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5.2 In Vitro Approach

To overcome the difficulty and expense associated with in vivo trials, significant research efforts have been directed toward developing bench-scale in vitro methods to predict RBA. The GI tract is an extremely complex system, so in vitro methods do not necessarily replicate GI tract conditions but instead mimic important biochemical parameters known to influence the release of contaminants from the soil matrix. Consequently, these assays determine the fraction of a soil contaminant that is solubilized following extraction and are therefore potentially available for absorption into the systemic circulation (termed "bioaccessibility").

For the purposes of predicting RBA for use in human health risk assessment, in vitro bioaccessibility (IVBA) is defined as:

IVBA (%) = mass of chemical solubilized/ (total mass of chemical in soil) * 100

Note that the total mass of chemical in the soil should be determined at the same particle size fraction that will be tested in vitro, which may vary from contaminant concentrations in bulk soils, as described in the Logistical Constraints section.

▼*Read more*

In vitro bioaccessibility (IVBA) methodologies were initially developed to predict iron absorption and to evaluate iron nutrition of food (Crews, Burrell, and McWeeney 1983). These IVBA methods were then adapted to evaluate the bioaccessibility of lead and arsenic from soil with subsequent development for other contaminants of interest (Ruby et al. 1992). IVBA methodologies may include gastric extraction alone or several phases to represent sequential dissolution from the mouth, stomach and small intestines. Physiologically based in vitro methods incorporate several key GI tract physiological factors that affect contaminant dissolution including pH, chemical composition of gastrointestinal fluids, gastrointestinal transit time, and the ratio of soil to gastrointestinal fluid. Key GI parameters for commonly reported methods for inorganic contaminants are listed in Table 5-2. A discussion of each parameter follows the table. Table 8-2 includes examples of in vitro Physiologically Based Extraction Test (PBET) parameters for PAH methods.

Table 5-2. Key Human GI physiology parameters used to develop in vitro bioaccessibility methods (Basta et al. 2016)

Parameter	Physiological Values	Reference
Gastric		
pH	1.5-2	(Magagelada et al. 1976)
pH	1.7 (1.5-2)	(Charman et al. 1997)
pH	1.3 (1 – 2)	(Horter and Dressman 2001)
Time	1 h	(Schwartz, Belko, and Wien 1982)
Time	2 h	(Culp and Rawitch 1973)
Soil:solution	1:800mL	(Magagelada et al. 1976)
Pepsin	1% (10 mg/mL)	(Crews, Burrell, and McWeeney 1983)
NaCl	0.15M	(Crews, Burrell, and McWeeney 1983)
AA	0.42 – 1.65 mg/h	(O'Connor et al. 1989)
Intestinal		
pH	5-6.5	(Horter and Dressman 2001)
pH	7.5	(Crews, Burrell, and McWeeney 1983)
pH	6.5	(Magagelada et al. 1976)
Time	>3-5h	(Guyton 1981)
Pancreatin	0.04%	(Crews, Burrell, and McWeeney 1983)

Parameter	Physiological Values	Reference
Bile	0.38%	(Crews, Burrell, and McWeeney 1983)

pH. *Read More*

The GI tract pH has an especially important effect on contaminant dissolution of metals from soil. In humans, stomach pH conditions range from 1.5 to 2.5 under fasting conditions and increase to pH 4 under fed conditions (Magagelada et al. 1976). Several in vitro studies have illustrated the importance of gastric phase pH in influencing lead and arsenic IVBA results. IVBA methodologies use a gastric phase pH value that represents a worst-case scenario (fasted state) for young children. Low pH stomach values are particularly prudent for the assessment of lead, arsenic, and other metal contaminants, because the pH may drive the dissolution of many metals and arsenic mineral phases, thereby controlling the fraction that is potentially available for uptake. In addition, small intestine pH conditions vary from 5.5 to 6.5 in the duodenum and jejunum, respectively, and from 6.5 to 7.5 in the ileum. These values affect organic chemicals through micelle formation, which is known to be critical for organic contaminant absorption (Rahman, Barrowman, and Rahimtula 1986) and is favored at a higher pH (Zhang et al. 2015).

