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Standard Operating Procedure for an *In Vitro* Bioaccessibility Assay for Lead in Soil

1.0 Scope and Application

The purpose of this standard operating procedure (SOP) is to define the proper analytical procedure for the validated *in vitro* bioaccessibility assay for lead in soil (U.S. EPA, 2007b), to describe the typical working range and limits of the assay, and to indicate potential interferences. At this time, the method described herein has only been validated for lead in soil (U.S. EPA, 2007b).

The SOP described herein is typically applicable for the characterization of lead bioaccessibility in soil. The assay may be varied or changed as required and dependent upon site conditions, equipment limitations, or limitations imposed by the procedure. Users are cautioned that deviations in the method from the assay described herein may impact the results (and the validity of the method). Users are strongly encouraged to document any deviations as well as the comparison and associated Quality Assurance (QA) in any report.

This document is intended to be used as reference for developing site-specific Quality Assurance Project Plans (QAPPs) and Sampling and Analysis Plans (SAPs), but not intended to be used as a substitute for a site-specific QAPP or a detailed SAP.

Mention of trade names or commercial products does not constitute endorsement or recommended use by U.S. EPA.

2.0 Method Summary

Reliable analysis of the potential hazard to children from ingestion of lead in the environment depends on accurate information on a number of key parameters, including (1) lead concentration in environmental media (soil, dust, water, food, air, paint, etc.), (2) childhood intake rates of each medium, and (3) the rate and extent of lead absorption from each medium ("bioavailability"). Knowledge of lead bioavailability is important because the amount of lead that actually enters the body from an ingested medium depends on the physical-chemical properties of the lead and of the medium. For example, lead in soil may exist, at least in part, as poorly water-soluble minerals, and may also exist inside particles of inert matrix such as rock or slag of variable size, shape, and association. These chemical and physical properties may tend to influence (usually decrease) the absorption (bioavailability) of lead when ingested. Thus, equal ingested doses of different forms of lead in different media may not be of equal health concern.

The bioavailability of lead in a particular medium may be expressed either in absolute terms (absolute bioavailability) or in relative terms (relative bioavailability).

- Absolute Bioavailability (ABA) is the ratio of the amount of lead absorbed compared to the amount ingested:

  ABA = (Absorbed Dose) / (Ingested Dose)

  This ratio is also referred to as the oral absorption fraction (AFo).

- Relative Bioavailability (RBA) is the ratio of the absolute bioavailability of lead present in some test material compared to the absolute bioavailability of lead in some appropriate reference material:

  RBA = ABA(test) / ABA(reference)

For example, if 100 µg of lead contained in soil were ingested and 30 µg entered the body, the ABA for soil would be:

30 (Absorbed Dose) /100 (Ingested Dose), or 0.30 (30%).

Likewise, if 100 micrograms (µg) of lead dissolved in drinking water were ingested and a total of 50 µg entered the body, the ABA would be:

50 (Absorbed Dose) /100 (Ingested Dose), or 0.50 (50%).

If the lead dissolved in water was used as the frame of reference for describing the relative amount of lead absorbed from soil, the RBA would be:

0.30 (test) / 0.50 (reference), or 0.60 (60%).

Usually the form of lead used as reference material is a soluble compound such as lead acetate that is expected to completely dissolve when ingested.

The *in vitro* bioaccessibility assay described in this SOP provides a rapid and relatively inexpensive alternative to *in vivo* assays for predicting RBA of lead in soils and soil-like materials. The method is based on the concept that lead solubilization in gastrointestinal fluid is likely to be an important determinant of lead bioavailability *in vivo*. The method measures the extent of lead solubilization in an extraction solvent that resembles gastric fluid. The fraction of lead which solubilizes in an *in vitro* system is referred to as *in vitro* bioaccessibility (IVBA), which may then be used as an indicator of *in vivo* RBA. Measurements of IVBA using this assay have been shown to be a reliable predictor of *in vivo* RBA of lead in a wide range of soil types and lead phases from a variety of different sites (U.S. EPA, 2007b).

