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In-vitro Methods for the Measurement of the Oral Bioaccessibility of Selected Metals and Metalloids in Soils: A Critical Review

R&D Technical Report P5-062/TR/01

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In-vitro Methods for the Measurement of the Oral Bioaccessibility of Selected Metals and Metalloids in Soils: A Critical Review

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Statement of Use
This technical report is a critical review of *in-vitro* methods for the measurement of the oral bioaccesibility of metals and metalloids in soils. The information in this document is for use by EA staff and others involved in assessing the risk to human health from contaminated land.

Keywords
bioavailability, bioaccessibility, *in vitro* methods, soil contamination, metals and metalloids, physiologically based extraction test, critical review.

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EXECUTIVE SUMMARY

In April 2000, the Contaminated Land Regulations in England (Environmental Protection Act 1990: Part IIA) came into force. This placed duties on local authorities to inspect their areas and to identify sites that fall into the definition of 'contaminated land', and required its assessment and remediation in line with the 'suitable for use' approach. Guidance has recently been published on the assessment of risks to human health from land that includes Soil Guideline Values (SGVs) for inorganic contaminants such as arsenic. Where the concentration is above the SGV, there is a 'need to consider whether the presence of the contaminant justifies taking remedial action.'

The aim of this review is to provide a summary of *in-vitro* tests that are currently in use for evaluating the ingestion bioaccessibility of selected metals and metalloids in contaminated soils. This report includes a brief outline of the methodologies, and a critical commentary on their robustness and validity for measuring bioaccessibility. The tests covered will only be for human/animal oral bioaccessibility (not phytoavailability) and the bioaccessibility of organic compounds is not covered.

Bioaccessibility tests can be divided into two categories:

- those using chemical extraction tests that equate the 'easily extractable metals', usually at low pH conditions, with those that are likely to be bioaccessible; and
- gastro or gastrointestinal analogue tests which attempt to mimic the biochemical conditions in the human/animal gastrointestinal tract.

In-vitro gastrointestinal protocols may be appropriate alternatives to animal testing as they provide a rapid and inexpensive means to determine the bioaccessibility of a given potentially harmful element present in soil.

There are advantages and disadvantages associated with all the methodologies currently available for estimating bioaccessibility. Decisions on the applicability of bioaccesibility estimates and the most appropriate method should consider the validation statues of any method, the ease of use and the degree to which the method mimics the human gastrointestinal environment.

There needs to be further research carried out to validate that the *in-vitro* test data relates to human bioavailability for a wider range of metals and soil types. An important adjunct to this work will be the measurement of the way in which simulated gastrointestinal solutions alter the chemical speciation of contaminant metals as this could play an important role in determining the risk to human health.

Acknowledgements

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CONTENTS **Page**

List of Figures

List of Tables

1. INTRODUCTION

In April 2000, the Contaminated Land Regulations in England (Environmental Protection Act 1990: Part IIA) came into force, and placed duties on local authorities to inspect their areas to identify sites which fall into the definition of 'contaminated land', and required its remediation in line with the 'suitable for use' approach. The objective of Part IIa is to identify and control threats to human health and the wider environment resulting from land contamination. The principles underlying the proposed regime are risk based with a suitable for use approach to remediation. The statutory duties that will be placed on local authorities under the Act will require them to:

- inspect their areas to identify contaminated land;
- prepare and serve notifications of contaminated land;
- establish whether sites should be designated as 'special sites' and thus become the responsibility of the environment Agency;
- serve remediation notices where necessary;
- undertake assessment of the best practical remediation option and test for reasonableness;
- consult other parties including the Environment Agency; and,
- compile and maintain registers of contaminated land.

The inspection step is an important activity for local authorities before the implementation of the other duties under the Act. The identification of contaminated land depends on whether or not there is a significant pollutant linkage between a source (the contaminant), a pathway (such as ingestion) and a receptor (for example, a human being).

6.2 Risk-based approach to identifying contaminated land

Until recently, assessing possible risk to human health from toxic metals in soils was based on the ICRCL trigger values (Interdepartmental Committee on the Redevelopment of Contaminated Land 1987) that were derived from permissible concentrations in sewage sludge applied to farmland at soil pH values of 6.5. Guidance has recently been published on the assessment of risks to human health from land contamination (Department for the Environment Food and Rural Affairs and the Environment Agency 2002a) that includes Soil Guideline Values (SGVs) for inorganic contaminants such as arsenic (Department for the Environment Food and Rural Affairs and the Environment Agency 2002d). Where the concentration is above the SGV, there is a 'need to consider whether the presence of the contaminant justifies taking remedial action.' Local authorities and other stakeholders use a tiered approach to assessing the risks from contaminated land (Department for the Environment Food and Rural Affairs and the Environment Agency 2002a). Scientifically derived generic SGVs are used in the risk-based approach to screen those sites that may pose a risk to human health and warrant further attention.

The SGVs have been derived on the basis of a particular land-use. Currently there are three types of land-use for which SGVs have been developed: residential; allotments; and commercial/industrial. SGVs for other types of land-use such as recreational open space and school playing fields may be developed in the future as part of the on-going research programme. This is intended to highlight that these categories are representative of a range of generic site conditions, taking into account studies of social behaviour, but also incorporating

the simplifying and precautionary assumptions necessary to derive SGVs that are broadly applicable to a range of different circumstances. The standard land-uses are not intended to reflect accurately either the conditions of a specific site or the behaviour of a particular individual. The assessor in judging the applicability of SGV to a specific site, must consider whether it is appropriate to apply the value for a standard land-use taking into account the limitations of the underlying conceptual exposure model.

The SGVs are often derived with the use of assessment models, these conceptual models are an essential element of any site-specific risk assessment (Department for the Environment Food and Rural Affairs and the Environment Agency, 2002a). In the context of environmental risk assessment they are often simple representations of the hypothesised relationships between sources, pathways and receptors (DETR *et al.* 2000). The conceptual exposure models used in the derivation of SGVs are based on three elements: land-use, fate and transport of contaminants, and contaminant toxicology.

Commonly used models are the Scotland and Northern Ireland forum for Environmental Research (SNIFFER) model (Land Quality Management SCHEME the University of Nottingham 2000) and the Contaminated Land Exposure Assessment (CLEA) model (Department for the Environment Food and Rural Affairs and the Environment Agency 2002a). The SNIFFER model is based on a deterministic mathematical approach rather than a probabilistic approach using single value input parameters. Both of these models produce values that are designed to establish whether a site poses actual or potential risks to human health, in the context of the existing or intended usage of the site. However, neither of these models take into account the concept of bioaccessibility (apart from for Lead in CLEA), as they assume that all of the potentially harmful chemicals present in the soil, are available to the biosphere.

The CLEA model is a computer-based application that combines information on the toxicity of soil contaminants with estimates of potential exposure by adults and children living, working and/or playing on land affected by contamination, over long periods of time. The CLEA model uses a variety of Monte Carlo simulations in order to examine the various different pathways by which humans become exposed to soil derived contaminants. It predicts the amount of contaminant to which they might be exposed based on a given soil contaminant concentration. By comparing predicted exposure with health criteria values on tolerable or acceptable contaminant intakes, the model is used to generate SGVs that establish a contaminant concentration in soil that is protective of human health. The advantages of CLEA derived guidelines are that they are based on risk assessment, they specifically provide for uncertainty and they provide an objective basis for decision making.

1.2 Scientific basis for the use of *in-vivo* and *in-vitro* tests

Quantitative guidelines for assessing risks are associated with several scientific problems. There are difficulties in establishing concentrations of contaminants beyond which risks from exposure to these contaminants would be “unacceptable”. This requires not only scientific (toxicological) information on the health effects, but also an element of judgement on what is “unacceptable” risk. In addition, soil is only one of the sources of contaminant exposure, and its effect, and the cost of dealing with it, needs to be kept in proportion with the total exposure to contaminants from all sources.

To be simple to use, guidelines that are generally applicable are needed. However, in the case of soil quality, it is particularly difficult to take account of the variety of soil types and particular site conditions, as well as the ranges of contaminants, contaminant species and contaminant mixtures. Complex assumptions are needed to establish the relationship between the concentration of a contaminant in soil and the effect on a human or other receptor of relevance. All these factors make it difficult to derive generally applicable criteria. "Worst-case" assumptions can be used with care to overcome some of the problems, but they can result in criteria that are impracticable.

In terms of human health risk assessment there are three main exposure pathways for a given contaminant present in soil. The largest area of concern is the oral/ingestion pathway followed by the dermal and respiratory exposure routes (Paustenbach 2000). Whether contaminated soils pose a human health risk depends on the potential of the contaminant to leave the soil and enter the human bloodstream. The use of total contaminant concentrations in soils provides a conservative approach as it assumes that all of the metal present in the soil can enter the bloodstream. Results from animal tests (Casteel *et al.* 1997, Freeman *et al.* 1996, Freeman *et al.* 1994) suggest that contaminants in a soil matrix maybe absorbed to a lesser extent and show fewer toxic effects compared to the same concentration of soluble salts of the contaminants in a food or liquid matrix.