Chemical composition of simulated GI fluids. *Read More*

The chemical composition of IVBA solutions range from simple gastric phase systems that contain limited constituents (for example, a simple buffered system at gastric pH using 0.4 M glycine) to highly complex solutions that contain multiple organic and inorganic components (see tables in arsenic and PAH chapters). Pepsin and glycine are base constituents in gastric phase solutions, while bile and pancreatin are often added when modifying solution conditions from the gastric to the intestinal phase. When organic chemicals in soil are the chemicals of interest, a sink must be included. The sink consists of a hydrophobic phase that organic contaminants can readily partition into, enhancing chemical dissolution by maintaining steep desorption gradients and preventing chemicals from partitioning back to the soil following dissolution. This sink can be represented by simple oils; recent efforts have been directed toward the use of solid phase sinks. Another consideration in determining the composition of simulated GI fluid is whether to include the presence of food.

Presence of food. *Read More*

Because the presence of food influences stomach pH values, the solubility of lead and other metals may decrease when food is added to an IVBA system (Ruby et al. 1996). Addition of the grain feed dosing vehicle used in the juvenile swine model to IVBA extraction fluid decreased mean IVBA for lead from 32.2% to 23.0% in gastric fluid (Schroder et al. 2004) and decreased mean IVBA cadmium from 63.0% to 38.2% in gastric fluid (Schroder et al. 2003). However, the addition of the grain feed dosing vehicle used in the juvenile swine model to the IVBA extraction approximately doubled IVBA arsenic for 5 of 10 mining waste soils (Basta et al. 2007). The increase was attributed to desorption of arsenate from the solid waste soils by phosphate released from the dosing vehicle. High phosphate foods (for example, dairy, powdered milk, soft drinks) could potentially increase measured IVBA arsenic.

For in vitro systems that include efforts to simulate conditions of the small intestine, addition of food additives to the in vitro solution has been shown to decrease the bioaccessibility of lead while increasing the bioaccessibility of arsenic, likely due to desorption from soil iron, aluminum, and manganese oxides (Rodriguez et al. 2003). For organic contaminants, bioaccessibility can increase with the addition of food components (such as powdered milk or porridge) (Hack and Selenka 1996). This effect is likely related to the increased presence of lipid in these systems which enhance the formation of bile acid micelles favoring the uptake of hydrophobic organic contaminants (Zhang et al. 2015). A review of studies evaluating different in vitro methods suggests methods based on simple acid dissolution generally underestimate the dissolution of organic chemicals from soil (Ruby et al. 2016).

Rate of emptying of the stomach and transit time in the small intestine. *Read More*

Small variations in extraction times are seen between IVBA methodologies (see tables in arsenic and PAH chapters). Gastric and GI extraction time frames, however, reflect nutrition studies that have demonstrated that stomach emptying occurs after 1 to 2 h, while between 3 and 5 h is required for constituents to pass from the small to the large intestine.

Ratio of soil/solid:gastrointestinal fluid. *Read More*

The soil:fluid ratio used in in vitro systems has the potential to influence IVBA results because of its effect on dissolution kinetics. At small soil:fluid ratios, IVBA may be underestimated as a result of solubility issues arising from diffusion-limited dissolution kinetics (Ruby et al. 1992). Some studies indicate that metal IVBA values may not vary significantly, however, when soil:liquid ratios vary from 1:100 to 1:5000 g ml^{-1} (Hamel, Buckley, and Lioy 1998). These results vary depending on the metal of interest, soil sample type, and specific solid:fluid ratio. For example, for barium, higher dissolution rates are found at higher relative fluid volumes, while aluminum bioaccessibility from the same samples was relatively insensitive to the solid:fluid ratio (Shock et al. 2007). These observations are consistent with a study by Richardson, Bright, and Dodd (2006) describing the effects of variable solid:fluid ratios on metals bioaccessibility, with particular focus on nickel and how,

in the case of nickel, the changes in bioaccessibility related to the solid:fluid ratio could be explained by the saturation of the solution. Together, the available information indicates that the potential uncertainty around this assumption should be considered in the development of IVBA methods.

