsenic or lead in soil (µg/g)

## 3.4 Dose Analysis

### 3.4.1 Dosed Feed Concentration and Stability

At least two extra dough balls (or sets of doughballs if more than one doughball is required to administer the soil) will be prepared for each dose “batch” (a “batch” is a group of doughballs sufficient for three days administration). After all doughballs in the batch are prepared, two will be selected at random, wrapped individually in plastic wrap, and placed together in a plastic bag labeled with the appropriate group/treatment identification number. All dose verification samples will be stored in the freezer until the end of the study. At the end of the study, at least 5% of verification samples will be randomly selected for analysis.

### 3.4.2 Test Article Homogeneity

It is expected that the bulk soil sample will be non-homogeneous with respect to particle size, and the concentration and form of lead and arsenic is expected to vary as a function of particle size. Therefore, it is important that the soil be well-mixed

prior to removal of the dose aliquots. This is achieved by placing the bottle containing the bulk soil sample on a roller operating at low speed for about 30 minutes. After rolling, the bottle should be further mixed by inverting five times. It is important that vigorous methods of mixing not be used, since this might lead to an alteration of the particle size distribution.

#### 3.4.3 Dose Administration

Animals will be dosed twice daily for 15 days at 9 a.m. and 3 p.m., 2 hours before feeding.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be male juvenile swine. Juvenile swine were selected for use in the study because the gastrointestinal physiology and overall size of young swine are similar to that of young children, who are the population of prime concern for exposure to metals in soil. Additionally, studies of the bioavailability of soil arsenic and lead have previously been conducted in swine. Young swine will be used because lead bioavailability decreases with age after weaning. Use of juveniles should maximize lead absorption. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

### 4.2 Test System

Intact male swine of a genetically defined line, approximately 5-6 weeks of age at initiation of dosing will be obtained from an appropriate vendor in sufficient numbers to provide the required number of healthy animals for testing (approximately 10% more than the number of animals to be tested). The target body weight at purchase will be 7-8 kg. The number of animals to be tested will be 50.

### 4.3 Animal Health and Quarantine

Animals will be held under quarantine to observe their health for one week before beginning exposure to test materials. Swine chosen for each investigation will be monitored throughout the investigation to identify any evidence of disease. The monitoring program will consist of the following elements:

- Daily observation by the Principal Investigator or designated assistant, with consultation as needed by a board-certified food-animal clinician. Observations for each animal will be recorded daily on a health-status chart attached to the cage of each animal. Observations will be generally similar to the "SOAP" (subjective, objective, analysis, plan) process. If any intervention is taken for an animal (e.g., administration of antibiotics), this action shall also be recorded on the chart for that animal.
- Any animal that dies during the study period will have a thorough post-mortem examination conducted to determine the cause of death. The post-mortem examination will include gross and histologic examinations and any ancillary tests,

such as microbiology, deemed appropriate by the veterinary pathologist. All observations and findings will be recorded.

- Veterinary records from the swine producer and the producer's veterinarian, including documentation of health status, will be available if needed to assess overall swine herd health, history of vaccinations, and other veterinary data.
- Blood samples will be collected for clinical chemistry and hematological analysis on days -4, 7, and 15 to assist in clinical health assessments. Any animals that do not appear healthy or are not growing at the same rate as the other animals will be excluded from the investigation. Animals judged to be seriously ill by the attending veterinarian will be removed from the study.

#### 4.4 Animal Housing

Animals will be individually housed in lead-free, stainless steel, metabolic cages. Metabolic cages are designed to collect and separate urine and feces.

#### 4.5 Diet and Water

Animals will be provided with 100% of their recommended daily food requirements. This will be achieved by supplying each animal with food equivalent to 5% of its body weight each day, in two equal portions at 11:00 AM and 5:00 PM. Since the animals are expected to grow significantly (0.3 to 0.8 kg/day) over the investigation period, the food portions must be constantly adjusted upward over time. Two samples of each batch of feed delivered will be analyzed prior to usage to confirm low lead and arsenic concentrations. A swine nutritionist will review the dietary composition. Feed will be purchased from Ziegler, Inc. (Gardners, PA) and detailed analysis of the composition will be provided with each lot purchased.

Food portions will be weighed every three days into disposable paper containers. The total number of portions weighed will be six times the number of animals in the study (two portions per day for each of three days). The mean body weight at each three-day interval will be used to calculate food intake for the following three days, adjusted by expected weight gain between weighings. Specifically, the twice daily food portion will be calculated as follows:

On the day of the weighing,

$$Portion (g) = \left(\frac{1}{2}\right)(0.05)(body\ weight\ in\ kg)\left(1,000\ \frac{g}{kg}\right)$$

This size portion will be used for the following three days, and then adjusted again in a similar manner.

Water will be provided to animals *ad libitum* via a pipe and nozzle which is activated by the animal. Laboratory technicians will check each day to ensure that all water delivery nozzles are functioning properly. The water source will be a municipal drinking water system. One water sample will be drawn at random from a drinking water nozzle once per week during the study and analyzed for lead and arsenic.



#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Animals will be randomly assigned to treatment groups by the following method:

- A list of animals will be prepared by ear tag number order.
- Random numbers will be generated by a computer and these numbers assigned to each animal's ear tag number.
- Animals will be sorted sequentially by assigned random number.
- The first five animals will be assigned to group 1, the next to group 2, etc...
- Animals will be sorted sequentially within assigned group by ear tag number.

## 5.0 EXPERIMENTAL DESIGN

This study involves subchronic oral administration of one soil arsenic source and one soil lead source mixed with diet as a means of characterizing the oral bioavailability of arsenic and lead in soil relative to soluble arsenic and lead. A non-treated group will serve as a control for determining background arsenic and lead levels. Five animals will be used in each treatment group. Specifically, the following groups will be studied.

Group	Number of Animals	Dose Material Administered	Target Dose ( $\mu\text{g}/\text{kg}\cdot\text{d}$ )
1	5	Control	0
2	5	Lead Acetate	Pb Dose 1
3	5	Lead Acetate	Pb Dose 2
4	5	Lead Acetate	Pb Dose 3
5	5	Sodium Arsenate	As Dose 1
6	5	Sodium Arsenate	As Dose 2
7	5	Sodium Arsenate	As Dose 3
8	5	Soil with As&Pb	As/Pb Dose 1
9	5	Soil with As&Pb	As/Pb Dose 2
10	5	Soil with As&Pb	As/Pb Dose 3

Doses will be administered in two equal portions given at 9:00 AM and 3:00 PM each day. Doses will be based on the mean weight of the animals in each group, and will be adjusted every three days to account for weight gain.