In many cases there is no distinction made between the intake for contaminants that are bound to soil and those which occur as a vapour or are released during processes like digestion into solution (the so-called bioaccessible fraction). For example, children may ingest arsenic-contaminated soil through deliberate mouthing of soil or inadvertent mouthing of dirty hands or soiled toys. Empirical studies have sought to demonstrate a relationship between the type of contaminated soil and the fraction of arsenic that can be dissolved by digestion (Ruby *et al.* 1999). Using such studies may improve our knowledge of the intake of bioaccessible organic and inorganic compounds in the future, as this parameter represents a better estimate of exposure than total concentration of soil contaminants.

There is, therefore, a clear need for a practical methodology that measures the fraction of the contaminant in the soil that, through oral ingestion, can enter the systemic circulation of the human body and cause toxic effects. This is known as the oral *bioavailability* and can be formally defined as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract (Paustenbach 2000). This term must not to be confused with the oral *bioaccessibility* of a substance, which is defined as the fraction that is soluble in the gastrointestinal environment and is available for absorption (Paustenbach 2000). There are other definitions of bioaccessibility that take into account all of the primary boundaries e.g. skin, lungs and gastrointestinal tract (Ruby *et al.* 1995). For the purposes of this report, however, it is only the oral ingestion route that is being considered.

Since bioavailability data is essentially related to the amount of contaminant in the animal/human bloodstream the data must be produced from the dosing of animals with contaminated soil and the subsequent measurement of the contaminant in the blood or organs of the animal; these are known as *in-vivo* animal models. Where animal trial models are carried out, there needs to be additional studies to show how the animal bioavailability can be related to human bioavailability. Usually animals with similar gastrointestinal tract characteristics to humans, such as immature swine, are preferred and have been shown to be reasonable analogues (Casteel *et al.* 1997). Bioaccessibility data, however, is normally determined in a test tube environment (*in-vitro*) and represents the amount of contaminant

dissolved in the gastrointestinal tract prior to crossing the mucosal walls. The amount of pollutant which is actually absorbed by an organism is generally less than or equal to the amount which is mobilised (Paustenbach 2000). *In-vivo* dosing trials have used a variety of animal species e.g. rats and rabbits. Species that have similar gastrointestinal tract characteristics to human children, such as immature swine are preferred and have been shown to be reasonable analogues for children (Dodds and Hsu, 1982). In this type of testing, known amounts of contaminant are added to the feed of the species being tested, in the form of soluble salts or contaminated materials. The test species are dosed to stimulate intermittent eating patterns. Blood and urine samples are extracted regularly prior to death and after necropsy the brain, heart, liver, lungs, gastrointestinal and urinary tracts are removed for analysis. Bioaccessibility extraction tests are generally based around the gastrointestinal parameters of young children (0-3 yr). This age group is thought to be at most risk from accidental ingestion of soil. Also, since children can absorb a higher percentage of contaminant through the digestive system than adults, they are more susceptible to adverse health effects (Hamel *et al.* 1998).

The determination of bioavailability of metals and metalloids from a number of solid media has been undertaken over a period of years, on several animal species. Although arsenic is a metalloid it will be referred to in this report as a metal. The species used for such testing regimes have included juvenile swine (Casteel *et al.* 1997), rats (Ellickson *et al.* 2001), rabbits (Davis *et al.* 1992, Freeman *et al.* 1993) and monkeys (Freeman *et al.* 1995). Data have been collected from blood and urine samples taken from the animal species under investigation. Data from *in-vivo* studies are difficult to interpret with respect to their relevance to human health because of the physiological differences between humans and the experimental species being used (Ruby *et al.* 1999).

Mammal dosing trials are time consuming and expensive. Also, none of the *in-vivo* testing regimes using animal models have been validated against estimates of metals absorption in either children or adults, due to the toxic nature of the metals of interest, hence data from such tests are difficult to interpret. The only trial to be completed on humans to date is a study of oral lead bioavailability by Maddaloni *et al*. (1998). This study used isotopic measurements to study the blood lead uptake from adults dosed with soil from a residential garden in an area contaminated by mining activity. Results of the analyses from these subjects indicate that, on average, 26.2% of the total lead in the soil was taken up into the systemic system. Six additional subjects that ingested soil immediately after a standardised breakfast, however, were found to absorb only 2.52% of the total lead in the soil. A consequence of the lack of testing on human models is that the animal models used to date may produce bioavailability estimates that are different from those produced in humans (Ruby *et al.* 1999).

To supplement or supersede the use of animals in determining the bioavailability of potentially harmful elements for human health risk assessment, or to estimate bioavailability where animal studies are not available, a potential alternative is the use of *in-vitro* tests. Since the bioaccessible fraction is usually greater than the bioavailable fraction (Paustenbach 2000), its use may provide a conservative measure of bioavailability and therefore laboratory based *in-vitro* tests, can be designed to predict oral bioaccessibility of metals. This area of testing could provide "a rapid and significantly less expensive method to determine the amount of pollutant that can be dissolved out of the contaminated soil by the juices of the upper digestive tract" (DIN 2000). A number of *in-vitro* tests have been used to assess the degree of metal solubility in a simulated gastrointestinal environment imitating the leaching of a solid matrix (Oomen *et al.* 2002). Predecessors of such systems were originally developed to

assess the bioavailability of iron in food for nutrition studies (Miller *et al.* 1981, Rodriguez and Basta 1999). Gastrointestinal processes are complex and difficult to simulate. *In-vitro* techniques have traditionally used various metal salts or soils that are incubated at low pH for a period intended to mimic the residence time in the stomach and followed by an increase in pH to mimic conditions in the small intestine. Enzymes and organic acids are used to simulate stomach and small intestinal fluids.

In-vitro testing regimes are used as predictors, as they do not provide absolute bioavailability data, this can only be done at present by *in-vivo* techniques. As the cost and time required to perform *in-vitro* techniques is small in comparison to *in-vivo* methods, a larger number of soils can be assessed to fully characterise a site. Currently there are several *in-vitro* methodologies available for a variety of soil media and potentially harmful elements. These tests range from simple one-stage extraction schemes to more elaborate multi-stage sequential chemical fractionation methods. To date reviews in this area have been quite broad based (Ruby *et al.* 1999, Tristan-Montero 2000) and have not discussed the practicalities and specific use of bioaccessibility tests for the provision of data for human health risk assessments.

The aim of this review is to provide a summary of tests that are currently in use for evaluating the ingestion bioaccessibility of selected metals and metalloids in contaminated soils. This report will include a brief outline of the methodologies, and a critical commentary on their robustness and validity for measuring bioaccessibility. The tests covered will only be for human/animal oral bioaccessibility (not phytoavailability i.e. that which is available for plant uptake) and bioaccessibility of organic compounds will not be covered.

2. BIOACCESSIBILITY TESTS IN CURRENT USE

Bioaccessibility tests can be divided into two categories:

- those using chemical extraction tests that equate the 'easily extractable metals', usually at low pH conditions, with those that are likely to be bioaccessible; and,
- gastro or gastrointestinal analogue tests which attempt to mimic the biochemical conditions in the human/animal gastrointestinal tract.

2.1 Chemical extraction tests

These tests generally fall into three categories:

i) Single extraction tests that simulate the leaching of potential contaminants from soil or waste by rainwater or landfill leachate;
ii) Single extraction tests that are designed to determine the phytoavailability of chemicals within the soil (Rauret 1998); and,
iii) Multiple extraction tests designed to either extract specific physico-chemical soil phases to determine the distribution of metals within the soil (often referred to as sequential extraction tests).

The first type of extraction test protocols have been set up to measure potential mobilisation of contaminants from soil or waste piles by rain or landfill leachate. The USEPA method 1311(USEPA 2000) Toxicity Characteristic Leaching Procedure (TCLP) uses two different

buffered solutions (pH 4.93 and 2.88) and was designed specifically to simulate landfill conditions. There is an Australian version of this test (Australian Standard 1997) that allows for the additional use of deionised water, tetraborate solution at pH 9.2 and local water as leaching media. The ASTM test D3987-85 (ASTM 1999) and the UK Environment Agency leaching test (Lewin *et al.* 1994) use deionised water. The USEPA has three further tests; the Synthetic Precipitation Leach Procedure (SPLP Method 1312), the Extraction Procedure Toxicity Test (EPTT Method 1310A) and the Multiple Extraction Procedure (MEP Method 1320) that are all designed to simulate leaching by acid rain under different conditions (USEPA 2000).

In the second type of test, single extractions are used to measure the phytoavailable fraction of an inorganic constituent of the soil. These tests are commonly used in agronomy and soil science applications and have been recently reviewed (Rauret 1998).

In the case of the sequential extraction tests, many of these are based on the method first proposed by Tessier *et al.* (1979). The distribution of the metals is methodologically defined into five categories:

- exchangeable;
- carbonates;
- reducible;
- oxidisable; and,
- residual.

