
Soil quality — Assessment of human exposure from ingestion of soil and soil material — Procedure for the estimation of the human bioaccessibility/bioavailability of metals in soil

Qualité du sol — Évaluation de l'exposition humaine par ingestion de sol et de matériaux du sol — Mode opératoire pour l'estimation de la bioaccessibilité/biodisponibilité pour l'homme de métaux dans le sol



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 7, *Impact assessment*.

This first edition of ISO 17924 cancels and replaces ISO/TS 17924:2007, which has been technically revised. The changes compared to the previous edition are as follows:

- 7.1 "General", 7.2 "Choosing an appropriate test", 7.3 "Description of applicable test methods" and 7.4 "Recommendations" have been deleted. 7.5 "Use and interpretation of *in vitro* tests for risk assessment" has been retained and renumbered to [Clause 7](#);
- [Clause 8](#) "Description of test method" has been added;
- [Clause 9](#) (formerly Clause 8) "Data handling, quality control and presentation of results" has been completely revised;
- Annex A "Human bioaccessibility testing" has been replaced by [Annex A](#) "Sample preparation procedure";
- the figures have been revised;
- the complete document has been editorially revised;
- the Scope has been adapted.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

When assessing soils contaminated with, for example, potentially harmful elements (e.g. arsenic), soil ingestion (especially by children) is often considered to be the most important exposure pathway. This assessment is often carried out on the basis of total content of the potentially harmful elements in question in the soil. However, several studies suggest that the availability of the potentially harmful elements (e.g. arsenic) in gastrointestinal tract is dependent on the form of the potentially harmful elements present and the site-specific soil chemistry. Test methods based on *in vivo* tests with, for example, juvenile swine or mini pigs are time consuming and expensive and not very compatible with the decision processes connected with the assessment and clean-up of contaminated sites. Test methods have thus been developed and validated, which involve *in vitro* laboratory tests aimed at simulating *in vivo* results. This will reduce the cost and practicalities related to the use of such testing on contaminated land.

Due to the large expenditure necessary for both private landowners and public funds set aside for the remediation of contaminated land, International Standards on the assessment of contaminated soil, especially with regard to human health, are in great demand. International Standards in this complex field will support a common scientific basis for the exchange of data, development of knowledge and sound evaluation. The aim of this document is to describe the elements of such an *in vitro* test system and give advice as to the appropriate combination and use of these elements in the specific situation. The method is based on the Bioaccessibility Research Group of Europe, Unified Bioaccessibility Method (BARGE UBM), which has been developed and agreed upon by the BARGE group.

In human health risk assessment, “bioavailability” is specifically used in reference to absorption into systemic circulation, consistent with the toxicological use of the term. This encompasses bioaccessibility, which again is a combined measure of the processes determining the interaction between the metal associated with the soil and the liquid in the human digestion system. Bioavailability furthermore includes the absorption of the contaminant through a physiological membrane and the metabolism in the liver. The bioavailable fraction is thus the fraction left after release into the human digestive liquid, transport across the intestinal epithelium and metabolism in the liver. Further description of these processes is given in [Clause 4](#).

When considering bioavailability as the fraction of the chemical that is absorbed into systemic circulation, two operational definitions are important: absolute and relative bioavailability. Absolute bioavailability is the fraction of the applied dose that is absorbed and reaches the systemic circulation (and can never be greater than 100 percent). Relative bioavailability represents a comparison of absorption under two different sets of conditions, for example from a soil sample vs. food or another matrix used in a toxicity study, and can be greater than or less than 1. This factor can be used in exposure assessments for exposure by direct ingestion of soil, for instance if the absolute bioavailability of the metal in the specific soil is suspected to differ significantly from the absolute bioavailability implicit in the toxicity value/quality criteria used.

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Soil quality — Assessment of human exposure from ingestion of soil and soil material — Procedure for the estimation of the human bioaccessibility/bioavailability of metals in soil

1 Scope

This document deals with the assessment of human exposure from ingestion of soil and soil materials. It specifies a physiologically based test procedure for the estimation of the human bioaccessibility of metals from contaminated soil in connection with the evaluation of the exposure related to human oral uptake.

The method is a sequential extraction using synthetic gastrointestinal fluids and can be used to estimate oral bioaccessibility. Soils or other geological materials, in sieved form, are extracted in an environment that simulates the basic physicochemical conditions of the human gastrointestinal tract.

This document describes a method to simulate the release of metals from soil and soil materials after passage through three compartments of the human gastrointestinal tract (mouth, stomach and small intestine). It produces extracts that are representative of the concentration of potentially harmful elements in the human gastrointestinal tract for subsequent chemical characterization.

NOTE 1 Bioaccessibility can be used to approximate oral bioavailability.

NOTE 2 The test has been validated for arsenic, cadmium and lead in an interlaboratory trial. The method has been *in vivo* validated to assess the oral bioavailability of arsenic, cadmium and lead.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 11074, *Soil quality — Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 11074 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

absorption

process by which a body takes in substance and makes it a part of itself

3.2

bioaccessibility

fraction of a substance in soil or soil material that is liberated in (human) gastrointestinal juices and thus available for absorption

3.3

bioavailability

fraction of a substance present in ingested soil that reaches the systemic circulation (blood stream)

3.4

contaminant

substance or agent present in the soil as a result of human activity

Note 1 to entry: There is no assumption in this definition that harm results from the presence of the contaminant.