*The main text describes appropriate target doses for arsenic and lead in the respective sections addressing each metal. It may not be possible to achieve targets for both metals in one soil sample.*

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

### 5.1 Clinical Observations

Animals will be examined daily as described in Section 4.3. Clinical observations of possible signs of toxicity will be recorded.

### 5.2 Food Consumption

Food portions will be calculated based on the mean body weight of all animals in the study. Any fraction of food or doughball not eaten will be recorded and dosage will be corrected to reflect actual intake after completion of the study.

### 5.3 Body Weights

Animals will be weighed every three days beginning at day -1 of the study. Animals will also be weighed on the day of sacrifice. All body weights will be recorded in the laboratory log book to the nearest 0.1 kg.

### 5.4 Tissue Collection

Blood samples will be collected at 8:00 AM from each animal four days prior to exposure (day -4), on the first day of exposure (day 0), and on days 1, 2, 3, 5, 7, 9, 12, and 15. All blood samples will be collected into purple-top Vacutainer tubes containing EDTA by vena-puncture of the anterior vena cava. Following euthanization on day 15, samples of liver, kidney, and bone (the right femur) will be removed and stored in lead-free plastic bags for lead analysis. Samples of all biological samples collected will be archived to allow for later reanalysis and verification, if necessary.

Urine and feces samples (48 hour composites) will be collected from each animal on days 6-7, 8-9, and 10-11 of the study, beginning at either 9:00 or 10:00 AM on the first day of the collection period. Urine will be collected by placing a stainless steel pan beneath each cage that drains into a plastic storage bottle. Each collection pan will be fitted with a nylon screen to minimize contamination with feces, spilled food, or other debris. Plastic diverters will be used to minimize urine dilution with drinking water spilled by the animals from the watering nozzle into the collection pan. During the collection period, urine will be removed from the collection pans at least twice daily and stored in a separate container for each animal.

## 6.0 SAMPLE PREPARATION

### 6.1 Blood

One mL of whole blood is removed from the Vacutainer and added to 9 mL of “matrix modifier”, a solution recommended by the Centers for Disease Control and Prevention (CDCP) for analysis of blood samples for lead. The composition of matrix modifier is 0.2% (v/v) ultrapure nitric acid, 0.5% (v/v) Triton X-100, and 0.2% (w/v) dibasic ammonium phosphate in deionized and ultrafiltered water. Samples of the matrix modifier will be analyzed for lead to ensure the absence of lead contamination.

## 6.2 Liver and kidney

One gram of tissue is placed in a lead-free screw-cap Teflon container with 2 mL of concentrated nitric acid and heated in an oven to 90°C overnight. After cooling, the digestate is transferred to a clean lead-free 10 mL volumetric flask and diluted to volume with deionized and ultrafiltered water.

## 6.3 Bone

The right femur of each animal is removed, defleshed, and dried overnight at 100°C. The dried bones are dry-ashed in a muffle furnace at 450°C for 48 hours. Following dry ashing, the bone is ground to a fine powder using lead-free mortar and pestle, and 200 mg is removed and dissolved in 10 mL of 1:1 (v:v) concentrated nitric acid:water. After the powdered bone is dissolved and mixed, 1 mL of the acid solution is removed and diluted to 10 mL by addition of 0.1% (w/v) lanthanum oxide (La<sub>2</sub>O<sub>3</sub>) in deionized and ultrafiltered water.

## 6.4 Urine

The 48-hour urine samples will be mixed by swirling in the collection vessels and the volume measured in a graduated cylinder. Three 60-mL aliquots of urine will be retrieved from the samples, placed in capped plastic urine storage bottles and acidified by addition of 0.6 mL of concentrated nitric acid. Two bottles will be archived in the refrigerator, the last sent to the laboratory for analysis.

## 6.5 Feces

*(Note: It may be advantageous to collect feces and analyze samples for arsenic concentrations. Sample preparation methods would need to be developed.)*

## 7.0 ANALYSIS OF SAMPLES

All urine, blood, tissue, and bone samples will be analyzed for arsenic (urine) or lead (blood, tissue and bone) by graphite furnace atomic absorption spectroscopy (GFAA). Internal quality assurance samples will be run every tenth sample, and the instrument recalibrated every 15<sup>th</sup> sample. A blank, duplicate and spiked sample will be run every 20<sup>th</sup> sample.

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation for each dose group. The relative bioavailability (RBA) of lead will be calculated for each dose group based on the blood lead results. The following method will be used to calculate an RBA:

1. Plot the biological responses of individual animals exposed to a series of doses of lead acetate. This is done by first calculating the area under the curve (AUC) of the blood lead vs. time response for each animal. Then calculate the dose group mean and standard error for each dose group. Plot group mean blood lead AUC vs. dose. Fit an equation that gives a smooth line through the observed data points.

2. Plot the biological responses of individual animals exposed to a series of doses of lead in the test material in the same way as for lead acetate. Fit an equation that gives a smooth line through the observed data.
3. Using the best fit equations for lead acetate and the test material, calculate RBA as the ratios of doses of test material and reference material which yield equal biological responses. Depending on the relative shape of the best-fit lines through the lead acetate and test material dose response curves, RBA may either be constant (dose-independent) or variable (dose-dependent). If both curves are linear, RBA equals the ratio of slopes of the test material curve to the lead acetate curve.

The amount of arsenic absorbed will be evaluated by measuring the amount of arsenic which was excreted in urine, the Urinary Excretion Fraction (UEF). UEF is estimated by plotting mass recovered in urine per 48 hours divided by the amount given per 48 hours. The RBA equals the ratio of the test material UEF to the sodium arsenate UEF:

$$\text{RBA} = \text{UEF}_{\text{test}} / \text{UEF}_{\text{NaAs}}$$

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on lead concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data

- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within a mutually agreed upon timeframe following completion of the dosing experiment.

## 11.0 REFERENCES

Casteel, S.W., R.P. Cowart, C.P. Weis, G.M. Henningsen, E. Hoffman, W.J. Brattin, R.E. Guzman, M.F. Starost, J.T. Payne, S.L. Stockham, S.V. Becker, J.W. Drexler, and J.R. Turk. 1997a. Bioavailability of lead to juvenile swine dosed with soil from the Smuggler Mountain NPL site of Aspen, Colorado. *Fund. Appl. Toxicol.* 36:177–187.