Many modifications of this test have appeared in the literature over the years. In an attempt to standardise the methodology, the European Standard Measurement and Testing programme has proposed a standardised leaching scheme commonly referred to as the BCR method (Ure *et al.* 1993). This consists of a 'cut-down' version of the Tessier method with methodologically defined extraction of exchangeable, reducible and oxidisable fractions, which, with some modifications, has been successfully used to produce reproducible inter-laboratory data for reference materials e.g. (Lopez-Sanchez *et al.* 1998, Quevauviller *et al.* 1997, Rauret *et al.* 1999, Sahuquillo *et al.* 1999, Sahuquillo *et al.* 2000). The more easily extractable phases of these tests (e.g. exchangeable and carbonates) are often equated to the bioavailable fraction (Kavanagh *et al.* 1997) although this is probably referring to phytoavailability not animal or human bioaccessibility.

Although the results of these tests give a broad idea of easily mobilised contaminants, the extraction conditions and the leaching reagents are not representative of those found in the human gastrointestinal tract. There has been no attempt to calibrate results for this type of test against human *in-vivo* or *in-vitro* studies (apart from the PBASE test Section 2.1.1) and therefore their use in assessing risk to human health is very limited.

2.1.1 Potentially Bioavailable Sequential Extraction (PBASE)

The PBASE methodology was developed to evaluate the relationship between metal fractionation and the bioaccessibility of metal contaminants in a soil (Basta and Gradwohl 2000). The PBASE test is essentially a four stage sequential extraction using a series of reagents based on metal solubilities; metals extracted earlier in the series are more soluble and therefore assumed to be more bioaccessible.

Table 2.1 PBASE extraction conditions (Basta and Gradwohl 2000)

Step	Media	Time (hr)	Temperature °C	Phase extracted
1	0.5M $Ca(NO_3)_2$	16	25	Exchangeable, readily soluble
2	1M NaOAc (pH 5)	5	25	Acid-soluble weak surface complexes
3	0.1M Na_2EDTA (pH 7)	6	25	Surface complexes and precipitates
4	4M HNO_3	16	80	Very insoluble

The protocol has been applied to 12 smelter-contaminated soils and to plant species, such as lettuce, for the determination of cadmium, lead and zinc contamination via two human exposure pathways. The pathways investigated were plant uptake (phytoavailability) and incidental ingestion (gastrointestinal availability). Data from these studies were calibrated against bioaccessibility data obtained from a Physiologically Based Extraction Test (PBET) extraction (Ruby *et al.* 1996) of the same materials. Cadmium and zinc appear to correlate well with the lettuce bioassays indicating that the PBASE test can provide phytoavailability data. The sum of all the fractions in the PBASE method was found to correlate well with the gastric phase from the PBET for lead (Basta and Gradwohl 2000).

Unlike many sequential extraction methods an attempt has been made to relate the results to *in-vitro* bioaccessibility measurements by comparison with the PBET test. The results showed a significant correlation with data from the PBET test only for lead. However, even though there was a good correlation for the lead data, the actual amount extracted was significantly different, *viz*. the sum of the PBASE test extracts was more than nine times as much as that extracted by the intestinal phase of the PBET test. In addition to this, the method is very time consuming (see Table 2.1) and, in common with the Tessier-style extract schemes, would not be practicable for large batches of samples.

2.1.2 European Standard for Safety of Toys

This method (European Standard EN 71-3 1994) provides a way to evaluate the bioaccessibilty of eight metals (antimony, arsenic, barium, cadmium, chromium, lead, mercury and selenium). The methodology is used for the extraction of a particular metal, from a toy material reduced to <500µm, at a liquid to solid ratio of 50:1 in HCl at pH 1.5 at 37°C for 2 hours. It has been in use since 1994 by 18 countries of the Comité European de Normalization for the regulation of the safety of toys (Ruby *et al.* 1999). However at present it has not been applied to any other solid material, such as contaminated soils, therefore its applicability and validity are unknown.

A recent test (Mercier *et al.* 2002), is very similar to European standard methodology and has been developed as a replacement for the USEPA TCLP method (Section 2.1). The extracting conditions (pH 5) used in the TCLP method were found to be too high to solubilise potentially bioaccessible metal contaminants particularly if gastric absorption were to be considered. This method extracts the soil samples at 37°C in HCl at pH 2. The method has not been validated against any other *in-vitro* or *in-vivo* models.

2.2 Gastrointestinal Analogues

The human gastrointestinal tract consists of a number of compartments where ingested soil undergoes a series of reactions in which fluid composition, pH and reaction time all vary. Table 2.2 summarises the order and conditions in each compartment (Oomen *et al.* 2002).

Table 2.2 Human Gastrointestinal Tract Compartments (Oomen *et al.* 2002)

Order	Compartment Name	Residence Time	pH
1	Oral Cavity	Seconds to minutes	6.5
2	Stomach	8-15 min (fasting, half emptying time) 0.5-3 hr (fed, half emptying time)	1-2 2-5
3	Small Intestine i) Duodenum ii) Jejunum iii) Ileum	 0.5-0.75 hr 1.5-2 hr 5-7 hr	 4-5.5 5.5-7 7-7.5
4	Colon	15-60 hr	6-7.5

The analogue digestive tract methods use extractions that mimic a combination of one or more of the compartments shown in Table 2.2. It is important to note, however, that the analogue methods discussed in this review only consider the biochemical environment and disregard the effect of active transport mechanisms and the role of micro-organisms in the gastrointestinal tract. Whether this omission is significant for ingestion of soils has yet to be established. The gut bacteria have been shown to have a significant effect on the metabolism of drugs (Dressman *et al.* 1993) although there does not seem to be much work carried out on their effect on toxic metal absorption.

The inclusion or omission of one of these compartments shown in Table 2.2 will affect the measured bioaccessibility of contaminant metals in different ways e.g. arsenic which is primarily anionic and lead which is cationic in solution will behave differently when extracted from the solid matrix under different pH regimes. There has also been discussion regarding the modelling of the affect of food in tests i.e should the test be carried out under fed or fasting conditions (Ruby *et al.* 1996). Work has shown that the presence of food can reduce the uptake of lead (James *et al.* 1985, Maddaloni *et al.* 1998, Ruby *et al.* 1996). Other workers (Oomen *et al.* 2002, Rodriguez and Basta 1999) found that the inclusion of food increased the bioaccessibility of some elements.

At present there is no consideration of the involvement of the lower gut in bioaccessibility assessment. Metals in insoluble particles would eventually pass into the colon. There are very different physico-chemical conditions and biochemical activities in the colon as a consequence of the huge numbers of bacteria. This could have an impact on the soil particles and hence mobilisation of toxic metals could take place (Rumney and Rowland 1992). If the current models, which ignore the colon compartment, are found to be producing bioaccessibility data which are consistently lower than bioavailability data then consideration should be given to the inclusion of a colon compartment.

The analogue digestive tract methods can be divided into two types: the batch test, where the sample and reagents are reacted in a single container with additional chemicals being added and samples removed as stated in each specified protocol; and the flow through reactor system in which the reaction takes place under flowing conditions, designed to simulate the actual transit characteristics of the gastrointestinal tract. An overview of the methods based on their main features is given in Table 2.3 and each is discussed individually in Sections 2.2.1 to 2.2.10. In addition to the main operating parameters, an ‘ease of use’ parameter has

also been included. This is a subjective judgement by the authors of this report, based on descriptions in the literature, and provides a simple measure to assess whether the methodology is suitable for large scale screening of soils for metal bioaccessibility.

2.2.1 The Physiologically Based Extraction Test (PBET)

The PBET simulates the leaching of a solid matrix in the human gastrointestinal tract, and determines the bioaccessibility of a particular element i.e. the total fraction that is available for adsorption during transit through the small intestine (Ruby *et al.* 1993). The PBET was designed around the paediatric gastrointestinal tract for a child of 2-3 years old. This age group was chosen because it is believed to be at greatest risk from accidental soil ingestion (Ruby *et al.* 1993). This test is essentially a two stage sequential extraction using various enzymes to simulate both gastric and the small intestine compartments with extraction carried out at 37°C. Potentially contaminated soils are introduced into the simulated gastric solution to solubilise any bioaccessible metal present. The conditions are then modified after a stomach sample has been collected to simulate the small intestine. The reaction vessels for the extraction are argon purged to keep the system under anoxic conditions. The metal content of the extracts was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

The data obtained have been linearly correlated with results from a Sprague-Dawley rat model (r^2=0.93) between *in-vitro* and *in-vivo* (n=7) and arsenic data were found to be over-predictive of bioavailability when compared to rabbit and primate models (Ruby *et al.* 1996). The stomach phase of the test has also been used for the determination of lead in house dusts. These data have been correlated to blood lead levels in the children living in the houses where the dusts were sampled (Ruby *et al.* 1999). All data collected at present indicate that the PBET is a "good predictor of oral lead bioaccessibility" (Basta and Gradwohl 2000). Ongoing research to further validate the PBET for arsenic is currently being undertaken by the Solubility/Bioavailability Research Consortium in the USA, which includes representatives from the United States Environment Protection Agency (USEPA), academia and consultants from the Exponent Environmental Group (Smith and Rawlins 1998).