3.5

dermal contact

contact with (touching) the skin

3.6

exposure

dose of a chemical that reaches the human body

3.7

exposure pathway

route a substance takes from its source to a receptor

3.8

ingestion

act of taking substances, such as soil and soil material, into the body by mouth

3.9

***in vitro* bioaccessibility test**

bioaccessibility test carried out outside a living organism

3.10

no observed adverse effect level

NOAEL

dose at which no adverse effect on a receptor can be observed

3.11

pica

eating habit where usually strange and unpalatable material such as soil material and stones are consumed

Note 1 to entry: The term pica stems from the Latin name *pica pica* for the raven bird magpie which picks up randomly any kind of material for nest construction.

3.12

provisional tolerable weekly intake

PTWI

provisional weekly tolerable amount of a substance which can be taken in by a human body during a lifetime through the food chain without affecting human health

3.13

receptor

<human> potentially exposed person

3.14

relative absorption fraction

RAF

ratio between the amount of a contaminant reaching systemic circulation when ingested with, for example, soil and the same amount obtained when ingested in the toxicity experiment underlying the criteria

3.15**species**

different forms of a substance always arising with each other in a reaction equilibrium

3.16**tolerable daily intake value****TDI**

daily tolerable amount of a substance which can be taken in by a human body during a lifetime through the food chain without effecting human health

4 Bioaccessibility/Bioavailability as a concept in assessment of soils and sites with respect to human exposure

The characterization of bioaccessibility/bioavailability is usually performed as a part of a risk and/or exposure assessment.

Risk assessment comprises the following elements:

- hazard identification;
- dose-response assessment;
- exposure assessment;
- and based on the above: risk characterization.

An exposure assessment is the process wherein the intensity, frequency, and duration of human exposure of a contaminant are estimated, and comprises:

- source identification and characterization;
- identification of exposure routes;
- identification of relevant receptors/target groups;
- and based on this: the actual exposure assessment.

For the assessment of possible effects on human health, an analysis of the exposure routes is a prerequisite. Where receptors are not directly exposed to a contaminant, exposure assessment needs to consider the various ways by which indirect exposure might occur and the significance of them.

Human exposure from soil contamination can occur through different media.

Directly from the soil, the following exposure routes exist:

- soil ingestion, both dietary and through adherence to hands and unwashed vegetables, etc.;
- dermal contact;
- ingestion of house dust that predominantly consists of soil material.

Airborne exposure comprises the following:

- inhalation and ingestion of fugitive dust;
- inhalation of elevated outdoor-concentrations;
- inhalation of vapours that have intruded into buildings.

Exposure through food chain comprises the following:

- consumption of plants including crops, wild plants and fungi;

- consumption of animals and animal products, including wild animals;
- consumption of contaminated water.

Within this document, direct uptake of soil via ingestion and/or ingestion of fugitive dust is considered. Oral ingestion is one of the most important exposure routes for humans to soil contaminants.

Quality criteria for soil (the maximum concentration limits for soil) are usually calculated on the basis of a tolerable daily intake value (TDI) or a provisionally tolerable weekly intake (PTWI), that can be derived from the no observed adverse effect level (NOAEL) found in human data or experimental animal data. For genotoxic carcinogens for which no lower threshold for increased risk for cancer is assumed, the TDI value is set at a level that corresponds to a tolerable low (negligible) cancer risk level.

For determining the TDI, data on oral toxicity are primarily considered. These data often pertain to animal experiments where the substance is administered to the animals mixed in the feed or in drinking water (the vehicle or transporter of the contaminant). The amount of contaminant needed to produce adverse health effects in the animal is then recorded. As an alternative, epidemiological studies relating observed human health effects to recorded exposures have been used. Most toxicological studies report the total ingested amount and seldom indicate exact values for the bioavailability of the substances administered.

When extrapolating from such experimental conditions to other conditions, e.g. to intake of contaminated soil, this approach assumes that the uptake efficiency is equal for all scenarios, i.e. that the absolute bioavailability of the contaminant is constant. The absolute oral bioavailability can be defined as the fraction of an orally ingested contaminant that reaches systemic circulation, i.e. enters the blood stream. The absolute oral bioavailability of a contaminant may range from close to 0 to almost 1 (i.e. 100 %) depending upon the physiochemical form of the contaminant. In this context, the use of the concept of absolute, oral bioavailability rests upon the assumption that adverse health effects are systemic and thus triggered by the contaminants reaching the blood stream, i.e. the internal exposure as opposed to the external exposure measured directly as intake of a contaminated medium multiplied by the concentration of the contaminant in the medium, see [Figure 1](#).

The absolute bioavailability can be measured as the ratio between amounts in the blood of animals or man after intravenous injection (100 % bioavailability) and after oral ingestion (uptake of bioavailable fraction).