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis*. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency. [www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).

**APPENDIX G**

**TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF CADMIUM IN  
SOIL FOLLOWING ORAL ADMINISTRATION USING SPRAGUE-DAWLEY  
RATS**





**TEMPLATE PROTOCOL**  
**for**  
**BIOAVAILABILITY STUDY OF CADMIUM IN SOIL FOLLOWING ORAL**  
**ADMINISTRATION USING SPRAGUE-DAWLEY RATS**

**1.0 PRINCIPALS**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVE**

The objective of this study will be to use Sprague-Dawley rats as a test system to determine the relative oral bioavailability of cadmium in soil compared to that of soluble cadmium forms. These relative bioavailability estimates will be used in human health risk assessments. Relative bioavailability of cadmium in soil will be estimated by comparing blood cadmium concentration data from the rats dosed with soil administered via gelatin capsules with similar data from rats administered a single oral (gavage) dose of cadmium chloride.

**3.0 TEST SUBSTANCES**

3.1 Test Substance Identification

The test substances for this study will be capsules of soil samples collected from test sites. Soil cadmium will be characterized, and concentration, stability and purity determined before the study.

An appropriate dose of CdCl<sub>2</sub> solution will be used as a reference standard for comparison with the test soil group.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of cadmium in the test substances will be determined. Analysis of the test substances will involve extracting cadmium from a sample of each test substance, digesting the extracted material, and measuring the concentrations of cadmium by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of cadmium in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of cadmium will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu\text{m}$  size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250  $\mu\text{m}$ . Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical*

*Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The cadmium chloride reference standard to be used for dosing the gavage study groups will be formulated into an aqueous solution. A sufficient quantity of cadmium chloride will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

### 3.4 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for cadmium by graphite furnace atomic absorption spectroscopy or, if the concentration of cadmium is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than  $\pm 10$  percent.

### 3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be Sprague-Dawley (Cd/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil cadmium have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (Cd/BR) rats, 8 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. (*Specify the number of animals here*)

#### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

#### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

#### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Feed will be provided *ad libitum* in glass jars. Feed will be withheld from animals for 16 hours prior to oral dosing. Two hours after dosing, the animals can be allowed free access to food. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed for cadmium, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for cadmium, calcium, magnesium, iron, zinc, and phosphorous.

#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined on Day -3. Randomization will be performed using a computer program that places animals in groups to ensure homogeneous group mean body weights at study onset.

### 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of two cadmium sources (soluble cadmium chloride and cadmium in soil), as a means of characterizing the oral bioavailability of cadmium in soil relative to soluble cadmium. A non-treated group will serve as a control for determining background cadmium levels. On Day -3, rats will be weighed in order to determine doses to be used. On Study Day 1, rats will be given the cadmium chloride solution by gavage using a stainless steel gavage needle. The soil-filled capsules will be administered using a capsule applicator (test soil group). Control group will receive orally administered saline only. For all groups, samples of whole blood will be collected from each animal at specified intervals for 6 days after administration of test article or control saline.

The following table summarizes the treatment groups.

Group	Treatment	Number of Animals <sup>a</sup>
1	Control	32 <sup>b</sup>
2	CaCl <sub>2</sub>	32 <sup>b</sup>
3	Soil	32 <sup>b</sup>

<sup>a</sup> One animal may be subjected to as many as 3 bleeds. (Example: one animal may be bled at 10 min, 24 hr and 72 hr.)

<sup>b</sup> Suggested number of animals.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

#### 5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under CO<sub>2</sub> anesthesia. Heparinized blood samples will be collected at 0, 10, 20, 30, 60, 120, 240 and 480 min on the first day and at 24, 48, 72, 96, 120 hr and stored at -20°C until analysis.

#### 5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done twice daily. Cage checks will be made once a day for moribundity and mortality.

#### 5.3 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion of the approximately 24-hour interval, feeders will be reweighed. The net difference

between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

#### 5.4 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination (Day 6).

### 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

### 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for cadmium by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for cadmium analysis.

Quality control (QC) samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for cadmium in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of cadmium from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for cadmium: *[Specify number of samples for each.]*

<i>I. Non-Biological Samples</i>
A. Test substance
B. Diet
C. Water

<i>II. Blood Samples</i>
Treatment Group:
A. Untreated control
B. Cd-saline
C. Capsule-Cd

<i>III. Quality Control Samples</i>
-------------------------------------

## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$F_{\text{rel}} = (AUC_{\text{soil}} / AUC_{\text{pure}}) \times \frac{\text{dose pure}}{\text{dose soil}}$$

where  $AUC_{\text{soil}}$  is area under the curve for the capsule-Cd group and  $AUC_{\text{pure}}$  is the area under the curve for the Cd-saline group.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on cadmium concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol

- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within \_\_ days (*specify*) of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency.  
[www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).



## **APPENDIX H**

**TEMPLATE PROTOCOL FOR PILOT STUDY:  
BIOAVAILABILITY OF CHROMIUM IN SOIL FOLLOWING  
ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS**



*(Note: Because no reliable study design has been developed for assessing bioavailability of chromium in soil, any planned study should begin with a pilot study using a small number of animals.)*

**TEMPLATE PROTOCOL FOR  
PILOT STUDY:**

**BIOAVAILABILITY OF CHROMIUM IN SOIL FOLLOWING ORAL  
ADMINISTRATION USING SPRAGUE-DAWLEY RATS**

**1.0 PRINCIPALS**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVE**

Several objectives will be accomplished in this study: a) To determine the half-life of chromium in weanling rats; b) To determine time to reach chromium peak plasma concentration in the weanling rat; c) To determine the relative oral bioavailability of chromium in soil compared to that of soluble chromium forms. These relative bioavailability estimates will be used in human health risk assessments.

**3.0 TEST SUBSTANCES**

3.1 Test Substance Identification

The test substances for this study will be capsules of soil samples collected from test sites. Soil chromium will be characterized, and concentration, stability and purity determined before the study.