The test as first described (Ruby *et al.* 1993) is cumbersome and difficult to carry out for large batches of samples for two reasons:

i) difficulties in obtaining reproducible mixing of the sample with argon gas whilst manipulating the samples in the temperature controlled water bath; and,
ii) the dialysis bag containing the sodium carbonate solution can be easily ruptured (Rodriguez and Basta, 1999) and it takes a long time for the pH to rise to 7 in the small intestine phase of the extraction (longer than the 4 h small intestine transit time recommended).

Table 2.3 Summary of the Main Features of Current Gastrointestinal Analogue Extraction Tests

Method	Type	Compartments	pH	T °C	Food	L/S ratio	Residence time	Analysis	Metals tested	Validation status	Ease of use
		Note a				Note b	Note c	Note d		Note e	Note f
PBET	Batch	2	2.5	37	n	100/1	1 hr	solution	As,Pb	V/swine,monkey(As, Pb)	original 3
		3	7	37	n		4 hr	Solution(2) *			modified 7
SBET	Batch	2	1.5	37	n	100/1	1 hr	solution	As,Cd,Pb	V/swine(Pb)	9
IVG	Batch	2	1.8	37	y	150/1	nr	solution	As	V/swine(As)	5
		3	5.5	37	y		nr	solution			
US P	Batch	2	ca.1	37	n	1000/1	2 hr	solution	Pb, Cr, As, Cd, Ni	NV	9
MB & SR	Batch	1		37	n	160/1	5 s		Pb, Cr, As, Cd	C/human(Pb)	3
		2	ca.1	37	n	2160/1	2 hr	solution			
		3		37	n	4770/1	4hr	solution +solid			
DIN	Batch	1	6.4	37	y	15/1	0.5		As,Cd,Pb,Cr,Hg	V/swine(unpublished)	5
		2	2	37	y	50/1	2 hr				
		3	7.5	37	y	100/1	6 hr	solution			
SHIME	Batch	2	5.2	37	y	2.5/1	3 hr		As,Cd,Pb	C	5
		3	6.5	37	y	4/1	5 hr	solution			
RIVM	Batch	1	6.5	37	n	15/1	5 m		As,Cd,Pb	C	6
		2	1.1	37	n	37.5/1	2 hr				
		3	5.5	37	n	97.5/1	2 hr	solution +solid			
TIM	Flow-Through	1	5	37	n	5/1	5m		As,Cd,Pb	C	2
		2	2	37	n	30/1	1.5 hr				
		3	7.2	37	n	51/1	6 hr	solution			
AOAC	Batch	2	1.12	37	n	150/1	16 hr	solution	Cu, Zn, Mn, Fe, Al	NV	9

Note a – 1 refers to Oral Cavity; 2 refers to Stomach; 3 refers to Small Intestine.
Note b – Liquid to solid ratio used in the extraction.
Note c – Time of reaction in each compartment; nr indicates that a time was not reported.
Note d – Indicates the nature of the sample taken for analysis from each compartment.
Note e – NV indicates no validation against other bioavailability or bioaccessibility methods.
V indicates the method has been validated against a bioavailability model (human or animal).
C indicates the method has not been validated but has been compared to other bioaccessibility tests.
Note f – Ease of use scale 1 to 10. Ten represents a relatively fast test with simple apparatus (suitable for large batches of samples) and 1 represents a relatively slow test with very complex apparatus (not suitable for large batches of samples).
* Two samples are taken from this compartment for this test.

Two modifications have been adopted by the British Geological Survey (Cave *et al.* 2002b, c) that make the test more reproducible and easier to carry out. Rodriguez and Basta (1999) showed that the use of the dialysis tubing containing sodium carbonate or bicarbonate used to raise the pH for the small intestine extraction could be replaced by titrating the stomach extract directly with saturated sodium carbonate or bicarbonate solution to bring the pH to 7. Other workers (Medlin 1997, Ruby *et al.* 1999) showed that it was not necessary to maintain anaerobic conditions in the extraction solutions and the extraction could be carried out in screw-top polypropylene vessels. Agitation of the soil solution mixture could then be reproducibly carried out by end over end shaking in a water bath (Medlin 1997).

2.2.2 Simplified Bioaccessibility Extraction Test (SBET)

A simplified form of the PBET extraction procedure was developed specifically for lead bioaccessibility measurements (Medlin 1997). The stomach phase of this technique has been shown to correlate well for lead in a series of young swine studies conducted by USEPA region VIII and the University of Missouri (Ruby *et al.* 1999). A correlation coefficient of 0.85 was obtained for 15 soils studied as shown in Figure 2.1 (Medlin 1997)). This indicated that the extent of lead dissolution in an acidic stomach environment was predictive of relative bioavailability in two animal models (weanling rats and young swine). This simplified method has been refined further (Drexler 1999) to produce the SBET test. The development of the SBET was in response to a request by the USEPA region VIII and from a need for other US laboratories to be able to use and apply simple bioaccessibility testing regimes. The procedure was developed to test soils that had previously been studied in swine and other animal studies. The method has recently undergone extensive validation for lead in the USA, as required by the USEPA, and is thought likely to be adopted as a standard procedure (Rawlins and Wragg 1999).

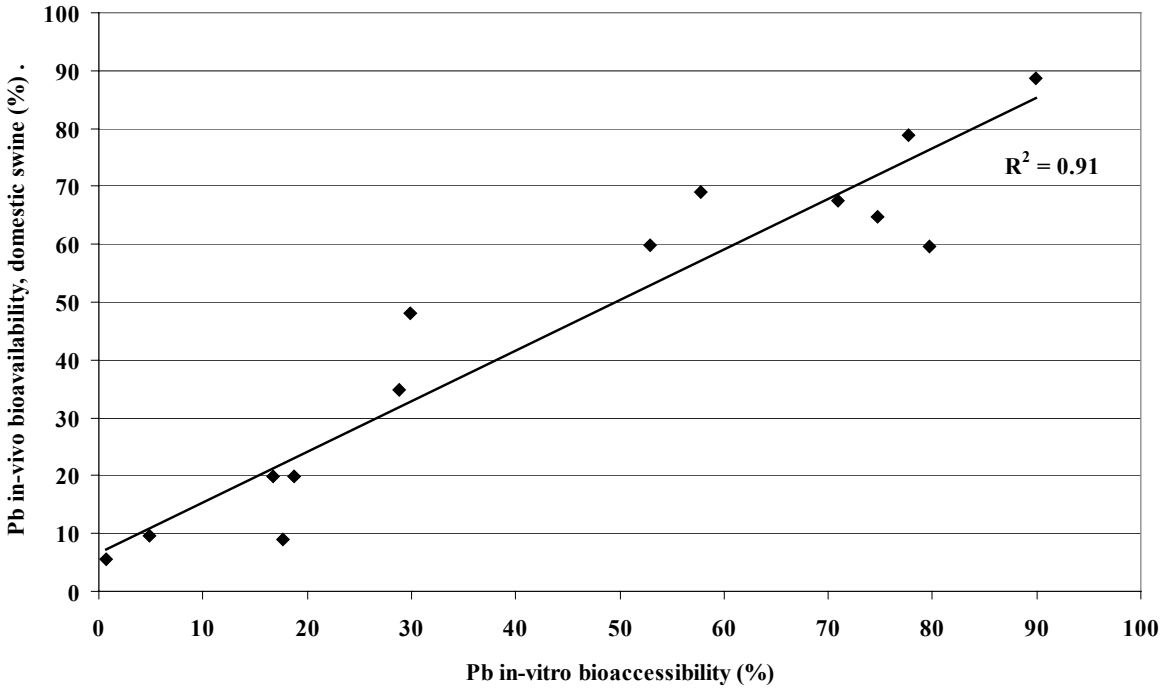


Figure 2.1 Correlation of the in-vitro SBET test with swine model bioavailability for lead (Medlin 1997).

The SBET extraction takes place at 37°C for one hour using a 0.4 M glycine solution adjusted to pH 1.5 with HCl. Constant agitation is used to simulate movement within the stomach. This method only considers uptake of lead from the stomach phase, the small intestine phase has been removed for lead, as at pH values above ca. 5.5 the lead is insoluble and would therefore be excreted with other solid matter. This choice of validation model is preferred as young swine have closer physiologic, anatomic, nutritional and metabolic similarities with humans (Dodds and Hsu 1982). The methodology is currently undergoing further validation because the *in-vivo* swine tests, mass balance studies show a net loss of arsenic with mass

recoveries of 23% and 36% for sodium arsenate and Grant-Kohrs tailings. The unknown nature of these losses leads to uncertainty in the accuracy of the swine model. In addition to these problems, there has not been as much work on arsenic compared to lead and there is a less comprehensive and reliable *in-vivo* database (Ruby *et al.* 1999). The next round of validation will include researching dust bioaccessibility and other elements such as cadmium, chromium and beryllium. It is hoped that the SBET will be widely adopted in the US, to replace the TCLP (USEPA 2000), once it has become a USEPA method. Currently it is only being used to refine risk assessments.