3.0 Sample Preparation, Preservation, Containers, Handling, and Storage

All test soils should be prepared by drying (<40°C) and sieving to <250 µm. The <250 µm size fraction was used because this particle size is representative of that which adheres to children's hands (U.S. EPA, 2000). Stainless steel sieves are recommended. Samples should be thoroughly mixed prior to use to ensure homogenization. Mixing and aliquoting of samples using a riffle splitter is recommended. Clean plastic bags or storage bottles are recommended. All samples should be archived after analysis and retained for further analysis for a period of six (6) months. No preservatives or special storage conditions are required.

4.0 Interferences and Potential Problems

At present, it appears that the relationship between IVBA and RBA is widely applicable, having been found to hold true for a wide range of different soil types and lead phases from a variety of different sites. However, the majority of the samples tested have been collected from mining and milling sites, and it is plausible that some forms of lead that do not occur at this type of site might not follow the observed correlation. Thus, whenever a sample containing an unusual and/or untested lead phase is evaluated by the IVBA protocol, this sample should be identified as a potential source of uncertainty. In the future, as additional samples with a variety of new and different lead forms are tested by both *in vivo* and *in vitro* methods, the applicability of the method will be more clearly defined. In addition, excess phosphate in the sample medium may result in interference (i.e., the assay is not suited to phosphate-amended soils). Interferences and potential problems are discussed under Procedures (Section 7).

5.0 Apparatus

The main piece of equipment used for this procedure is the extraction device shown in Figure 1. An electric motor (the same motor as is used in the Toxicity Characteristic Leaching Procedure, or TCLP) drives a flywheel, which in turn drives a Plexiglass block situated inside a temperature-controlled water bath. The Plexiglass block contains ten 5-centimeter holes with stainless steel screw clamps, each of which is designed to hold a 125-mL wide-mouth high density polyethylene (HDPE) bottle. The water bath should be filled such that the extraction bottles are completely immersed. Temperature in the water bath should be maintained at 37±2 °C using an immersion circulator heater. The 125-mL HDPE bottles should have air-tight screw-cap seals, and care should be taken to ensure that the bottles do not leak during the extraction procedure. All equipment should be properly cleaned, acid washed, and rinsed with deionized water prior to use.