A mixture of chromium oxide and potassium chromate in the same proportions as  $\text{Cr}^{+3}$  +  $\text{Cr}^{+6}$  in soil will be used as the reference standard.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of chromium in the test substances will be determined. Analysis of the test substances will involve extracting chromium from a sample of each test substance, digesting the extracted material, and measuring the concentrations of chromium by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of chromium in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of chromium will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu$ m size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250- $\mu$ m. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical*

*Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

Particle size distribution of the test soil will be determined using an electrozone sensor in an Elzone<sup>®</sup> 280PC System (Particle Data Laboratories, Elmhurst, Illinois) or similar instrument. Briefly, particles will be suspended in an electrolyte solution and drawn through an orifice in which a constant current has been established. As the particle traverses the orifice, it will displace a quantity of suspended electrolyte proportional to the volume of the particle. The resulting change in electrical resistance across the orifice will create a voltage pulse. These pulses will be amplified, scaled, and counted. From these data, particle size distributions will be generated.

### 3.3 Dose Preparation

Gelatin capsules will be used to administer the test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The reference standard will be formulated as an aqueous solution and will be administered by gavage to the animals. A sufficient quantity of a mixture of chromium oxide and potassium chromate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

### 3.5 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for chromium by graphite furnace atomic absorption spectroscopy or, if the concentration of chromium is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than  $\pm 10$  percent.

### 3.6 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (Cd/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the chromium uptake and distribution have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (Cd/BR) rats, 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. A total of 24 rats will be used for this study.

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Feed will be provided *ad libitum* in glass jars. Feed will be withheld from animals for 16 hours prior to oral dosing. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed for chromium, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for chromium, calcium, magnesium, iron, zinc, and phosphorous.

#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined on Day -3. Randomization will be performed using a computer program that places animals in groups to ensure homogeneous group mean body weights at study onset.

### 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of two chromium sources [a mixture of potassium chromate and chromium oxide (the reference standard) and chromium in soil], as a means of characterizing the oral bioavailability of chromium in soil relative to soluble chromium. On Day -3, rats will be weighed in order to determine doses to be used. Rats will be given the potassium chromate/chromium oxide solution by gavage using a stainless steel gavage needle for 35 days. The soil-filled capsules will be administered using a capsule applicator (test soil group). For all groups, samples of whole blood will be collected from each animal at specified intervals for approximately 2 days after the last day of administration of test article or reference standard.

The following table summarizes the treatment groups.

Group	Treatment	Number of Animals <sup>a</sup>
1	Reference Standard	12
2	Soil	12

<sup>a</sup>One animal may be subjected to as many as 2 bleeds. (Example: one animal may be bled at 0 hr and 24 hr.)

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

#### 5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under CO<sub>2</sub> anesthesia. Heparinized blood samples will be collected at 2, 4, 8, 16, and 24 hr post-test article administration and stored at -20°C until analysis.

Other tissues that can be collected and saved for later analysis include liver, kidney, spleen and bone (femur).

#### 5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done during the study period.

#### 5.3 Food Consumption

No food consumption estimates will be made.

#### 5.4 Body Weights

Body weights will be taken on all rats at the start of the study.

### 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

### 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for chromium by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for chromium analysis.

Quality control samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for chromium in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of chromium from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for chromium: (*Specify number of samples for each*)

<i>I. Non-Biological Samples</i>
A. Test substance
B. Diet
C. Water

<i>II. Blood Samples</i>
Treatment Group
A. Reference standard
B. Chromium (soil-filled capsule)

<i>III. Quality Control Samples</i>
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## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$Rf = \frac{C_{\max} \text{ (soluble chromium)} - BKG_{\text{Dose (soluble chromium)}}}{C_{\max} \text{ (soil chromium)} - BKG_{\text{Dose (soil chromium)}}}$$

where BKG = overall background value from feed and water analysis and  $C_{\max}$  = peak chromium concentration.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on chromium concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol

- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within \_\_ days (*specify days*) of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis*. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency.  
[www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).

**APPENDIX I**

**TEMPLATE PROTOCOL FOR BIOAVAILABILITY OF  
LEAD IN SOIL FOLLOWING ORAL ADMINISTRATION  
USING WEANLING SPRAGUE-DAWLEY RATS**



**TEMPLATE PROTOCOL**  
**for**  
**BIOAVAILABILITY STUDY OF LEAD IN SOIL FOLLOWING**  
**ORAL ADMINISTRATION USING WEANLING**  
**SPRAGUE-DAWLEY RATS**

**1.0 PRINCIPALS**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVES**

The objectives of this study will be to use weanling Sprague-Dawley rats as a test system to determine the relative oral bioavailability of lead in soil compared to that of soluble lead forms. These relative bioavailability estimates will be used in human health risk assessments. Relative bioavailability of lead in soil will be estimated by comparing blood lead concentrations in rats dosed orally with lead-containing soil in capsules for 48 days to similar data from rats administered lead acetate in capsules for approximately 48 days.

**3.0 TEST SUBSTANCES**

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil lead will be characterized, and concentration, stability and purity determined before the study.

Reference standard consists of soluble lead (II) acetate trihydrate ( $[\text{CH}_3\text{CO}_2]_2\text{Pb}\cdot 3\text{H}_2\text{O}$ ) administered in gelatin capsules. A non-treated group will be used as a control for determining background lead levels.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of lead in the test substances will be determined. Analysis of the test substances will involve extracting lead from a sample of each test substance, digesting the extracted material, and measuring the concentrations of lead by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of lead in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of lead will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu$ m size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250  $\mu$ m. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

A specified amount of the reference standard will be transferred into gelatin capsules and assembled as described above.

### 3.4 Dose Administration

Capsules will be administered to the animals once per day for 48 days using a small animal capsule applicator. The dose will be based upon individual animal body weights, which will be determined just prior to dosing (fasted body weights). Animals will be dosed just prior to the lights out cycle to minimize the amount of food in the stomach at the time of dosing.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil lead have previously been conducted in rats. Weanlings (approximately 4 weeks old) will be used because lead bioavailability decreases with age after weaning. Use of weanlings should maximize lead absorption. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

Since the absolute bioavailability of lead is much lower in rats than in humans, this test system is only useful in estimating relative bioavailability when used in human health risk assessments.

### 4.2 Test System

Male and female Sprague-Dawley (CD/BR) rats, approximately 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. The number of animals on test will be 40 males and 40 females.