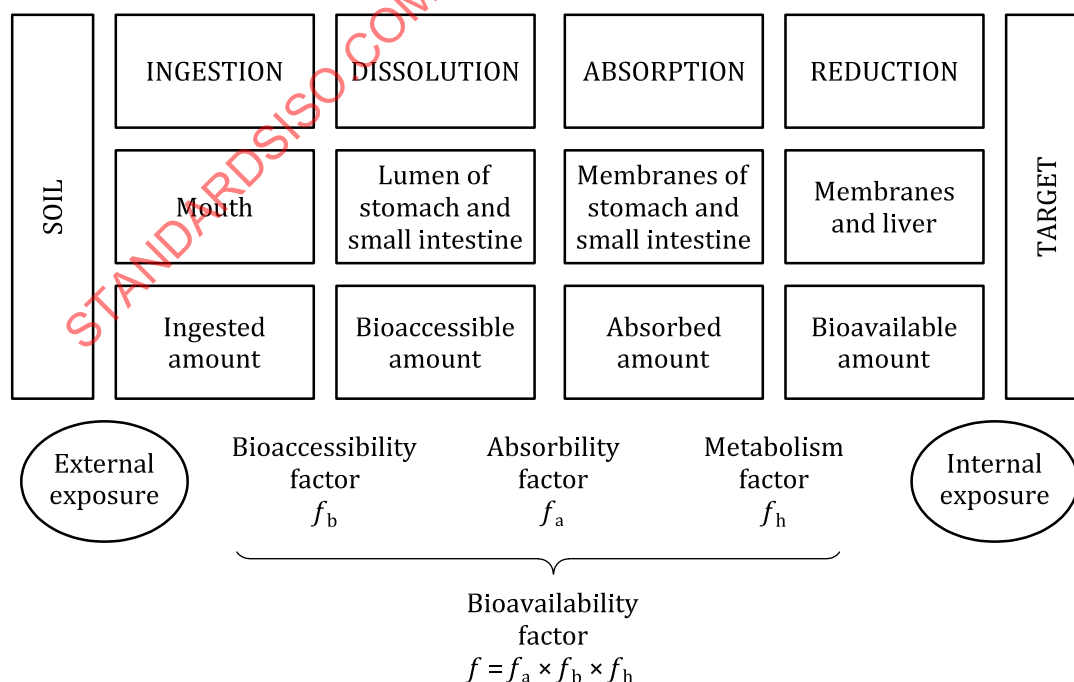


Figure 1 — Schematic presentation of oral uptake processes

A more feasible approach is to measure the relative bioavailability or relative absorption fraction (RAF), which is the ratio between the amount of a contaminant reaching systemic circulation when ingested with, for example, soil and the same amount obtained when ingested in the toxicity experiment underlying the criterion.

It should be noted that although most relative bioavailabilities are less than 1 and would result in an increased acceptable levels, RAF values above 1 could be found that would result in a demand for a decreased acceptable level.

5 Description of the mechanisms of human contaminant uptake

A series of compartments are involved in human bioavailability of ingested soil contaminants, as described in [Clause 4](#).

The overall pathway leads the food and soil with contaminants from the mechanical grinding in the mouth through a series of chemical and microbiological processes to partial dissolution through the entire gastrointestinal tract (bioaccessibility processes). The dissolved components are transported through the membranes of the gastrointestinal epithelium (absorption) and into the blood stream. During transport through the membranes, degradation can occur (metabolism). The blood passes the liver before entering the systemic circulation, allowing for degradation or removal of unwanted compounds in the liver (metabolism, first pass effect). Most of the dissolution processes are completed before the material leaves the small intestine, and it is generally accepted that most of the uptake takes place in the small intestine. To which extent uptake takes place in the stomach depends on the compound. The environment in the compartments differs and accordingly impacts the bioaccessibility process differently, see [Table 1](#).

Table 1 — Functions and conditions in the compartments involved in bioaccessibility processes

Compartment	Primary digestion functions	Main added "reagents"	pH	Residence time	Contaminant dissolution function
Mouth	Grinding Cleavage of starch	Moisture Amylase	6,5	Seconds to minutes	Grinding enhances subsequent dissolution
Gullet	Transport	None	6,5	Seconds	None
Stomach	Cleavage of proteins and fats	Hydrochloric acid Proteases Lipases	1 - 5	8 min to 3 h	Acid dissolves labile mineral oxides, sulphides and carbonates to release metals.
Small intestine	Cleavage of oligosaccharides, proteins, fats and other constituents Solubilization of fats	Bicarbonate Bile Proteases Lipases Oligosaccharases Phosphatases	4 - 7,5	2 h to 10 h	Organic matter is dissolved and bound contaminants released Cationic metals are solubilised by complexation with bile acids Some metals are precipitated by the high pH or by phosphate

The pH in the stomach may vary from close to 1 under fasted conditions to as high as 5 after feeding. Residence time (1/2-time for emptying) in the stomach varies similarly from 8 min to 15 min and 30 min to 3 h for fasted and average fed conditions, respectively. Furthermore, bile release varies as well, with high releases under fed conditions. Finally, the pH in the stomach can be lower for small children than for adults.

The gastrointestinal tract constitutes a complex ecosystem with aerobic and anaerobic microorganisms. The density of microorganisms is less in the human stomach and in the upper part of the small intestine but increases towards and in the large intestine. Anaerobic microorganisms dominate in human faeces, whereas aerobic bacteria are found in high densities in the large intestine. Sulfate reducing

bacteria have been detected in the human large intestine while high concentrations of oxygen have been detected throughout the gastrointestinal tract of pigs. Overall, dominating aerobic conditions and microorganisms would be expected in the stomach, but with increasingly anaerobic conditions from the small intestine to the large intestine.

Absorption requires that the contaminants are dissolved (free or bound to a dissolved carrier such as bile), transported to the gastrointestinal wall and, if bound to a carrier, released at the surface of the gastrointestinal membrane for absorption. The carrier mechanisms can be complexation of cationic metals by bile acids. Bile acids, proteins and other complexing agents can enhance exposure for cationic metals. Also, lipids and other soluble organic matter in the diet can add to the carrier effect of the bile.