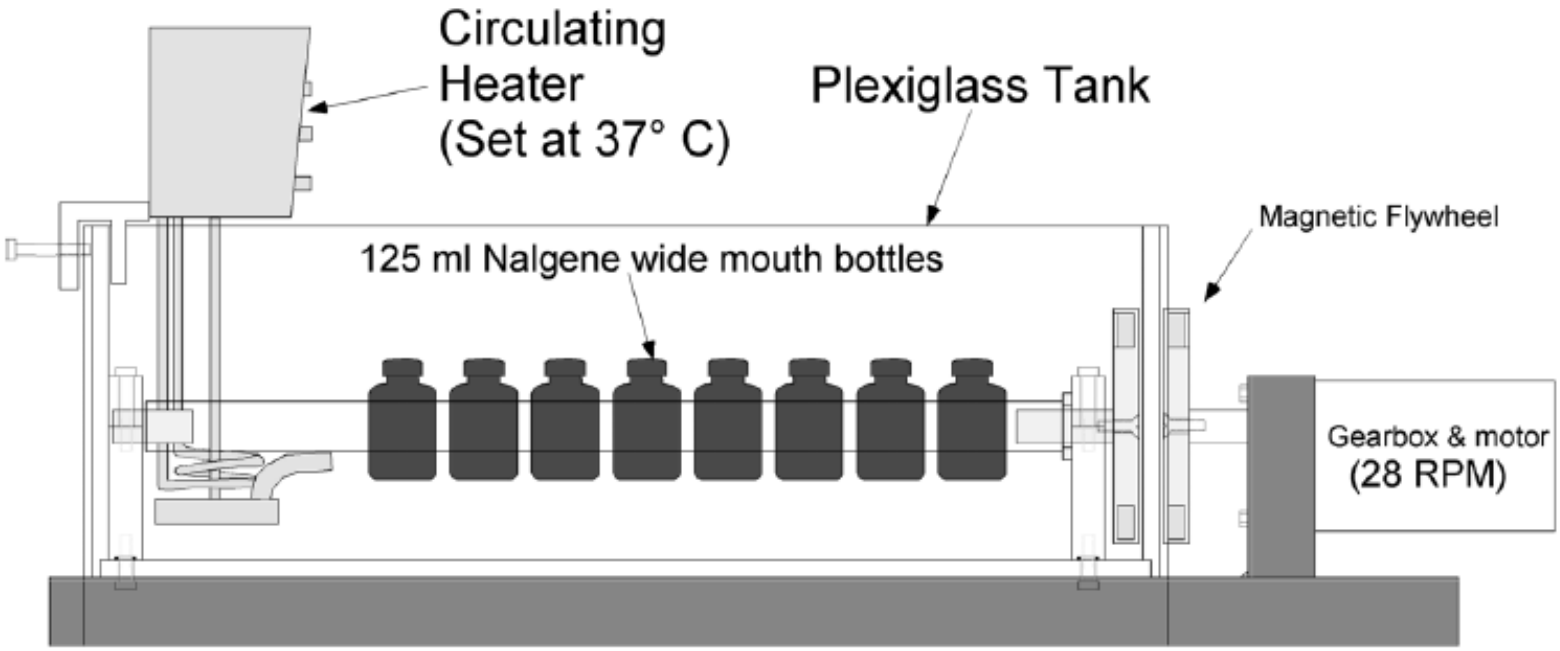

Figure 1. In Vitro Bioaccessibility Extraction Apparatus.

6.0 Reagents

All reagents should be free of lead and the final fluid should be tested to confirm that lead concentrations are <¼ (<one-fourth) the project required detection limit (PRDL) of 10 µg/L (i.e., <2 µg/L lead in the final fluid). Cleanliness of all materials used to prepare and/or store the extraction fluid and buffer is essential; all glassware and equipment used to prepare standards and reagents should be properly cleaned, acid washed, and triple-rinsed with deionized water prior to use.

7.0 Procedures

The dissolution of lead from a test material into the extraction fluid depends on a number of variables including extraction fluid composition, temperature, time, agitation, solid/fluid ratio, and pH. Any alterations in these parameters should be evaluated to determine the optimum values for maximizing sensitivity, stability, and the correlation between *in vitro* and *in vivo* values. Additional discussion of these procedures is available in U.S. EPA (2007b) and Drexler and Brattin (2007).

7.1 Extraction Fluid

The extraction fluid for this procedure is 0.4 M glycine (free base, reagent grade glycine in deionized water), adjusted to a pH of 1.50±0.05 at 37°C using trace metal grade concentrated hydrochloric acid (HCl).[1]

7.2 Temperature

A temperature of 37°C should be used because this is approximately the temperature of gastric fluid *in vivo*.

7.3 Extraction Time

The time that ingested material is present in the stomach (i.e., stomach-emptying time) is about 1 hour for a child, particularly when a fasted state is assumed (see U.S. EPA 2007a, Appendix A). Thus, an extraction time of 1 hour should be used. It was found that allowing the bottles to stand at room temperature for up to 4 hours after rotation at 37°C caused no significant variation (<10%) in lead concentration.

7.4 pH

Human gastric pH values tend to range from about 1 to 4 during fasting (see U.S. EPA 2007b, Appendix A). For the IVBA, a pH of 1.5 should be used.