The SBET uses simple reagents in a single extraction test for a relatively short period of time making it practically simple to carry out and therefore ideal for large batches of samples. As yet, it has only been validated for lead using animal models. Results of this method have been published in an inter-comparison of bioaccessibility tests (Oomen *et al.* 2002); bioaccessible results for three contaminated soils for arsenic, cadmium and lead for this test were comparable to the other methods with a tendency to produce slightly higher values for lead. The reason for the latter observation is that all the other methods studied used both a stomach compartment extraction and a lower intestine compartment extraction, whereas the SBET only uses a stomach compartment extraction. Therefore the soil in the SBET method only experiences acid conditions but in the other tests the soil is extracted under acid conditions followed by the near neutral pH conditions similar to the lower intestine. This change in pH from low to neutral is likely to cause some of the lead to be precipitated from solution and therefore give a lower bioaccessibility than the SBET method (Medlin 1997).

2.2.3 *In-Vitro* Gastrointestinal Method (IVG)

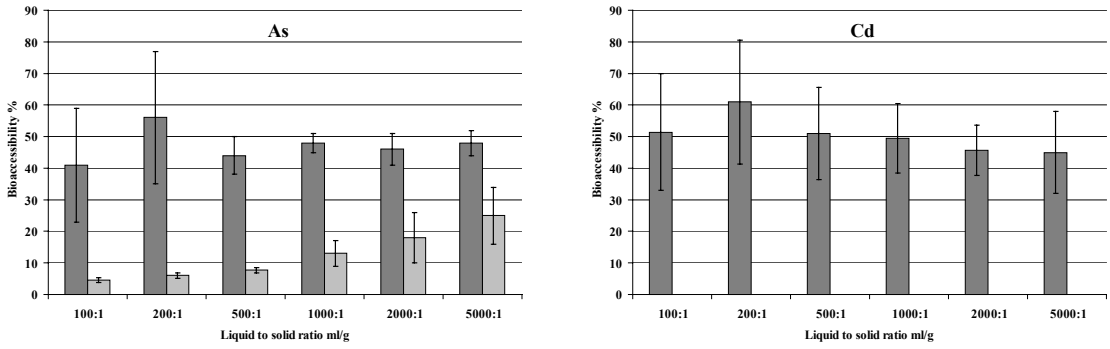
The *in-vitro* gastrointestinal method was developed to simulate the human gastrointestinal environment and estimate the bioaccessibility of arsenic in soil and soil media (Rodriguez and Basta 1999). The samples used for this study were not natural soils but aged (50 yr) calcine material, a waste product formed from roasting arsenopyrite ore and an iron ore slag material which was a waste product from smelting lead ore. The samples contained high arsenic concentrations ranging from ca. 300-18000 mg kg^{-1} and high concentrations of lead, zinc and copper. In this method, arsenic was sequentially extracted with simulated gastric and small intestinal fluids under anaerobic conditions at 37°C. The method was developed to address the limitations of the PBET test, as the ability to accurately predict arsenic bioaccessibility is not as good as it appears to be for lead (see Section 2.2.1). Unlike the PBET method, the IVG simulated gastric solution is prepared in a 0.15 M sodium chloride matrix and uses different concentrations of reagents and a lower pH (pH 1.8 for the stomach phase and 5.5 for the intestine phase). In addition to these differences, simulated food was added to the initial gastric solution in the form of dough. This test has also been further developed to include an iron-hydroxide gel phase, (IVG-AB method), to simulate the intestinal absorption of arsenic. Comparison of results against those collected from dosing trials using immature swine have shown that the data are not statistically different from the *in-vivo* method (Rodriguez and Basta, 1999). There was no increase in bioaccessible arsenic during the intestinal extraction phase and the addition of the iron-hydroxide gel did not affect the arsenic bioaccessibility. A comparison of results with and without the food in the simulated gastric fluid showed no change for a slag sample. However, for a calcine sample, there was increased extraction of arsenic in the presence of food, although the mechanism for this is unknown (Rodriguez and Basta 1999). The results of the PBET tests used in this work showed that the bioaccessible arsenic was generally lower than IVG method. This is probably due to the lower pH of the

IVG method. Despite the favourable results of this study, the method is practically time consuming to carry out and does not seem to have been adopted in subsequent studies. There are no data as to its performance on other metals.

2.2.4 US Pharmacopoeia Method (US P)

This *in-vitro* method was developed to simulate drug dissolution (USP XII 1990), but has been utilised by a number of workers to determine the bioaccessibility of heavy metals in contaminated soils (Hamel *et al.* 1998). The methodology uses a synthetic gastric solution of sodium chloride, hydrochloric acid and pepsin, which is used to extract the heavy metals in a given soil at 37°C over a period of two hours. From varying the solid to solution ratio it was found that a ratio of 1:1000 was the most appropriate for the test. However, the amount of solid compared to the volume of fluid used is very small in reality compared to other testing protocols highlighting difficulties in standardising the amount for incidental ingestion. The method is similar to the PBET, and although the human digestive system does contain hydrochloric acid, it is dilute compared to the volume used in this test. The PBET methodology contains ca. 1 ml per litre whereas this methodology contains 7 ml.

The US Pharmacopoeia method was used to obtain bioaccessibility data for National Institute of Standards (NIST) SRM 2710 Montana soil and a Jersey City composite soil for arsenic, cadmium, chromium, lead and nickel (Hamel *et al.* 1998). A summary of the bioaccessible fraction of the metals in the NIST 2710 Montana soil and the Jersey City Soil at different liquid to solid ratios are shown in Figure 2.2. The results showed that, within the uncertainty of the measurement, only arsenic in the Jersey City soil showed a significant change in bioaccessibility; increasing by a factor of ca. 5 from the lowest to the highest solid to solution ratios. However, it is difficult to put too much weight on the evidence of this work, as the high uncertainty in the measurement masked the effect of changing the solid to solution ratio. No results on how this method relates to bioavailability measurements have been presented.



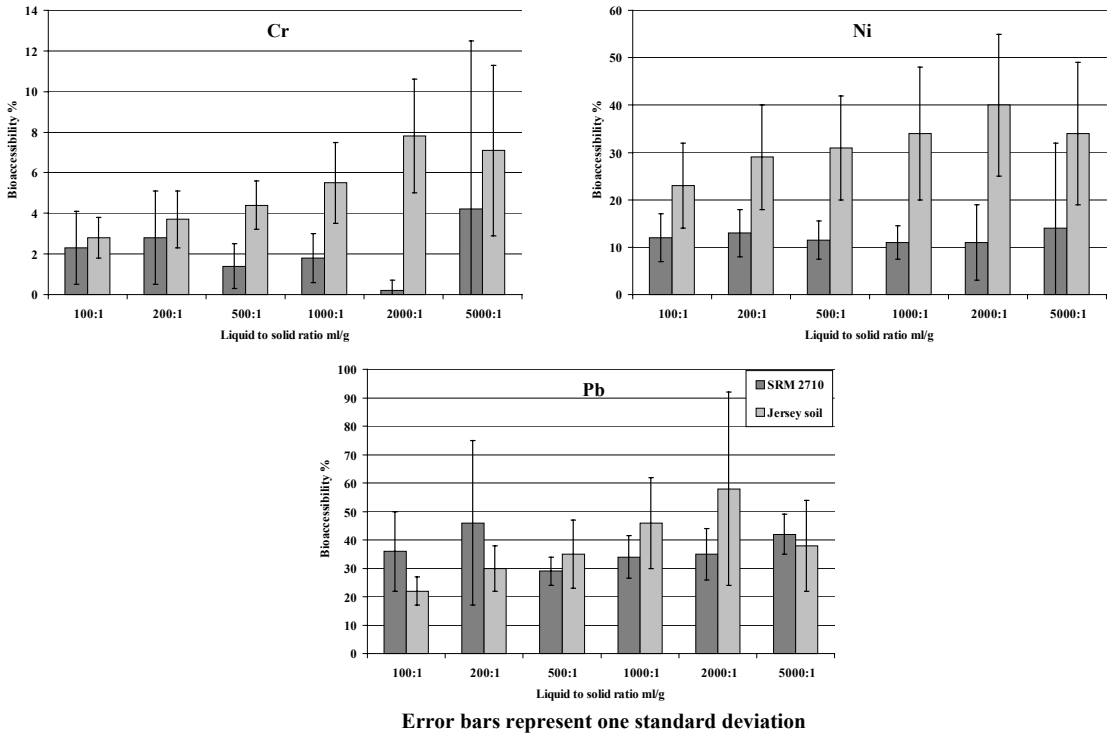


Figure 2.2 Effect of different solid to solution ratios on relative bioaccessibility (Hamel *et al.* 1998)

2.2.5 Mass Balance & Soil Recapture method (MB & SR)

In the development of the US Pharmacopoeia Method given in Section 2.2.4 Hamel *et al.* (1999) produced an *in-vitro* protocol which simulates three compartments of the gastrointestinal tract using artificial saliva as described by Fusayama *et al*. (1963), gastric fluid (US Pharmacopoeia formula, (USP XII 1990) and intestinal fluid (0.2 M sodium carbonate) to sequentially extract the soluble metal fraction from any given soil at 37°C. The three fluid types used are intended to reasonably characterise the processes that precede *in-vivo* absorption of a given contaminant. In this method not only was the concentration of extracted metal determined in each of the extraction stages, but also the final metal content of the soil was determined after extraction. There was good agreement between the extracted metal content and the decrease in metal concentration in the extracted soil. Measurement of the metal content of the recaptured soil gave better precision than summing the metal content of the extracts. The authors pointed out that measurement of metals in the recaptured soil has a number of other advantages:

- only one analysis has to be carried out other than determining the initial total metal content of the soil;
- analytical difficulties in the measurement of metals in complex gastric solution simulants are avoided; and,
- when the bioaccessible fraction is extracted into solution it is close to the instrumental detection limit, measurement of the difference between total metal content and non-bioaccessible metal content of the soil is likely to provide a more robust result.