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory

animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

#### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 64-79°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

#### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. The complete AIN-93G™ meal feed will be refrigerated at approximately 4°C and will have expiration dates of approximately 4 months after milling. Feed will be provided *ad libitum* in glass jars. Feeders with fresh feed will be provided at least biweekly. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at a designated laboratory for lead, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for lead, calcium, magnesium, iron, zinc, and phosphorous.

#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.



## 5.0 EXPERIMENTAL DESIGN

This study involves subchronic daily administration of lead (soluble lead acetate) and test soil (soil lead) administered in gelatin capsules, as a means of characterizing the relative bioavailability of lead. A non-treated group will serve as a control for determining background lead levels. Forty rats per sex will be used in each treatment group. Animals will be administered test soil or soluble level for 48 days.

Treatment Group	Dose Levels <sup>a</sup>	
	Pb (µg)	mg Pb/kg BW
Untreated (control)	NA	NA
Soluble lead in capsule	Low	TBD
	Medium	TBD
	High	TBD
Test soil in capsule	Low	TBD
	Medium	TBD
	High	TBD
<sup>a</sup> The estimated doses of lead will be based on estimated soil lead concentrations. NA = Not applicable. TBD = To be determined.		

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

### 5.1 Clinical Observations

Clinical observations of any possible signs of toxicity will be recorded. Otherwise, cage checks will be made once a day for moribundity and mortality.

### 5.2 Food Consumption

Food consumption will be determined weekly for each rat. Known amounts of feed will be provided in cage feeders, and at the conclusion of the approximately 7-day interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

### 5.3 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination.

### 5.4 Tissue Collection

At termination, and prior to cessation of heart contractions following an injection of sodium pentobarbital, a whole blood sample will be collected from each rat by cardiac puncture. The blood will be transferred to an appropriate container and stored frozen approximately

(-20°C) until prepared for analysis. The kidneys will be removed and stored in polyethylene tissue bags or liquid scintillation vials with plastic tops at approximately -20°C until removed for preparation and analysis. In addition, the femur and liver will be removed and stored in polyethylene tissue bags or scintillation vials at approximately -20°C until removed for possible preparation and analysis at a later date if needed. A wet weight of the tissues upon removal will be collected. The residual carcass will be saved and stored frozen for possible additional tissue sample collection and analysis. Samples will be shipped to (*specify name of test facility*) for analysis.

## 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for lead by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for lead analysis.

Quality control samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for lead in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of lead from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for lead: (*Specify number of samples for each.*)

<i>I. Non-Biological Samples</i>
A. Test substance (soil lead)
B. Soluble lead
C. Diet
D. Water

<i>II. Biological Samples</i>
Treatment Group:
A. Untreated control
B. Soluble lead
C. Test soil

<i>III. Quality Control Samples</i>
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## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation.

Relative bioavailability can be calculated by the following equation:

$$Rf = \frac{C_{\min (\text{soluble lead})} - \text{BKG}_{\text{Dose (soluble lead)}}}{C_{\min (\text{soil lead})} - \text{BKG}_{\text{Dose (soil lead)}}}$$

where:  $C_{\min}$  = mean minimum concentration of lead  
BKG = overall background value from feed and water analysis.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on lead concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories

- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within days (specify) of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency.  
[www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).

**APPENDIX J**

**TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF MERCURY IN  
SOIL FOLLOWING DOSED FEED ADMINISTRATION USING WEANLING  
SPRAGUE-DAWLEY RATS**



**TEMPLATE PROTOCOL**  
**for**  
**BIOAVAILABILITY STUDY OF MERCURY IN SOIL FOLLOWING**  
**DOSED FEED ADMINISTRATION USING WEANLING SPRAGUE-DAWLEY RATS**

**1.0 PRINCIPALS**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVES**

The objectives of this mercury study will be to use blood mercury concentrations to determine the relative bioavailability of mercury in weanling Sprague-Dawley rats as a test system to determine the relative oral bioavailability of mercury in soil compared to that of soluble mercury forms. These relative bioavailability estimates will be used in human health risk. Relative bioavailability of mercury in soil will be estimated by comparing blood mercury concentrations in rats dosed orally for 30 days with mercury-containing soil mixed with feed to similar data from rats fed mercuric chloride-containing feed for approximately 30 days.

**3.0 TEST SUBSTANCES**

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil mercury will be characterized and concentration, stability and purity determined before the study.

For the soluble mercury-dosed feed group, HgCl<sub>2</sub> will be used to administer appropriate doses of water-soluble forms of mercury.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of mercury in the test substances will be determined. Analysis of the test substances will involve extracting mercury from a sample of each test substance, digesting the extracted material, and measuring the concentrations of mercury by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of mercury in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of mercury will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until constant weight or after heating for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu$ m size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250  $\mu$ m. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical*



*Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dosing Formulation Preparation

The appropriate amount of test soil (dosed feed test soil group) or HgCl<sub>2</sub> to be mixed with AIN-93G™ complete meal to make approximately 20-25 kg of diet will be determined for each dose group. Approximately 2 kg premix will be prepared by adding the calculated amount of test soil or HgCl<sub>2</sub> to an equal amount of AIN-93G™ complete meal feed. A sufficient amount of the HgCl<sub>2</sub> to be used for the dosed feed soluble mercury group may be dissolved in deionized water. The mixture will be stirred with a spatula, and more of the AIN-93G™ feed will be added until the premix totals nearly 2 kg. Approximately 10 kg of the AIN-93G™ feed will be placed in the bottom of a Patterson-Kelley blender (or equivalent), the premix will be layered roughly equally between the two blender ports, and approximately 12 kg more of the AIN-93G™ feed will be added to the blender. The blender will be operated for 5 minutes with the intensifier bar on, and an additional 10 minutes with the intensifier bar off. The corners of the blender will be tapped during preparation to minimize the possibility that feed will be compacted in the corners and prevent proper mixing. An archive sample (approximately 150 g) will be taken from each dosed-feed batch at the time of preparation and will be stored in individually labeled, sealed containers at approximately -20°C.

Mixed feed preparations will be refrigerated in sealed containers at approximately 4°C and protected from light.