[1] Most previous *in vitro* test systems have employed a more complex fluid intended to simulate gastric fluid. For example, Medlin (1997) used a fluid that contained pepsin and a mixture of citric, malic, lactic, acetic, and hydrochloric acids. When the bioaccessibility of a series of test substances were compared using 0.4 M glycine buffer (pH 1.5) with and without the inclusion of these enzymes and metabolic acids, no significant difference was observed (p=0.196). This indicates that the simplified buffer employed in the procedure is appropriate, even though it lacks some constituents known to be present in gastric fluid.

7.5 Agitation

If the test material is allowed to accumulate at the bottom of the extraction apparatus, the effective surface area of contact between the extraction fluid and the test material may be reduced, and this may influence the extent of lead solubilization. Depending on which theory of dissolution is relevant (Nernst and Brunner, 1904, or Dankwerts, 1951), agitation will greatly affect either the diffusion layer thickness or the rate of production of fresh surface. Previous workers have noted problems associated with both stirring and argon bubbling methods (Medlin and Drexler, 1995; Drexler, 1997). Although no systematic comparison of agitation methods was performed, an end-over-end method of agitation is recommended.

7.6 Solid/Fluid Ratio and Mass of Test Material

A solid-to-fluid ratio of 1/100 (mass per unit volume) should be used to reduce the effects of metal dissolution as noted by Sorenson *et al*. (1971) when lower ratios (1/5 and 1/25) were used. Tests using Standard Reference Materials (SRM 2710a) showed no significant variation (within ±1% of control means) in the fraction of lead extracted with soil masses as low as 0.2 gram (g) per 100 mL. However, use of low masses of test material could introduce variability due to small scale heterogeneity in the sample and/or to weighing errors. Therefore, the final method employs 1.0 g of test material in 100 mL of extraction fluid.

In special cases, the mass of test material may need to be <1.0 g to avoid the potential for saturation of the extraction solution. Tests performed using lead acetate, lead oxide, and lead carbonate indicate that if the bulk concentration of a test material containing these relatively soluble forms of lead exceed approximately 50,000 ppm, the extraction fluid becomes saturated at 37°C and, upon cooling to room temperature and below, lead chloride crystals will precipitate. To prevent this from occurring, the concentration of lead in the test material should not exceed 50,000 ppm, or the mass of the test material should be reduced to 0.50±0.01 g.

7.7 Summary of Final Leaching Protocol

The extraction procedure is begun by placing 1.00±0.05 g of sieved test material (<250 μm) and 100±0.5 mL of the buffered extraction fluid (0.4 M glycine, pH 1.5) into a 125-mL wide-mouth HDPE bottle. Care should be taken to ensure that static electricity does not cause soil particles to adhere to the lip or outside threads of the bottle; if necessary, an antistatic brush can be used to eliminate static electricity prior to adding the test substrate. The bottle should be tightly sealed and then shaken or inverted to ensure that there is no leakage and that no soil is caked on the bottom of the bottle.

Each bottle should be placed into the modified TCLP extractor (water temperature 37±2°C). Samples are extracted by rotating the samples end-over-end at 30±2 rpm for 1 hour. After 1 hour, the bottles should be removed, dried, and placed upright on the bench top to allow the soil to settle to the bottom. A 15-mL sample of supernatant fluid is removed directly from the extraction bottle into a disposable 20-cc syringe. After withdrawal of the sample into the syringe, a Luer-Lok attachment fitted with a 0.45-μm cellulose acetate disk filter (25 mm diameter) is attached, and the 15 mL aliquot of fluid is filtered through the attachment to remove any particulate matter. This filtered sample of extraction fluid is then analyzed for lead, as

described below. If the total time elapsed for the extraction process exceeds 90 minutes, the test must be repeated.