The simple dissolution/transport/absorption processes can be complicated by chemical kinetics resulting from the sequential change in the chemical environment of the gastrointestinal tract, as well as by soil and contaminant chemistry. As an example, lead found in soil as the common contaminant anglesite (PbSO_4) will dissolve in the stomach and will stay in solution here at the low pH and high chloride concentration, see Figure 2. Entering the higher pH in the presence of dissolved phosphate in the small intestine, the dissolved lead ions (Pb^{2+}) will precipitate very quickly as lead chlorophosphate [chloropyromorphite, $\text{Pb}_5(\text{PO}_4)_3\text{Cl}$]. The phosphate can originate from digested food or from the soil. Phosphate minerals, such as hydroxyapatite, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, will dissolve in the low pH of the stomach, but dissolution will be slower and less complete at higher pH in the stomach (as occurring after food ingestion). If stomach transit is fast (as occurring under fasting conditions), the hydroxyapatite may not dissolve in the stomach and reach the small intestine where the neutral to slightly alkaline pH will prevent further dissolution and thus also precipitation of released lead as lead chlorophosphate. Conversely, just after transit from the stomach to the small intestine, the pH is still low and absorption of lead can take place driven by the high dissolved lead concentration possible in acidic pH. Overall, the *de facto* dissolution of lead from soil will depend upon interacting conditions such as soil composition, simultaneously ingested food and feeding conditions of the human. This also means that cultural factors that affect the type of food typically consumed can have an influence on the actual uptake.

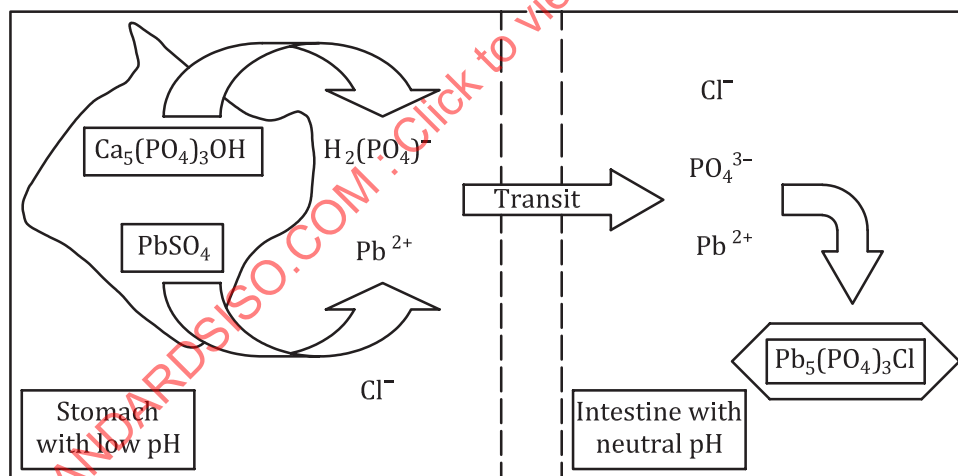


Figure 2 — Example of dissolution of a lead mineral (lead sulfate) in the stomach and subsequent precipitation in the small intestine

The absorption of dissolved contaminants predominantly occurs through the epithelium of the stomach and the small intestine (the intestinal epithelium) either through the cells (transcellular transport) or between the cells (paracellular transport). The pathway between the cells is primarily taken by polar or ionic contaminants (e.g. some metals).

Metals are absorbed by passive paracellular transport, by passive transcellular diffusion or by active transcellular transport fitting into a transport system already present. One example is that cadmium can be absorbed by both the passive paracellular route and the passive diffusive route. Another example is lead that is probably absorbed via the calcium uptake system(s), including both active and passive transcellular transport, as well as by paracellular transport.

Metabolism of the absorbed contaminant concentrations takes place in the epithelium membranes (binding and exclusion), as well as in the liver (transformation of metals, and secretion of metals with bile). Contaminants entering systemic circulation via the lymph will be less efficiently reduced, as the liver is bypassed for this route. Finally, the contaminants are diluted when entering systemic circulation in the blood stream.

If the sensitivity to changes of the processes of dissolution, absorption and reduction is considered to be caused by varying “vehicles” (i.e. ingestion with soil, food or in solution) and chemical forms (i.e. different metal salts ingested), it is expected that dissolution will be highly sensitive, absorption sensitive and reduction slightly sensitive (chemical form) or insensitive (vehicle). In applying the concept of relative bioavailability (see [Clause 4](#)), the most important factor to assess would thus be the bioaccessibility factor f_b (see [Figure 1](#)) followed by the absorbability factor f_a .

Estimation of the relative bioavailability factor is thus reduced to an estimation of how the two potentially rate limiting processes of dissolution and absorption respond to variations in vehicle and chemical form of the contaminants.

If the dissolution process is rate limiting (i.e. if dissolution is slower than absorption), changes in f_b will determine the relative bioavailability. If the absorption process is rate limiting (i.e. absorption of dissolved contaminants is too slow to be completed before transit), f_a will be “in charge” of relative bioavailability. *In vitro* tests are generally based on the measurement of bioaccessibility, and are thus based on the assumption that absorption is not rate limiting, or at least that the absorption of a compound dissolved from soil is no different than the absorption of the compound when administered in a fairly soluble form.

6 Description of metal binding mechanisms (speciation of metals) in soil

In assessing the bioaccessibility of metals in soil, three major obstacles are encountered.

- Most metals occur naturally at varying concentrations and in varying physical and chemical forms.
- Chemical forms (species) of the original metal (source) may vary from solid metal to aqueous solution of a salt.
- Chemical forms are interchangeable depending upon the soil conditions and history.

Assessment of bioaccessibility data for metals in soil therefore needs to reflect the varying geochemical conditions. Due to their different physical-chemical properties, the mechanisms for reduced bioaccessibility differ among the metals. An example of distribution between phases and chemical forms (species) in soils is shown for copper in [Figure 3](#). The bioaccessibility of the three solid species of copper, free metal (Cu), copper sulphide (CuS), and copper cations bound by ion exchange mechanisms, will differ. Similarly, the absorption of the three dissolved species of copper, free copper ions, copper ions in inorganic complexes, and copper in organic complexes with, for example, humic substances or organic acids, may differ, depending upon the stability of the complexes in the gastrointestinal lumen, see [Clause 4](#).