5.2.1 Other Factors in Developing In Vitro Methods for Predicting Bioavailability

Several additional factors should be considered in the development of in vitro methods in addition to physiological parameters, including:

Soil particle size.▼*Read more*

Soil particle size can influence the dissolution of chemicals from soil using IVBA test systems (Siciliano et al. 2009). For a given soil type and contaminant, generally higher dissolution rates may be expected with smaller particles. This effect is likely due to the larger surface area of the smaller particles, providing greater contact between the soil particles and extraction fluid. The effect can be confounded by chemical enrichment in different particle sizes. Conversely, in some instances different soil particle sizes reflect different soil chemistries, and thus control soil-chemical interactions. For metals in soil, higher dissolution rates are generally observed from smaller particle sizes (for example, Davis et al. 1993; 1996; Ruby et al. 1996; 1999; Shock et al. 2007). For organic chemicals, at least one study (Siciliano, Laird, and Lemeieux 2010) found higher dissolution from a very large particle size fraction (<4000 µm) than from smaller particles. Conversely, an oral bioavailability study (Rozett et al. 1996) reported the opposite phenomenon, concluding that bioavailability is enhanced in the finest particle size fraction. Given the variable information regarding the influence of particle size, it is generally appropriate that both animal and IVBA studies should focus on the particle size fraction of interest for the exposed population of concern.

Appropriate control samples.▼*Read more*

Development of every in vitro method and application to test soils should include appropriate positive and negative control samples. The negative control sample is included to demonstrate lack of contamination in the test system. This control is important because many of the chemicals of interest for IVBA testing are naturally occurring or are common environmental or laboratory contaminants. A positive control sample should be included to demonstrate good recovery of the target analyte. Without a positive control sample, poor recovery could be misinterpreted as low bioaccessibility. Replicates represent quality control samples that allow for a meaningful assessment of reproducibility within a sample run. Ideally an in vitro method also includes a standard reference material (SRM), or certified reference material (CRM), both in method development and in the application of the method to site soils. Including the SRM or CRM ensures consistent results across time or between laboratories.

Costs of IVBA tests.▼*Read more*

The specific costs of in vitro tests are variable and depend both on the target analyte (inorganics are less expensive than organic chemicals) and the complexity of the specific IVBA method used. Simple extraction tests, such as the Relative Bioaccessibility Leaching Procedure (RBALP) developed by USEPA for estimating the RBA of lead, are relatively inexpensive (see Table 4-1 in section 4.4.1.2, for cost information). More complex IVBA systems with data from multiple phases (gastric, intestinal) increase costs, as do the complex analytical demands of studying IVBA of organic chemicals from soil. Additionally, meaningful study design and applying findings to specific sites frequently requires efforts, as discussed in the decision process chapter, in addition to the simple extraction and analytical work, which results in additional costs that vary with the level of effort.

Complexity in model development.▼*Read more*

Some in vitro methods developed to predict the RBA of chemicals from soil are simple, while others are highly complex. For example, the RBALP recommended by USEPA for estimating the RBA of lead is a single-phase extraction using a simple HCl solution buffered to pH 1.5 with glycine. Conversely, systems such as the Gastro-Intestinal Model (TIM) developed by The Netherlands Organization (TNO) Nutrition (Minekus 2015), and to a lesser extent the BioAccessibility Research Group of Europe (BARGE) and Simulator of the Human Intestinal Microbial Ecosystem (SHIME) methods, more closely replicate the chemistries and processes of the full gastrointestinal tract. Because little is known about the factors that control dissolution of chemicals from soil and subsequent absorption, it can be argued that the physiologically based approaches should provide data with a higher confidence for bioavailability in humans. In practice, however, the higher degree of complexity results in more resource-intensive testing and can also result in more variability in the data and poorer reproducibility across different laboratories.