### 3.4 Dose Analysis

#### 3.4.1 Dosed Feed Concentration and Stability

The stability of mercury and HgCl<sub>2</sub> in feed will be determined prior to study initiation. For each mixed dosed feed preparation, a sample of each dose level will be removed at the time of preparation for analysis of mercury concentrations. At the conclusion of the in-life phase, one dosed feed preparation from each treatment group will be sampled and analyzed for mercury. The actual dosed feed concentrations of mercury as determined at the time of preparation will not differ from the target concentration by more than ±20 percent. A comparison between the sample removed at preparation and the sample removed at the conclusion of the in-life phase will serve to evaluate the stability of the dosed feed preparation for mercury. Duplicate samples of the dosed feed preparation will be digested and single aliquots of the digestate will be analyzed for mercury by GFAA or ICP-AES.

#### 3.4.2 Dosed Feed Homogeneity

An analysis of homogeneity of mixing will be performed prior to study initiation for the high and low dose levels of each treatment group. Samples will be taken from the top right, top left, and bottom part of the twin-shell blender. Triplicate samples will be digested and analyzed for mercury by GFAA or ICP-AES. The preparation will be considered to be homogeneous if the relative standard deviation (RSD) is less than 15 percent. The mean value from the three areas of the blender will be used as the concentration of these specific dosed feed samples.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be weanling Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by the EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, mercury absorption studies have previously been conducted in rats. The oral route of administration was selected as the route of exposure since this is the most likely human route of exposure.

### 4.2 Test System

Male and female Sprague-Dawley (CD/BR) rats, approximately 4 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing.

### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 64-79°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

#### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) to which the appropriate amount of soil, or HgCl<sub>2</sub> has been added will be used for this study. Complete AIN-93G™ meal feed will be given to the control group. The complete AIN-93G™ meal feed will be refrigerated at approximately 4°C and will have expiration dates of approximately 4 months after milling. Feed will be provided *ad libitum* in glass jars. Feeders with fresh feed will be provided at least biweekly. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at a designated laboratory for acetate, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals *ad libitum* via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for acetate, calcium, magnesium, iron, zinc, and phosphorous.

#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.

### 5.0 EXPERIMENTAL DESIGN

This study involves subchronic daily oral administration of mercury (soluble HgCl<sub>2</sub> mixed in feed) as a means of characterizing the oral relative bioavailability of mercury in soil relative to soluble mercury. A non-treated group will serve as a control for determining background mercury levels. Five rats per sex will be used in each treatment group. Specifically, the following groups (five per sex per group) will be studied. Soluble HgCl<sub>2</sub> will be given by intravenous administration using the tail vein for 30 days in a group of rats. A group of rats will receive daily oral administration of dosed feed-soluble mercury for 30 days. A second group will receive daily dosed feed soil from a contaminated site for 30 days. Untreated controls will be fed complete AIN-93G™ meal feed for 30 days.

Treatment Group	Dose Levels <sup>a</sup>		Soil Concentration in the feed g soil/kg feed
	µg Hg/g feed	mg Hg/kg BW	
Untreated (control)	NA	NA	NA
Dosed feed-soluble HgCl <sub>2</sub>	Low	TBD	NA
	Medium	TBD	NA
	High	TBD	NA
Dosed feed-soil	Low	TBD	TBD
	Medium	TBD	TBD
	High	TBD	TBD
<sup>a</sup> The estimated doses of mercury for the dosed feed soil groups will be based on estimated soil mercury concentrations. NA = Not applicable. TBD = To be determined.			

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

#### 5.1 Clinical Observations

Clinical observations of any possible signs of toxicity will be recorded. Otherwise, cage checks will be made once a day for moribundity and mortality.

#### 5.2 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion of the approximately 24-hour interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

#### 5.3 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1), weekly thereafter, and at termination.

#### 5.4 Tissue Collection

At termination, and prior to cessation of heart contractions following an injection of sodium pentobarbital, a whole blood sample will be collected from each rat by cardiac puncture. The kidneys will be removed and stored in polyethylene tissue bags or liquid scintillation vials with plastic tops at approximately -20°C until removed for preparation and analysis. A wet weight of the tissues upon removal will be collected. The residual carcass will be saved and stored frozen for possible additional tissue sample collection and analysis. Samples will be shipped to (*specify name of test facility here*) for analysis.

## 6.0 SAMPLE PREPARATION

The kidney will be digested and prepared according to the testing facility standard operating procedure.

## 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for mercury by GFAA, ICP-AES, or ICP-MS.

Quality control samples will be analyzed at the beginning and end of each daily analysis. These will be digested and analyzed along with study samples. Recovery of mercury from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for mercury: (*specify number of samples for each.*)

<i>I. Non-Biological Samples</i>
A. Test substance
B. Mixed feed
1. Homogeneity
2. Concentration
C. Diet
D. Water

<i>II. Biological Samples</i>
Treatment Group
A. Untreated control
B. Dosed feed soluble mercury
C. Dosed feed soil
D. Water

<i>III. Quality Control Samples</i>
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## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. The test laboratory will also calculate actual administered doses based on actual feed mercury concentrations, food consumption, and body weight data. The relative bioavailability (Rf) ratio for each dose level will be determined using the following equation:

$$Rf = \frac{C_{(\text{soluble mercury})} - BKG_{\text{Dose (soil mercury)}}}{C_{(\text{soil mercury})} - BKG_{\text{Dose (soluble mercury)}}$$

where

BKG = overall background value from feed and water analysis and

C = mean concentration of mercury in kidney.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on mercury concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories
- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within \_\_\_ days (*specify*) of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency. [www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).





**APPENDIX K**  
**TEMPLATE PROTOCOL FOR BIOAVAILABILITY STUDY OF NICKEL IN SOIL FOLLOWING**  
**ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS**



**TEMPLATE PROTOCOL**  
**for**  
**BIOAVAILABILITY STUDY OF NICKEL IN SOIL FOLLOWING**  
**ORAL ADMINISTRATION USING SPRAGUE-DAWLEY RATS**

**1.0 PRINCIPALS**

Sponsor: *(Specify)*

Sponsor's Project Monitor: *(Specify)*

1.1 Testing Facility: *(Specify)*

Study Director: *(Specify)*

**2.0 OBJECTIVES**

The objectives of this study will be to use Sprague-Dawley rats as a test system to determine the relative oral bioavailability of nickel in soil compared to that of soluble nickel forms. These relative bioavailability estimates will be used in human health risk. Relative bioavailability of nickel in soil will be estimated by comparing blood nickel concentrations in rats dosed orally a single time with nickel-containing soil with similar data from rats administered nickel sulfate hexahydrate by gavage.