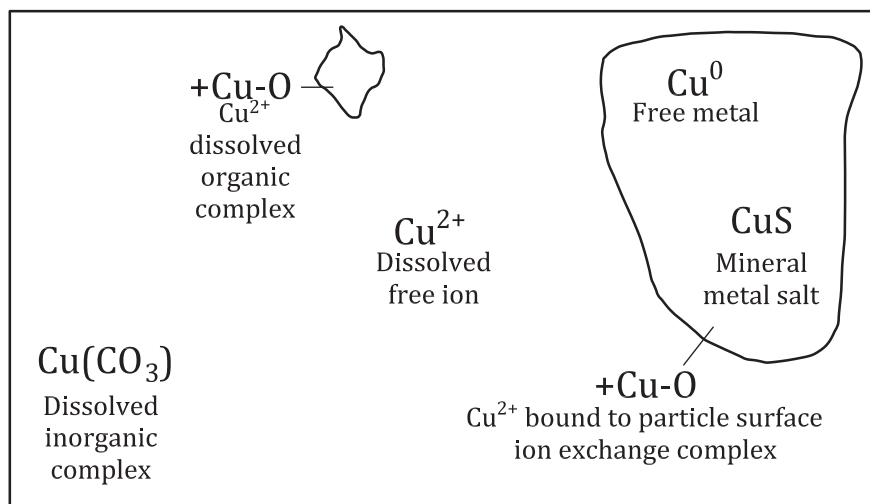


Figure 3 — Example of distribution of metals (copper) in soil

Ageing effect in soils has been observed for As(V) and Cr(VI), which may lead to different accessibility over time. On the other hand, some heavy-metal-bearing minerals have resisted weathering and dissolution over geological time scales. Whether the aggressive chemical conditions in the human digestive tract nevertheless will cause dissolution depends upon the mineral.

7 Use and interpretation of *in vitro* tests for risk assessment

When deciding whether or not to make use of physiologically based extraction methods for the estimation of actual bioavailability, the following considerations should be made.

- Is the site considered to be at risk using conventional methods (comparison of total concentrations to the appropriate quality criteria)? If no, it can reasonably be expected that including bioavailability considerations will not alter this conclusion.
- If yes, does the available data imply that the metals in question are likely to be less bioavailable in soil? This could be information on type of contamination source, type of soil/soil material?
- Is soil ingestion considered to be a major pathway of exposure? If no, studies on oral bioavailability of the contaminants from soil would not be meaningful.
- Would the result of the site-specific risk assessment be sufficiently sensitive to the suspected variation in bioavailability?
- Would the decisions regarding remediation at the site be impacted, or would the collection of the sufficient new data be over costly in relation to the expected change in remedial costs?
- Is a sufficiently validated method available for the contaminants and context in question taking into account the relevant cultural setting?

NOTE Guidance is provided in ISO 18400-104 on what constitutes a sufficiently representative sample and the need, when deciding where and how to sample, to take variability at different scales into account.

If all the above questions can be answered with a yes, the use of *in vitro* tests is justifiable. Furthermore, a judgement should be made about how and where samples are to be taken, and also how large an area or volume a sample can be considered representative of based on the knowledge of the uniformity or otherwise of the distribution of the substance in question.

The relative bioaccessibility is determined as the ratio between the results obtained for the soil in question to the results obtained in the test applying the contaminant in a way consistent with the toxicity evaluation that is the basis for the soil quality criteria used in the risk assessment. This relative

bioaccessibility or relative absorption fraction (RAF) can then be multiplied with the total contaminant concentration in the soil to obtain the bioaccessible soil concentration. This concentration can then be used in the risk assessment instead of the total concentration, when evaluating the soil ingestion pathway.

8 Description of test method

8.1 Test principle

The method bioaccessibility is assessed using two different mixed solutions (i.e. gastric and gastrointestinal).

When an *in vitro* test is to be used for the evaluation of the relative bioavailability, a number of conditions shall be met.

- The relative bioavailability of the metal in question can be reasonably estimated through the estimation of the relative bioaccessibility.
- The test is based on simulation of physiological conditions that influence bioaccessibility.
- A reasonable correlation can be proven between the test results and a relevant *in vivo* animal model that is relevant and validated and has a sufficient data set.

It should be noted that *in vivo* models do not necessarily give a correct estimate of the bioavailability in humans depending on the difference in uptake and metabolism between the animal in question and humans, and is also dependent on in which matrix the compounds are measured (e.g. faeces, blood, organs). It should also be noted that sufficient *in vivo* data do not exist for all relevant metals.

The method is a sequential leaching test using synthetic gastrointestinal fluids and can be used to estimate oral bioaccessibility. Bioaccessibility can then be used to approximate bioavailability. The method has been validated for arsenic, cadmium and lead in an international interlaboratory trial. The method has been *in vivo* validated to assess the bioaccessibility of arsenic, cadmium and lead.

8.2 Apparatus

The apparatus specified shall be checked before use for proper operation and absence of interfering elements that may affect the result of the test. The apparatus shall also be calibrated where relevant.

All glassware and centrifuge tubes shall be cleaned prior to use using a suitable acid rinsing protocol.