In developing the RBALP, the technical approach entailed iterative comparison against animal data for paired soil samples and optimizing specifically for predicting the RBA of lead from soils. So, while reflecting less of the complexity of the physiological conditions of the gastrointestinal tract, this comparison of the IVBA data against animal RBA data allowed for the extraction method to be narrowed down to only the critical components. This approach to developing a parsimonious method (one that includes the critical components but nothing more) is discussed further below. Applying a method that is optimized for one contaminant may or may not be optimal for other chemicals in soil.

5.2.2 Components and Documentation of IVBA Methods

▼*Read more*

Documentation of an IVBA study should include, at a minimum:

- a detailed protocol for the test method with a description of the materials needed, a description of what is measured and how it is measured, acceptable test performance criteria (for example, positive and negative control responses), the method of bioavailability calculation, and a description of the statistical treatment of the data
- a description of the known limitations of the test including a description of the classes of materials that the test can and cannot accurately assess
- the extent of within-test variability and the demonstrated reproducibility of the test within and among laboratories
- the degree to which sample variability affects test reproducibility
- the extent to which test method performance has been demonstrated using reference materials or test materials representative of the types of substances for which the test method is applied
- the number of soils tested, their sampling locations, and the concentrations of the chemicals being evaluated
- the method of bioavailability calculation and a description of the statistical treatment of the data
- appropriate control samples to ensure precision and accuracy (positive and negative controls, replicates, and an SRM)

USEPA(2012b) recently updated their guidance document for methods development and validation. The document provides the structure and principles for demonstrating proof of concept and for formal validation of analytical methods to be added to SW-846, *Compendium of Hazardous Waste Test Methods*.

The IVBA method for assessing the RBA of lead from soil was added to the Validated Methods list, an important stage in the formal process of adding methods to SW-846, as EPA Method 1340 in 2013. This method is currently undergoing the public comment period required to formally add it to SW-846. In April 2017, Method 1340 was updated to include the analysis of lead and arsenic IVBA simultaneously, and that version is available on the Validated Methods list of SW-846 (USEPA 2017e). Among other components, this update formalized in vitro bioaccessibility assay for lead in soils (Method 1340) as follows:

- scope and application of the method
- summary and details of the method
- safety
- equipment and supplies
- requirements for sample collection, preservation and storage,
- quality control requirements
- necessary calibration and standardization
- method for data analysis and calculations
- method performance
- references
- tables, diagrams, flowcharts and validation data.

5.2.3 In Vivo – In Vitro Correlation (IVIVC)

The goal of IVIVC is to promote an in vitro IVBA test method to replace in vivo RBA feeding studies. Successful IVIVC has been established when the RBA of a test soil can be determined using a predictive model (for example, simple linear regression), and meet the USEPA requirement (2007b) that “the in vitro result (entered as input) will yield an estimate of the in vivo value (as output).” If a good IVIVC has been established, then the in vitro data for soils can be used as the sole basis for adjusting RBA in a human health risk assessment.

▼*Read more*

In addition to providing RBA values that can be obtained more rapidly and inexpensively than through animal experiments, an advantage of having an IVIVC for a chemical in soil is that the in vitro data may then be used to extrapolate RBA outside the limits of the animal testing model. For example, although analytical detection limits for arsenic are low, animal models are generally unreliable for estimating the RBA of arsenic for soils with arsenic concentrations less than 100 mg/kg. This unreliability results from issues in variability, statistical power, and background exposures from the diet (even under controlled laboratory conditions). This limitation may be significant, especially because risk and remediation decisions are frequently made for soils with arsenic concentrations below 100 mg/kg. For arsenic in soil, an in vitro method may be the only way to estimate the RBA for concentrations in this range of interest.