**3.0 TEST SUBSTANCES**

3.1 Test Substance Identification

The test substances for this study will be samples of soil collected from test sites. Soil nickel will be characterized, and concentration, stability and purity determined before the study.

For the soluble nickel group animals, nickel sulfate hexahydrate will be used to administer appropriate doses of water soluble nickel.

## 3.2 Test Substance Analysis

### 3.2.1 Concentration

The concentrations of nickel in the test substances will be determined. Analysis of the test substances will involve extracting nickel from a sample of each test substance, digesting the extracted material, and measuring the concentrations of nickel by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or graphite furnace atomic absorption (GFAA) (EPA SW-846, U.S. EPA, 2001). The concentrations of nickel in the soil will be determined by digesting duplicate aliquots of the sample and analyzing single aliquots of the digestate. No attempt to identify the different chemical forms of nickel will be conducted at the test laboratory.

### 3.2.2 Moisture

The percent water in the test substance will be determined by measuring the moisture content. Moisture content will be determined by weighing and drying an approximately 5-g sample at 105-110°C for 2 hours, followed by reweighing. Percent moisture will be determined by calculating the difference between the pre-dried and dried samples.

### 3.2.3 Organic Matter

The organic matter in the test substance will be determined. Organic matter content will be determined using the method of loss-on-ignition at 430°C until sample reaches constant weight or has been heated for 24 hours (Davies, 1974).

### 3.2.4 Total Element Content

Total element content will be determined using EPA SW 846 Method 6010 (U.S. EPA, 2001). This method will use ICP-AES for the determination of 25 elements after appropriate sample preparation.

### 3.2.5 pH

The pH of the soil will be determined using EPA SW 846 Method 9045 (U.S. EPA, 2001). The soil will be mixed with either Type II water or a calcium chloride solution (depending on whether the soil is calcareous or noncalcareous), and the pH will be measured with a pH meter.

### 3.2.6 Particle Size Determination

For bioavailability studies, only the fraction of the soil sample in the <250- $\mu$ m size range will be used. Therefore, this fraction will be separated from the remainder of the soil sample by performing a standard sieve analysis using the American Society for Testing and Materials (ASTM) No. 60 Sieve (60 openings/inch of surface area). Soil passing the No. 60 sieve corresponds to the fraction <250  $\mu$ m. Sample preparation for sieve analysis should be performed in accordance with the procedure outlined in *Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis* (Gee and Bauder, 1986) or the ASTM Method D422-63 (Standard Test Method for Particle Size Analysis of Soils).

### 3.3 Dose Preparation

Gelatin capsules will be used to administer test substance (soil). Soil will be transferred into one-half of the gelatin capsule until the desired weight of soil to be placed into a single capsule has been achieved. The capsule will then be reassembled by attaching the other half of the capsule to the soil-filled capsule. Each capsule will be weighed and enough capsules will be prepared for each animal to achieve the desired amount of soil to be administered. The total weight of soil contained in the set of capsules will be within 5 percent of the targeted dose. After preparation, the capsules will be stored at room temperature until used.

The nickel sulfate hexahydrate to be used for dosing the gavage study groups will be formulated into an aqueous solution. A sufficient quantity of nickel sulfate hexahydrate will be dissolved in deionized water (vehicle) to produce the target concentration of the dosing solution. The solution for oral dosing will be prepared at a concentration such that the dosing volumes do not exceed 5 mL/kg.

### 3.4 Dose Analysis

The dosing solution for the gavage administration groups will be analyzed for nickel by graphite furnace atomic absorption spectroscopy or, if the concentration of nickel is sufficiently high, the analysis will be conducted by ICP-Atomic Emission Spectroscopy (AES). A sample of the dosing solution will be taken at the time of preparation. Duplicate aliquots of the dosing solution will be analyzed. The actual dosing solution concentration will not differ from the target concentration by more than  $\pm 10$  percent.

### 3.5 Dose Administration

Capsules will be administered to the animals using a small animal capsule applicator. The dose will be based upon individual animal body weights which will be determined just prior to dosing (fasted body weights).

For the gavage study group animals, the dosing solution will be administered using a stainless steel gavage needle.

## 4.0 EXPERIMENTAL ANIMALS

### 4.1 Justification

The test system for this study will be Sprague-Dawley (CD/BR) rats. The rat was selected as the test system because it is recognized by EPA for use in chemical safety evaluation testing and a large historical database exists for this animal. Additionally, studies of the bioavailability of soil nickel have previously been conducted in rats.

### 4.2 Test System

Male Sprague-Dawley (CD/BR) rats, 8 weeks of age at initiation of dosing will be obtained from a licensed USDA vendor in sufficient numbers to provide the required number of healthy animals for testing. *(Specify the number of animals here.)*

#### 4.3 Animal Health and Quarantine

All animals will be quarantined under environmental conditions simulating those of the study. Each animal will be examined and its health status determined by a laboratory animal veterinarian within 48 hours of receipt. Upon arrival, the rats will be given complete AIN-93G™ diet. At the end of a 48-hour quarantine period, animals of questionable health (as determined by a laboratory animal veterinarian) will be excluded from the study.

#### 4.4 Animal Housing

Animals will be individually housed in polycarbonate cages. All housing and care will conform to ILAR standards and those published in the *Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23*. The environmental conditions of the animal study room will conform to the following specifications:

- The light/dark cycle will be set for 12 hours of light and 12 hours of dark each day during the study.
- The room temperature will be 67-77°F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range.
- The relative humidity will be 40-70 percent and will be recorded twice daily. At least 90 percent of the total recordings will be within the specified range.
- Fresh air will be supplied to the room at sufficient rate to provide a minimum of 10 changes of room air per hour.

#### 4.5 Diet and Water

AIN-93G™ meal feed (Zeigler Brothers, Gardners, PA) will be used for this study. Complete AIN-93G™ meal feed will be given to the control group. Feed will be held from animals for 16 hours prior to oral dosing. Analysis of each feed lot will be provided by the vendor. Also, prior to dosing, feed will be analyzed at (insert name of laboratory here) for nickel, calcium, magnesium, iron, zinc, and phosphorous.

Water will be provided to animals ad libitum via glass bottle reservoirs fitted with stainless-steel sipper tubes. Animals will be given deionized water. During the in-life period, a sufficient volume of deionized water will be available so that all animals receive water from the same source. A sample of the water will be removed at the start and after testing, which will be analyzed for nickel, calcium, magnesium, iron, zinc, and phosphorous.