8.2.1 Oven, water bath or incubator, capable of maintaining a temperature of $(37 \pm 2) ^\circ\text{C}$.

8.2.2 End-over-end rotator or tumbler, capable of 30 r/min at $37 ^\circ\text{C}$.

8.2.3 Centrifuge, capable of reaching 4 500*g*.

8.2.4 pH meter, with an accuracy of at least 0,05 pH units. pH is an important parameter of the test, controlling the leaching from the matrix. It is important that the pH meter is precisely calibrated at room temperature.

8.2.5 Analytical balance, with an accuracy of at least 1 mg.

8.2.6 Volumetric flasks, suitable grades.

8.2.7 Auto pipettes.

8.2.8 Magnetic agitator.

8.2.9 Polycarbonate centrifuge tubes, with polypropylene screw cap (50 ml).

8.2.10 HDPE screw top bottle, volume capacity of 2 l.

8.2.11 Sieving equipment, with sieves of 250 µm nominal screen size.

8.3 Reagents

Use only reagents of recognized analytical grade.

8.3.1 Distilled water, demineralized water, deionized water or water of equivalent purity, ($5 < \text{pH} < 7$) with conductivity $> 0,1 \text{ mS/m}$ according to grade 2 specified in ISO 3696.

8.3.2 Sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), CAS-Nr 13472-35-0.

8.3.3 D-Glucuronic acid ($\text{C}_6\text{H}_{10}\text{O}_7$), CAS-Nr 6556-12-3.

8.3.4 Sodium chloride (NaCl), CAS-Nr 7647-14-5.

8.3.5 Potassium thiocyanate (KSCN), CAS-Nr 333-20-0.

8.3.6 Sodium sulfate, anhydrous (Na_2SO_4) CAS-Nr 7757-82-6.

8.3.7 Potassium chloride (KCl), CAS-Nr 7447-40-7.

8.3.8 Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), CAS-Nr 10035-04-8.

8.3.9 Ammonium chloride (NH_4Cl), CAS-Nr 12125-02-9.

8.3.10 Sodium bicarbonate (NaHCO_3), CAS-Nr 144-55-8.

8.3.11 Potassium dihydrogen phosphate (KH_2PO_4), CAS-Nr 7778-77-0.

8.3.12 Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), CAS-Nr 7796-30-3.

8.3.13 Sodium hydroxide (NaOH), CAS-Nr 1310-73-2, $c(\text{NaOH}) = 1 \text{ mol/l}$ to 5 mol/l .

8.3.14 Hydrogen chloride (HCl), CAS-Nr 7647-01-0, $c(\text{HCl}) = 37 \%$.

8.3.15 Urea, ($\text{CH}_4\text{N}_2\text{O}$), CAS-Nr 57-13-6.

8.3.16 D(+)-Glucose ($\text{C}_6\text{H}_{12}\text{O}_6$), CAS-Nr 50-99-7.

8.3.17 D(+)-glucosamine hydrochloride ($\text{C}_6\text{H}_{13}\text{NO}_5 \cdot \text{HCl}$), CAS-Nr 66-84-2.

8.3.18 Pepsin (porcine), CAS-Nr 9001-75-6.

8.3.19 Bovine serum albumin (BSA), CAS-Nr 90604-29-8.

8.3.20 Mucin (procine), CAS-Nr 84082-64-4.

8.3.21 Uric acid ($C_5H_4N_4O_3$), CAS-Nr 69-93-2.

8.3.22 Pancreatin (porcine), CAS-Nr 8049-47-6.

8.3.23 α -Amylase (bacillus species), CAS-Nr 9000-90-2.

8.3.24 Lipase (porcine), CAS-Nr 9001-62-1.

8.3.25 Bile salts (bovine), CAS-Nr 8008-63-7.

8.3.26 Nitric acid (HNO_3), CAS-Nr 7697-37-2, $c(HNO_3) = 69\%$, 0,1 M.

The concentrations of the reagents used for pH adjustment are listed in [Table 2](#).

Table 2 — pH adjustment reagent concentrations

Reagent	Volume/Mass in 1 000 ml
0,1 M HNO_3	6,30 ml
1,0 M NaOH	40,0 g

8.4 Preparation of simulated fluids

8.4.1 General

Four different digestive fluids are prepared.

- simulated saliva fluid (see [8.4.2](#));
- simulated gastric fluid (see [8.4.3](#));
- simulated duodenal fluid (see [8.4.4](#));
- simulated bile fluid (see [8.4.5](#)).

All simulated gastrointestinal fluids are combinations of separately prepared 500 ml solutions with either inorganic and organic reagents and an addition of solid constituents. The preparation of the digestive fluids is described in [8.4.2](#) to [8.4.5](#).

All simulated gastric and intestinal fluids are prepared one day prior to carrying out bioaccessibility extractions. Mix thoroughly for at least 3 h or until the reagents are completely dissolved.

The solutions are made according to detailed instructions given on the day before the extractions. The pH-values of the solutions are adjusted to the correct ranges on the day of use.

8.4.2 Simulated saliva fluid (1 000 ml)

The chemical composition of the inorganic solution, the organic solution and the solid chemical reagents required to prepare 1 000 ml of simulated saliva fluid are given in [Tables 3, 4](#) and [5](#), respectively.