Defining what constitutes an acceptable IVIVC is critical for determining whether an IVBA is sufficiently predictive of bioavailability in vivo that it can be used for decision making. There is currently no universally accepted set of criteria for IVIVC as it pertains to bioavailability from soil. Regulatory agencies faced with deciding whether to accept an IVBA method might consider the following questions:

- *Has the method been evaluated for a wide range of soil types, including soils that are relevant to your jurisdiction?* The regression model should be based on a robust set of test soils for which reliable in vivo RBA and IVBA values are both available. Regulatory agencies with a preference as to animal models for in vivo RBA determination will presumably be most interested in datasets with comparisons to RBA values generated with their accepted models. While there is no specific number of soils that results in a robust dataset for IVIVC, generally it is better to include a diversity of soil types, contaminant concentrations, and sources. Usually, a larger number of test soils provides greater power to assess the IVIVC. If linear regression models are developed for site- or region-specific use (such as for mining impacted environments), then sample sets should reflect the pertinent physicochemical properties across the site or region.

- *When in vitro bioaccessibility is plotted against in vivo RBA and the data is regressed, what is the goodness of fit?* Currently, USEPA (2007b) has not specifically defined the criteria for the goodness of fit parameters for the IVIVC. A regression model with a slope close to 1 and y-intercept of zero would indicate that the IVBA value is a direct surrogate for RBA, but these criteria are not required for establishing a predictive model. Based upon guidance developed by the FDA, (Wragg et al. 2011) proposed that a linear relationship between in vivo and in vitro data should be observed with a correlation coefficient of (r) >0.8 and a slope >0.8 and <1.2. However, IVIVC evaluated by the FDA differ in some ways from IVIVC for soil bioavailability, and other criteria for correlation coefficient and slope could be reasonably adopted.

- *To what extent are results from the IVBA test known to be repeatable both within and among laboratories*? A method that can yield consistent results provides greater confidence that substantial error in test results will not occur. Wragg et al. (2011) proposed intralaboratory repeatability of $\leq$10% relative standard deviation (RSD) and interlaboratory repeatability of $\leq$ 20% as criteria based upon FDA guidance, although in practice these criteria may be difficult to meet for IVBA test data. USEPA (2009c) discusses the importance of within-laboratory and interlaboratory reproducibility of IVBA methods and includes an example of the application of these principles to the development of the IVBA for predicting the RBA of lead (USEPA Method 1340). Similarly, Brattin et al. (2013) provide measured data on interlaboratory variability that illustrates the potential difference between laboratories when measuring arsenic IVBA.

- *Does the test produce results that are biased toward over- or underprediction of the RBA?* This bias cannot be deduced directly from the correlation coefficient, yet can be an important matter for regulatory agencies. How often does the test overpredict versus underpredict the RBA? Some agencies may be willing to accept random or frequent underestimation of RBA, while others might choose to have assurance that underestimation of RBA will occur infrequently to avoid underestimating risk from soil exposure.

- *What is the magnitude of prediction error possible with this method?*There are three ways to evaluate prediction error:

1. Prediction limits for the regression model show how well a single prediction agrees with a measured RBA.
2. External validation shows whether predictions for soils not used to derive the RBA are within the prediction limits of the model.
3. Wide prediction limits may not be acceptable to the regulatory agency. Although large errors in prediction affect the correlation coefficient, the correlation coefficient alone does not indicate how badly a test might over- or under-predict RBA in a limited set of soil samples from a given site. This error can, however, be determined from the plot of paired in vitro estimates and RBA values for individual soils and reviewing soil samples for which

magnitude of error was largest. Agencies might be particularly interested in the magnitude of the largest underpredictions of bioavailability, as well as how often and in what soil/site conditions these underpredictions occurred. An underprediction is less protective than an overprediction.

Considering each of these questions allows an assessment of frequency, direction, and magnitude of error associated with using an in vitro test to predict RBA. With this information, an agency can decide whether the error is acceptable for the management goals of a site.

Examples of IVIVC for lead, arsenic, and PAHs are shown and discussed in their respective chapters.