Using this approach, the technique should provide the same information as other bioaccessibility experiments but with considerably less analytical effort. It therefore has the

potential to incorporate more complex fluids and be a more rapid estimation than other *in-vitro* techniques such as the PBET. However the overall method has many steps and is quite complex in its design.

The study by Hamel *et al.* (1999) to study four soils: the National Institute of Standards (NIST) SRM 2710 Montana soil; a soil from Liberty State Park, NJ USA; a soil from Bunker Hill ID, USA (obtained from a USEPA special investigation site residential garden in a mining impacted area); and a soil from Califon, NJ, USA. Results for lead, arsenic, cadmium and chromium were presented. The authors were able to make a direct comparison with their measured bioaccessibility data for lead in the Bunker Hill soil with human bioavailability data obtained from a study which had been carried out on the same sample (Maddaloni *et al.* 1998). The bioaccessible fraction of lead in the soil was found to be 70% ± 11 (95% confidence limit), which is significantly higher than the bioavailable fraction found from the human study (26.2% ± 8.1), suggesting that this method provides more aggressive extraction conditions than those found in the human gastrointestinal tract. The relatively high bioaccessibility compared to the bioavailability value may be due to the high concentration of HCl used in the stomach phase (see Section 2.2.4). In addition, there are a number of enzymes and other gastrointestinal constituents (e.g. bile salts, pancreatin) omitted from this procedure compared to the PBET method.

2.2.6 German DIN 00 19738 (DIN)

The *in-vitro* method used at the University of Bochum, Germany (DIN 2000), is "a test system for mobilising pollutants in contaminated materials using synthetic digestive juices". To date the method has been partially validated for both organic and inorganic contaminants under standardised conditions that are physiologically close to humans (Hack and Selenka 1996). Validation studies for mini-pigs are being performed and will be published in the future (Oomen *et al.* 2002). The DIN method has been used for anthropogenically and geologically contaminated soils. Other work has been completed on a variety of sample types, including fly ash, blasting sand, sewage sludge, sediments and foundry waste (DIN 2000). The synthetic juices employed contain both electrolytes and organic chemical components. The sample under investigation is treated with gastric juice (pH 2.0 ± 0.3) for two hours, followed by a 6-hour small intestine phase (pH 7.5 ± 0.3). The use of nitrogen to create anaerobic conditions is optional for special purposes and the whole system is kept agitated at 37°C. It has been found that the incorporation of a saliva phase has only a negligible effect on the level of mobilisation for organics and is an optional step depending on the nature of the sample (DIN 2000). The influence of foodstuffs has also been investigated as part of the development of the methodology; this was achieved by the addition of whole milk powder or other food. The use of whole milk powder is used to substitute the average high fat and protein constituents in human food. This highlights the effect of food on the mobilisation of pollutants in the gastrointestinal tract of babies and small children (Hack and Selenka 1996, Oomen *et al.* 2002). In a comparative study of soils (Oomen *et al.* 2002), the DIN method gave values within the bioaccessibility range obtained by the other methods. In the presence of milk powder, simulating fed conditions in the stomach, the bioaccessibility for arsenic, cadmium and lead increased for all soils. However, the DIN model does not take into account the probable increase in pH in the presence of food and therefore assumes the worst-case scenario.

2.2.7 Simulator of Human Intestinal Microbial Ecosystem of Infants (SHIME)

The VITO institute, Belgium, is using an *in-vitro* digestion method in conjunction with the University of Ghent for use in contaminated land studies. The original system was designed to simulate the gastrointestinal microbial ecosystem of humans (Molly *et al.* 1993). The SHIME method includes both stomach and small intestine phases with incubation at 37°C for 3 and 5 hours respectively. The gastric solution contains a more complex mixture of reagents than the PBET method including cream to simulate nutrition of young children. The stomach phase is adjusted to pH 5.2 and the small intestine phase to pH 6.5. For soil samples, a comparison has been made with other bioaccessibility tests for arsenic, lead and cadmium (Oomen *et al.* 2002). The stomach conditions for this study were adjusted to pH 4.0, which is more realistic of fed conditions. The results for all metals were consistently lower by a factor of five to tenfold. This was thought to be due to the relatively high pH of the stomach compared to the other methods. In addition to this, the method uses a much higher solid to liquid ratio than the other methods considered, although it was thought that this was not the cause of the low bioaccessibility values (Oomen *et al.* 2002). There are no validation data for this test against *in-vivo* studies for metals in soils, but the test has been successfully validated against human studies on polysaccharides (Molly *et al.* 1994).

2.2.8 RIVM in-vitro Digestion Model (RIVM)

An *in-vitro* digestion model being used and refined by the National Institute of Public Health and the Environment (RIVM) in the Netherlands is based on a method originally used for measuring the bioaccessibility of organics from slag material (Rotard *et al.* 1995). This was subsequently modified for soil samples (Sips *et al.* 1998) and used for studies of organics and lead bioaccessibility in soils by Oomen *et al.*(2000). This is a three-stage sequential extraction method using a five minute saliva phase at pH 6.5, followed by a two hour stomach extraction at pH 1.07 and a two hour small intestine extract at pH 5.5 (Oomen *et al.* 2002). For soil samples, a comparison has been made with other bioaccessibility tests for arsenic, lead and cadmium (Oomen *et al.* 2002). Results for the three metals were in the middle of the range found for the other tests. However mass balance measurements gave recoveries significantly higher than 100% for some combinations of soils and metals. This was thought to be a problem with the total digestion procedure used by this laboratory that was shown to give low recoveries (Oomen *et al.* 2002). This highlights the problem of reporting bioaccessibility values relative to the total metal concentration. Clearly, if different laboratories use different total metal measurement techniques (e.g. aqua regia digest, HF digests, XRF analysis), the apparent relative bioaccessibilities will not be comparable. There are no validation data for this test against *in-vivo* studies for metals in soils.

2.2.9 TNO Gastrointestinal Model (TIM)

The current method employed by TNO Nutrition, at Zeist in the Netherlands is a complex *in-vitro* test system involving a number of gastrointestinal solutions (Minekus *et al.* 1995). A number of fractions are produced from this method over a period of 6 hours. After the initial saliva phase, the pH of the stomach extraction is reduced from 5 down to 2 over a period of 1.5 hours. As the system is dynamic, the mixture from the stomach phase passes through three intestinal phases that represent the duodenum at pH 6.5, the jejunum at pH 6.8 and the ileum at pH 7.2. Mathematical modelling of gastric and ileal delivery with power exponential

equations was used for the computer control of meal transit. The model was shown to reproduce accurately the pre-set data on meal transit, pH and bile salt concentrations in the different gastrointestinal compartments. The model has been validated by comparing the dissolution profile of drugs *in-vivo* with and without food components. For soil samples, a comparison has been made with other bioaccessibility tests for arsenic, lead and cadmium (Oomen *et al.* 2002). Arsenic and cadmium bioaccessibility were similar to the other methods and the lead bioaccessibility was relatively low in comparison. The differences in lead bioaccessibility could be attributed to the dynamic design of the TIM model or to the differences between the filtration methods used in the different tests. Other factors may also be involved and require further investigation. The TIM method has been designed to be a much closer analogue of the human gastrointestinal tract than static extraction tests. The results should, therefore, be a better approximation to the true bioaccessibility than the static methods. The system is, however, quite complex and not suited to the analysis of large batches of samples. It is likely that the role of this type of test would be as a reference method for validation of new procedures.

Another computer controlled dynamic system has been designed and built by the Nutrition and Food Technology group of the University of Auvergne in France. This system has been used for food and drug bioaccessibility studies (Blanquet *et al.* 2001) but has not yet been used for soil bioaccessibility testing.

2.2.10 Association of Analytical Communities (AOAC) Pepsin Digestibility Test

The AOAC Pepsin Digestibility Test 971.09 was originally designed to measure the digestible protein in animal foods (AOAC 2000). The method mimics the conditions of a chicken's metabolism. A sample is agitated in a flask with 0.075 N hydrochloric acid (the pH of a chicken's stomach) and 0.2 % pepsin for 16 hours at 37°C. Porcine pepsin is used because it is easily isolated and reliably reproducible from batch to batch. In recent work, this test has been applied to contaminated estuarine sediments (Turner *et al.* 2001, Turner and Olsen 2000) to obtain bioaccessibility data for aluminium, copper, iron, manganese and zinc. Variations of the concentrations of the reagents were reported and results were compared to extractions carried out using the gut fluid of plaice. Results were quite variable for the different extraction solutions and there was clear evidence that the bioaccessibility is dependent on the concentration of enzymes in the system (Turner *et al.* 2001). In order to check the relevance of this method to human bioaccessibility, further comparisons of the data produced with other bioaccessibility and bioavailability studies need to be carried out.