Table 3 — Inorganic saliva phase reagent (500 ml)

Reagent	Volume/Mass made up to 500 ml	Final concentration mg/l
KCl	0,896 g	1 792
NaH ₂ PO ₄ · 2H ₂ O	0,888 g	1 776
KSCN	0,200 g	400
Na ₂ SO ₄	0,570 g	1 140
NaCl	0,298 g	596
NaOH	1,80 ml of 1,0 M	144

Table 4 — Organic saliva phase reagent (500 ml)

Reagent	Mass made up to 500 ml g	Final concentration mg/l
Urea	0,200	400

Table 5 — Additional constituents for saliva phase reagent

Reagent	Mass made up to 1 000 ml g	Final concentration mg/l
α-Amylase	0,145	145
Mucin	0,050	50,0
Uric acid	0,015	15,0

Add the solid chemical reagents to a suitable container according to the masses given in [Table 5](#). Simultaneously pour the separately prepared 500 ml volumes of the inorganic and organic saliva phase reagents into the bottle ([8.2.10](#)) containing the solid reagents and mix thoroughly with a magnetic agitator ([8.2.8](#)) for at least 3 h or until the reagents are completely dissolved. The fluid is then stored at room temperature overnight prior to completion of the bioaccessibility extractions.

8.4.3 Simulated gastric fluid (1 000 ml)

The chemical composition of the inorganic solution, the organic solution and the solid chemical reagents required to prepare 1 000 ml of simulated gastric fluid are given in [Tables 6, 7](#) and [8](#), respectively.

Table 6 — Inorganic gastric phase reagent (500 ml)

Reagent	Volume/Mass made up to 500 ml	Final concentration
NaCl	2,752 g	5 504 mg/l
NaH ₂ PO ₄ · 2H ₂ O	0,266 g	533 mg/l
KCl	0,824 g	1 649 mg/l
CaCl ₂ · 2H ₂ O	0,400 g	799 mg/l
NH ₄ Cl	0,306 g	612 mg/l
HCl	8,3 ml of 37 % HCl	0,31 %

Table 7 — Organic gastric phase reagent (500 ml)

Reagent	Mass made up to 500 ml g	Final concentration mg/l
D(+)-Glucose	0,650	1 300
D-Glucuronic acid	0,020	40,0
Urea	0,085	170
D(+)-Glucosamine hydrochloride	0,330	660

Table 8 — Additional constituents for gastric phase reagent

Reagent	Mass made up to 1 000 ml g	Final concentration mg/l
Bovine serum albumin	1,000 g	1 000
Mucin	3,000 g	3 000
Pepsin	1,000 g	1 000

Add the solid chemical reagents to a suitable container (preferably [8.2.10](#)) according to the masses given in [Table 8](#). Simultaneously pour the separately prepared 500 ml volumes of the inorganic and organic gastric phase reagents into the bottle ([8.2.10](#)) containing the solid reagents and mix thoroughly for at least 3 h with a magnetic agitator ([8.2.8](#)) until the reagents are completely dissolved. The fluid is then stored at room temperature overnight prior to completion of the bioaccessibility extractions.

8.4.4 Simulated duodenal fluid (1 000 ml)

The chemical composition of the inorganic solution, the organic solution and the solid chemical reagents required to prepare 1 000 ml of simulated duodenal fluid are given in [Tables 9, 10](#) and [11](#), respectively.

Table 9 — Inorganic duodenal phase reagent (500 ml)

Reagent	Volume/Mass made up to 500 ml	Final concentration
NaCl	7,012 g	14 024 mg/l
KH ₂ PO ₄	0,080 g	160 mg/l
KCl	0,564 g	1 129 mg/l
MgCl ₂ · 6H ₂ O	0,050 g	100 mg/l
HCl	180 µl of 37 % HCl	0,01 %

Table 10 — Organic duodenal phase reagent (500 ml)

Reagent	Mass made up to 500 ml g	Final concentration mg/l
Urea	0,100	200

Table 11 — Additional constituents for duodenal phase reagent

Reagent	Mass made up to 1 000 ml g	Final concentration mg/l
CaCl ₂ ·2H ₂ O	0,200	200
Bovine Serum Albumin	1,000	1 000
Pancreatin	3,000	3 000
Lipase	0,500	500

Add the solid chemical reagents to a suitable container (preferably 8.2.10) according to the masses given in Table 11. Simultaneously pour the separately prepared 500 ml volumes of the inorganic and organic duodenal phase reagents into the bottle (8.2.10) containing the solid reagents and mix thoroughly for 3 h with a magnetic agitator (8.2.8). Measure the pH of the simulated duodenal fluid, which should be at pH = 7,4 ± 0,2. If required, adjust the pH of the duodenal fluid to the correct pH with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14). The fluid is then stored at room temperature overnight prior to completion of the bioaccessibility extractions.

8.4.5 Simulated bile fluid (1 000 ml)

The chemical composition of the inorganic solution, the organic solution and the solid chemical reagents required to prepare 1 000 ml of simulated bile fluid are given in Tables 12, 13 and 14, respectively.

Table 12 — Inorganic bile phase reagent

Reagent	Volume/Mass made up to 500 ml	Final concentration
NaCl	5,259 g	10 518 mg/l
NaHCO ₃	5,785 g	11 570 mg/l
KCl	0,376 g	753 mg/l
HCl	180 µl of 37 % HCl	0,01 %

Table 13 — Organic bile phase reagent

Reagent	Mass made up to 500 ml g	Final concentration mg/l
Urea	0,250	500

Table 14 — Additional constituents for bile phase reagent

Reagent	Mass made up to 1 000 ml g	Final concentration mg/l
CaCl ₂ ·2H ₂ O	0,222	222
Bovine Serum Albumin	1,800	1 800
Bile	6,000	6 000

Add the solid chemical reagents to a suitable container (preferably 8.2.10) according to the masses given in Table 14. Simultaneously pour the separately prepared 500 ml volumes of the inorganic and organic bile phase reagents into the bottle (8.2.10) containing the solid reagents and mix thoroughly. Mix thoroughly for at least 3 h with a magnetic agitator to allow for the complete dissolution of all reagents

and measure the pH of the simulated bile fluid. The simulated bile fluid should be at $\text{pH} = 8,0 \pm 0,2$. If required, adjust the pH of the bile fluid to the correct pH with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14).