#### 4.6 Animal Identification

All of the animals will be uniquely identified by ear tag and cage card. Each cage will be labeled with the number that corresponds with the ear marking of the animal in the cage.

#### 4.7 Randomization

Rats will be randomly assigned to treatment groups on the basis of body weights. Body weights will be determined the day before dosing. Randomization will be performed using a computer program that places animals in groups to ensure similar group mean body weights at study onset.

### 5.0 EXPERIMENTAL DESIGN

This study involves oral administration of nickel from two sources (soluble nickel sulfate hexahydrate and nickel in soil) as a means of characterizing the oral bioavailability of nickel in soil relative to soluble nickel. A non-treated group will serve as a control for determining background nickel levels. On Day -1, rats will be weighed in order to determine doses to be used. On Study Day 1, rats will be given the nickel-saline solution by gavage using a stainless steel gavage needle. The soil-filled capsules will be administered using a capsule applicator (test soil group). Control group will receive orally administered saline only. For all groups, samples of whole blood will be collected from each animal at specified intervals, for 6 days after administration of test article or control saline.

The following table summarizes the treatment groups.

Group	Treatment	Number of Animals <sup>a</sup>
1	Control	32 <sup>b</sup>
2	NiSO <sub>4</sub> ·6H <sub>2</sub> O	32 <sup>b</sup>
3	Soil	32 <sup>b</sup>

<sup>a</sup> One animal may be subjected to as many as 3 bleeds. (Example: one animal may be bled at 10 min, 24 hr and 72 hr.)

<sup>b</sup> Suggested number of animals.

During the in-life phase, clinical observation, food consumption and body weight determinations will be made as described below.

#### 5.1 Blood Collection

Serial samples of whole blood (approximately 1 mL) will be collected by orbital puncture under CO<sub>2</sub> anesthesia. Heparinized blood samples will be collected at 0, 10, 20, 30, 60, 120, 240 and 480 min on the first day and at 24, 48, 72, 96 and 120 hr and stored at -20°C until analysis.

#### 5.2 Clinical Observations

Clinical observations of any possible signs of toxicity will be done twice daily. Cage checks will be made once a day for moribundity and mortality.

#### 5.3 Food Consumption

Food consumption will be determined once a day at approximately the same time each day for each rat. Known amounts of feed will be provided in cage feeders and at the conclusion

of the approximately 24-hour interval, feeders will be reweighed. The net difference between the original and final feeder weight will serve as a measure of the feed consumed. Procedures for evaluating feed spillage will be specified.

#### 5.4 Body Weights

Body weights will be taken on all rats at the start of the study (Day 1) and at termination.

### 6.0 SAMPLE PREPARATION

Blood will be digested in concentrated nitric acid. One or more internal standards may be added, and the digestate diluted for analysis.

### 7.0 ANALYSIS OF SAMPLES

All samples will be analyzed for nickel by GFAA, ICP-AES, or ICP-MS. Single analysis of whole blood will be conducted. Blood samples will be digested in acid, as necessary, prior to removal of the aliquot for nickel analysis.

Quality control (QC) samples will be analyzed at the beginning and end of each daily analysis. Quality control samples for nickel in blood will be certified blood standards obtained from a specified commercial laboratory. These will be digested and analyzed along with study samples. Recovery of nickel from QC samples will be considered adequate if they are within 25 percent of the certified or prepared value.

The following table summarizes the approximate numbers of samples per sample type that will be analyzed for nickel: *(Specify numbers of samples for each)*

<i>I. Non-Biological Samples</i>
A. Test substance
B. Mixed feed
1. Homogeneity
2. Concentration
C. Water

<i>II. Biological Samples</i>
Treatment Group:
A. Untreated control
B. Nickel-saline
C. Capsule (soil nickel)

<i>III. Quality Control Samples</i>
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## 8.0 STATISTICS

All individual raw data will be summarized and reported as the mean and standard deviation. Relative bioavailability ratio will be determined using the following equation:

$$F_{\text{rel}} = (AUC_{\text{soil}} / AUC_{\text{pure}}) \times \frac{\text{dose}_{\text{pure}}}{\text{dose}_{\text{soil}}}$$

where  $AUC_{\text{soil}}$  is area under the curve for the capsule-Ni group and  $AUC_{\text{pure}}$  is the area under the curve for the Ni-saline group.

## 9.0 RECORD AND SAMPLE RETENTION

### 9.1 Sample Retention

All samples will be frozen (approximately -20°C) and retained at the test laboratory or its designated facility until acceptance of the final report. At that time, all samples will be disposed of or shipped to the Sponsor as directed by the Sponsor.

### 9.2 Records

All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained at the test laboratory. The stipulations of this protocol will be implemented in conformance with EPA's Good Laboratory Practice Standards (40 CFR Part 792). This study will be listed on the test laboratory's Master Study Schedule. All appropriate records will be maintained and will include, but not be limited to, the following:

- Quarantine and acclimation period information pertaining to daily housing and environmental conditions
- Animal body weights at randomization, animal identification, source of animal supply
- Test substance inventory, receipt, and storage conditions
- Original raw and reduced data on nickel concentrations from all samples
- Dosing and sample collection times
- Food consumption, body weight, and clinical observation data
- Original raw and reduced data from test substance analysis upon receipt, dose preparation, and dose analysis
- Disposition of all samples, including chain-of-custody documentation for transfer of samples between laboratories

- A copy of the signed protocol
- All letters, memos, or notes that pertain to the study
- Original signed final report.

### 9.3 Report

A written draft final report of this study will be submitted to the Sponsor within (*specify*) days of the sacrifice date of the last animals.

## 10.0 REFERENCES

Davies, B.W. 1974. Loss-on-ignition as an estimate of soil organic matter. *Soil Sci. Soc. Am. Proc.*, **38**: 150.

Gee, G.W., and J.W. Bauder. 1986. *Particle-Size Analysis*, in Methods of Soil Analysis, Volume I: Physical and Mineralogical Analysis. American Society of Agronomy, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 383-411.

U.S. EPA. 2001. SW-846. On-Line Test Methods for Evaluating Solid Waster Physical/Chemical Methods. U.S. Environmental Protection Agency. [www.epa.gov/epaoswer/haswaste/test/txmain.htm](http://www.epa.gov/epaoswer/haswaste/test/txmain.htm).