3. CONTROLLING FACTORS IN BIOACCESSIBILITY MEASUREMENTS

3.1 Physico-Chemical controls on bioaccessibility

Most of the work on bioaccessibility testing has concentrated on the development of the extraction methods that mimic the human digestive system. This research is mainly concerned with the gastrointestinal uptake of a few toxic contaminants. However, there has been relatively little work on the physical and chemical processes that govern the

bioaccessibility of these contaminants in soils and why they are solubilised under conditions found in the gastrointestinal tract of animals and humans.

Davis *et al.* (1996) studied the mineralogical constraints on the bioaccessibility of arsenic in mining sites in the Anaconda soils. They concluded that the arsenic bioaccessibility compared to the total arsenic content in the soils was constrained by:

- encapsulation in insoluble matrices e.g. energite in quartz;
- formation of insoluble alteration or precipitation rinds e.g. authigenic iron hydroxide and silicate rinds precipitating on arsenic phosphate grains; and,
- formation of iron-arsenic oxide and arsenic phosphate cements that reduce the arsenic–bearing surface area available for dissolution.

In a previous study on lead in Montana soils Davis *et al*. (1993) found similar results in which the solubility was constrained by alteration and encapsulation which limited the available Pb-bearing surface area. Ruby *et al.* (1996) diagrammatically summarised how the chemical and mineralogical forms of arsenic and lead relate to their bioaccessibility. Figure 3.1 shows the possible physico-chemical processes governing the bioaccessibility of arsenic in a contaminated soil.

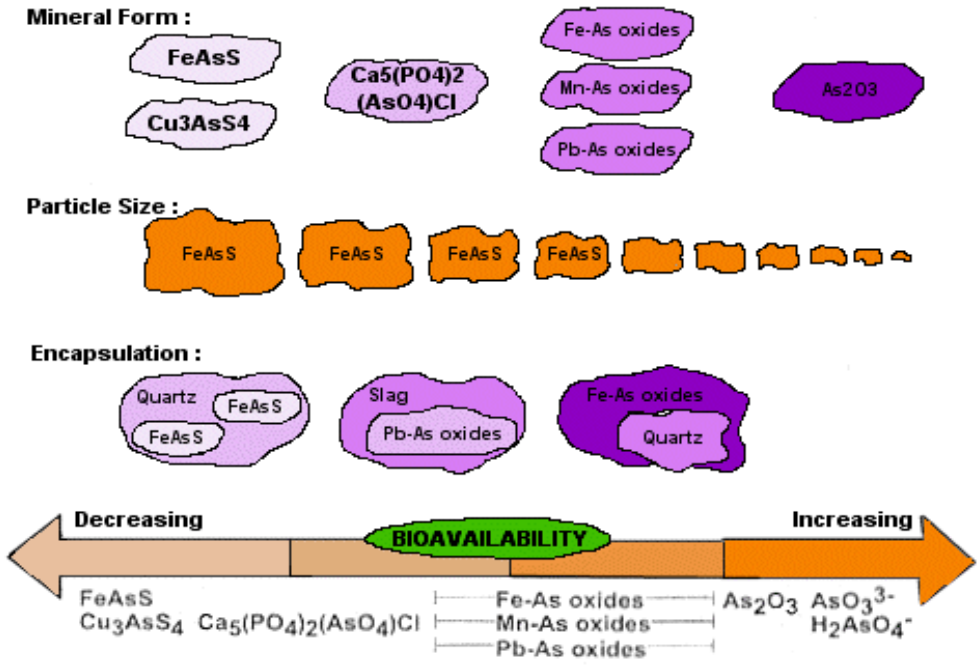


Figure 3.1 Schematic diagram of how different arsenic species, particle size and morphologies affect arsenic bioavailability (Ruby *et al.* 1996).

In addition to these geochemical and physical limitations, the kinetic effects (Ruby *et al.* 1992) associated with dissolution and transit times in the gastrointestinal tract limit the amount of contaminant likely to be bioaccessible.

Rieuwerts *et al.* (1998) have looked at the possibility of modelling the soil solution availability of a number of metals by considering the physical and chemical properties of soils. They suggest that an empirical model using soil parameters (e.g. total metal concentration, pH, redox potential, soil texture, clay content, organic matter content, iron and manganese oxide and the presence of cations and anions in soil solution) could be used to

predict bioavailability. The author's definition of bioavailability in this instance is 'the soil solution concentration', which is not necessarily related to human bioaccessibility.

Recent studies (Cave *et al.* 2002b, c) have compared major and trace element data from PBET and SBET tests, total soil concentrations and a new sequential extraction methodology. The method uses multivariate statistical analysis of chemical concentration data from a sequential extraction using increasing concentrations of nitric acid. The information obtained allows distribution of the trace elements between the physico-chemical components of the soil to be determined (Cave *et al.* 2002a). This holistic approach provides an understanding of how the toxic metals bind to the soil matrix and what forms of the metals are likely to be bioaccessible. The results of the sequential extraction test have been confirmed by XRD and SEM studies (Cave *et al.* 2002a, Cave and Wragg 2002, Pearce *et al.* 2002). Initial results from the arsenic study from a variety of UK sites (Cave *et al.* 2002b, c) show that the majority of the arsenic is associated with iron oxide-related components. The form of the iron oxide e.g. amorphous or crystalline appears to be one of the controlling factors that influence arsenic bioaccessibility. Clearly, this highlights the need to consider the geochemical forms of the potential contaminant and to carry out a multi-element approach to allow informed decisions to be made.

3.2 Experimental factors affecting bioaccessibility

In order to carry out successful bioaccessibility testing, it is necessary to have a full understanding of the factors within the chain of sample collection, preparation, analysis and data processing that are most influential to the final results obtained.

3.2.1 Sampling and sample preparation

As with all soil analysis applications, the final results are only meaningful if the soils are representative of the sampling location. The soil sampling exercise must, therefore, take into account: the history of the site (e.g. likelihood of hotspots and effect of the underlying geology); use a well designed sampling protocol that includes provision for the collection of duplicate samples (Lee and Ramsey 2001, Ramsey and Argyraki 1997); use clean high quality sampling tools and sample containers to avoid contamination and cross contamination (particularly for sites with a high contrast in contamination); and maintain good written records of sample locations, sample numbers, date and time of sampling, descriptions of the samples taken and any field measurements taken on site.

When considering the choice of size fraction of soils, studies have used <2mm (Oomen *et al.* 2002), <250µm (Davis *et al.* 1997a, Davis *et al.* 1993, Davis *et al.* 1992, Maddaloni *et al.* 1998), <150µm (Casteel *et al.* 1997) and <125µm (Hamel *et al.* 1998). As the methodologies are concerned with accidental ingestion of a given soil there needs to be uniformity as to the size fraction that adheres to hands. It is therefore inappropriate to use the <2mm size fraction that is commonly used for soil analysis. The size fraction of choice in the majority of cases is the <250µm grain size, because it is considered to be the optimum size to adhere to children's hands (Duggan *et al.* 1985). Another reason for using this fraction is that it is routinely used in electron microprobe investigations that have supported human health risk assessments (Davis *et al.* 1997b).

3.2.2 Extraction conditions

A number of the methods used are based on similar extraction techniques incorporating a combination of mouth cavity, stomach and small intestine phases. There seems to be evidence that the mouth cavity compartment has little effect on the final bioaccessibility and in some methods it is an optional step (DIN 2000). A number of methods only collect one solution sample for analysis after completing the two or three stage extraction. This has some potential disadvantages as highlighted in Oomen *et al.* (2002). With only one sample at the final stage it is not possible to monitor how the concentration of metals changes in the preceding steps. For example, the stomach phase has a low pH that is changed to a higher pH in the small intestine, causing the concentration of some metals to be higher in the stomach and because of precipitation reactions to be lower in the intestine phase (Ruby *et al.* 1996). It may be more appropriate for the purposes of risk assessment to calculate a bioaccessibility in both compartments and, if the stomach concentration is very much higher than in the intestine, it may be better to use the more conservative estimate of the two. This is why the one step SBET test gives higher lead bioaccessibility than some of the multi-step methods (See Section 2.2.2). The PBET method uses this multi-sampling approach but this could also be used in other methods. The analysis of the soil after the bioaccessibility extraction test is useful as it allows the extraction process to be checked by calculation of a mass balance. Although this procedure is only reported for two methods (Hamel *et al.* 1999, Oomen 2000) there is no practical reason why it cannot be applied to the other methods.

In a comparative study of five bioaccessibility methods Oomen *et al.* (2002) concludes that pH is probably the one single factor that has the most influence on the final result; low pH in the stomach phase leading to higher bioaccessibility values. It is therefore wise not to consider methods that use too high or too low pH conditions in the stomach. The US Pharmacopoeia and the Mass Balance & Soil Recapture methods use too much hydrochloric acid and are not representative of physiological conditions (Ruby *et al.* 1999). Whereas the SHIME method probably uses a pH value of 5.2, which is too high (Oomen *et al.* 2002).