The fluid is then stored at room temperature overnight prior to completion of the bioaccessibility extractions.

8.4.6 pH control of mixed fluids

Check that the final pH of the mixed saliva and gastric phase (1 ml of saliva and 1,5 ml of gastric) is $\text{pH} = 1,20 \pm 0,05$. If the mixture is not within specification adjust the pH of the gastric fluid with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14) and recheck an aliquot of the mixed saliva-gastric phase.

Check that the final pH of the mixed gastrointestinal fluid (1,0 ml of saliva, 1,5 ml of gastric, 3,0 ml of duodenal and 1,0 ml of bile) is $\text{pH} = 6,3 \pm 0,5$. If the mixture is not within specification adjust the pH of the duodenal fluid to with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14) and recheck an aliquot of the mixed gastrointestinal phase.

The solutions shall be heated to 37 °C prior to use on the day of extraction.

8.5 Sample pre-treatment

8.5.1 General

All samples prepared for bioaccessibility testing are dried in a fan assisted oven at <40 °C and sieved to <250 μm .

8.5.2 Preparation of test samples

Weigh four 0,6 g test samples of each test soil (after drying) accurately into uniquely labelled suitable centrifuge tubes capable of undergoing centrifugation. Label two of the test samples of the soil for the gastric phase of the extraction and the other two test samples gastrointestinal phase (the test is carried out in duplicate). Store the weighed test samples at room temperature prior to completion of the extraction methodology. For every 10 unknown test samples, extract a blank sample and two reference material samples.

8.5.3 Typical analysis protocol

Consider a sample batch of 22 samples for bioaccessibility testing. Table 15 gives a typical protocol with duplicates and blanks which would be run in a randomized order.

Table 15 — Typical analysis protocol for bioaccessibility testing

	Sample type	Sample number	Sample type	Sample number	Sample type	Sample number	Sample type
1	Sample	0001 Dup	Duplicate	BGS102 A	Reference Soil	Blank A	Blank
2	Sample			BGS102 B	Reference Soil	Blank B	Blank
3	Sample			BGS102 C	Reference Soil	Blank C	Blank
4	Sample	0004 Dup	Duplicate	BGS102 D	Reference Soil	Blank D	Blank
5	Sample	0005 Dup	Duplicate				
6	Sample						
7	Sample	0007 Dup	Duplicate				
8	Sample						
9	Sample	0009 Dup	Duplicate				
10	Sample						

Table 15 (continued)

	Sample type	Sample number	Sample type	Sample number	Sample type	Sample number	Sample type
11	Sample	0011 Dup	Duplicate				
12	Sample						
13	Sample						
14	Sample						
15	Sample						
16	Sample	0016 Dup	Duplicate				
17	Sample	0017 Dup	Duplicate				
18	Sample						
19	Sample						
20	Sample						
21	Sample						
22	Sample	0022 Dup	Duplicate				

There are a few reference soils that can be used. The BGS guidance soil BGS102 available from the British Geological Survey^[11] and NIST 2710A and 2711A available from the National Institute of Standards and Technology^[12].

8.6 Sample preparation procedure

8.6.1 Switch on the incubator (8.2.1) at least 2 h prior to beginning the bioaccessibility extraction and set the temperature to $(37 \pm 2) ^\circ\text{C}$.

8.6.2 Warm the simulated gastrointestinal fluids (prepared on the previous day) to $(37 \pm 2) ^\circ\text{C}$ prior to their use in the bioaccessibility extraction method.

8.6.3 Check that the final pH of the mixed saliva and gastric phase (1 ml of saliva and 1,5 ml of gastric) is $\text{pH} = 1,20 \pm 0,05$ and the mixed gastrointestinal fluid (1,0 ml of saliva, 1,5 ml of gastric, 3,0 ml of duodenal and 1,0 ml of bile) is $\text{pH} = 6,3 \pm 0,5$. If the saliva-gastric mixture is not within specification, adjust the pH of the gastric fluid with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14) and recheck an aliquot of the mixed saliva-gastric phase. If the gastrointestinal mixture is not within specification, adjust the pH of the duodenal fluid to with either 1,0 M NaOH (8.3.13) or 37 % HCl (8.3.14) and recheck an aliquot of the mixed gastrointestinal phase.

8.6.4 Once the incubator set-up (8.2.1) and simulated gastrointestinal fluids have reached an operating temperature of $(37 \pm 2) ^\circ\text{C}$, carry out the following extraction protocol (8.6.5 to 8.6.18).

8.6.5 Accurately add, to each centrifuge tube (8.2.9), via pipette (8.2.7) 9,0 ml of simulated saliva fluid.

8.6.6 Cap each centrifuge tube (8.2.9) and manually shake to thoroughly mix the soil and simulated fluids.

8.6.7 Accurately add to each centrifuge tube, via pipette (8.2.7) 13,5 ml of simulated gastric fluid, 5 min to 15 min after the addition of the simulated saliva fluid.

Check the pH (this should be stable at $1,20 \pm 0,05$) and adjust with either NaOH (8.3.13) or HCl (8.3.14).

8.6.8 Place the centrifuge tubes (8.2.9) in the incubator (8.2.1) and incubate the samples using end-over-end rotation, at $(37 \pm 2) ^\circ\text{C}$ for 1 h.

8.6.9 Remove both the gastric and gastrointestinal centrifuge tubes (8.2.9).