As noted above, in some cases (mainly slag soils), the test material can increase the pH of the extraction buffer, and this could influence the results of the bioaccessibility measurement. To guard against this, the pH of the fluid should be measured at the end of the extraction step (just after a sample was withdrawn for filtration and analysis). If the pH is not within 0.5 pH units of the starting pH (1.5), the sample should be re-analyzed. If the second test also resulted in an increase in pH of >0.5 units, it is reasonable to conclude that the test material is buffering the solution. In these cases, the test should be repeated using manual pH adjustment during the extraction process, stopping the extraction at 5, 10, 15, and 30 minutes and manually adjusting the pH down to pH 1.5 at each interval by drop-wise addition of HCl.

7.8 Analysis of Extraction Fluid for Lead

The filtered samples of extraction fluid should be stored in a refrigerator at 4°C until they are analyzed (within 1 week of extraction). Once received by the laboratory, all media should be maintained under standard chain-of-custody. The samples should be analyzed for lead by ICP-AES or ICP-MS (U.S. EPA Method 6010 or 6020, U.S. EPA, 1986). The method detection limit (MDL) in extraction fluid should be approximately 20 µg/L for Method 6010 and 0.1-0.3 µg/L for Method 6020.

8.0 Calculations

In order for an *in vitro* bioaccessibility test system to be useful in predicting the *in vivo* RBA of a test material, it is necessary to establish empirically that a strong correlation exists between the *in vivo* and the *in vitro* results across many different samples. Because there is measurement error not only in RBA but also in IVBA, linear fitting was also performed taking the error in both RBA and IVBA into account. There was nearly no difference in fit, so the results of the weighted linear regression were selected for simplicity (U.S. EPA, 2007b). This decision may be revisited as more data become available. Based on this decision, the currently preferred model is:

$$RBA = 0.878 \bullet IVBA - 0.028$$

It is important to recognize that use of this equation to calculate RBA from a given IVBA measurement will yield the "typical" RBA value expected for a test material with that IVBA, and the true RBA may be somewhat different (either higher or lower).

9.0 Quality Control/Quality Assurance

Recommended quality assurance for the extraction procedure are as follows:

- Reagent Blank — extraction fluid analyzed once per batch.
- Bottle Blank — extraction fluid only (no test soil) run through the complete procedure at a frequency of 1 in 20 samples (minimum of 1 per batch).

- Blank Spike — extraction fluid spiked at 10 mg/L lead, and run through the complete procedure at a frequency of 1 in 20 samples (minimum of 1 per batch).

- Matrix Spikes — subsample of each material used for duplicate analyses used as a matrix spike. The matrix spike should be prepared at 10 mg/L lead and run through the extraction procedure at a frequency of 1 in 10 samples (minimum of 1 per batch).

- Duplicate Sample — duplicate sample extractions performed on 1 in 10 samples (minimum of 1 per batch).

- Control Soil — National Institute of Standards and Testing (NIST) Standard Reference Material (SRM) 2711 (Montana Soil) used as a control soil. The SRM should be analyzed at a frequency of 1 in 20 samples (minimum 1 per batch).

Recommended control limits for these quality control samples:

Analysis	Frequency	Control Limits
Reagent blank	once per batch	<25 µg/L lead
Bottle blank	5%*	<50 µg/L lead
Blank spike (10 mg/L)	5%*	85-115% recovery
Matrix spike (10 mg/L)	10%*	75-125% recovery
Duplicate sample	10%*	±20% RPD
Control soil (NIST 2711)	5%*	±10% RPD

RPD = Relative percent difference
*Minimum of once per batch

10.0 Data Validation

NIST SRM 2711 should be used as a control soil. To evaluate the precision of the *in vitro* bioaccessibility extraction protocol, replicate analyses of standard reference materials (NIST SRM 2710 or 2711) should be used. The SRM will be analyzed at a frequency of 1 in 20 samples (minimum 1 per batch).

11.0 Health and Safety

When working with potentially hazardous materials, follow U.S. EPA, OSHA, or corporate health and safety procedures.

12.0 References

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