The solid to solution ratios of the physiologically based tests varies from 2.5:1 to 5000:1 (Table 2). Hamel *et al*. (1998) suggest that the ratio is not very critical, but it is better to choose conditions that are more closely aligned to a child's (2-3yr) gastrointestinal system. (Ruby *et al.* 1996) suggests this should be ca 100:1.

The median stomach small intestine residence times for the methods in Table 2 are 2 and 4.5 hr respectively which are in the range of values found for a 2-3 yr old child (Ruby *et al.* 1996). The exception to this is the AOAC method (16 hr) that is based on a chicken's physiology for the stomach phase only and is therefore not a practical choice for human health risk assessment. There is very little information in the literature regarding the sensitivity of the methods to residence time but it is thought that for those tests based on human physiology, the relatively small variations in methodologies have little effect on the final bioaccessibility values obtained.

Some of the physiologically based methodologies specify the use of food simulants in the extraction mixture, (see Table 2) to assess the effect of fed or fasted conditions on the bioaccessibility measurement. Milk powder or milk products (DIN 2000, Molly *et al.* 1993) and dough (Rodriguez and Basta 1999) have been used as food types. The choice and the amount of food used could potentially have a significant effect on the measured bioaccessibility for example by changing the pH or coating the soil particles. From a practical

view it is one less variable to deal with when carrying out the extraction process if only fasted conditions are considered. From the point of view of risk assessment, however, some workers using bioaccessibility tests (Oomen *et al.* 2002, Rodriguez and Basta 1999) found that the inclusion of food increased the bioaccessibility of some elements; whilst others working on a human model (Maddaloni *et al.* 1998) found that under fed condition bioavailability of lead decreased. Until further work has been carried out, testing of both fed and fasted conditions should be considered to ensure that representative bioaccessibility data are obtained. Because of the variety of foods, the differences between diets in different areas and cultures as well as different metal species reacting differently to different food types it is likely that this could be a complex task.

As already discussed (Section 2.1) chemical sequential extraction and single extraction test systems have a number of limitations for bioaccessibility testing. These include non-specificity and, as each extraction is intended to define the specific phases present by the reagents used, determining bioaccessibility from such systems has no physiological basis. At present there is little or no information available on the ability of chemical fractionation methods to measure the bioaccessibility of heavy metals from incidental ingestion of contaminated soils (Basta and Gradwohl 2000).

4. QUALITY ASSURANCE CONSIDERATIONS

Bioaccessibility testing is still in an early stage of development. There are no internationally recognised standard methods, although there is a German standard procedure (DIN 2000). The most comprehensive comparison of techniques has been carried out by the Bioaccessibility Research Group of Europe (BARGE) as described by Oomen *et al*. (2002), which has highlighted some of the variables that could have an effect on bioaccessibility. Collaborative research under the auspices of BARGE is continuing (Oomen *et al.* 2002). Information regarding the quality of data produced by the methods is very sparse. Method precision data is limited to within laboratory repeatability, with little or no data on between laboratory reproducibility. The production of information on the quality of results is hampered by the lack of any standard material for laboratories to test their methods against and the existence of any proficiency testing schemes to monitor continuing performance. There are some data on soils certified for their total metal content (e.g. NIST 2710 and 2711) (Ellickson *et al.* 2001, Hamel *et al.* 1998, Hamel *et al.* 1999, Oomen *et al.* 2002) but improvements in the quality of data from bioaccessibility testing will not be able to move forward unless proficiency testing schemes are set up and reference materials are characterised and made available.

It is quite common that bioaccessibility measurements are reported as relative bioaccessibility (the contaminant mobilised from the soil during digestion relative to the amount of contaminant in the soil before digestion, expressed as a percentage (Oomen *et al.* 2002)). The problem with this definition for metals is that there are a number of methods available for the determination of the total metal in the soil prior to digestion. Many laboratories use 'pseudo-total' methods, e.g. aqua regia or nitric acid extraction (Oomen *et al.* 2002), whereas other laboratories use digestion techniques that break down the whole soil matrix using a combination of hydrofluoric and mineral acids (Thompson and Walsh 1983). Other analysis techniques such as XRF also give total concentrations in the soil (Johnson *et al.* 1996; Revenko 1994). Relative bioaccessibility results will, therefore, depend on the method used for determination of the metal before the bioaccessibility digestion as well as the

bioaccessible content itself. This makes comparisons between methods very ambiguous. To avoid this confusion, it is recommended that bioaccessibility results be reported as mg kg^{-1} in the solid along with the total concentration including a description of the method used to obtain the total value. It is the authors' belief that methods that measure the 'true totals' in the soil are to be recommended, as this helps to give a better overall picture of the extent of contamination and greatly aids subsequent risk assessment.

5. CURRENT INITIATIVES

There are groups such as the BARGE that are starting to address the problems associated with standardisation of methods and inter-laboratory comparisons of techniques (BARGE). In addition to this, BARGE is also looking at cross validation studies with the work of Maddaloni *et al*. (1998). This work has been conducted on humans for lead bioaccessibility/bioavailability. This study is using a round robin trial in which the methods outlined in Oomen *et al.* (2002) and the modified PBET test (see Section 2.2.1) are being used in both fed and fasted conditions on the Bunker Hill soil used by Maddaloni *et al*. (1998). Other work currently being carried out is on the use of different bile salts for the small intestine phase. The work started using porcine bile salts and has progressed to chicken and bovine derived salts. Preliminary results have indicated that the use of chicken bile produces increased bioaccessibility values compared to bovine for a Butte Montana reference material (BARGE).

In the USA, ongoing research to further validate the PBET for arsenic is currently being undertaken by the Solubility/Bioavailability Research Consortium (SBRC), which includes representatives from the USEPA, academia and consultants from the Exponent Environmental Group (Smith and Rawlins 1998). A list of current topics of research can be found the Exponent Environmental Group website (Exponent Environmental Group).

From the review to date, it has been noted that there is very little research into the speciation of arsenic and lead in the simulated gastrointestinal systems (Oomen *et al.* 2002). There are well known differences between the toxicity of the different forms of metal contaminants e.g. arsenic III/V oxidation states (Department for the Environment Food and Rural Affairs and the Environment Agency 2002b). The way in which simulated gastrointestinal solutions alter the species present could also play an important role in assessing risk to human health. This topic will need to be addressed more rigorously in future investigations.

6. CONCLUSIONS AND RECOMMENDATIONS

In order to use bioaccessibility measurements successfully, there are a number of key areas that must be addressed:

- A well designed sampling strategy and soil sampling programme must be carried out. If sampling is not carried out correctly, no matter how good the analysis and testing of the soils, the data will not adequately represent the site under investigation;

- The bioaccessibility extraction test must be physiologically based and preferably validated against an animal or human study. It is also sensible to choose a test that tends to be over predictive of bioaccessibility so that the results provide more conservative values for inclusion in risk assessments;

- A holistic approach to bioaccessibility measurement should be adopted. The results must be put into the context of the whole geochemistry, previous land use and proposed use of the area under study;

- Single metal studies do not supply enough information to aid the final assessment of risk. Studies of both major and trace element concentration in the soil and the bioaccessible fraction are needed to interpret the data in a meaningful way (Cave *et al.* 2002c);

- Reference soil material(s) certified for total metal content should be analysed with the samples. A reference soil for which there is bioaccessibility data from other methods should be analysed by the bioaccessibility test being used for the study; and,

- The final report of the data should provide: the total trace element and major metal content (and the method used for analysis); the bioaccessible values (expressed as mg kg^{-1} in the soil) put into the context of the method used, the values obtained from the reference soil compared to other methods; and the uncertainty on the total and bioaccessible values obtained including both sampling and analysis contributions.

6.1 Recommended choice of a bioaccessibility test method

There are advantages and disadvantages associated with all of the methodologies available, sensibly it is most informative to use a method that is based around the human gastrointestinal environment. The validation status of any methodology is also an important factor, although the differences in human and animal physiology must be taken into consideration. The ease of use of the method will also be an important consideration, particularly when large batches of samples are to be analysed (See Table 2.3 for overview of methodologies).

The TIM method (see Section 2.2.9) requires the use of very specialised laboratory equipment and is therefore not useful as a routine procedure. However, of all of the procedures discussed, this method most closely monitors the human digestive system. This method should therefore be considered as reference method and outside of full human/animal testing should be regarded as an interim procedure for validating the simpler routine *in-vitro* methods.

6.2 Recommendations for further work

For the immediate future, the most important task is set up proficiency testing schemes and to identify and characterise potential reference materials to allow the quality of the data from *in-vitro* tests to be monitored. In conjunction with this, there needs to be further research carried out to validate that the *in-vitro* test data relates to human bioavailability for a wider range of metals and soil types. An important adjunct to this work will be the measurement of the way in which simulated gastrointestinal solutions alter the chemical speciation of contaminant metals, as this could play an important role in assessing risk to human health (See Section 5).

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